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Fetal and Neonatal Physiology

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Fetal and Neonatal PHYSIOLOGY

FOURTH EDITION / VOLUME 1

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Dedicated to

Our spouses –
Helene Polin, Laurie Kilpatrick, and Carolyn Abman

Our children –
Allison Polin Steinbrenner, Mitchell Polin, Jessica Moseley, and Gregory Polin
Will Fox, Jon Fox, James Fox, Lauren Fox, and James Hunt
Ryan Abman, Lauren Abman, Mark Abman, and Megan Abman

And our grandchildren –
Lindsey Steinbrenner, Eli Steinbrenner, Willa Polin, Jasper Polin, and Casey Moseley
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Pathophysiology of Acute Renal Failure in the Neonatal Period

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Calcium, Phosphorus, and Magnesium Transport Across the Placenta

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Vitamin K Metabolism in the Fetus and Neonate

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Pathophysiology of Respiratory Distress Syndrome and Surfactant Metabolism

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Intraventricular Hemorrhage in the Neonate

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Pathophysiology of Respiratory Distress Syndrome and Surfactant Metabolism
Surfactant Treatment

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Host-Fungi Interactions Relevant to the Newborn Infant

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The Extracellular Matrix in Development

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Postnatal Maturation of Renal Blood Flow

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Bile Formation and Cholestasis

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Lipids as an Energy Source for the Premature and Full-Term Neonate
Protein and Amino Acid Metabolism and Requirements

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Growth Factor Regulation of Fetal Growth

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Host Defense Mechanisms Against Viruses

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Physiology of Nitric Oxide in the Developing Lung

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Pathophysiology of Acute Renal Failure in the Neonatal Period

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Maternal Drug Abuse: Effects on the Fetus and Neonate

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Maternal Drug Abuse: Effects on the Fetus and Neonate

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Zinc in the Fetus and Neonate

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Physiology of Congenital Heart Disease in the Neonate

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Role of the Kidney in Calcium and Phosphorus Homeostasis

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Pathophysiology of Gastroesophageal Reflux

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Concentration and Dilution of the Urine

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Organogenesis and Histologic Development of the Liver

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Structure and Development of Skin and Cutaneous Appendages

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Development of the Renin-Angiotensin System

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Fetal and Neonatal Body Water Compartment Volumes With Reference to Growth and Development

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Growth Hormone and Prolactin

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Basic Pharmacokinetic Principles

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Functional Development of Respiratory Muscles

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Integrins and Cell Adhesion Molecules

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Host-Fungi Interactions Relevant to the Newborn Infant
T-Cell Development

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Fetal and Placental Circulation During Labor

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Regulation of Lower Airway Function
Pathophysiology of Apnea of Prematurity

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General Concepts of Protein Metabolism

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Development of the Exocrine Pancreas

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Oxygen Transport and Delivery

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Physiology of Lactation

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Apoptotic Cell Death

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Development of Taste and Smell in the Neonate

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Pathophysiology of Apnea of Prematurity

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Retinal Development and the Pathophysiology of Retinopathy of Prematurity

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Developmental Hemostasis

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Bile Acid Metabolism During Development

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Mechanics of Breathing

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Lipoprotein Metabolism and Nutritional Programming in the Fetus and Neonate

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Digestive-Absorption Functions in Fetuses, Infants, and Children

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Role of the Kidney in Calcium and Phosphorus Homeostasis

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Development of the Gastrointestinal Circulation in the Fetus and Newborn

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Regulation of Umbilical Blood Flow

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Neonatal Calcium, Phosphorus, and Magnesium Homeostasis

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Luteinizing Hormone and Follicle-Stimulating Hormone Secretion in the Fetus and Newborn

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Physicochemical and Structural Properties Regulating Placental Drug Transfer

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Brown Adipose Tissue: Development and Function

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Digestive-Absorption Functions in Fetuses, Infants, and Children

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Homeobox Genes

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Developmental Basis of Congenital Limb Differences

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Genetics and Physiology of Surfactant Protein Deficiencies

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Pathophysiology of Shock in the Fetus and Neonate

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Metabolism of Glucose and Methods of Investigation in the Fetus and Newborn

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Neonatal Neutrophil Normal and Abnormal Physiology

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Cerebral Blood Flow in Premature Infants: Regulation, Measurement, and Pathophysiology of Intraventricular Hemorrhage

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Oxygen Consumption and General Carbohydrate Metabolism of the Fetus

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Developmental Electrophysiology in the Fetus and Neonate

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Trophic Factor and Nutritional and Hormonal Regulation of Brain Development

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Trophic Factor and Nutritional and Hormonal Regulation of Brain Development

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Physicochemical and Structural Properties Regulating Placental Drug Transfer

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Fetal and Neonatal Thyroid Physiology

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Physicochemical Aspects of Pulmonary Surfactant

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Molecular Mechanisms of Lung Development and Lung Branching Morphogenesis

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The Extracellular Matrix in Development

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Lipids as an Energy Source for the Premature and Full-Term Neonate

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Impaired Lung Growth After Injury in Premature Lung

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Developmental Biology of the Pulmonary Vasculature

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Structure of Alveolar Epithelial Cells and the Surface Layer During Development

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Angiogenesis
Fetal Requirements and Placental Transfer of Nitrogenous Compounds

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Control of Breathing in Fetal Life and Onset and Control of Breathing in the Neonate

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Fetal and Maternal Responses to Intrauterine Infection

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Regulation of Surfactant-Associated Phospholipid Synthesis and Secretion

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Development of the Corticotropin-Releasing Hormone–Corticotropin System in the Mammalian Fetus

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Regulation of the Placental Circulation

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Organogenesis of the Gastrointestinal Tract

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Molecular Mechanisms of Lung Development and Lung Branching Morphogenesis

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Mechanisms of Bilirubin-Induced Brain Injury

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Temperature Control in Newborn Infants

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Developmental Biology of the Hematopoietic Growth Factors
Mononuclear Phagocyte System

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Temperature Control in Newborn Infants

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Development of the Corticotropin-Releasing Hormone–Corticotropin System in the Mammalian Fetus

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Physics and Physiology of Human Neonatal Incubation

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Neural Regulation of Blood Pressure During Fetal and Newborn Life

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Regulation of Acid-Base Balance in the Fetus and Neonate

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Upper Airway Structure: Function, Regulation, and Development
Liquid Ventilation

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Physiology of Nitric Oxide in the Developing Lung

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Vitamin A Metabolism in the Fetus and Neonate

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Development of the Blood-Brain Barrier

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Structure of Alveolar Epithelial Cells and the Surface Layer During Development

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Basic Mechanisms of Oxygen Sensing and Response to Hypoxia

PREFACE

It has been nearly 7 years since the third edition of *Fetal and Neonatal Physiology* was published. During that interval, thousands of publications have focused on various aspects of fetal or neonatal physiology. The challenge faced in the preparation of this edition was to provide an exhaustive update without allowing the size (and cost) of the textbook to escalate. To further this end, each of the authors has been asked to incorporate relevant new material, but to remain within the page limitations of the previous edition. We have also eliminated clinical information that can easily be found in one of the many standard textbooks on neonatology, without sacrificing our goal of making the book a comprehensive reference text. Notably, there are several new chapters in this edition, and all chapters from the previous edition have been extensively revised.

As with any large textbook, there are a number of individuals we need to acknowledge. First, we wish to thank the hundreds of authors who contributed chapters to this fourth edition. We realize that the "academic" benefits of writing a chapter are limited, and that both new chapters and revisions require enormous time and effort. Only through the generosity of these contributors and their desire to educate has this edition been able to go forward. We wish to thank Heidi Kleinbart for her superb editorial assistance and Melissa Dudlick at Elsevier for her tremendous help in the development and organization of the book. Finally, we want to thank Judy Fletcher at Elsevier, who has been our senior editor since the first edition of *Fetal and Neonatal Physiology*, and who has challenged us to make this edition ever better.

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ACKNOWLEDGMENTS

We acknowledge with great sadness the passing of Michael Linshaw, MD, the renowned pediatric nephrologist who contributed the chapter "Concentration and Dilution of the Urine" to this text for several editions. He was a beloved teacher and mentor who enjoyed solving the mysteries of the kidneys. His passion and tenacity for doing so improved the lives of many children and their families.

We also wish to acknowledge the many scientific contributions of Karl Bauer, MD, whose research on energy expenditure and body composition were fundamental to understanding changes in body water compartments that occur in the transition to postnatal life. Dr. Bauer was awarded the Czerny Prize of the German Society of Pediatrics for his research on the genetics of lymphocytes in the newborn. He served as director of the department of neonatology of the University of Frankfurt and was president of the German Society of Perinatal Medicine.

SECTION I

Genetics and Embryology

1

Fred Levine

Basic Genetic Principles

PRIMARY STRUCTURE OF NUCLEIC ACID

The two kinds of nucleic acid—deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)—both are composed of recurring monomeric units called *nucleotides.* Each nucleotide has three components (Figure 1-1, *A*): (1) a phosphate group linked to (2) a five-carbon atom cyclic sugar group, which, in turn, is joined to (3) a purine or a pyrimidine base. DNA and RNA are distinguished by their base components and the makeup of their sugar-phosphate backbones. DNA consists of four deoxyribonucleotides that differ in their base components. The four bases are the purine derivatives adenine (A) and guanine (G) and the pyrimidine derivatives cytosine (C) and thymine (T) (see Figure 1-1, *B* and *C*). Similarly, four different ribonucleotides are the major components of RNA; they contain the purine bases adenine and guanine and the pyrimidine bases cytosine and uracil (U). Thus the major difference in base composition between RNA and DNA is that RNA contains uracil, whereas DNA contains thymine. The other difference between RNA and DNA is in their sugar-phosphate backbones: RNA contains ribose, and DNA contains 2-deoxyribose. In both DNA and RNA, the nucleotides are joined together by phosphodiester bonds linking the phosphate group of one nucleotide to a hydroxyl group on the sugar of the adjacent nucleotide. The purine and pyrimidine bases of the nucleotide constitute distinctive side chains and are not present in the backbone structure of nucleic acids.

By analyzing x-ray diffraction patterns of purified DNA and by building models, Watson and Crick deduced that native DNA consists of two antiparallel chains in a structure that can be conceptualized as resembling a spiral staircase, the *double helix* (Figure 1-2, *A*). The two strands are held together by hydrogen bonds between pairs of bases on the opposing strands, similar to the steps on the staircase (see Figure 1-2, *B*). The bonding is specific: A always pairs with T, and C always pairs with G. As Watson and Crick noted, a pair of purines would be rather large to fit inside a double helix (which has a thickness of 2 nm), and a pair of pyrimidines would be too far apart to form stable hydrogen bonds with each other. The base pairs AT and GC, however, proved to be similar not only in size but also in shape. Overall, the discovery of the structure of DNA was one of the most important events in biology because it not only provided an explanation for how genetic information is carried but also indicated how this information is propagated. As a consequence of the hydrogen bonding between the two DNA strands, also known as *base pairing* or *hybridization,* a DNA molecule can replicate precisely by separation of the two chains followed by synthesis of two new complementary strands.

In contrast with the regular structure of DNA, most RNA molecules are single-stranded. Base pairing, however, occurs between regions of an RNA strand with complementary sequences, with AU pairs instead of AT, giving RNA molecules a complex secondary structure that is poorly understood in most cases but plays an important role in cellular metabolism (e.g., as has been described for transfer RNA [tRNA]).

GENOMIC ORGANIZATION

Chromosomes

Each cell in a human being contains an enormous amount of DNA. With the completion of the human genome sequence, we know now that the genome contains 3.24475×10^9 base pairs per haploid genome. Obviously, the cell must be able to organize such a large amount of DNA in a compact manner. This organization is accomplished by packaging the DNA into large macromolecular complexes called *chromosomes.* A major distinguishing feature of eukaryotic organisms such as humans versus prokaryotic organisms such as bacteria is the presence of a nucleus, the main function of which is to contain the chromosomes.

Of the 46 chromosomes in the human nucleus, 44 are *autosomes;* the remaining 2 are the *sex chromosomes*—those involved in sex determination. In the human karyotype (an organized microphotographic array of all of the chromosomes), the autosomes can be seen to exist in homologous pairs, which are numbered from 1 to 22 in order of decreasing size, with one member of each pair inherited from one parent. The two sex chromosomes are designated X and Y. Normally, females have two X chromosomes, whereas males have one X and one Y. A small segment of the Y chromosome includes the gene for the testis-determining factor (TDF) responsible for male development. The lack of this factor results in female development, so it is the presence or absence of the Y chromosome that actually determines gender. The *TDF* gene encodes a sequence-specific DNA binding protein named SRY (for "sex-determining region Y").

Each chromosome consists of one large DNA molecule complexed with large amounts of two types of protein, called *histone* and *nonhistone chromosomal proteins,* which serve to condense the DNA into an orderly, compact structure. The five principal histone types—H1, H2a, H2b, H3, and H4—interact specifically with one another and with DNA to form structures called *nucleosomes.* Each nucleosome consists of a disk-shaped histone core plus a segment of DNA that winds around the core. The core contains two copies of H2a, H2b, H3, and H4, and the DNA wrapped around the histone core is approximately 140 base pairs in length. H1 binds to the DNA just next to the nucleosomes. The nonhistone proteins are much more diverse and complex; 12 to 18 major and many additional minor species have been identified. Nonhistone proteins play important roles, including as chromosomal structural elements and in the control of gene expression, for example, posttranslational modification of histones by acetylation and deacetylation. The complex of DNA and histones, called *chromatin,* forms coils to produce a fiber with a larger diameter. Histone-depleted metaphase chromosomes have been shown to consist of a nonhistone protein scaffold that has the shape characteristic of a metaphase chromosome surrounded by a halo of DNA. In this model of chromosome organization, the nucleoprotein fibers form radially oriented loops that converge to the central scaffolding. Most chromatin fibers

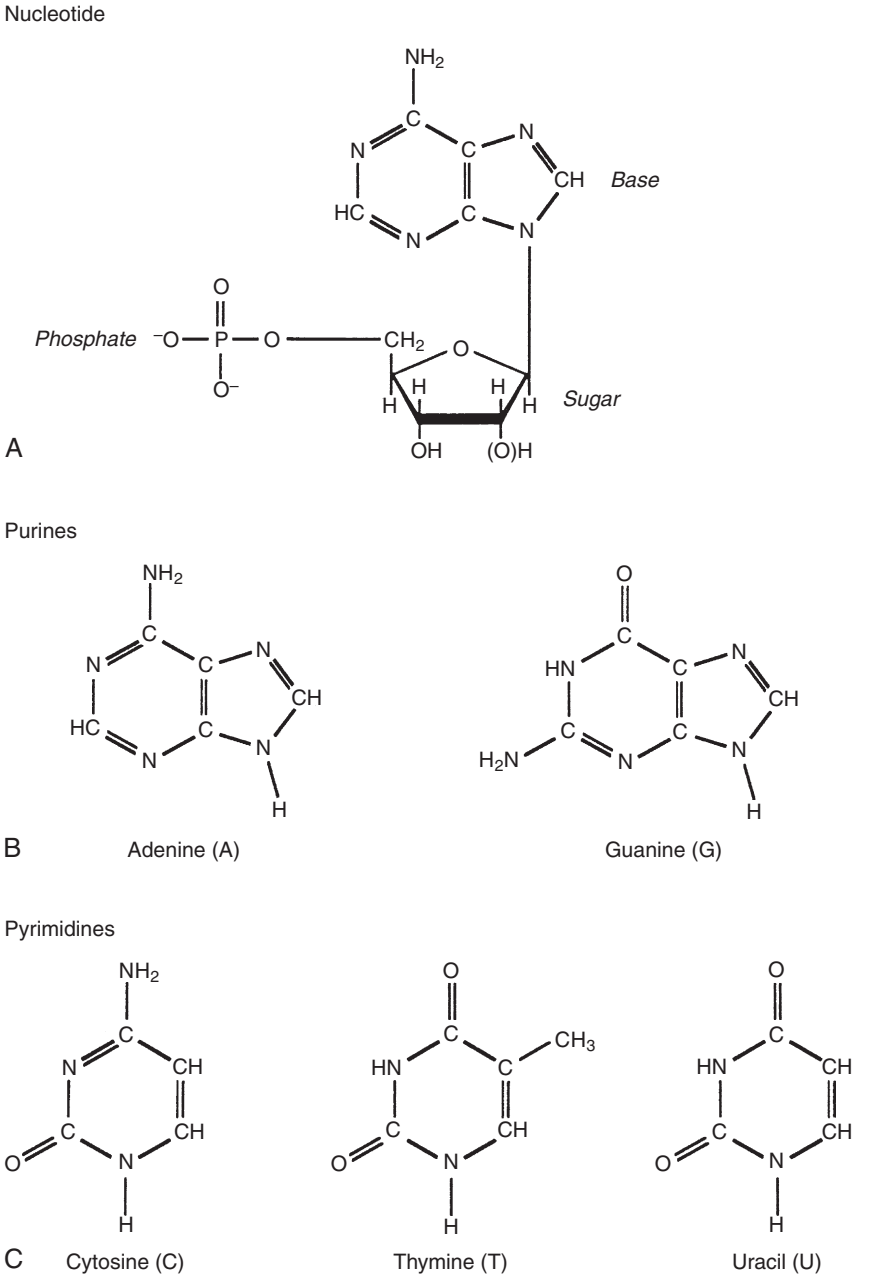


Figure 1-1 Nucleotide structure. A, General structure of nucleotides, consisting of a purine or pyrimidine base (in this case, adenine), a five-carbon atom sugar, and a phosphate group. The oxygen in *parentheses* in the sugar is present in the ribose of RNA but is absent in the deoxyribose of DNA. The plane of the sugar is perpendicular to that of the other subunits. **B,** Structures of the two purines in DNA and RNA. **C,** Structures of the three pyrimidines in nucleic acids. Cytosine is found in both DNA and RNA, thymine is unique to DNA, and uracil is unique to RNA.

undergo a transition between dispersed and condensed configurations during the cell cycle. Before cell division, most chromatin is in the condensed form, with limited but functionally important transcriptional activity. Between cell divisions, the bulk of the chromatin in most cells is dispersed within the nucleus for events such as DNA replication and gene transcription.

Gene Structure

All hereditary information is transmitted from parent to offspring through the inheritance of genes, which are defined as the DNA sequences necessary to produce a functional protein or RNA sequence. It is thought that approximately 25,000 genes are present on human chromosomes, although this number is the subject of controversy, even with the completion of the human genome sequence. A surprising finding on completion of the genome sequence was that the number of genes in human beings is not substantially greater than that in lower organisms. Thus, evidently it is not the number of genes that accounts for increased complexity, but rather the manner in which they are regulated.

Genes in eukaryotic cells are divided into regions called *expressed sequences (exons)* and *intervening sequences (introns),* of which only the exon sequences are present in mature messenger RNA (mRNA) and actually code for proteins (Figure 1-3, *top*). Although some introns play a role in the control of gene expression, the function of most is unknown, but they can greatly expand

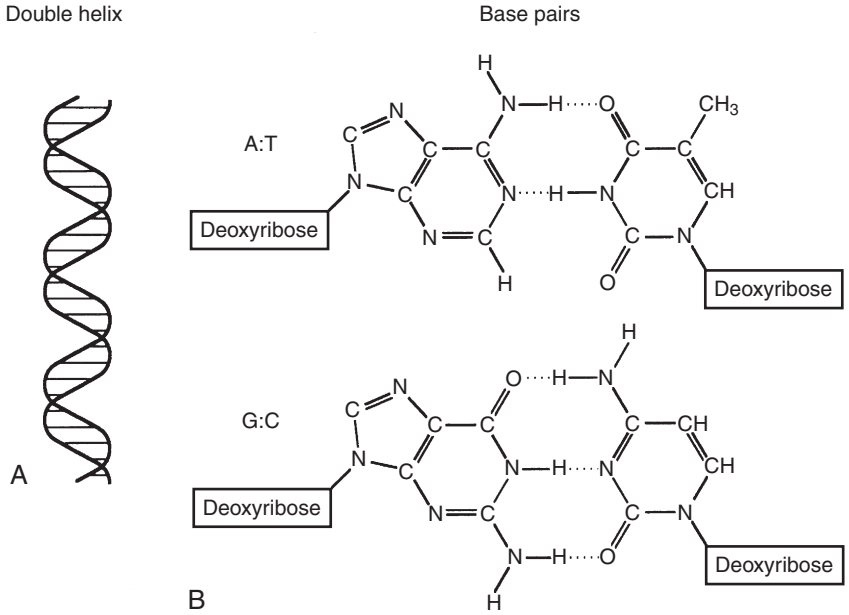


Figure 1-2 DNA structure and base pairing. A, Schematic representation of the double helical DNA molecule. **B,** Base pairing of purines and pyrimidines in DNA. Hydrogen bonding between the pairs is indicated by *dotted lines.* The AT and GC base pairs are identical in size and nearly identical in shape. Note that the GC base pair has an additional hydrogen bond and therefore is held together more strongly.

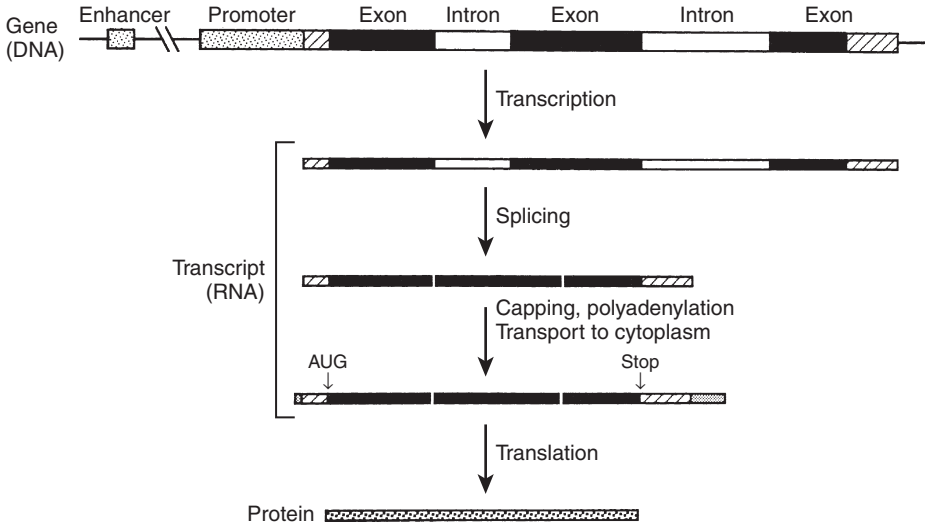


Figure 1-3 Gene structure *(top)* and the flow of genetic information from DNA to protein. *Cross-hatched boxes* indicate the regions of exons that do not encode amino acid sequences; *shaded boxes* indicate posttranscriptional modifications. AUG is a codon that specifies the amino acid methionine and is also used to specify the first amino acid of a protein.

the size of genes. For example, the dystrophin gene, involved in Duchenne muscular dystrophy, is approximately 2300 kilobase (kB) pairs of DNA in length and includes 79 exons accounting for only 14 kB, with the remainder consisting of introns, some more than 100 kB long. By contrast, the α-globin gene, 835 base pairs long, includes two introns that total 261 base pairs.

In addition to the exons and introns of genes, most of the eukaryotic genome consists of long DNA stretches that are not part of genes. In fact, it has been estimated that genes make up only 10% of the human genome, and the actual protein-coding regions account for only 1% of the genome. It is not understood why these long DNA stretches are present. Although some of them may play a role in DNA replication, chromosome pairing, and recombination, most of these stretches apparently serve no obvious function.

Interspersed throughout the noncoding DNA stretches are many repeated sequences, which are either clustered together or evenly distributed throughout the genome. These sequences can be short and consist only of 5 to 10 nucleotides, or they can be as long as 5000 to 6000 nucleotides. Whether these repeats have important functions or are mere players in an intragenomic evolutionary battle for self-preservation and expansion remains largely a mystery. Some of the longer repeats, such as the so-called Alu sequences or long interspersed nuclear elements (LINEs), have features similar to those of viruses and have been shown to be able to move from place to place in the genome. Other repeats are short, consisting of a stretch of two bases, such as CACACA. It has been known for some time that short repeats exhibit extreme variability from individual to individual. This variability

can be used to generate a type of fingerprint to uniquely identify any given individual. Such genetic "fingerprinting" is a powerful tool for gene mapping studies as well as in forensic applications such as paternity testing and criminal investigations. More recently, longer repeats and large regions of chromosomes have been found to exhibit copy number variation. This large-scale variation will almost certainly be found to contribute to genetically determined individual variation in human populations.

HOW GENES FUNCTION

Flow of Genetic Information

Transcription

Because DNA stores genetic information in the nucleus of eukaryotic cells, whereas protein synthesis occurs in the cytoplasm, a mechanism by which the information is carried to the cytoplasm is essential. The first step in gene expression is the production of an RNA molecule from the DNA template. This RNA acts as a molecular messenger, carrying the genetic information out of the nucleus. The synthesis of mRNA is called *transcription* because the genetic information in DNA is transcribed without being changed into a new language. During the process of transcription, the two DNA strands separate, and one functions as a template for the synthesis of single-stranded RNA molecules by the action of enzymes called *RNA polymerases.* The initial RNA transcripts are quite long because they include both intron and exon sequences from the gene (see Figure 1-3). The intron sequences are cut out by specific enzymes, and the remaining exons are spliced together. To form the mature mRNAs that leave the nucleus, a methylated guanine nucleotide called a *cap* is added to the beginning; a string of 200 to 250 adenine bases usually is added to the end (see Figure 1-3). The cap is important for ribosomal binding in the initiation of protein synthesis, whereas the polyadenosine stretch at the end of the mRNA plays a role in the stability of the mRNA.

In addition to mRNA, other major classes of RNA are transcribed from DNA: ribosomal RNA (rRNA), tRNA, and micro-RNAs (miRNAs). In contrast with mRNA, these classes of RNA do not code for proteins. Ribosomal RNAs and tRNAs are required for protein synthesis. The recently discovered miRNAs play complex and as yet poorly understood roles in posttranscriptional regulation of gene expression. A subclass of small RNA molecules, termed small interfering RNAs (siRNAs), have homology to specific mRNAs in the cell. Binding of a target mRNA by an siRNA results in its being degraded, with a consequent decrease in the production of the encoded protein. This phenomenon is widely used by researchers to shut down the expression of target genes. Ultimately, the hope is that this approach can be used therapeutically to target genes that may be involved in diseases such as cancer; as with other forms of gene therapy, however, the major problem remains efficient delivery to specific target cells in the body.

Unlike prokaryotic cells, in which a single RNA polymerase makes all types of RNA, eukaryotic cells have three different RNA polymerases that transcribe different classes of RNA: The precursors to 18S and 28S rRNA are made by RNA polymerase I; the precursors to mRNA are made by RNA polymerase II; and 5S rRNA and tRNA are synthesized by RNA polymerase III. RNA polymerase I functions in a specialized region within the nucleus called the *nucleolus.*

Translation and the Genetic Code

The production of protein from an mRNA template is called *translation* because the genetic information that is stored in DNA as a sequence of nucleotides is translated into a sequence of amino acids. The method of storing genetic information is called the *genetic code.* Each member of the code, called a codon, consists of three adjacent bases. Each codon specifies a particular amino acid. Thus the linear nature of the codons in a DNA sequence specifies the sequence of amino acids in a protein. Because each of the three sites in a codon can be one of four possible nucleotides, a total of 4^3, or 64, different codons can be generated. Three of these 64 possible codons, UAA, UAG, and UGA, are called nonsense or termination codons because they do not code for amino acids, but rather serve to mark the end of a protein, that is a stop signal. The remaining 61 codons specify one of the 20 amino acids. Because each amino acid is specify by more than codon, the genetic code is said to be degenerate. A consequence of degeneracy in the genetic code is that some mutations do not result in a change in the amino acid sequence.

The three types of rRNA—28S, 18S, and 5S—associate with more than 50 proteins to form the ribosomes, which are the cytoplasmic sites of translation. The tRNAs are small molecules approximately 80 nucleotides long whose function is to position the correct amino acid for incorporation into the polypeptide. Before an amino acid can be incorporated into a polypeptide chain, it is first coupled to an appropriate tRNA by an aminoacyl-tRNA synthetase, which is specific for each amino acid–tRNA combination. A three-nucleotide region of each tRNA, designated the *anticodon,* includes a base sequence complementary to the appropriate mRNA codon and therefore hybridizes to it. In this way, each amino acid is brought into proper position and is added sequentially to the growing polypeptide chain by peptidyl transferase, an enzyme that is an integral part of the ribosome. Initiation of transcription almost always occurs at an AUG codon, which codes for methionine (see Figure 1-3). In many proteins, the initiation methionine is removed posttranslationally. Translation is terminated when a ribosome encounters a nonsense codon (see Figure 1-3). In the presence of the appropriate factors, the polypeptide chain is released from the last tRNA, and the ribosome disengages from the mRNA to start the cycle of protein synthesis over again.

Regulation of Gene Expression

The ability to control the production of proteins is central to the development and functioning of every organism. Although this control occurs at every stage of protein production, the most important level of control occurs at the level of mRNA production (i.e., transcriptional control). Transcriptional regulation is accomplished via the action of proteins that act on DNA, either by modifying it (e.g., cytosine methylation) or by binding to specific DNA sequences to activate or repress transcription from a gene.

In higher eukaryotes such as humans, two major types of DNA sequences regulate gene expression: promoters and enhancers. *Promoters* are located immediately adjacent to the start site of transcription, whereas enhancers can be located at large distances from the transcribed regions of the gene (see Figure 1-3). Several types of regulatory sequences have been identified in promoters that are important in transcriptional initiation by RNA polymerase II, including the *TATA box,* so called because it consists of a run of T and A base pairs. The TATA box is located approximately 30 bases before the transcription start site and functions as the binding site for a large, multisubunit complex of transcription factors, which includes RNA polymerase. Other sequence elements that are common to many genes transcribed by RNA polymerase II include the CAAT sequence, or *CAT box,* which lies between 50 and 100 bases before the transcription start site, and the GGGCGG sequence, or *GC box.* As with the TATA box, the function of these sequence elements is to serve as binding sites for proteins that are involved in transcriptional control.

Enhancer sequences serve to increase the level of transcription from promoters and have been shown to play pivotal roles in gene regulation, particularly for highly expressed genes such as viral genes and the globins. Many mammalian enhancers are tissue specific in their action. For example, enhancers from the pancreatic amylase, chymotrypsin, and trypsin genes function only in exocrine pancreas, and enhancers from immunoglobulin genes function only in lymphoid cells. As noted, in contrast with promoters, which have fixed positions relative to the transcription start site, enhancers exist in widely varying positions,

including before the promoter, after the coding region, within introns, and at great distances from the coding sequence, as in the case of the globin enhancer. Another property of enhancers that distinguishes them from promoters is their ability to function in a position-independent and orientation-independent manner relative to the gene. In addition to enhancer elements, which increase the levels of transcription, similar elements called *silencers* decrease the level of transcription. Combinations of these elements allow for precise control of the level of gene transcription.

Transcription is regulated by interactions among proteins bound to enhancer and promoter sequences. Such proteins can have either stimulatory or inhibitory functions. For example, the receptors for steroid hormones such as the glucocorticoids, estrogen, and androgens have been isolated and shown to bind to specific sequences near steroid-responsive genes such as vitellogenin and lactalbumin. In addition to the classic transcription factors that bind to specific sequence elements in genes, other proteins play important roles in the control of gene expression. For example, methylases and demethylases regulate the methylation of cytosine residues to affect gene expression. Mutations in MeCP2, a protein that binds to methylated DNA, cause Rett syndrome, an X-linked neurodegenerative disease. Rubinstein-Taybi syndrome is caused by mutations in a protein called CREB-binding protein (CBP), which acts to acetylate the histone proteins that are major components of chromatin, illustrating the fact that posttranslational modifications of chromatin proteins play central roles in regulating gene expression. Overall, it is the complex interplay of positive and negative effects between factors bound to different sites, both near and far from the coding region of a gene, that determines the pattern of gene expression.

In addition to transcriptional control, posttranscriptional mechanisms play important roles in controlling the level of gene product. Regulation takes place at virtually every level, including alternative splicing, transport of RNA from the nucleus to the cytoplasm, persistence of mRNA in the cytoplasm, translational efficiency, and regulation of the rate of protein degradation. Individual mRNA species differ widely with respect to metabolic stability. The half-lives of some mRNAs span several hours or even days, whereas those of others are extremely short. The rate of turnover of some mRNAs can vary dramatically in response to changes in the cell cycle and in response to treatment with certain hormones. Protein binding and mRNA structural features also have been shown to influence susceptibility to decay. The significance of posttranscriptional influences on protein levels has given rise to the field of *proteomics,* in which techniques for measuring protein levels on a global and high-throughput scale are used to define the *proteome* (i.e., the total complement of proteins) within a cell.

Stem Cells and Development

Development, in general terms, is the process by which a single fertilized egg becomes a complete organism. Central to development is the process of *differentiation,* whereby cells acquire different properties to carry out specific functions in separate tissues. To a large extent, differentiation is reflected in the production of tissue-specific proteins that, of course, is the result of specific gene expression. A fundamental question in development is how a group of genetically identical cells comes to express sets of genes in a tissue-specific manner. Much progress in this area has come from the striking similarities between the process of development in organisms such as *Drosophila* and *Caenorhabditis elegans* and in humans. Important principles of development, such as the role of morphogen gradients, were first described in lower organisms but have proved to be relevant in humans as well. A number of transcription factors are recognized to form concentration gradients that are critical in determining the pattern of body formation.

In addition to knowledge gained from lower organisms, a revolution in understanding of human development and human genetic disease has come from advances in the ability to manipulate mice genetically. Originally, this was done by introducing genetic material into mice by microinjection of fertilized eggs. The injected eggs give rise to mature animals that integrate the injected genes into their genome; such transgenic animals will pass the introduced genetic material through the germline to their offspring. In many cases, the introduced genes are expressed, allowing the effect of overexpression or ectopic expression of a gene product to be studied.

Inactivation of specific genes in mammals can be done by genetically manipulating embryonic stem (ES) cells derived from the blastocyst (Figure 1-4). A *stem cell,* by definition, is any cell that can both self-renew (i.e., replicate) and also give rise to more differentiated progeny. ES cells usually are derived from blastocysts, although ES cell lines have been derived even from eight-cell embryos. They can be grown in culture while retaining the ability to differentiate into all somatic tissues and the germline of mature mice. Thus a single cell grown in culture can be used to create a living mouse. Because ES cells can be grown in culture and genetically manipulated, mutations can be created in DNA using recombinant techniques, the resulting DNA introduced into ES cells where it is incorporated into DNA by homologous recombination (Figure 1-5), and the resulting ES cells carrying a specific mutation used to create mutant mice. To do that, the genetically manipulated ES cells are injected into blastocysts, giving rise to chimeric adult mice (Figure 1-4). Some of these mice contain the genetically manipulated cells in their germline and will produce genetically altered, nonchimeric offspring.

In many instances, gene mutations result in nonviable organisms; such genes are said to possess embryonic lethality. This characteristic is useful in genetic studies. For example, a common investigational technique is to knock out genes in a particular

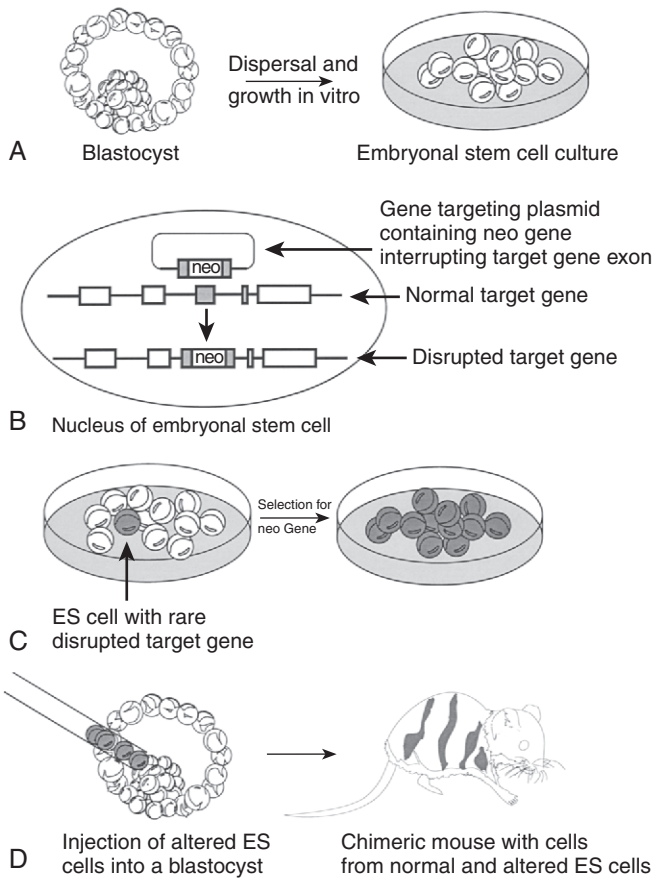


Figure 1-4 Genetic alteration of mice by homologous recombination.

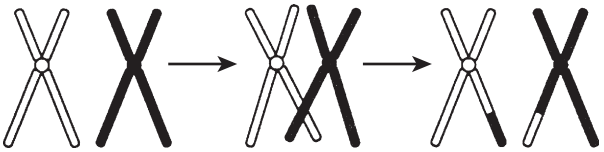


Figure 1-5 Recombination. In this simplified view of recombination, the two members of a homologous pair of chromosomes line up during the first meiotic prophase. Segments of the two chromosomes "cross over," and breakage and rejoining of the DNA strands occur.

tissue through modification of the target gene by flanking it with 34–base pair DNA sequences called *lox sites* that are the substrates for a recombinase enzyme derived from the bacteriophage P1 Cre recombinase. In mice, when Cre (for "cyclization recombination") is expressed from a tissue-specific promoter element, the affected mouse will demonstrate deletion of the target gene only in cells expressing the tissue-specific promoter.

CELL DIVISION AND RECOMBINATION

Cell Cycle and Mitosis

The *cell cycle* is the process by which the cell divides to form two daughter cells. In *mitotic division,* the normal complement of 46 chromosomes is maintained through a process of DNA replication and subsequent separation of the chromosomes. Additionally, the cytoplasm of the eukaryotic cell cleaves into two approximately equal halves. Mitotic division takes up only a small part of the cell cycle. A complete cell cycle consists of four phases: G_1 (growth or gap 1), S (synthesis), G_2, and M (mitosis). The cycle of each type of cell varies considerably in total duration, from minutes in some cells to weeks or months in others. The G_1 phase begins immediately after a cell division. It is followed by the S phase, during which DNA replication occurs, as described earlier. Immediately after the S phase, the cell has a tetraploid chromosome content, instead of the usual diploid number, so there are 92 chromosomes divided into 46 pairs of sister chromatids. Cells then pass into the premitotic G_2 phase, which ends with the onset of mitosis or actual cell division. The G_1, S, and G_2 phases are called *interphase* because despite continued growth and synthesis of macromolecules, such as DNA, RNA, and proteins, cell division takes place only during mitosis. DNA and the histone components of the chromatin are synthesized only during the S phase, whereas RNA, the cytoplasmic proteins, and organelles are synthesized continuously during all of interphase. The cell finally divides in the M phase, during which the synthesis of RNA and protein is greatly reduced.

During interphase, chromosomes are not visible by light microscopy because chromatin is dispersed throughout the nucleoplasm. The beginning of mitosis is signaled by the appearance of chromosomes as thin threads inside the nucleus. Mitosis is divided somewhat arbitrarily into four phases: prophase, metaphase, anaphase, and telophase. During *prophase,* the nuclear envelope begins to break up, and each chromosome can be seen to consist of two identical or sister chromatids held together at specific regions called *centromeres.* Another structure that is important for proper segregation of chromosomes is the centriole, an organelle just outside the nuclear membrane. Each cell normally has a pair of centrioles, arranged opposite one other, but they are duplicated early in the S phase. During mitotic prophase, the two pairs of centrioles separate and migrate to define the poles of the cell. During *metaphase,* the chromosomes move to the equatorial plane of the cell and become attached to the spindle fiber apparatus, which is a structure consisting of microtubules of protein that radiate from the centrioles at either pole and extend from pole to pole. Each chromatid has near its centromere a dense granule called a *kinetochore* to which the spindle fibers attach. Because of the kinetochores and attached spindle fibers, the two sister chromatids are pulled toward opposite poles. During *anaphase,* the centromeres divide, and the two chromatids of each pair, now free of each other, move toward their respective poles by the contraction of spindle fibers. In *telophase,* the chromosomes and spindle fibers disperse and disappear, and new nuclear envelopes are assembled to surround the two sets of daughter chromosomes. Simultaneously, separation and segregation of the cell cytoplasm occur, a process called *cytokinesis,* which results in the formation of a complete membrane around the cell and constitutes the end of the process of forming a new cell.

Progress through the cell is controlled by a complex set of phosphorylation and dephosphorylation steps, mediated by many interacting kinases and phosphatases. Proteins called *cyclins,* so called because their expression is limited to specific stages of the cell cycle, control the initiation of the kinase-phosphatase cascade. That cascade, in turn, ultimately controls the ability of the cell to progress through the major cell cycle control points called *checkpoints,* at the G_1-S and G_2-M boundaries. Many of the proteins involved in cell cycle control are, not surprisingly, involved in the loss of cell cycle control that is the hallmark of carcinogenesis and so can be classified as oncogenes or tumor suppressor genes, depending whether their normal role is to promote or inhibit proliferation. In addition to the proteins that are directly involved in cell cycle control, proteins that are important in DNA repair also have been found to be important in carcinogenesis. Most prominently, the *TP53* tumor suppressor gene, which seems to be the central monitor of genomic damage, is also the most commonly mutated gene in human cancer, and it has a role in a high percentage of all human cancers.

Meiosis

Meiosis is the process by which germline cells form gametes. In contrast with mitosis, in which a single cell division and an exact duplication of the genetic material in the parent cell occur, meiosis involves two separate cell divisions from a diploid parent cell and a random reassortment and reduction of genetic material so that each of the four daughter cells has a haploid DNA content (i.e., 23 chromosomes). In this way, meiosis yields four haploid gametes, the sperm and the egg cells, which support sexual reproduction and a new generation of diploid organisms.

The first meiotic division, as with mitosis, is separated into four stages: prophase, metaphase, anaphase, and telophase. Before meiosis begins, the chromosomes in the cell are replicated to produce two pairs of sister chromatids, and each pair of sister chromatids remains together throughout the first meiotic division. In metaphase, the spindle fibers attach to chromosomes, and the paired chromosomes align themselves in the equatorial plane of the cell. In anaphase, the paired homologous chromosomes separate and move toward their respective poles. The daughter chromatids, however, remain attached to their centromeres. In telophase, the chromosomes arrive at the poles. One of the sets of chromosomes forms the first polar body, which eventually is lost. Because the number of chromosomes in each daughter cell is reduced by half, the first mitotic division is called the *reduction division.* This polar body can be removed from the unfertilized egg and the purified DNA amplified by polymerase chain reaction (PCR) techniques (described further on) and used for preimplantation genetic diagnosis.

The second meiotic division is completed after fertilization and occurs without DNA replication. A second polar body, containing a complete set of chromosomes, is extruded, leaving the egg with a single remaining set (i.e., it is haploid). The second polar body is also useful for preimplantation genetic diagnosis, particularly when recombination has occurred during the first meiotic division.

Recombination

During prophase of the first meiotic division, homologous pairs of chromosomes are held together by a protein-containing framework called a *synaptonemal complex,* which extends along the entire length of the paired chromosomes. Recombination between chromatids of the homologous chromosomes occurs at this stage, resultin in the exchange of DNA between the original parental chromosomes (see Figure 1-5). In males, the X and Y chromosomes are associated only at the tips of their short arms during meiotic prophase. This short associated region is called the *pseudoautosomal region* because recombination between the X and Y chromosomes occurs there (so it behaves as an autosome in terms of mendelian inheritance). This region probably plays an important role in sex chromosome pairing and segregation, as well as in male fertility. Recombination, in conjunction with mutation (see later), is important for generating genetic diversity through the exchange of DNA between different chromosomes, and it plays a critical role in gene mapping studies.

MUTATION AND GENETIC HETEROGENEITY

Mutation is defined broadly as any change in the sequence of DNA. Because most of the human genome does not consist of genes, most mutations are of no apparent functional consequence and thus are termed *silent.* Only mutations that affect the expression or function of a gene or its product are phenotypically apparent. Of these, many are not clinically relevant but instead contribute to normal population heterogeneity. Variations in hair and eye color, for example, originally arose through mutation. Thus the term *mutation* may be defined differently at the molecular genetic, biologic, and clinical levels. Along with recombination, mutation is a central element in producing the population diversity that is the substrate for evolution. The mutation rate in humans has been measured at 2.5×10^{-8} mutation per nucleotide site per generation. This translates to 175 new mutations in every person. The mutation rate and the types of mutations that occur, however, can vary dramatically among different loci. The consequence of this rate of mutation is that variations in the human genome occur, on average, approximately once per 1000 base pairs. Hence, each genome differs from others at millions of sites. Although mutations occur in both germline and somatic (nongermline) cells, only mutations affecting the germline are inherited. Somatic cell mutations also are of major medical importance, particularly in the development of cancer.

Single-Gene Mutations

Mutations can range from those affecting only a single base pair to major alterations in chromosome structure. Mutations that affect only one nucleotide are called *point mutations* and involve the substitution of one nucleotide for another (Figure 1-6, *A*). Accordingly, such mutations also are referred to as single-nucleotide polymorphisms (SNPs). When a point mutation occurs in a part of the gene that codes for a protein and alters the protein by changing the codon of which it is a part, it is called a *missense mutation.* Because the genetic code is degenerate, it is possible to have a point mutation that does not change the amino acid that is encoded. This is called a *silent mutation.* Insertion or deletion of a nucleotide in the protein-coding portion of a gene is called a *frameshift mutation* because it changes the entire reading frame of the gene at every codon distal to the site of the mutation. *Nonsense mutations* are those point mutations that result in the formation of one of the three codons (UAA, UAG, UGA) that do not code for an amino acid and so produce truncated proteins, which usually have little or no activity. Point mutations occurring near the boundaries between introns and exons

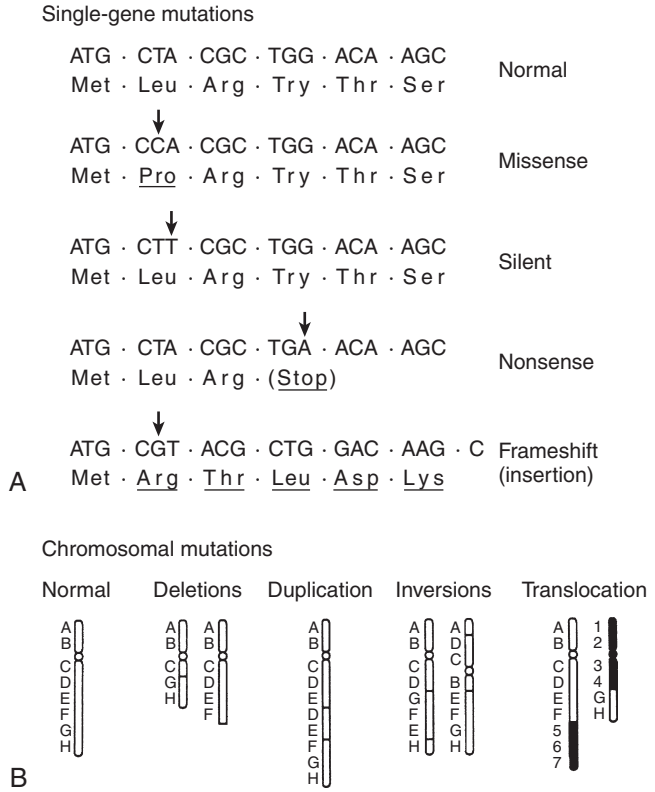


Figure 1-6 Mutation. A, Single-gene mutations. A prototypical normal gene sequence is shown on the first line, with the corresponding amino acid sequence. Examples of four types of common mutations also are shown. The substituted or inserted nucleotides are indicated by *arrows,* and the affected amino acids are *underlined.* **B,** Chromosomal mutations. A prototype normal chromosome is shown, with genes A through H. Examples of gross chromosomal mutations are shown to the *right,* and their effects on gene content and arrangement are indicated. In the translocation example, the two chromosomes are not members of a homologous pair.

can cause improper splicing of mRNA precursors, resulting in RNA instability or the production of truncated proteins, or both.

Regulation of gene expression also can be affected by mutations occurring in control elements such as promoters and enhancers. Although the effect of such mutations usually isthe production of less protein, such as occurs in some forms of thalassemia, some mutations also result in the increased production of a gene product, as in hereditary persistence of fetal hemoglobin.

An interesting mutational mechanism involves the expansion of triplet repeat sequences. More than 10 disorders have been found to be caused by an increase in the number of copies of CCG or AGC repeats in or near a gene, including myotonic dystrophy, fragile X syndrome, and Huntington disease. In these disorders, the repeat number tends to increase with succeeding generations. As the repeat number increases, so does the severity of the disease, giving rise to the phenomenon of *anticipation.* First described in myotonic dystrophy, anticipation refers to an increase in disease severity within succeeding generations of an affected family.

Chromosomal Mutations

Mutations involving large alterations in chromosome structure are visible microscopically by karyotypic analysis (see Figure 1-6, *B*). These include deletions, duplications, inversions, and

translocations from one chromosome to another. Because chromosomal aberrations usually result in the disruption of multiple genes, they often have profound clinical consequences. *Terminal chromosomal deletions* result from a single chromosomal break with subsequent loss of the piece of chromosome without a centromere. *Duplications* occur when a segment of a chromosome is repeated, either from inappropriate recombination or as a result of meiosis involving chromosomes with inversions or translocations. Most other chromosomal rearrangements, such as interstitial deletions, require multiple break-and-reunion events, so they usually are less common. Some common genetic diseases, however, such as Duchenne muscular dystrophy, result primarily from small interstitial deletions—demonstrating that chromosomal regions vary greatly in their propensity to undergo different types of mutational events.

Inversions result from two chromosome breaks followed by reversal of the broken piece of chromosome and subsequent rejoining to form an intact but rearranged chromosome. Although the inversion in and of itself does not have any clinical consequences unless one of the breakpoints affects gene expression, significant effects are seen in subsequent generations. When chromosomes with inversions (either pericentric, in which the inverted region includes the centromere, or paracentric, in which the centromere is not involved) go through meiosis and recombination with normal homologues, gametes may be formed that contain duplications and deletions of parts of the involved chromosomes. *Translocations* result from the exchange of genetic material between two nonhomologous chromosomes. Similar to inversions, they do not cause any clinical disease unless the breakpoints occur in a gene. Persons in whom a translocation is present but who have a normal amount of genetic material are called *balanced translocation carriers.* Like inversions, however, translocations can have severe effects in offspring, resulting from the consequent duplication or deficiency syndromes (or both).

GENETIC DISORDERS

Approximately 4500 inherited disorders have been described thus far. More than half of these have been genetically mapped, and the molecular defect is being identified in a rapidly increasing number. Genetic disorders can be classified into three general categories: simple mendelian, chromosomal, and multifactorial. This section discusses these categories of disease as well as factors that contribute to disease heterogeneity.

Mendelian Disorders

Mendelian disorders are caused by mutation of a single gene, so they display the simple patterns of inheritance dictated by the mendelian laws. Mendelian disorders can be classified as being of either autosomal dominant (AD), autosomal recessive (AR), or X-linked inheritance. X-linked disorders result from mutations on the X chromosome; by contrast, AD and AR disorders are the result of mutations on the autosomes. The demonstration that a particular disease or syndrome exhibits simple mendelian inheritance implies that the pathogenesis, no matter how seemingly complex it may be, is an alteration at a single genetic locus. In many cases, a single mutation can lead to numerous, seemingly unrelated defects, a phenomenon known as *pleiotropy.* It has been estimated that approximately 1% of people in the general population have a monogenic disorder. The simple pattern of inheritance has made it relatively straightforward to isolate genes for monogenic disorders. Virtually all common monogenic disorders and an increasingly large number of quite rare disorders have had the responsible genes isolated and studied.

Autosomal Dominant Disorders

AD disorders are those in which a patient manifests clinical symptoms when only a single copy of the mutant gene is present (i.e., the patient is heterozygous for the mutation). Inheritance of AD disorders follows several general principles (Figure 1-7, *A*):

- Each affected person has an affected parent.
- Affected persons, on average, have equal numbers of affected and unaffected children.
- Normal children of affected parents have only unaffected children.
- Males and females are affected in equal proportions.
- Each sex is equally likely to transmit the disorder to male and female children.
- Vertical transmission of the disorder occurs through successive generations.

These general rules of AD inheritance are based on the assumption, not always valid, that no new mutations occur. In fact, in some disorders the incidence of new mutations is quite high. For example, up to 50% of the cases of von Recklinghausen neurofibromatosis are estimated to result from new mutations.

Dominant mutations involve types of gene products in which an abnormality in 50% of the protein leads to disease. Although mutation of genes with many different functions results in AD inheritance, three classes of proteins frequently are involved: (1) proteins that regulate complex metabolic pathways, such as membrane receptors and rate-limiting enzymes in pathways under feedback control; (2) structural proteins; and (3) proteins with alterations that cause a dominant negative function, that is, in which the mutant protein interferes with the function of the protein expressed from the normal allele. Examples of AD disorders are familial hypercholesterolemia, which is caused by mutations in the low-density lipoprotein receptor; osteogenesis imperfecta, caused by mutations in some members of the collagen gene family; and Huntington disease, caused by a triplet repeat expansion in the Huntington gene.

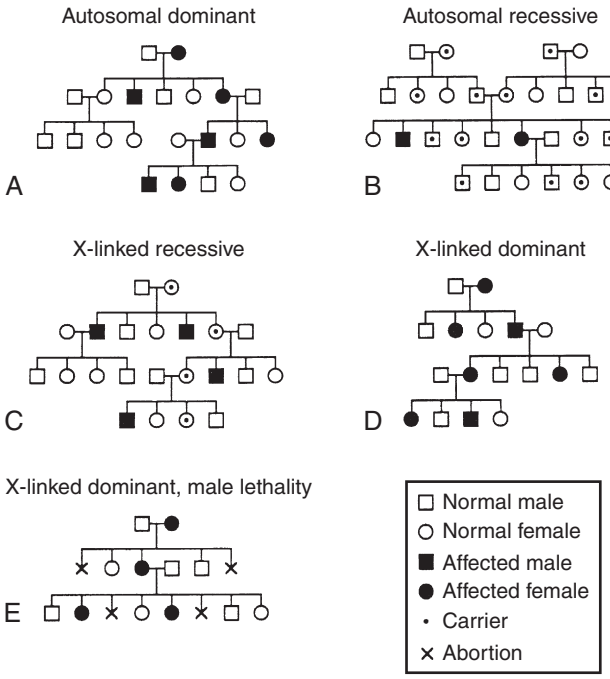


Figure 1-7 A-E, Pedigrees for disorders exhibiting the various mendelian modes of inheritance. These are idealized pedigrees, assuming full penetrance and no new mutations.

A characteristic of many AD disorders is *incomplete penetrance,* whereby not all persons carrying the relevant gene(s) exhibit a specific trait. A particular gene defect can therefore manifest with widely variable severity. For example, tuberous sclerosis, one of the neurocutaneous disorders, can be clinically silent. Some persons are diagnosed with this disorder only when they have multiple severely affected children. At that point, careful examination may reveal subtle evidence of tuberous sclerosis, such as a minor abnormality on a computed tomography scan of the head. Similar observations have been made for many different dominant diseases. Incomplete penetrance is a manifestation of the interaction of other gene products with the product of the disease gene. Increasingly, this phenomenon is being recognized as a step in the continuum between simple completely penetrant monogenic disorders and so-called complex disorders in which no single gene mutation is sufficient to cause disease.

The phenomenon of *germline mosaicism* is a complicating factor in incomplete penetrance. Germline mosaicism occurs when a mutation is present in some of the germ cells but not in most other cells. The affected person is completely healthy but is at risk for having multiple affected children. Germline mosaicism is fairly common in Duchenne muscular dystrophy and occurs in other disorders as well. It can sometimes be difficult to distinguish between germline mosaicism and incomplete penetrance, thereby making accurate genetic counseling and prenatal diagnosis difficult unless a direct assay for the presence of a mutated gene is available.

Autosomal Recessive Disorders

AR disorders are those that are clinically apparent only when the patient is homozygous for the disease (i.e., both copies of the gene are mutant). The following general principles of inheritance are recognized for AR disorders (see Figure 1-7, *B*):

- The parents of affected children may be clinically normal (i.e., carriers).
- Assuming that the carrier frequency in the population is low, only siblings are affected, and vertical transmission does not occur; the pattern therefore tends to appear horizontal.
- Males and females are affected in equal proportions.
- When both parents are heterozygous carriers of the mutation, 25% of their children are affected, 50% are carriers, and 25% are normal.

Every person is a carrier of certain AR mutations. Fortunately, the carrier frequency for most of these mutations is so low that the likelihood that carriers will have affected children is low.

Recessive mutations frequently involve enzymes, as opposed to regulatory and structural proteins. This is because 50% of the normal level of enzyme activity usually is sufficient for normal function. Complete enzyme deficiency produces an accumulation of one or more metabolites preceding the enzymatic block, such as the buildup of phenylalanine in phenylketonuria, and a deficiency of metabolites distal to the block. Either, or both, of these abnormalities may be responsible for the disease phenotype. Although many recessive disorders involve enzymes, two of the most common disorders with AR inheritance are cystic fibrosis, resulting from a mutation in a chloride channel, and sickle cell anemia, resulting from a mutation in the β-globin gene.

The terms *dominant* and *recessive* refer to phenotypes only and have their greatest application at the clinical level. At the gene level, "dominance" and "recessiveness" do not exist. Persons heterozygous for a recessive disorder may be clinically normal, but the reduced level of functional or immunoreactive protein usually is detectable analytically and may lead to other biochemical abnormalities that have no obvious effect on the person's health. In addition, patients homozygous for dominant mutations usually are more severely affected than are heterozygous patients, as is true in familial hypercholesterolemia. In many cases, the homozygous condition results in embryonic lethality, so the clinical disorder is never seen. Huntington disease stands out as a major exception in that homozygous patients are not clinically different from heterozygous patients.

X-Linked Disorders

With X-linked disorders, the mutation occurs on the X chromosome, of which females have two copies and males have only one. Thus, for these disorders, clinical risk and disease severity will differ for males and for females.

The following rules of inheritance apply for X-linked recessive disorders (see Figure 1-7, *C*):

- In contrast with the vertical pattern of inheritance seen on pedigrees for AD disorders and the horizontal pattern for AR disorders, the inheritance pattern tends to be oblique, because patients have unaffected parents but affected cousins and uncles.
- Male-to-male transmission of the disorder does not occur, because fathers transmit X chromosomes only to daughters.
- Male children of carrier women have a 50% chance of being affected.
- All female children of affected men are heterozygous carriers.
- Unaffected men do not transmit the disease to any children.

Examples of X-linked recessive disorders are hemophilia, color blindness, and Lesch-Nyhan syndrome (hypoxanthine-guanine phosphoribosyltransferase [HPRT] deficiency).

Although X-linked recessive disorders generally are observed only in male patients, X-linked dominant disorders are approximately twice as frequent in females as in males and are characterized by transmission of the disorder from affected men to all daughters but to no sons (see Figure 1-7, *D*). Relatively few X-linked dominant disorders have been described, but one example is hypophosphatemia (vitamin D–resistant rickets), in which males and females are equally affected even though females carry a normal as well as an abnormal gene. Several X-linked dominant disorders demonstrate embryonic lethality in hemizygous males (and presumably in homozygous females). In these disorders, affected mothers transmit the trait to half of their daughters but to no sons (see Figure 1-7, *E*). A high incidence of spontaneous abortions has been recognized, and the male-to-female ratio in children is significantly less than 1. Disorders that appear to have this mode of inheritance include focal dermal hypoplasia, incontinentia pigmenti, and orofaciodigital syndrome type I. Aicardi syndrome is a neurodegenerative disorder consisting of agenesis of the corpus callosum with chorioretinal abnormalities that has been described only in female patients, suggesting X-linked dominant inheritance. Because of the severity of the phenotype and low reproductive fitness of affected persons, however, it is difficult to define the pattern of inheritance. Presumably, virtually all cases result from new mutations.

An important feature of X-linked disorders is the wide range of clinical expression in heterozygous females. In contrast with the incomplete penetrance of AD disorders, which probably results from interactions among different genes, variability in X-linked disorders is significantly affected by the process of X-inactivation, or lyonization (after its discoverer, Mary Lyon). Because females carry two copies of the X chromosome and males carry only one, a mechanism called *dosage compensation* has evolved to equalize the amount of gene product that is produced from genes on the X chromosome. It is possible to envision many ways in which this could be accomplished. For example, X-linked genes in females could be transcribed at half the rate of those genes in males, as occurs in insects. The mechanism that operates in humans, however, involves the random inactivation of one of the X chromosomes in every cell of the body. Therefore, only one X chromosome is active in each cell. This observation has major implications for X-linked diseases. Because the process of

inactivation is random, on average, half of the cells inactivate the X chromosome carrying the normal gene and half inactivate the abnormal X chromosome. Unfortunately, in some cases, significant deviation from an equal ratio may be seen. Female patients in whom X chromosome inactivation has occurred in a high percentage of the body's normal cells may demonstrate significant symptoms. This may be one reason for the significant percentage of female carriers of the fragile X syndrome who exhibit some degree of mental retardation.

It also is important to distinguish between actual X-linked disorders and sex-influenced disorders. The latter category consists of disorders encoded by autosomal genes that are differentially expressed in the two sexes. Male pattern baldness, for example, is the result of an AD mutation that requires high testosterone levels to become manifest.

Chromosomal Disorders

Chromosomal disorders fall into two general categories: those involving incorrect chromosome number, called *aneuploidy,* and those that result from large chromosomal mutations, as described earlier. Aneuploidy is the result of nondisjunction during meiosis, in which both members of a homologous pair of chromosomes move to the same daughter cell. As a result of nondisjunction, the fertilized egg receives either one or three copies of the chromosome instead of the usual two. Because they involve numerous genes, with disturbance in the normal genomic balance, most disorders affecting chromosome number are lethal in the embryonic or fetal period, particularly if the defect is loss of a chromosome. Disorders that are not lethal usually result in sterility, because they prevent meiosis from proceeding normally. The best known and most common chromosomal disorder is Down syndrome, which generally results from trisomy of chromosome 21 but also can be caused by a duplication or translocation of a specific region of chromosome 21. Trisomies of chromosome 13 or 18 also occur but are much less common than Down syndrome. Turner syndrome occurs in women who receive only a single X chromosome, whereas Klinefelter syndrome occurs in men who receive two X chromosomes in addition to the Y chromosome.

Deletions that are too small to be visible using standard cytogenetic techniques (but that still result in the deletion of multiple genes) are called *microdeletions,* and the resulting disorder is termed a microdeletion syndrome or *contiguous gene syndrome.* Such deletions sometimes can be detected using advanced cytogenetic techniques, such as prometaphase banding, in which cells are arrested in mitosis at an earlier stage, so that instead of the usual 300 to 400 bands (each containing about 1 megabase of DNA), more than 1000 bands can be visualized. With the availability of cloned genetic markers covering much of the genome, the technique of fluorescence in situ hybridization (FISH) can be used to detect small deletions. In the FISH technique, a cloned DNA probe is labeled with a fluorescent molecule and is then hybridized to a standard chromosome preparation on a microscope slide. The presence of two normal chromosomes can be visualized by the appearance of two fluorescent dots, whereas a heterozygous microdeletion appears as a single dot. Examples of microdeletion syndromes are the DiGeorge syndrome, characterized by T cell immunodeficiency and cardiac anomalies and caused by a microdeletion of chromosome 22, and Prader-Willi syndrome, characterized by mental retardation, infantile hypotonia, and a compulsive eating disorder and frequently caused by a microdeletion of chromosome 15. A clinically unrelated disorder, Angelman syndrome, characterized by severe mental retardation, seizures, and a movement disorder, also can be caused by a microdeletion in the same region of chromosome 15 as that affected in Prader-Willi syndrome. In Prader-Willi syndrome, the deletion is always on the chromosome inherited from the father, whereas in Angelman syndrome, the deletion is always on the maternally inherited chromosome. Both Prader-Willi and Angelman syndromes can arise from *uniparental disomy,* which means that both chromosomal homologues are derived from one parent, with no contribution from the other. For example, in approximately 15% of patients with Prader-Willi syndrome, both copies of chromosome 15 are maternally derived, whereas in Angelman syndrome, both copies can be from the father.

Parent-of-origin effects on the occurrence of a genetic disease are a reflection of the phenomenon of *imprinting.* Imprinting refers to a process of transcriptional inactivation of a region of a chromosome derived from only one parent. The mechanism of this transcriptional inactivation involves methylation of cytosine residues during development. The reason for the existence of imprinting is not known, but it is clear that proper imprinting is necessary for normal development.

Mitochondrial Disorders

Mitochondria, cytoplasmic organelles whose major function is to serve as the sites of oxidative phosphorylation and energy production for the cell, also contain their own genetic material in the form of a small circular piece of DNA. The structure of the mitochondrial genome is similar in this way to that of bacterial genomes—a finding contributing to the theory that mitochondria originally developed from bacteria that established a symbiotic relationship within eukaryotic cells.

Many different clinical entities are now recognized to be caused by mutations in mitochondrial DNA. Mitochondrial diseases frequently affect organs that are highly dependent on energy production and use, such as the central nervous system, muscle, and also pancreatic beta cells. Examples of mitochondrial diseases are myoclonic epilepsy with ragged red fibers (MERRF) syndrome; mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) syndrome; and Kearns-Sayre syndrome, which has heart block and retinal disease as prominent components. Many of the mutations in the mitochondrial DNA have been defined, with both point mutations and deletions described. The inheritance of mitochondrial DNA is unique because only maternal mitochondria are transmitted to the zygote. Therefore, although males and females can be equally affected by disorders caused by defects in mitochondrial DNA, the mutation can be passed only through the maternal lineage. This is called *maternal* or *cytoplasmic inheritance,* because the mitochondria are located in the cytoplasm.

Multifactorial Disorders

Multifactorial disorders, which are by far the most common form of genetic disease, do not show clear-cut mendelian patterns of inheritance but tend to run in families. These disorders include common chronic diseases of adults, such as atherosclerosis, hypertension, diabetes, peptic ulcers, and schizophrenia, as well as birth defects, including cleft lip and palate, spina bifida, and congenital heart disease. Multifactorial disorders are thought to result from the interaction of multiple genes with environmental factors, leading to the observed familial clustering. The polygenic component of these disorders consists of a series of genes interacting in a cumulative manner. A particular combination of genetic and environmental factors pushes vunerable persons past a threshold at which they are at risk for the disease. The genes that contribute to a particular polygenic trait such as atherosclerosis can be difficult to define, but advances in gene mapping techniques, combined with increasing understanding of the pathophysiology of these disorders, are leading to rapid progress in elucidation of the ways in which genes and the environment interact to cause multifactorial disorders.

Heterogeneity in Genetic Disorders

As discussed earlier, genetic disorders are quite heterogeneous as a result of the complex interactions between multiple genetic loci and environmental factors. This is true for diseases that segregate as simple mendelian traits as well as for multifactorial disorders. In numerous single-gene disorders, identical mutations are recognized to cause widely varying phenotypes. Nongenetic factors also can play roles in mendelian disorders, leading to heterogeneity. Several disorders result from interactions between a mutant gene and an environmental factor. For example, patients with xeroderma pigmentosum are unusually sensitive to sunlight, whereas persons with α_1-antitrypsin deficiency, who have a predisposition to develop emphysema, are more sensitive to the deleterious effects of tobacco smoke.

Of particular interest for the clinician, inherited single-gene mutations may produce potentially serious inappropriate responses to certain drugs. These pharmacogenetic disorders exhibit all three mendelian modes of inheritance. The most common is glucose-6-phosphate dehydrogenase deficiency, inherited as an X-linked recessive disorder, which may induce hemolytic anemia in response to various drugs. Without exposure to these drugs, such patients otherwise appear to be normal. Genetic differences in drug metabolism are increasingly recognized as important in determining pharmacokinetics. This finding has spurred interest of pharmaceutical companies in the burgeoning field of pharmacogenomics, which is directed at understanding the genetic contribution to pharmacology. Genetic testing for certain alleles of cytochrome P-450 that affect drug metabolism is already commercially available.

In addition to the interactions between genetic and nongenetic components in both mendelian and multifactorial disorders, other factors serve to increase the heterogeneity of genetic disorders. As stated earlier, AD disorders often are characterized by varying severity and incomplete penetrance. It is likely that the specific disease locus interacts with the genetic background of the individual patient. Some combinations of genes at other loci may minimize the pathologic consequences of the mutation, whereas other combinations may accentuate them. In addition, disease heterogeneity results from multiple mutant alleles for a single locus. For example, Duchenne muscular dystrophy is caused by mutations in the dystrophin gene that usually lead to complete absence of the protein. The less severe Becker muscular dystrophy results from mutations at the same locus that lead to shortened dystrophin molecules. In addition, mutations that lead to partial deficiency of hypoxanthine-guanine phosphoribosyltransferase (HPRT) activity cause gout, whereas mutations that abolish HPRT activity lead to the severe neurologic manifestations of the Lesch-Nyhan syndrome. These examples of allelic variation and disease are akin to the normal allelic variation that gives rise to the rich diversity of life.

An additional reason for heterogeneity in genetic disease is that mutations in different genes can sometimes have quite similar clinical manifestations. Certain forms of hemophilia, for example, are caused by mutations in either the gene for factor VIII, responsible for classic hemophilia, or the gene for factor IX, the cause of Christmas disease. Both of these genes are on the X chromosome, and both conditions are inherited as X-linked recessive disorders. Additional bleeding disorders result from mutations in other genes. Other diseases caused by mutations in multiple gene loci have different modes of inheritance in different families. For example, spastic paraplegia, Charcot-Marie-Tooth disease, and retinitis pigmentosa all have AD, AR, and X-linked recessive inheritance forms.

MOLECULAR GENETICS AND MEDICINE

Molecular Genetic Methods

The field of molecular genetics has developed explosively since Watson and Crick's discovery of the structure of DNA in 1953. Two developments in particular made possible recombinant DNA research: the discovery of bacterial restriction enzymes and the invention of blot hybridization methods. Subsequently, the development of DNA and RNA amplification techniques based on PCR revolutionized virtually every area of biology dealing with nucleic acids and had far-reaching effects on society as a whole; for example, forensic DNA testing has had a dramatic effect on criminal investigations.

Restriction Enzymes

Restriction enzymes recognize a specific DNA sequence, most commonly 4 or 6 base pairs long, and they cleave DNA at this site (Figure 1-8). Restriction enzymes recognizing many different sequences have been isolated from numerous species and strains of bacteria. Most of these recognition sites are inverted palindromes, meaning that they have the same sequence on both DNA strands. In addition, most restriction enzymes cut the two strands of DNA in a staggered fashion, leaving two to four bases of single-stranded DNA on both segments of DNA (see Figure 1-8).

Because of the inverted palindromic nature of most recognition sites, these single-stranded regions are complementary and hybridize to each other whenever pieces of DNA cut with the same enzyme are mixed together, temporarily joining two segments. If an enzyme called *DNA ligase* also is present, it covalently links the hybridized segments. Thus the discovery and isolation of restriction enzymes allowed molecular geneticists to cleave DNA at specific sites and to reconnect the segments in specific arrangements, thus allowing for the creation of *recombinant DNA,* popularly known as genetic engineering.

Blot Hybridization

Various methods, collectively known as *blot hybridization,* have been developed to allow the rapid identification of a segment of nucleic acid containing a particular nucleotide sequence. As first described by Southern in 1975, in blot hybridization, DNA is digested with a restriction enzyme. The now fragmented DNA is separated according to size by electrophoresis through an agarose gel matrix, in which smaller fragments move faster than larger ones. The DNA is then transferred to a filter under conditions that cause the two DNA strands to come apart. The filter is incubated with a radiolabeled RNA or DNA probe including the desired nucleotide sequence. The probe hybridizes to the

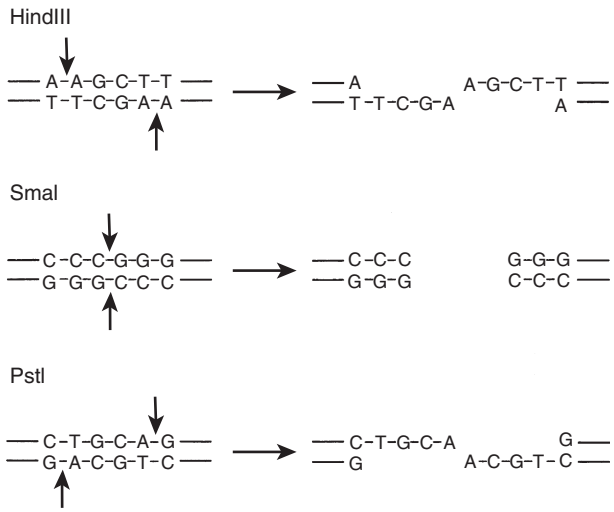


Figure 1-8 Restriction enzymes. The recognition sequences for three common restriction enzymes are shown at *left.* The *vertical arrows* indicate the sites at which each strand is cleaved to produce two ends, depicted on the *right.* There are three types of cut ends, differing in which strand, if any, is single-stranded.

complementary sequence on the filter, and the labeled DNA band is then visualized by autoradiography (i.e., exposing x-ray film to the filter). This method of identifying DNA sequences is called *Southern blotting*, after its inventor (Figure 1-9). A similar method has been developed to identify RNA sequences and is called, by playful analogy, *Northern blotting.* (*Western blotting,* in which antibodies directed against specific proteins are used to detect those proteins on blots of cellular extracts, also exists.)

Cloning

The ability to clone genes has greatly facilitated the ability to understand the pathogenesis of inherited disease at the molecular level. In molecular genetics, *cloning* means isolating a particular gene in a form that allows its DNA to be amplified and manipulated. A major breakthrough in the ability to clone genes came with the discovery of the enzyme reverse transcriptase, which is essential in the life cycle of a class of viruses called *retroviruses.* These viruses, which have single-stranded RNA genomes, include the human immunodeficiency virus (HIV). The function of reverse transcriptase is to make a DNA copy of the viral genome that can integrate into the cellular DNA and can be replicated. The designation *reverse transcriptase* was chosen because the enzyme functions counter to the normal DNA-to-RNA direction of transcription. Researchers commonly use reverse transcriptase to make single-stranded DNA molecules complementary to mRNA sequences and therefore called *complementary DNA* (*cDNA).* The single-stranded molecules are converted to normal double-stranded cDNA using DNA polymerase and are then inserted into cloning vectors. This permits the isolation or cloning, in the form of cDNA, of any region of the genome that is transcribed into RNA. Even with recently acquired knowledge of the complete human genome sequence, the technology of generating cDNA from RNA remains invaluable for determining the complement of genes that are expressed in a particular cell type. The general approach to cloning gene sequences is summarized in Figure 1-10.

Polymerase Chain Reaction

The development of the *PCR technique,* which allows amplification of selected pieces of DNA by many orders of magnitude, revolutionized both basic and clinical genetics (Figure 1–11). Oligonucleotide primers that flank the section of DNA to be amplified are hybridized to a DNA-containing sample. The intervening segment of DNA is then filled in by a heat-stable DNA polymerase derived from bacteria that are adapted to extremely hot environments. The sample is then heated to separate the strands and is cooled, and the cycle is repeated. In this way, the desired segment of DNA is exponentially amplified, resulting in million-fold increases in the number of copies of a desired segment of DNA after 30 to 40 cycles. This technology has become central to virtually every aspect of genetics and many areas of medicine and biology, including gene mapping, gene cloning, analysis of gene transcription (using reverse-transcribed mRNA as a starting material), prenatal diagnosis (including preimplantation diagnosis from a single cell of an eight-cell embryo), infectious disease detection, and forensic medicine.

Microarrays

The ability to produce nucleic acids in large quantities using amplification technologies such as PCR or direct chemical synthesis, combined with technology such as photolithography and nanodroplet deposition used in the computer industry, has enabled the production of high-density arrays of nucleic acids (Figure 1-12). In this way, arrays can be produced that are composed of up to a million distinct nucleic acids, each with a different sequence, and each placed in a distinct location. These nucleic acid "targets," which are tethered to the surface of the array (composed of glass or a variety of other materials), can be hybridized with a nucleic acid "probe," consisting of a biologic sample that has been modified to incorporate a fluorescent label.

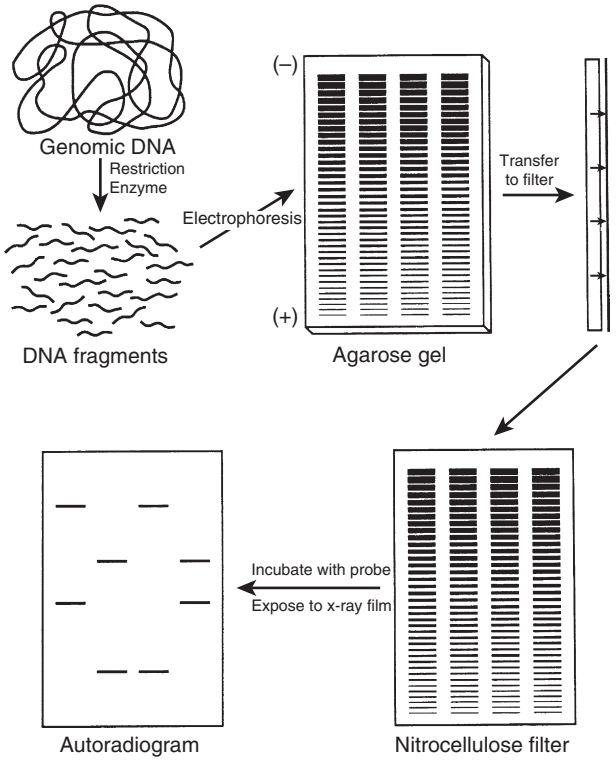


Figure 1-9 Southern blotting. Cellular DNA is cut with a restriction enzyme to produce small fragments. The cut DNA is separated by size by means of electrophoresis through an agarose gel. Because DNA is an acid and therefore has a negative charge, it migrates toward the positive electrode, the smaller fragments migrating faster than the larger. The DNA is transferred to a nitrocellulose (or charged nylon) filter, usually by buffer flow (as shown in an *edge view*), to produce a replica of the gel. The filter is incubated with a radioactive DNA or RNA probe and then washed, followed by exposure of x-ray film to the filter. The resulting autoradiogram indicates DNA bands that have sequence homology with the probe.

Uses of microarrays are many and varied, depending on the sequences of the nucleic acids that are used to produce the array. Diagnostic arrays containing nucleic acid targets from pathogenic microorganisms as well as research arrays containing sequences from all known genes of a particular organism are available. Probes can range from diagnostic samples such as blood that may contain a virus to mRNA isolated from a particular cell type to determine the pattern of genes expressed in that cell. Use of microarrays that distinguish between different SNPs in the genome is now the predominant technology for genetic mapping studies. Microarrays are even being used in conjunction with a technique called comparative genomic hybridization (CGH) to measure the number of copies of particular genes. This technology has the potential to supplant conventional chromosomal karyotypes and to detect microdeletions that are below the limit of sensitivity of current banding techniques.

Biomedical Applications of Molecular Genetics

Production of Clinically Useful Proteins

Once a gene has been cloned, it is possible to produce virtually unlimited quantities of the encoded protein by introducing the clone (in an appropriate expression vector) into bacterial, yeast,

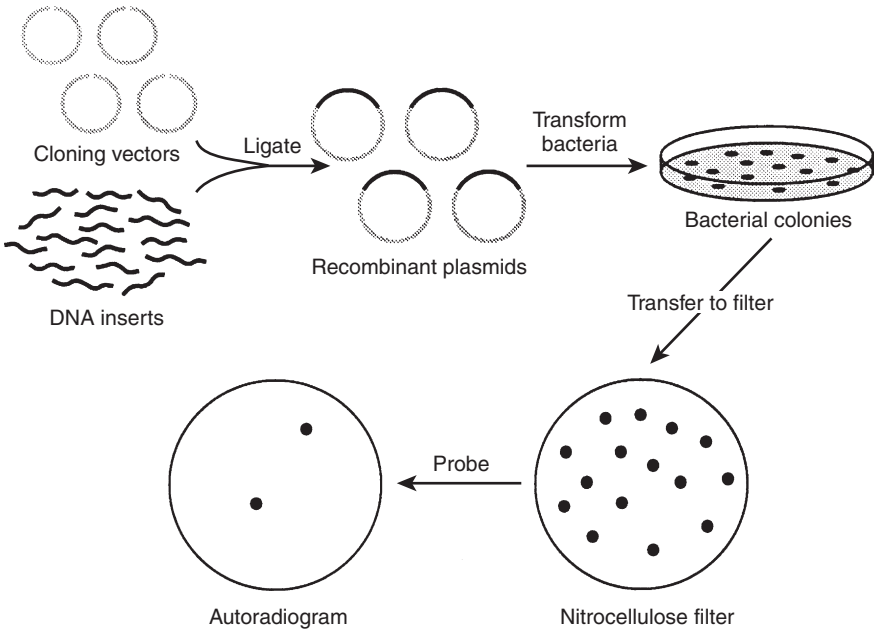


Figure 1-10 Cloning. The desired DNA inserts, either genomic DNA cut with a restriction enzyme or cDNA produced from mRNA, are ligated into cloning vectors that have been cut to accept the inserts. The resulting recombinant plasmids are introduced into bacteria, a process called *genetic transformation,* producing a genetic library. Bacterial colonies grown from the library are transferred to a nitrocellulose filter by simply overlaying the plate. The filter is then probed, either with DNA or RNA, as described for Southern blotting, to look for colonies with homologous sequences, or with labeled antibodies, to look for specific protein production. The resulting autoradiogram uses x-ray film to indicate the position of colonies containing the desired sequence.

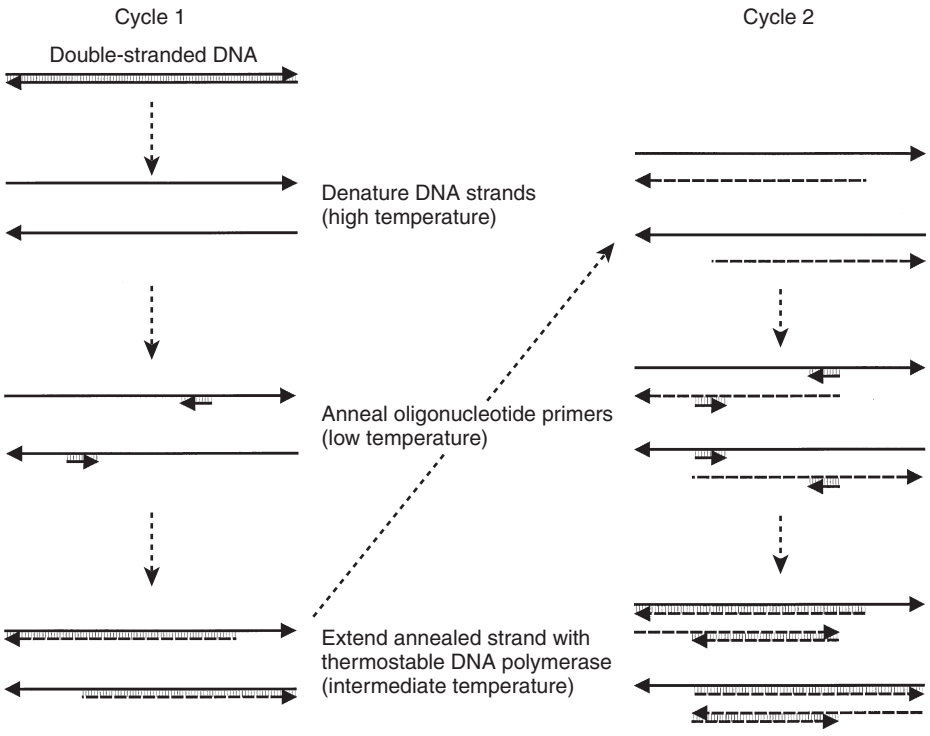


Figure 1-11 Polymerase chain reaction (PCR) amplification of DNA. Double-stranded DNA is subjected to cycles of denaturation, annealing with short, single-stranded, oligonucleotide DNA primers, and extension using a thermostable DNA polymerase. Typically, 20 to 40 cycles of PCR are performed, resulting in exponential amplification of the DNA sequence between the two primers. The high specificity of primer annealing allows a short sequence (typically <1 kilobase [kB]) to be amplified from the 3 million kB present in the human genome. The advantage of a thermostable polymerase is that it can withstand the extreme temperatures (close to boiling) necessary to denature the DNA strands.

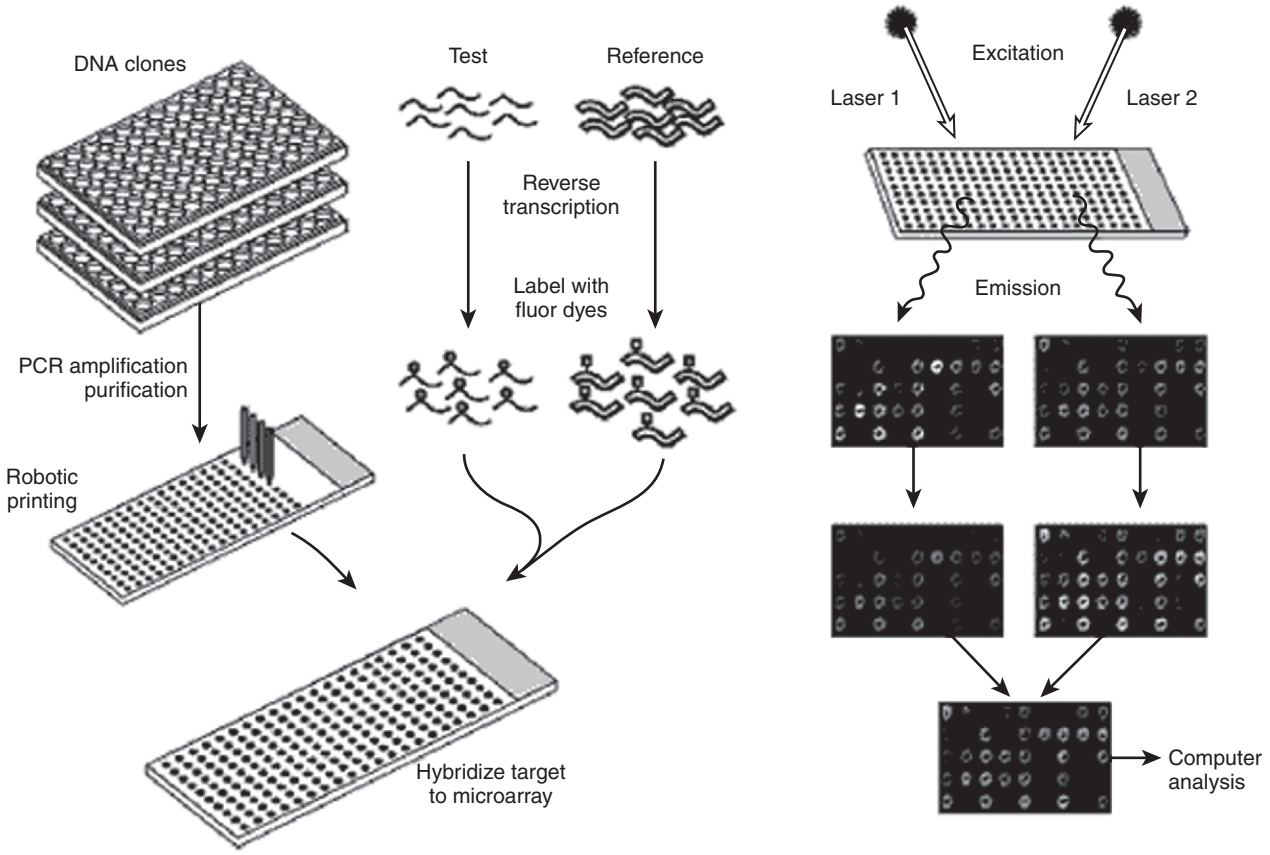


Figure 1-12 Complementary DNA (cDNA) microarray schema. Templates for genes of interest are obtained and amplified by PCR. After purification and quality control, aliquots (~5 nL) are printed on coated glass microscope slides using a computer-controlled, high-speed robot. Total RNA from both the test and reference samples is fluorescently labeled with either Cye3–dUTP or Cye5–dUTP using a single round of reverse transcription. The fluorescent targets are pooled and allowed to hybridize under stringent conditions to the clones on the array. Laser excitation of the incorporated targets yields an emission with a characteristic spectrum, which is measured using a scanning confocal laser microscope. Monochrome images from the scanner are imported into software in which the images are pseudo-colored and merged. Information about the clones, including gene name, clone identifier, intensity values, intensity ratios, normalization constant, and confidence intervals, is attached to each target. Data from a single hybridization experiment are viewed as a normalized ratio (that is, Cye3/Cye5) in which significant deviations from 1 (no change) are indicative of increased (>1) or decreased (<1) levels of gene expression relative to the reference sample. In addition, data from multiple experiments can be examined using any number of data mining tools. *dUTP,* deoxyuridine-5′-triphosphate; *PCR,* polymerase chain reaction. *(From Duggan DJ, Bittner M, Chen Y, et al: Expression profiling using cDNA microarrays,* Nat Genet *21:10-14, 1999.)*

mammalian, or other cells. Because many eukaryotic proteins undergo posttranslational modification, including glycosylation, phosphorylation, acylation, and carboxylation, which frequently are required for normal protein function, it is often preferable to express proteins in mammalian cells. Rapidly increasing numbers of recombinant human proteins are in clinical use, including insulin, growth hormone, clotting factors VIII and IX, erythropoietin, hematopoietic colony-stimulating factors, and tissue plasminogen activator, among others. A number of lysosomal storage diseases such as Gaucher disease are now treated by infusion of recombinant enzymes. In addition to simple replacement therapy, monoclonal antibodies directed against cell surface proteins expressed on tumors are revolutionizing cancer treatment.

Genomics and the Genome Project

Perhaps one of the most significant scientific achievements in human history is the complete sequencing of the human genome. The *Human Genome Project* is still ongoing, and further sequencing and gene identification remain to be done before the sequence will be truly complete—a process that may take many more years. This genome project was made possible by development of the techniques of molecular genetics described earlier as well as automated sequencing machines. Such machines made it possible to achieve the economies of scale necessary to sequence the 3 billion base pairs in the human genome.

In addition to the human genome, the genomes of other organisms, including many important human pathogens such as *Mycobacterium tuberculosis,* have been sequenced. Sequencing the genomes of other mammalian and nonmammalian species has led to the recognition that most human genes have homologues in other species, including lower organisms such as yeast and worms, thereby justifying the enormous effort that has been devoted to studying those model organisms over the years. The ultimate impact of the genomics revolution has yet to be felt. Much more DNA sequencing and analysis will be required to obtain a complete understanding of how organisms differ from one another at the interspecies and intraspecies level. Undoubtedly, however, the end result, an understanding of the genetic basis of human variability and predisposition to disease, will produce enormous benefits for human health.

Gene Mapping

Linkage mapping in humans involves examining pedigrees to determine the frequency of co-inheritance of specific traits. As described previously, recombination between chromatids of

homologous chromosomes occurs in meiosis, so sections of chromosomes are exchanged with each other. Thus, recombinant chromosomes are produced that contain a segment derived from each of the original homologous chromosomes. The closer together two genes are located on the same chromosome, the less likely it is that recombination will occur between them during meiosis, and the more likely it is that particular alleles of these genes will be inherited together. Thus, it is possible to use the recombination frequency between two genes to determine their distance from each other and hence to construct a genetic map.

A 1% recombination frequency between two loci is defined as 1 map unit, or 1 centimorgan (cM). Genes located on separate chromosomes are inherited independently and are therefore unlinked. Genes located on the same chromosome but that are so far apart that recombination occurs between them 50% of the time (i.e., 50 cM apart) are also considered unlinked. Although 1 cM corresponds to approximately 1 million base pairs, this physical distance must be considered to represent only a rough estimate, because recombination does not occur purely randomly throughout the genome—instead, the genome has "hot" and "cold" spots. Regions of decreased recombination give rise to the phenomenon of *linkage disequilibrium,* in which alleles of linked loci are found on the same chromosome more frequently than one would expect with normal levels of recombination occurring over time. Linkage disequilibrium also can arise from selective pressure for the maintenance of linked alleles, as seems to occur for the human leukocyte antigen (HLA) major histocompatibility complex. The implication of linkage disequilibrium is that two alleles that are not in close physical proximity may nonetheless be co-inherited. A set of alleles inherited as if on a single locus is referred to as a *haplotype.*

The ability to detect and to assay for sequence variations in DNA made feasible the construction of sets of markers that cover the entire genome. Current gene mapping strategies take advantage of SNPs. Because each person differs from every other at millions of individual loci, increasingly dense SNP maps have been constructed. As the number of polymorphic markers increases and the expense of genotyping decreases, diseases can be mapped with increasing resolution and complex polygenic diseases can be attacked. Once an approximate map location is determined for a gene, the genome sequence can be used to search for candidate genes known to have sequence elements or biologic functions suggesting that they may be involved in the disease being studied. Moreover, the approximate map locations can serve as the starting point for more detailed studies using a higher density of polymorphic markers from the specific region being studied. Both approaches have been greatly facilitated by the completion of the human genome sequence.

Linkage analysis and, more recently, direct mutation detection have revolutionized prenatal diagnosis. Fetal cells are obtained from amniotic fluid, and blood or skin samples are obtained from parents and siblings. Analysis of DNA from these samples can then determine in many cases whether the fetus has inherited the disease. This approach was first used for the prenatal diagnosis of sickle cell anemia. It is now used for diagnosis of various other inherited disorders and can be applied to any condition for which a linked polymorphic marker or, even better, the pathogenic mutation has been identified. The same principles involved in prenatal diagnosis also apply to presymptomatic diagnosis in disorders with late onset and to carrier detection in X-linked and AR disorders. The most sophisticated application of prenatal diagnosis is preimplantation genetic diagnosis, in which genetic material from an early embryo or even from the polar bodies of the egg are used to detect the presence of a particular genetic trait.

Gene and Cell Therapy

The available therapies for most human diseases, and particularly genetic diseases, are inadequate. Treatments such as enzyme replacement for Gaucher disease or dietary restriction as in disorders of amino acid metabolism have serious limitations, such as high cost or low efficacy. Additionally, protein replacement is applicable to only a few diseases in which delivery into the circulation is efficacious; this approach is problematic when it is necessary to deliver a missing component directly to cells, particularly when tissue specificity is necessary. The ultimate treatment for many inherited disorders is to use DNA itself as a pharmacologic agent to treat the actual defect—the gene mutation. The development of sophisticated gene transfer methods has made this gene therapy approach a reality.

It is important to distinguish between gene therapy affecting the germline and therapy affecting only somatic tissues. Germline gene therapy would allow genetic modifications to be transmitted to future generations. Even if such modifications were limited to treating specific diseases, serious moral and ethical questions would arise. This situation is further complicated because it would theoretically be possible to alter traits to produce more "desirable" children. Because somatic cell therapy affects only the person treated, the ethical considerations are greatly simplified. All current gene therapy protocols in humans are limited to somatic cell experiments.

Although inherited mutations exist in every cell in the body, most genes are expressed in specific tissues, and, in most cases, gene therapy needs to be directed only toward the proper tissue. Even when a gene is expressed in all tissues, only one tissue may need be treated to ameliorate the disease phenotype. The effectiveness of this approach has already been proved for maple syrup urine disease, in which liver transplantation is effective despite the fact that the enzyme is expressed in other tissues as well. For some diseases, such as hemophilia, any cell that can secrete a protein into the circulation is a potential target for gene therapy, even if it is not the normal site of protein production.

The most desirable methods of gene therapy allow for gene transfer directly into the patient (i.e., in vivo gene therapy) (Figure 1-13). For some tissues, such as the central nervous system, this is likely to be the major mode of gene transfer. For organs such as the bone marrow, however, cells can be removed easily, manipulated in vitro, and reintroduced into the patient (i.e., ex vivo gene therapy) (see Figure 1-13).

In the ideal gene therapy system, the normal gene would be introduced into cells in vivo in such a way that it would replace the defective gene through site-specific recombination. This would result in the gene's being in the proper position relative to the various transcriptional regulatory elements. Unfortunately, efficient techniques for site-specific integration are not yet available, and all current methods of gene transfer result in predominantly random integration. Because of this limitation, gene *replacement* therapy is not yet possible. Instead, gene *augmentation* therapy has been pursued, in which the normal gene supplements the defective gene from a distal site. Although simpler, this method suffers from the risk of insertional mutagenesis, in which the transducing vector affects the expression of genes in the region in which it integrates. This phenomenon led to the development of leukemia in a number of patients enrolled in a gene therapy trial for severe combined immunodeficiency in which bone marrow was removed and transduced with a retroviral vector expressing the adenosine deaminase gene and the transduced cells were reintroduced into the patient. Although the augmentation therapy achieved its goal, the severe complication of leukemic transformation by insertional mutagenesis has raised questions about the risk-benefit ratio of the procedure.

Various biochemical and physical methods have been developed to introduce DNA into mammalian cells, referred to as *transfection* (i.e., *trans*formation + in*fection*). These methods generally suffer from the serious limitation that only a few of the treated cells are permanently genetically modified. Direct injection of DNA into some tissues such as muscle results in gene transfer and expression, but this method is inefficient, with only

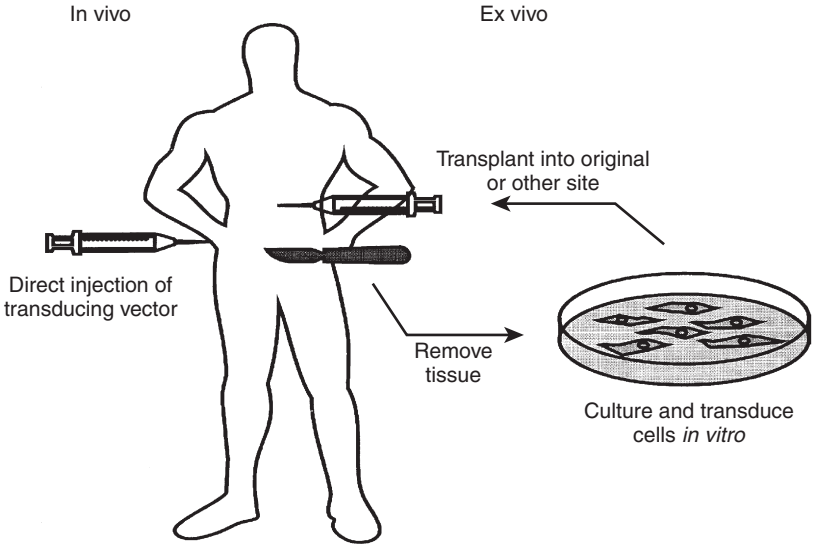


Figure 1-13 Models for human gene therapy. In vivo gene therapy involves direct introduction of a transducing vector into the patient. Ex vivo gene therapy involves removal of tissue and transduction in vitro.

a small number of cells taking up the injected DNA. Nevertheless, this approach is well suited by virtue of simplicity and low cost to vaccine development.

To increase the efficiency of gene transfer, most gene therapy studies have instead used replication-defective genetic transducing vectors derived from viruses. Numerous different viruses have been used, including adenovirus adeno-associated virus, murine retroviruses, and even lentiviruses such as HIV. Viral vectors can infect various cell types from several species at efficiencies often approaching 100%.

Although the initial thrust of research in gene therapy was to correct single-gene defects such as in hemophilia and adenosine deaminase deficiency, work in the field has shifted toward applying gene transfer technology to more common polygenic diseases such as cancer and coronary artery disease. Viral vectors are being used to deliver cytotoxic genes to cancer cells. In some cases, the vectors are designed specifically to target the cancer cells—a so-called magic bullet. Other experiments are using gene transfer techniques to enhance angiogenesis in ischemic cardiac tissue. The appeal of using gene therapy for common polygenic diseases is that these techniques can be applied to many more patients than would be the case if gene therapy were limited to the much rarer monogenic disorders.

Stem Cells

As described previously, ES cells from mice have revolutionized the study of mammalian development and disease. In addition to basic science applications, stem cells are now being studied to determine if they can be used to treat diseases in which replacement of malfunctioning cells or cells that have been destroyed is an attractive approach. Examples are type 1 diabetes, in which the pancreatic beta cells are destroyed, and Parkinson disease, in which the dopaminergic neurons die. The isolation and extended culture in vitro of human ES cells in 2000 by Thompson and colleagues at the University of Wisconsin raised the hope that such cell-based therapies could be developed and marked the advent of a new area of medicine termed *regenerative medicine.*

Although ES cells have enormous potential in terms of being able to differentiate into any cell type in the body, major problems are still to be overcome. One such problem is achieving the ability to direct ES cells along a particular lineage so that only the desired cell types are produced. Another is to find ways to stimulate the cells that are formed to incorporate functionally into the target organ. For instance, dopaminergic neurons derived from ES cells must be properly integrated into the substantia nigra so that dopamine release is properly regulated. Finally, transplanted cells will be rejected by host immune responses unless they are genetically identical to the host's. This fact has stimulated interest in a technique called somatic cell nuclear transfer, in which the nucleus from an adult is transferred into an egg in place of the egg nucleus, resulting, albeit with great inefficiency, in the formation of an ES cell line that is genetically identical to that of the donor of the adult nucleus. This technology has been used to create a variety of cloned mammals, most notably Dolly the sheep. Thus far, it has not been done successfully with human cells, and serious ethical issues continue to swirl around the entire field.

In addition to ES cells, which are not found postnatally, so-called adult stem cells have been isolated from a number of tissues. By far the best characterized is the hematopoietic stem cell, which gives rise specifically to blood cells. Other widely studied types of adult stem cells include neural stem cells and mesenchymal stem cells. In general, adult stem cells give rise to a restricted range of mature cell types—for example, a neural stem cell can give rise to neurons but not cardiomyocytes. An advantage of using adult stem cells is that they are already programmed to give rise to specific cell types. Also, because they exist normally in many tissues and function in tissue regeneration, they already "know" how to incorporate functionally into the organ to which they give rise. Significant problems include the fact that adult stem cells have not been identified for many organs, and the signals that control the regenerative process are poorly understood for the most part—the hematopoietic system being a notable exception.

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WEB-BASED RESOURCES

Genetics Home Reference: http://ghr.nlm.nih.gov/

GeneTests•GeneClinics: http://www.genetests.org/

Online Mendelian Inheritance in Man: http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim/

2

George A. Diaz and Kurt Hirschhorn

Advances in Molecular Genetics: Developmental and Clinical Implications

MOLECULAR GENETICS TOOLS

The explosive advances in human genetics of the past few decades have been fueled by a powerful set of tools built on a small number of critical scientific developments. The more important of these techniques and principles are reviewed briefly in this opening section.

Recombinant DNA Technology

The identification and characterization of enzymes involved in DNA replication, repair, and sequence-specific endonuclease digestion (Table 2-1) have led to the development of tools that allow convenient manipulation of DNA. Restriction endonucleases are a class of enzymes that recognize specific DNA sequences, typically 6– to 8–base-pair palindromes, and usually cut the DNA backbone at a symmetric point within the recognition sequence on both strands. Digestion of purified genomic DNA with a restriction enzyme yields a collection of fragments with identical end sequences and discrete sizes corresponding to the distances between recognition sites in the DNA sequence. The termini of fragments resulting from a restriction digestion may be single-stranded and self-complementary over several bases or completely double-stranded, depending on the cleavage site. By using a different enzyme, DNA ligase, digested fragments with using can be spliced together, even if the two pieces were not initially contiguous, generating a recombinant molecule.

Library Construction

The enzymatic tools just described allow the direct manipulation of DNA, but additional methods had to be developed to permit efficient amplification of specific fragments. Small, circular DNA molecules that replicate autonomously in bacterial cells (plasmids) have been characterized and developed as vectors to propagate DNA sequences of interest. These reagents make it possible to ligate restriction enzyme–digested DNA fragments into digested plasmids, introduce the recombinant plasmid into bacterial cells, and then select for the presence of a plasmid-encoded antibiotic resistance gene. DNA isolated from the resulting colonies is then analyzed by restriction analysis or sequencing to identify plasmids containing DNA of interest. Cells containing the desired plasmid can then be propagated or stored frozen indefinitely. This process is known as *cloning*.

When complex mixtures of DNA are ligated into a recipient plasmid, the resulting recombinant molecules represent a *library* of plasmids containing different fragments. Libraries can be generated from digested genomic DNA or from complementary DNA (cDNA) produced by reverse transcription from mRNA. Genomic DNA libraries were critical reagents in the sequencing of the human genome, with improvements made over time in the cloning vectors used (yeast or bacterial artificial chromosomes [YACs or BACs, respectively]) and in the stability of the cloned inserts. In contrast with the goal that genomic libraries represent the full genetic complement of an organism, cDNA libraries are designed to represent the tissue-specific spectrum of genes expressed in the mRNA source tissue. In the creation of such libraries, hybridization methods (see further on) are used to normalize the contribution of highly expressed ubiquitous housekeeping genes.

Libraries have been generated from a wide range of tissue types and from samples obtained in different disease states. Sequence data from clones derived from different libraries, so-called expressed sequence tags (ESTs), have been catalogued in publicly available databases. Identification of transcripts whose expression is restricted to a specific tissue type is a powerful tool for understanding the genetic basis of tissue-specific function. The ability to work with nucleic acids, which are easily manipulated, rather than with the encoded proteins, allowed a significant acceleration in the understanding of many biologic phenomena.

Polymerase Chain Reaction

The use of thermostable DNA polymerases derived from bacteria that live in extremely high-temperature environments represented a revolutionary twist on the use of nucleic acid polymerases. These enzymes retain activity even after heating to temperatures sufficient to denature DNA strands (higher than 96° C), allowing multiple cycles of primer annealing and chain elongation to be carried out in a single reaction. Each cycle of primer annealing and chain elongation results in double the number of template molecules for the next cycle of primer binding and extension, so amplification of the input template DNA occurs exponentially.

Polymerase chain reaction (PCR) methodology thus makes possible the amplification of large amounts of product from trace amounts of starting material, overcoming the technical limitations imposed by the relatively limited number of copies of genomic DNA available in a given human tissue specimen. The extensive range of uses for which PCR amplification is essential, from basic molecular biology research to infectious disease diagnostics and forensic applications, attests to the power of this technique.

Nucleic Acid Hybridization

Alterations in thermal or ionic conditions can disrupt the hydrogen bonding interactions in a DNA double helix, resulting in single-stranded DNA. Complementary strands can reanneal with great specificity when reaction conditions are adjusted appropriately, a property that has been exploited to use a cloned DNA as a probe for the presence of related sequences among a mixture of single-stranded DNA or RNA. Target molecules are first separated based on size by gel electrophoresis, transferred to a support matrix, such as a nylon membrane, and then probed with a DNA fragment labeled with a radioisotope or some other reporter molecule. This procedure is known as a Southern blot (Figure 2-1) after its inventor, Edward Southern. When applied to RNA, the procedure is called a "Northern blot." Alternatively, a collection of bacterial colonies representing a cDNA library grown

on agar plates can be transferred and lysed directly on nylon filters, allowing screening of the entire library for sequences of interest. The thermal energy required to disrupt DNA hydrogen bond interactions is related to the base composition of the nucleic acid duplex, allowing approximate calculations of the melting and annealing temperature of a known sequence. Under appropriate hybridization conditions, even small synthetic oligonucleotides can be used as high-specificity probes to detect single-base mismatches.

Differences between single- and double-stranded nucleic acids in binding to positively charged matrices have been exploited in subtractive hybridization approaches. Single-stranded cDNA from a differentiated or diseased tissue can be hybridized with excess cDNA strands from nondifferentiated precursor or normal tissue. Genes transcribed in both states will be readily available for hybridization with the excess cDNA pool, whereas those induced only in the differentiated or disease condition will remain single-stranded. By binding double-stranded molecules on an affinity column, state-specific transcripts can be isolated and enriched in the final library. Modifications of the principles of subtractive hybridization also allow identification of low abundance transcripts whose expression is associated with developmental changes or disease states.

Hybridization-based approaches continue to be used in a variety of high-throughput methods, such as those based on so-called DNA chips, in which the hybridization reaction occurs in parallel at an enormous number of individual sites on a small solid support (Figure 2-2, *A*). Technologies involving the performance of massive numbers of hybridization reactions in a small volume, with data obtained in a digital format are being applied in research settings to assess differences in transcription patterns between normal and diseased tissue or to score for the presence or absence of polymorphisms in genomic DNA. The introduction of such techniques to the clinical arena offers the promise of therapeutic interventions tailored to an individual patient's genotype—for example, by aggressive measures for disease prevention (e.g., testing for BRCA1/BRCA2 and breast and ovarian cancer) or by adjusting medication exposure or dosing based on the presence or absence of polymorphisms affecting the function of metabolic enzymes. This type of approach also has been used to perform molecular characterization of pathologically indistinguishable cancerous cells by the pattern of gene expression exhibited by different specimens on microarray analysis, suggesting a potentially useful application as a diagnostic and prognostic tool (Figure 2-2, *B*).

TABLE 2-1

Examples of Gene Dosage Effects

Gene	Genome Rearrangement	Disease	Point Mutation	Mouse Model
PMP22	Duplication	CMT1A	+	+
	Deletion	HNPP	+	+
PLP1	Duplication	PMD	+	+
	Deletion	PMD	+	+
ELN	Deletion	WS/SVAS	+	+
LIMK1	Deletion	WS	–	–
TBX1	Deletion	DGS/VCFS	–	+
CREB	Deletion	RTS	+	+
JAG1	Deletion	AGS	+	+
LIS1	Deletion	MDLS	+ (lissencephaly)	+
EXT1	Deletion	LGS/exostoses	+ (exostoses)	+
TRPS1	Deletion	LGS/TRPS1	+ (TRPS1)	–
WT1	Deletion	WAGR	+ (Denys-Drash syndrome)	–
PAX6	Deletion	WAGR	+ (aniridia)	+
NSD1	Deletion	Sotos syndrome	+	–

AGS, Aicardi-Goutieres syndrome; *CMT1A,* Charcot-Marie-Tooth disease type 1A; *DGS,* DiGeorge syndrome; *HNPP,* hereditary neuropathy with liability to pressure palsies; *LGS,* Lennox-Gastaut syndrome; *MDLS,* Miller-Dieker lissencephaly syndrome; *PMD,* Pelizaeus-Merzbacher disease; *RTS,* Rubinstein-Taybi syndrome; *SVAS,* supravalvular aortic stenosis; *TRPS1,* trichorhinophalangeal syndrome type 1 (Langer-Giedion syndrome); *VCFS,* velocardiofacial syndrome; *WAGR,* Wilms tumor, aniridia, genitourinary abnormalities/gonadoblastoma, retardation; *WS,* Williams syndrome.

Expression Libraries

An early effort in the effort to catalogue the complete set of human genes involved the generation of libraries of reverse-transcribed and cloned transcripts from various tissues. These clones were subjected to limited sequencing and the resulting data were stored in a repository of transcript sequences (expressed sequence tags [ESTs]). An important limitation of this approach is the difficulty of obtaining cDNA from all tissues, as well as in all developmental stages, but this repository (dbEST) has proved extremely useful in mapping exonic sequences onto the complete genome sequence data.

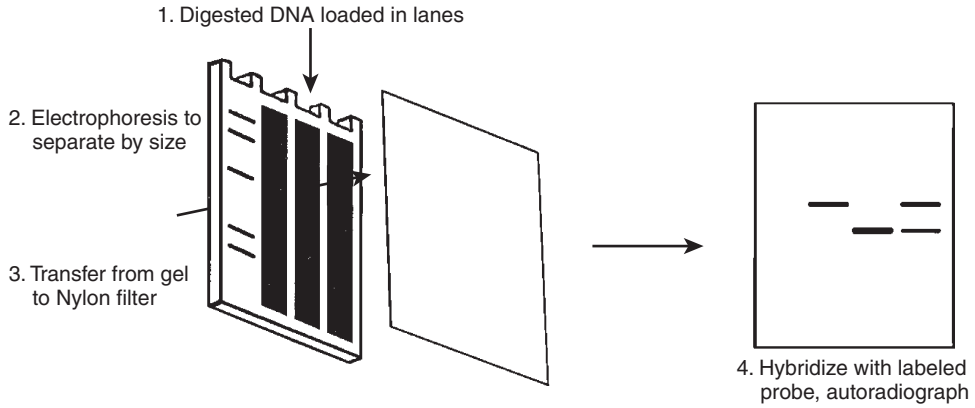


Figure 2-1 Southern blot analysis. Hybridization of labeled DNA probes to immobilized DNA affixed to a solid support was developed by Dr. Edward Southern. The technique involves separating different-sized DNA fragments produced by restriction enzyme digestion by electrophoresis through a porous gel. After the fragments are separated, the DNA is migrated out of the gel in a perpendicular direction by capillary flow, or in a second electrophoretic event, and onto a positively charged membrane (nitrocellulose or nylon). DNA affixed to this membrane can then be hybridized with a rabiolabeled probe and the filter used to expose x-ray film in order to detect bands containing DNA sequences related to the probe.

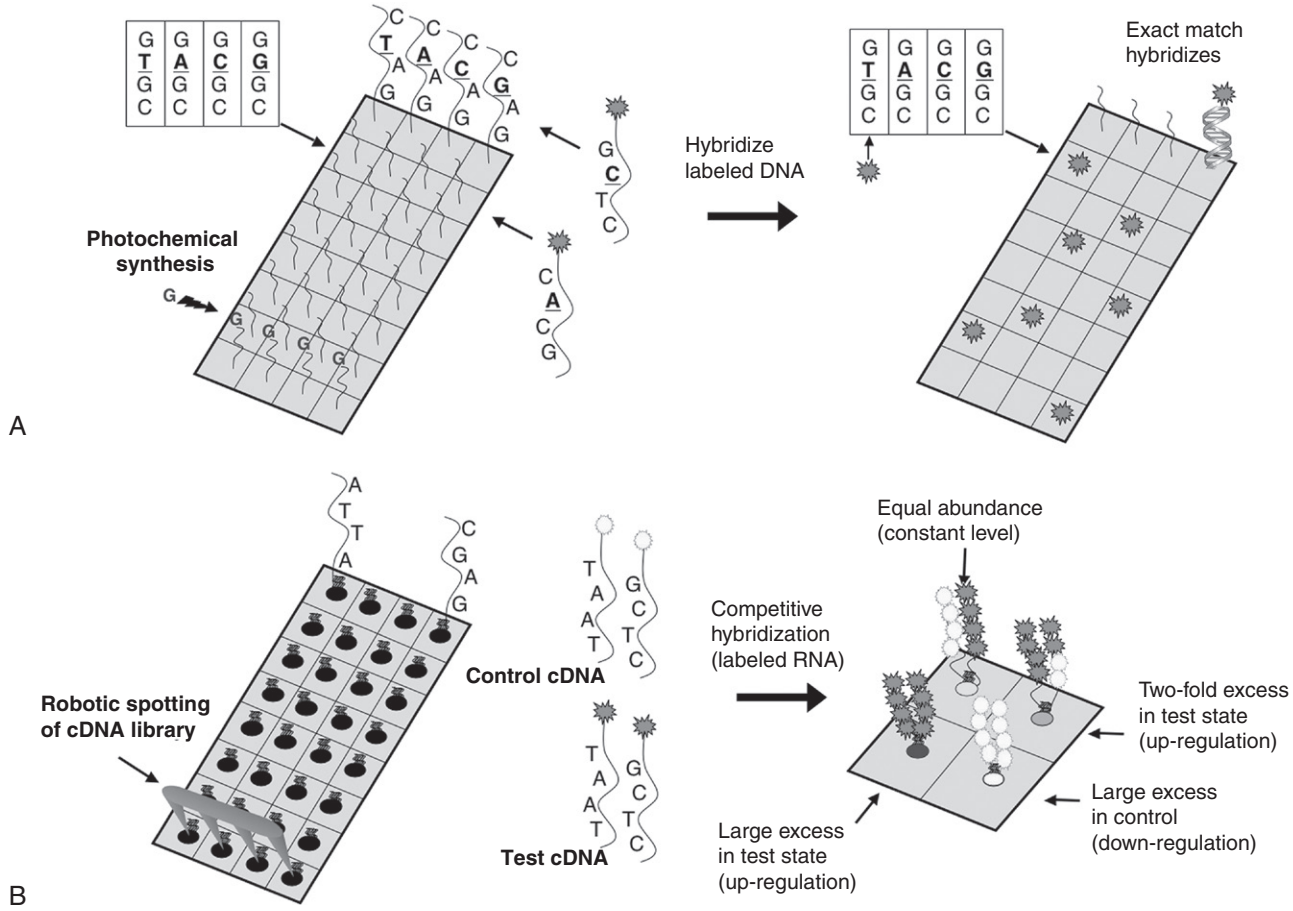


Figure 2-2 Microarrays. The general principles behind two different types of microarrays. **A,** Oligonucleotides are synthesized directly on a chip surface by photochemical techniques, allowing a series of overlapping sequences to be produced with each row containing all possible single base substitutions introduced at each given position. The DNA sequence to be analyzed (resequenced) is labeled with a fluorochrome and hybridized to the array, producing a pattern of hybridization that allows the detection and identification of any point mutations occurring within the portion of a gene represented by the overlapping oligonucleotide array. **B,** Complementary DNA (cDNA) arrays are produced by spotting characterized cDNA clones onto slides at high density. The expression levels of the genes placed on the array are then assayed by generating cDNA from RNA samples from a tissue of interest and from a matched control sample (e.g., normal versus malignant). The two samples are labeled fluorescently in two different colors, and equivalent amounts of the cDNAs are mixed and used for competitive hybridization to the targets on the microarray. The expression level of a gene in the test sample relative to the control can be calculated by determining the intensity of each of the fluorescent label at spots corresponding to hybridization targets for that cDNA.

HUMAN GENOME PROJECT

The most significant development in molecular genetics of the late 20th century was the commitment to sequence and then identify all of the genes in the complete genome of humans and other important model organisms and pathogens. Use of the basic tools described previously at an industrial level culminated in the development of highly detailed linkage, physical, and sequence maps of the human genome. This section describes some of the methods that were required to complete the mapping and sequencing of the human genome.

Polymorphic Markers

Although identity at the DNA sequence level between any two humans is greater than 99.9%, a large number of interindividual differences exist. These sequence polymorphisms have been exploited as tools to track the segregation of chromosomal loci through generations in human pedigrees. The first such markers were single-base changes or small insertions or deletions that created or destroyed a restriction enzyme site (restriction fragment length polymorphisms [RFLPs]), detectable by Southern blot analysis as bands of altered mobility. The discovery of variable number tandem repeat (VNTR) markers provided reagents that were more abundant and more informative (with more alleles, corresponding to differences in the number of repeats found in the population). The size of the repeat units typically identified was such that different alleles could differ in size by thousands of base pairs, making Southern blotting a requisite step for genotyping. The type of marker used most extensively in the creation of human genetic linkage maps was the short tandem repeat (STR), in which runs of two to four nucleotides (e.g., CA, AAT, GATA) were repeated approximately 10 to 30 times. Size differences in alleles as little as two base pairs in PCR products ranging from approximately 80 to 300 base pairs in length were resolved easily by electrophoresis in polyacrylamide sequencing gels. Compared with Southern blotting, this process required significantly less labor and sample DNA, substantially facilitating large-scale genotyping.

The most recently developed type of polymorphic marker is the *single-nucleotide polymorphism* (SNP). SNPs are single-base

changes that occur on average once every 750 base pairs. SNPs have fewer alleles and are therefore less informative than the repeat-type markers, but their sheer abundance is anticipated to make them powerful tools in the effort to elucidate the genetic basis of common complex diseases, such as hypertension and asthma, as well as quantitative traits that have an impact on fetal health, such as birth weight, at the molecular genetic level.

Linkage Analysis

The ability to map genes to specific chromosomal locations was developed in experimental organisms such as *Drosophila melanogaster* in the early 20th century. The construction of genetic maps in model systems required the identification of individuals with traits of interest (e.g., mutations in eye color or wing shape) and subsequent selective mating to generate numbers of progeny suitable for quantitative analysis. This approach was not applicable to humans, limiting the success of early mapping studies to those diseases in which the genetic locus was tightly linked to the few available polymorphic loci, such as the ABO blood group antigen locus. The discovery of abundant DNA polymorphisms with significant levels of interindividual variation made linkage mapping feasible in humans.

Linkage analysis is a statistical method in which genotyping data from a pedigree are evaluated for the probability that two loci (markers) are inherited together because they are in physical and thus genetic proximity, compared with the probability that the data can be explained by the null hypothesis that the markers are not linked and are being inherited independent of each other. The statistic commonly used is the LOD ("logarithmic odds") score, which represents the logarithm of the probability of linkage divided by the probability of no linkage. An LOD score greater than 3.3 traditionally is taken as the threshold to declare linkage, because it represents a 1000:1 probability ratio in favor of linkage, corresponding to a p value of approximately .05.

Genetic and Physical Map

An extensive set of polymorphic markers was used to genotype a collection of large multigenerational pedigrees for the construction of a human genetic linkage map. In contrast with the mapping of genes responsible for physical traits in model organisms, the human genetic map established genetic distances between anonymous polymorphic markers. These markers were concurrently mapped to physical locations by a variety of methods such as fluorescence in situ hybridization (FISH) mapping (see later on), allowing the coordination of genetic loci to physical chromosomal locations. The mapping of markers onto genomic clones—YACs or BACs—by hybridization or PCR amplification allowed the identification of clones containing common markers that were therefore overlapping. Collections of overlapping clones were organized by computational analysis into large contiguous assemblages ("contigs") that allowed the ordering of data generated by sequencing the cloned DNA.

Complexity in Eukaryotic Genomes

Among the interesting facts to emerge from the Human Genome Project was that the total number of genes, as defined by transcribed open reading frames, was not significantly higher in humans than in lower organisms such as the fruitfly, *D. melanogaster,* or the roundworm, *C. elegans.* The greater complexity of mammals is attributable in part to more flexible utilization of the available exons in a transcriptional unit. By alternatively splicing a common set of exons, transcripts encoding proteins with distinct or even opposing activities can be generated. In addition, proteins can be modified in numerous ways that may affect localization or function (e.g., glycosylation, phosphorylation, lipidation, proteolysis). Thus, the complement of proteins generated from the genome—the proteome—is much larger than the number of genes, or discrete transcriptional units.

The study of the proteome, or *proteomics,* has become a new focus of interest in the "postgenomic" world. Recent innovations in protein separation technologies—tandem mass spectrometry, multidimensional gel electrophoresis, and protein tissue array analysis—have accelerated the ability to identify and characterize the protein complement of cells or tissues of interest, as well as their modifications, localization, and interactions. The ability to isolate and characterize individual proteins and to profile protein changes qualitatively and quantitatively from tissues of interest (healthy versus diseased), in conjunction with genome-scale characterization of diseased and normal tissue (e.g., expression profiling, cataloguing sequence polymorphisms), is anticipated to facilitate the identification of protein biomarkers, the discovery of candidate therapeutic drug targets, and discrimination of individual patient responses to a given treatment. Proteomics complements other functional genomics approaches, including microarray-based profiles, systematic phenotypic profiles at the cell and organism level, systematic genetics, and small molecule–based arrays. As these approaches migrate from the research laboratory to the clinic, they will provide the tools for the practice of more personalized medicine.

Regulatory RNA

Efforts to characterize the complete set of expressed RNAs in humans and higher mammals identified a set of transcripts in the EST database that did not contain identifiable open reading frames (ORFs). These noncoding RNAs, which initially were thought to represent truncated transcripts or cloning artifacts, more recently have been recognized to correspond to a significant fraction of mammalian transcribed sequences, and to play important roles in the regulation of gene expression.[1] Regulatory RNAs in mammalian cells, particularly micro-RNA and small interfering RNA (siRNA) sequences, are now known to possess the ability to down-regulate gene expression—something previously observed only in simpler eukaryotes. This property has been widely exploited at the level of preclinical basic research, and RNA-mediated down-regulation is now being considered as a therapeutic approach for a number of diseases.

GENETIC BASIS OF HUMAN DISEASE

Identification of Disease-Associated Gene Mutations

Currently available molecular tools allow the efficient mapping and identification of loci underlying single-gene (mendelian) disorders. Families transmitting a disease of interest can be genotyped using polymorphic markers spaced at equal intervals (approximately 10 cM) across all chromosomes to determine the inheritance of maternal and paternal alleles. The probability that a given marker is close to the disease locus can then be calculated by comparing the inheritance of marker alleles and disease affectation status by linkage analysis. To identify the genetic basis of the trait under study, candidate genes near the disease locus are analyzed for mutations, with priority given to genes having an expression pattern or known function relevant to the disease manifestations. Before the availability of sequence and map information produced by the Human Genome Project, this latter stage required the construction of a physical map of a YAC or BAC contig spanning the minimal genetic interval containing the locus. These clones were then used to identify new polymorphic markers and to map candidate genes onto the region of interest. By eliminating the need to perform these last two laborious and time-intensive steps, the sequence and map information produced by the Human Genome Project has dramatically accelerated the rate at which genes underlying mendelian diseases

are being identified by this positional cloning approach. These endeavors have resulted in the identification of disease-causing mutations both in well-studied and well-characterized genes as well as in genes without previously known functions.

Atypical Inheritance

As increasing numbers of genetic disease loci have been characterized at the molecular level, it has become apparent that numerous diseases can result from mutations at any of several genes; such diseases are said to possess genetic heterogeneity. Mutation analysis studies of genetically heterogeneous diseases have led to the discovery of interesting exceptions to traditional mendelian inheritance. Aganglionic megacolon (Hirschsprung disease) is a heterogeneous disease that can show autosomal dominant, recessive, or complex inheritance. In rare cases, inheritance has been found to result from double heterozygosity for single mutant alleles at each of two different genes that can independently cause the phenotype in a mendelian fashion.[2,3] In some cases of Bardet-Biedl syndrome, a syndrome of mental retardation and obesity, disease expression requires mutation of both alleles of one gene and a single mutant allele at one of two other genes—an example of triallelic inheritance.[4] In both disorders, genetic complexity is intermediate between simple monogenic disease inheritance and multigene complex inheritance.

Complex Inheritance

The inheritance of complex traits is thought to involve multiple loci, with environmental factors contributing to phenotypic expression. Trait expression can involve quantitative features like weight and blood pressure that are distributed in a gaussian fashion in the population, or qualitative features that are either present or absent, such as cleft lip and palate. The former are thought to result from interactions between multiple genes with alleles that contribute positively or negatively to the expression of the final phenotype. The favored model for the latter involves the contributions of multiple liability loci, in which variants at each locus contribute additively to the expression of the disease phenotype until a critical liability threshold is passed and the trait is expressed. Examples of such traits are spina bifida and pyloric stenosis. In this model, traits with marked gender differences in incidence are assumed to reflect gender-specific differences in the liability threshold for disease expression. Occurrence of the condition in the less commonly affected gender implies inheritance of a greater burden of liability alleles, so the recurrence risk for subsequent siblings is higher than if the affected child is of the more commonly affected gender.

Because of the involvement of multiple loci whose relative contribution may differ from pedigree to pedigree, linkage studies have been largely unsuccessful in dissecting the genetic basis of complex traits. However, if a single locus contributes substantially to the heritability of a trait, the locus may be detectable by standard linkage analysis. The first successful example of disease gene discovery for a complex trait was the identification of variants in the gene *NOD2* contributing to the expression of Crohn disease.[5,6] Additional complex disease-associated variants have been successfully identified by association and linkage studies, particularly by investigators focusing on relatively genetically isolated populations. These successes are setting the stage for future diagnostic and therapeutic advances in the future.

The International HapMap Project

After the completion of the Human Genome Project, a new effort was organized to characterize the genetic variations present in different human populations as discrete blocks of co-inherited polymorphisms, or haplotypes (Figure 2-3). This effort, the International HapMap Project, is a multinational collaborative effort to collect genetic data from four populations derived from Africa, Asia, and Europe. By determining which polymorphisms are likely to be inherited as a distinct haplotype in these populations, the HapMap holds the potential to streamline the discovery of genetic variants that contribute to the susceptibility for complex disorders. Recently, HapMap data were used in documenting the rapid spread of polymorphisms that confer survival advantages through human populations (e.g., the evolutionary origin of lactose tolerance in Northern Europeans and isolated tribes in Africa), illustrating that the potential benefits of the effort are rapidly becoming a reality.[7,8]

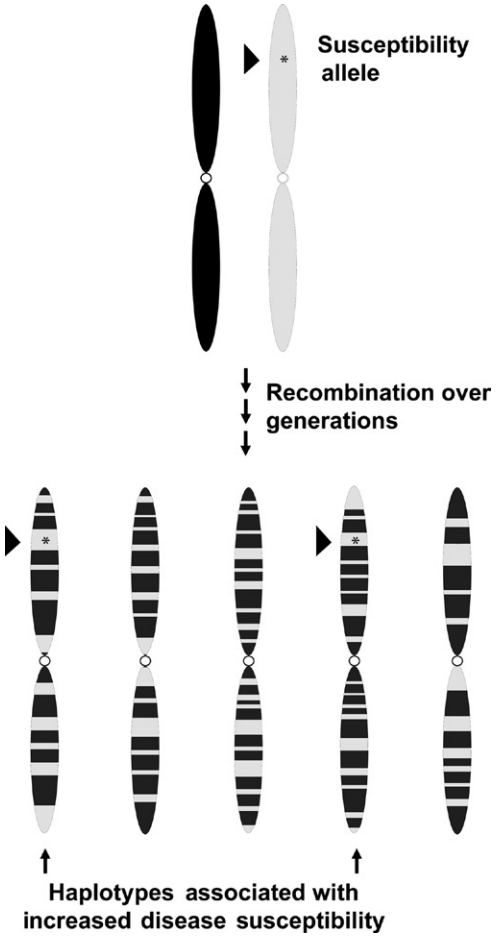


Figure 2-3 Ancestral disease susceptibility alleles exist on conserved haplotype blocks. Genetic variants predisposing to disease susceptibility (*, indicated by arrows) arose on ancestral chromosomes that have subsequently undergone recombination through meiosis over succeeding generations. In contemporary populations, the disease-predisposing variants occur on haplotype blocks conserved from the ancestral chromosomes.

DISEASE INHERITANCE AND MOLECULAR PATHOGENESIS

Diseases caused by mutations of single genes may be inherited in either dominant or recessive fashion. The observed inheritance pattern will depend on the effect of the mutation on the encoded protein. Mutations may inactivate, dysregulate, or enhance protein function. By cataloguing the distribution and types of mutations (e.g., missense, nonsense) causing a given disease phenotype, the effect on protein function can sometimes be deduced.

Null Alleles

Mutations that abolish protein expression or function produce a *null allele.* Potential mechanisms include mutations that interfere with transcription (promoter mutations), prematurely terminate the open reading frame (nonsense, frameshift, splicing mutations), or alter catalytically or structurally critical residues (point mutations, small in-frame insertion or deletions). If cellular function is not compromised by the diminished abundance of wild type protein, a recessive inheritance pattern is seen. Carriers of a single null allele of a gene encoding an enzyme are usually healthy, but mutation of single alleles encoding structural proteins or transcription factors may cause disease, as discussed further on.

Familial cancer syndromes caused by mutations in tumor suppressor genes are an interesting exception to the rule that mutations in genes for which a single allele is sufficient for normal cellular function will manifest recessive inheritance. Cells carrying a null mutation in a tumor suppressor gene grow normally. Uncontrolled growth occurs when the remaining wild type allele is mutated by a second, independent event. Because both alleles must be impaired for expression of the phenotype, this is an example of a recessive mechanism at the cellular level. At the organismal level, the large number of total cells makes the probability of a second mutation event in at least one cell quite high. Thus, despite the recessive mechanism observed at the cellular level, familial cancer syndromes caused by loss of tumor suppressor genes usually are inherited in a dominant fashion.

Haploinsufficiency

For some proteins, such as those expressed as part of highly regulated developmental programs, total abundance may be critical to the execution of their function. When a single copy of an allele is inadequate for normal function or development, carriers of null mutations are symptomatic—a mechanism known as *haploinsufficiency*. Examples are transcription factor mutations, in which decreased transcript abundance results in secondary diminution of downstream effector protein levels, and heteromeric structural protein mutations, such as in collagens, in which the proper stoichiometry of the component subunits is important. Transmission of the null allele is sufficient to cause disease, so dominant inheritance is observed.

Dominant Negative Mutations

Mutations may abolish protein function in a domain-specific manner so that some activities are lost, while others are maintained. Potential mechanisms include missense mutations or in-frame deletions that affect a single functional domain and truncations that ablate distal domains. Mutant proteins retaining some aspect of function can be more deleterious for cellular function than complete absence of an allele if the mutant prevents the available wild type protein from performing its function. This mechanism is exemplified by mutation of a transcription factor so that transactivation is lost but DNA binding is normal, thereby preventing the protein encoded by the wild type allele from binding to the recognition sequence and activating transcription. Proteins that are components of a multimeric complex, such as the individual collagen chains forming a heterotrimeric collagen fibril, can have dominant negative effects when the incorporation of a mutant subunit impairs the assembly of the functional unit.

Activating Mutations

Mutations can directly (by active site alteration) or indirectly (by disabling a regulatory domain) enhance the activity of a protein. In the case of signal transduction proteins such as kinases, inappropriately regulated enzymatic activity may initiate a signaling cascade that stimulates cell division and sets the stage for cellular transformation. As the abnormal activity of the mutant allele overrides the properly regulated activity of the wild type allele, dominant inheritance is observed.

TRADITIONAL AND MOLECULAR CYTOGENETICS

Chromosomal abnormalities, both numeric and structural, are the leading cause of fetal loss, developmental and congenital abnormalities, and apparent infertility. Although some of these result in live births, many are responsible for lack of embryonic implantation, growth, or survival. Of the numeric abnormalities, the only ones associated with the potential for survival are trisomies 21, 13, 18, 22, and rarely 9, along with the sex chromosome abnormalities (XXX, XXY, XYY). The only monosomy that permits survival (although more than 95% of affected fetuses abort in the first trimester) is 45,X. Because virtually all of these abnormalities are the result of meiotic nondisjunction, observation oftrisomies and monosomies of every chromosome would be expected. A few trisomies can be found among spontaneous abortions, especially trisomy 16. Calculation of the expected frequency of the discovered and the nonviable trisomies and monosomies predicts that approximately 75% of fertilized ova are chromosomally abnormal, most of which never implant or result in miscarriage early in pregnancy, appearing as "late" menstrual periods. In a study by Wells and colleagues,[9] 9 of 10 polar bodies studied by comparative genomic hybridization (CGH) (discussed further on) had either one too many (i.e., 24) or one too few (i.e., 22) chromosomes—a proportion not dissimilar from the theoretical prediction. Among the chromosomes involved, some were identified that are never seen in viable fetuses, even those studied after spontaneous abortions. Another form of numeric abnormality leading to multiple congenital malformations and fetal demise, stillbirth, or, rarely, neonatal death is triploidy (69 chromosomes). This abnormality can result from dispermy in a single ovum or one sperm with two female germ cells, probably an ovum and a polar body.

Numeric abnormalities also can result from mitotic, postfertilization events, which generally lead to mosaicism. *Mosaicism* should be distinguished from *chimerism,* resulting from fusion of two separate embryos into one individual embryo. In the latter condition, the affected infant is born alive and exhibits generally normal development but can be infertile or a true hermaphrodite if the two initial embryos are of different genders. Mosaicism, on the other hand, results from either mitotic nondisjunction or anaphase lag. If the abnormal event occurs in the first cell division after fertilization, an embryo with two cell lines will result: In the case of mitotic nondisjunction, they both will be abnormal (45 and 47 chromosomes each, with monosomy and trisomy for the nondisjoined chromosome); in the case of anaphase lag, one cell line will be normal and the other will be missing a chromosome. Anaphase lag is the cause of many cases of mosaic Turner syndrome, when the involved chromosome is an X or a Y. The source of the nondisjoined chromosome has clinical significance because if it was a Y, the resulting mosaic individual may have Turner syndrome with testicular rests leading to a risk for gonadal tumors. If mitotic nondisjunction occurs later in embryonic development, the affected individual will have three cell lines—normal, trisomic, and monosomic. This mechanism accounts for mosaic Down syndrome cases (46 and 47 chromosomes, the latter with trisomy 21); in affected persons, the predicted monosomy 21 cell line dies off because of nonviability of autosomal monosomic cells.

The other major category of chromosomal abnormalities is structural alteration of one or more chromosomes. A single chromosome may be abnormal as a consequence of a deletion or a duplication of a piece of chromosomal material ranging in

size from a few genes to whole chromosomal arms. The resulting abnormalities and viability of the fetus will vary depending on the amount and genetic content of the material deleted and duplicated. Breakage and recombination of fragments between two or occasionally more chromosomes also will result in structural variation. If the exchange is achieved without loss or gain of material, the translocation is balanced and should not result in abnormalities unless a gene is damaged at the breakpoints. Of note, however, a normal carrier of such a balanced translocation can produce gametes that have duplications of one of the exchanged fragments, along with deficiency of the other (unbalanced translocation). Because the number of possible rearrangements is virtually infinite, the phenotype can range from one characterized by essentially normal features or mild anomalies to one featuring severe anomalies and death in utero or after birth.

Chromosomal Basis of Nonmendelian Disease

Over the past several years it has become apparent that nonmendelian behavior of chromosomes and their genes can be the result of a variety of mechanisms. Some of these mechanisms and their alterations lead to a variety of developmental defects. Among these are genetic imprinting, uniparental disomy, the formation of neocentromeres, and mitochondrial inheritance.

Imprinting results in the expression of certain genes exclusively from the chromosome derived from one of the two parents in a gender-specific fashion. The allele derived from the parent of the opposite gender is silent (imprinted). This parent of origin–specific expression appears to involve differential methylation of the regions controlling imprinted genes during formation of gametes. It is this phenomenon that accounts for the lack of viability of embryos derived from a single parent. Embryos with a chromosomal complement derived only from the male have poorly functioning placentas, whereas those with entirely female-derived chromosomes do not develop properly develop fetal tissues which may lead to formation of a hydatidiform mole. Absence of the active allele by deletion or mutation results in abnormalities of the offspring. The type of abnormality is determined by which of the imprinted genes is absent. For example, the proximal long arm of chromosome 15 has two adjacent areas, one of which is paternally imprinted and the other maternally imprinted. Deletion of the paternal imprinted genes results in Prader-Willi syndrome, whereas absence of the maternal genes results in Angelman syndrome, both associated with distinct patterns of developmental defects. Disturbances in an imprinted gene in the short arm of chromosome 11, most commonly caused by relaxing imprinting and allowing both alleles to be expressed in an imprinted area, results in an overgrowth syndrome, Beckwith-Wiedemann syndrome, which also is associated with tumor formation and other abnormalities. This disorder appears to be due to overexpression of a growth factor in the usually imprinted region. Chromosomal regions known to contain imprinted genes are shown in Figure 2-4.

One of the mechanisms by which absence of the active allele at an imprinted locus or a double expression of the active allele can occur is *uniparental disomy*. In uniparental disomy, both members of a chromosome, in some cases, a part of a pair of chromosomes, are derived from the same parent. If imprinted genes exist in this pair, the phenotypic result will depend on whether the pair derives from the mother or the father, and on which parent contributes the imprinted gene. As an example, with maternal uniparental disomy of chromosome 15, the active allele that is derived from the father will be absent, and the result will be Prader-Willi syndrome, as if the paternal allele had been deleted. Similarly, with paternal uniparental disomy of chromosome 15, the active maternal allele at the Angelman locus will be absent, and Angelman syndrome will result, just as if the maternal allele had been deleted.

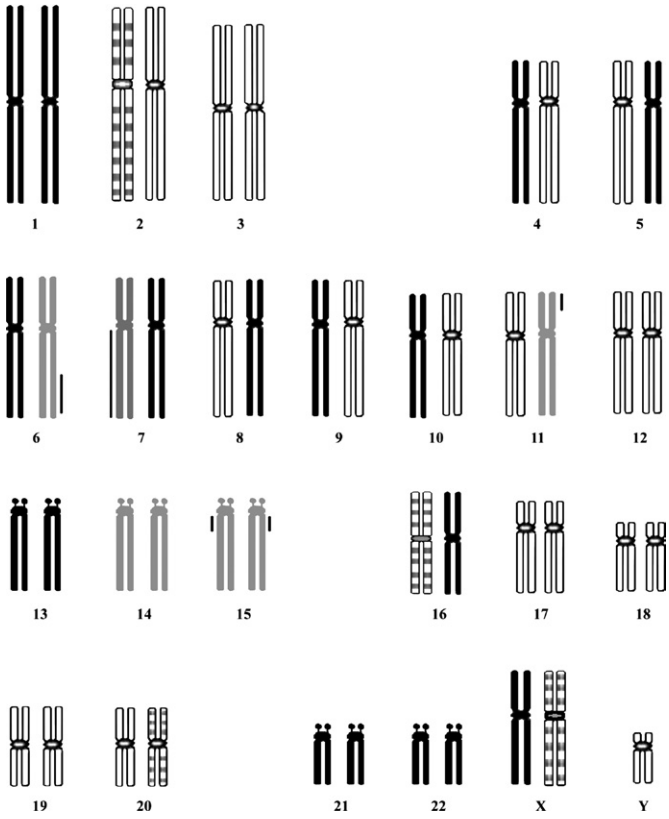


Figure 2-4 Imprinted chromosomal regions. Data are from a survey summarizing areas of human chromosomes that are known to be imprinted *(gray)* with the imprinted region indicated by adjacent bars is applicable. Chromosomes that are possibly imprinted are hatched, those unlikely to be imprinted are indicated in *shaded in black*, and those without any UPD cases reported are *unshaded*. Maternal chromosomes are shown at left of each pair. The figure can be viewed as an interactive webpage document (http://genes.uchicago.edu/upd/) with links to citations supporting the summary data. *(Courtesy Dr. David Ledbetter, with permisssion.)*

Uniparental disomy comes in two forms. Uniparental *heterodisomy* occurs if the embryo inherits both members of a pair of chromosomes from one parent, whereas in uniparental *isodisomy,* two copies of a single chromosome from the pair are transmitted. In addition to the effect on imprinted genes, uniparental isodisomy also results in homozygosity for the entire chromosome or for the part that is disomic, including any mutant genes that happen to be on that chromosome. In fact, the first proven case of human uniparental isodisomy was in a child with cystic fibrosis who had received both members of a single maternal chromosome 7 that happened to contain the cystic fibrosis mutation. The child also was unusually short, because of the presence of an imprinted gene with a strong impact on growth.

None of these observations would be possible without the technical advances allowing detailed examination of the chromosomes. After discovery of methods to produce specific banding patterns in the early 1970s, permitting identification of each chromosome and its major parts, additional breakthroughs led to the ability identify increasingly smaller parts of chromosomes by fluorescence in situ hybridization (FISH) (Figure 2-5), identification of each chromosome in a single karyotype by multicolor FISH, and most recently, CGH. CGH is done by double hybridization of patient DNA labeled with one fluorochrome and normal DNA labeled with a different fluorochrome with normal

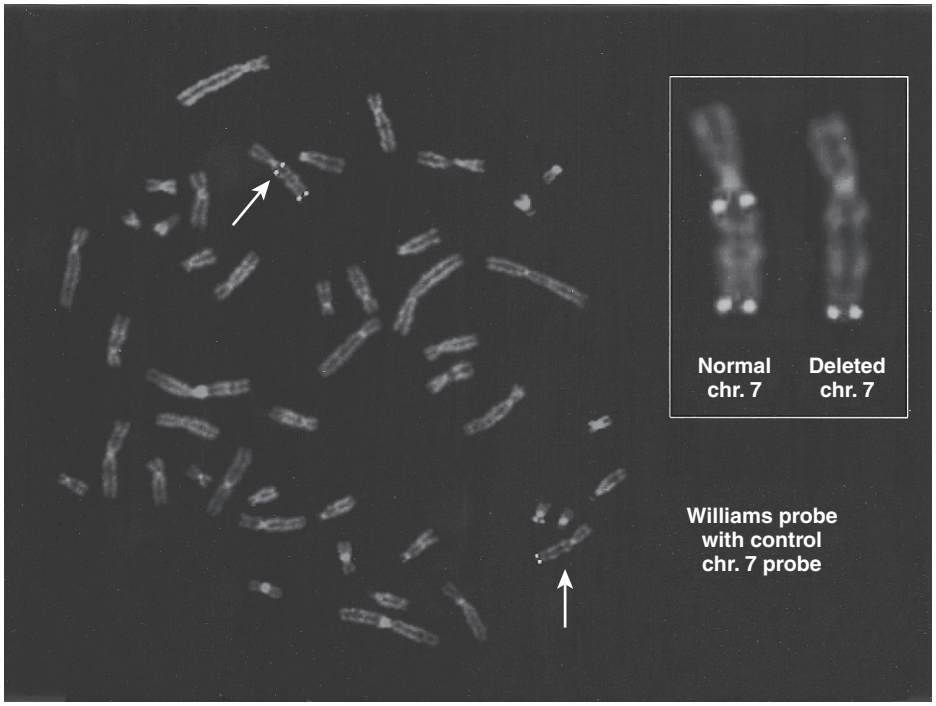


Figure 2-5 Fluorescence in situ hybridization (FISH). Diagnosis of chromosomal microdeletion syndromes has been revolutionized by molecular cytogenetic techniques allowing metaphase chromosomes to be analyzed for the presence of specific sequences using fluorescently labeled cloned DNA as probes. In this example, a telomeric probe is seen hybridizing to each replicated pair of chromosome 7; a probe specific for a region that is recurrently deleted in patients with Williams syndrome is absent from one pair of sister chromatids *(arrows). (Courtesy Dr. Brynn Levy.)*

metaphase chromosomes on a slide. Using a computerized camera and microscope, this technique permits the discovery and exact identification of missing and extra material in any sample, even when the sample cannot be grown in culture (as with products of conception, for example). More recent application of CGH to DNA probes on microarrays has allowed the discovery of the smallest duplications and deletions, even from a single cell. These new techniques have rapidly led to far more accurate delineation of the causes of developmental and congenital abnormalities.

Genomic Disorders and Gene Dosage

A number of human genetic disorders are now known to result from loss or gain of genetic material on a smaller scale than that for disorders caused by cytogenetically observable events (i.e., in the range of hundreds of kilobases to several megabases). Deletions and duplications as small as 100 base pairs were rendered easily detectable with the advent of FISH technology. This advance set the stage for the molecular characterization of diseases in which a recurrent deletion of a specific genomic interval caused a characteristic phenotype, such as in DiGeorge syndrome. Studies of this syndrome and other diseases caused by deletion or duplication of genomic segments (see Table 2-1) have identified the features in the genomic DNA that predispose to these events.

The presence of low-copy DNA repeats having relatively high levels of sequence conservation may predispose to deletion or duplication events if the repeats occur in the same orientation (tandem repeats) or to inversion events if the repeats are in opposite orientations (inverted repeats). Unlike the more abundant high-copy repeats present in human DNA (e.g., *Alu* retrotransposon insertions), low-copy repeats exist as duplicated segments with extensive homology over relatively long distances (up to tens of thousands of base pairs). Inappropriate pairing between repeat elements at meiosis could thus result in the loss or gain of intervening genomic DNA segments as long as several million base pairs. Such an event would not necessarily change the coding sequence of genes on the deleted chromosome but would change the copy number of genes in the affected interval, leading to haploinsufficiency for all of the genes contained within the deleted interval. Conversely, in a duplication event, the dosage for all of the affected genes would be increased. Both aspects of this principle have been well illustrated in the case of neurologic disease caused by gain or loss of the peripheral myelin gene, *PMP22* (reviewed by Inoue and Lupski[10]). *Deletion* of the locus results in type 1 Charcot-Marie-Tooth disease. *Duplication* of the locus leads to increased dosage of *PMP22,* resulting in hereditary susceptibility to neuropathy and pressure palsies (HNPP). Of interest, persons who are homozygous for such a duplication (gene dose of four instead of two) are more severely affected than those heterozygous for the mutation. Proof of the principle is further provided by gain-of-function mutations in *PMP22,* in which the gene dose is unchanged but the clinical phenotype mirrors the effect of a duplication.

The change in gene copy number is necessarily deleterious in events detected in patients with genetic diseases, but copy number changes also have been found to occur as polymorphisms in human populations. The contribution of these copy number changes to disease burden is not yet known, but in at least one case, gene copy number polymorphisms have been shown to modulate the course of a common human disease. The copy number of the inflammatory chemokine gene, *CCR3L1,* is polymorphic across and within different human populations, resulting in differences in serum CCR3L-1 protein levels. Cohort studies of humsn immunodeficinecy virus (HIV)-infected patients have revealed that *CCR3L1* copy number higher than the mean observed in a given population was associated with decreased infection rates and slower progression of HIV disease and acquired immunodeficiency syndrome (AIDS). This effect, presumably mediated by increased CCR3L-1 ligand occupancy of the HIV co-receptor, CCR5, was most evident in the setting of CCR5 alleles associated with enhanced disease progression.[10] This first example of gene copy number polymorphisms modulating human pathologic states is unlikely to be the last.

CONCLUSION AND FUTURE DIRECTIONS

The massive amount of information made available by the Human Genome Project has already shown its power in allowing the mapping of many hundreds of disease genes and the cloning of

a high proportion of these. The methods developed by the project, such as high-throughput genotyping, have become widely used and are beginning to be applied to a much broader set of genes associated with susceptibility to common diseases, such as cardiovascular disease, diabetes, and obesity. Clearly, a number of conditions applicable to fetal development will fall into this effort, including prematurity, intrauterine growth restriction (IUGR), and birth weight. The search for such genes until now has been conducted primarily by association studies, many of which have not been replicated or replicable. As noted in a review by Hirschhorn and colleagues,[12] of approximately 200 such associations found in more than 600 references in the literature, only 5 were clearly replicable, with highly suggestive results for another 20 or so. Nevertheless, with application of increasingly powerful methodologies to the search for associations between alleles for specific genes and disease susceptibility, this number is likely to grow steadily.

Another potentially highly useful application of such studies is in the field of pharmacogenomics. A number of genes have already been identified in which polymorphisms lead to large differences in individual responses to therapeutic agents. It is suspected that some of these may even be related to whether or not a particular drug is teratogenic in a particular fetus. The ability to tailor types and amounts of medications to different patients would be an enormous benefit to therapeutic interventions by reducing risks of drug reactions or side effects.

Finally, the long-hoped-for practical use of gene therapy may grow in the near future. The successful treatment of several children with X-linked severe combined immunodeficiency[12] has given hope for the application of treatment of deleterious mutations by gene replacement. The first success has definitely been helped by the fact that corrected immune cells have a selective advantage over the abnormal cells and therefore replace them to produce a cure of the disease. This success was predictable from previous findings that reversion of deleterious mutations in such cells led to a reversion to relative normality in an otherwise fatal illness.[14] Prenatal gene therapy has been attempted in immunodeficiency with some apparent success, but the occurrence of lymphoproliferative disease caused by insertion of transgenes upstream of proto-oncogenes has tempered the initial enthusiasm for this approach. Until methods for gene replacement are worked out for many other diseases, advances in product replacement, particularly for lysosomal storage diseases, are expanding.

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WEB-BASED RESOURCES

Online Mendelian Inheritance in Man: http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim
A database of human genetic diseases providing a clinical overview and survey of relevant literature.

National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/
A collection of links to literature, genome maps, sequence repositories and genomic analysis tools.

HapMap Homepage: http://www.hapmap.org/
Link to background information and publicly available genetic analysis data, genome maps, and genomic analysis tools.

3 Prenatal Diagnosis

Louise Wilkins-Haug

Currently, the prenatal diagnosis of a fetal genetic disorder or a chromosome abnormality requires invasive testing; all of the tests in use carry small but recognized risks of miscarriage (0.5% to 2%). Accordingly, an important aspect of prenatal care is screening to identify those women who face an increased risk of a pregnancy complicated by a genetic disease or congenital malformation. Such screening modalities include review of the clinical history for both the patient and her partner, evaluation of maternal serum markers, and ultrasound examination in both the first and second trimesters. Ultimately, however, the definitive diagnosis of a genetic condition or chromosome abnormality in the fetus requires fetal nucleic acids obtained by chorionic villus sampling (CVS), amniocentesis, or percutaneous umbilical blood sampling (PUBS). Under investigation are new methods of assessing fetal DNA in the maternal serum, which could provide, for the first time, prenatal diagnosis without the risk for miscarriage.

SCREENING

Because women with "positive screens" (risk greater than a predetermined cutoff), indicating increased risk, often proceed to an invasive prenatal diagnostic test with an inherent risk of miscarriage, screening methods should strive for a high level of detection with the lowest screen-positive rate. Concepts such as *screen-positive rate* (the number of women with an increased risk among those undergoing testing identified on the screening test), *positive predictive value* (the chances of an abnormal result among the screen-positive group), and *detection rate* (number of abnormal fetuses identified from within the screened population) provide useful parameters to compare screening approaches. Additionally, knowledge of the gestational age at which screening can be performed is important and may influence pregnancy options.

Parental Clinical History

Parental Age

A long-recognized increase in aneuploidy as women become older is a cornerstone of prenatal diagnosis. For women who are 35 years of age at delivery, the chance of having a newborn with Down syndrome (trisomy 21) frequency is approximately 1 in 308 pregancies. Because trisomy 21 is associated with increased risk for miscarriage and stillbirth, for a 35-year-old woman the chances are actually higher that at amniocentesis (1 in 258) or CVS (1 in 175) Down syndrome will be diagnosed. Although maternal age was the first screening criterion for Down syndrome, it performs poorly when assessed at a population level. Approximately 15% of women have children at age 35 years or older (screen-positive rate), and the likelihood in this subgroup women that a pregnancy is complicated by Down syndrome (positive predictive value) is only 1% to 3%. Furthermore, the detection rate is only approximately 20%; less than one fourth of Down syndrome infants are born to women in this older maternal age subcategory. When evaluated by these screening parameters, the utility of maternal age over 35 years alone as an indication for an invasive prenatal diagnostic test has been challenged.[1] Genetic conditions associated with the father's age are more difficult to delineate but include an increased risk for dominant mutations as exemplified by achondroplasia.[2]

Reproductive History

Assessment of the couple's reproductive history also may signal an increased genetic risk to the pregnancy. A history of repeat miscarriages (2 or more) is associated with an increased risk for parental balanced translocation (6.8%). Other reproductive outcomes such as a previous malformed stillbirth along with a single miscarriage also are associated with an increased risk for a parental balanced translocation, (5.4%).[3] A history of three or more consecutive first-trimester abortions carries a 9.6% risk of a parental balanced translocation. [4] Similarly, repeated failure of vitro fertilization cycles (for more than 10 cycles) attributable to poor implantation is associated with an increased risk of a parental balanced translocation of 2.5%.[4] By comparison, the overall rate of balanced translocations in newborns is 0.2%.[5] A balanced translocation increases the person's risk that offspring may inherit an unbalanced complement of chromosomes, with associated implications for mental and physical delays.

In addition to previous pregnancies, a diagnosis of infertility warrants closer examination of the identified etiologic disorder and the possible recommendation of prenatal diagnostic testing. Balanced translocations and sex chromosome aneuploidy occur in 14.3% and 6.5% of men with absent and low sperm counts, respectively.[6] In addition, with male factor infertility related to obstructive azoospermia, congenital absence of the vas deferens (CBAVD) is a common diagnosis. Of men with CBAVD, almost two thirds carry at least one mutation in the gene responsible for classic cystic fibrosis (i.e., the cystic fibrosis transmembrane receptor gene [*CFTR*]). Almost half (54.5%) of the men are double heterozygotes, possessing two mutations for classic cystic fibrosis, although most often the second mutation is a variant specifically associated with infertility and not classic cystic fibrosis.[7] Because men with CBAVD can father children through assisted reproduction using intracytoplasmic sperm injection, carrier screening of the female partner is critical in view of the relatively high carrier frequency—1 in 25 in the white population. Couples in which both members carry a *CFTR* mutation face a 25% risk of having a child with cystic fibrosis; this finding emphasizes the importance of delineating the specifics of male factor infertility.

Female factor infertility also may have an underlying genetic etiology with subsequent risk to the offspring. In particular, poor ovarian reserve and oligomenorrhea or amenorrhea may reflect a premutation of fragile X. Classically, 3% of cases of sporadic premature ovarian failure (POF) and 13% of cases of familial POF are associated with a premutation fragile X.[8] Of significance for female factor infertility, earlier menopause in women with a premutation of fragile X heightens the possibility that these women will seek infertility evaluation and treatment with a diagnosis of poor ovarian reserve.[9] In view of an overall frequency of fragile X premutations in the general population of approximately 1 in 200 women, many infertility centers now are more likely to offer screening for fragile X to women. For fragile X premutation carriers, the implications for the offspring reflect the degree of expansion of the fragile X site (as discussed next under family history screening).

Family History

Finally, review of the clinical history for both parents includes an assessment of family history. Ethnicity and country of origin are now routinely ascertained at preconception and prenatal visits. Various diseases of an autosomal recessive nature occur with increased frequency among specific populations reflecting historical physical or cultural constraints to gene migration. For some disorders, disease distribution is widespread and warrants screening in essentially all individuals. Cystic fibrosis is an example of such an autosomal disease. Current recommendations are to offer *CFTR* carrier screening to all women, ideally in the preconception period, with education regarding disease frequency and sensitivity of testing within the patient's specific ethnicity (Table 3-1). In persons of Northern European heritage, the carrier frequency is 1 in 25, with screening detecting 88% of the carriers. However, in populations in which cystic fibrosis is less common, such as Asians (carrier frequency of 1 in 94), screening detects only 49% of carriers. In any population, screening can reduce but not totally negate the presence of a *CFTR* carrier.

Several autosomal recessive disorders occur more frequently within specific populations, and screening is then specifically directed by the individual patient's race or ethnicity. For example, hemoglobinopathies are more common in people of African, Mediterranean, or Asian origin. The carrier state for sickle cell occurs in approximately 1 in 12 persons of African American ancestry, and hemoglobin electrophoresis is the preferred method of screening. In some populations, further assessment for a hemoglobinopathy is warranted in the presence of a low mean corpuscular volume (MCV). β-Thalassemia will be detected by hemoglobin electrophoresis, whereas low MCV without iron deficiency and with a normal hemoglobin electrophoresis is suggestive of α-thalassemia. Further diagnosis of this carrier state would require molecular diagnostic testing based on the individual patient's country of origin. Among persons of Ashkenazi Jewish heritage, the American College of Obstetrics and Gynecology recommends carrier screening for Tay-Sachs disease, cystic fibrosis, Canavan disease, and familial dysautonomia.[10] For each of these disorders, the carrier frequency is sufficiently increased and the molecular diagnostic tests are sufficiently sensitive to meet the criteria for a prenatal screening test. As with all autosomal recessive disorders, any offspring would have a 25% chance of inheriting the disease in question if both parents are carriers.

For couples in which only one person is of Eastern European Ashkenazi Jewish heritage, the recommendation remains to offer screening but with the knowledge that in non–Ashkenazi Jewish populations the carrier frequency is lower and typically is not established. Furthermore, among non–Ashkenazi Jewish persons, the sensitivity of the molecular diagnostic tests for specific disease mutations is substantially less. For example, in screening for Tay-Sachs disease carriers in a non–Ashkenazi Jewish population, the recommendation is to use a functional assay with 98% detection, compared with detection by molecular diagnostic tests of only 50%. Persons of Ashkenazi Jewish heritage also may avail themselves of information regarding additional autosomal recessive diseases (Table 3-2). For couples in which only one member is of Ashkenazi Jewish heritage, however, the constraints of accurate screening in the non–Ashkenazi Jewish persons remain, with functional assays available only for Tay-Sachs disease. For couples in which both members are carriers for these autosomal recessive disorders, prenatal diagnosis is possible with use of the same molecular diagnostic tests on fetal cells obtained by either amniocentesis or CVS.[11]

Of relevance to prenatal diagnosis, a family history of mental retardation of unknown etiology or significant developmental delay or autism represents a positive screen for fragile X syndrome. Fragile X syndrome is the most common inherited cause of mental retardation and manifests with a spectrum of mental disability. The specific characteristics result from expansion of the fragile X mental retardation (FMR-1) region on the X chromosome. In most cases, 40 or fewer CGG repeats are present within FMR-1, and the region remains stable when passed from either parent to their offspring. Of note, however, some persons have inherited expansions of this repeat region, either slight (41 to 60—"intermediate range") or larger (61 to 200—"premutation range").[12] Approximately 1 in 200 women (1 in 113 to 1 in 350) carry a premutation for fragile X syndrome. When this unstable CGG repeat region expands to greater than 200 repeats ("full mutation"), increased methylation impairs translation, resulting in lack of production of the fragile X mental retardation protein (FMRP). The size of the maternal premutation allele directly influences whether further expansion occurs during meiosis.[13] (Table 3-3). Sons who inherit a full mutation have characteristics of typical fragile X syndrome. In daughters who inherit the full mutation, features of the syndrome are unpredictable because of the normal random silencing of one X chromosome (Lyon hypothesis). As many as two thirds of daughters with a full fragile X mutation may have mild to moderate retardation. Although general population screening for the premutation carrier state in women is not currently advocated, with a relatively high frequency of premutation carriers (1 in 200) and implications for disability, judicious review of the family history for characteristics of fragile X syndrome is encouraged.[14]

The family history of both partners also can yield important information regarding adult-onset dominant disorders such as Marfan syndrome, polycystic kidney disease, myotonic dystrophy, and Huntington disease. Such dominant, adult-onset disorders may be noted in one or more seemingly remote family members, with no perception of the significance for the current pregnancy. Non–disease-related death of affected persons before disease manifestation and the later age at onset of symptoms can cloud the inheritance pattern in a family. Additionally, especially in women with myotonic dystrophy, the most common adult-onset muscular dystrophy, the occurrence of congenital myotonic dystrophy with symptoms more severe than those typical of the adult-onset disease should be addressed. Disorders of recessive inheritance require attention with testing of the individual at risk for the specific DNA mutation known to be segregating within the family. Lack of knowledge of the specific mutation within a family does not prevent testing the person at risk but will limit the assurance of exclusion of the carrier state. For example, in a woman with a brother who has cystic fibrosis arising from the most common mutation, homozygosity for delta508, a negative result. for the most common cystic fibrosis mutations (which include delta508) changes her risk of being a carrier from 2 in 3 (unaffected sibling of a patient with an autosomal recessive disease) to 1 in 208 (background residual risk that any Northern European individual has for undetected carrier status for cystic fibrosis). By comparison, if her brother had not been tested, perhaps dying before molecular diagnosis, and neither parent was available, then her two-thirds empiric carrier risk could be reduced to only 1 in 15 (as a result of residual undetectable cystic fibrosis mutations in a Northern European person).

TABLE 3-1

Cystic Fibrosis Detection and Carrier Rates Before and After Testing

Racial or Ethnic Group	Detection Rate %	Carrier Rate (Before Testing)	Approximate Carrier Risk (After Negative Test Result)
Ashkenazi Jewish	94	1/24	1/400
Non-Hispanic white	88	1/25	1/208
Hispanic American	72	1/46	1/164
African American	65	1/65	1/186
Asian American	49	1/94	1/184

TABLE 3-2

Autosomal Diseases With Increased Frequency Among Persons of Ashkenazi Jewish Heritage

		ASHKENAZI JEWISH		NON–ASHKENAZI JEWISH	
Disease	**Description**	**Carrier Rate**	**Carrier Detection**	**Carrier Rate**	**Carrier Detection**
Tay-Sachs	Neurologic deterioration, death in early childhood; juvenile- and late-onset forms	1/30	98% by Hex A testing 94% by DNA	1/300	98% by Hex A testing 50% by DNA
Canavan	Neurologic deterioration; death during early childhood, with some survivors into teens	1/40	98% by DNA	Undetermined	60% by DNA
Cystic fibrosis	Chronic pulmonary disease, pancreatic insufficiency, variable survivorship	1/29	97% by DNA	Varies by ethnicity	Varies by ethnicity
Familial dysautonomia	Impairment of sensory and autonomic nervous systems	1/32	99% by DNA	Unknown	Unknown
Fanconi anemia group C	Pancytopenia; developmental delay and failure to thrive	1/89	99% by DNA	Unknown	Unknown
Niemann-Pick type A	Lysosomal storage disease with degenerative course similar to that in Tay-Sachs	1/90	95% by DNA	Unknown	Unique mutations, enzymatic levels poorly discriminate normal and carrier states
Mucolipidosis IV	Neurodegenerative disorder with marked developmental and growth retardation	1/127	95% by DNA	Unknown	Unknown
Bloom	Pre- and postnatal growth restriction, susceptibility to malignancies	1/100	95% by DNA	Unknown	Unknown
Gaucher	Type 1—variable severity secondary to deposition in spleen, liver, and bones; presentation from chronic illness to asymptomatic	1/15	95% by DNA	Unknown	70% by >30 mutations

Data from ACOG Committee on Genetics: ACOG committee opinion. Number 298, August 2004. Prenatal and preconceptional carrier screening for genetic diseases in individuals of Eastern European Jewish descent, *Obstet Gynecol* 104:425-428, 2004; and Preconception and Prenatal Genetic Screening Pocket Facts. March of Dimes, 2001. Available at: http://www.marchofdimes.com/catalog/product.

TABLE 3-3

Maternal Premutation Allele Size and Risk for Expansion to Full Mutation

	FULL MUTATION RISK: % (NO. OF FETUSES AFFECTED)			
Maternal Repeat Size*	**Nolin, 1996**	**Pesso, 2000**	**Toledano-Alhadef, 2001**	**Nolin et al, 2003**
55-59	13 (3/22)	0 (0/11)	0 (0/22)	4 (1/27)
60-69	21 (7/34)	12(1/8)	10 (2/20)	5 (6/113)
70-79	58 (59/102)	50 (1/2)	17 (1/6)	31 (28/90)
80-89	73 (78/107)	50 (1/2)	—	58 (81/140)
90-99	94 (83/88)	100 (1/1)	—	80 (89/111)
100-200	99 (177/179)	75 (3/4)	—	98 (194/197)

Data from Nolin SL, Brown WT, Glicksman A, et al: Expansion of the fragile X CGG repeat in females with premutation or intermediate alleles, *Am J Hum Genet* 72:454-464, 2003.

*With fewer than 200 repeats.

TABLE 3-4

Screening for Down Syndrome in the Second Trimester

	Screen-Positive Rate	**Detection Rate**
Maternal age >35 years	15%	20%
Triple test*	5%	75%
Quad test†	5%	80%
"Soft marker"‡ on fetal survey ultrasound examination	13%	50%
≥ two markers, thickened nuchal fold, or structural anomaly	6%	75%

*Triple test: maternal age + alpha-fetoprotein, β-human chorionic gonadotropin (β-hCG), and estriol assays.

†Quad test: triple test + inhibin assay.

‡Defined as increased nuchal fold, echogenic bowel, shortened femur, echogenic cardiac focus, or renal pelvis dilatation.

Maternal Serum Screening for Aneuploidy Risk

With recognition of maternal age as a poor screening instrument for the risk of aneuploidy in a pregnancy, various approaches evolved to refine an individual woman's risk for having an infant with Down syndrome. These approaches consist of identification of protein and hormonal markers from the fetus or the placenta and can be assessed in a sample of the mother's blood. On average, when the fetus has trisomy 21, lower levels of alpha fetoprotein and estriol and increased levels of human chorionic gonadotropin (hCG) are present. As these values change during gestation, standardization to a specific week is achieved with multiples of the median (MoM) for each gestational week from 15 to 22 weeks. For an individual woman, the maternal age risk is modified by the likelihood that changes in each of these three independent markers are more likely to occur in a Down syndrome fetus. Determination of levels of these three markers, in combination with the maternal age, constitutes a "triple test." Approximately 5% of women will have a positive triple test result, with detection of 75% of fetuses with Down syndrome within this screen-positive group. Addition of a fourth marker, inhibin, converts the screen to a "quad test," with a 5% screen-positive rate and detection of 80% of trisomy 21 fetuses. These results are markedly improved over those for maternal age alone, which yields a 15% screen-positive rate and a 20% detection rate (Table 3-4).

TABLE 3-5

Common Ultrasound Findings in Fetuses With Chromosome Abnormalities

Abnormality	Ultrasound Findings
Trisomy 21	Ventriculomegaly, brachycephaly Nuchal thickening Cardiac defect—AV canal Duodenal atresia, echogenic bowel Renal pyelectasis Shortened femur/humerus; clinodactyly involving fifth digit, sandalfoot
Trisomy 18	CNS—agenesis of corpus callosum, meningomyelocele, ventriculomegaly Cystic hygroma Cardiac anomalies Congenital diaphragmatic hernia Omphalocele Clenched hands with overlapping digits IUGR with polyhydramnios
Trisomy 13	CNS—holoprosencephaly, agenesis of corpus callosum, meningomyelocele, microcephaly Cleft lip/palate, midface hypoplasia, cyclopia, microophthalmia Nuchal thickening Cardiac anomalies Omphalocele, echogenic bowel Echogenic kidneys Radial aplasia, polydactyly
Turner	Cystic hygroma Cardiac defects (coarctation of the aorta) Horseshoe kidneys Hydrops
Triploidy	CNS—holoprosencephaly, agenesis of corpus callosum, meningomyelocele, Dandy-Walker malformation Hypertelorism, micrognathia Syndactyly involving third and fourth fingers Cardiac defects Omphalocele Early-onset IUGR affecting skeleton more than head Placental abnormalities—enlarged, or small and calcified

From Benacerraf B (ed): *Ultrasound of Fetal Syndromes*, New York, 1998, Churchill Livingstone.

AV, Atrioventricular; *CNS,* central nervous system; *IUGR,* intrauterine growth retardation.

TABLE 3-6

Aneuploidy Risk Among Isolated Major Anomalies

Anomaly	Risk (%)*	Most Common
High-Risk Category		
Cystic hygroma	>50	45,X
Hydrops	>50	13, 21, 18, 45,X
Holoprosencepahly	50	13, 18, 18p−
Complete AV canal	40	21
Omphalocele	30	13, 18
Duodenal atresia	30	21
Bladder outlet obstruction	20	13, 18
Lower-Risk Category		
Hydrocephaly/ ventriculomegaly	10	21, 13, 18, triploidy
Cardiac defects	10	21, 18, 13, 22−, 8, 9
Meningomyeloceles	7	18
Anencephaly	2	
Encephalocele	10	
Limb reduction	8	18
Clubfoot	6	47,XXY, 47,XXX, 18, 21
Facial clefts	1	13, 18, 22q−
Minimal-Risk Category		
Gastroschisis—must be differentiated from ruptured omphalocele		
Hydranencephaly		
Single umbilical artery		

*Data from Nyberg D, Mahony B, Pretorius D (eds): *Diagnostic Ultrasound of Fetal Anomalies: Text and Atlas*, St. Louis, 1990, Mosby; and Sanders R, Hogge W, Spevak P, Wulfsberg E (eds): *Structural Fetal Abnormalities:The Total Picture*, St Louis, 2002, Mosby.

Risk data are estimates, which are influenced by gestational age at detection and the resolution of ultrasound images in reported studies.

AV, Atrioventricular.

Prenatal Ultrasound Examination

As maternal serum screening during the second trimester gained popularity during the 1990s, advances in ultrasound resolution also occurred. For the more common aneuploidies, characteristic patterns of ultrasound findings emerged (Table 3-5). Even when isolated malformations exist, the risk for aneuploidy can range from a few percent to greater than 50% (Table 3-6). Some of the increased risk associated with isolated ultrasonography-detected malformations is attributable to the possibility that further subtle dysmorphia or other minor malformations undetectable even by high-resolution ultrasound techniques will be present in the newborn.

As ultrasound survey in the second trimester increasingly became used for assessment of structural malformations, various minor ultrasound features gained new importance. "Soft markers" for aneuploidy include findings such as increased dimension to the nuchal fold, echogenic bowel, shortened femur, echogenic cardiac focus, choroid plexus cysts, and renal pelvis dilatation. These findings typically are transient and if not associated with aneuploidy have no functional significance in the fetus. The one exception is echogenic bowel, which may be associated with cystic fibrosis or fetal infection (typically cytomegalovirus infection). The use of a genetic sonogram, with recommendation for amniocentesis if any one of these soft markers is present, is evolving. When this modality is used as a screening tool for aneuploidy, the screen-positive rate (based on the presence of any one of these subtle markers as a positive result) is relatively high (13.0%), with a detection rate for Down syndrome of approximately 50%.[15] One modification of this approach is to offer amniocentesis if any of the following is present: two or more markers, an increased nuchal fold, or a structural anomaly. This approach decreases the screen-positive rate to 5.7% with a 75% detection rate for Down syndrome.[16] Incorporation of maternal age into this approach increases the screen-positive rate to 27.1% but also increases the detection rate for Down syndrome to 86.8%.[17]

First-Trimester Screening for Aneuploidy

With improvements in ultrasound resolution, greater clarity of fetal structures in the first trimester emerged. Between 11 and 14 weeks of gestation, visualization of additional fluid collection at the nape of the fetal neck (nuchal lucency) is a sensitive indicator of aneuploidy in the fetus. In initial studies, 70% detection of Down syndrome was predicted, with only 5% of the population considered screen-positive (increased nuchal lucency more than 2 standard deviations for gestational age). The addition of maternal serum markers, primarily pregnancy-associated plasma

TABLE 3-7

Detection Rates for Down Syndrome With First-Trimester Screening

	FIRST TRIMESTER		SECOND TRIMESTER			
Finding	Screen-Positive Rate (%)	Detection Rate (%)	Screen-Positive Rate (%)*	Detection Rate (%)	Total Screen-Positive Rate (%)	Total Detection Rate (%)
Nuchal lucency alone	4	77	NA	NA	4	77
Nuchal lucency + serum markers	5	87	NA	NA	5	87
Integrated	NA	NA	5	97	5	97
Sequential						
Stepwise	2	65	3	30	5	95
Contingent	2	65	3	28	5	93

*In the first trimester.
NA, Not applicable.

protein-A and hCG, brought the screen-positive rate in line with 5%, with a detection rate of 80% for trisomy 21 in the fetus.[18] The striking difference is that this screening can be performed in the first trimester, allowing earlier diagnosis. Ductus venosus measurements and the assessment of the nasal bone are two additional first-trimester ultrasound parameters to further decrease screen-positive rates without loss of detection.[19,20]

Although first-trimester screening with a combination of ultrasound examination and maternal serum screen yields high detection and low screen-positive rates, an alternative approach of so-called *integrated testing* also has been supported. Integrated testing consists of first-trimester ultrasound examination and measurement of serum markers in combination with select second-trimester serum markers. With an integrated testing, the screen positive rate is lower by a few percent and the detection rate is above 95%.[21] This methodology, however, delays screening results until the second trimester. A compromise between first-trimester screening alone and integrated testing is reached with *sequential screening*. Sequential approaches may be stepwise or contingent; both release high-risk screen results in the first trimester. Further screening is done in the second trimester for all (in the stepwise approach) or only a proportion of the women (in the contingent approach)[22,23] (Table 3-7). Either sequential approach maintains a, low screen-positive rate with (<5.0%) with a detection rate for Down syndrome above 90%. Currently, the American College of Obstetrics and Gynecology recommends offering women of all ages screening for aneuploidy in pregnancy, with initiation of screening in the first trimester optimal.[1]

Of fetuses with increased nuchal lucency but a normal karyotype, a majority proceed through gestation without complication. As the degree of first-trimester nuchal edema increases, however, the risk of other structural malformations detected on the second-trimester ultrasound or at birth also increases. Although a relatively low risk of 2.7% is present for fetuses with mild nuchal edema (nuchal latency 3.0 mm), risk reaches 35.6% for those fetuses with markedly enlarged nuchal translucency (7.0 mm).[24] Although many of the identified abnormalities are cardiac malformations, other disorders such as congenital diaphragmatic hernia, skeletal dysplasias, fetal akinesis, and metabolic storage disease also are reported.[25] Persistence of markedly increased nuchal edema into the second trimester represents perhaps the greatest risk, with almost one half (40.9%) of fetuses experiencing an adverse outcome such as structural malformation, hydrops, or in utero demise.[26] Conversely, for fetuses with mild increases (2.5-3.0 mm) in first-trimester nuchal fold, a majority continue through gestation without adverse outcome, and the risk for structural abnormalities or disability in the newborn period or early childhood is not significantly increased.[27]

DIAGNOSTIC TESTING

Although use of screening modalities for genetic disease and aneuploidy during pregnancy avoids the risk of miscarriage, none of the available tests provide a definitive answer. For diagnosis, fetal DNA needs to be studied; currently, appropriate samples can be obtained only through invasive testing: The placenta (CVS), amniotic fluid (amniocentesis), or fetal blood (percutaneous blood sampling [i.e., PUBS]) may be used. When patients are counseled regarding each of the diagnostic modalities, the miscarriage rate frequently is quoted, but other concerns such as fetal morbidity from the procedure and the likelihood of technical complications also should be considered.

Chorionic Villus Sampling

Available from 10 weeks of pregnancy onward, CVS currently affords the earliest diagnostic possibility. Trophoblast cells obtained from the placenta can be studied for specific genetic mutations as well as chromosome analysis. CVS is performed under ultrasound guidance with the passage of a catheter into the placenta, either transcervically or through the maternal abdomen. Based on large multicenter collaborative studies from the 1990s, with either transcervical or transabdominal CVS, an approximate 1.0% risk of miscarriage has been reported.[28] Concerns related to fetal morbidity include fetal infection (from maternal disease such as HIV), isoimunization, and fetal damage. With regard to fetal injury, initial reports of higher rates of limb reduction defects among newborns after first-trimester CVS sampling have been extensively investigated. At this time, the risk of such fetal complications is considered rare, 5.2 to 5.7 per 10,000 after CVS, compared with 4.8 to 5.97 per 10,000 without CVS.[29] Technical concerns also have surfaced with CVS. The procurement of the sample is technically different from an amniocentesis, and the learning curve is longer. In addition, with multifetal pregnancies, assurance of sampling each individual fetus is possible but can be more problematic than with amniocentesis. Finally, although both CVS and amniocentesis will provide information concerning the chromosomal makeup of the fetus, CVS also may detect aneuploidy, which is confined to the placenta. Occurring in approximately 1% to 2% of CVS samples, confined placental mosaicism reflects a combination of karyotypically normal and abnormal cells. In two thirds of the cases, the abnormal cell line is confined to the placenta, although further evaluation of the fetus by amniocentesis or PUBS is warranted. An additional outcome associated with confined placental mosaicism is fetal growth restriction, which is likely to be dependent on the specific chromosome abnormality.[30]

Early Amniocentesis (at 9 to 11 Weeks of Gestation)

In order to provide diagnostic results in the first trimester, early amniocentesis was investigated. From 10 to 18 weeks of gestation, however, the amniotic fluid volume is rapidly expanding such that the removal of 20 mL at 11 weeks represents as much as a third of the total volume. The same 20 mL removed at 18 weeks represents less than 10% of the total volume. An initial trial to assess the safety of early amniocentesis was halted owing to an increased miscarriage rate.[31] When compared with CVS, early removal of amniotic fluid carries a significantly higher relative risk for miscarriage of 1.92 (95% confidence interval [CI], 1.14 to 3.23).[32] Additionally, increased fetal morbidity was noted, with a higher rate of clubfoot, which was unrelated to whether or not rupture of membranes had occurred.[33] Technical concerns also arose, with increased rates of culture failure, and efforts to decrease the amount of amniotic fluid removed were associated with an even greater chance of technical culture failure.

Standard Amniocentesis at 15 Weeks or Later

Removal of 15 to 20 mL of amniotic fluid in the second trimester is associated with a small risk of miscarriage. Collaborative studies in the 1990s noted a miscarriage rate of approximately 0.5% above the background rate. The definitive prospective, randomized study by Tabor in 1986 revealed a 1.0% risk of miscarriage above the background rate. This study was performed in low-risk women with both case and control groups of subjects of comparable gestational ages. In the amniocentesis and control groups, miscarriage rates between 16 and 24 weeks were 1.7% and 0.7%, respectively, with a relative risk of 1.72 (95% CI, 1.06 to 2.81). [34] More recently, analyses of data perhaps more reflective of the current practice of amniocentesis suggest that the loss rate may be less than that previously noted. Additionally, risk factors may be present that increase a particular woman's miscarriage rate after amniocentesis. When women with repeated miscarriages or bleeding in the current pregnancy are compared with women without these risk factors, increased odds ratios of 3.03 and 2.4, respectively, have been reported.[35] If such predisposing risk factors are considered, the risk of miscarriage in the woman without significant history is only minimally and not significantly increased above the background rate.[36]

Fetal morbidity after amniocentesis also should be considered. In pregnant women who are Rh-negative, the combination of an Rh-positive fetus and an invasive diagnostic test carries a small risk for fetal-maternal hemorrhage. For this reason, RhoGAM is given as a prophylactic measure to all Rh-negative women unless the partner is known to be Rh-negative and the woman declines RhoGAM. For women with other red blood cell antigen incompatibilities with their partners, any invasive diagnostic test increases the risk for antibody formation and the effects of isoimmunization in the fetus. These risks also exist for CVS, although they are theoretically lower, because amniocentesis can avoid the placenta. Similarly, infectious disease in the mother poses a theoretical risk. Transmission of hepatitis B in women who are chronic carriers does not appear to increase the risk for subsequent carriage in their infants. Likewise, although transmission of HIV through amniocentesis remains a theoretical possibility, testing by amniocentesis in women with low viral load is considered reasonable.

Fetal morbidity also is associated with rupture of membranes occurring after amniocentesis. In approximately 1% of procedures, the amniotic membranes do not promptly reseal, and amniotic fluid leakage occurs. In a majority of such instances (90%), the amniotic fluid will reaccumulate to normal levels, although the mean duration of the recovery period has been reported to be 3 weeks. Amniocentesis-related rupture of membranes with failure to reseal is associated with increased risk for intrauterine growth restriction and prematurity.[37] By contrast, spontaneous rupture of the membranes in the second trimester carries a poor prognosis, with only a limited chance of regaining normal fluid levels.

Technical issues arise with amniocentesis when the genetic diagnosis being pursued relies on molecular genetic studies. All amniotic fluid samples contain a small number of maternal cells, probably obtained as the needle passes through the maternal skin and uterus. When cultured amniotic fluid cells are evaluated, the chance of overgrowth by the maternal cells, with consequent assessment of the karyotype of the mother and not the fetus, is low. Nevertheless, when molecular diagnostic studies are initiated on direct, uncultured amniotic fluid, certainty that the fetal genome is being assessed is needed. This can be accomplished either by (1) clearing the initial 1 or 2 mL from the needle before obtaining the amniotic fluid sample or (2) simultaneous evaluation of maternal blood for additional polymorphic markers to ensure that the amniotic fluid sample represents a discretely different genome (maternal cell contamination study).

Percutaneous Umbilical Blood Sampling

Percutaneous umbilical blood sampling (PUBS) is performed at 18 weeks or later in gestation by the removal of a blood sample from the umbilical cord under ultrasound guidance. The procedure is accompanied by an approximate 1% to 2% risk of pregnancy loss, which is higher with fetuses with other risk factors such as hydrops. Nevertheless, PUBS can provide rapid and often more extensive information than that obtained by CVS or amniocentesis. Karyotype analysis yields results within 48 hours, because the rapidly dividing peripheral blood cells do not require the extensive, often 2-week period of culture needed for both CVS and amniocytes. Additionally, molecular genetic studies are possible on the extracted DNA as well as functional assessment of the bone marrow, immune system, and hepatic system. Fetal anemia can be diagnosed and treated by umbilical vessel transfusion in situations in which the hematologic suppression is expected to be transitory, such as parvovirus infection. Polymerase chain reaction (PCR) analysis for specific viruses such as parvovirus and cytomegalovirus also can be performed using fetal blood. Altered hepatic or bone marrow function may point to an underlying metabolic disease, guiding more specific molecular testing in the fetus.

NEW AVENUES FOR PRENATAL DIAGNOSIS

Preimplantation Genetic Diagnosis

After in vitro fertilization, directed assessment of a single cell from a blastocyst before transfer to the uterus—preimplantation genetic diagnosis (PGD)—can reveal single-gene disorders as well as chromosome abnormalities. For persons with a balanced chromosome translocation, single blastomeres can be studied with fluorescence in situ hybridization (FISH)—a method to determine copy number for specific, predefined segments of DNA in nondividing cells. With probe combinations designed for the specific translocation carried by the individual, couples proceed through assisted reproduction and in vitro fertilization. On day 3 after fertilization, single cells are removed from each blastocyst and assessed within 12 to 24 hours with the translocation-specific FISH probes. On completion of the FISH analysis, the information obtained is used to guide selection of blastocysts to transfer to the woman. This methodology also allows determination of chromosome copy number and was initially applied as preimplantation genetic screening (PGS) in women at increased risk related to increased maternal age, previous anueploidy, or repeat miscarriage. Development of the optimal panel of probes remains challenging, because increases in the number of chromosomes studied are associated with higher technical error rates and the potential for eliminating from

consideration for transfer a blastocyst that may indeed be chromosomally normal.[38] As currently performed, PGD is a screening method to decrease the chance that the pregnancy will be complicated by a chromosome abnormality but does not improve outcomes for women of advanced age or habitual miscarriage. Application of CGH array technology may be helpful in this area.

Various factors can contribute to the approximate 10% error rate, including the lack of possibility of retesting owing to use of a single cell, the existence of mosaicism in the early blastocyst, and the inherent technical difficulties of resolution with FISH probes. Approaches using a molecular approach to chromosome number (such as microarray or PCR methods) are likely to address this concern in the future.

Similarly, individual patients or couples at risk for a single-gene disorder that is inherited in either a recessive or dominant fashion may be candidates for PGD. As the technology continues to progress and the accuracy of PGD improves, current indications range from diagnosis of autosomal recessive disorders associated with childhood lethality to autosomal dominant disorders of adult onset. Uses of PGD that represent areas of ethical controversy in this field are screening for cancer genes, human leukocyte antigen (HLA) matching for an affected sibling, and family balancing.

Cell-Free Fetal DNA

In the late 1990s, Lo and colleagues identified the presence of cell-free fragments of fetal DNA (cff DNA) in the circulation of pregnant women.[39] As opposed to the earlier search for fetal cells in the maternal circulation, methods for retrieving cff DNA are proving to be reproducible and sensitive in numerous laboratories. Additionally, cff DNA has the advantage of rapid clearance from the maternal circulation, with essentially none remaining within hours of the infant's delivery.

Applications for this methodology initially focused on settings in which the paternal and, potentially, the fetal genotypes differ from the mother's. The primary example of such a condition is Rh isoimmunization. Because the mother's genotype is Rh-negative, the presence of any Rh-positive DNA in her circulation denotes a fetus with the Rh-positive gene from the father and for whom the risks of isoimmunization exist.[40] The methodology is reproducible and sensitive such that cff DNA is used in the management of Rh disease. When a woman is isoimmunized to Rh antigen and produces antibodies, the Rh status of the fetus becomes an important piece of information in the management of this disease. An Rh-negative fetus would not require the extensive surveillance and testing indicated for the Rh-positive fetus with other risk factors.[41] Before the advent of cff DNA, fetal Rh status could be established only by determining the genotype of the fetal cells, typically obtained by amniocentesis. Further studies are under way assessing cff DNA for RhD as a component of determining which women should receive RhoGAM. Additional uses of cff DNA for aneuploidy assessment and determination of other molecular genetic disorders will follow as specific markers for differentiation of fetal DNA from that of the mother emerge.[42] Knowledge of quantitative alterations in cell-free nucleic acids in the maternal circulation as a reflection of placental integrity is growing and is likely to form the basis for important tools for the presymptomatic assessment of a range of pregnancy complications such as preeclampsia.[43]

SUMMARY

Prenatal genetic diagnosis currently is possible only by means of invasive procedures, with their inherent risk of miscarriage as well as fetal morbidity and technical constraints. CVS, amniocentesis, and PUBS each can provide the fetal DNA needed for the assessment of a wide range of genetic and chromosome disorders. However, although the risks from any one of these procedures is relatively low, preconception and prenatal screening to identify which women face an increased risk of genetic disease is appropriate. Clinical history including parental ages, reproductive history, and family history each have value in screening for genetic and chromosome risks. During pregnancy, both maternal serum markers and ultrasound examination are productive screening tools used primarily to assess Down syndrome risk. Ideally, such screening should be initiated in the first trimester. Finally, new methods of prenatal diagnosis are emerging. Most important, further developments in the assessment of cell free fetal DNA will allow prenatal genetic diagnosis from a maternal blood sample and remove the need for more invasive testing.

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4 Basic Embryology

David L. Bolender and Stanley Kaplan

The human embryo begins as a single large cell, approximately 0.1 mm in diameter, just visible to the unaided eye. During the 266 days of gestation following fertilization, this cell increases in size, weight, and surface area in a rapid and markedly nonlinear fashion. From newly fertilized egg to newborn, length increases by a factor of 5000, surface area by a factor of 61 million, and weight by a factor of nearly 6 billion.[1] During this process the fertilized egg divides and differentiates into more than 200 different morphologically recognizable cell types. Orchestration of the increase in size and specialization in cellular function is a complex process about which much remains unknown. It has been argued, however, that the principles of development have been established and that details are missing only at the molecular level.[2] This claim is undoubtedly an overstatement; nevertheless, during the last decade or so, understanding of the molecular control of development has increased substantially. That human embryonic development occurs normally in most pregnancies is a tribute to the design of the control mechanisms that are operating. This chapter presents a brief description of the growth and differentiation of the human embryo, along with a limited discussion of certain factors that play a part in control of these activities.

GAMETES AND THEIR MATURATION

The human egg and sperm are two highly specialized cells that share little in common with the other cells of the adult body. They are different in both form and function. Like other cells, however, they must achieve a degree of maturity before they can perform their function (i.e., combining to form the zygote). The steps as well as the chronology leading to this maturation are quite different in the male and in the female, and such differences reflect the diverse pathways of the two sexes beginning early in human development.[3]

Origin of the Gametes

The human egg and sperm are derived from large, round primordial germ cells that can be identified in the wall of the yolk sac as early as 24 days after fertilization.[4] As the yolk sac begins to be incorporated into the embryo, the germ cells migrate along the dorsal mesentery of the hindgut to the gonadal ridges, which they reach by the end of the fourth or early fifth week (Figure 4-1). This migration has been observed in vitro in pieces of hindgut, mesentery, and gonadal ridges of mouse embryos.[5] It is facilitated in humans by a striking ameboid shape (which persists even after the cells have reached the gonad[6]) and pseudopodia typical of those found in ameboid cells. The pseudopodia disappear later after the migration is complete.[5,7] In humans, these cells contain glycogen stores that diminish over time and disappear when the cells have reached their destination in the gonad, suggesting that the glycogen may be the energy source for their journey.[8]

Organization of the Gonad

The coelomic epithelium covering the medial aspect of the gonadal ridges undergoes proliferation about week 7 of gestation. As these cells multiply, they grow into the underlying mesenchyme in a series of fingerlike cords of cells called primitive sex cords. The primordial germ cells associate with these cords. If the embryo is to become a male, these cords continue to be prominent and eventually develop into the seminiferous tubules and rete testis. The early male gonad can also be recognized by the separation of the cords from their parent epithelial covering by a fibrous connective tissue layer, the tunica albuginea, that forms just under the epithelium. If the gonad is to become an ovary, the primitive sex cords remain rudimentary. The origin of the follicle cells of the ovary remains unclear, but likely candidates are cells from the coelomic

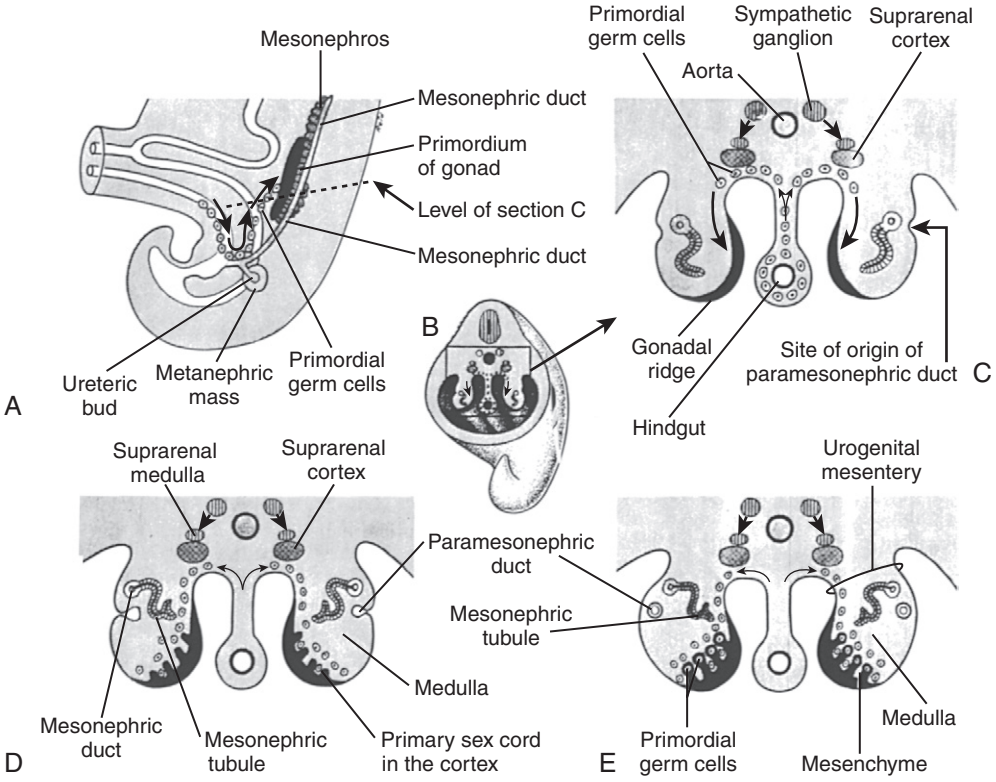


Figure 4-1 **A,** Sketch of a 5-week embryo, illustrating the migration of primordial germ cells from the yolk sac. **B,** Three-dimensional sketch of the caudal region of a 5-week embryo, showing the location and extent of the gonadal ridges on the medial aspect of the urogenital ridges. **C,** Transverse section showing the primordium of the suprarenal (adrenal) glands, the gonadal ridges, and the migration of primordial germ cells into the developing gonads. **D,** Transverse section through a 6-week embryo, showing the primary sex cords and the developing paramesonephric ducts. **E,** Similar section at a later stage showing the indifferent gonads and the mesonephric and paramesonephric ducts. *(From Moore KL, Persaud TVN: The Developing Human: Clinically Oriented Embryology, 5th ed, Philadelphia, WB Saunders, 1993, p 281.)*

epithelium and the mesonephros. The follicle cells associate with the primordial germ cells to form primordial ovarian follicles.[8]

Development of the Female Gamete (See Chapter 184)

If the gonad develops into an ovary, the primordial germ cells become oogonia and continue mitotic division.[9] Mitotic division of these cells has been observed in humans up to the seventh fetal month[10] but ceases sometime shortly before birth. No oogonia form subsequent to the birth of the infant after a normal full-term pregnancy.

In both males and females, the germ cells form a syncytium while dividing.[11,12] These intercellular connections permit communication and facilitate the high degree of synchrony that has been observed during both mitotic and meiotic divisions.[13-15]

By the eighth or ninth week after fertilization, some oogonia enter prophase of meiosis I and become primary oocytes.[9-16] Meiosis begins first deep to the surface of the human ovary and then expands toward the surface. Thus, at an appropriate fetal stage, oogonia are found superficially, oocytes deep to the surface, and small follicles at the inner part of the ovarian cortex.[9] It has been suggested that a diffusible meiosis-activating substance is secreted by rete cells (derived from the mesonephros), which lie in the center of the ovary, and good experimental evidence is available to support this hypothesis.[17-20]

The oocyte goes through the leptotene, zygotene, and pachytene stages of meiosis I, and it then arrests at the diplotene stage. At this point the oocyte becomes surrounded by a single, incomplete layer of flat follicular cells[9]; this unit is called a *primordial follicle.* The follicle's large central nucleus is known as the germinal vesicle. A crescent-shaped assembly of cellular organelles containing mitochondria, endoplasmic reticulum, the Golgi complex, lysosomes, and annulate lamellae (stacked parallel membrane arrays with pores) remain clustered adjacent to the nucleus.[15,21] Once it has been incorporated into a primordial follicle, the oocyte enters a long period of quiescence, beginning before birth in humans and ending in either atresia or ovulation.

Once sexual maturity is attained, a small number of oocytes begins the process of folliculogenesis or follicle maturation during each menstrual cycle.[22] The oocyte grows and eventually becomes one of the largest cells in the human body.[23] The organelles disperse throughout the cytoplasm and the germinal vesicle (nucleus) enlarges. It increases its complement of nuclear pores, facilitating transport of molecules between nucleoplasm and cytoplasm. The follicular cells resume mitosis and increase markedly in size, changing in shape from squamous to cuboidal, and the follicle becomes surrounded by a basement membrane. Those follicles containing an oocyte surrounded by a single layer of cuboidal follicular cells are known as unilaminar primary follicles, to distinguish them from earlier or later stages.[3]

During further growth of the primary follicle, a thick, acellular coat, the *zona pellucida,* begins to form between the oocyte and the follicular cells. Mitotic activity increases the number of follicle cell layers, and the follicle is now called a multilaminar primary follicle. The expanding follicle compresses the surrounding ovarian stoma, which organizes into a compact layer adjacent to the basement membrane of the follicle. This layer of stromal cells is called the theca interna, and its cells have the capacity to produce androgens when stimulated by luteinizing hormone activity (Figure 4-2). The theca interna is vascularized, but the epithelial layers of follicle cells remain avascular.

The zona pellucida is important in the process of fertilization because it contains sperm receptors, takes part in induction of the acrosome reaction, and becomes a block to polyspermy. It also may act after fertilization as a smooth, slippery envelope to contain the sticky ball of cells of the morula-stage embryo; these are free to adhere to the uterine endothelium when the zona breaks down, just before implantation.

The zona pellucida is made up of three separate filamentous glycoproteins, ZP1 through ZP3, which differ in molecular weight and isoelectric point and account for virtually all protein in the zona pellucida. ZP1 cross-links these filaments, resulting in a three-dimensional matrix that is permeable to large macromolecules. ZP3 serves as a species-specific sperm receptor and also

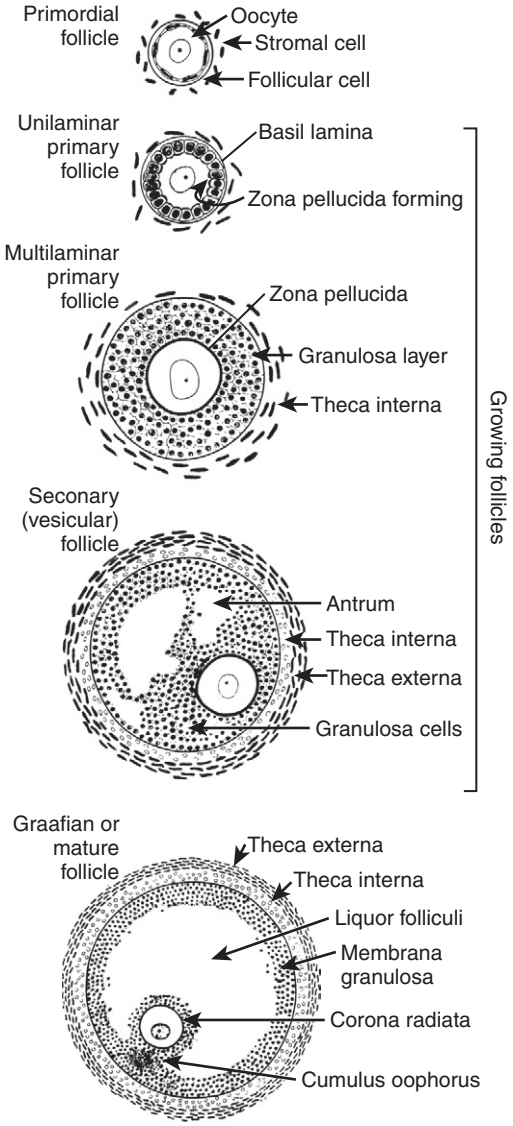


Figure 4-2 Schematic drawing of development of ovarian follicles, starting with the primordial follicle and ending with mature follicles. *(From Junqueira LC, et al:* Basic Histology, *7th ed, Norwalk, CT, Appleton & Lange, 1992.)*

induces the acrosome reaction in sperm at contact. At or shortly after fertilization, these two characteristics are lost, reducing the likelihood of polyspermy.[24-26] The ZP3 gene has been cloned; it is expressed only in oocytes, and then only during the growth phase of oogenesis.[27] The human genes for ZP2 and ZP3 are located on chromosomes 16 and 7, respectively,[28] but the location of ZP1 remains unknown. The interesting story of these zona pellucida proteins has been the subject of a popularized account[29] as well as several reviews.[28,30,31] Radiolabeling studies in mice indicate that all three glycoproteins are synthesized by the oocyte itself, rather than by the follicular cells.[32] Furthermore, immunofluorescence studies show that zona pellucida antigens are present within human oocytes but not in follicular cells.[33] Studies in species other than the mouse, however, suggest that the granulosa cells that surround the oocyte also may play a role in the synthesis of zona pellucida components.[30]

Numerous cytoplasmic projections of the follicular cells penetrate the zona pellucida to contact the cell membrane of the oocyte. In humans, these filopodial extensions of the follicular cell may actually lie deeply buried in the oocyte, in straight invaginations or pits.[34] These pits are lined by the oocyte cell membrane; however, no cytoplasmic continuity exists between the two cell types. Animal studies have demonstrated the presence of gap junctions along the association of these two cell membranes, permitting transfer of small molecules (molecular weight of approximately 1000) between them.[22]

As the primary follicle enlarges, the follicular cells begin to produce follicular fluid, which collects within the intercellular spaces between follicle cells. These spaces coalesce to form a large fluid-filled cavity called the antrum, which is characteristic of the secondary (vesicular) follicle. The antrum expands, and the oocyte becomes located on one side of the follicle, where it is embedded within a mound of follicle cells known as the cumulus oophorus. The layers of follicular cells immediately surrounding the oocyte are termed the corona radiata. Because of its increased size, the follicle further compresses the surrounding ovarian stroma. A looser, less organized layer of flattened stromal cells encircles the follicle superficial to the theca interna. This is called the theca externa, and its cells have no steroid-secreting activity (see Figure 4-2). A few days before ovulation, one secondary follicle becomes dominant and inhibits the growth of the remaining secondary follicles. The dominant follicle, now called a *graafian follicle,* can reach several centimeters in diameter. The oocyte is approximately 100 μm in diameter at this stage. Approximately a day before ovulation, its nuclear membrane breaks down, the nucleolus disappears, and the first polar body forms, containing one of the two sets of chromosomes. Meiosis I is completed, and the oocyte proceeds to meiosis II, but it again arrests on reaching metaphase. In most mammalian species including humans, meiosis II resumes only after the oocyte is penetrated by a sperm.[35,36] Completion of meiosis in the fertilized oocyte results in production of the second polar body.

Follicles of any stage can undergo atresia. Atresia begins in the fetus and continues into menopause until all follicles have disappeared. At birth, approximately 2 million primordial follicles are present within the two ovaries. It has been estimated that half of the 2 million follicles present at birth are atretic at that time.[10] In humans, follicular growth starts before birth, and the newborn ovary contains multilaminar primary follicles as well as primordial follicles. Follicular growth and subsequent atresia are continuous during human childhood, and it has been clearly stated that "quiescent ovaries in which follicular growth is absent do not occur in normal children."[37]

Little is known about control of atresia. For example, it is not known whether atresia is initiated by action of the follicular cells, by that of the oocyte, or by both.[22] The process of atresia, however, can be manipulated experimentally.[37] Approximately 40,000 follicles are present in the two ovaries of a young adult woman, indicating a reduction to 2% of the pool originally present at birth.[38]

These stages, up to and including the newly fertilized mature ovum, are summarized in Table 4-1. Most or all of the RNA and protein found in a mature oocyte are synthesized during oocyte growth. Those macromolecules present in the oocyte of an atretic follicle are degraded and the degradation products subsequently used for new synthesis.[23]

As estimated from an assumed fertility span of 30 years, approximately 400 eggs are shed during a woman's lifetime.[35] Thus, approximately 1 in every 100 of the eggs present in a young adult completes maturation and is ovulated; the rest degenerate. A human female has her full complement of eggs, albeit immature, on the day she is born. This is not the case for sperm development in males.

Early Development of Male Gametes (See Chapter 185)

In male humans, the primordial germ cells migrate into the gonadal ridges as outlined earlier (see Figure 4-1). Once they have reached the gonad, they divide to form a pool of spermatogonia.[39]

TABLE 4-1

Summary of the Developmental Characteristics of the Human Female Germ Cell

Name	Approximate First Recognizable Time (Postfertilization)	Location	Approximate Total Number (Both Ovaries)	Size, Shape, Characteristics	Relevant Study(ies)
Primordial germ cell	During 4th week	Caudal yolk sac, among endoderm cells	500	Large, round, 15-20 μm in diameter	Witschi, 1948[4]; Fujimoto et al, 1977[7]
	During 5th week	Dorsal mesentery of hindgut and gonadal ridges	500	Ameboid shape, migrating, with pseudopodia; >20-30 μm in long axis; alkaline phosphatase–positive	Witschi, 1948[4]; Fujimoto et al, 1977[7]
Oogonium	During 6th week	Sexually indifferent gonad	100,000	Rapid mitosis increases numbers (mitosis signals name change)	Witschi, 1948[4]; Byskov, 1980[9]
	During 7th week	Gonad recognizable as ovary	100,000	Mitosis continues; almost all primordial germ cells are now in the gonad	Fujimoto et al, 1977[7]; Moore and Persaud, 1998[38]
Oocyte (in primordial follicle)	Weeks 8-9	Ovary	?	Meiosis begins; ~19 μm diameter	Dvorak and Tesarik, 1980[16]
Quiescent oocyte in primordial follicle	16 weeks	Ovary	?	Arrest of meiosis I at diplotene; 50-70 μm; round to ovoid; vitelline body present	Baca and Zamboni, 1967[34]
	2 months	Ovary	600,000		Baker, 1963[10]
	5 months	Ovary	6,800,000		Baker, 1963[10]
	7 months	Ovary		Mitosis of oogonia ceases	Baker, 1963[10]
	Birth	Ovary	2,000,000 (50% atretic)		Baker, 1963[10]; Moore and Persaud, 1998[38]
	7 years	Ovary	300,000		Baker, 1963[10]
Primary to mature (graafian) follicles	Puberty on	Ovary	40,000 and declining	From oocyte to mature: comes out of meiotic arrest and enters metaphase of meiosis II, then arrests again	Moore and Persaud, 1998[38]
Mature ovum	Puberty on	Uterine tube	1 per month	Meiosis is completed and the 2nd polar body is extruded when penetrated by a sperm	Moore and Persaud, 1998[38]

Both spermatogonia and their supporting cells—the Sertoli cells—can be identified as early as 48 days after fertilization.[40] The germ cells and the supporting cells combine to form seminiferous tubules.

Spermatogonia are located next to the basement membrane of the seminiferous tubule, where they lie quiescent until puberty. Experimental studies with mice have shown that male primordial germ cells are kept in that state by a meiosis-preventing substance, which also can arrest female germ cells in meiosis. Conversely, the female gonad secretes a meiosis-inducing substance, which can induce male germ cells to enter meiosis.[19]

At puberty, the spermatogonia begin differentiation into sperm (spermatogenesis). The process of spermatogenesis occurs in three phases. In the first phase, the spermatogonia divide mitotically. In the second phase, some spermatogonia differentiate into primary spermatocytes and undergo meiosis. In the third phase, spermatids proceed through spermiogenesis to form spermatozoa. In contrast with the female, the cycle of differentiation of gametes in men is essentially continuous throughout life. Studies in which tritiated thymidine was injected into healthy male volunteers indicate that the complete cycle takes approximately 74 days.[41] The various stages of spermatogenesis, however, are not synchronized along the length of the coiled seminiferous tubule in humans: Different stages are found at different positions.

FERTILIZATION

Development begins with the fusion of the male and female gametes at fertilization, which takes place in the distal third of the oviduct. Although fertilization is an "internal" process in humans and other mammals, the development of culture systems that support fertilization has made detailed study of the sperm-oocyte interaction possible, as well as providing a basis for in vitro fertilization for clinical ends. As a result, the precisely ordered events comprising a "fertilization pathway" have been identified (Figure 4-3). The mechanisms involved in the fertilization pathway have been the subject of investigation at the molecular level.[29,31,42] Most studies on fertilization have been done using mice, but comparative data suggest that the pathway is similar in all mammals, including humans.

The pathway of fertilization begins with binding of the sperm to the surface of the zona pellucida (see Figure 4-3). On the surface of every sperm are thousands of copies of an egg-binding protein; these are recognized by thousands of copies of sperm receptors on the zona pellucida.[43] Binding is relatively species-specific and requires a complete plasma membrane (i.e., an acrosome-intact sperm).[44,45] Once bound to the zona surface, the sperm undergoes a series of dynamic membrane fusions known as the acrosome reaction (see Figure 4-3).[46] During this phase, the plasma membrane at the apical end of the sperm fuses with the outer membrane of the acrosome, forming a series of membrane-bound vesicles. These are eventually sloughed, which exposes the inner acrosomal membrane and its complement of enzymes.[31,47]

As a result of enzyme modification of the zona, the sperm is able to tunnel its way through. The first sperm to penetrate the perivitelline space (between the zona pellucida and the oocyte plasma membrane) and fuse with the plasma membrane triggers activation of the egg (see Figure 4–3). Oocyte activation is a dynamic, multistep process that includes mechanisms to prevent polyspermy, completion of meiosis by the oocyte, engulfment of the sperm, formation of male and female pronuclei and initiation of the first mitotic division of the embryo. Prevention of fertilization by more than one sperm (polyspermy—a potentially lethal condition) is thought to be a biphasic reaction, although the first phase is not well documented in humans. The first phase is rapid and consists of hyperpolarization of the oocyte plasma membrane. The second phase may take several minutes and involves the release of enzymes from the cortical granules that alter the structure of the zona pellucida. As a result, the plasma membrane and the zona pellucida become refractory to further penetration by other sperm.

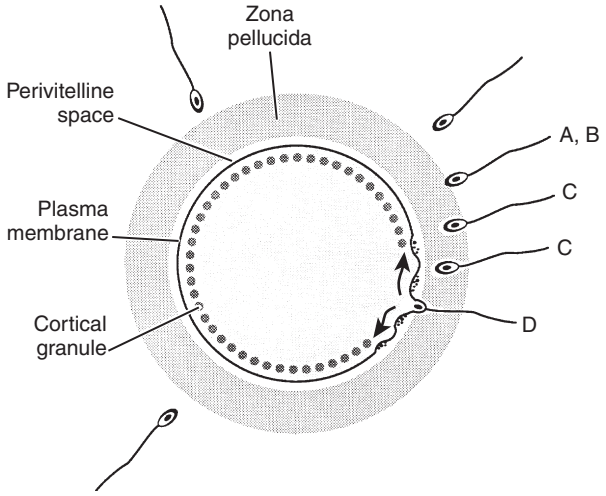


Figure 4-3 Pathway of fertilization. *A* and *B* show sperm binding and the acrosome reaction, exposing the zona to acrosomal enzymes. *C* depicts penetration of the enzyme-modified zona pellucida by the sperm. *D* shows activation of the egg, including oocyte membrane hyperpolarization and release of enzymes by the cortical granules. *(Modified from Wassarman PM: Fertilization in Mammals,* Sci Am *259:82, 1988.)*

Studies at the molecular level have revealed that a component of the zona pellucida is a key substance in the fertilization pathway. As mentioned earlier, the zona pellucida is an acellular coat that surrounds the oocyte and consists of three glycoproteins—ZP1, ZP2, and ZP3—arranged in an interlacing filamentous network.[43,48] Of interest, one of them, ZP3, functions as the sperm receptor, initiates the acrosome reaction, and participates in the zona reaction.[48] Sperm binding is mediated by a subset of the *O*-linked oligosaccharides associated with ZP3, whereas a segment of the polypeptide backbone is needed to induce the acrosome reaction.[29,49]

Several important events necessary for development of the embryo—initially called a *zygote* after fertilization—are accomplished as a result of fertilization.[38] First, the diploid chromosome number is restored by fusion of the two haploid gametes. Normally, half of the chromosomes come from each parent, and the new complement of chromosomes in the zygote promotes species variation. In addition, the genetic sex of the zygote is determined by the type of sperm that participates in fertilization. Sperm that bear a Y chromosome produce a genetically male zygote (XY), whereas an X-bearing sperm produces a female zygote (XX). Finally, fertilization initiates cleavage, the mitotic division of the zygote. Apposition of the male and female pronuclei forms a metaphase plate, and the first cleavage soon begins. In contrast with some animal species, the human male and female pronuclei never fuse (i.e., form a complete nucleus). Instead, they immediately enter mitotic metaphase.[50]

Parthenogenesis is activation of the unfertilized oocyte, leading thereafter to varying degrees of successful development of the zygote and embryo. In some animal species, this process is well known to occur and may even produce viable offspring. No verified human cases, however, have been reported in the scientific literature.[38]

MORPHOGENESIS

The mechanisms mediating the transformation of a fertilized oocyte into a three-dimensional embryo are complex and still not completely understood. Studies on human embryos have been, for the most part, limited to observations of static images or serial reconstructions on preserved specimens of different developmental stages. Therefore most knowledge of the mechanisms controlling development has come from animal studies.

During development, cells of different genetic backgrounds are constantly interacting with each other and with a variety of molecules within their extracellular environment. The processes involved in these interactions consist of many well-recognized phenomena of cell biology, including cell division, adhesion, secretion, cytodifferentiation, motility, and cell death. Although the complex interactions that occur during morphogenesis may appear to be unorganized, they are recognized to occur not stochastically but rather in a precisely ordered sequence of events resulting in recognizable patterns of histogenesis and organogenesis. In the past decade, an abundance of molecular studies have provided a much clearer picture of the complex signaling activity that controls embryonic development.[8]

As a result of fertilization, the zygote undergoes a series of mitotic divisions termed *cleavage.* The cells derived from these repeated mitotic divisions are called *blastomeres.* The first divisions result in a solid mass of blastomeres that are still surrounded by the zona pellucida. Starting at the 8- to 16-cell stage, intercellular spaces between blastomeres coalesce to form a central cavity. The embryo, now termed a *blastocyst,* consists of a regionalized clump of cells termed the *inner cell mass,* that projects into the blastocyst cavity and is surrounded by an outer layer of trophoblast cells (Figure 4-4, *A* and *B*). Initially the blastocyst floats freely within the uterine cavity. After shedding the zona pellucida, the blastocyst attaches and implants within the uterine endometrium.[38]

Studies on embryos suggest that the earliest cleavage divisions appear to be driven by maternal messages stored within the oocyte cytoplasm.[51] In mammals (including humans), the embryonic genome is activated by the two- to four-cell stage and begins to synthesize proteins on its own. This functional maturation is reflected in the steady rise in the synthesis of many intracellular proteins such as actin.[52-54]

During cleavage, several changes fundamental to embryonic development occur at the molecular level. One of the most important processes is the generation of cell diversity. Initially, all blastomeres express a specific transcription factor, oct-4, which reflects the undifferentiated state of these cells. If separated from the others at this stage, each of the blastomeres has the capacity to form a complete embryo. By the 8- and 16-cell stage the embryo is a solid mass of cells called a *morula.* At this time, the outer cells of the morula are distinguishable from the inner cells because the outer cells no longer express oct-4.[8] The outer cells, now designated the *trophoblast,* also begin to exhibit epithelial polarity.[55] As a result, the first embryonic tissue (trophoblast epithelium) is formed. Subsequent cytodifferentiation of the trophoblast results in a double-layered membrane, which is a progenitor tissue of the chorion, the fetal portion of the placenta (see Figure 4-4, *C*). The inner cellular layer is called the *cytotrophoblast,* and the outer layer the *syncytiotrophoblast.* The latter structure, which secretes human chorionic gonadotropin (hCG) and proteolytic enzymes, is critical to implantation.[38]

During the second week of development, the cells of the inner cell mass that face the blastocyst cavity become flattened, forming a second layer of epithelium.[38] The upper layer, located next to the trophoblast, is now designated the epiblast, whereas the bottom layer of flattened cells is called the primary endoderm or hypoblast (see Figure 4-4, *C*). Cells of the epiblast become organized into an epithelial disk, the progenitor of all embryonic tissues, as well as the extraembryonic mesoderm, amnion, and yolk sac.[53] The cells forming the extraembryonic mesoderm apparently arise from the presumptive caudal end of the epiblast and coat the internal surface of the cytotrophoblast.[56] The extraembryonic mesoderm combined with the trophoblast constitutes the chorion. Even at this early stage of development, it is possible to determine that the surface of the epiblast adjacent to the trophoblast represents the dorsal side of the embryo.[57]

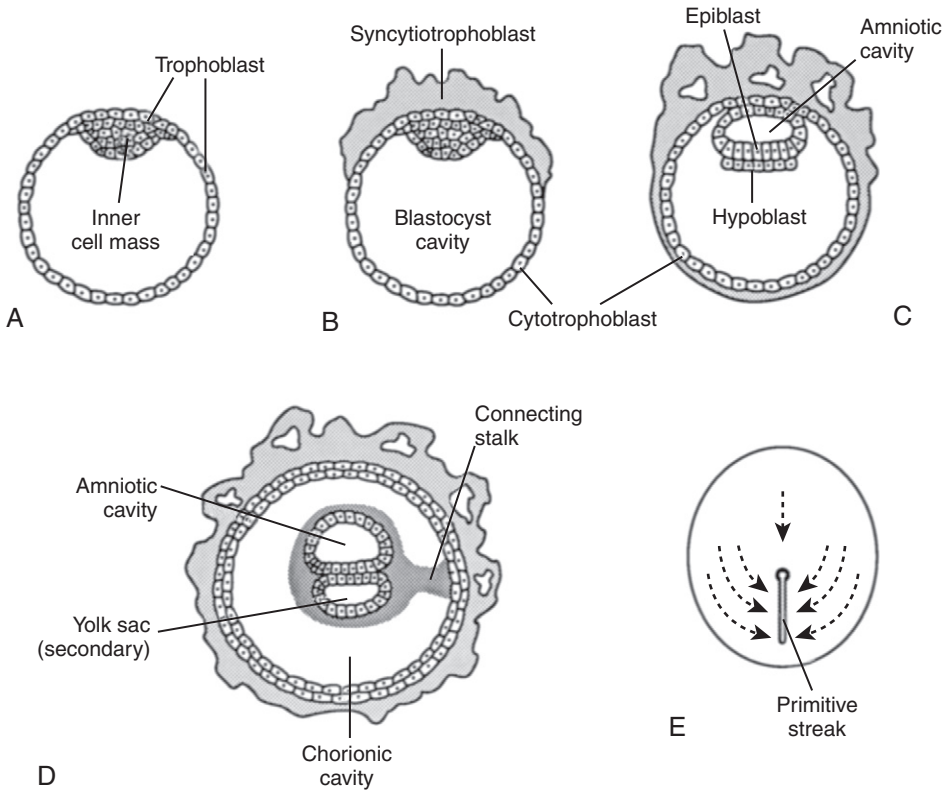


Figure 4-4 Development of the blastocyst embryonic disk. **A,** Blastocyst stage human embryo, exhibiting the inner cell mass as a regionalized mass of cells projecting into the blastocyst cavity. The cavity is surrounded by trophoblast cells. **B,** Slightly older blastocyst than that seen in **A.** At this time, the inner cell mass and trophoblast cells are capped by cells of the syncytiotrophoblast, which are buried in the endometrial stroma (not shown). The cellular layer of trophoblast is now called the cytotrophoblast. **C,** An older blastocyst stage human embryo. The cavity is now surrounded by a double-layered membrane, and the inner cell mass has developed into a bilayered embryonic disk capped by a cavity (amniotic cavity). **D,** This somewhat older, completely implanted embryo now exhibits cavities both above and below the embryonic disk, the amniotic cavity, and the yolk sac. No axial features are present in the disk at this time; cephalic and caudal regions cannot be discerned. **E,** Surface view of the embryonic disk, showing the primitive streak. This midline thickening of epiblast cells occurs during the early part of the third week after fertilization and produces a landmark (the primitive streak) that delineates the midline of the embryo and reveals the future cephalic and caudal ends. The thickening of epiblast cells in the midline (primitive streak) is actually an increased population of cells in this region, resulting from both a high mitotic rate in the midline and migration of a subpopulation of epiblast cells to a midline position. The *arrows* indicate migration of the epiblast cells.

Rearrangement of some of the epiblast cells results in the formation of a small amniotic cavity.[58] It is unclear whether the amnion is derived from epiblast cells adjacent to the newly formed cavity[58] or from the cytotrophoblast.[59] Primary endoderm cells of the hypoblast proliferate and migrate onto the inner surface of the cytotrophoblast, forming the yolk sac or umbilical vesicle.[60] Therefore, by the end of the second week of development, the embryo consists of a circular bilaminar disk located between two fluid-filled cavities (see Figure 4–4, *C* and *D*). At this time, no axial features are visible within the embryonic disk.

At the outset of the third week of development, dynamic cell movements result in extensive rearrangement of the epiblast cells. In most species, this period, called *gastrulation,* is characterized by morphogenetic movements and the changes resulting from them. A midline thickening of the now elongated epiblast becomes visible, designating the future posterior end of the embryo.[53] This thickening is the *primitive streak* (see Figure 4-4, *E*). Cellular activity at the streak results in another fundamental process of morphogenesis, epithelial-mesenchymal transformation. This process begins when some epiblast cells enter the streak while others remain within the epiblast to become the embryonic ectoderm.[61] The transformation from epithelium to mesenchyme consists of a cascade of cellular dynamics, including loss of intercellular connections, cell shape changes, and eventual freedom from the confines of the epiblast. Thus, at the primitive streak, subsets of polarized epithelial cells within the epiblast transform into nonpolarized free cells termed *mesenchyme,* the second embryonic tissue. These events are thought to be mediated by modulation of adhesive molecules located on the cell surface,[62] as well as by cytoskeletal rearrangements. In addition, variable expression of homeobox genes as well as of many other signaling molecules also occurs during gastrulation,[8] leading to patterning of axial and nonaxial structures

The primitive streak provides a means by which subsets of epiblast cells can ingress and be distributed to more ventral regions of the embryo as the endoderm and the mesoderm.[61] The first cells through the streak probably represent the definitive embryonic endoderm. These are followed by a solid cord of cells, the notochordal process, which extends cranially from the streak. These cells form the notochord, which defines the axis of the embryo and plays a significant role in the induction of the nervous system. Studies suggest that the notochord is an important signaling center for organizing the embryo. It secretes several important morphogenetic signaling molecules such as retinoic acid and Sonic hedgehog.[8,62] Another important signaling center, the prechordal plate, forms just cranial to the notochord. The prechordal plate is an important organizing center for the head of the embryo.[8] Just cranial to the prechordal plate, the endoderm fuses to the to the

overlying ectoderm. This region of fused ectoderm and endoderm is the site of the future mouth.[63] The remainder of the cells that pass through the streak become the intraembryonic mesoderm and come to lie between the endoderm and the ectoderm. Thus, the primitive streak provides the embryo with a means to organize epiblast cells, perhaps already partially fate-specified, into three primary germ layers—ectoderm, mesoderm, and endoderm.

As a consequence of cleavage and gastrulation, subpopulations of cells in various states of determination are brought together in new spatial relationships, which permits new tissue interactions. Subsequent histogenesis and organogenesis are driven by these tissue interactions, defined as the action of one dissimilar group of cells on another, resulting in the alteration of cell behavior of one of the component groups in a developmentally significant direction.[64] Tissue interactions often result in induction in which signals from one cell group mediate the change in developmental direction of another group of cells that are competent to respond to the inductive signals. These interactions are mediated by a variety of signaling molecules such as growth factors, secreted factors, and transcription factors, produced by cells and often concentrated in the extracellular matrix.[8]

EMBRYOLOGY OF THE ORGAN SYSTEMS

The following brief account of the development of some of the major organs provides an overview of some of the complex processes that occur as the embryo is built from raw materials. It is an amazing and precisely timed process. That it happens properly in most conceptions is even more remarkable. For a much more complete account, several other excellent and current texts are recommended,[8,38,63] as well as appropriate chapters elsewhere in this book.

Nervous System (See Chapter 160)

The human nervous system begins to form approximately 18 days after fertilization,[64,65] making it the first of the organ systems to initiate development. It begins as a thickening of the ectodermal layer along the craniocaudal axis of the embryo in the area destined to become the cervical region (Figure 4-5, *B*). This thickening is the result of an increase in the height of the ectodermal cells as they change shape from cuboidal to tall columnar, as well as intercalatory movements within the local population of cells. The result is an oval or keyhole-shaped area of thickened ectoderm known as the *neural plate.* Two ridges of this neural plate on each side of the midline undergo accelerated growth giving rise to two longitudinal neural folds with a neural groove between. Before this folding, a mesencephalic flexure forms in the cranial portion of the neural plate.[63] This flexure demarcates the future prosencephalon, mesencephalon, and rhombencephalon. These neural folds increase in height, curve toward each other, touch, and fuse to form the rudiment of the neural tube midway along the embryonic axis (see Figure 4-5, *B*). This fusion then proceeds in both cranial and caudal directions, as if two zipper fasteners were operating simultaneously but in different directions.

The remaining unfused ends of the neural folds at each end of the embryo are called the *cranial* and *caudal neuropores* because the neural tube is open at these sites. The cranial neuropore closes on day 25 and the caudal neuropore on day 27 of development.[64,65] This folding and shaping of the neural tube occur through both intrinsic (cell cycle, cell shape) and extrinsic (proliferation of adjacent tissue) mechanisms.[66,67] Shortly after fusion of the neural folds in a given region of the embryo, the neural tube separates from the ectoderm and becomes buried in the mesenchyme below the surface.

During this process of neural tube formation, an epithelial-mesenchymal transformation occurs, resulting in formation of a group of cells derived from the crests of the neural folds. These

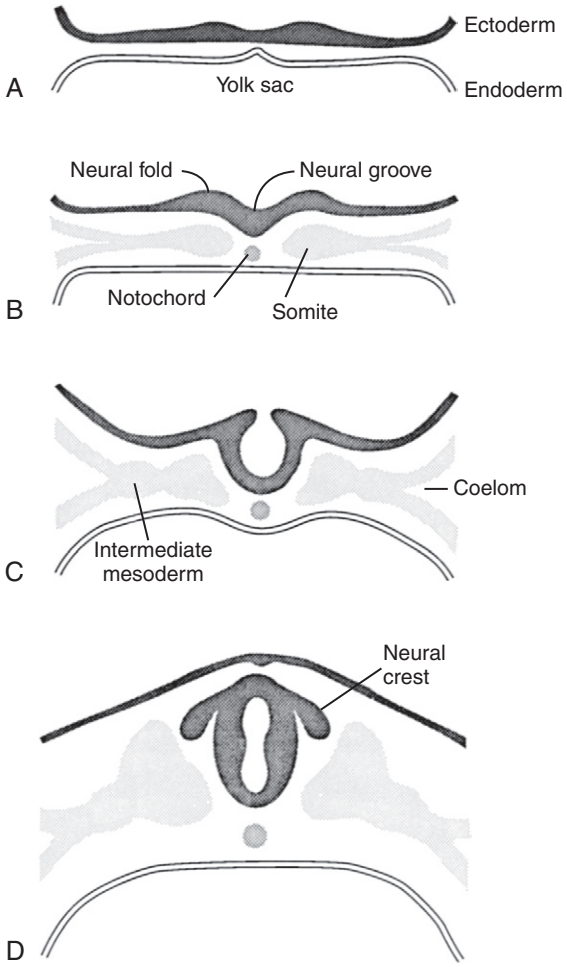


Figure 4-5 Development of the neural tube. Approximate ages: **A,** 15 days; **B,** 18 days; **C,** 20 days; **D,** 23 days. All sections are transverse, approximately midway along the embryonic axis. Note that although the neural tube is completely closed in the section shown in **D,** both the rostral and the caudal neuropores remain open in an embryo of this age. They do not close until near the end of the fourth week.

neural crest cells come to lie on the superolateral margins of the tube. The neural tube proper goes on to form the central nervous system, which consists of the brain and spinal cord, whereas the neural crest forms much of the peripheral nervous system, consisting of portions of autonomic, cranial, and spinal ganglia and nerves. The lumen of the neural tube becomes the central canal of the spinal cord and the ventricles of the brain.

Further Development of the Central Nervous System

The spinal cord develops from that part of the neural tube caudal to the cervical flexure, one of two unambiguous bends in an embryo about 30 days old that give the embryonic axis a C-shaped profile in lateral views (Figure 4-6). The wall of the tube thickens and soon stratifies into a ventricular zone that borders the central canal, an intermediate zone, and a marginal zone. The intermediate zone is created by migration of neurons from the ventricular zone. These neurons then send out processes that create the marginal zone, later to become the white matter of the cord.

Proliferation of the cells of these zones is greatly influenced by the somites, mesodermal structures that lie lateral to the neural tube along its craniocaudal axis. Later, the roof and floor of the neural tube become thin, while the lateral walls thicken. Studies

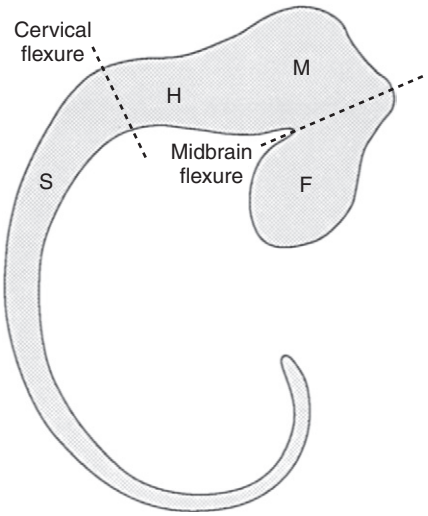


Figure 4-6 Lateral view of the isolated central nervous system of a 28- to 30-day embryo, showing the two flexures *(dotted lines)* and the resulting divisions of the brain and spinal cord at this age. *F,* Forebrain; *H,* hindbrain; *M,* midbrain; *S,* spinal cord.

in animals show that this particular organization around the circumference of the neural tube is under the influence of an inductive substance from the somites.[68] Experimental manipulation of the number, size, or position of somites influences the cross-sectional profile of the tube, including the keyhole-shaped profile of the neural canal (Figure 4-7).

The sulcus limitans is a groove that divides the more dorsal alar plate from the ventral basal plate. The former develops into the dorsal gray horns associated with sensory (afferent) input. The basal plate gives rise to the ventral and lateral gray horns, which function in motor (efferent) output. The floor plate secretes morphogenetic molecules (e.g., retinoic acid, Sonic hedgehog), which control the patterning of dorsal sensory and ventral motor elements.[8,69]

The spinal cord extends the entire length of the developing vertebral column during the embryonic period. The spinal cord grows more slowly than the vertebral column, however, so that in the fetal period and beyond, these two structures change position with respect to each other. At 24 weeks, the spinal cord extends caudally to the level of the first sacral (S1) vertebral body. In the newborn, it extends to the third lumbar vertebral body (L3), and in the adult to L1. This aspect of spinal development is important clinically with respect to lumbar punctures and other procedures that require knowledge about where the cord ends.

The brain is divided into forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon) when the midbrain flexure appears (see Figure 4-6). These three divisions of the brain quickly become five by the partitioning of the forebrain into telencephalon and diencephalon and the separation of the hindbrain into metencephalon and myelencephalon. The formation of divisions and their adult derivatives are summarized in Table 4-2. Two distinct signaling centers apparently organize the cranial portion of the neural tube. One center organizes the forebrain and the other the hindbrain; each produces its own set of signaling factors. Another important source of signaling molecules for brain regionalization comes from the midbrain-hindbrain signaling center.[8]

The Eye (See Chapter 170)

At approximately 4 weeks of development, two outpouchings of the forebrain (diencephalon) expand laterally to form the optic vesicles (Figure 4-8, *A* and *B*), progenitors of the eyes. Inductive

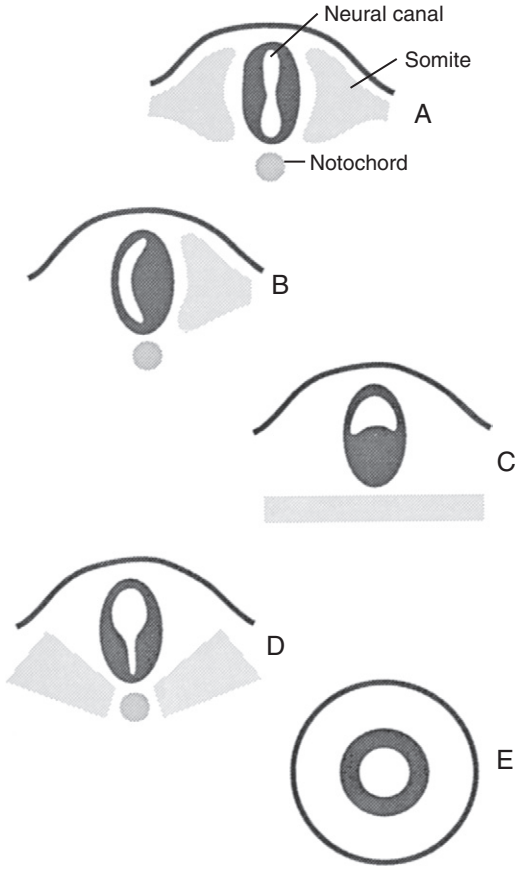


Figure 4-7 Influence of somite mesoderm *(lightly stippled areas)* **on neural tube and neural canal shape. A,** Normal. **B,** One somite removed. **C,** Somite mesoderm transplanted to the ventral surface of the neural tube. **D,** Somite mesoderm transplanted to the ventrolateral surface of the neural tube. **E,** Complete absence of somite mesoderm. *Clear areas* between structures contain non-somite mesoderm. *(Modified from Saxen L, Toivonen S:* Primary Embryonic Induction, *London, 1962, Logos Press.)*

interaction takes place between the optic vesicles and the head ectoderm, which lies closest to the vesicles.[70] The first manifestation of this interaction is the formation of a thickened plate in the head ectoderm, the lens placode (see Figure 4-8, *B*). At approximately the same time, the lateral surface of the optic vesicles begins to invaginate to form the double-layered optic cup. The lens placode also invaginates creating a lens pit (see Figure 4-8, *C*). The pit continues to deepen as it follows the profile of the increasingly concave optic cup. Finally the deeply pitted placode pinches off, forming the lens vesicle (see Figure 4-8, *D*). By this time point (approximately 30 days), the double walls of the optic cup appose each other; fusion of these two layers is completed during the fetal period. The inner layer forms the neural retina, and the outer becomes the pigment epithelium of the retina. The peripheral margin of the optic cup forms the iris and ciliary apparatus, whereas the optic nerve is formed from the optic stalk. Mesoderm surrounding the optic cup forms the inner vascular choroid and the fibrous outer sclera.

The Ear (See Chapter 171)

The inner ear begins to develop in the fourth week after fertilization as a recognizable thickening of surface ectoderm on either side of the myelencephalon, known as the otic placode (Figure 4-9, *A*). As with the lens vesicle, the placode invaginates to form

TABLE 4-2

Brain Vesicles and Their Derivatives

Early Division	Later Divisions	Wall Derivatives	Lumen Derivatives
Forebrain (prosencephalon)	Telencephalon	Cerebral hemispheres	Lateral ventricles, rostral part of 3rd ventricle
	Diencephalon	Epithalamus, thalamus, hypothalamus, pineal	Most of 3rd ventricle
Midbrain (metencephalon)	Mesencephalon	Midbrain	Cerebral aqueduct
Hindbrain (rhombencephalon)	Metencephalon	Pons, cerebellum	Superior part of 4th ventricle
	Myelencephalon	Medulla	Inferior part of 4th ventricle

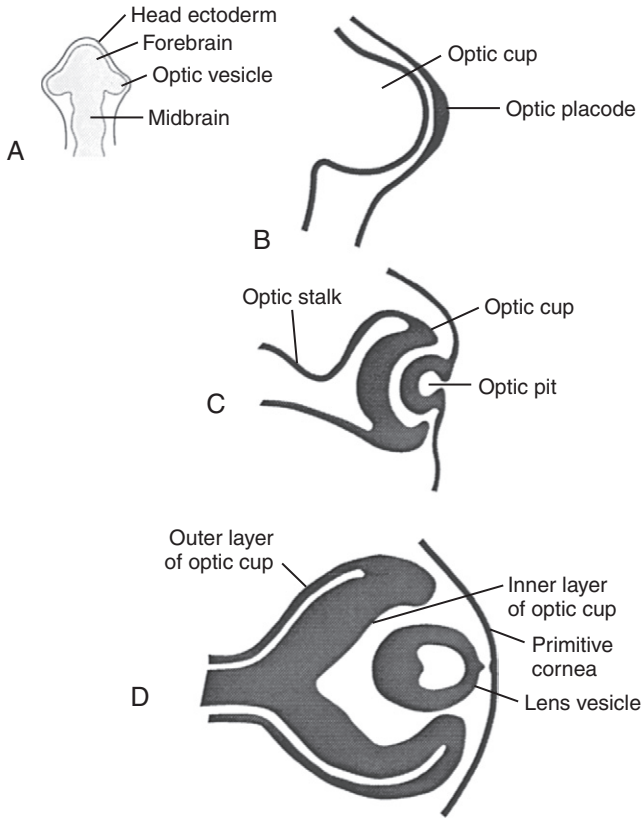


Figure 4-8 Development of the eye. A, Dorsal view showing the head, optic vesicles, and overlying head ectoderm. **B-D,** Magnified view of the right optic vesicle at successively older stages, illustrating the development of the lens placode, pit and vesicle, and the optic cup.

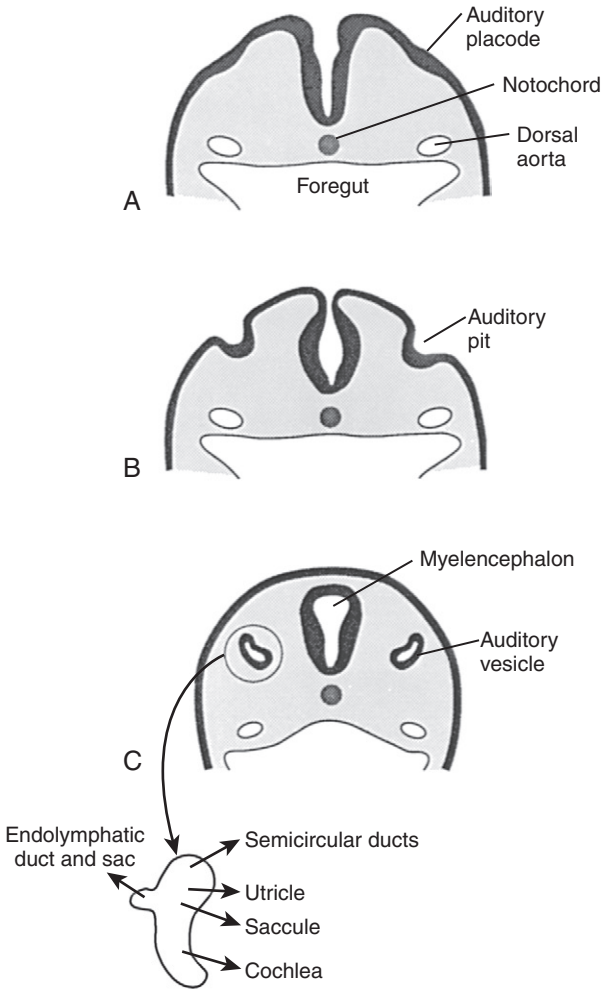


Figure 4-9 Successively older stages **(A-C)** showing development of the inner ear from the auditory placode. Derivative adult structures are shown in the *inset* in **C.**

the otic pit) (see Figure 4-9, *B*) and pinches off to form the otic vesicle (see Figure 4-9, *C*). Small diverticula soon bud from each vesicle to form the endolymphatic sac. As growth of the hollow vesicle proceeds, additional regional morphogenesis yields the utricle, semicircular ducts, saccule, and cochlear ducts, as well as other sensory receptors and primary sensory neurons (see Figure 4-9, *C*).

Cardiovascular System (See Chapters 63 and 64)

Heart

Cells destined to form the primitive heart tube are located within two oval areas of mesoderm (Figure 4-10) on either side of the midline of the embryo.[71-73] Organized as discrete clusters of cells between the ectodermal and endodermal layers, this precardiac mesoderm migrates cranially and fuses in the midline just cranial to the oral membrane. The cell clusters form two more or less parallel solid cords of cells on reaching the area just forward of the oral plate (see Figure 4-10). These soon canalize to form endocardial tubes. The tubes swing together because of lateral body folding and fuse to form the primitive heart tube, which then undergoes additional growth, morphogenesis, and changes in position with time. Initially the primitive chambers of the heart tube are arranged in a linear series, with inflow at the caudal end and outflow at the cranial end.

The heart begins beating approximately 22 days after fertilization, and weak circulatory movement of fluid begins in adjacent vessels about a day later. At this time, the embryo can be compared to an aggregate of cells approximately 1 × 1 × 2 mm. Tissue culture studies indicate that cells in the center of a mass thicker than approximately 0.5 mm die of oxygen deprivation, because

the simple diffusion of oxygen into the core is not sufficient to support cell metabolism. Thus it appears that the formation of a functional circulatory system is requisite for the continued life of the embryo. Indeed, the circulatory system is the first organ system to become functional in the human embryo.[74,75]

Good evidence from animal experiments indicates that the first contractions of the embryonic heart are initiated by stretching of the heart tube as fluid pressure builds in the developing circulatory system. Isolated hearts do not begin beating on time unless artificially produced fluid pressure is maintained within the lumen of the heart. Moreover, the heart in intact embryos can be induced to begin beating earlier than normal by the introduction of small amounts of fluid into the lumen, thereby increasing intraluminal pressure and causing stretching of the walls of the heart.[76,77]

Almost as soon as the primitive heart tube forms, sulci on its external surface deepen markedly. This begins a process called *cardiac looping,* in which the inflow and outflow regions are brought into approximation. The mechanisms behind the control of looping are poorly understood.

Several concurrent events occur after looping that lead to partitioning of the heart into separate chambers and outflow channels. The sinus venosus, which initially receives all incoming venous blood from the embryo, is remodeled so that all blood enters what will later become the right atrium. Resorption of the sinus venosus forms the smooth area of the definitive right atrium and contributes to atrial septation. Much of the definitive left atrium is formed by resorption of the pulmonary veins.

Separation of the atria begins during week 5, when a sickle-shaped membrane, the septum primum, grows from the roof of the atrium toward the atrioventricular canal. This canal is simultaneously being divided into right and left channels by the fusion of the enlarging superior and inferior endocardial cushions. The opening between right and left sides of the common atrium, now partially divided by the growing septum primum, is called the *foramen* (or *osteum*) *primum.*

Soon the lower edges of the septum primum fuse with the endocardial cushions, and further growth of both the septum and the cushions closes the foramen primum. Before it closes completely, however, several perforations appear in the superior portion of the septum primum. These perforations fuse to form the foramen secundum. Thus, communication between the right and left atrial cavities is maintained during this complex morphogenetic process.

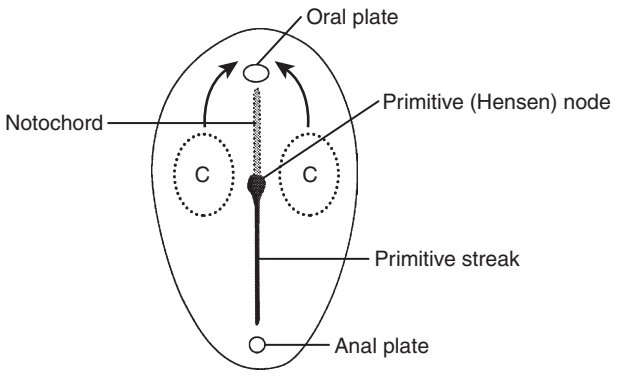


Figure 4-10 Surface view of an embryo at approximately 16 to 17 days after fertilization. This view is of the ectodermal surface. Structures below the surface are outlined with *dotted lines.* The anal and oral plates are areas in which the ectoderm and endoderm are in tight contact, as in a "spot weld," with no mesoderm between. Mesoderm is present in other areas. At *C* lie the mesodermal cell clusters that migrate *(arrows)* to an area forward of the oral plate, to form the cardiogenic cords. These two *oval areas* contain precardiac mesoderm cells.

Another septum (the septum secundum) begins to form at about the time the foramen secundum becomes well defined. This septum forms to the right of the septum primum as a crescentic ridge. Further growth produces a thick membrane, but the oval opening in this membrane persists as the foramen ovale. This arrangement of two parallel septa (primum and secundum) with offset holes (secundum and ovale) produces a flap valve between the two atria, ensuring unidirectional flow of blood from right to left.

Fusion of the endocardial cushions (composed of cardiac mesenchyme) forms the septum intermedium. This structure not only divides the atrioventricular canal into right and left portions but also acts as a central attachment point and reference center for several septation events, as already described. The cushion tissue of the septum intermedium contributes to the formation of the membranous portion of the interventricular septum, as well as to the development of the atrioventricular valves and to the fibrous cardiac skeleton. It also serves as a fusion point for the cushion tissue–derived ridges, which divide the proximal outflow region of the heart.

Even though the heart is enlarging while septation proceeds, the dimensions in this central region around the septum intermedium remain constant. A significant enlargement in this region at this time could lead to congenital heart defects.

Partitioning of the ventricle is accomplished primarily by fusion of trabeculae, which form a muscular interventricular septum. Ventricular septation is completed by formation of the membranous portion of the septum from an outgrowth of endocardial cushion tissue. This morphogenetic process occurs simultaneously with division of the proximal outflow area. Initially, no direct communication is present between the right atrium and the right ventricle. Remodeling activities in the inner curvature of the heart create this communication by mechanisms which are still obscure.

Division of the outflow tract is complex and still not completely understood. The proximal portion of the outflow tract is subdivided into the conus and truncus regions, both of which are divided into left and right halves by ridges of endocardial cushion tissue called the conotruncal or bulbar ridges. Septation of the conus region forms an outflow segment for each ventricle, whereas in the truncus region, distinct aortic and pulmonary valves develop. A portion of the conal septum extends down and attaches to the muscular interventricular septum, partially closing the interventricular foramen. Complete closure of the foramen is accomplished by downgrowth of cushion tissue from the septum intermedium. In the distal outflow tract (the aortic sac), a wedge of mesenchyme (the aorticopulmonary septum) develops between the fourth and sixth aortic arches. This mesenchyme (thought to be of neural crest origin[78]) grows downward and then penetrates and fuses with the conotruncal ridges.

Vessels

During the third week of development, groups of extraembryonic mesodermal cells in the yolk sac and chorion, known as *angioblasts,* aggregate to form isolated, solid masses called *angioblastic clusters* or *blood islands.* These soon cavitate (Figure 4-11), and those cells on the periphery of the hollow blood islands differentiate into endothelial cells. Blood cells are formed from those angioblasts remaining in the lumen, as well as by cell division and budding from the primitive endothelial lining. Growth and fusion of the isolated hollow blood islands form tubes, and the tubes fuse to form long interconnected channels. Lateral buds from tubes and channels also may extend the developing vasculature into adjacent areas.

This sequence, based on observations of sectioned embryos, constitutes the basic template for how vessels form. It does not explain how the pattern of vessels develops. Little is known concerning how the intricate pattern of anastomosing vessels is established in the early embryo.

The Musculoskeletal System (See Chapter 175)

Muscular System

The three types of muscle tissue—skeletal, smooth, and cardiac—are largely derived from cells of the mesodermal germ layer. Skeletal muscle forms from paraxial mesoderm, whereas cardiac muscle is a derivative of splanchnic mesoderm. Most smooth muscle is derived from splanchnic mesoderm. It seems likely, however, that all mesenchyme, whether derived from mesoderm or from neural crest (as in head mesenchyme), has the potential to form vascular smooth muscle. The dilator and sphincter smooth muscles of the iris as well as the myoepithelial cells that surround sweat glands and mammary glands are thought to be of neural crest origin.

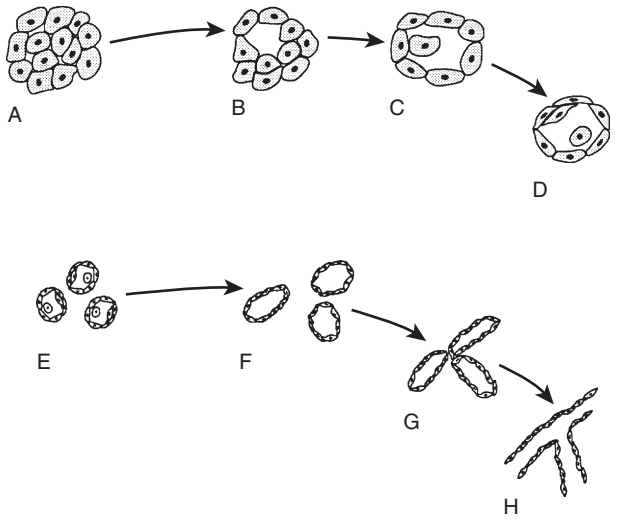


Figure 4-11 Vasculogenesis. A-D, Formation of hollow blood islands from solid clusters of angioblasts. **E-H,** Formation of a portion of a primitive vascular network by fusion of blood islands. Budding *(not shown)* also contributes to vascular pattern growth.

Skeletal Muscle

Mesenchyme cells that are to develop into skeletal muscle elongate and lose their multiple processes. The earliest cell that can be identified as a skeletal muscle precursor is a fusiform cell called a *myoblast* (Figure 4-12). These cells become postmitotic and fuse into cylindric, multinucleated myotubes. These myotubes start producing actin and myosin myofilaments, which adds to girth. Growth of myotubes also may occur by fusion of additional myoblasts. The skeletal muscles of the head and neck develop from paraxial mesoderm represented by somitomeres in the early embryo. Trunk musculature is derived from the myotome portion of the somites (Figure 4-13).

Somites themselves are interesting structures—serially repeated paired blocks of condensed (closely packed) paraxial mesoderm cells. Approximately 44 pairs of somites eventually develop in humans, beginning between days 19 and 21.[64] These can be seen in a surface view of the embryo because they produce bulges in the overlying sheet of ectoderm. Each member of a pair of somites lies lateral to the neural tube (see Figure 4-13). Myoblasts of the myotome portion of each somite divide and spread out deep to the embryonic skin, where they form the musculature. Various morphologic processes including fusion, tangential splitting of layers, reorientation of muscle fibers, and formation of tendon intersections are responsible for the final morphologic form of the named muscles.

The musculature of the limb also is derived from the myotome portion of the somites. These myoblasts migrate into the elongating limb buds and arrange into dorsal and ventral muscle masses, which later become subdivided into the definitive limb muscles.

Cardiac Muscle

Cardiac type of muscle develops from splanchnic mesoderm adjacent to the pericardial or transverse portion of the intraembryonic coelom. Myocardial precursor cells undergo mass migration and do not fuse to form myotubes. Rather, the myoblasts differentiate as discrete cells, with closely applied end-to-end junctions, which persist as the adult intercalated disk. They form a layer around the endothelial tube of the heart, which eventually becomes the myocardial or muscular wall of the heart.

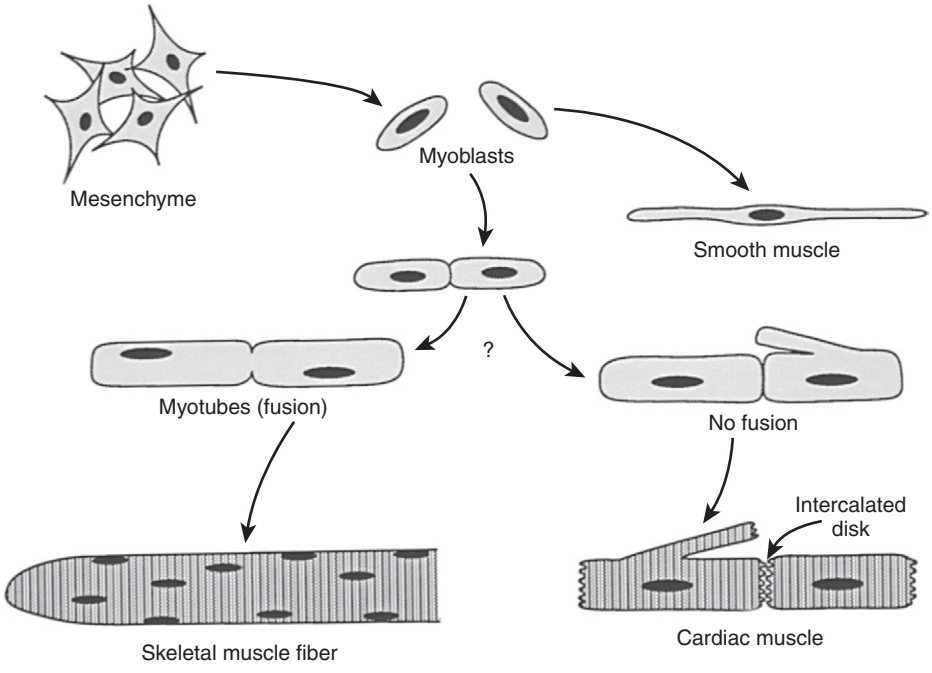


Figure 4-12 Schematic representation of the development of the three types of muscle cells—skeletal, smooth, and cardiac. Some question exists regarding the precursor cells—that is, a current hypothesis is that mesenchyme gives rise to a cell type that develops into cardiac and smooth muscle, but that it gives rise to another cell type that develops into skeletal muscle.

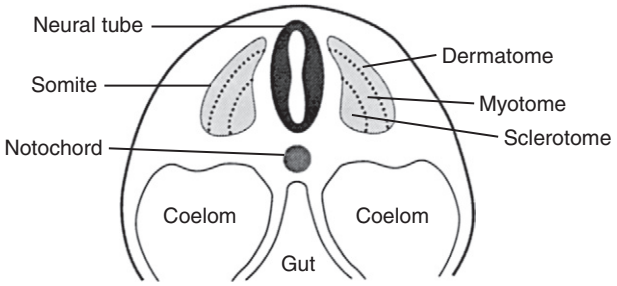


Figure 4-13 Fate map of the somite, showing regions from which cells developing into dermis, muscle, and cartilage and bone are derived.

Smooth Muscle

Smooth muscle cells form from myoblasts derived primarily from splanchnic mesoderm (see Figure 4-12).

Skeletal System (See Chapter 173)

Cartilage develops from mesodermally derived mesenchyme, except in some areas of the head and neck, where it is of neural crest origin. Chondroblasts aggregate, condense, and begin to produce collagen fibers and ground substance. In the embryo, bone tissue is formed in either of two distinct ways, depending on the site and type of bone growth. The first is de novo bone formation, in which mesoderm cells (or, in the case of some skull bones, neural crest cells) first condense (pack) into sheets or membranes. Cells in these sheets then differentiate into osteoblasts, which secrete prebone or osteoid. Osteoid is the extracellular matrix onto which hydroxyapatite crystals (a unique calcium phosphate mineral) form. Once the mineral is present and integrated into the collagen of the matrix, the tissue is considered to be bone. This type of bone formation is called intramembranous ossification because the osteogenesis occurs within these sheets or membranes of condensed mesenchyme. Some of the bones of the cranial vault, face, and jaws form in this way.

The second manner in which bone forms in the embryo is called *endochondral ossification.* As implied by the name, bone forms in this case only in sites where preexisting cartilage models are found. The cartilage does not turn into bone. Rather, bone replaces the preexisting cartilage model in a sequence of steps, as follows. Cartilage cells hypertrophy and calcify in an area where bone is to form, that is, they become large and undergo metabolic changes, which lead to infiltration of the surrounding cartilage extracellular matrix with insoluble calcium salts. In this way, the cartilage cells become partitioned from their surroundings by an environment that is presumed to cut off their supply of oxygen and nutrients, and they die. At the same time, local osteoblasts begin to produce osteoid, which then becomes mineralized as described in intramembranous ossification. Once bone has formed in the embryo, it can be remodeled by changes in the balance between addition of more bone by osteoblasts and removal of bone by osteoclasts.

Respiratory System (See Chapter 78)

During the fourth week of development, the embryo forms a C shape, and the primitive gastrointestinal tube is already divided into a foregut, midgut, and hindgut (Figure 4-14). A groove, the respiratory (laryngotracheal) diverticulum, arises as an evagination of the ventral surface of the foregut, close to the region destined later to become the stomach. As the foregut elongates a wedge of mesoderm, the tracheoesophageal septum, separates the foregut (portion that will become esophagus) from the future lungs. This early association of developing lung and stomach leads to the occasional finding of ectopic lung and cartilage tissue near the esophageal-gastric junction.[79]

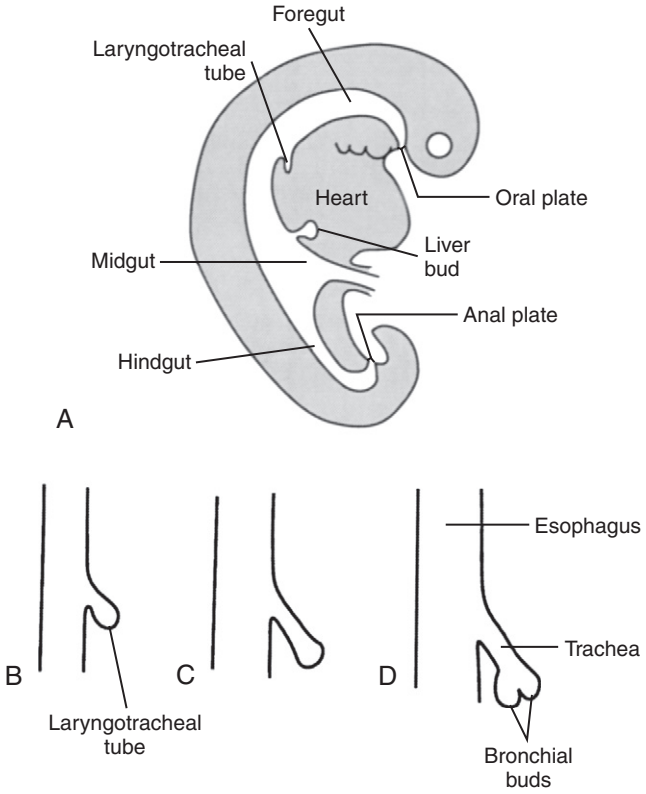


Figure 4-14 Development of the respiratory system. The laryngotracheal (respiratory) tube buds off from the ventral surface of the foregut, as shown in **A.** The tube elongates and splits into two bronchial buds **(B-D),** each of which ultimately gives rise to the epithelial lining of one lung.

The respiratory diverticulum grows caudally and soon splits into two bronchial buds. Each of these buds subdivides to form primitive secondary or lobar bronchi. On the left side, two secondary bronchi supply the developing superior and inferior lobes of the left lung. On the right, the inferior secondary bronchus divides into two, providing a total of three secondary bronchi to supply the three lobes on this side. The third-order branches form the bronchopulmonary segments. Altogether, approximately 17 generations of branches form. Differentiation of the respiratory passages begins in the fetal stage and proceeds from distal to proximal along the branches of the respiratory diverticulum. Alveolar formation begins toward the end of the fetal period and continues until the age of 2 to 3 years.[53]

Of note, the endoderm of the foregut gives rise to the epithelial lining of the trachea, bronchi, and lungs, including the alveoli. The surrounding splanchnic mesoderm develops into the cartilage and fibrous connective tissue of the larger airways as well as the blood vessels and supporting tissues of the smaller airways and alveoli.

Digestive System (See Chapters 108 and 109)

The lining of the gastrointestinal tube arises primarily from endoderm, whereas the muscle coats and connective tissue elements usually are derived from splanchnic mesoderm. Toward the end

of the third week the embryo folds craniocaudally and laterally, forming a inner gut tube of endoderm that is subdivided into the foregut, midgut (still connected to the degenerating yolk sac), and the hindgut.

Oral Cavity and Anal Regions

Throughout most of the developing gastrointestinal tube, the epithelium and derivative glands arise from the endodermal germ layer, which lines the foregut, midgut, and hindgut (see Figure 4-14). Cranial to the foregut and caudal to the hindgut, the relationship of embryonic structure to adult derivatives is less obvious. For example, the oral membrane, which is a fusion of ectoderm and endoderm, is the cranial boundary of the foregut (in the adult this boundary is at the level of the tonsillar fauces). The oral cavity develops cranial to the oral membrane from an ectodermal depression, the stomodeum, bounded by the first pharyngeal arch and the frontal process. Three groups of salivary glands develop in this region. The sublingual and submaxillary glands develop from endoderm, as might be expected, but the parotid is derived from head ectoderm.[38] Similarly, structures below the pectinate line in the adult anal canal are derivatives of the proctodeum, an ectodermal depression in the caudal end of the embryo that is sealed off from the gut tube by the anal plate until the end of the eighth or beginning of the ninth week. Thus the anal columns (of Morgagni), located above the pectinate line, are of endodermal origin, whereas the anocutaneous (white) line and surrounding epithelial structures are from ectoderm.

Liver (See Chapter 118)

The hepatic diverticulum (or liver bud) arises as an outpouching of the lumen of the distal foregut during the fourth week of development (see Figure 4-14). The liver bud gives rise to the gallbladder and bile ducts as well as to the parenchyma of the liver. The liver bud grows toward the anterior body wall, at first completely buried in the mesenchyme of the septum transversum. Rapid growth causes the liver bud to bulge into the abdominal cavity, freeing it on all but the cephalic surface. There it remains in contact with the septum transversum as the latter forms part of the diaphragm. After development is complete, this area of contact between the liver and the diaphragm is known as the "bare area of the liver" because it is not covered with capsule or peritoneum.

The liver is connected on its anterior surface to the anterior body wall by the ventral mesentery, which becomes the falciform ligament. The hepatocytes are derived from endoderm, as are the bile canaliculi and the epithelial linings of the intrahepatic biliary ducts. The liver sinusoids, larger blood vessels, and connective tissue stroma of the liver all are derived from the mesenchyme of the septum transversum, which is of mesodermal origin.

Pancreas (See Chapter 114)

Most of the pancreas develops from the dorsal pancreatic bud, which arises as an outpouch of the dorsal surface of the future duodenum at the caudal end of the foregut. It grows into the mesenchyme of the dorsal mesentery at this site. The ventral pancreatic bud starts as an outgrowth of the future common bile duct, but it fuses with the dorsal pancreatic bud when rotation of the gut brings these two components of the pancreas into contact. Both exocrine and endocrine (islets of Langerhans) elements of the pancreas are derived from endoderm, whereas the blood vessels and connective tissue components arise from splanchnic mesoderm.

Esophagus

The esophagus is a short regional specialization of the embryonic foregut tube that elongates with growth of the body. During the middle of the embryonic period, its lumen is obliterated by proliferation of the endoderm-derived lining cells, but cell death (a normal developmental process in many areas of the human embryo) reestablishes the lumen by early fetal life.

Stomach

The stomach begins as a simple fusiform dilation of the foregut. Growth of the dorsal wall of the stomach surpasses that of the ventral wall, leading to the greater curvature and driving a clockwise rotation of the gut that brings the greater curvature (original dorsal surface) to the left and the lesser curvature (original ventral surface) to the right. As a result of differential growth, the cardiac region (cranial) of the stomach moves inferiorly and to the left, and the pyloric (caudal) end moves superiorly and to the right. Ultimately, this rearrangement results in the almost horizontal axis seen in the adult stomach.

Duodenum

The duodenum develops from both the caudal end of the foregut and the cranial end of the midgut. It rapidly elongates into a loop, the bend of which is toward the ventral body wall. As the stomach rotates to the right, so does the duodenal loop, bringing it to lie against the dorsal wall of the body cavity. There it fuses with the dorsal wall, and most of it comes to lie in a retroperitoneal position. As in the esophagus, the duodenal lumen is transiently obliterated by growth of the endoderm-derived lining cells until the beginning of the fetal period.

Lower Gastrointestinal Tract

Much of the remaining gastrointestinal tube—jejunum, ileum, cecum, ascending colon, and half of the transverse colon—develops from the midgut. The elongating midgut also forms a distinct loop the of which is closest to the anterior body wall. This U-shaped loop herniates (normally) into the extraembryonic coelom of the umbilical cord. While herniated, the loop rotates 90 degrees counterclockwise. Thus the cranial branch of the U-shaped loop moves to the right. At the same time, growth and elongation create several additional subloops in the cranial portion of the U-shaped loop; these later give rise to the jejunum and ileum.

The herniated small intestine with its subloops returns to the body cavity first. As the large intestine follows, it rotates an additional 180 degrees, bringing the cecum and appendix to their final position in the lower right quadrant of the abdomen.[80] Little is known about how these complex morphogenetic events are controlled. Nevertheless, a thorough knowledge of these events as they occur in a normal embryo can help to explain the numerous anatomic variations and congenital malformations that have been observed. The hindgut contributes to the distal half of the transverse colon, the descending and sigmoid colon, the rectum, and the anal canal down to the white line.

Urinary System (See Chapter 123)

Near the end of the third week after fertilization, the intermediate mesoderm appears as a solid cord of condensed mesenchyme just lateral to the paraxial (somite-forming) mesoderm (see Figure 4-5, *C*). The intermediate mesoderm is bilateral and extends along the length of the body axis as far as the somites do; it gives rise to components of the urinary and genital system.

The intermediate mesoderm is displaced ventrally to a position lateral to the dorsal aorta and notochord when the embryo becomes tubular, owing to growth and formation of the lateral body folds. During this movement, the connection between the somites and intermediate mesoderm is broken. Once this connection is lost, the two bars of intermediate mesoderm are called nephrogenic cords.

The traditional description of kidney development suggests that three successive sets of kidneys form in the embryo—the

pronephros, mesonephros, and metanephros. The concept of a pronephros, however, is not relevant in humans.[81] Beginning at the level of the eighth or ninth somite,[82] the mesonephric kidney contains primitive tubules associated with glomeruli that empty into a mesonephric (wolffian) duct. The cephalic tubules begin to degenerate even before the more caudal tubules are starting their development. In males, the mesonephric duct becomes associated with the primitive gonad and gives rise to the epididymis, ductus deferens, and ejaculatory ducts; some of the mesonephric tubules persist as the efferent ductules. In females, the mesonephric duct distal to the ureteric bud degenerates. The mesonephros actively produces urine as early as the sixth week of development and continues into the early fetal period.

During the fifth week, the metanephros begins as a small diverticulum near the caudal end of the mesonephric duct—the ureteric bud. The mesoderm at the caudal aspect of the nephrogenic cord condenses around the ureteric bud, forming a metanephric blastema. The ureteric bud plus the metanephric blastema form the metanephric or definitive kidney (see Figure 4-1, *A*). The nephron from the renal corpuscle to the collecting duct is derived from the metanephric blastema whereas the collecting system and ureter are derived from the ureteric bud. Development of the metanephric kidney depends on mutual inductive signals from the metanephric blastema and the ureteric bud.[68] Signals from the bud result in mesenchymal-epithelial transformation of the metanephric blastema mesenchyme, leading to formation of an epithelial vesicle. The vesicle remodels into a tubule, the proximal end of which attaches to the ureteric bud, whereas the distal end becomes associated with vascular precursors of the glomerulus. Signals from the metanephric mesoderm cause the ureteric bud to branch, forming the collecting system. If for some reason the ureteric bud does not grow to meet the metanephric blastema, the blastema does not develop nephrons, and renal agenesis results. If the blastema is absent, the ureteric bud does not develop into the collecting system of the kidney.[83]

By the end of the first trimester, the fetal kidneys begin to produce urine.[84] At that time, they have been displaced from their initial pelvic location to the abdomen, and they lie in a retroperitoneal position close to the adrenal glands. The latter develop from coelomic mesoderm (cortex) and neural crest cells (medulla) that have become associated in the abdominal cavity, cephalic to the final position of the kidneys.

The urogenital sinus is an endoderm-lined cavity from which are derived the epithelium of the urinary bladder, all of the female urethra, and most of the male urethra. The muscular coats and connective tissue elements of all of these structures are of splanchnic mesoderm origin.

GROWTH AND MATURATION OF THE EMBRYO AND FETUS

Human embryonic development is a continuous process that averages 266 days, or 9½ months, when counted from the day of fertilization. Clinically, the start of gestation is determined by counting from the date of the last menstrual period (LMP). Estimated this way, it averages 280 days or 10 lunar months.[85] Human prenatal development commonly is divided into two periods or phases: the embryonic period and the fetal period (Figure 4-15). The first 8 weeks after fertilization constitute the *embryonic period.* It has been subdivided into 23 developmental stages (Carnegie stages).[53] During the embryonic period, the single-celled zygote is transformed into an embryo. With respect to human embryology, the term *embryo* means "an unborn human in the first 8 weeks" from fertilization.[53] This period is characterized by several developmental milestones, including cell division of the zygote, formation of a blastocyst, implantation, formation of three primary germ layers, segmentation and axis formation, and initial morphogenesis of organ systems.

It is thought that during the first 2 weeks of development, the embryo is relatively insensitive to the action of agents that cause congenital malformations (teratogens). Retrospective human studies as well as studies in animals suggest that low to medium doses of teratogens will not cause abnormal development during the first 2 weeks, but that a large dose will kill the embryo. Duringthis 2-week period, defects also may develop

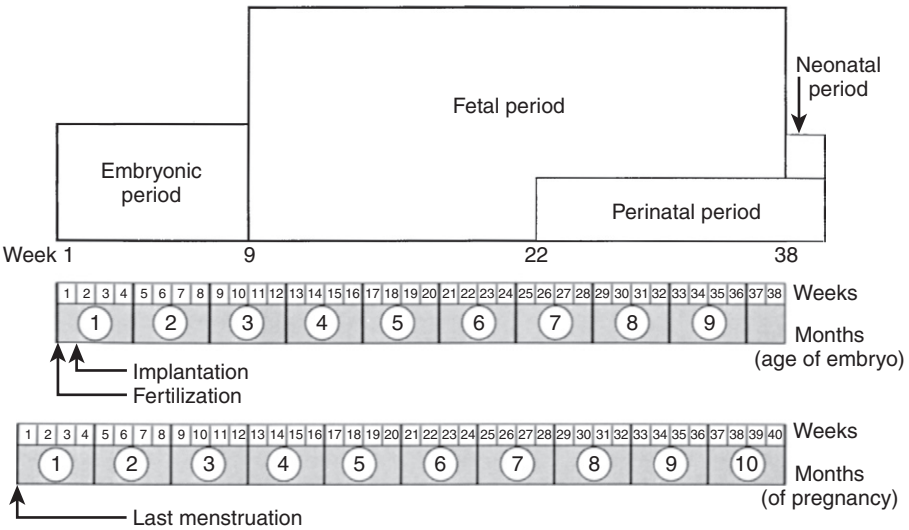


Figure 4-15 Human development. The *upper portion* of the schematic diagram shows human development divided into periods. The embryonic period extends from fertilization through week 8. The remainder of pregnancy (week 9 through birth) is termed the fetal period. The perinatal period extends from the prenatal week 22 until 4 weeks after birth. The neonatal period is the first 4 weeks after birth. In the *bottom portion* of the diagram, the ages of the embryo/fetus are shown on two time scales. The lower of the two is a clinical scale, with *pregnancy* counted from the date of the last menstrual period. Above it are weeks and months of *development,* counted from fertilization. Note that the 2-week difference, with pregnancy lasting 40 weeks. *(Modified from Kaplan S:* Congenital Defects: An Overview: An Introduction to the Principles of Teratology, *Chapel Hill, NC, 1981, Health Sciences Consortium.)*

after treatment with the teratogenic agent if for some reason it remains available to the embryo throughout the period—for example, because of slow metabolism or excretion. It is during the third to eighth week of development, however, that morphogenesis of most organ systems begins and, for many systems, ends. This is the portion of the embryonic period in which the organs, and therefore the embryo as a whole, are most sensitive to the actions of teratogens. For example, a kidney will fail to develop only if, during morphogenesis, the ureteric bud fails to send the proper signals to the metanephric blastema. Moreover, a limb will be abnormally foreshortened only if long bones fail to form during that time when the bony elements are due to undergo their primary morphogenesis. Therefore weeks 3 to 8 of the embryonic period constitute the critical period in human development with regard to the action of teratogenic agents.

During the *fetal period* (beyond the end of the eighth week after fertilization, or beyond the tenth week of post-LMP pregnancy—see Figure 4-15), the fetus becomes increasingly resistant to the action of teratogens. This decreasing susceptibility does not mean that no organ system can become malformed during fetal life. An example of such an organ is the brain, which exhibits the longest developmental period of any organ; it continues both physical and functional development throughout the fetal period and beyond, well after birth.[86] This extended development also prolongs its period of susceptibility; more major congenital defects occur in the brain than any other organ.[87]

Organ systems other than the brain also continue morphogenesis into the fetal period (e.g., palate, ear, external genitalia, lungs). In contrast with the brain, however, most other organ systems essentially complete morphogenesis by the end of the embryonic period.

The fetal period is characterized by rapid growth in weight and size of the conceptus as a whole (Figure 4-16), which reflects growth in individual organs. All organs expand and undergo histogenesis, or differentiation of the cell populations of which they are composed, but the particular pattern of increase in weight may vary from organ to organ. Hematopoiesis begins in the liver during the late embryonic period, making the liver a functional organ relatively early in development.[85] Accordingly, it expands rapidly to make up approximately 10% of total body weight by the beginning of fetal life.[38] Thereafter, its growth rate follows

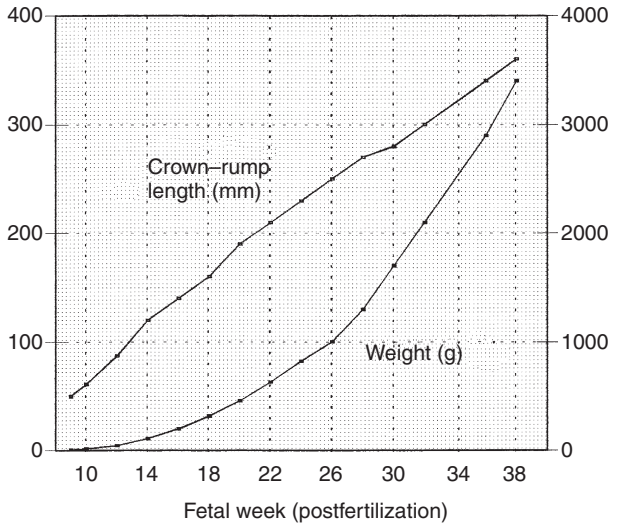


Figure 4-16 Weight and crown–rump length during human development. *(Data from Moore KL, Persaud TVN: The Developing Human: Clinically Oriented Embryology, 6th ed, Philadelphia, 1998, WB Saunders.)*

that of the whole body, so that in proportion to body weight, liver size remains virtually constant.[88]

The same proportional growth pattern is seen in heart and kidney, but growth of the head is quite different. At the beginning of the fetal period, the head is approximately half the crown-rump length; at 12 weeks, it is approximately one-third this value. Thereafter, the rate of growth continues to slow in relation to the rest of the body.

An important point is that the establishment of the gross form of an organ does not necessarily correspond to initiation of function. The human fetal gastrointestinal tube closely resembles that of the newborn infant as early as the middle of the sixth month. By contrast, cell differentiation and the development of complex enzyme systems necessary for the digestive process continue through birth and well beyond.[89]

The early human fetus is approximately 95% water, and a progressive increase in solids occurs throughout fetal growth.[88] Most of the water (in a ratio of 4:1) is extracellular in early fetal life, but more is found within cells as the fetus matures, so that by birth the ratio is approximately 1:1.[90] Protein is accumulated most rapidly before the fetus weighs 1 kg, with the accumulation rate slowly declining thereafter. Fat deposition rate is low until the middle of gestation, when the proportion of fat (especially subcutaneous fat) to total body weight increases exponentially.[91]

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5 Regulation of Embryogenesis

Matthew K. Lee, Yang Chai, David Warburton, and Harold C. Slavkin

Vertebrate body plans result from the divergent proliferation, migration, survival, and differentiation of cells derived from a single fertilized egg. This divergence is induced and sustained by regulatory signals within the embryo that modulate cell behavior. Behavioral changes accumulate over successive generations of cells, so the same stimulus may have disparate effects on cells at different developmental stages, with different lineages, or in different physical contexts. Because a cell's response to a specific stimulus is altered by its historical and physical context, regulatory processes can be used iteratively to control development. In this way, the mechanisms regulating early embryogenesis are recapitulated during the development of limbs, organs, and craniofacial structures in an essentially fractal process. This recapitulation underlies the disrupted organogenesis that accompanies somatic malformation in many human congenital syndromes. Conversely, the same mechanisms guide tissue regeneration after injury to the adult organism. The elucidation of embryonic regulatory mechanisms therefore represents an area of enormous therapeutic importance as well as a signal accomplishment of modern biology and medicine.

This chapter introduces some of the mechanisms by which key embryonic events are initiated. The regulation of initial axis determination in the oocyte, mesodermal induction in the blastula, and mesodermal patterning during gastrulation is discussed for the African frog *Xenopus laevis,* long a leading model of vertebrate embryology. Among vertebrates, the mechanisms underlying initial embryonic patterning are most completely elucidated for the frog. By comparison, the initial patterning of birds and mammals is more complex and incompletely defined.[1] Mouse and chicken development are discussed as excellent models of left-right axis determination and segmentation. For each developmental stage, a regulatory mechanism that is important in guiding embryogenesis during that period is introduced. In addition, some examples of disrupted late embryonic regulation are correlated with specific clinical syndromes to demonstrate how regulatory pathways are reiterated temporally and spatially within the embryo and fetus.

Critical events in embryogenesis are initiated by the expression of key genes. Gene transcription, in turn, is regulated by transcription factors that bind the promoter region immediately upstream of the protein coding region and by enhancers and suppressors that bind at variable distances upstream or downstream of the coding region. Transcriptional regulators typically bind many different genes, some of which also may encode transcriptional regulators. In this way, dozens of genes may be controlled by a single stimulus. The activity of a transcription factor is not dependent solely on its expression; the affinity of a transcription factor for its binding site may depend on its posttranslational modification, the presence or absence of cofactors, or the packing state of the targeted chromatin. The same variables also determine whether that factor increases or decreases the activity of a particular gene. Gene expression is thus subject to regulation by a variety of molecular mechanisms, many of which are implicated in embryonic regulation. Some of this variety is evident in the following examples.

THE SPATIAL ORIENTATION OF THE OOCYTE IS INITIATED BY EGG ROTATION

Vertebrate embryos arise from radially symmetric spheres or cups, and their development requires the establishment of spatial references. Vertebrates develop asymmetrically along three spatial axes: anterior-posterior (anteroposterior), dorsal-ventral (dorsoventral), and left-right. These axes become visible during gastrulation but are established earlier, in the blastula. In the frog *Xenopus,* oocytes are intrinsically divided into animal and vegetal hemispheres. Fertilization occurs in the animal hemisphere, where sperm entry induces formation of an aster centered on the sperm centriole as well as an array of vegetal cortical microtubules.[2] The aster induces the egg cortex to rotate through a 30-degree angle with respect to the vegetal hemisphere, thereby displacing some of the clear vegetal cortex into the animal hemisphere.[3] The intersection of vegetal cortex with animal cytoplasm is visible as a gray crescent that becomes the dorsal pole of the embryo.[4,5] Conversely, the ventral pole forms at the area of contact between animal cortex and vegetal cytoplasm.[6]

How does cortical rotation regulate gene transcription? In *Xenopus,* the convergence of vegetal cortex with animal cytoplasm results in localized activation of Wnt signaling at the gray crescent. The Wnt pathway regulates many cellular processes in both adult and developing organisms and centers on β-catenin, a membrane-bound multifunctional protein that participates in transcriptional activation.[7] In the absence of Wnt signaling, β-catenin is continuously produced and destroyed by ubiquitin-mediated proteolysis. Two inhibitors of this proteolysis, Dishevelled (Dsh) and GSK-binding protein (GBP), are expressed in the vegetal hemisphere and attached to kinesin light chains.[8] Kinesins are cargo-carrying molecular motors that hydrolyze ATP to move along microtubules toward the *plus* end, and the vegetal cortex microtubules that assemble after sperm entry are aligned with the plus ends directed toward the dorsal pole.[2] Kinesin activity therefore results in transportation of Dsh and GBP into the gray crescent, with attendant dorsal accumulation of β-catenin (Figure 5-1). As the oocyte undergoes successive rounds of cleavage, β-catenin becomes increasingly localized to the cytoplasm and then the nuclei of cells on the dorsal aspect,[9] where it binds and modulates the activity of transcription factors that, in turn, regulate the transcription of a large array of genes.

MESODERMAL INDUCTION IN THE BLASTULA IS REGULATED BY Nodal SIGNALING

In the preceding example, β-catenin signaling is dependent on extrinsic factors: the maternally derived β-catenin protein and a microtubule-dependent mechanical contraction that is initiated by sperm aster. Once the oocyte begins to cleave, embryonic regulation is increasingly dominated by intercellular communications.

This is apparent during the differentiation of the oocyte into endoderm, mesoderm, and ectoderm. Whereas cells from the vegetal pole form endodermal derivatives and cells from the animal pole form ectodermal derivatives, the mesoderm arises from the ectoderm in response to signals from the endoderm. The mesoderm, in turn, is divided into dorsal and ventral regions that give

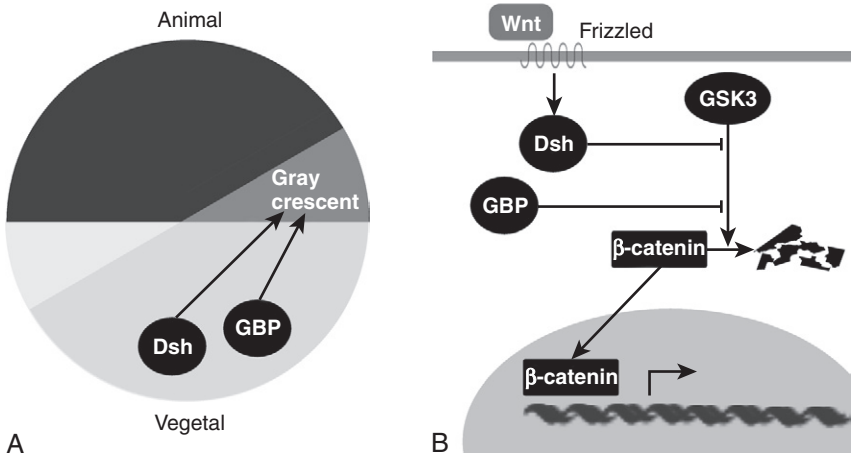


Figure 5-1 Cortical rotation in the *Xenopus* oocyte up-regulates β-catenin. A, Disheveled (Dsh) and GSK3-binding protein (GBP) are distributed in the vegetal hemisphere before fertilization. Fertilization induces cortical rotation and microtubule formation; Dsh and GBP are transported along these microtubules to the intersection of vegetal cortex and animal cytoplasm (the gray crescent). **B,** Dsh and GBP are components of the canonical Wnt signaling pathway. Wnt ligands are glycoproteins expressed on cell surfaces. In the absence of Wnt ligand, GSK3 phosphorylates β-catenin, leading to its ubiquitination and destruction. Wnt binding to its receptor Frizzled activates Dsh and thereby inhibits GSK3. In the oocyte, sequestration of Dsh and GBP inhibits GSK activity, thereby allowing β-catenin to accumulate and regulate gene transcription in the gray crescent.

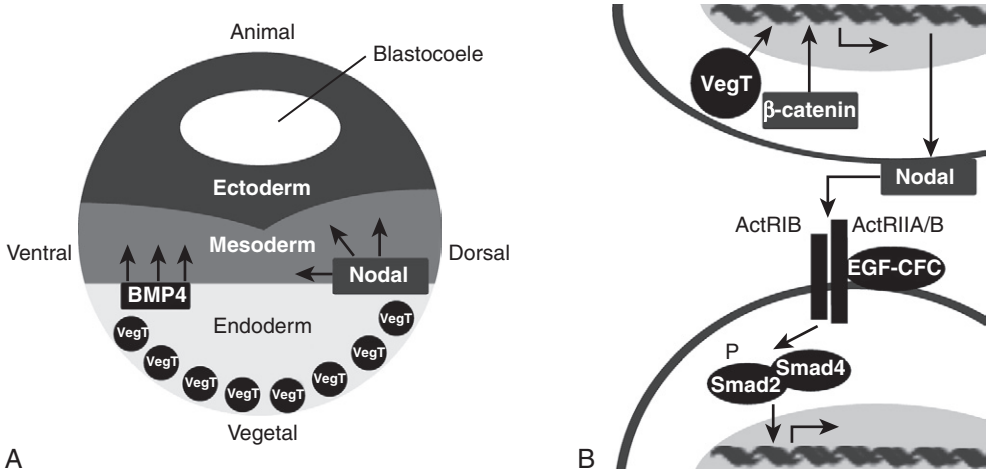


Figure 5-2 Vegetal cells secrete Nodal to induce mesoderm formation in the *Xenopus* blastula. A, Maternally derived VegT is sequestered in the vegetal cytoplasm and functions as a transcription factor that interacts with β-catenin to induce Nodal expression at the prospective dorsal pole. **B,** Secreted Nodal binds and activates cognate cell surface receptor complexes composed of TGF-β family type I and type II receptors, the activin receptors (ActRIB and ActRIIA or ActRIIB, respectively). This activation requires a cofactor of the EGF-CFC family (see text). The activated receptor complex phosphorylates the intracellular signaling proteins Smad2 and Smad4, which associate, translocate to the nucleus, and regulate gene expression. BMP-4, another TGF-β family peptide, has a similar inductive function at the ventral pole. *BMP-4,* Bone morphogenetic protein-4; *TGF-β,* transforming growth factor-β.

rise to distinct tissues. In *Xenopus,* dorsal mesodermal induction is initiated by expression of the transcription factor VegT from maternally derived mRNA sequestered in the vegetal cortex[10] (Figure 5-2); for example, the notochord comprises dorsal mesoderm, whereas the hematopoietic precursors are derived from ventral mesoderm. VegT initiates endodermal differentiation[11] and interacts with β-catenin at the gray crescent to induce the expression of Nodal, a member of the transforming growth factor-β (TGF-β) peptide family that also includes activin and the bone morphogenetic proteins (BMPs). Like other TGF-β family proteins, Nodal is secreted as an inactive precursor that is processed into a mature active dimer by an extracellular enzyme.[12] Nodal then diffuses through the intercellular space, its concentration falling and hence its stimulatory effects decreasing with increasing diffusion distance. Thus far only one Nodal protein has been characterized in mice; five Nodal-related proteins are recognized to participate in *Xenopus* mesodermal induction.[13] In the *Xenopus* blastula, Nodal is most highly expressed in the dorsal endoderm adjacent to the gray crescent[14] in a region known as the Nieuwkoop center that will confer important organizing functions during gastrulation.

When a Nodal peptide encounters a target cell, its presence is detected by cell surface receptors. Like other TGF-β family receptor complexes, the Nodal receptor complex is composed of type I and type II receptors. Both receptors are transmembrane proteins with extracellular ligand-binding domains and intracellular serine-threonine kinase domains. The activin type IB receptor (ActRIB) protein functions as the Nodal type I receptor, and either the activin type IIA or type IIB receptor (ActRIIA or ActRIIB) can

serve as the Nodal type II receptor. Upon ligand binding, the type II receptors bind, phosphorylate, and activate the type I receptors, which in turn bind, phosphorylate, and activate cytoplasmic signaling proteins. The association of Nodal with its receptor complex also requires a novel secreted coreceptor that demonstrates homology to epidermal growth factor (EGF)[15]; this family of EGF-CFC proteins includes FRL-1 in *Xenopus,* Cripto and Cryptic in mammals, and One-eyed pinhead in zebrafish (in the designation *EGF-CFC,* CFC = Cripto–FRL-1–Cryptic).

The primary substrates of ActRIB are the transcriptional regulators Smad2 and, possibly, Smad3. Of the eight mammalian Smad proteins (named for their similarity to proteins encoded by *Drosophila* Sma and *Caenorhabditis elegans* MAD ["mothers against decapentaplegic"] genes), five are TGF-β receptor family substrates.[16] One of the three remaining Smads, Smad4, is a cofactor for all receptor-activated Smads; the other two, Smad6 and Smad7, inhibit Smad signaling by functioning as false receptor substrates. Inactive receptor-activated Smads are sequestered in the cytoplasm in close proximity to the receptor by anchor proteins such as SARA ("Smad anchor for receptor activation").[17] Upon phosphorylation, Smad2 detaches from SARA to associate with Smad4 and a cofactor that confers specificity to the complex. In Nodal signaling, Smad 2 forms a complex with Smad4 and the forkhead family transcription factor FoxH1 (previously termed FAST-1).[18] The complex is transported into the nucleus, where it binds and activates the promoter for Mix2, a homeodomain transcription factor (these are discussed later). Similarly, Smad 3 partners with FoxO1, FoxO2, and FoxO3 to up-regulate the cyclin-dependent kinase inhibitor p21Cip1.[19] Modulated expression of these proteins initiates or terminates their associated cellular activities.

Among the proteins induced by FoxH1 is Nodal itself.[20] Nodal autoinduction represents positive feedback regulation that may allow its expression to spread out of the endoderm and into the marginal zone. FoxH1 also induces the expression of the secreted Nodal inhibitors Cerberus and Lefty2; this induction represents negative feedback regulation. Nodal can therefore both inhibit and sustain its own activity, and the balance between these conflicting influences determines its expression and localization.

Nodal is necessary for anterior mesoderm specification, but Nodal-deficient animals develop ventral mesoderm. The two regions have divergent differentiation potentials: Dorsal mesoderm will give rise to the head structures, whereas ventral mesoderm will differentiate into hematopoietic and renal derivatives.[14] Ventral mesoderm is induced by endodermally produced BMP-4, another member of the TGF-β family. BMP-4 signaling is transduced by the BMP type II receptor (BMPR-II) in concert with ActR-IA, BMP-RIA, or BMP-RIB type I receptors, and intracellular BMP-4 signaling is mediated by Smad1, Smad5, and Smad8.[16] BMP-4 is produced in a ventral organizing center located opposite the gray crescent. Although both BMP-4 and Nodal induce mesoderm, they appear to have antagonistic effects on mesoderm dorsalization[21] and can combine into mutually inhibitory heterodimers.[22] The overall effect of the two organizing centers is to establish a dorsal-ventral Nodal activity gradient in the endoderm that induces and patterns the mesoderm.[20]

DURING GASTRULATION, THE EMBRYO IS PATTERNED BY Nodal ANTAGONISTS

A number of generalizations about intercellular regulation are apparent from the *Nodal* signaling example. First, intercellular signaling processes can be regulated at many points. Nodal activity may be modulated by altering its transcription, translation, secretion, activation, diffusion, and degradation; its receptors, coreceptors, and cytoplasmic mediators are subject to similar control. Second, multiple factors may have similar activities. As noted, mesoderm is induced by both BMP-4 and Nodal. Moreover, receptors may bind multiple ligands with variable affinity. ActRIB also is activated by alternative TGF-β peptides such as Vg1, Derriére, and Activin,[23] and the biologic activity of Nodal may be at least partially replicated by these proteins. Conversely, Nodal also binds promiscuously with another receptor, activin-like kinase 7.[24] Moreover, the intracellular pathways of different receptors may regulate one another.[25] Finally, multiple receptors often activate the same intracellular proteins. Smad2 is activated by numerous TGF-β type I receptors, and both the activin type IB and activin-like kinase 7 receptors interact with a variety of TGF-β type II receptors to activate Smad2.[26]

Posttranscriptional regulation of Nodal signaling is an important mechanism in embryonic patterning. During *Xenopus* gastrulation, the newly formed mesoderm curls into the blastopore and expands along the inner surface of the ectoderm (Figure 5-3). This process, known as involution, places the dorsal mesoderm of the future head at a point on the embryo that is roughly opposite to the initial location of the gray crescent. This reorganization is critically regulated by a specific area of the dorsal mesodermal lip characterized by Hans Spemann and Hilde Mangold in 1923,[27] who found that implantation of the anterior dorsal lip from one newt embryo into the ventral mesoderm of another induced the formation of a complete second body axis, including a second head. Because the major portion of this material was derived from recipient tissue, it was apparent that the anterior lip, named the "organizer" by Spemann, induced ventral tissue to undergo dorsal differentiation, including the induction of neural, heart, kidney, and somite tissues, as well as head formation. The Spemann organizer is established in the dorsal mesoderm directly adjacent to the Nieuwkoop center as a result of stimulation by β-catenin– and Nodal-related signals.[13,28]

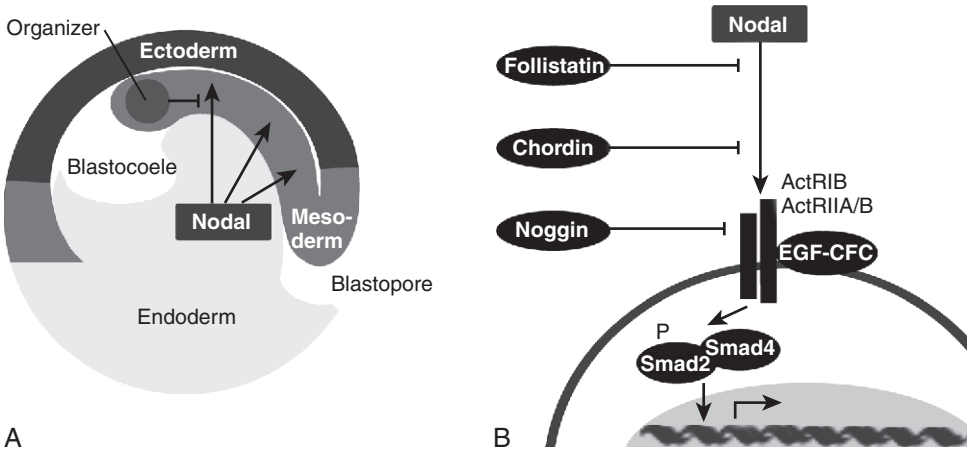


Figure 5-3 Nodal antagonists pattern the gastrulating mesoderm. A, During *Xenopus* gastrulation, the mesoderm involutes by expanding and migrating into the blastocoel through the blastopore. The leading edge contains Spemann's organizer, which is responsible for imparting dorsal characteristics to adjoining tissues. **B,** The dorsalizing activity is conferred by the secreted proteins follistatin and Chordin, which bind and neutralize Nodal, and Noggin, which binds and inactivates the Nodal receptor.

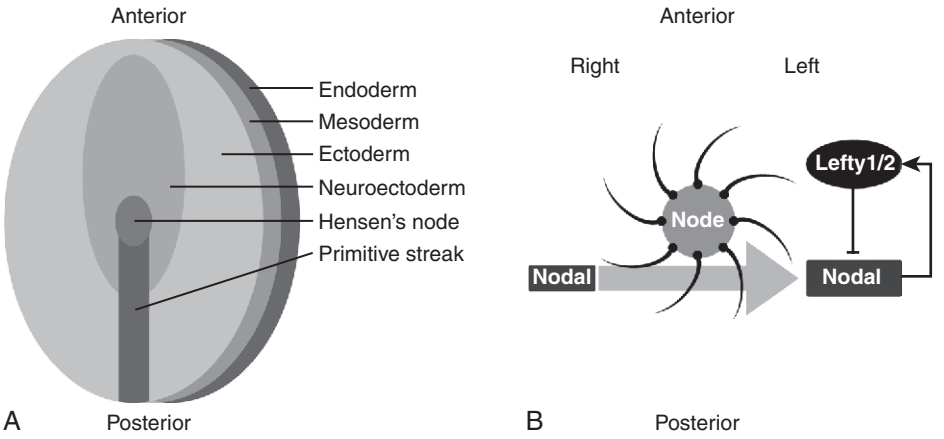


Figure 5-4 Left-right asymmetry in the mouse is established by ciliary distribution of Nodal. A, Hensen's node becomes visible in late gastrulation at the anterior margin of the primitive streak. **B,** The ventral surface is covered with cilia that propel the extraembryonic fluid in a clockwise motion, establishing a higher Nodal concentration on the left side. This asymmetry is reinforced by the induced expression of Nodal inhibitors including Lefty1 and Lefty2.

Spemann's organizer alters the fate of nearby cells by secreting proteins that diffuse into neighboring tissues to interrupt Nodal and BMP signaling. Noggin, chordin, follistatin, and Cerberus all are antagonists of BMP signaling that are secreted by Spemann's organizer, and their depletion results in failed dorsalization.[29,30] Noggin binds BMP receptors and prevents their activation,[31] whereas chordin and follistatin bind and neutralize BMPs.[32,33] Cerberus binds and neutralizes both BMPs and Nodal.[30] These interactions suggest that Nodal and BMP-4 prevent dorsalization, and that antagonists secreted by Spemann's organizer permit cells to dorsalize.

VERTEBRATE LEFT-RIGHT ASYMMETRY IS ESTABLISHED DURING LATE GASTRULATION

The early development of birds and mammals is more complicated and less well characterized than that of *Xenopus.* This is due in part to the presence of extraembryonic tissues that assume some of the organizing functions intrinsic to the *Xenopus* oocyte, thereby adding considerable complexity to the system.[34] For example, cortical rotation does not occur in chicken eggs and in mouse embryos. In these species, dorsoventral axis specification is not completely understood but is apparently mediated by gravitational influences and extraembryonic oocyte derivatives, respectively.[35,36] Mouse and chick embryos are more planar, with no blastopore; the analogous structure is the primitive streak, with Hensen's node at its anterior end. During gastrulation, tissue accumulates anterior to the node, and the node (or streak) regresses or moves to a posterior position. Somites form in the wake of node regression. Moreover, dorsoventral axis determination is not apparent in the mouse until gestation is one-third completed. Nonetheless, Nodal signaling patterns mice and birds as it does *Xenopus.*

In the frog *Xenopus,* left-right axis determination is concomitant with dorsal-ventral specification.[37] In mammals, however, establishment of left-right asymmetry occurs much later, during the one-somite stage.[38] In mice, left-right asymmetry centers on the ventral node, a transient structure located on the ventral aspect of Hensen's node. The ventral surface of the node is a pitlike structure lined with specialized cilia that are driven by kinesin motor proteins. The combination of the orientation of these cilia and their clockwise unidirectional rotation results in a corresponding leftward fluid movement called nodal flow that represents the initial break in symmetry[39] (Figure 5-4). Artificial reversal of this flow reverses the orientation of the internal organs.[38] This fluid flow appears to establish a concentration gradient of secreted factors including Nodal (initially characterized in this context[39]) or, alternatively, to induce asymmetric signaling by sensory cilia.[40] In either case, asymmetric Nodal expression is established and sustained in the left lateral plate mesoderm, and its asymmetric expression is augmented by similarly asymmetric expression of the Nodal inhibitors Lefty1 and Lefty2.[37]

The mechanism underlying left-right axis determination serves to illustrate how defective early embryogenic regulation can have subsequent pathologic consequences. One example of a disorder resulting from such regulatory disturbance is Kartagener syndrome, a human congenital syndrome in which half of the affected patients exhibit *situs inversus,* a mirror-image reversal of the internal organs.[41] The underlying dysfunction is impaired ciliary motility, which precludes establishment of the nodal flow. The lack of nodal flow results in random Nodal localization and correspondingly random organ lateralization. The same motor proteins are used in the adult, in whom ciliary akinesis manifests as immotile sperm and impaired pulmonary clearance leading to recurrent pneumonias.

NEURULATION IS INITIATED BY RECEPTOR TYROSINE KINASE SIGNALING

Neurulation follows gastrulation and represents the second major embryonic inductive event, after mesoderm induction. Once again, tissue induction is coordinately regulated by secreted agonists and antagonists. In *Xenopus,* the prospective neural plate forms in the ectoderm overlying the paraxial mesoderm, whereas in mice and chicks, the neural plate arises in the ectodermal tissue straddling the primitive streak. The newly differentiated neuroectoderm will receive anterior-posterior guidance from the Spemann organizer and adjacent tissues to give rise to the somites, notochord, and nervous tissue.[42]

Although incompletely defined, neurulation apparently is initiated by fibroblast growth factor-8 (FGF-8), which is secreted by the mesoderm to induce the differentiation of ectodermal cells[43] (Figure 5-5). FGF-8 is a member of a peptide family comprising at least 24 different FGF peptides that signal through four closely related receptor tyrosine kinases. Related receptors transduce signaling by peptides such as epidermal growth factor, platelet-derived growth factor, and nerve growth factor (peptide growth factors are named upon initial characterization, and subsequent work often reveals additional functions that are not reflected in the nomenclature).

FGF receptors are transmembrane proteins that dimerize (i.e., associate to form pairs of identical subunits) in the presence of FGF ligand. As with TGF-β receptors, FGF receptor dimerization brings the cytoplasmic tyrosine kinase domains of the two receptors into close proximity and facilitates their mutual phosphorylation and activation. Activated FGF receptors directly phosphorylate cytosolic phospholipase C gamma (PLC-γ), FGF receptor substrate 2 (FRS-2), and Grb2.[44] FRS2 and Grb2 are nonenzymatic adapter proteins that associate with each other and with the guanosine triphosphate (GTP) exchange protein Sos upon phosphorylation.

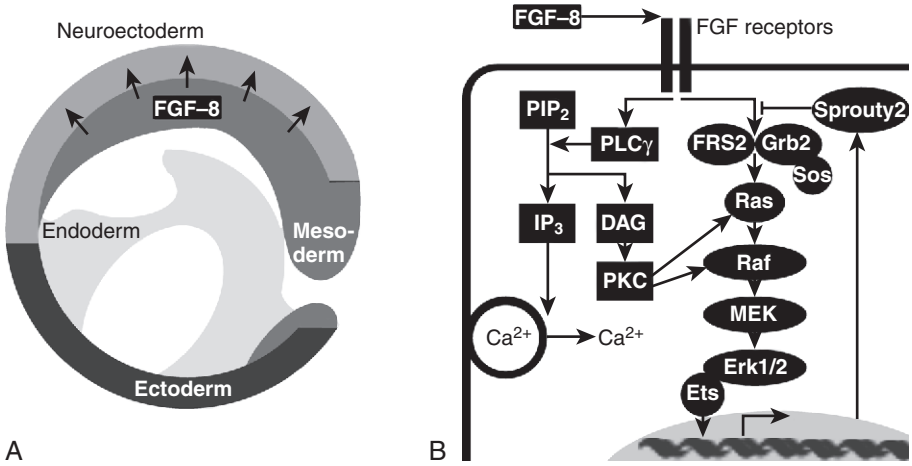


Figure 5-5 Neurulation in *Xenopus* is initiated by receptor tyrosine kinase signaling. A, FGF-8 is secreted by the involuted mesoderm to induce neuroectoderm differentiation. **B,** FGFs bind and activates dimeric transmembrane receptor tyrosine kinases that phosphorylate cytosolic FGF receptor substrate (FRS) 2, and Grb2. Activated FRS2 and Grb2 associate with Sos to activate Ras and, subsequently, Raf, MEK, and the MAP kinases Erk1 and Erk2. Erk MAP kinases phosphorylate Ets family transcription factors that regulate numerous genes. FGF-mediated Erk activation is subject to inhibitory feedback by Sprouty2, which prevents FRS2 from associating with Grb2. FGF receptors also phosphorylate phospholipase C γ (PLC-γ), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces calcium release from the endoplasmic reticulum into the cytosol, whereas DAG activates protein kinase C and the Erk MAP kinase pathway. *FGF,* Fibroblast growth factor; *MAP,* mitogen-activated protein; *MEK,* mitogen-activated protein kinase kinase.

The resulting heterotrimer activates the GTP-binding protein Ras, leading to sequential activation of Raf, MEK, and the mitogen-activation protein (MAP) kinases Erk1 and Erk2.[45] Erk1 and Erk2 phosphorylate Ets family transcription factors; on phosphorylation, these factors enter the nucleus and associate with serum response elements and other cofactors to bind specific sites in the promoters of numerous genes. Each step in this pathway represents a node at which the FGF-8 signal may be amplified, attenuated, or conditioned by other signaling pathways. The FGF pathway, like Nodal signaling, also incorporates a negative feedback mechanism; Sprouty2 is a cytosolic protein that, on phosphorylation by FGF receptors, inhibits the association of FRS2 with Grb2, thereby interrupting FGF-8–mediated Erk activation.[46]

FGF receptors also phosphorylate PLC-γ, an enzyme that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 binds to cognate receptors embedded in the membrane of the endoplasmic reticulum, thereby releasing calcium from the endoplasmic reticulum into the cytosol.[47] Intracellular calcium signaling regulates cellular activities as diverse as muscle contraction, nuclear breakdown, and egg fertilization. Diacylglycerol activates protein kinase C, which activates the Erk MAP kinase pathway through Ras or Raf.[48] The FGF-8 signaling pathway, with its extracellular ligand, transmembrane receptor, and intracellular mediators, is roughly analogous to the Nodal signaling pathway, albeit with additional intracellular signaling branches.

SEGMENTATION IS CONTROLLED BY SIGNALING GRADIENTS AND CYCLING GENES

Vertebrates, like arthropods, are segmental organisms. Segmentation becomes visible after gastrulation, when paired somites condense from the paraxial mesoderm on both sides of the midline. Somites give rise to the axial musculature, ribs, and vertebrae. Segmental boundaries also define the migration patterns for neural crest derivatives including epithelial and neural precursors. Somite size, number, and position are closely regulated, and irregularities in somite formation result in corresponding somatic malformations.[49]

In the chick embryo, somitogenesis progresses concomitantly with the regression of Hensen's node. New somites appear at regular 90-minute intervals, beginning at the anterior end and proceeding posteriorly. The leading model of somite formation was proposed by Cooke and Zeeman in 1976[50] and postulates two sets of regulatory signals: (1) a maturation wavefront that sweeps through the embryo from anterior to posterior and (2) a clock signal expressed synchronously throughout the presomitic mesoderm with a 90-minute periodicity[51] (Figure 5-6). Signaling processes initiated by the advancing maturation wavefront interact with those of the segmentation clock to establish the anterior and posterior margins of each somite.[52]

The segmentation clock is incompletely characterized, but its existence is supported by several sets of cyclically expressed genes. The most completely defined pathway centers on Notch, a single-pass transmembrane receptor whose extracellular domain contains 36 tandem repetitions of a motif that resembles epidermal growth factor.[53] Its ligands include Serrate and Jagged, proteins that are expressed on the surface of adjoining cells. On binding ligand, Notch is cleaved by proteases embedded in the membrane of the target cell.[53,54] The cleaved cytoplasmic fragment translocates to the nucleus, where it associates with the DNA binding proteins C promoter-binding factor 1 (CBF1) and recombination signal-binding protein 1 for J-kappa (RBPJK), thereby converting them from transcriptional repressors to transcriptional activators. Notch activation is antagonized by the glycosyl transferase Lunatic fringe (Lfng).[55] Lfng is a short-lived protein whose expression is up-regulated by Notch signaling. Inhibitory feedback conferred by Lfng results in cyclic Notch activation and expression of Notch-regulated transcription factors, notably members of the HES ("hairy and enhancer of split") family that share a common basic helix-loop-helix DNA binding motif.[56] At least some of the Hes factors also are unstable, and their degradation is necessary for the cyclic expression of Notch.[57]

A second set of molecular oscillators acts through the *Wnt* pathway. *Wnt* genes encode a large family of secreted lipid-modified proteins with important functions in many developmental contexts.[58,59] *Wnt* signals are transduced by Frizzled, a receptor with seven transmembrane domains that otherwise has little

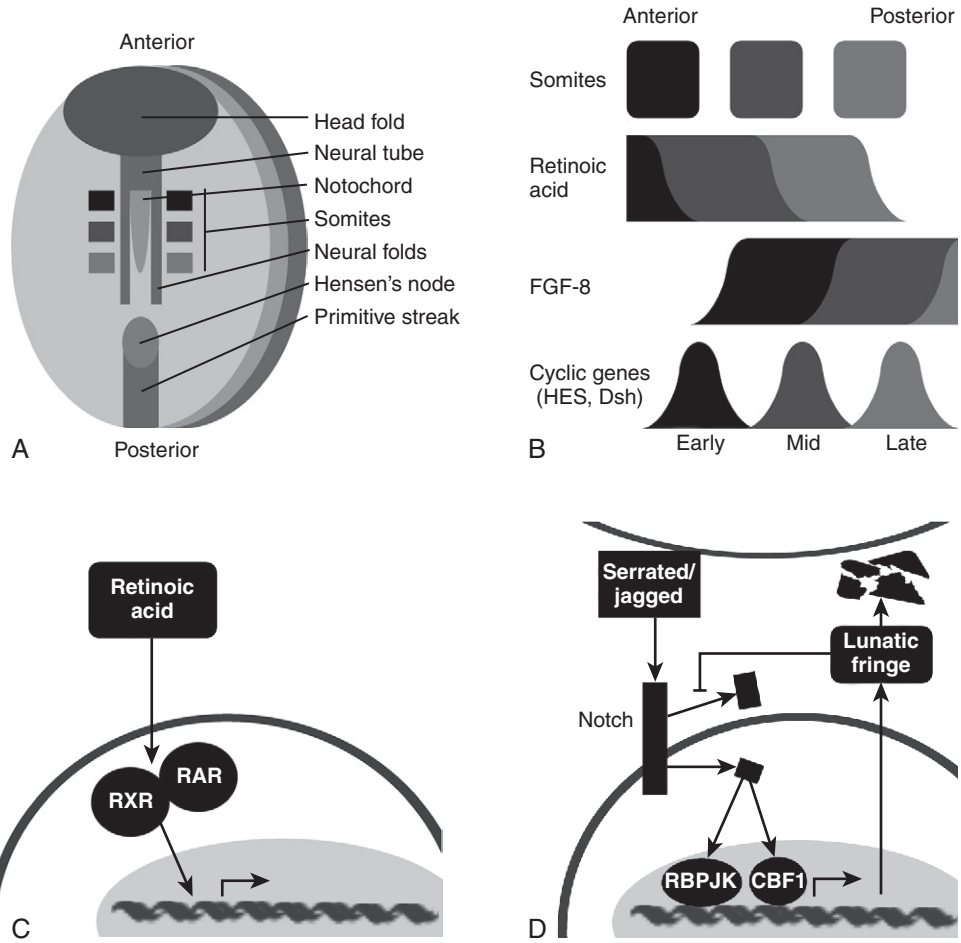


Figure 5-6 Somitogenesis is regulated by a maturation wavefront and a mesodermal clock signal. A, In chicks, somites arise from paraxial mesoderm at 90 minute intervals in the wake of node regression. **B,** The boundaries of each somite are established by one or more oscillating genes interacting with a maturational wavefront that is the product of countervailing retinoic acid and FGF-8 concentration gradients. As new somites are formed, this wavefront sweeps posteriorly. **C,** Retinoic acid, unlike Nodal or FGF, enters the cytoplasm of targeted cells, where it binds homodimers or heterodimers of retinoic acid receptors (RAR) or retinoid X receptors (RXR). **D,** The resulting complex enters the nucleus to bind specific genes. Cyclic genes include components of the Notch pathway, which is initiated by Serrate and Jagged expressed on adjoining cells. Upon binding ligand, Notch is cleaved, and the cytoplasmic fragment translocates to the nucleus to bind the centromere binding proteins CBF1 and RBPJK and to activate gene transcription. One of the induced proteins, the glycosyl transferase Lunatic fringe, inhibits Notch cleavage. Lunatic fringe also is short-lived, and the resulting oscillating feedback loop results in cyclic Notch activation. *FGF,* Fibroblast growth factor.

homology with the seven-transmembrane GTP-protein–coupled receptors that constitute the largest and most diverse class of signaling receptors in metazoans.[60] Frizzled signaling results in activation of the cytoplasmic intermediate Dsh. Dsh, as noted previously, antagonizes the ubiquitin-mediated breakdown of β-catenin. β-Catenin then accumulates in the nucleus, where it regulates gene transcription. One of the targeted genes encodes axin2, an inhibitor of *Wnt* signaling whose expression oscillates in parallel with segmentation.[61] *Wnt* cycles out of phase with Notch cycling, and the coordination between the two signaling loops is not well understood. Nonetheless, the cycling of both gene expression loops is apparently important for coordinated somite development.

The maturation wavefront appears to be defined by opposing gradients of FGF-8 and retinoic acid. FGF-8 is secreted from the posterior presomitic mesoderm,[62] whereas retinoic acid is produced in the segmented anterior mesoderm and diffuses in a posterior direction to form a gradient that antagonizes FGF signaling.[63] Unlike with Nodal and FGF-8, retinoic acid signaling is not mediated by a membrane-bound receptor. Retinoic acid is a vitamin A derivative and belongs to the steroid/thyroid hormone receptor superfamily that includes sex hormones and growth hormone. These molecules penetrate the plasma membrane and enter the cytoplasm, where they bind specific nuclear receptors composed of homo- or heterodimers of two cytosolic proteins, the retinoic acid receptors and the retinoid X receptors. The resulting complexes enter the nucleus, where they bind specific promoter response elements, thereby regulating gene transcription.

The antagonism between FGF and retinoic acid signals establishes the maturation wavefront, which moves posteriorly at a steady rate as new somites are added. The position of the wavefront at each cycle of the segmentation clock defines the border of each segment. Clock signals and wavefront signals interact to transiently express the transcription factor Mesp2 in a one-somite-wide band,[64] and presumably Mesp2 or factors coregulated with Mesp2 stabilize the boundaries and initiate the differentiation of the new somite. The mechanism by which these signals interact is incompletely defined but has been modeled mathematically.[65]

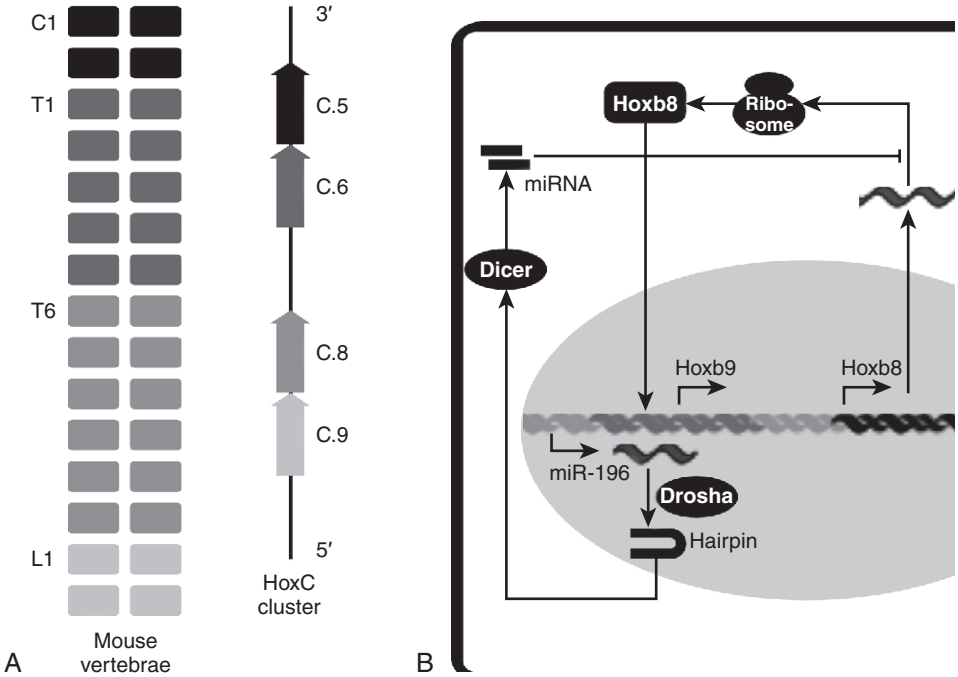


Figure 5-7 Homeobox (Hox) genes confer positional identity to differentiating segments. Hox genes are organized in four clusters, and the genes within each cluster are arranged in the same order as they are expressed. **A,** In this example, the arrangement of genes within the *Hoxc* cluster correlates with their expression within a portion of the mouse vertebral column. The *arrows* indicate the direction of transcription. **B,** One model of *Hox* expression, wherein one Hox protein, Hoxb8, facilitates the expression of the next, Hoxb9. Also shown is the regulatory function of the miR-196 miRNA sequence embedded within the *Hoxb* cluster. These sequences are processed by Drosha and Dicer into miRNAs that enable RNA-induced silencing complexes to target and inactivate Hoxb8. *miRNAs,* Micro-RNAs.

SEGMENT POSITIONAL IDENTITY IS CONFERRED BY HOMEOBOX GENES

Metazoan body plans are dependent on the divergent morphogenesis of individual segments. This, in turn, presupposes that each segment has a positional identity that determines how it develops. The mechanism by which this identity is conferred was first elucidated in the fruit fly *Drosophila melanogaster,* wherein single mutations resulted in duplication of entire body segments. The genes that controlled these so-called homeotic mutations were recognized as master switches that controlled the function of numerous genes within each segment. Sequencing revealed that the genes responsible for homeotic mutations were positioned one after another on the same chromosome, oriented in the same 5′ to 3′ transcriptional direction, and positioned in the same order as for the body domains regulated by them.[66] Each gene contained a highly conserved sequence of 180 bases, the homeobox, that encoded a 60–amino acid motif called the homeodomain. The homeodomain is a helix-turn-helix DNA-binding motif, indicating a role in transcriptional regulation. The genes themselves are called homeobox *(Hox)* genes.[67]

The mouse and human *Hox* gene complexes comprise 38 genes organized in four chromosomal complexes of approximately 120 kb.[68] As in fruit flies, the physical order of the *Hox* genes within these clusters corresponds to their expression from anterior to posterior (Figure 5-7). *Hox* expression boundaries, however, correspond to anatomic regions and not to individual somites[69]; multiple somites may be regulated by a single set of *Hox* genes to assume a similar phenotype. This coregulation underlies the similarity, for example, between the 7 human cervical vertebrae, relative to the 12 thoracic vertebrae. Moreover, differences in the size and placement of homologous structures (such as the number of cervical vertebrae in mice, humans, and chickens) correspond to differences in the distribution of the analogous *Hox* gene products.

The regulation of *Hox* expression is incompletely understood, but several mutually compatible mechanisms are supported by the evidence.[70] First, the colinear placement of *Hox* genes within each cluster suggests that transcription of one gene facilitates the transcription of the next. This may involve transcription factors encoded by the preceding *Hox* gene[71] or mechanical unpacking of the chromatin that permits transcription factors to associate with the gene.[72] Second, *Hox* expression may be regulated by the Notch segmentation clock, thereby synchronizing *Hox* genes within each somite.[73] Finally, anterioposterior gradients of FGF, retinoic acid, or other factors may confer positional regulation of *Hox* domains.[74]

The morphological development of a somite or anteroposterior region is determined by the combination of *Hox* genes, or *Hox* code, expressed within those tissues.[75] *Hox* genes regulate arrays of genes by binding *Hox*-responsive enhancers within DNA regulatory regions. Individual Hox proteins can modulate as many as 68 distinct genes,[76] some of which also are transcription factors.

In addition to transcriptional regulation, *Hox* clusters also regulate gene expression through RNA interference,[77] a mechanism in which specific mRNA strands in the cytoplasm are degraded. This degradation reduces the translation and, hence, expression of the encoded proteins. *Hox* clusters encode precursor microRNA (miRNA) transcripts that contain complementary inverted repeat sequences; the internal allowergetically favorable for the transcripts to fold back on themselves to form hairpin structures that are cleaved within the nucleus by the double-stranded RNA (dsRNA) endonuclease Drosha.[78] The cleavage products are exported to the cytoplasm where they are processed by the dsRNA endonuclease Dicer into 21-base duplexes that are then incorporated into RNA-induced silencing complexes (RISCs) whose specificity is defined by the now single-stranded miRNA. The RISC targets and cleaves mRNA strands that contain sequences complementary to the miRNA, resulting in the post-transcriptional down-regulation of the targeted gene product.

Sequence analyses predict that many *Hox* gene products are targets of the miRNA sequences encoded within *Hox* clusters.[79] One, Hoxb8, has been experimentally silenced by an miRNA (miR-196) encoded by the mouse *Hoxb* cluster.[80] Like the *Hox* genes, the miRNAs encoded by the *Hox* cluster appear to have a regional distribution that may have developmental implications.[81] Although the functional significance of Hox regulation by miRNA is unclear, accumulating evidence suggests that such regulation is widespread and highly conserved throughout metazoans.[82]

NEUROECTODERMAL REGULATION PATTERNS CRANIOFACIAL STRUCTURES

After neurulation, differentiation of the craniofacial structures begins with invagination of the neural plate to form the neural folds. The subsequent approximation and closure of these folds results in formation of the neural tube.[83] Cells dissociate from the neural folds and undergo epithelial-to-mesenchymal transformation to become the neural crest cells that migrate throughout the embryo along defined routes. The developmental contributions of neural crest cells correlate with their original position, and this identity is conferred by ectodermally secreted Wnt proteins,[84] BMP-4, and the BMP antagonists chordin and Noggin secreted by the anterior mesoderm.[85] BMP-4 and its antagonists establish an activity gradient that determines the fate of neuroectodermal derivatives, with the level required for neural crest induction greater than that required for neural plate induction and lower than that required for ectoderm specification.[86] This gradient is associated with regional expression of specific transcriptional regulators[83,87,88]: The zinc-finger transcription factors of the *Zic* family are expressed throughout the neural plate and crest; the homeobox transcriptional repressors Msx1 and Msx2 are expressed in the neural plate border; the paired-box transcriptional activators Pax3 and Pax7 are expressed at the neural plate border; and the transcriptional activators Sox9 and Sox10 localize to premigratory and migratory neural crest, respectively.

After closure, the anterior neural tube differentiates into forebrain, midbrain, and hindbrain components. The hindbrain is of particular clinical interest because hindbrain derivatives are major contributors to facial formation, and craniofacial anomalies comprise one third of all human congenital defects.[89] During early vertebrate hindbrain development, seven transient transverse bulges become apparent. These divide the hindbrain into eight developmental domains called *rhombomeres.* In contrast with somites, whose numbers vary considerably across species, the number of rhombomeres is quite stable across vertebrate classes.[90] As with the somites, the divergent differentiation of these rhombomeres is regulated by *Hox* genes whose expression, in turn, is controlled by FGF-8 and retinoic acid.[91,92] Rhombomeres give rise to cranial neural crest cells that migrate out of the neuroectoderm, proliferate, coalesce into the first three branchial arches, and ultimately coalesce into the craniofacial structures[93] (Figure 5-8). Subtle differences in rhombomere regulation are reflected in variations in cell migration and proliferation that contribute to each person's unique facial structure, whereas more disruptive mutations result in overtly dysmorphic features that often are pathognomonic for those mutations. The developmental contribution of each rhombomere is regulated by *Hox* gene expression. For example, rhombomeres 3 and 5 express Msx2, and cranial neural crest cells that express this factor undergo apoptosis when stimulated with BMP-4.[94] Similarly, Sox9 expression regulates BMP-4–mediated chondrogenesis.[95] The profile of Hox genes expressed within each rhombomere therefore guides the behavior of its derivatives.

DISRUPTED CRANIOFACIAL DEVELOPMENT IS ASSOCIATED WITH VISCERAL MALFORMATIONS

As noted previously, embryonic regulatory mechanisms also are used to control somatic and visceral development as well as tissue regeneration after injury. For example, FGF signaling regulates both limb formation and lung morphogenesis.[96-98] Disruption of FGF signaling reduces lung branching,[99] whereas abrogation of its feedback inhibitor Sprouty[100,101] has the opposite effect. Similarly, components in the FGF pathway are regulated in response to lung injury.[102,103]

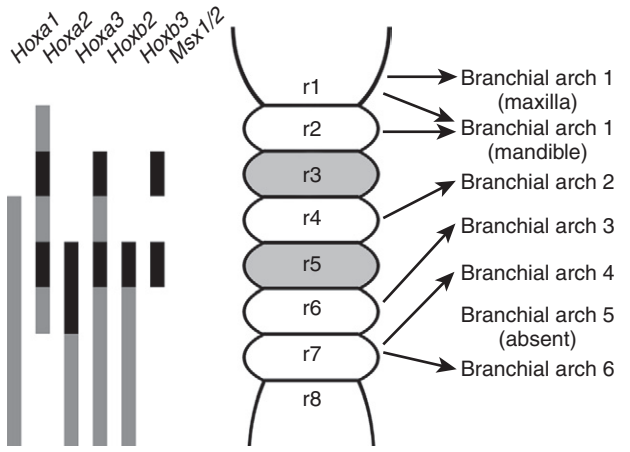


Figure 5-8 The expression of *Hox* genes within each rhombomere determines the developmental contribution of its derivatives. The distribution of selected *Hox* genes is shown, together with the branchial arches derived from the migrating neural crest cells of each rhombomere. Many neural crest cells from r3 and r5 *(shaded)* undergo apoptosis, a process that appears to be initiated by bone morphogenetic protein-4 (BMP-4) stimulation.

Because craniofacial structures are derived from cranial neural crest cells, disruptions in rhombomere regulation are associated with characteristic craniofacial anomalies. Many of the same pathways are reiterated later in fetal development, so these craniofacial malformations often are associated with additional somatic or visceral anomalies. Mutations in human Pax3 (which, as noted previously, appears to define the neural plate border) are associated with type I and type III Waardenburg syndrome, characterized by congenital deafness and partial albinism with or without limb anomalies.[104] Mutations in Sox10 are associated with type IV Waardenburg syndrome, characterized by deafness, partial albinism, and aganglionic megacolon.[105] Additional examples are listed in Table 5-1.

SUMMARY

Embryonic development is regulated by intercellular communications that are mediated by secreted molecules. These factors are expressed in organizing centers and diffuse outward to establish concentration gradients that induce tissue differentiation and confer positional and behavioral identity. Signaling factors may interact cooperatively or antagonistically with other secreted proteins to transmit signals that are transduced into targeted cells by families of related receptors with overlapping functions and specificities. Receptor families prominent in early embryogenesis include TGF-β receptors (Nodal and BMP), receptor tyrosine kinases (FGF), Notch receptors (Serrate and Jagged), Frizzled receptors (Wnt), and nuclear receptors (retinoic acid). These receptors initiate chains of intracellular kinase reactions that regulate and are regulated by other signaling pathways. Consequently, cell behaviors are specified by combinations of signals that interact in both the intracellular and extracellular domains. These pathways modulate the activity of DNA-binding proteins that alter gene transcription. Certain transcription factors (notably the Hox proteins) serve as master switches that regulate batteries of genes. These genes also may encode transcriptional regulators or micro-RNAs, so that dozens of proteins are modulated by a single stimulus, thereby initiating complex cell behaviors in a coordinated fashion. Most signaling pathways incorporate positive feedback loops that maintain and propogate pathway activity, and negative feedback loops that stabilize

TABLE 5-1

Gene Product Mutations With Associated Craniofacial and Visceral Malformations

Mutated Regulatory Mediator	Associated Syndrome/ Disorder	Craniofacial Manifestations	Somatic/Visceral Manifestations
FGF receptor-1	Pfeiffer syndrome	Microcephaly, sensorineural deafness	Broad, short thumbs and great toes; webbed hands/feet
FGF receptor-2	Crouzon syndrome	Craniosynostosis, maxillary hypoplasia, hypertelorism	Cervical fusion (18%), acanthosis nigra (5%)
FGF receptor-3	Costello syndrome	Macrocephaly, coarse facies, brain atrophy, macroglossia	Cardiomyopathy, cryptorchidism, hyperkeratosis
FGF receptor-3	Thanatophoric dwarfism	Macrocephaly, frontal bossing	Short limbs, pulmonary hypoplasia
Ubiquitin ligase E3A	Angelman syndrome	Microcephaly, developmental delay, seizures, ataxia	Fair skin and hair
Msx1	Witkop syndrome	Hypodontia	Hypoplastic nails
Pax3	Waardenburg syndrome types I and III	Hearing loss, hypopigmented hair patch and eye(s)	Limb anomalies, aganglionic megacolon
Sox9	Campomelic dysplasia	Cleft palate, macrocephaly, micrognathia, retroglossia	Skeletal anomalies, cardiac and renal malformations, sex reversal
Sox10	Waardenburg syndrome type IV	Hearing loss, hypopigmented hair patch and eye(s), central demyelinating leukodystrophy	Limb anomalies, aganglionic megacolon, peripheral demyelinating neuropathy
Noggin	Facioaudiosymphalangism syndrome	Broad hemicylindrical nose, conductive hearing loss	Joint dysplasia and synostosis

FGF, Fibroblast growth factor.

and limit signaling. Short-lived components within these feedback loops induce oscillating signals that are exploited to form repeated structures. Most signaling mechanisms are reiterated in different contexts to regulate development, homeostasis, and regeneration and are therefore relevant to the induction and amelioration of pathologic processes.

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6

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The Extracellular Matrix in Development

As a single fertilized egg develops into a fully patterned embryo, individual cells divide, migrate, change their shape, and respond to environmental cues. Throughout these processes, cells remain in constant contact with extracellular matrix (ECM), a three-dimensional latticework of glycoproteins that directs morphogenesis. The ECM surrounding individual cells provides a specialized microenvironment that regulates behavior through interacting with cell surface receptors. In this regard, integrins have emerged as the predominant cell surface receptors. The ECM and integrins regulate extracellular and intracellular signals emanating from extrinsic factors, including growth factors, hormones, and biomechanical forces. The ECM acts as a reservoir for growth factors, collaborating with integrins and ECM-degrading proteinases to present these factors to cells within developing tissues. In addition, proteolysis of the ECM generates neoepitopes that confer functions on cells and tissues distinct from those specified by their nonproteolyzed counterparts. In this chapter, as background for explicating these and other points, a review of the complex structure of the ECM, and of how integrins convey signals from the ECM to the cell interior, is presented first. To illustrate these processes in more detail, how ECM regulates angiogenesis, a key process during embryonic development, also is described.

MULTIFUNCTIONAL NATURE OF THE EXTRACELLULAR MATRIX

Normal development requires precise temporal and spatial coordination of cellular proliferation, differentiation, migration, and apoptosis. Deciding which of these programs a cell will ultimately elect is determined, to a large extent, by the ECM. Promotion or suppression of cellular proliferation by the ECM, for example, results in either stimulation or inhibition of genes involved in the regulation of the cell cycle.[1-4] To counteract runaway cellular proliferation and to sculpt or refine developing tissue structures, select cells also must be eliminated from developing tissues. To this end, loss of cell contact with the ECM leads to apoptosis during development.[5,6] Tissue-specific ECM components also regulate the transcription of genes associated with specialized differentiated functions, including alkaline phosphatase expression in osteoblasts,[7] albumin production in hepatocytes,[8] and intermediate filament protein expression in keratinocytes.[9] These observations are supported by the identification of ECM-responsive transcription factors and *cis* elements within gene promoter elements.[10,11] The mechanical properties of ECM also have been shown to regulate mesenchymal stem cell differentiation into various cell lineages.[12] Throughout development, cells move from their point of origin to other positions within the embryo, and the ECM provides the template on which migration occurs. Moreover, stem cell maintenance, self-renewal, and cell fate determination depend on the ECM.[13-16] To decipher how the ECM confers so many different functions, an appreciation of its complex structure, reviewed next, is essential.

EXTRACELLULAR MATRIX IS STRUCTURALLY DIVERSE

The ECM is an oligomeric, three-dimensional network composed of four major protein components: the collagens, structural glycoproteins (e.g., fibronectin, laminin, tenascin-C), proteoglycans (e.g., heparan sulfate proteoglycans, syndecans), and elastic fibers (e.g., elastin, microfibrillar proteins). Not every ECM network contains all of these proteins, however, nor does the composition of the ECM remain constant within any particular tissue. Rather, the distribution and organization of the ECM are both dynamic and tissue-specific. For example, mesenchymal cells are surrounded by an interstitial stromal ECM, which includes type I collagen, type II collagen, fibronectin, and proteoglycans. The basement membrane represents another specialized ECM that is composed predominantly of laminin, type IV collagen, heparan sulfate proteoglycan, and entactin. Some basement membranes exist as an amorphous sheetlike structure connected to an underlying collagenous stroma by anchoring fibrils composed of type VII collagen. Alternatively, basement membrane material may separate distinct cell layers, as is the case in the kidney glomerulus, where the basement membrane separating epithelial and endothelial cells also functions as a filter. ECM concentrates specific receptors and channels during neuronal synapse formation and maturation.[17] Cells within certain tissues (e.g., skin, arteries) need to be especially resilient to the effects of certain environmental factors such as mechanical force. In this respect, ECM reinforced with elastin fibers provides structural stability.

Within these different extracellular matrices, additional structural and functional diversity is generated through the use of alternative gene promoters and RNA splicing, and by posttranslational modifications, including glycosylation and sulfation of newly synthesized ECM proteins. Once secreted into the extracellular space, ECM proteins require integration into a functional network. Identifying binding partners for a specific ECM protein is therefore a prerequisite to ascertaining its biochemical and cell-signaling properties. For example, the ECM glycoprotein tenascin-C can interact in a tissue-specific manner with fibronectin, perlecan, neurocan, heparin, phosphacan, syndecan, glypican, and periostin.[18,19] Accordingly, understanding the biology of a single ECM component requires an appreciation of the structure and functions of numerous other affiliated proteins. In view of the number of steps involved in coordinating ECM expression, secretion, and assembly, deciphering how individual ECM proteins contribute to structural morphogenesis during development represents a formidable task.

GENETIC APPROACHES TO DISSECTING EXTRACELLULAR MATRIX FUNCTIONS

Both mapping and identifying gene mutations that lead to heritable connective tissue disorders and the generation of animal models in which ECM genes have been mutated or ablated have been successfully used to ascertain the functions of individual ECM proteins within specific tissues. Many of the diseases resulting from ECM gene mutations result from defective structural integrity of specific tissues. Mutations in collagen type VII, collagen type XVII and laminin 332 genes can cause the skin-blistering disease epidermolysis bullosa.[20,21] Mutations in type I collagen genes cause osteogenesis imperfecta,[22] and mutations in both collagen I and tenascin-X genes can cause Ehlers-Danlos syndrome.[23] In addition to gene defects that alter mechanical properties of the ECM, mutations in the fibrillin-1 gene that cause

Marfan syndrome appear to increase TGF-β signaling, leading to the cellular disease phenotype.[24]

Although mutations in ECM genes can produce heritable disorders, animal studies suggest that many structural ECM glycoproteins are essential for embryonic or fetal development. As a result, mutations in these genes often cause lethality early in development, complicating the study of gene function in vivo. Inactivation of the fibronectin gene in mice results in embryonic death due to mesodermal, neural tube, and vascular developmental defects.[25] In addition, fibronectin also has been shown to be required for normal gastrulation.[26] More targeted studies have provided data on the role of fibronectin in several developmental processes. Injection of inhibitory peptides or antibodies into postgastrulation embryos prevents fibronectin-cellular interactions and disrupts neural crest migration.[27] In contrast with fibronectin, knockout of the tenascin-C gene in mice results in viable and fertile adults,[28] suggesting that other ECM proteins may be able to compensate for tenascin-C deficiency. Consistent with this notion, experiments using isolated adult hypertensive pulmonary arteries, in which tenascin-C expression has been suppressed, indicate that osteopontin substitutes for tenascin-C in promoting smooth muscle cell proliferation.[29]

Mutations in ECM component genes may also cause adult onset phenotypes. Some of these phenotypes may result from abnormalities not grossly visible at birth, and many of these animals have not been analyzed in sufficient detail to rule out developmental defects. For example, tenascin-C gene–knockout mice suffer from several neurologic defects, including hyperactivity, poor sensorimotor coordination, clinging, and freezing behavior, as well as poor performance in passive avoidance tests.[30] Other defects in tenascin-C gene–knockout animals have subsequently been detected, including reduced corneal wound healing and hematopoiesis.[31,32] Tenascin-C gene–knockout mice also exhibit less severe inflammation in an arthritis model, suggesting that ECM components participate in the inflammatory response.[33]

Reverse genetics also has been useful in revealing unexpected functions for other ECM genes. For instance, ablation of the elastin gene was predicted to cause structural defects in the three-dimensional structure of blood vessels. Elastin-null animals, however, die within days of birth as a result of obstructive arterial disease characterized by proliferation of subendothelial smooth muscle.[34] Thus, elastin exerts an unexpected growth inhibitory role during normal vascular morphogenesis. Adult elastin–haploinsufficient mice are hypertensive, also as a result of abnormal vascular development and remodeling.[35] Results from ECM-mutant animals therefore demonstrate the important roles ECM components play both in development and in response to stressful challenge or injury.

INTEGRINS AS EXTRACELLULAR MATRIX RECEPTORS

Studying ECM receptors provides another approach to understanding how the ECM controls development and tissue homeostasis. Cell adhesion or release from the ECM is mediated by cell surface proteoglycans and integrins. The most-studied class of ECM receptors, integrins are noncovalently linked, heterodimeric, transmembrane proteins composed of one α- and one β-subunit.[36,37] At the structural level, the α- and β-subunits are distinct, with separate proteins functions. The large extracellular domains of integrins interact with ECM ligands in the extracellular space, whereas their small intracellular domains associate with the actin cytoskeleton by means of adapter signaling proteins, including vinculin, talin, and α-actinin.[38] The α6β4 integrin is an exception to this rule: It possesses an unusually long cytoplasmic domain that interacts with intermediate filaments within hemidesmosomes, rather than with actin microfilaments. The cytoplasmic domains of integrins interact with multiple signaling molecules, included focal adhesion kinase (FAK), integrin-linked kinase (ILK), kindlins, filamins, and migflilin.[39-41] Thus integrins connect the extracellular environment to a cell's internal signaling pathways.

To date, 18 α- and 8 β-subunits have been identified, and these assemble into 24 distinct αβ integrins that recognize different ECM ligands. For example, certain integrins recognize specific ECM proteins, including fibronectin, vitronectin, and tenascin-C, which contain a small tripeptide sequence designated RGD (Arg-Gly-Asp). By contrast, β4 integrins interacting with α3-, α6-, and α7-subunits recognize laminins, whereas integrins composed of the β1-integrin subunit and the α1, α2, α10, or α11 subunit bind collagen. This apparent redundancy in ligand binding specificity suggests that integrins might have overlapping functions. Gene knockout experiments in mice, however, have shown that individual integrin subunits possess unique functions.[36,37,42] For example, although the α3β1 and α7β1 integrins both recognize laminins, knockout of the α3 receptor subunit leads to kidney defects and reduced lung branching morphogenesis, whereas α7-null animals develop muscular dystrophy. The integrins α5β1 and α8β1 both bind RGD peptides including fibronectin, but whereas deletion of the α5 subunit is associated with embryonal lethality, deletion of the α8 subunit causes renal dysplasia and abnormal lung morphogenesis.[43,44] The αvβ3 and αvβ8 integrins also interact with RGD-containing ECM proteins. Ablation of the β3-subunit causes defects in platelet aggregation and osteoclast function, resulting in hemorrhage and osteosclerosis,[45,46] whereas β8-null–mice die during embryogenesis because of abnormal placental and neurovascular development.[47] Keratinocytes use both α3β1 and α6β4 integrins to adhere to laminin. Mice lacking the integrin α3β1 exhibit a poorly organized basement membrane[48] but maintain keratinocyte adherence through integrin α6β4. Further studies suggest that the α3β1 laminin receptor can organize the basement membrane, whereas integrin α6β4 functions primarily to stabilize adhesion through hemidesmosomes.[49] Overall, these studies indicate that each member of the integrin family has evolved to subserve specialized and distinct functions throughout development. Determining how integrins transmit signals to the cell interior is clearly fundamental to understanding how this specificity is attained.

Signaling Through Integrins

Binding ECM ligands promotes integrin clustering and formation of cytoplasmic protein complexes that are nucleated by integrin cytoplasmic domains. These complexes are composed of cytoskeletal proteins and numerous intracellular signaling proteins at the inner surface of the plasma membrane.[50] These complexes congregate within specialized signaling centers termed *focal adhesions.* Different types of focal adhesions form depending on whether cells are cultured on two-dimensional surfaces or within three-dimensional matrices.[51] In this chapter, all of these structures are collectively referred to as focal adhesions.

On binding to ECM, signals initiating from the cytoplasmic tails of integrin receptors rapidly initiate recruitment of proteins to focal adhesions. Focal adhesion kinase, a 120-kDa nonreceptor tyrosine kinase, is a key regulator of signaling events downstream of focal adhesion formation. On initial formation of the focal adhesion, FAK, paxillin, talin, and Arp 2/3 mediate connection of ECM and integrins with the intracellular actin cytoskeleton. FAK activation then recruits an additional wave of signaling molecules to focal adhesions, which leads to dynamic changes in membrane dynamics, directional cell migration, and focal adhesion turnover.[52]

Many observations strongly suggest that activation of FAK by integrins plays a central role in initiating cell proliferation. For example, mutation of tyrosine residues critical for FAK activation prevents integrin-mediated proliferation.[53] Additionally, oncogenic

transformation of cells activates FAK, abolishing the requirement for anchorage-dependent growth.[54] Consistent with this, introduction of constitutively active FAK mutant leads to cell transformation, anchorage-independent cell division, and the suppression of apoptosis.[55] FAK deficiency in mice causes embryonic lethality, with delayed embryonic cell migration, impaired organogenesis, and vascular defects. This phenotype also is reminiscent of fibronectin- and α5-integrin–deficient mice, supporting the notion that ECM, integrins, and FAK are intimately linked. Unexpectedly, fibroblasts isolated from FAK-null mice are able to assemble focal adhesions and display increased adherence to the ECM substrates in tissue culture. This finding may explain why FAK-deficient cells exhibit reduced migration in vivo. Of interest, a truncated form of FAK is expressed in the brain, a highly differentiated, largely nonproliferative tissue.[56] Future studies aimed at determining the nature of the signaling molecules that lie downstream from FAK may reveal how this major integrin-responsive kinase specifies so many functions in different tissues.

Inside cells, ILK and a group of integrin tail-binding proteins called kindlins regulate how signals from the ECM affect cell behavior. ILK binds the cytoplasmic tail of β-integrin subunits and regulates actin dynamics through interaction with the actin regulatory proteins α- and β-parvin. Mutations in ILK that disrupt α-parvin binding prevent normal cell migration and cause renal agenesis in mice.[57] The three kindlin proteins—kindlin-1, -2, and -3—have different expression patterns but are required for normal cell-matrix adhesion. Kindlin-2 is most widely expressed, and loss of kindlin-2 expression disrupts adhesion of endoderm and epiblast cells to their basement membranes, leading to early embryonic death.[58]

Cross-Talk Between Integrins and Growth Factors

Various extracellular growth factors regulate cell migration and dynamics by means of integrin-mediated signaling. Growth factor stimulation can activate FAK, indicating that integrin and growth factor signaling pathways intersect at focal adhesions.[59] Consistent with this notion, integrin clustering promotes recruitment and activation of growth-factor receptors within focal adhesion complexes. For example, treatment of endothelial cells with beads coated either with RGD tripeptide or with fibronectin leads to coaggregation of β1-integrins and FAK, as well as with high-affinity fibroblast growth factor (FGF) receptors within newly formed focal adhesions.[60] Parallel studies using similar approaches have extended these findings to show that integrin activation also can lead to recruitment of a more extensive repertoire of growth factor receptors, including those for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF).[61]

It is not fully understood how integrin ligation leads to the recruitment of receptor tyrosine kinases to the focal adhesion site. However, the fact that high-affinity EGF receptors can directly bind actin, which in turn enhances EGF-dependent autophosphorylation and activation of downstream substrates, indicates a critical role for the F-actin cytoskeleton in coordinating signaling between integrins and growth factors.[62-64] Not surprisingly, pharmacologic disruption of the actin cytoskeleton not only prevents focal adhesion formation but also leads to reduced growth factor receptor activation.[65,66] Thus the actin cytoskeleton and associated proteins may act as a solid-state scaffold that spatially and biochemically coordinates cross-talk between integrins and growth factor receptor tyrosine kinases.

Integrins also can facilitate extracellular binding of growth factors to their receptors.[67] Transforming growth factor-β (TGF-β) is synthesized as a prepropeptide and cleaved by furin proteases before secretion. Once released in the extracellular environment, TGF-β remains associated with its propeptide—latency-associated peptide (LAP)—in a large latent complex (LLC) within the ECM. LAP binds fibrillin and fibronectin, and TGF-β as part of the LLC is inactive. ECM degradation can facilitate TGF-β receptor binding if components of the LLC-fibronectin-fibrillin complex are broken down.[68] In the absence of ECM degradation, the cell surface integrins αvβ6 and αvβ8 can bind LAP, present the active TGF-β peptide to its receptor, and promote downstream signaling. Integrin-mediated TGF-β activation increases in the presence of mechanical strain between ECM and adjacent cells, providing a potential mechanosensory signaling mechanism within developing tissues.[69]

EXTRACELLULAR MATRIX AND ANGIOGENESIS

The studies described already fully support the idea that signals generated by the ECM control many aspects of cell behavior. Instead of describing all of the developmental events in which ECM plays a role, this chapter focuses on angiogenesis, the growth of new capillaries from preexisting blood vessels. Angiogenesis also has been studied extensively in a variety of pathologic conditions, including neovascularization of solid tumors and the growth of new vessels into the retina, as occurs in diabetic retinopathy. Although developmental angiogenesis and pathologic angiogenesis differ somewhat, changes in endothelial cell adhesion to the ECM are critical in both instances. For example, in response to angiogenic factors, endothelia degrade their underlying basement membrane and invade the adjacent perivascular ECM. Proteolysis of the ECM also allows soluble serum proteins, including vitronectin, fibronectin, and fibrinogen, to extravasate into the extracellular space, thereby providing additional ECM components for endothelial cell migration. Next, endothelial cells begin to proliferate and to align themselves into angiogenic sprouts, columns of endothelial cells that orient toward sources of growth factor expression within tissues. Forming a new sprouting stalk of endothelia requires precise coordination of both cell-cell and cell-ECM adhesion. Finally, to form a patent vessel, lumina form within the column of sprouting endothelial cells and connect with existing vessels to establish blood flow and circulation. Endothelia in these new vessels then recruit pericytes and establish a new basement membrane. Interaction with pericytes and ECM further instructs endothelia within newly formed vessels to exit the cell cycle and become quiescent.

Integrins and Angiogenesis

Although vascular endothelia express a variety of integrins, including α6β1, α1β1, α2β1, α3β1, and α6β4, a great deal of attention has been focused on the αv-subunit–containing receptors. Treatment of endothelial cells with angiogenic factors, including vascular endothelial growth factor (VEGF), stimulates the expression of these integrins.[70] Furthermore, VEGF has been shown to promote EC adhesion to the ECM through αvβ3 and αvβ5 integrins, demonstrating that VEGF controls integrin activity.[71] In addition, immunoprecipitation experiments have shown that VEGF receptors (e.g., VEGF-R2) associate with αvβ3 integrins.[72] At a functional level, blockade of the αvβ3 integrins prevents angiogenesis induced by VEGF, whereas inactivating the αvβ5 integrin inhibits the angiogenic effects of FGF-2.[73] These latter studies not only demonstrate that soluble angiogenic factors and endothelial integrins communicate with one another but also suggest that different soluble angiogenic factors control different aspects of angiogenesis. In another example of differential mechanisms regarding angiogenesis, inhibition of protein kinase C (PKC)[73] disrupts VEGF-induced angiogenesis, yet PKC inhibition has no effect on FGF-2–dependent stimulation of endothelial cells.

A proangiogenic role for the αvβ3 integrin also is suggested by a number of other studies. For instance, the αvβ3 integrin localizes to newly formed embryonic blood vessels, suggesting an important role in vivo.[74] This notion is upheld by the finding that blocking the αvβ3 integrin receptor prevents angiogenesis

in various tissue types including the chick chorioallantoic membrane, mouse retina, quail embryo, and rabbit cornea.[74-76] Endothelial survival requires adhesion to the ECM,[77] and the αvβ3 integrin receptor has been shown to regulate this process.[78,79] Of interest, blocking αvβ3 integrins in vivo selectively induces apoptosis in newly formed vessels, but this treatment has no effect on preexisting vessels. In terms of the actual mechanism involved in promoting EC survival, ligation of endothelial αvβ3 integrins suppresses the activity or expression of the cell death mediators p53 and p21 WAF1/CIP1.[80]

Endothelial cell motility also requires the αvβ3 integrin receptor. To migrate, endothelial cells must reorganize their cytoskeleton in a manner that allows for the generation of intracellular forces that exert traction on the underlying ECM. Activation of the αvβ3 integrin receptor is required for sustained activation of the mitogen-activated protein kinases ERK1 and ERK2,[81] which in turn leads to phosphorylation of myosin light chains, components of the cell motility machinery required for endothelial cell motility.[82]

Null mutations of αv integrin subunits also have provided insights about how these receptors function during developmental angiogenesis. Contrary to expectations, vascular development in the αv-null mouse proceeds in a normal manner, and approximately 20% of the animals are born live.[83] After birth, however, these animals succumb to severe intracerebral and intestinal hemorrhages. These studies either reflect redundant and degenerate roles for αv integrins or indicate that αv integrins are more essential for vascular integrity than for blood vessel formation.

Matrix Metalloproteinases and Angiogenesis

Movement of angiogenic endothelial cells through the basement membrane and the perivascular tissue microenvironment requires catabolism of the ECM. The finding that matrix metalloproteinase-2 (MMP-2)-deficient mice show reduced rates of angiogenesis supports this idea.[84] Once again, the αvβ3 integrin appears to be involved, because interaction of this receptor with MMP-2 leads to MMP-2 activation on the surface of invasive endothelial cells.[85] Furthermore, treatment of angiogenic endothelia with a peptide that prevents binding of MMP-2 to αvβ3 integrins not only blocks collagenolytic activity but also inhibits angiogenesis.[86]

During angiogenesis, MMP-mediated degradation of ECM reveals neoepitopes that provoke alternative forms of endothelial cell behavior. For example, proteolytic cleavage of type IV collagen by MMP-9 results in the exposure of a functionally important proangiogenic αvβ3 integrin-binding site that usually is hidden within the native collagen structure.[87] This mechanism may allow endothelial cells to modify and respond to microenvironmental cues rapidly without de novo gene expression.

Experiments involving tissues isolated from MMP-deficient mice have provided additional clues as to how MMPs promote angiogenesis. Ablation of the gene encoding membrane-type matrix metalloproteinase-1 (MT1-MMP), an enzyme required for MMP-2 activation, leads to defective angiogenesis within remodeling cartilage and an impairment of skeletal development.[88] Similarly, homozygous mice with a null mutation in MMP-9 exhibit an abnormal pattern of skeletal growth plate vascularization and ossification.[89] In each case, tissues explanted from these null mice show delayed release of angiogenic factors, including FGF-2 and VEGF, reinforcing the idea that the MMP-cleavage of the endothelial ECM promotes the bioavailability of angiogenic growth factors.

Biomechanical Force and Angiogenesis

Cells within developing embryos are exposed to mechanical forces that maintain and modify their biologic behavior. In continuous contact with blood flow, endothelial cells are not exempt from this situation. For example, stretching endothelia increases β3 integrin mRNA levels, as well as reorganization of β1, α5, and α2 integrins.[90,91] Studies in vascular smooth muscle cells demonstrate that biomechanical-dependent cellular proliferation requires β3 and αvβ5 integrins.[92] The idea that integrins detect biomechanical signals is further supported by the recent finding that FAK is involved in mechanosensing during cell migration.[93] Biomechanical force also modulates the expression and activities of ECM components and proteases including tenascin-C and MMP-2, positive regulators of angiogenesis.[94] Collectively, these studies indicate that not only do biomechanical signals influence the ECM and its receptors, but also downstream signals generated by mechanical force modulate cell adhesion components. Additional studies are clearly needed to determine how local force differentials modulate endothelial cell behavior during angiogenesis within the developing embryo.

Extracellular Matrix–Dependent Changes in Cell Shape and Angiogenesis

A frequently overlooked function of the ECM is its ability to maintain or modify cell morphology within developing, remodeling, and differentiated tissues. Moreover, the way in which cell shape imposes itself on signaling pathways generated by the ECM and integrins is not well understood. Nevertheless, a seminal study by Folkman and Moscona in 1978[95] showed that aortic endothelial cell proliferation is tightly coupled to cell shape. In essence, the greater the extent of endothelial cell spreading, the greater the extent of proliferation. Subsequently, a role for cell shape affecting integrin-dependent signaling pathways has been demonstrated in angiogenic endothelia. The proliferation and subsequent survival of endothelial cells require interactions between ECM and αvβ3 integrin. Blocking integrin ECM binding reduces endothelial proliferation and increases apoptosis.[75] If cell spreading is prevented, however, occupation and ligation of αvβ3 integrins using antiintegrin antibodies fail to support endothelial proliferation and survival.[96] Endothelial cell rounding may therefore lead to apoptosis as a consequence of decreased integrin ligation, with correspondingly less signaling through integrins, FAK, and other downstream components. In a study using endothelia cultured in a defined concentration and area of different ECM ligands, however, cell spreading alone was conducive to proliferation, whereas cellular rounding was associated with apoptosis.[97] Thus endothelial cell shape, specified by the ECM, appears to profoundly modulate signals generated by identical ECM-integrin interactions.

To elucidate further how ECM- or integrin-dependent endothelial cell shape changes operate to control angiogenesis, it will be necessary to identify additional proteins that are able to coordinate both intracellular signaling pathways and cell morphology. To this end, the discovery that the Rho family of small GTPases (e.g., RhoA, Rac1, Cdc42) are able to relay integrin-derived signals, as well as organizing the cytoskeleton,[98,99] suggested that these proteins are well poised to integrate endothelial cell shape and function. In fact, subsequent studies showed that VEGF-dependent endothelial motility is critically dependent on Rac1,[100] and that Rac1 integrates signaling through the actin and microtubule cytoskeleton to promote capillary tube assembly in a tissue culture model of angiogenesis.[101]

CONCLUSION

Clearly, cell-ECM interactions play a fundamental role in development, yet many questions remain. The phenotypic analysis of mice bearing mutations in ECM genes and receptors is providing new insights into ECM function. A basic understanding of how different combinations of ECM proteins generate tissue-specific forms of cell behavior will be critical to translate current

molecular and cellular knowledge into improved approaches for tissue engineering and regeneration. Regardless of the outcomes of such experiments, the emergence of rapid screening techniques for the detection of ECM gene mutations, the development of diagnostic DNA and protein microarrays, and whole-genome sequencing will no doubt aid in determining whether the results gleaned from experimental systems are relevant to defects in human embryogenesis and development.

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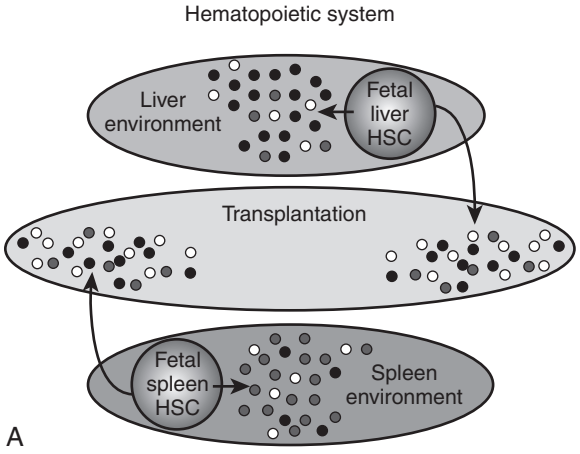
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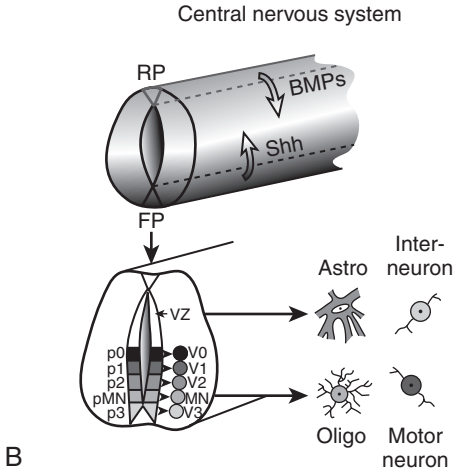
7 Stem Cell Biology

Evan Y. Snyder and Stephen Yip

CONCEPTS AND NOMENCLATURE

Stem cells have entered the consciousness of both the medical community and the lay public because their tremendous regenerative capabilities may hold great therapeutic potential.[1,2] It probably is most instructive, however, to view these cells in their original natural context: playing fundamental roles in development as the building blocks of organogenesis and the mediators of organ homeostasis.[3] Their therapeutic potential rests essentially on the ability to harness these fundamental developmental actions for therapeutic ends—"translational developmental biology," so to speak. Restoration of organ integrity and function through the replacement of damaged or defective cells is an extremely powerful concept, if such replacement can realized. It represents a paradigm shift in medical science; indeed, the stem cell concept helped give birth to the emerging field of *regenerative medicine*. Of interest, it may prove that the perinatal period—during which developmental processes are at their crescendo—is when stem cell–based approaches may find their most successful applications. To the extent that their utility can be applied to "postdevelopmental" organ dysfunction probably





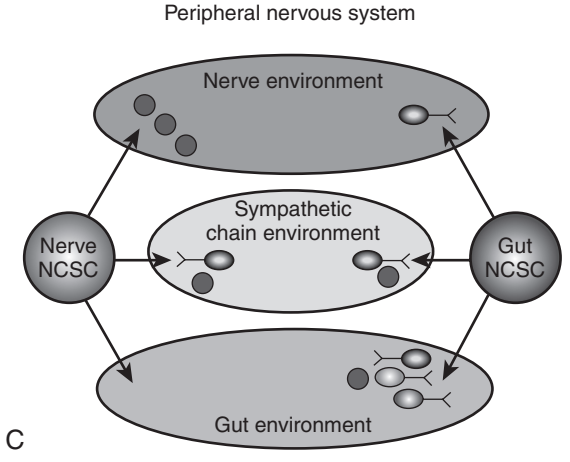


Figure 7-1 Determination of stem cell fate is governed by both internal (intracellular) and external (microenvironment or niche) signals. However, inherent difference in the relative susceptibility to these signals exists in different tissues, and this is often essential and unique to the tissue in question. **A,** Development of the hematopoietic system is reliant on signals from the microenvironment. **B** and **C,** However, progenitor cells in the developing central nervous system and the peripheral nervous system appear to rely on differential intrinsic responsiveness to external soluble factors and developmental cues. This strategy allows the nervous system to acquire distinct regional identities, as is crucial for proper development and functioning of the organism. *BMPs,* bone morphogenetic proteins; *FP,* floor plate; *HSC,* hematopoietic stem cell; *NCSC,* neural crest stem cells; *RP,* roof plate; *Shh,* Sonic hedgehog protein; *VZ,* ventricular zone. *(From Joseph NM, Morrison SJ: Toward an understanding of the physiological function of mammalian stem cells,* Dev Cell *9:173-183, 2005.)*

will depend on how well the developmental microenvironment is mimicked either naturally by the insult itself or artificially by the clinician. A partial glimpse at the potential of stem cell biology in this regard is offered by the current success of hematopoietic stem cell–mediated reconstitution of an ablated bone marrow in the context of bone marrow transplantation (BMT) for selected diseases.[4] Nevertheless, apart from the therapeutic potential of stem cells, every perinatologist—the clinical counterpart of the developmental biologist—should be familiar with basic stem cell concepts.

A stem cell is the most primitive cell of a given organ or tissue that is able to differentiate into all of the more mature and specialized daughter cells of that tissue (a property called multipotency) and to give rise to some daughter cells with exactly that same potential (a property called self-renewal). Stem cells seem to undergo *symmetric* cell division—splitting into two identical daughter cells that are themselves stem cells—when the stem cell pool must be expanded rapidly, as during the first stages of organogenesis or in the first response to extrinsic stimuli such as stress. They undergo *asymmetric* cell division—giving rise to

a more specialized daughter cell and only one new stem cell—when the stem cell pool simply needs to be maintained at a constant size. Very often, this cell division can be quite slow, because the stem cell largely exists in a quiescent state.[5] In some organ systems, it is hypothesized that when tissue turnover is required to be at its zenith, the stem cell gives rise to an even more rapidly dividing "transient amplifying cell." Stem cells have the ability to undergo both symmetric and asymmetric divisions, and normal development of the organism requires a balanced switching between the two.[6] Teleologically, this balance is significant for the persistence of stem cell pools into adulthood and for maintaining homeostasis throughout life. In fact, experiments have demonstrated that aberrant skewing toward symmetric division may be associated with malignant transformation in vivo.[7] Taking advantage of this biologic attribute, however, should permit indefinite expansion of stem cells in vitro once they have been isolated from a tissue and placed in culture.

In vivo, the microenvironment surrounding the stem cell, including the extracellular matrix and the supporting stromal cells, in conjunction with intrinsic stem cell developmental programs, appears to control the various stem cell decision pathways that ultimately lead to the creation of a given organ.[8,9] The concept of the stem cell niche was first explored in hematopoiesis but has now been extended to stem cell biology in general (Figure 7-1).[10] Cellular and extracellular constituents of the niche have come to be regarded as equal partners with stem cells themselves in the execution of proper developmental programs and in maintaining a system that can respond to dynamic needs.[11] Dysregulation of this developmental milieu may potentially lead to oncogenesis.[12] Ongoing attempts to recreate those signals on command and to master the molecular controls of the niche microenvironment are expected to provide important tools of regenerative medicine.

The coming together of two *gametes* (sperm and oocyte, respectively) yields a fertilized egg, or *zygote,* which begins to divide, yielding daughter cells that initially are *totipotent*—that is, they have the potential to give rise to the complete embryo as well as all of the extraembryonic tissues, including the trophoblast (Figure 7-2). (Each zygote is not self-renewing and therefore is not itself regarded as a "stem cell.")

The fertilized cells go through two-cell, four-cell, and then eight-cell stages, ultimately yielding a morula and then a blastocyst. The blastocyst becomes asymmetric in its appearance and function. Toward one pole of the blastocyst, a group of stem cells begin to accumulate to form the *inner cell mass* (ICM); that portion of the blastocyst is called the *fetal pole* because it is within this group of cells that the nascent embryo will begin to emerge. The ICM will then differentiate into an *epiblast,* the actual precursor of the embryo, and *extraembryonic endoderm,* a tissue that will help support the epiblast. If the ICM is extracted from the blastocyst and cultured in a dish under appropriate conditions that support their continued cell division while arresting any further differentiation, a cell population known as *embryonic stem cells* (ESCs) will result. These cells are regarded as being *pluripotent* because they have the potential to give rise to all cell types of the embryo but not to trophoblast. In vitro, much as is seen in the epiblast in vivo, ESCs, if permitted to differentiate, will begin to generate daughter cells that become partitioned to one of three fundamental germ layers: *ectodermal, mesodermal,* or *endodermal.* This occurs within 1 week and is most classically promoted experimentally by allowing the ESCs to aggregate in a floating clump of cells called an *embryoid body* (EB), so termed because it mimics—albeit in a very haphazard, random, and disorganized fashion—the emerging cell types of the embryo in vivo. A fundamental difference between an EB and an actual embryo is that a blastocyst, if implanted into a uterus (as during the process of assisted reproduction by *in vitro fertilization* [IVF]), will give rise to a complete mammalian organism after an appropriate gestational period; an EB will never do so under any circumstances. The corollary of this biologic fact is that

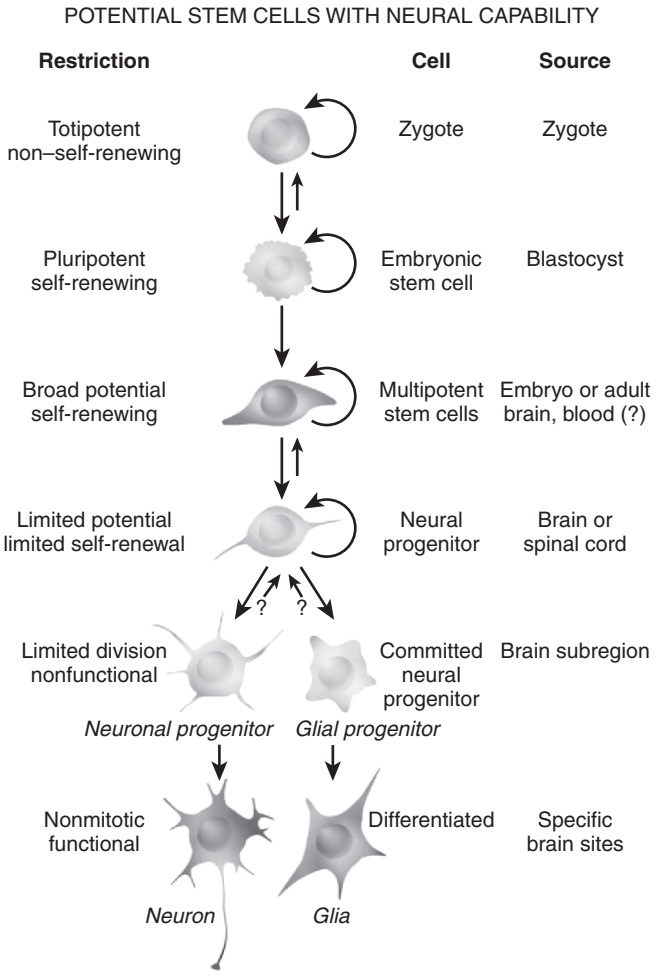


Figure 7-2 The developmental potential of mammalian stem cells into neurons. The developmental hierarchy of the stem cells is shown; pluripotentiality becomes much more restricted with maturation of the stem cell. *(From Gage FH: Mammalian neural stem cells,* Science, *287:1433, 2000.)*

even the *potential* for continued development beyond the blastocyst stage requires the environment of an intact female reproductive system. ESCs do not have the potential in vitro to give rise to an organism. Furthermore, other than for purposes of fertility treatment, it is considered unethical to implant any experimentally manipulated zygote.

The *ectoderm* ultimately gives rise to cells of the skin and the nervous system. The ectoderm differentiates into the *neuroectoderm,* which begins to roll up into a tube (called the *neural tube*) that will become the central nervous system (CNS). As the neural tube begins to close, a population of cells on the superior surface of the tube begins to migrate. These cells are collectively called the *neural crest* and will give rise to peripheral, autonomic, and enteric nervous systems as well as a host of other tissues (including the facial bones, the great arteries of the heart, the thymus, and melanocytes). The *mesoderm* gives rise to muscle, bone marrow, blood vessels, and the urogenital system, as well as the connective tissues of the body. The *endoderm* gives rise to the epithelial lining of the gastrointestinal tract, parenchymal pancreas, thyroid, parathyroid, and liver. Although ESCs in reality are derived only in artificial tissue culture, they are placed conceptually at the apex of the stem cell hierarchy because of their pluripotency (Figure 7-3). As discussed later, once a cell has been assigned to a given germ layer and even more narrowly to a given tissue or organ lineage, its potency becomes somewhat restricted,

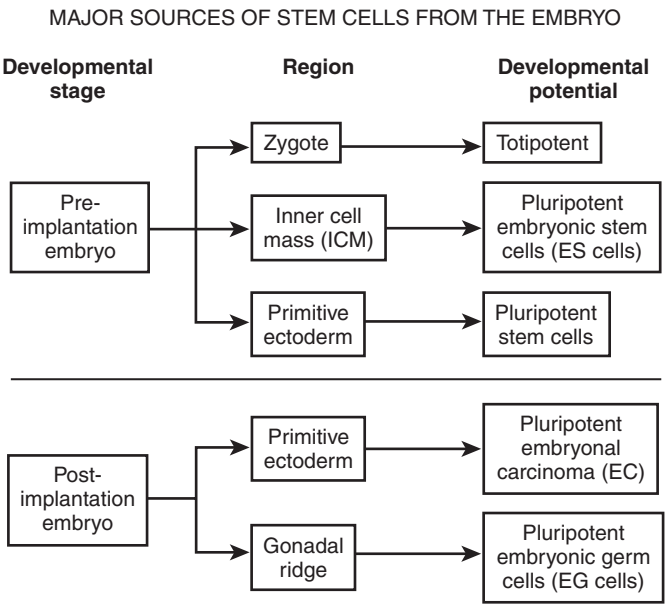


Figure 7-3 Examples of tissue-derived stem cells from different stages of development. Stem cells can be obtained from many different tissues, but their abundance and developmental potential follow a strict hierarchy in which embryo-derived stem cells have the highest pluripotentiality.

a state reflected by the term *multipotency.* It is at this stage that such cells are called *somatic stem cells* or *tissue-resident stem cells* or *tissue-derived stem cells,* distinct from *embryonic stem cells.* (The term "adult stem cells" often applied erroneously to such cells and should be avoided because of the confusion it creates.) As development proceeds, a subset of these multipotent stem cells become even more restricted in their natural fate, a condition reflected by use of the term *oligopotent* (if a few cell type options remain, often used synonymously with *progenitor cell*) or *unipotent* (if only one cell type can emerge, sometimes inferred by use of the term *precursor*).

One of the most vexing and controversial aspects of stem cell biology is the question of where pluripotent stem cells (PSCs) may reside in the body. Clearly, they can be isolated from the ICM of the blastocyst (discussed later), giving rise to cells of the three primary germ layers but not trophoblasts. ICM-derived cells remain the prototype for pluripotence. Indeed, pluripotent cells also can be derived from some neoplastic states, such as teratocarcinomas and teratomas. It was the study of such tumors in the 1970s that ultimately guided developmental biologists to search for ESCs in the normal ICM. Stem cell researchers noted more than 50 years ago that teratocarcinomas contained differentiated cells belonging to all three primary germ layers as well as a population of pluripotent cells that, when transplanted to a receptive host, would form a second tumor. In addition, teratocarcinomas also could proliferate in vitro as undifferentiated embryonal carcinoma (EC) cell lines in the presence of a feeder cell layer.[13] Subsequently, it was noted that similar tumors could be generated by implanting cells from the ICM into immune sanctuary sites, such as the testis or inside the kidney capsule. Later, cells from the postimplantation embryonic gonadal ridge were found to have a similar capacity. To this day, the ability to form a teratoma remains part of the operative definition of a pluripotent cell. EC cell lines appear to straddle the line between normal embryonic development and oncogenesis. Blelloch and colleagues have performed nuclear transplantations of nuclei from EC cell lines into normal blastocysts and further examined the genetic profiles of the former, thus establishing genetic lesions with potential for tumor development.[14]

Whether vestiges of ICM-like or gonadal ridge–like cells can still be isolated from more mature "postembryonic" or even adult tissues remains extremely controversial. Also uncertain is whether exceedingly small populations of pluripotent cells can be found in the bone marrow mesenchyme, in the umbilical cord blood, or in the amniotic fluid and placenta. A fascinating observation has recently expanded the potential source of pluripotent cells—the well-supported notion that seemingly lineage-committed cells can be artificially "dedifferentiated" or reprogrammed back to a pluripotent state through the transduction of a panel of oncogenic-type genes. These experiments are briefly discussed further on.

It does seem likely that *multipotent* stem cells persist throughout life in many organs. As noted earlier, these are stem cells that have "learned their address" and have been allocated to residence within a given tissue. They begin to appear in embryonic and fetal organs, are present throughout gestation and birth, and, in most organs, persist to some extent into adolescence and adulthood (although their number and degree of responsiveness in later life in some tissues remains controversial—such as heart, pancreas, and CNS). Such multipotent stem cells, when present, are restricted to developmental pathways characteristic of their particular organ of residence and certainly to their fundamental germ layer of derivation. Although for a brief period the stem cell field entertained the possibility that multipotent stem cells could "transdifferentiate" into cells of another organ system, the data suggesting that possibility were found to be the misinterpretation of an experimental artifact and the notion has fallen into disfavor in most circles. In essence, a multipotent stem cell of ectodermal origin can give rise to daughter cells that belong only to this germ layer; neural stem cells (NSCs) give rise only to neural tissue; hematopoietic stem cells give rise only to blood-derived cells. Blood will not give rise to brain, and brain will not give rise to blood. Nevertheless, within a given organ, the degree of plasticity and the scope of fate options remain broad. Committed progenitor cells, such as the colony-forming unit granulocyte-macrophage (CFU-GM), are more restricted in their developmental potential. These cells are limited in their ability for self-renewal and are programmed to give rise to a very limited repertoire of specialized cells within a germ layer.

To summarize, stem cells can be divided into *ICM-derived pluripotent cells* and *somatic, tissue-derived,* or *tissue-resident multipotent stem cells.* The latter designation can be further modified by specifying the particular tissue and the particular age or developmental stage from which the stem cell was observed or isolated, (e.g., a fetal NSC, an adult NSC, or a newborn hematopoietic stem cell).

These concepts are discussed in greater detail in the following sections.

EMBRYO-DERIVED STEM CELLS

Embryonic Development

A 3- to-5-day-old preimplantation human embryo known as the *blastocyst* contains the ICM and the trophectoderm.[15] The trophectoderm lacks stem cells but contains *trophoblastic precursor cells* that later become the fetal part of the placenta. During the preimplantation stage, the outer trophectodermal epithelium pumps fluid to form the blastocoel, a hollow cystic structure that gives the blastocyst its name.[16] The ICM, located at one end of the blastocoel, consists of approximately 30 pluripotent cells that can give rise to all tissue types of the fetus (Figure 7-4). (By convention, before 8 weeks gestation, the developing human is called an *embryo*; after 8 weeks gestation, it begins to be called a *fetus*.) Implantation of the embryo (embryonic days 5.5 to 7 in a mouse and days 7 to 12 in humans) is associated with further cellular differentiation and the impending disappearance of the pluripotent state. This is followed by the establishment of

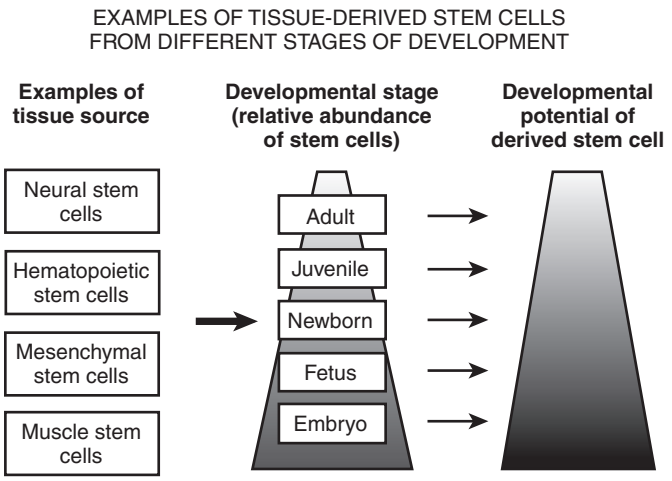


Figure 7-4 Major sources of stem cells from the embryo. Embryo-derived stem cells can be classified as being from the preimplantation or postimplantation stages. They can be further subcategorized according to region of origin, which ultimately will affect their developmental potential.

the primitive endoderm that contains precursors to cells of the embryonic yolk sac, a nutritive organ for the developing embryo.

ESCs are derived from the in vitro expansion of ICM cells from the preimplantation blastocyst stage, classically accomplished by plating the cells on specialized feeder cell layers, often mouse or human embryonic fibroblasts. (Much research is ongoing to determine whether ESCs can be derived under entirely defined conditions, including without need of a feeder layer.) Immunosurgery is sometimes used to isolate cells from the ICM and to deplete it of contaminating trophoblasts. Alternatively, the ICM can be mechanically dissected from the blastocyst. If the ICM cells begin to divide and maintain their undifferentiated state (marked by the presence of a panel of proteins—Oct4, Nanog, SSEA-4, Tra-1-80), for at least 12 passages, they are termed ESCs. The final proof that they have become an ESC line, however, depends on their ability to differentiate into all three germ layers. This capacity is routinely tested in two ways: First, the cells should yield teratomas when implanted into immunoincompetent mice (teratomas, by definition, contain representatives of all three germ layers); second, they should be able to yield cell types of these same germ layers when aggregated as the aforementioned EBs.[17] Indeed, the ability to form teratomas is part of the definition of an ESC. ESCs from primates (both monkey and human) are much harder to grow and to keep in an undifferentiated, proliferative state than are ESCs from mice.

Once established, ESCs should be capable of being maintained and expanded in an undifferentiated state in the laboratory indefinitely as stable cell lines. At this point, the goal next becomes to differentiate the ESCs in a more controlled, lineage-specific manner. Typically this process involves removing the ESCs from a feeder layer if present and often omitting mitogens (such as basic fibroblast growth factor) from the medium while adding such inducing molecules as retinoic acid or Sonic hedgehog. Novel protocols are constantly being devised for efficiently directing ESCs toward becoming different cell types. A review of these specific "recipes" is beyond the scope of this discussion; the reader is directed to such source material as *Current Protocols in Stem Cell Biology*.

Although ESC research initially was built on years of empirical observations and trial and error, its future will clearly depend on the rigorous dissection of the complex molecular mechanisms involved in maintenance of pluripotency and controlled cellular development.[18,19]

Embryonic Germ Cells

Another source of embryo-derived pluripotent stem cells is the gonadal and germinal ridge from the 7- to-10-day-old embryo. These primordial germ cells (PGCs) can be expanded in vitro to generate embryonic germ cell (EGC) lines. Transplantation of mouse PGCs (which persist through midgestation) into permissive recipients can give rise to teratocarcinomas, as noted previously. Shamblott and associates isolated human PGCs and characterized the subsequently derived EGC line.[20] They harvested and isolated gonadal ridges and mesenteries from 5– to 9–postfertilization week embryos. Extensive characterization of the resultant EGCs demonstrated that they satisfied all of the criteria previously established for hESC lines. Specifically, they could be maintained as unspecialized cells in vitro in cocultures with mouse feeder layers and, when induced to differentiate, were able to generate daughter cells belonging to all three germ layers. The EGCs have normal karyotypes and demonstrate immunohistochemical profiles similar to those of ICM-derived stem cells. The differentiated progeny of EGC lines express the broad range of developmental markers associated with neuronal, vascular, hematopoietic, muscle, and endodermal lineages.[21] As with ESCs, differentiated EGCs are characterized by a heterogeneity of lineages; differentiated cells from multiple lineages coexist, persist, and even reemerge after application of ostensibly lineage-specific instructive protocols. Obtaining pure homogeneous populations of a single cell type from pluripotent cells remains a challenge to the field. Figure 7-2 illustrates the source of pluripotent ESCs and EGCs from the preimplantation embryonic ICM and the postimplantation embryonic gonadal ridges, respectively. Both cell populations retain pluripotency and many other stem cell characteristics. Cells from the reproductive system are apparently unique in that they maintain pluripotent capacity longer than would normally be expected in any other tissue. For example, developmental pluripotency has been observed in cells isolated from neonatal mouse testis and in adult murine spermatogonial cells.[22,23] Of interest, however, the ability to demonstrate therapeutic efficacy and safety using EGCs has been less forthcoming than those for ESCs. It is unclear whether this disparity reflects a fundamental difference between ESCs and EGCs or merely the greater number of investigators focusing their efforts on working out the technical hurdles of ESC use.

Embryonic Stem Cells of Mouse Origin

Murine-derived ESCs (mESCs) originally were isolated in 1981 by Evans and Kaufman.[24] Since that time, many research teams have refined the isolation and culture techniques. Stable mESC lines were generated by the successive replating and numeric expansion of the undifferentiated subpopulation of a previous mESC culture, essentially selecting for the most proliferative cells. The use of a suitable feeder cell layer initially was indispensable to the maintenance of an undifferentiated state. It was subsequently learned that (LIF) could be used in place of the feeder cell layer for mESCs; this cytokine appears to be important in maintaining the viability of PSCs in the preimplantation mouse embryo.[16,25] LIF also has pleiotrophic actions on the organism, including effects on hematopoiesis, osteogenesis, lipid transport in adipocytes, neurogenesis, and neuronal survival.[26] The feeder cell layer appears to provide a mixture of secreted soluble cytokines, membrane-associated factors, and factors associated with the extracellular matrix. These factors, in turn, maintain the growth of the mESC lines in an undifferentiated state. Attempts have been made to analyze systemically the biochemical composition of these factors.[27]

Mouse ESC lines not only have been the object of study in themselves but have also made possible a valuable biologic tool,

the *transgenic mouse*. Specific genes of interest can be "knocked out" or "knocked in" within mESCs, which then can be placed into the ICM of a blastocyst-stage mouse. The altered mESCs now contribute to all organs of the developing mouse at its earliest stages of embryogenesis including the germ cells, allowing the traits to enter the germline. A strain of mice ultimately develops with altered expression of the gene of interest—either overexpressed or suppressed. Such transgenic mouse technology has revolutionized studying the molecular basis of both development and disease and was recognized by the 2007 Nobel Prize for Physiology and Medicine.

To take full advantage of ESCs, the mechanisms underlying cellular differentiation and lineage development must be understood. To date, the greatest advances in this regard have come from the use of mESCs, although similar protocols for hESCs are rapidly being devised; for some cell types, extrapolation from mESCs to hESCs is possible; for other cell types, the fundamental differences between mESCs and hESCs demand different strategies. Various methods have been devised to control the fate of differentiating mESCs. Obviously, it is important to avoid transplantation of pluripotential ESCs that could potentially form tumors (e.g., teratomas or teratocarcinomas) or give rise to cell types inappropriate to a given organ. Restricting the potential of these very pluripotent cells also has been a challenge. Often the strategy entails recapitulating in vitro the step-by-step molecular process by which a given cell type is generated during normal development in vivo. For example, motor neurons have been generated from mESCs by first neuralizing the cells with retinoic acid and then applying a ventralizing morphogen such as Sonic hedgehog. It is thought that, in directing an ESC toward becoming a mature cell type, the cell passes—fleetingly—through a stage of being a tissue-derived stem cell. For example, when an ESC is directed to become a neuron, it might first pass through a stage of becoming an NSC, a cell equivalent to those populating the neuroectoderm and lining the neural tube. It was this type of reasoning that helped facilitate the selective expansion of apparently multipotent neuroepithelial stem or progenitor cells from mESCs through the use of bFGF.[28] Such NSCs were then used to generate postmitotic functional neurons and glial cells. In one set of experiments, oligodendroglial and astrocyte precursors generated in this manner from mESCs were capable of myelinating host neurons in a dysmyelinated rodent.[29,30] Similarly, mESC-derived dopaminergic neurons have been able to reverse some deficits in rodent models of Parkinson disease. Long-term engraftment of lymphoid-myeloid precursors from genetically modified mESCs has been achievable.[31] mESCs also have been induced to differentiate into adipocytes, islet cells, hepatocytes, and osteoblasts.[32]

Embryonic Stem Cells of Human Origin

The isolation, growth, and characterization of human ESCs (hESCs) were reported in 1998.[33] The lag time between the routine generation and use of murine ESCs (mESCs) in the early 1980s and the success in creating primate ESCs nearly 20 years later suggests the difficulty in simply extrapolating from rodent to human and reflects the unanticipated fundamental biologic differences between the two types of ESCs in terms of growth requirements, culture conditions, and signaling pathways involved in pluripotency and differentiation. Some differences may be attributable to the fact that hESCs appear to represent a somewhat later stage in development than that characteristic of mESCs. Whereas mESCs reflect the ICM stage of murine embryonic development, hESCs, although generated from the ICM, appear to be more representative of the epiblast stage in human development.

Even the appearance of hESCs and mESCs differ: hESCs grow as flat colonies, whereas mESCs grow in round, amorphous masses. Although some of the same molecules characterize the undifferentiated pluripotent state in both species of ESCs, such as the transcription factors Oct-4 (which blocks trophectoderm formation) and Nanog (which blocks primitive endoderm differentiation), other markers differ. For example, although both species of ESCs express stage-specific embryonic antigens (SSEAs) on their cell surfaces, mESCs express SSEA-3 and hESCs express SSEA-4; indeed, SSEA-3 in hESCs is associated with the differentiated state, not the undifferentiated. In addition, mESCs grow more rapidly and robustly in vitro. hESCs are more difficult to manipulate and, to date, grow less reliably without a mouse embryonic fibroblast (MEF) feeder layer. Whereas mESCs are dependent on leukemia inhibitory factor (LIF) for feeder-free growth, hESCs have no such LIF dependence, suggesting that different signal transduction pathways are operative. Because hESCs may incorporate and express on their cell surface certain glycoproteins taken up from the feeder cells, there is the theoretical concern that immunocompatibility following transplantation of such cells into a human host might be compromised. Advances in the understanding of hESC biology have begun to suggest better conditions for the in vitro propagation of hESCs, including eliminating the need for murine feeder cells.[34-36]

Despite their differences, it is the similarities between murine and human ESCs that is most compelling and has stimulated the imagination of the regenerative medicine community. hESCs, like their murine counterparts, have the ability for indefinite self-renewal yet can differentiate into cells representing all three germ layers.[37] Indeed, a similar antigen expression profile has been demonstrated between hESCs and preimplantation human embryos.[38] Of course, the ultimate test of pluripotence as applied to murine ESCS—the ability to generate a totally chimeric mouse[39]—cannot be used for assessing hESCs, for obvious ethical reasons. Surrogate tests, however, can be used and typically consist of determination of the ability of the cells to form teratomas in immunoincompetent mice and the ability to yield multiple differentiated cell types after EB formation.

Significant cross-pollination of knowledge and technical expertise occurs between the fields of hESC research and the rapidly evolving area of assisted reproductive research, especially IVF.[16] The rapid development of preimplantation genetic diagnosis (PGD) at the eight-cell blastomere stage is an example of the union between scientific and technical advances.[40] PGD represents a significant advance in the prenatal diagnosis of genetic diseases.[41] Often, PGD indicates that a blastocyst generated by IVF in the context of assisted reproduction bears a lethal or devastating disease, rendering it morally unacceptable to implant the blastocyst. The defective blastocysts, however, can be used to generate hESC lines that then might faithfully model cellular aspects of that human disease. Such cellular models are particularly useful for conditions in which no good representative and predictive animal model exists. the resultant "disease lines" can then be used to test pharmaceuticals, to identify novel drug targets, to gain mechanistic insights at the cellular and molecular levels, and to identify better prognostic or diagnostic markers. This use of hESCs is potentially quite powerful and offers prospects for therapeutic applications apart from direct cell-mediated repair. In a similar vein, hESCs may serve as an in vitro model or surrogate for dissecting the earliest stages of human embryogenesis that, for obvious ethical reasons, cannot be directly observed in situ.

The ability to manipulate hESCs genetically (particularly developmentally important genes), although initially quite difficult, is becoming somewhat easier, thereby enhancing the value of hESCs as models of human embryogenesis and disease. Initial manipulations were through homologous recombination,[42] a technically challenging technique. More recently, lentiviral vector–mediated manipulation has proved effective for inserting reporter genes to act as biosensors (particularly when under the control of developmental stage–specific promoters), overexpressing genes, or

suppressing the expression of genes (e.g., through RNA interference). If a therapeutic gene is placed into hESCs and the hESCs are subsequently differentiated toward engraftable somatic cells, genetic defects in a host also may be correctable by this means,[543] much as has been achieved using somatic stem cell transplantation.[44,45,46,47]

Progress is being made in devising culture conditions that may potentially permit hESCs to be directed toward particular lineages in vitro. In some instances these recipes have successfully borrowed from the murine literature (e.g., generating spinal motor neurons). In other instances the strategy must differ from that in mouse ESCs (e.g., creating pancreatic beta cells). Mesenchymal precursors have been derived from hESCs with the capacity to differentiate into skeletal muscle cells, fat, cartilage, and bone.[48] Neuroectoderm, vascular endothelium, and beating cardiomyocytes emerge seemingly spontaneously and constitutively, although not as pure populations in large numbers with high or predictable efficiency. Some hESC-derived cell types have been shown to integrate successfully into rodent organs after transplantation—for example, hESC-derived neural progenitor cells implanted into the cerebral ventricles of developing brain[44,49,50]—and have even proved to be therapeutic in murine models of lethal human disease.[44] A fascinating observation is that human stem cells are capable of developing and differentiating in a murine environment, clearly responding to highly conserved even if xenogeneic signals. The hESC-derived cells can migrate extensively along established host migratory pathways, integrate into host NSC pools, contribute to adult neurogenesis in the olfactory bulb, and yield cells of all three fundamental neural lineages—neurons, astrocytes, and oligodendrocytes—as well as participate in repair.[44,50] The creation of such chimeric animals has generated considerable scientific interest but also controversy related to the admixture of human brain cells with those of a nonhuman nervous system.[51] Nevertheless, such models are required preclinically to establish proof-of-concept of repair, and to establish protocols for cell preparation and administration that achieve efficacy while ensuring safety. Supporters of this line of research are confident that hESCs, because they are "naturally immortalized" and can therefore provide an inexhaustible supply of undifferentiated cells in vitro for subsequent differentiation along numerous lineages, hold enormous therapeutic potential if the biology can be "tamed"[52] (Figure 7-5).

One of the technical challenges that must be surmounted is the anticipated immunogenicity of allogeneic hESCs when implanted into an unmatched immunocompetent host.[53,54] The degree to which such immunoincompatibility presents a de facto problem remains an area of both active investigation and debate. Some investigators have found that hESCs actually exhibit a degree of immune-"tolerizing" in mixed cultures with alloreactive human T cells.[55] In a similar vein, other reports have shown that cells from a murine ESC line are capable of reconstituting normal hematopoiesis and functional adaptive T and B cell immunity in an allogeneic graft recipient without a graft-versus-host response.[56] Nevertheless, it remains prudent to assume that overcoming immunologic barriers will still require careful consideration and could present a formidable scientific and technical hurdle to the successful clinical application of ESCs.

Another potential technical and biologic obstacle is the difficulty of achieving pure populations of single hESC-derived cell lineage. Even when various combinations of cytokines have been found to force the differentiation of hESCs toward a particular lineage, cell type heterogeneity spontaneously reemerges.[57,58] Such heterogeneity may be a fundamental property of a stem cell that cannot (and should not) be suppressed. Repair of a particular organ system may actually require the replacement of multiple collaborating cell types. On the other hand, the emergence of cell types inappropriate to a given organ is a risk and may prove dangerous. For a given disease process, only rarely has it been possible to achieve a full understanding of the array of cell types that may actually need to be replaced and in what ratio, and with what timing, and anatomic relationships. An additional conundrum lies in the recognition that treating an hESC such that its progeny are so fully mature and committed that alternate cell types are not possible also compromises the ability of that cell to integrate into a host system after transplantation. Incomplete restriction of the potential of an hESC, however, leaves open the possibility of tumor formation or the emergence of undesired cell types in the host after engraftment.

SOMATIC STEM CELLS (TISSUE-RESIDENT OR TISSUE-DERIVED STEM CELLS)

Somatic stem cells, also called tissue-derived or tissue-resident stem cells, possess the ability for (1) self-renewal and (2) differentiation into most of the specialized cells of the tissue, organ, or germ layer of their origin. Because they cannot give rise to all cell types of the organism (a property for which the term *pluripotence* is reserved), these cells are designated as *multipotent*. In the lay literature, somatic stem cells often are erroneously named "adult stem cells" to distinguish them from pluripotent cells obtained from blastocysts (i.e., hESCs), a politically controversial source; however, that misnomer is never used by true stem cell biologists unless they have obtained the cells from a fully adult organ or tissue. Typically, the most useful and robust "adult" stem cells are obtained from fetal sources.

Indeed, somatic stem cells are found in abundance during fetal development, and their number is thought to decrease as the organism ages (see Figure 7-3). The first solid-organ somatic stem cell to be described, isolated, engrafted, and exploited for therapeutic purposes and developmental insights was the NSC.[47,59,60] In fact, recognition of the existence of the NSC suggested a greater degree of inherent plasticity within the CNS than had been classically taught. The isolation and expansion of human NSCs (hNSCs)—including those with the ability to be engrafted, to respond to developmental cues, to replace missing neural cell types, and to transport in foreign genes—were first reported in 1998.[61] These hNSCs were obtained from a germinal zone (the ventricular zone) in the human fetal telencephalon. These somatic stem cells, at least those in the human, are less robust in their proliferative potential than their embryonic counterparts; in other words, whereas hESCs are "naturally immortalized," such somatic stem cells as hNSCs typically require tonic exposure to a mitogen or augmentation with manipulation of cell cycle regulatory or "stemness" genes (Figure 7-6).

NSCs (or, at least, neural progenitor cells) have been isolated from the adult human brain, but they are much less robust than their fetal and neonatal counterparts in vitro.[62] The adult cells senesce sooner than neonatal cells, an observation investigators have attributed in part to shorter telomere lengths in the older cell resulting from differences in telomerase activity. The biology and molecular pathways underlying the behavior of adult NSCs or neural progenitor cells continue to be an active area of investigation.[63-65]

The discovery of somatic stem cells in the brain helped stimulate the search for similar stem cells in other solid organs,[66,67] including heart, muscle, pancreas, and liver. When such cells have been found, those obtained from embryo and fetal organs appear to retain stem cell characteristics with greater vigor than that typical of cells derived from the same tissues at adulthood, much as observed in the brain.

Probably the prototypically ideal somatic stem cell derived from an adult tissue is the *hematopoietic stem cell*. A single hematopoietic stem cell is able to give rise to the entire spectrum of blood cells during development and to reconstitute the entire hematopoietic system of a lethally irradiated adult animal

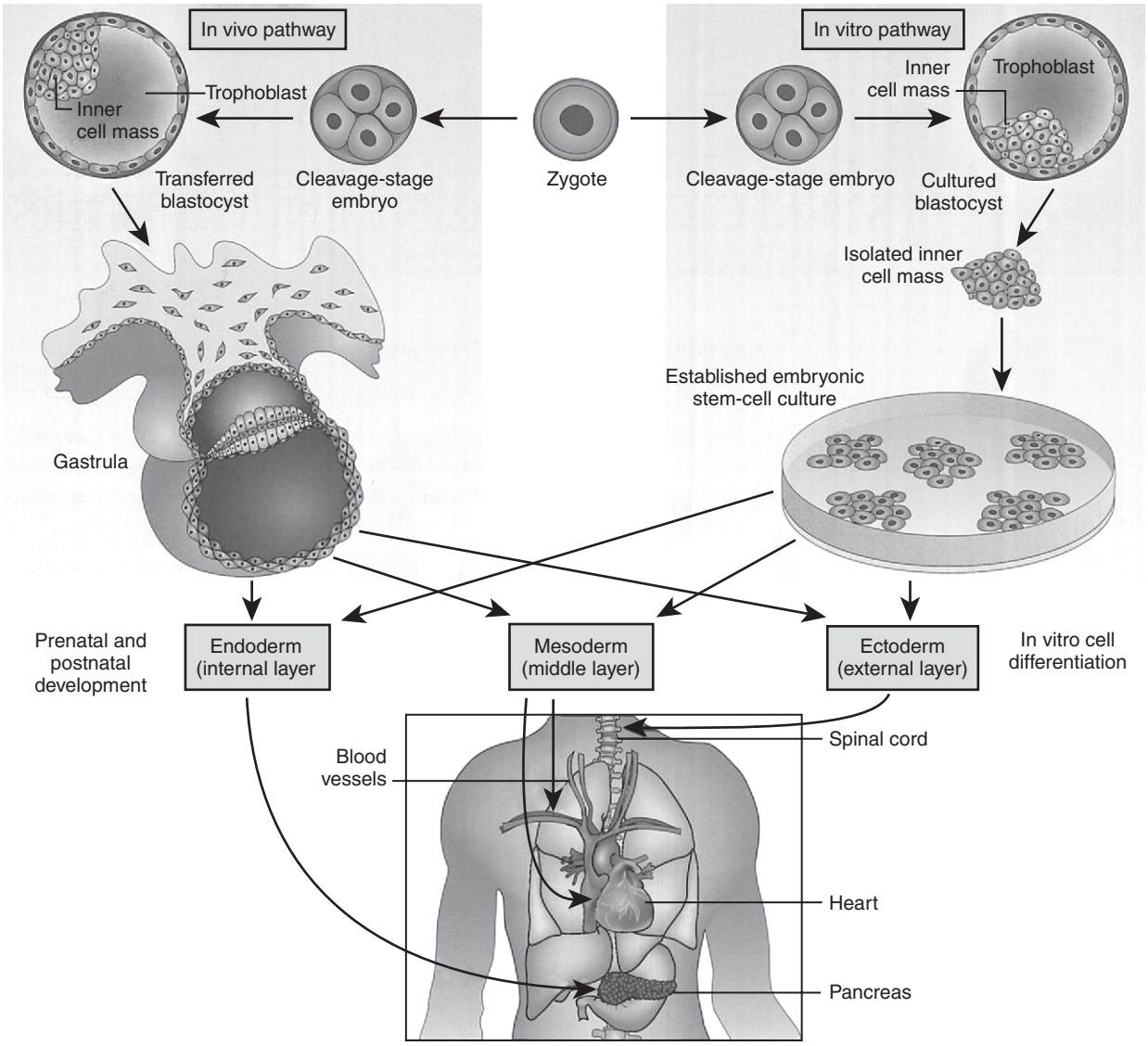


Figure 7-5 Alternative fates for an in vitro fertilized zygote: Intrauterine (in vivo) versus in vitro development. Either of two developmental pathways is possible for an embryo generated by in vitro fertilization: When an embryo is transferred to the uterus at the cleavage or blastocyst stage, its development can result in the birth of a child. When an embryo is cultured exclusively in vitro, it can result in the derivation of stem cells. These two divergent pathways may ultimately converge in the use of stem cells and their derivatives for cell-based transplantation therapies. In vitro fertilization was used first as a treatment for infertility, wherein the resulting embryos are transplanted to the uterus at the early cleavage or blastocyst stage. This in vivo pathway can result (in approximately one third of cases) in the implantation of the embryo into the wall of the uterus, where it undergoes gastrulation and subsequent prenatal development. Recent successes in the derivation of human embryonic stem cells (hESCs) have opened up a second, in vitro pathway for the development of human zygotes. Isolation and culture of the inner cell mass can result (in up to 50% of cases) in the prolonged growth of cells that have the capacity to differentiate into all three cell types of the body—endoderm, mesoderm, and ectoderm. Human ESCs have been shown to be capable of differentiating in vitro into insulin-producing cells, heart cells, blood vessel cells, and nerve cells, as have their mouse counterparts. Therefore hESCs are a promising source of tissue for transplantation for the treatment of, for example, diabetes, lung disease, cardiovascular disease, anemia and other diseases of the blood, and diseases or injuries of the central nervous system. *(From Bradley JA, Bolton EM, Pedersen RA: Stem cell medicine encounters the immune system,* Nat Rev Immunol *2:859-871, 2002.)*

or human.[68] Indeed, these capacities have come to be regarded as part of the operational definition of a somatic *stem* (as opposed to a *progenitor*) cell for any organ. For obvious reasons, most somatic stem cells cannot be tested for the important and defining attribute of reconstitution—for example, it is not possible to ablate the nervous system in the same way as for the hematopoietic system. Substantial lesions can be experimentally created in the nervous system, however. In such cases, NSCs have been found to reconstitute the missing parenchyma with a broad range of missing cell types.[45,69,70] As mentioned previously, the fate and potential of these somatic stem cells are restricted to those lineages that constitute the tissue or germ layer from which the cells were isolated. Surprisingly, the precise role for these tissue-resident stem cells in some solid organs is not yet entirely certain. For example, the existence of NSCs has been proved beyond any doubt, yet their purpose is still a matter of speculation. It

is presumed that these reservoirs of immature cells that persist into adulthood are designed to maintain a given organ's homeostasis by buffering the forces that cause cell death and dysfunction (toxins, senescence, infection, trauma) throughout the life of the organism by providing a small ready supply of "replacement" cells. If this is so, it is postulated (indeed, hoped) that their role can be exploited for even more extensive repair on demand. A growing appreciation is emerging that these pools of immature cells also restore a perturbed system to equipoise not solely by replacing missing cells, but also by providing molecular support for extant host cells (e.g., protecting and nourishing those cells and their connections or detoxifying the microenvironment).[71-73]

As noted previously, the possibility that a somatic stem cell from one organ or germ layer could "transdifferentiate" into cell types of another—as with NSCs yielding blood cells or blood-forming stem cells yielding neural or other solid organ cells—was a hotly debated topic.[74-79] More recent studies, however, have shown these initial conclusions to be due to an experimental artifact: the misinterpretation of the fusion of a donor stem cell with a preexisting host cell of another lineage.[78-84] For example, a transplanted blood cell, including its nuclear contents (often bearing a reporter transgene), would merge with a large host neuron (e.g., a Purkinje neuron), giving the misimpression that the neuron was of donor origin. Therefore, the notion of transdifferentiation has fallen into disrepute. Interesting developments in this field now revolve around whether fusion itself may play an adaptive biologic or reparative role. It appears that fusion between hematopoietic stem cells and nonhematopoietic cells is enhanced in the presence of injurious inflammation; under such conditions, the nuclear program of the host cell comes to supersede that of the donor hematopoietic cell while possibly coopting some of its genetic machinery.[85-87] This revisionist view of the process of cell fusion, which heretofore has simply been regarded as an experimental inconvenience, is now fueling new investigations into this area.[87]

In view of the recent reports[88-94] (discussed further on) that mature somatic cells may be dedifferentiated or reprogrammed experimentally in vitro to display pluripotence (through a combination of defined "stem"-ness and oncogenic genes), the possibility remains that an aberrant form of reprogramming may occur in vivo to account for the reports of transdifferentiation or lineage reassignment of somatic stem cells.[84] Such rare occurrences would need to be viewed as similar to (or at least as the first step in) metaplasia and neoplastic transformation.[95] Much more work is required in this regard.[96]

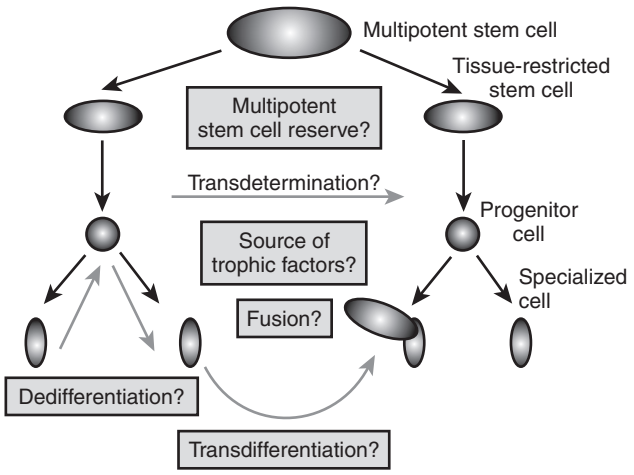


Figure 7-6 The six potential mechanisms in which adult stem cells can function in tissue repair: transdifferentiation, dedifferentiation, transdetermination, cell fusion, secretion of trophic factors, and the participation of true adult pluripotent stem cells in renewal and regeneration. *(From Rice CM, Scolding NJ: Adult stem cells—reprogramming neurological repair?* Lancet *364:193-199, 2004.)*

The allure of finding adult somatic stem cells that could change their lineage assignments in vivo—particularly cells that could be taken from the actual transplant recipient—was rooted in two concerns in the stem cell field that still prevail (the same concerns that have fueled a search for pluripotent cells in repositories other than the blastocyst): (1) avoiding the ethical minefield of obtaining pluripotent stem cells from preimplantation embryos and (2) obviating the potential for immunorejection of allogeneic grafts, which otherwise might require long-term immunosuppressive therapy or even such morally and technically thorny procedures as nuclear transfer (NT)—in lay parlance, "therapeutic cloning" (discussed later on). Of interest, in more recent reports, reprogramming the epigenome in vitro[88-94] (discussed later) appears to be a strategy with much more scientific credibility for approaching these dual problems.

An additional lesson to be learned from some of the misleading reports in the somatic stem cell field is that the expression of immunocytochemically detectable markers associated with a particular cell lineage is *not* sufficient to impute functional equivalence with that cell type. Additional evidence indicates that ostensibly cell type–specific markers can actually be expressed much more promiscuously than had been assumed, particularly when they are being assayed in systems in which their specificity was never tested. For example, a cell cannot be assumed to have become a neuron simply because it expresses a few neuron-associated markers; a cell is a neuron only if it has the functional properties of a neuron, which include electrical excitability and the ability to form synapses and spike action potentials.[82-84] Similarly, a cardiomyocyte must contract rhythmically, and a pancreatic beta cell must produce insulin in a glucose-regulated manner.

Germline Stem Cells

Spermatogenesis occurs throughout life in the mammalian male, yet oogenesis ceases before birth in most mammalian females.[97] In fact, isolation of spermatogonial stem cells from the adult murine testis[23] that may retain pluripotency has been reported. Such cells might be adult remnants of embryonic germ cells. Obviously, the possibility of harvesting cells with ESC-like properties from the adult human testis using minimally invasive techniques offers intriguing clinical opportunities.

The evidence for germline stem cells in adult murine ovaries remains more controversial.[98] Even more provocative are reports of a pool of cells within the bone marrow and in the peripheral circulation that may continually seed postnatal ovaries and contribute to ongoing oogenesis.[99] If such reports are accurate, the ability to isolate and expand bone marrow–derived germline stem cells may potentially be an important adjunct for infertility treatment. Unfortunately, other subsequent studies appear to disprove the existence of such bone marrow–derived stem cells.[100]

SOMATIC CELL NUCLEAR TRANSFER

Somatic cell nuclear transfer (SCNT), or "therapeutic cloning," is a potentially powerful technique that allows for the generation of autologous ESCs from adult somatic cells.[43] In SCNT, the nucleus from a mature somatic cell, such as a skin fibroblast, is transferred into an enucleated oocyte which is then stimulated to divide. The oocyte cytoplasm reprograms the nucleus of the somatic cell to begin reexpressing genes that mediate pluripotence and self-renewal. Furthermore, as this oocyte with this foreign nucleus begins to divide, it begins to develop along the normal pathways consistent with any preimplantation embryo,

including proceeding to the blastocyst stage from which ESCs may be derived from the ICM[43,101] (Figure 7-7). Because these ESCs possess the nuclear content of the donor skin cells—presumably obtained from the patient to undergo transplantation—the risk of immunorejection of the grafted ESCs is obviated.

It is important to differentiate the "therapeutic cloning" of SCNT from "reproductive cloning," which aims to implant the blastocyst into a womb and generate a complete organism that is genetically identical to the organism that donated the nucleus. In SCNT, only cell populations that are patient-specific are intended for diagnostic and therapeutic goals. It is illegal in virtually all countries to implant a human egg so manipulated. Although SCNT is routinely used for cloning farm animals, it remains a technical challenge using human eggs—particularly the steps of achieving an ICM and generating hESCs from it. SCNT using human material is extremely inefficient. Even in mice, the frequency of developmental abnormalities in subsequent clones can be high.[102] Despite initial misleading reports.[103-105] the generation of hESCs after SCNT remains unachieved. The hESCs that were reported were not clones of the nucleus donor but rather hESCs derived from parthenogenesis.[106] The recent report describing the derivation of ESCs from cloned primate embryos[107] suggests that success using human material, as technically daunting as it appears, may be on the not-too-distant horizon.

Of interest, recently published more tractable alternative techniques for reprogramming the nuclei of somatic cells using defined genes, rather than the undefined contents of the cytosol of an unfertilized oocyte, may sideline continued efforts to achieve human SCNT.

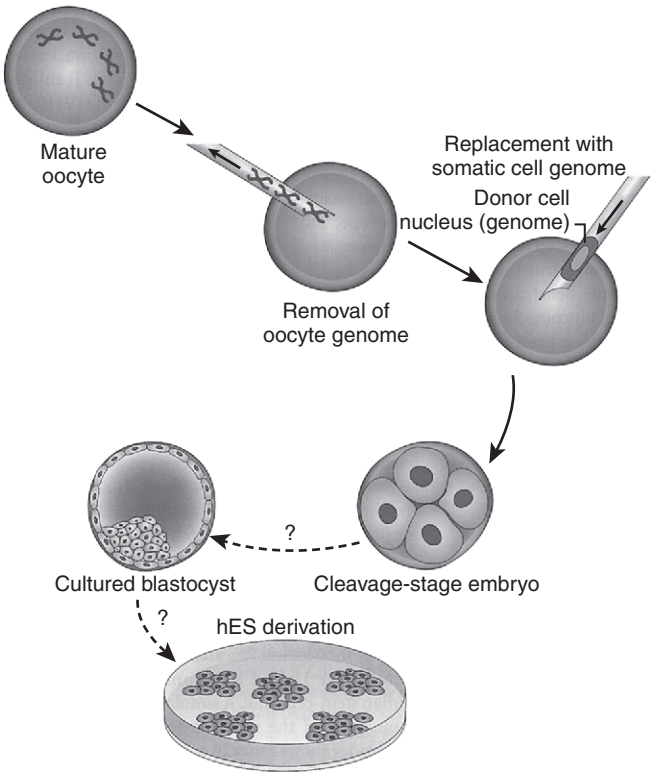


Figure 7-7 Genomic replacement (somatic cell nuclear transfer) as a way of matching stem cell–derived tissues with their intended recipient. Replacing the genetic material (genome) of an oocyte with that of a somatic cell generates a near-perfect match between the resulting donor cells and the graft recipient (donor of the somatic cell genome). For this purpose, the oocyte genome is removed and replaced with the nucleus of a cell from any one of several possible somatic (body) tissues of the graft recipient, using a micropipette. The oocyte cytoplasm enables the donor genome from a somatic cell to support embryonic development by a poorly understood process known as reprogramming. The resulting embryo is cultured to the blastocyst stage, from which embryonic stem cells are derived. Of note, owing to minor histocompatibility antigens encoded by the DNA of egg-derived mitochondria, grafts derived from stem cells grown from such embryos might not be strictly identical to the graft recipient. *hESC,* Human embryonic stem cell. *(From Bradley JA, Bolton EM, Pedersen RA: Stem cell medicine encounters the immune system,* Nat Rev Immunol *2:859-871, 2002.)*

NUCLEAR FUSION AND REPROGRAMMING ("INDUCED PLURIPOTENT STEM CELLS")

Although SCNT may be the best-studied modality for reprogramming the nucleus of an adult somatic cell to a embryonic pluripotent state, a number of other techniques for nuclear reprogramming are being actively investigated, many less technically challenging.[108-110]

Fusion of a somatic cell with an ESC generates a tetraploid hybrid that has the molecular characteristics and biologic behavior of a stem cell.[111] Soluble factors from the ESCs have the aforementioned ability to reprogram the nucleus of the somatic cell (i.e., to induce its reversion to a pluripotent embryonic state). The identity of these factors is being investigated. The transcription factor NANOG probably plays a prominent role in mediating this action.[112] A systems-based analysis of differential gene and proteomic expression and epigenetic (e.g., methylation) status is likely to be helpful in identifying other factors important in such nuclear reprogramming (Figure 7-8).[113-116]

As noted earlier, although undefined factors from an unfertilized oocyte cytosol or from the cytoplasm of an hESC appear to provide the benchmark for nuclear reprogramming, the recent identification of a set of four genes and their gene products that may do so as effectively has captured the imagination of the stem cell field and is likely to define research efforts for the next few years.

From a list of 24 genes found to be highly differentially expressed between differentiated and undifferentiated hESCs, an

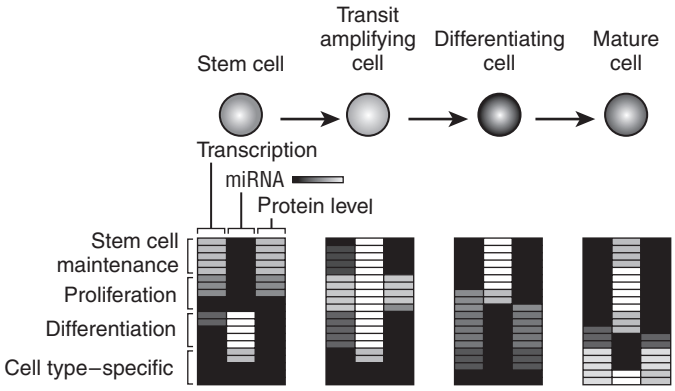


Figure 7-8 Epigenetic control mechanism, in this case microRNA (miRNA)–mediated suppression, acts in conjunction with differential transcription of genes to fine tune the expression of proteins responsible for maintenance of pluripotency, cell proliferation, and differentiation. Expression of selected genes is activated or suppressed by mechanisms such as promoter methylation and miRNA; the pattern of expression of these genes is implicit in the determination of cell fate. *(From Cheng LC, Tavazoie M, Doetsch F: Stem cells: From epigenetics to microRNAs,* Neuron *46:363-367, 2005.)*

empiric trial-and-error unbiased approach identified a combination of four from that list were sufficient to "turn back the clock" on first murine and then human somatic cells (adult, neonatal, and fetal skin fibroblasts; gastric epithelium; hepatocytes).[88-92] The four genes are *Oct3/4, Sox2, c-Myc,* and *Klf4*, introduced into fibroblasts using retroviral or lentiviral vector–mediated gene transfer under ESC culture conditions. Because the somatic cells not only expressed genes associated with pluripotence but could also form teratomas and EBs and self-renew, they were called *induced pluripotent somatic cells* (iPSCs or iPCs). When compared with ESCs, iPSCs appear to evince a similar developmental potential, gene expression pattern, and epigenetic profile.[89] Other "rejuvenating" cocktails are being devised independently by various investigators—for example, *Oct3/4, Sox2, NANOG,* and *LIN28*.[91] Of note, certain factors appear to remain common across reports and are therefore likely to be necessary; others may have actions that are redundant with those of other genes. In some cocktails, genes associated with neoplastic transformation, such as c-Myc, play a prominent role. Efforts to eliminate that gene, as well as others that may potentially have undesirable consequences, are ongoing and seem promising,[91,92] although Myc may add a degree of efficiency not seen in its absence. (The continuum between "normal" reprogramming and oncogenesis is highlighted by these types of studies.) Of interest, it appears that long-term expression of these factors is not required, which suggests that transient, non–virus-mediated provision of the proteins to the somatic cells may be sufficient to trigger resetting of the genome and epigenome. Subsequently, improvements in our understanding of the molecular basis of reprogramming has led to much more efficient techniques and some which do not rely on viral vectors.[117-120] In addition, several recent studies have highlighted the tremendous potentials of iPSCs as faithful disease models for unprecedented appreciation of pathophysiology and ideal test platforms for molecular correction of the underlying genetic defects.[121-125] Significant progress has been made since the initial descrition of reprogramming technique by Yamanaka and Blau,[120] as demonstrated by recent reports of iPSC generation from terminally differentiated T cells from the peripheral circulation.[125,126] These findings open up tremendous possibilities with such a ready source as peripheral blood for iPSC production. Another recent report demonstrated direct reprogramming of fibroblasts into functional cardiomyocytes, thereby showing practical and functionally directed instead of ontogenically directed reprogramming is possible.[126] Along with the promising potentials of reprogramming also comes new questions and cautions highlighted by reports showing prolonged epigenetic memory and influence of cell type of origin on the functional properties of the iPSCs.[127-128] In summary, much progress has been made in understanding the molecular mechanisms and technical advances in iPSC production yet many unanswered questions remain. Nevertheless, the tremendous stride of scientific and technical advances in this field bode well for its clinical translation.

These technologic advances not only will provide intriguing insights into development but also hold significant implications for the generation of patient-specific pluripotent cells for the study of the underlying mechanisms of different diseases, as well as for individualized therapeutics. Murine iPSCs already have been shown to be efficacious in treating a mouse model of sickle cell anemia[93] and a rat model of Parkinson disease.[94] Among the challenges for the iPSC strategy are making the reprogramming process more efficient and eliminating the neoplastic risk (tumors are prevalent in some animals receiving iPSC transplants).

CANCER STEM CELLS

Cancer stem cells are the malignant equivalent of normal somatic stem cells. They have the ability to maintain quiescence yet undergo symmetric division for self- renewal to sustain the growth and propagation of a tumor, as well as asymmetric division to repopulate the heterogeneous tumor cell population within the neoplasm.[129-133] The cancer stem cell, however, is autonomous and not governed by the intrinsic regulatory controls of normal stem cells, nor is it responsive to the controlled growth signals emanating from the local microenvironment. To date, cancer stem cells have been isolated principally from preexisting solid tumors (e.g., brain tumors) and appear to be responsible for maintenance of the malignancy and therefore tumor relapse.[131,132] It remains uncertain whether similar cells also are the originators of the tumor in the premalignant state, although the notion of a "normal stem cell gone bad" is appealing. The premorbid state of solid organ tumors is being investigated to address this possibility. In some leukemias, an aberration of a hematopoietic progenitor seems a likely initiator.[133]

Although the cancer stem cell—certainly in the morbid state and perhaps even in the premalignant state—would be a very desirable therapeutic target, its scarcity, growth characteristics, and ambiguous identifying characteristics make it elusive. A key achievement will be identification of a molecular marker that distinguishes, with sensitivity and specificity, cancer stem cells from normal somatic stem cells.[131]

POTENTIAL CLINICAL UTILITY OF STEM CELLS

Replacement Cell and Gene Therapies: Neural Stem or Progenitor Cells as a Prototype

NSCs, by definition, have the potential to undergo self-renewal (by both symmetric and asymmetric division) and to generate intermediate and mature cells of all glial and neuronal lineages throughout the neuraxis and at all stages of life.[60,134-136] NSCs can populate a region during its initial development or repopulate it after injury or disease. Although NSCs can migrate extensively within the CNS, they have a particular predilection for areas of pathology.[137] When the NSC engages the area of injury or developmental niche, it will respond specifically to local microenvironmental cues and alter its fate accordingly.[58,60]

More often than not, pathologic processes in CNS disease are widespread—for example, as seen in inherited and adult-onset neurodegenerative conditions, diffuse axonal injury after trauma and ischemia, inflammatory conditions, and brain neoplasms. These disorders present a clinical challenge because of their diffuse nature as well as the intermingling of diseased cells with normal brain cytoarchitecture. Targeting such a wide and complex terrain typically has been the province of pharmaceutical rather than transplantation-based therapies. Unfortunately, restorative and regenerative actions are beyond the capabilities of known pharmacologic agents. The properties of the NSC, however, make it uniquely suited to such interventions.[60,138] Transplanted NSCs have the capacity to migrate to developmental or diseased "post-developmental" niches in the mammalian (including primate) CNS and then integrate in an appropriate fashion.*

As proof of concept, unmodified and engineered NSCs have been implanted into several disease models. For example, when implanted into a globally dysmyelinated mouse brain, NSCs of both mouse and human origin will yield myelinating cells[30,142]; indeed, the implanted NSCs shift their fate to yield a greater proportion of myelinating oligodendrocytes than NSCs would in a wild type environment. Implanted mouse or human NSCs can similarly distribute missing gene products throughout the brain; for example, they can effect histologic and functional changes in the mucopolysaccharidosis type VII (MPS VII) and Sandhoff mouse models of defective lysosomal processing and storage[44,47]

*References 46, 60, 69, 134, 139-141.

performing the equivalent of a "bone marrow transplant to the brain." In an adult rodent neocortex with selective regions of neuronal apoptosis and in the substantia nigra of parkinsonian monkey, a multipotent NSC will shift its developmental fate only within those narrow neuron-deficient regions to yield pyramidal neurons or dopaminergic neurons, respectively, outside the normal temporal window of that cell type's birth.[60,73]

Many other examples of repopulation by multipotential NSCs of a diseased, traumatically injured, or developmentally deranged CNS have been reported.[71,143,144] In addition to their use for cell replacement, NSCs can be engineered to deliver therapeutic genes and viral vectors throughout the CNS.[45,72,145] Because NSCs have a strong tropism for sites of pathology, they can be quite effective in that role. For example, this ability has made them a potentially valuable adjunct in the treatment of aggressive disseminating gliomas if armed with oncolytic gene products.[145,146] The NSCs can track down even distantly invading glioma cells and deliver therapeutic genes directly to these widely dispersed and infiltrating neoplastic cells. Indeed, it was the pathotropism of exogenous NSCs[69,145] (including for brain tumors) that prompted some investigators to look for similar attributes in endogenous neural progenitors; such "glioma-tropic" behavior in pools of endogenous cells was detected,[147] helping to vouchsafe the representative nature of the implanted NSCs and their value for revealing fundamental NSC properties.

Apropos to a major problem in perinatology and neonatology, both exogenous and endogenous NSCs appear to be useful in attempting to reconstitute cerebral tissue compromised by hypoxic-ischemic insult.[69,70,134]

Finally, it has been recognized that NSCs—even when they do not differentiate into replacement cells—appear to have, even in their undifferentiated state, an intrinsic ability to rescue or protect degenerating host neurons as a result of complex host–stem cell cross-talk and reciprocal interactions.* This so-called chaperone effect has become a powerful force within the stem cell field and probably is the most tractable near-term application for stem cells against a variety of neural disorders, with extrapolation to stem cells and diseases of other organ systems (including heart, pancreas, lung, and bone).

In short, NSCs (a prototype for the somatic stem cell) possess biologic attributes that allow their use in the treatment of various diseases by a variety of mechanisms, all of which are components of fundamental stem cell biology.[148] It bears emphasizing that, to realize the therapeutic potential of pluripotent stem cells such as the ESC, they need to be directed to become somatic-like stem cells; then they too will evince the beneficial properties described here. Some of the potential uses for hESCs in cardiac cell replacement therapy have been reviewed elsewhere.[149]

SUMMARY AND FUTURE DIRECTIONS

Stem cells have the capability to undergo extensive self-renewal and to differentiate into committed progenitor cells and mature specialized cells of multiple organ systems. Stem cells can be isolated from the mammalian embryo and populations expanded in vitro. These ESCs are quite proliferative and have the capability to yield cells of all tissues of the organism. ESCs then give rise to somatic (tissue-derived or tissue-resident) stem cells, which also can be used as a source for lineage-specific cell replacement therapy. It remains to be determined how the degree of plasticity of these types of stem cells changes as the organism ages: Do vestigial embryo–like or tissue-resident stem cells persist throughout life in various reservoirs throughout the organism? Can they be isolated and used with equal efficiency, efficacy, and safety at all stages? Are the barriers between cell lineages rigid and inviolate? Many of these questions are beginning to be answered. Recent advances in understanding the biology of stem cells as well as the molecular controls of cellular pluripotency have significantly extended current understanding of the fundamental biology, with obvious practical implications. For the present, the most useful solid organ stem cells have been those derived from the ICM of the blastocyst and from embryonic and fetal tissues. Stem cells, endowed with their special biologic characteristics, also provide an invaluable tool for the study of developmental cell biology, as well as serving as cellular models of disease (for the elucidation of new pathophysiologic mechanisms and the identification of novel drug targets and diagnostic or prognostic markers). Indeed, any therapeutic use of stem cells should be viewed as "translational developmental biology."[150]

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8 Homeobox Genes

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In 1894 Bateson coined the term "homeosis" to describe inherited mutations in *Drosophila melanogaster* in which significant alterations of morphogenesis were observed.[1] Evidence that these alterations were the result of single-gene mutations led to the definition of homeotic genes—genes that control the formation of the body plan. With the development of gene cloning, these genes were among the first to be defined and sequenced. Early studies showed that homeotic genes are transcription factors, many of which contain within the gene a conserved 180–base pair sequence. This internal 180–base pair sequence codes for a 60–amino acid DNA binding region within the resulting protein. The protein binds to DNA, allowing the transcription factor to regulate the expression of other genes. This specific conserved region is known as the *homeobox.* Genes containing the homeobox are therefore called homeobox genes (reviewed by Gehring[2]). Generally, homeobox genes are expressed in cells of mesodermal origin, but expression of certain homeobox genes in cells of epithelial origin also has been reported.[3-5]

The discovery that the homeobox is found in many genes throughout the vertebrate kingdom created intense interest in the role of homeobox genes as master control genes of many aspects of embryonic development. A large number of *Drosophila* homeobox genes have mammalian counterparts that frequently are closely conserved in their gene or protein sequence relative to the *Drosophila* "parent" gene. Generally the homeobox portion of the gene is either 100% or very close to 100% conserved in the DNA or the amino acid sequence. Additional mammalian genes containing a strongly conserved homeobox sequence but with no counterpart in *Drosophila* also have been discovered. Well over 100 homeobox genes have been identified in humans; this number has been estimated to represent only half of all such genes within the human genome.[6] Thus homeobox genes may account for some 0.5% of the approximately 30,000 genes in the human genome. In this era of the Human Genome Project, studies using gene array technology are now uncovering evidence for a wider role for homeobox genes in development than has been previously appreciated.[3,7-9] Studies provide evidence of more complex combinatorial expression patterns within the establishment and maintenance of mature cell phenotypes, and that alterations in these combinatorial patterns may underlie the development of diseases.[9-11]

Homeobox genes function as regulators active in the development of embryonic cell fate and organ morphogenesis of all vertebrate species. Most studies of mammalian homeobox genes have been done in mice because of the ability to manipulate and study the mouse genome; however, available evidence indicates conservation of function across diverse species. Homeobox genes can be classified into two large groups: the *clustered* group, called *Hox* genes, located in specific regions on certain chromosomes and the *diverse* group of homeobox genes that are dispersed throughout the genome but are similar to the clustered group in genomic organization and conserved sequences. Some homeobox genes in the diverse group also exist in clusters, such as the so-called *Para-Hox* cluster that contains *Gsh1, Pdx1,* and *Cdx2.* Others can be grouped into families on the basis of sequence similarities outside of the homeobox, such as the *Nkx* family of genes, or their similarity to other *Drosophila* homeobox genes, such as the *Pax* family. Because of the large number of homeobox and homeobox-like genes and the affected developmental processes, this chapter focuses on the role of *Hox* genes and other specific homeobox genes in relation to development of foregut organs and limb, renal, and vascular development.

The unique and interesting genomic and genetic characteristics of the *Hox* genes have received particular attention from developmental biologists. In *Drosophila* the eight *Hox* genes are organized in a specific cluster termed the HOM-C complex, where they are arranged according to their spatial and temporal expression patterns during larval development (Figure 8-1). In the HOM-C complex, the *Hox* genes are organized on the *Drosophila* chromosome from 3′ to 5′ corresponding to their anterior (rostral) to posterior (caudal) larval expression pattern (spatial colinearity) and the timing in larval development when expression begins (temporal colinearity) (reviewed by Coletta and associates[12]). Although identical to each other within the homeobox region, the *Hox* genes also possess sequence similarities outside of the homeobox region that are important for Hox gene interactions with other transcriptional regulatory proteins. The degree of similarity is related to the proximity of genes within the cluster. Hox genes are expressed with a definitive anterior boundary. The level of this boundary is progressively more caudal for each successive gene in the cluster going in the 5′ direction.[13,14] *Hox* genes act to define specific body segments of the developing *Drosophila* larvae by the process of posteriorization, in which the most 5′ gene expressed in a given body region actively identifies that region as more posterior than the region just rostral to it. This *posterior dominance* explains why, in the presence of overlapping expression, one gene is instructive in determining body segment identity.[15] Within a given area, however, overlap in expression of the primary proximal gene with more distal genes is fairly common. Such overlap allows the potential for modification of tissue-specific or organ-specific action of the primary proximal gene through *Hox* gene interactions. In fact, two different studies—of a mouse model of the VATER Association and of humans with Currarino syndrome (which involves anomalies of the sacral and anorectal structures)—have shown that combinations of homeobox genes are important for normal structural development.[16,17]

Mutations causing ectopic expression of individual *Hox* genes result in altered body segmental identity (homeotic transformation), producing seemingly bizarre deformities in *Drosophila.* Such mutations "posteriorize" an anterior segment through ectopic expression of a posterior gene in an anterior position.[6] Classic examples of posterior homeotic transformation are represented by the Antennapedia and Ultrabithorax mutations. In the Antennapedia mutant, the *Antennapedia* gene is misexpressed in a more rostral position, causing identification of part of the head structure as mesothorax, such that the fly grows a leg instead of an antenna from the head. In the Ultrabithorax mutant, ectopic expression of the *Ultrabithorax* gene in the second thoracic segment redefines this segment as a duplication of the third thoracic segment, and the fly grows a second pair of halteres, instead of the wings that would normally develop from this body area. It is important to recognize that *Hox* genes and the other homeobox genes do not actually code for specific body structures. Antennapedia, for example, does not code for a leg. Rather, these genes

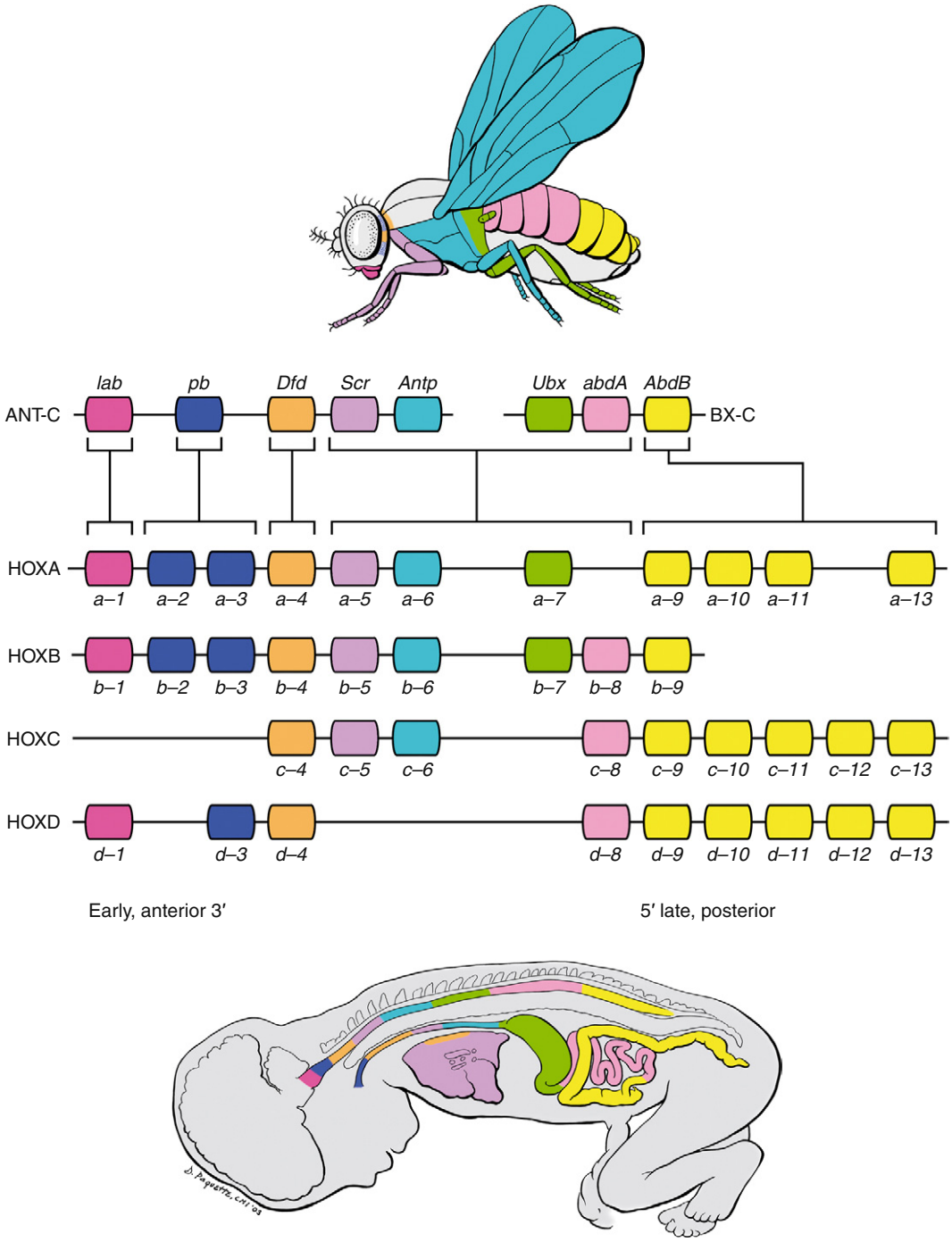


Figure 8-1 Relationship of the *Drosophila* HOM-C homeobox gene cluster and the mammalian *Hox* complex. Genes are depicted in their 3' to 5' organization on the *Drosophila* and each of the mammalian chromosomes. *Missing boxes* on the mammalian chart reflect that the specific gene is not present within that cluster. *Specific patterns* denote the mammalian genes paralogous to their *Drosophila* counterparts. Patterns also illustrate in the adult fly the body regions affected by expression of that specific *Hox* gene during embryonic development. In general, these regions correspond to the most anterior area of expression of that gene in the embryo. Loss-of-function mutations alter embryonic morphogenesis, resulting in defects of that particular body region. An exception is the pattern of the *pb* gene, which is *stippled* to indicate that although this is the most anterior region of expression in the embryo, no apparent phenotype for this region when the gene is mutated. Patterns in the human infant similarly indicate the anterior region in which that group of paralogous genes is expressed during embryonic development. For simplicity, only the nervous system, lungs, and intestinal tract are represented. These are not the only systems in which Hox genes are important. Data for the human infant are derived mostly from studies of mouse embryogenesis. *(Figure drawn after data and charts in Roberts DJ, Johnson RL, Burke AC, et al: Sonic hedgehog is an endodermal signal inducing BMP-4 and Hox genes during induction and regionalization of the chick hindgut,* Development *121:3163-1374, 1995; and in Coletta et al, 1994[12]; McGinnis and Krumlauf, 1992[68]; Mavilio, 1993[32]; Mark et al, 1997[6]; and Bogue et al, 1996.[41] © 2003 by Darisse Paquette, CMI, and Heber C. Nielsen, MD.)*

act as master control genes, giving specific embryonic cells positional and spatial cues that define their regional identity and fate and regulating the expression of genes that do code for the development of structures specific to the region defined by that homeobox gene.

In mammals, the *Drosophila* HOM-C gene cluster was duplicated onto four separate chromosomes and is called the HOM-C cluster. Although the genes are located on separate chromosomes, the unique organization and expression patterns, including spatial and temporal co-linearity, are completely retained (see Figure 8-1). Just as in *Drosophila,* the order of 3′ to 5′ chromosomal organization corresponds precisely with the anterior limit of gene expression within the embryo somite stage and ultimate organ structural development.The more 3′ the chromosomal location, the more rostral is the anterior limit of gene expression.[18] The mammalian *Hox* genes retain identity (homology) of the homeobox region at the amino acid level and near or complete identity at the DNA level. Chromosomal order within the mammalian clusters is identical to that of the *Drosophila* complex, allowing the classification of genes within the HOX-C complex as homologues of their *Drosophila* counterpart. Counterpart genes in each of the four complexes—for example, *Hoxa4, Hoxb4, Hoxc4,* and *Hoxd4*—are termed paralogous genes, or paralogs. Of great interest is that the HOX-C complex genes also retain characteristics of expression and apparent function of their *Drosophila* homologues.Thus body segments that are likely to be controlled by mammalian *Hox* genes are similar to the segments controlled by their corresponding *Drosophila* counterpart.[12]

Homeobox genes, in particular the Hox group, appear to exhibit three phases of expression during mammalian development.[19] First is expression in the very early embryo at gastrulation, which is likely to induce initial patterning.The second phase is during organ morphogenesis, with organ-specific expression that helps establish both organ structure and development of specific cell types. The third phase may begin in late fetal development or later in postnatal life. Expression generally is limited to specific cell types within an organ, such as the insulin-producing beta cell of the pancreatic islets. Evidence is increasing, however, that the specific cell type within an organ expressing a particular *Hox* gene may change over time during development. The cellular expression appears necessary to maintain a differentiated functional phenotype of the particular cell. During these phases of expression, *Hox* genes appear to have two distinct functions. One involves *homeosis,* whereby *Hox* genes impose a certain fate on groups of cells in one body region, a function active predominantly during initial patterning of the embryo but maintained throughout life.The ability of progenitor cells to "remember" or maintain homeobox gene activity appears to be unique to homeobox genes and allows these genes to provide segment identity to an organism's body plan.The other function, probably active during organogenesis and in postnatal life, is to serve as "realizator" genes or controlling genes that affect other realizator genes. Realizator genes directly influence a cell's specific identity and activity through processes of cell adhesion, cell division, survival, and apoptosis—events that control tissue and organ shape.[20,21] Homeobox genes regulate the process of transdifferentiation of specific precursor cells to a different cell type, an important event in morphogenesis.[22] In many instances the homeobox genes are subsequently down-regulated with further maturation, posing a significant biologic question: How are homeobox genes down-regulated in a development-specific fashion? A study of *Hoxb5* in liver development showed one mechanism, that of progressive gene-specific and tissue-specific methylation with advancing development.[23] More information is needed on this issue to elucidate further the mechanistic controls of organ and cellular identity and function.

Various knockout and transgenic mice models have been created to identify the developmental function of many HOM-C and other homeobox genes. Compared with the dramatic homeotic alterations caused by mutations in *Drosophila,* the results in mammals often have been surprisingly much more modest, especially in view of the significant developmental role these genes are believed to play. Knockout models of individual *Hox* genes frequently exhibit changes in vertebral body identity (for example, transformation of a cervical vertebra into a thoracic vertebra), changes in rib number or identity, and shoulder girdle alterations.[13] The reason for such comparatively restricted homeotic changes is unclear but may relate to the fact that the *Hox* genes are reproduced four times in mammals. This redundancy may allow paralogs or homologues of the targeted gene to replace some of the missing function, allowing a more normal morphogenesis.[24] This explanation is consistent with the proposed role of the HOM-C genes. In other words, nature has provided multiple backup strategies to allow normal development. Indeed, studies in which multiple *Hox* genes have been targeted show more significant homeotic changes in the developing mouse.[25-28] Thus the rule of posterior dominance used in *Drosophila* to determine which *Hox* gene is instructive within a particular segment becomes more complex in mammals. In addition to posterior dominance, it is likely that combinatorial and gradient rules influence gene dominance, a feature called *quantitative colinearity.*[25] Gene ablation studies in mice show that the phenotypic severity of loss of one *Hox* gene is amplified in combined ablation models that feature defects in two or more *Hox* genes.[27,28] Overall, these studies suggest that *Hox* genes help set up morphogen gradients in the developing organism and in developing organs to influence which combination of genes are active for development of body segments and organ regions.[20,29,30]

Of interest to those caring for the human fetus and newborn is the fact that many homeobox genes, and *Hox* genes in particular, are stimulated by retinoic acid.[31,32] Retinoic acid stimulation of *Hox* gene expression preserves temporal colinearity (the more 5′ *Hox* genes require progressively longer exposure times for stimulation) but spatial colinearity can be violated. Retinoic acid may induce *Hox* gene expression in body regions and cell types where they are not normally expressed. An in vitro example of this phenomenon in lung tissue is described further on. Alternatively, lack of appropriate retinoic acid concentrations at critical times in the developing fetus and newborn also can cause developmental defects.This is shown by an example in rats where vitamin A deficiency during the first 10 days of a 21-day gestation leads to inappropriate expression of certain *Hox* genes in more posterior regions of the embryo, where their expression normally would be repressed. In the example given, this *anterior transformation* led to anterior homeotic transformations of the vertebrae in the cervical to lumbosacral regions.[33] With current interest in the potential use of retinoic acid to assist maturation or effect repair from injury in the premature human infant, it is clear that careful and thorough examination of developmental effects must be made before such treatment should be clinically tested. Many of the teratogenic effects of retinoic acid are postulated to be mediated by induced *Hox* expression.

Much of the work on HOM-C genes has focused on development of the nervous system, because this organ system requires spatial identification, patterning, and determination of cell identity and fate that are unique to different somite regions.The anterior-posterior expression gradients of each of the four HOM-C gene clusters are relatively easily demonstrated within the developing neural system, and the importance of these genes for normal nervous system morphogenesis and development is becoming well established.[34,35] The development of other embryonic structures, however, also requires spatial identification, patterning, and determination of cell identity, for example limb morphogenesis and the development of the embryonic foregut into different structures including the lung, stomach, intestinal tract, liver, and

pancreas. In these systems the unique expression features of the HOM-C gene clusters are similarly evident.

The focus of this chapter is on the role of HOM-C and other homeobox genes in determination and morphogenesis of the foregut-derived organs, and in formation of the limbs, kidney, and cardiovascular system. This emphasis does not mean that more is known about these developing systems, or that development of these systems is more heavily dependent on homeobox genes. Rather, these systems are of particular interest to neonatologists because many neonatal diseases involve foregut-derived organs, as well as limb, kidney, and cardiovascular system. An understanding of how these developing systems are controlled may provide insights into development of new therapeutic strategies. In addition, more general information is presented on human diseases either caused by or associated with misexpression of homeobox genes.

HOMEOBOX GENES AND FOREGUT AND HINDGUT DEVELOPMENT

Lung

The embryonic lung develops as an evaginating diverticulum from the embryonic foregut. The diverticulum then becomes bilobate to form the early right and left lungs. Branching morphogenesis, driven by a number of growth factor signals and regulatory molecules, establishes the respiratory tree. The initial branching of the right and left lung primordia involves fibroblast growth factor family members (FGFs). The development of continued branching involves a complex interaction of FGFs and morphogenetic proteins such as Sonic hedgehog and bone morphogenetic protein-4 (BMP4)[36] (reviewed by Warburton and co-workers[37]). The complex pulmonary structure is populated by more than 40 different cell types arising from foregut, endoderm, and surrounding mesoderm. This complexity of structure and cell determination and differentiation clearly requires master control genes to establish positional identity and cell fate. Thus homeobox genes are prime candidates for significant roles in many aspects of lung formation, including a role in modulating the effects of other regulatory pathways, including but not limited to members of the FGF and BMP families, in the developing lung.

Early surveys of individual Hox gene expression in mouse embryos using in situ hybridization revealed expression of a few genes in the mesenchyme of developing lung, especially of *Hoxa5* and *Hoxb5,* homologues of the *Drosophila Scr* ("sex combs reduced") gene.[38,39] Among other functions in the *Drosophila* larva, *Scr* identifies the pharyngeal and upper respiratory region. Several groups of investigators interested in lung morphogenesis are focusing on these Scr paralogs and their close neighbors within the HOM-C complex in lung development. Bogue and co-workers surveyed *Hox* genes expressed in developing mouse lung using polymerase chain reaction (PCR) with degenerate primers.[40] They found 15 *Hox* genes, with *Hoxa5* and *Hoxb5* most prominently expressed. Many of these genes were stimulated by retinoic acid treatment in vivo. In another study using in situ hybridization, Bogue and associates evaluated the temporal and spatial expression of *Hoxb* cluster genes along the foregut just proximal and distal to the developing lung primordium, as well as within the developing lung itself.[41] *Hoxb3* and *Hoxb4* showed anterior expression boundaries in the foregut proximal to the position of the budding lung diverticulum, and *Hoxb5,* just at the position, in agreement with the general principles of proximal-distal patterning and overlapping expression of *Hox* genes. Within the lung, anterior-posterior expression gradients also were observed, with *Hoxb3* and *Hoxb4* expressed in mesenchyme underlying tracheal, proximal, and distal airway structures, but Hoxb5 only in mesenchyme of distal airway structures and in the pleura. Curiously, Hoxb2 also was expressed in distal lung mesenchyme, so in this respect spatial colinearity was not completely conserved.

Hoxb5

Earlier studies by Wall and associates showed that the fetal organ with highest *Hoxb5* protein expression was the spinal cord, followed closely by the lung.[42] Volpe and colleagues identified the developmental expression of *Hoxb5* protein in embryonic mouse lung.[43] Expression increases during branching morphogenesis, becoming maximal at day 15 (female lung) and day 16 (male lung), declining afterward to become very low at term and only minimally detectable in adult lung. The importance of the observed male-versus-female difference in timing of peak expression is unclear. Structurally, Hoxb5 protein was located diffusely in mesoderm during branching morphogenesis, then became localized to mesenchyme underlying conducting airways, especially at apparent developing branch points. Of interest, at late gestation Hoxb5 protein was also seen in occasional columnar epithelial cells. These findings in mouse lung development have been confirmed in human lung development with maximal expression in the pseudoglandular phase of lung morphogenesis; waning expression and localization to mesenchyme under conducting airways and at branch points in the canalicular stage; further decreasing to occasional mesenchymal cells in the saccular and alveolar stages; and no apparent expression in the adult lung.[44] The function of *Hoxb5* was studied in vitro using embryonic lung cultures. Overexpression of Hoxb5 protein (using retinoic acid stimulation) and knockout of *Hoxb5* (using antisense oligonucleotides or siRNA molecules) each altered the development of branching morphogenesis. Knockout in the early or mid-pseudoglandular period during active airway branching morphogenesis produced reduced proximal branching, and overexpression resulted in elongated proximal airways and reduced ability to form distal branches.[45] Additional studies in mouse lung organotypic cultures showed that growth factors and hormones that prematurely induce the differentiation of distal airway epithelium also significantly reduce expression of Hoxb5 protein in distal lung mesenchyme.[46,47] Dihydrotestosterone, which stimulates branching morphogenesis and delays lung maturation in male fetuses,[48,49] also up-regulates Hoxb5.[50] Some of these effects of Hoxb5 may be caused by Hoxb5 regulation of cell adhesion molecules and spatial expression pattern of FGF-10. Additionally, in those experiments Hoxb5 down-regulation affected expression of Hoxb4 and Hoxb6, suggesting that Hoxb5 may contribute to the lung phenotype for *Hoxb5* by cross-regulation of other *Hox* genes.

Results from the overexpression studies suggested a relationship of *Hoxb5* to congenital lung malformations. Studies of bronchopulmonary sequestrations and congenital cystic adenomatoid malformations show significantly increased Hoxb5 expression underlying the abnormal airways, which persisted in tissue removed at surgery during infancy.[44,45] It remains to be determined whether this abnormal persistence of high Hoxb5 protein expression is causal to the development of these lung malformations or whether some other signal initiated the formative process and Hoxb5 expression participated in determining the structural development of the already initiated malformation.

In a mouse model of pulmonary hypoplasia with congenital diaphragmatic hernia (CDH) that mimics human pulmonary hypoplasia with CDH, the Hoxb5 protein expression pattern at term resembles that at an earlier developmental stage, suggesting that modified Hoxb5 expression also may contribute to abnormal lung development in this condition.[51] In animal models of CDH in which in utero tracheal occlusion was used to accelerate lung development, Hoxb5 protein levels are prematurely down-regulated, suggesting that duration and timing of tracheal occlusion are important to modulate lung growth versus lung maturation.[52]

Hoxb2, Hoxb3, Hoxb4, and Hoxb6

Although *Hoxb2, Hoxb3,* and *Hoxb4* genes are expressed at critical time points in lung development and in specific spatial distributions, gene knockout studies involving abnormal expression

of one or more of these *Hox* genes have either not described or not evaluated lung structural or functional changes.[40,53-55] Lung abnormalities in these experimental studies remain unknown, partly because of embryonic death before birth to onset of lung development and the lack of research studies conducted to date on the mutant mice. Mice expressing mutant alleles of *Hoxb4* have severe abnormalities in closure of ventral body wall, including the sternum, that lead to a 50% rate of embryonic lethality on or before day 14.5 of gestation. Of mice that survive until birth, greater than 90% die in perinatal period apparently secondary to defects in chest cavity structure. In the *Hoxb2* mutants the abnormalities are similar to but less severe than those seen in the *Hoxb4* mutants. Genetic analyses suggest that the abnormalities seen in these mice are due to abnormal expression of both Hoxb2 and Hoxb4. *Hoxb2* mutants have decreased Hoxb3 and Hoxb4 expression, whereas *Hoxb4* mutants have decreased to absent Hoxb2 expression in neural crest cells migrating to thoracic region but unchanged Hoxb3 expression. The chest and body wall defects seen in the *Hoxb2* and *Hoxb4* mutants resemble the human condition termed the pentalogy of Cantrell. Genetic defects have been suggested but not confirmed in pentalogy of Cantrell. If two genes are involved, however, genetic diagnoses in human infants may be difficult to identify.

Recent studies show decreased Hoxb6 expression in a mouse model of pulmonary hypoplasia with CDH. In this study, Hoxb4 and Hoxa5 expression were increased at the same time that Hoxb6 was decreased, suggesting that altered relationships of the levels of these Hox proteins (and potentially others) in pulmonary hypoplasia could lead to altered transcriptional regulation of downstream genes specific for lung morphogenesis.[4]

Hoxa5

Studies of *Hoxa5* in lung development have concentrated on gene expression, although some data on protein expression are available. Like its homologue *Hoxb5,* for messenger RNA (mRNA) *Hoxa5* is diffusely and strongly expressed in developing lung mesenchyme during the embryonic to pseudoglandular stages of lung morphogenesis. Some studies described a decrease in late gestation, whereas others reported that both mRNA and protein levels remain high throughout gestation.[38,40,56] In vitro block of Hoxa5 expression using antisense oligonucleotide technology causes abnormal branching morphogenesis of the embryonic lung, resulting in foreshortened and aberrant branches.[57] A transgenic knockout of the *Hoxa5* gene results in the most significant in vivo pulmonary phenotype of any *Hox* gene knockout. The newborn pups homozygous for the *Hoxa5* deletion died an early neonatal death of respiratory failure.[58] The lung is present but structurally abnormal in these animals. Abnormalities observed include upper airway obstruction associated with cricoid cartilage abnormalities and abnormal tracheal rings, altered bronchial epithelium, and decreased production of surfactant proteins, particularly surfactant protein B. It is unclear whether the lethal respiratory failure is due to upper airway obstruction, malformed lung airway or saccular formation, or abnormal type II cell surfactant production, or possibly is secondary to alterations in rib cage or skeletal muscle that make respirations ineffective. Originally these effects on epithelial gene expression and lung function were interpreted as evidence of altered mesenchymal-epithelial cell interactions secondary to absent Hoxa5 in pulmonary mesenchyme. However, subsequent studies showing expression of *Hoxa5* mRNA and protein in bronchiolar and alveolar epithelial cells on or after day 16 of gestation in the mouse and in the adult human support a more direct role for Hoxa5 on lung epithelial cell function.[34] Studies in cell culture confirm the ability of retinoic acid to strongly stimulate Hoxa5 expression in fetal lung fibroblasts.[57] Of potential concern, this study also found that retinoic acid strongly stimulated Hoxa5 expression in the type II cell-like MLE-12 cell line, in which Hoxa5 expression normally is not apparent. This finding suggests the possibility that high doses of retinoic acid may have an undesirable effect on developing pulmonary epithelium by induction of *Hox* genes at inappropriate sites or developmental time points. More recent studies show that other *Hox* genes are expressed in developing pulmonary epithelium and pulmonary vascular cells, supporting the possibility that retinoic acid may induce both beneficial and unwanted effects on developing lung.

A study of *Hox* gene expression in developing human lung and in adults with emphysema and pulmonary hypertension showed *Hoxa5* mRNA expression in epithelial cells and possibly type 2 cells at the pseudoglandular and canalicular stages of development. Expression was increased in adult lungs with pulmonary hypertension and decreased in emphysema. The decreased expression of *Hoxa5* in alveolar epithelial cells in adults with emphysema is consistent with findings in surviving *Hoxa5*-knockout mice, which have abnormal alveolar septal development and lung morphology resembling that seen in emphysema. These mice also have ventilatory abnormalities consistent with the altered morphology.[59] This finding is supported by recent work showing that *Hoxa5* protein expression changes from mesenchymal to epithelial cells in the mid-pseudoglandular period and by studies of mRNA expression in human lungs.[43] Furthermore, as noted earlier, Hoxa5 expression is increased in a mouse model of pulmonary hypoplasia and CDH, especially in alveolar septal tips, suggesting an attempt to compensate for abnormal alveolar development in pulmonary hypoplasia.

Nkx2.1

This divergent homeobox gene clearly has importance in both lung branching morphogenesis and determination of cell differentiation. Blockade of Nkx2.1 significantly inhibits lung branching morphogenesis, both in vitro and in vivo (especially of distal branching).[5,60] In addition, Nkx2.1 is an important transcription factor regulating the differentiation and function of pulmonary epithelium. It specifically activates transcription of the surfactant protein C and surfactant protein B genes in type 2 cells and the Clara cell secretory protein gene in Clara cells. It appears also to control the determination of these cell types (reviewed by Warburton and associates[61]). An interesting case report described an infant with unexplained respiratory distress, hypothyroidism (Nkx2.1 also is involved in thyroid morphogenesis), and a haplotypic deletion of chromosomal region 14q13-21, which included the *Nkx2.1* gene.[62] The infant's problems may have been due to loss of *Nkx2.1,* but because the deletion included many more genes, such a conclusion remains speculative.

Iroquois (Irx) Genes

This strongly conserved group of divergent homeobox genes appears important in pattern specification and organ-specific development in both *Drosophila* and in mammals. In mammals, six Iroquois genes are known. During development, Irx1 through *Irx5* are expressed in multiple sites, but *Irx6* is highly restricted with weak expression. *Irx1, Irx2, Irx3,* and *Irx5,* but not *Irx4*, are found in the branching lung epithelium. In vitro knockout of each lung-expressed *Irx* gene decreased branching morphogenesis. This was associated with a loss of distal epithelial phenotype markers (surfactant protein C mRNA) and an increase in proximal epithelial markers (Clara cell secretory protein mRNA, class IV beta-tubulin mRNA), indicative of a role for *Irx* genes in the proximal-distal determination of epithelial fate.[63] *Irx5* is an immediate downstream target of Hoxb4, demonstrating the interactive hierarchy of homeobox gene functions.[64]

Liver

Much as in the lung, the hepatocytes in the embryonic liver develop from the endoderm of the foregut, invading the surrounding mesenchyme to form the developing liver structure.

The process of hepatocyte determination is driven by FGFs produced by the closely appositioned embryonic heart.[65] The target genes for these FGFs are not known but may include specific homeobox genes. Homeobox genes are important in the morphogenesis of the liver. In particular, the divergent homeobox gene *Hex* is necessary for liver formation. Hex protein normally is expressed in the third pharyngeal pouch and subsequently in developing forebrain, thymus, thyroid, lung, pancreas, and liver, and in adult thyroid, lung, and liver.[66-68] Hex promotes endoderm development into the primordial liver bud after the initial identification by FGFs of these cells for a liver fate.[69] A murine knockout of the *Hex* gene results in embryonic lethality around embryonic day 12.5.[70] Examination of *Hex*-knockout embryos shows the absence of hepatocytes and the absence of liver formation but the presence of the liver capsule. The lack of commitment of foregut endoderm to hepatocyte endoderm indicates that Hex is necessary for this commitment process and suggests that *Hex* is a target, either directly or through a pathway, of the cardiac FGFs that initiate liver formation.

In addition to *Hex,* other homeobox genes contribute to the cellular expansion and specification of cell fate. *Lhx2* is important for proper liver cellular organization, expansion and differentiation, but not for early cell fate.[71,72] *Prox1* specifies the differentiation of hepatoblasts into hepatocytes.[73] Prox1 also regulates bile synthesis and gluconeogenesis in adult liver, making it a good example of a homeobox gene that acts both in cell development and later in maintenance of differentiated phenotype.[74] Conversely, overexpression of Hoxb7 is a clinical marker for, and may be a pathogenic cause of, bile duct carcinoma in humans.[75]

Although specific roles of *Hox* genes from the HOM-C cluster remain to be demonstrated, evidence suggests they are involved and important in regulation of hepatocyte maturation and function. For example, overexpression of *Hoxa5* in transgenic mice (using regulatory elements from *Hoxb2,* a more proximal gene) caused significant growth repression in the first few postnatal weeks, resulting in a proportionate dwarf-like phenotype.[76] This growth disturbance was associated with a profound increase in liver expression of the insulin-like growth factor binding protein-1 (IGFBP-1) and with marked decreases in both liver expression of insulin-like growth factor 1 (IGF-1) mRNA and circulating levels of IGF-1. Additional studies in cultured cells showed that Hoxa5 interacts with other transcription proteins to strongly enhance IGFBP-1 gene expression. This example demonstrates how Hox proteins may act cooperatively in the developed animal to maintain cell differentiation in a tissue-specific manner.

Because the liver is a hematopoetic organ during in utero development, studies of homeobox genes in developing hepatic cells also demonstrate that specific homeobox genes have important roles for establishing lineage fates in hepatic hematopoetic multipotent stem cells and T cell progenitors.[9,77]

Pancreas

A number of homeobox genes are known to be critical in pancreas morphogenesis and cellular differentiation and function (reviewed by Ashizawa and colleagues[78] and Jensen[79]). In addition, combinations of tissue-specific and spatially restricted homeobox genes are active in both pancreatic development and in maintaining the identity of differentiated cells.[7,11,80] The current interest in developing transplant and gene therapies to treat diabetes has produced a recent explosion in the understanding of homeobox genes in pancreas development, regulation of cell fate, and maintenance of cell identity.

The pancreas develops from ventral and dorsal buds that evaginate from the distal foregut endoderm. During differentiation of the foregut endoderm in the normal embryo the ventral pancreas bud and the liver bud arise from adjacent non-overlapping regions of the ventral foregut endoderm.[65] It is the proximity to the embryonic heart that drives the foregut endoderm towards the liver primordium through action of FGFs produced by cardiac mesoderm. If removed from the presence of the heart, this region of the foregut endoderm will default to expression of Pdx1 and engender pancreatic tissue.[81] Initiation of pancreatic bud identity in the foregut involves the control of at least two homeobox genes, *Hex* and an Mnx-class homeobox gene, *Hb9.*[82,83] The further development of pancreatic tissue from the dorsal and ventral buds is directed primarily by the divergent homeobox gene *Pdx1,* a member of the Para-Hox cluster. The expression of *Pdx1* requires the action of at least two other homeobox genes, *Pax4* and *Nkx2.2.*[84] Although it was originally thought that *Pdx1* was only necessary for ventral and dorsal bud growth, recent studies make it clear that *Pdx1* is necessary for bud formation, growth and morphogenesis, and cell differentiation. Using a transgenic approach in which the *Pdx1* gene was placed under the control of a gene promoter that can be turned off by administration of doxycycline, Holland and co-workers interrupted Pdx1 expression at various stages of pancreatic development, as well as in the adult after normal pancreatic development and function was established.[81] Interruption of Pdx1 expression at developmental intervals blocked the processes of bud formation, organ growth and morphogenesis, and the identification, differentiation, and function of the islets including the endocrine and exocrine cell lineages within the islets.

Pdx1 regulates several genes involved in islet cell identity and function including insulin, glucose transporter 2, glucokinase, islet amyloid polypeptide, and somatostatin expression (reviewed by Hui and Perfetti[85]). Pdx1 is in turn regulated by glucose. In type 2 diabetes, characterized by insulin resistance and increased glucose concentrations, Pdx1 expression is elevated.[86] By contrast, loss of persistent Pdx1 expression is associated with the development of type 1 diabetes, decreased insulin production, and decreased numbers of pancreatic islet beta cells.[87] Work directed at finding transplant[88,89] or stem cell approaches to treat type 1 diabetes by restoring islet beta cells demonstrates the importance of induction of Pdx1 to generate the beta cell phenotype.[90-92]

Pdx1 gene abnormalities have been described in several human diseases. The extremely rare condition of congenital agenesis of the pancreas was found to be associated with a homozygous mutation in *Pdx1* that produced a truncated, non-functional protein product.[93] Family members heterozygous for this mutation manifested an autosomal dominant form of maturity-onset diabetes of the young (MODY type 4). Other studies have documented different mutations in *Pdx1* in families manifesting MODY type 4.[94] In all cases, the mutations alter the ability of Pdx1 to regulate the maintenance of beta cell identity and function.

Other divergent homeobox genes also exhibit functional regulation of pancreatic islet cell function. *Nkx2.2,* a member of the *Nkx* homeobox family, is expressed in three of the types of islet cells—namely, alpha, beta, and PP (pancreatic polypeptide–producing) cells—but not in delta cells. Mice in which the *Nkx2.2* gene has been knocked out lack beta cells and have marked reductions in the glucagon-producing alpha and PP cells.[95] In addition, there are many islet cells that do not produce any of the four pancreatic endocrine hormones. In other studies, deletion or disruption of the homeobox genes *Pax4* and *Pax6* shows that these genes are vital for islet endocrine cell differentiation—specifically, insulin- and glucagon-producing cells, respectively.[96,97]

Intestinal Tract

A number of *Hox* and divergent homeobox genes are expressed to influence intestinal development (reviewed by Morell[98]). As in the CNS, the development of stomach, small intestine, and large intestine exhibits a detailed representation of the spatial and temporal colinearity of expression of genes in the HOM-C complex.

Several studies have documented groups of *Hox* gene expression in the developing gut.[99,100] Expression is seen early in development of the foregut and hindgut structures and is confined primarily to the mesoderm. The developing esophagus, stomach, and cloacal region all exhibit evidence of endodermal cell Hox expression. Functions of *Hox* genes in intestinal development are less clear. Some evidence suggests that they interact with other homeobox genes in providing positional information to direct development of region-specific gut endoderm and structural morphogenesis. The temporal expression of the *Hox* genes also reflects the three waves of expression, with peaks in early embryonic mesoderm as the gut tube forms, in midgestation as determination of stomach, duodenum, small intestine, and large intestine is accomplished, and late gestation/early postnatal life as villous structures develop and are propagated. Although the specific functions of the *Hox* genes in intestinal development are not understood, several clues have been obtained from gene knockout and transgenic overexpression mouse models. Inactivation of *Hoxc4* caused disruption of esophageal muscle development and blockage of the esophageal lumen, suggestive but not identical to esophageal atresia of the human.[101] Overexpression of *Hoxa4* created an extended region of high *Hoxa4* expression into the large bowel and resulted in mice with megacolon suggestive of that found in Hirschprung disease.[102] Anterior overexpression of *Hoxc8* created the development of multiple hamartomas within the gastric mucosa.[103] The epithelium of these hamartomas resembled that seen in colonic mucosa, suggesting that anterior expression of this posterior *Hox* gene caused development of posterior mucosal characteristics. Studies have suggested that D cluster *Hox* genes are important in the development of intestinal sphincters. A multiple transgenic mouse, in which the D cluster of Hox genes from *Hoxd4* to *Hoxd13* was deleted, exhibited abnormalities of both pyloric and anal sphincter development.[104] Subsequent studies showed that deletions of either *Hoxd12* or *Hoxd13* caused anal sphincter abnormalities and rectal prolapse.[105]

Divergent homeobox genes also are critical for intestinal development. These include the members of the Para-Hox cluster *Cdx2* and *Pdx1*, as well as *Nkx2.3*. *Cdx2* is a mammalian homologue of the *Drosophila* homeobox gene *caudal*, which acts to posteriorize the rostral *Drosophila* larva. Cdx2 protein is expressed in the early embryo in the trophoblast cells; homozygous mutant embryos lacking Cdx2 fail to implant and so do not survive.[106] After day 12 of mouse development expression of Cdx2 is confined to gut epithelium where it appears responsible for posterior axial specification of the developing epithelium. Expression begins just anterior to the developing liver bud and extends posterior to the distal colon. In adult animals, high-level expression present in the proximal colon decreases with either rostral progression into the ileum and jejunum or caudal progression into the terminal colon.[107,108] Studies of *Hox* genes in context with *Cdx2* suggest that Cdx proteins may participate in establishing Hox expression boundaries in the developing gut.[109,110] Cdx2 induces several extracellular matrix proteins consistent with a proposed role of inducing enterocyte maturation on the intestinal villus.[110] Cdx2 also acts as a tumor suppressor in the adult colon.[111]

Although homozygous deletion of *Cdx2* causes lethality at implantation, it has been possible to use gene deletion to demonstrate the role of *Cdx2* in gut development in vivo. Transgenic mice that are heterozygous for the *Cdx2* deletion develop multiple polyp-like intestinal lesions that are most evident in the cecum, where Cdx2 is prominently expressed. The polyps extend proximally and distally with decreasing frequency and are found only within the normal expression domain of the *Cdx2* gene.[112] Histologically these lesions consist of normal gastric mucosa. Examined both proximally and distally from the cecum, the lesions demonstrate an orderly progression of cell type from stratified squamous epithelium (typical of the fore stomach) through the mucous glands of the cardia, the gastric glands of the corpus, to mucous cells with the appearance antral cells. Between polyp lesions is an intercalation of villi and Paneth cells, structures that lack Cdx2 expression. As Beck and colleagues have pointed out,[112] this is a remarkable presentation of an anterior homeotic shift, the only example of homeosis that to date has been demonstrated for the intestine, and also is the first report of intercalary regeneration in mammals. Another example of an anterior homeotic shift may occur in Barrett's esophagus, in which ectopic Cdx2 expression is found in the esophageal lesions.[113] Thus, Cdx2 works within the developing gut similar to the function of its *Drosophila* homologue to create a posterior phenotype that replaces a more proximal default program.

Patients with Hirschprung disease–associated enterocolitis were found to have significantly reduced expression of both Cdx2 and Cdx1 in the enterocolitis lesions. In contrast, other intestinal colitis diseases such as necrotizing enterocolitis did not display such a reduction, nor did Hirschprung disease intestine without enterocolitis.[114] Another possible clinical correlate is that of intestinal-type gastric cancer, which often is preceded in humans by metaplasia of gastric-type mucosa in the intestinal tract. The anterior homeosis observed in the heterozygous *Cdx2*-knockout mice resembles that in these types of lesions. Studies in humans suggest that reduced Cdx2 expression may be an antecedent cause of this form of intestinal cancer.[108]

The important role of the Para-Hox gene *Pdx1* in pancreatic development has already been discussed. *Pdx1* is expressed in distal foregut, where it not only defines pancreatic endoderm but also affects duodenal development. In Pdx1-deletion animals the rostral duodenum does not form villi or Brunner's glands and remains lined by a cuboidal epithelium.[115] These abnormalities are very closely restricted to the specific domain of Pdx1 expression in the distal foregut.

Members of the *Nkx* group of homeobox genes are expressed in intestinal mesoderm, where they appear to act on the overlying epithelium to direct its differentiation. For example, inactivation of *Nkx2.3,* which is expressed in gut mesoderm, causes abnormalities of adult intestinal epithelium.[116] These abnormalities include delayed villus development, increased growth of crypt cells, and hyperproliferation of epithelium, primarily in the jejunum. These findings support the general hypothesis that one important function of mesodermally expressed homeobox genes in general is to provide positional cues for overlying epithelium that defines their function and structural organization.

In addition, the divergent homeobox genes *Cdx1* and *Cdx4*, relatives of *Cdx2* but not members of the Para-Hox cluster, are expressed in developing gut endoderm and mesoderm, respectively.[117] It is not known if they are critical for development. Deletion of the divergent homeobox gene *Ncx/Hox11L.1* in mice causes a syndrome of mega ileocecal colon with intestinal dysmotility.[118]

HOMEOBOX GENES AND LIMB DEVELOPMENT

The mammalian limb provides scientists and clinicians with an outward example of embryonic structural patterning that requires a multitude of positional signals in the developing embryo to create the asymmetric and unique definition of structure along the anterior to posterior and dorsal to ventral axes. Multiple cellular processes are programmed in time and place, including a balance between rapid cell growth, cell proliferation, and apoptosis and the development of cell and structural differentiation. The easy visibility of structural changes in the limb and presence of human limb malformations in approximately 0.1% of newborns also has made limb development one of the most-studied processes for *Hox* gene involvement in both embryogenesis and malformations.

For orientation of events potentially controlled by Hox genes in limb development it is important to define the proximal to distal structures and certain regions of the developing limb. The limb has four segments that define the initial structural patterning events: the root or zonoskeleton, the proximal segment or stylopodium (one bone: either humerus or femur), the middle segment or zeugopodium (two bones: radius plus ulna or tibia plus fibula), and the distal segment or autopodium (several bones of the hands or feet with definite structural polarity). Additionally, the developing limb buds have zones that are partially defined by their location and the different influences they exert on patterning. The *apical ectodermal ridge* (AER) is located at the tip of the growing limb bud. It contains many members of the FGF family and maintains proliferation of the rapidly growing *zone of progression*, the region of mesenchymal cells that eventually become committed to the different regions of the final limb. The zone of progression determines the outward growth of limb along the proximal to distal axis. Another developmental zone is the *zone of polarizing activity* (ZPA), a region that contains posteriorizing signals and is located on the posterior edge of the outward growing limb bud. This region determines the anterior-to-posterior axis of the limb and is named for its ability to induce the formation of posterior structures when transplanted to the anterior edge of developing limbs, creating a mirror image limb.[28,119,120]

Over the past decade molecular biologists have began to unravel the molecules responsible for polarizing the developing limbs into three axes: anterior-posterior, proximal-distal, and ventral-dorsal. These molecules in general involve transcriptional regulators and signaling proteins, with the transcriptional regulators defining clear domains in the developing limb before visual evidence exists of structural definition. Much evidence now exists both from experimental studies and analysis of anomalies seen in humans that genes from the HOM-C cluster are a major group of transcriptional regulators necessary for defining regional and structural identity during limb morphogenesis. *Hox* genes subdivide the limb along two axes: the proximal-to-distal axis and the anterior-to-posterior axis. They activate downstream targets according to their position within these axes in agreement with the 3′-to-5′ proximal-distal principle of activity described at the beginning of this chapter. In addition, the principles of temporal and spatial colinearity of Hox gene expression described earlier also are maintained within the developing limb bud along the anterior-posterior and proximal-distal axes.

Many gene ablation studies have shown that mice lacking a certain *Hox* gene display a phenotype in which a specific skeletal element is missing from the region that would normally express the ablated gene. The phenotypic severity is amplified by combined ablation of two or more *Hox* genes.[27,28] Limb development also provides an example of the importance of quantitative or dose dependent regulation by *Hox* genes in addition to contributions from a Hox code in development.[121] For example, the size and number of digits depends on quantitative levels of genes from the 5′ paralog groups 11, 12, and 13 in the HOM-C complex. Limb development also shows how combinations of *Hox* genes help define a particular proximal-to-distal limb segment. For example genes from paralog groups 9 and 10 define the upper arm or the stylopodium, groups 10, 11, and 12 define the lower arm or zeugopodium, and groups 11, 12, and 13 define the digits or autopodium.[28] Restricted expression of groups of *Hox* genes from the different clusters also helps specify the anterior-posterior nature of the limb, with Hoxc4 and Hoxc5 specifying the forelimbs, Hoxc9, Hoxc10, and Hoxc11 defining the hind limb, and Hoxc6 and Hoxc8 being expressed in both fore and hind limbs. There are also specific functions within regions. For example, Hoxb5 is expressed early in the formation of the upper limb buds and is important to proper positioning of the upper limb bud along the anterior to posterior vertebral axis. Mice lacking *Hoxb5* have shoulder girdle deformities resembling Sprengel anomaly in humans where the forelimb arises more anteriorly leading to asymmetric abnormalities of shoulder position in reference to vertebral column.[122] Hoxb8 is also expressed very early in upper limb development and is necessary for inductive activity of the ZPA. Finally, non-clustered divergent homeobox genes such as *Lmx1* and *Engrailed1* are implicated in dorsal/ventral axis determination in limb development.[28,119]

Of the many *Hox* genes expressed during limb development, several are involved in limb anomalies in humans. Most notable are anomalies seen with mutations in *Hoxd13, Hoxa13,* or *Hoxa11,* which are required for normal development of the autopod. In humans with the autosomal dominant condition of synpolydactyly, mutations of the 5′ region of *Hoxd13* have been found.[123] Frameshift mutations of *Hoxd13* in humans are associated with a particular foot phenotype.[124] The autosomal dominant human hand-foot-genital syndrome has mutations in *Hoxa13*.[125] Similar anomalies are seen in mice homozygous for *Hoxa13* mutations. *Hoxa11* mutations have been found in persons with radioulnar synostosis.[126]

HOMEOBOX GENES AND KIDNEY DEVELOPMENT

Mammalian kidney development passes through three stages, the pronephros, mesonephros and metanephros, important to developmental morphogenesis and evolutionary comparisons. Given that the kidney is derived from intermediate mesoderm with a specific anterior to posterior definition and involves timely organized mesodermal-epithelial interactions for proper cellular determination, it is understandable that this process is regulated by homeobox genes. As indicated by studies in homeobox gene mouse mutants, homeobox genes help control downstream expression of other key transcriptional regulators and growth factor pathways in kidney development, including Gata 6, transforming growth factor-β_2 (TGF-β_2), and others.[8] Homeobox genes from the HOM-C clusters and the divergent *Hox* genes are both involved. Colinearity of *Hox* expression is preserved in kidney development with HOM-C genes 5′ in the clusters expressed in the nephrogenic regions and expression of 3′ genes restricted to the ureteric bud, which is formed from anterior intermediate mesoderm and pronephric duct.[127] A few *Hox* genes, including *Hoxb7, Hoxa11,* and *Hoxd11,* are necessary for kidney development.[128] Hoxb7 is expressed in the urogenital system in the mesonephros, ureter, and collecting system. Overexpression of *Hoxb7* in mice causes renal duplications.[127,129] Hoxa11 and Hoxd11 appear to act collectively in kidney organogenesis. In mice, knockout of just one of these *Hox* genes does not perturb kidney development, but double mutant mice have one hypoplastic kidney consistent with branching morphogenesis abnormalities or no kidneys.[130] These mice also lack a radius and ulna, underscoring the importance of *Hoxd11* for development of diverse structures. Additionally, *Hoxb13* gene misexpression causes renal agenesis, but whether other *Hox* genes of group 11 also are abnormally expressed in these mouse mutants is unknown.[131]

Early expression of several divergent *Hox* genes is found in intermediate mesoderm, including the *Lim1* and *Pax2* genes, suggesting they may be important in early patterning of the kidney, possibly at earlier stages than those for *Hoxa11* or *Hoxd11. Lim1*-knockout mice fail to develop kidneys as a result of failure of nephric duct formation.[132] Studies also show a sequential need for Lim1 for formation of the ureteric buds and patterning of the renal vesicles for nephron formation. This sequential function arises from action of Lim1 in distinct tissue compartments of the developing metanephros. Emx2, encoded by another divergent homeobox gene, probably is important later in kidney development during metanephros development after ureteric

bud formation. In *Emx2*-knockout mice, a ureteric bud develops but no tubular differentiation occurs.[133]

HOMEOBOX GENES AND CARDIOVASCULAR DEVELOPMENT

Homeobox genes from the clustered and nonclustered groups are implicated in cardiac morphogenesis, vasculogenesis of the great vessels, and angiogenesis and in related aspects of cellular differentiation. The clustered homeobox genes involved in these processes are derived predominantly from the *HoxA, HoxB,* and *HoxD* clusters, and the Para-Hox clusters (*Pdx* genes). Other nonclustered homeobox genes that are important to cardiovascular development include the *Nkx* family, *Prx,* and *Pitx* genes.

Hox Genes and Great Vessel Development

Morphogenetic events during the formation of the major chambers of the heart occur simultaneously with the formation of the great vessels from the pharyngeal arches and their corresponding pharyngeal arch arteries. Events in great vessel morphogenesis influence cardiac development including formation of the outflow tracts of the developing heart.

Each pharyngeal arch has a unique combination of expression of genes in the HOM-C cluster. The expression patterns of *Hox* genes in pharyngeal arches 2 through 5 is established by neural crest cells that migrate from rhombomeres expressing specific combinations of *Hox* genes. These neural crest cells also migrate to the aortic arches and ultimately contribute to the formation of the aorticopulmonary septum that divides the aortic and pulmonary outflow tracts and the endocardial cushions.[134-136]

Certain populations of neural crest cells express specific *Hox* gene combinations corresponding to the anterior-posterior origin of the cells, suggesting that *Hox* gene interactions with other key regulators help pattern the great vessels. For example, Wnt5a, which is expressed in similar regions to those for *Hox* genes in pharyngeal mesoderm and in pharyngeal neural crest cells, is necessary for cardiac outflow tract septation in mice.[137,138] Mutant mice lacking expression of the *Hoxa3* gene have many of the features seen in humans with DiGeorge syndrome/velocardiofacial syndrome (DGS/VCFS), with cardiac disease involving the great vessels as well as facial, thymic, and parathyroid abnormalities. These structures are derived from rhombomeres 5 to 7 and neural crest cells of pharyngeal arches 3 to 6, all of which have significant Hoxa3 expression.[134,136,139] Although gene defects have been seen mainly on human chromosome 22 in DGS/VCFS, Hox interactions with many cofactors during transcriptional regulation of downstream genes makes it likely that *Hox* gene mutations play a role in some cases of this syndrome. The molecular level at which *Hox* gene interactions with genes that are deleted from chromosome 22 in DGS/VCFS occurs remains to be determined.[135,140] A role for abnormal expression of Hoxa3 in formation of the great vessels and other features of VCFS is further supported by a case report of a child with a deletion in chromosome 7 leading to haploinsufficiency of genes from the entire *HoxA* cluster. This child exhibited abnormalities consistent with hand-foot-genital syndrome (see earlier section on limb development), which is known to be caused by mutations in *Hoxa13,* and also features of VCFS, including a striking resemblance to the defects described in the *Hoxa3*-knockout mice.[136,139,141]

Some studies suggest that loss of some other *HoxA* genes along with their paralogs in the other *Hox* clusters can lead to more severe cardiac defects resembling abnormalities seen in human infants. The contribution of collective groups of paralog genes from the *HoxA, HoxB* and *HoxC* clusters to the patterning of the pharyngeal arch arteries has been studied in chick embryos. For example, blocking expression of Hoxa3 and Hoxb3 proteins with antisense oligonucleotides in neural crest cells that contribute to the pharyngeal arches leads to regression of aortic arch 3, so that it is more similar in appearance to aortic arch 2. This homeotic transformation fits with the expression pattern of *Hox* genes in each pharyngeal arch. Blockade of pharyngeal arch neural crest expression of Hoxa5, Hoxb5 and Hoxc5 led to an additional pharyngeal arch and another aortic arch. In addition, isolated blockade of Hoxb5 resulted in an even more pronounced development of an additional aortic arch. This is particularly interesting because Hoxb5 is not expressed in neural crest cells during migration but becomes expressed in these cells when they reach the site of pharyngeal arch 6. The presence of Hoxb5 in this pharyngeal arch could thus contribute significantly to the appropriate patterning of arch derivatives from this region.[134,142, 143]

The paired related homeobox genes are also implicated in development of the great vessels. *Prx1* and *Prx2* gene products are expressed in mesoderm of developing great arteries and veins, in the endocardial cushions, and valves in the epicardium. Prx1 expression increases in the adventitia and outer media of developing great vessels. *Prx1*-knockout mice have normal intracardiac anatomy but have abnormal positioning and curvature of the aortic arch and a misdirected and elongated ductus arteriosus. Prx1 may exert its effects on great vessel development partly through regulation of tenascin-C, an extracellular matrix molecule localized to similar regions within the developing cardiovascular system.[144] Prx2 expression is intensely enriched in the ductus arteriosus beginning early in development, but *Prx2*-knockout mice do not show cardiovascular malformations. A combined role of Prx1 and Prx2 in cardiovascular development is suggested by the increased severity of anomalies in mice with combined null mutations for *Prx1* and *Prx2.* These mice have an unusually long ductus arteriosus, tortuous great vessels, and a retroesophageal right subclavian artery.[145-147]

Homeobox Genes and the Heart

The primitive heart tube in developing vertebrates forms from anterior lateral plate mesoderm. Through a complex process of heart rotation and folding during embryogenesis, the anterior regions of this heart tube ultimately form the ventricles, while the posterior portion of the heart tube form the atria. Even before formation of the heart tube, cell position in the heart-forming region of the lateral plate mesoderm helps determine specific anterior and posterior cell lineages that ultimately define which cells contribute to atria or ventricles of the developing heart. *Hox* genes from clusters A and D have distinct regional expression patterns during early heart development, suggesting that they participate in determining the anterior (ventricle) or posterior (atrial) phenotype of cells. *Hox* genes from paralog groups 1 to 5 are expressed in the heart-forming region and primitive heart tube of the developing chick heart, with both temporal and regional differences in expression. *Hoxa4, Hoxd3,* and *Hoxd4* are more highly expressed in posterior than in anterior regions of the primitive heart tube and primitive heart, followed by down-regulation during the stage of heart looping.[148] Other homeobox genes—*Nkx2.5, Iroquois,* and *Pitx2*—are expressed at similar or later times in heart formation, but the functional relationship of these Hox genes during heart development has not been studied. Some evidence exists for the ability of the clustered *Hox* genes to regulate *Nkx* and *Iroquois*[64,149] (reviewed by Epstein and Buck[135]). Certain homeobox genes are down-regulated during or after the stage of heart looping, suggesting that groups of clustered *Hox* genes are needed in early heart morphogenesis to specify or initiate expression of other factors for later stages of development.

Numerous studies have investigated the role of *Nkx2.5* in cardiac development. *Nkx2.5* is the mammalian homologue of the *Drosophila* gene *tinman*—so named because flies lacking *tinman* do not form the dorsal vessel, the structure similar to

the mammalian heart (thereby resembling the fabled Tin Man of Oz, who also lacked a heart).[135,150] Heart looping is prevented by inactivation of *Nkx2.5* in mice. Mutant mice form a primitive heart tube but then die in mid-gestation as a result of lack of heart looping. The fact that several of the clustered *Hox* genes are expressed in the primitive heart tube but are down-regulated at the heart looping stage suggests a possible hierarchy of gene interactions during heart development, with specific *Hox* genes being necessary early in heart morphogenesis and divergent homeobox genes such as Nkx2.5 being necessary later in development and possibly being activated by the earlier-expressed Hox genes.[149] At least 26 mutations have been found in the *Nkx2.5* gene in humans, some of which are in the homeodomain binding regions and others in regions of the gene that are involved in nuclear localization or interactions with other transcriptional regulators. Despite the variability in these mutations, recent studies suggest that the final common pathway for phenotypes seen with *Nkx2.5* abnormalities is influenced by to the total dose of Nkx2.5 protien available for DNA binding. Heterozygous loss-of-function mutations and hypomorphic alleles for *Nkx2.5* in humans are associated with atrial septal defects, conduction disturbances, ventricular septal defects, tetralogy of Fallot, and Ebstein's anomaly.[151-155]

The recently discovered homeodomain-only protein HOP is a downstream target of Nkx2.5 and also modulates Nkx2.5 function by inhibiting transcriptional activation of other cardiac specific genes by Nkx2.5. Homeodomain-only proteins such as HOP lack DNA binding activity but localize to the nucleus. HOP is expressed in cardiac muscle early in mouse development and in cells of pharyngeal arches that contribute to cardiac outflow tract. HOP expression continues in atrial and ventricular myocytes throughout development and in the adult. It probably contributes to control of cell proliferation, release of myocytes from cell cycle, and cardiac myocyte maturation. HOP also is expressed in other organs including lung, intestine, brain and kidney, but the regulatory interactions in these organs are less well defined to date.[156,157]

Another important aspect of cardiac morphogenesis is determination of appropriate left-right axis asymmetry. Situs anomalies occur in approximately 1 in 5000 live births, with equal incidence of situs inversus and situs ambiguus. Pitx2 (also called REIG, Otlx2, and Brx2) is a nonclustered homeodomain transcription factor originally identified as the gene responsible for Rieger syndrome, a disorder of autosomal dominant inheritance in which affected persons have haploinsufficiency of *Pitx2*.[158] Its potential role in left-right axis asymmetry is suggested by its expression pattern in left lateral plate mesoderm and the induction of its expression by other key mediators of left-right axis formation including Sonic hedgehog and Nodal. Mice with targeted mutations in *Pitx2* causing lack of Pitx2 protein on the left side of the embryo have cardiac defects including abnormal cardiac positioning, hypoplastic right ventricle, atrioventricular septal defects, and right pulmonary isomerism. Humans with *Pitx2* haploinsufficiency in Rieger syndrome, however, have not demonstrated left-right axis malformations in asymmetric organs such as the heart. The differences between human and mouse malformations with Pitx2 malformations can be explained by a gene dosage effect, because mice heterozygous for null *Pitx2* alleles have abnormalities similar to those seen in human Rieger syndrome without axis malformations.[159,160]

Vasculogenesis and Angiogenesis

Vasculogenesis, the formation of blood vessels from progenitor cells, and *angiogenesis,* the sprouting of new vessels from existing vessels, are processes that are active from the earliest stages of embryogenesis and organogenesis. Abnormal regulation of angiogenesis plays a role in many disease processes including but not limited to retinopathy of prematurity, bronchopulmonary dysplasia, and the progression and metastatic spread of cancer. Thus an understanding of how these processes are controlled in development and altered in newborn diseases is essential. The role of homeobox genes in pulmonary vasculogenesis and disease has been reviewed in detail.[161]

HoxA Cluster

Available evidence suggests that HoxA genes are involved in vasculogenesis. In addition to the abnormalities in large vessels discussed earlier, *Hoxa3*-knockout mice have enlarged pulmonary veins with an abnormal outer layer of cardiac cells, suggesting that Hoxa3 limits cardiac cell migration during pulmonary vein development.[139,161,162] Hoxa5 is abnormally increased in alveolar epithelial cells of humans with pulmonary hypertension, but whether this increase is causal or represents a downstream response is unknown.[3] Other *HoxA* genes are expressed in human fetal and adult vascular smooth muscle, including *Hoxa2, Hoxa4,* and *Hoxa11;* however, their role in vasculogenesis has not yet been investigated.

HoxB Cluster

Studies to date show that *HoxB* genes are the predominant Hox cluster expressed in endothelial cells. Belotti and co-workers evaluated the expression patterns of genes from the *HoxB* cluster in human umbilical vein endothelial cells (HUVECs).[163] These investigators showed that mRNA from all *HoxB* genes except *Hoxb1* are expressed in HUVECs, with low levels of *Hoxb7* and *Hoxb9;* high levels of *Hoxb2, Hoxb3,* and *Hoxb4;* and intermediate levels of *Hoxb5, Hoxb6,* and *Hoxb8.* Tissue plasminogen activator (TPA), which induces endothelial cells to change phenotype to angioblasts that create new vessel formation, increased the expression of all of these *HoxB* genes. This finding suggests that the pattern of *HoxB* expression in endothelial cells may specify this cell lineage and that *HoxB* genes may play a role in sprouting of new blood vessels in the process of angiogenesis.[163] Additionally, studies in tumor cell lines showed that Hoxb7 can increase levels of vascular endothelial growth factor (VEGF) isoforms, basic FGF, and matrix metalloproteinase-9 (MMP-9) proteins. An increase in Hoxb7 expression in tumor blood vessels suggests that this *Hox* gene could be involved in promoting vasculogenesis in the developing fetus.[164,165] Hoxb3, which is expressed in fetal lung, promotes capillary morphogenesis in cultured fetal lung cell lines by controlling ephrin A1 and by activation of the ephrin receptor EphA2.[166,167] Consistent with angiogenic and hematopoetic progenitor cells arising from the same stem cell population, erythroid progenitor cells also express *Hox* cluster genes, primarily *Hoxb2* through *Hoxb9*. Increased Hoxb4 and decreased Hoxb6 act to push embryonic stem cells or erythroid/myeloid stem cells toward an erythroid phenotype, suggesting that the combination of Hox proteins within cells helps determine their ultimate phenotype (reviewed by van Oostveen and colleagues[168]).

Divergent Hox Genes

Gax, a divergent homeobox gene, is expressed in mesoderm and muscular cell precursors in developing and adult mice. Its expression is restricted to the cardiovascular system and kidney. Gax inhibits vascular smooth muscle cell migration through changes in integrin expression, suggesting involvement in regulating the angiogenic balance. Gax also is expressed in endothelial cells, where it inhibits cell proliferation and in vitro VEGF-induced endothelial tube formation.[169,170]

In addition to the probable role of Prx1 in heart and great vessel development, Prx1 also probably is involved in pulmonary vasculogenesis. Prx1 is expressed in mesenchymal cells clusters during early lung development. It induces the expression of genes involved in the formation of adherens junctions, likely helping to stabilize cell-cell contacts in these clusters of mesenchymal cells.

Prx1 induces an endothelial cell phenotype in fetal lung mesenchymal cell lines, indicating that Prx1 is involved in mesenchymal to endothelial cell transformation.[22] Of note, not all cells expressing Prx1 differentiate into blood vessels. Thus the ultimate formation of blood vessels from aggregates of Prx1-expressing cells is likely to involve other factors including homeobox proteins that act in concert with Prx1. More recently Prx1 was found to directly regulate tenascin-C, an extracellular matrix (ECM) molecule that is an important component of the ECM around developing vessels.[171] *Prx1*-null mice have decreased amounts of tenascin-C and other markers of endothelial cells, Flk-1 and vascular endothelial cell adhesion molecule-1 (VCAM-1), further suggesting a role for *Prx1* in endothelial cell differentiation.[22] These mice also have hypoplastic lungs, supporting the hypothesis that lung vascular development plays a significant role in patterning alveolar development.[172]

Hex, whose importance for liver development is discussed earlier in this chapter, also may be involved in initiating processes of vasculogenesis and hematopoiesis. In the embryo, Hex is expressed in many multipotent progenitor cells. Expression appears first in primitive endoderm of the blastocyst and later in foregut endoderm and mesoderm. In mesoderm, Hex is transiently expressed in nascent blood islands of the yolk sac.[40,161,173] Hex expression in the distal mesenchyme of the developing lung colocalizes with Flk-, the receptor for VEGF, suggesting that Hex is an early marker of endothelial cells in the developing lung, where it may commit these cells to the vasculogenesis process initiated in the regions around developing airways. Unlike loss of Flk-1 expression, however, disruption of Hex expression does not appear to affect vasculogenesis and hematopoiesis.[161]

Hox GENES AND HEMATOPOIESIS

Blood cells of all lineages are derived from pluripotent stem cells in the process of hematopoiesis. In the developing embryo, hematopoietic stem cells arise from two main sites: (1) ventral mesoderm cells, which migrate to the yolk sac and differentiate primarily into erythroid cells and sometimes monocytes and megakaryocytes to contribute to embryonic hematopoiesis, and (2) cells in the dorsal mesentery or aorta gonad mesonephros region, which initiate definite hematopoiesis beginning in fetal liver and spleen and eventually populating the bone marrow.[168]

Studies addressing *Hox* genes in hematopoiesis have been done both in vivo and in vitro, but much work remains to be done on this topic, especially regarding the initiation of these processes in the developing human embryo and fetus.[168,174] Several *Hox* genes from the *HoxA, HoxB,* and *HoxC* but not *HoxD* clusters are expressed in hematopoietic stem cells during development, in the adult, and in certain disease states. In fact, some of the best studied diseases associated with aberrant *Hox* gene expression are the cancers of the blood cell lineages.

The number and expression level of *Hox* genes from each cluster are greatest for *HoxA.* Moreover, a change in colinear expression of *Hox* genes occurs with cell maturation. *Hox* genes located more 3′ within the clusters are down-regulated as cells mature, and higher levels of more 5′ genes are seen. It appears that certain *Hox* genes help determine specific lineage commitment, with some *Hox* genes expressed in all cell lineages but others showing a lineage-restricted expression pattern. For example, *Hoxc8, Hoxa7,* and *Hoxb7* are expressed in all hematopoietic lineages, but *Hoxc4* determines the lymphoid cell line, *Hoxa9* and *Hoxa10* determine the myelomonocytic lineage, and *Hoxb4* along with *Hoxb2* and *Hoxb12* determines the erythroid cell lineage. The combination of genes expressed depends on the cell type and on the stage of development or differentiation. Furthermore, down-regulation or up-regulation of particular *Hox* genes will produce transformation of a cell's phenotype to that of a different lineage.

Unlike congenital anomalies that are associated with Hox gene mutations, blood diseases associated with *Hox* gene abnormalities are due to translocations that lead to abnormal expression levels of a particular Hox protein. Experimental studies with decreased or over-expressed *Hox* genes are beginning to reveal significant roles for specific *Hox* genes in normal hematopoiesis. For example, *Hoxa9*-knockout mice have altered differentiation of myeloid and megakaryocytic cells and abnormal early T cell development with decreased circulating numbers of granulocytes and lymphocytes.[175] The T cells in these mice have decreased cell surface receptors including E-cadherin. Conversely, activation of *Hoxa7* or *Hoxa9* in mice leads to myeloid leukemia.[176] Similar results are seen in mice with bone marrow cell overexpression of *Hoxa9, Hoxa10, Hoxb3,* or *Hoxb8.* These findings in mice correlate with a poor prognosis in patients with acute myeloid leukemia whose cells express Hoxa9.[136,177] Some of the oncogenic potential of overexpressed Hoxa9 may relate to cooperative interactions with PBX and Meis family transcription factors (reviewed by Goodman[136]).

Hoxa10 expression is similar but not identical to Hoxa9 in hematopoietic cells and is further restricted to myeloid cells. Overexpression of Hoxa10 in mouse bone marrow leads to increased numbers of stem cells and myeloid progenitors. These mice are easily susceptible to the development of a myeloproliferative disorder resembling acute myeloblastic leukemia.[168,178]

B and T cell development may be partially determined by transcriptional regulation by Hoxa3, Hoxb3, and Hoxd3. Mice in which Hoxa3, Hoxb3, and Hoxd3 expression all are ablated have abnormal thymus, parathyroid, and thyroid glands. The *Hoxa3*-knockout mouse has decreased *Pax1* gene expression, and *Pax1*-knockout mice also are athymic, suggesting that Hoxa3 regulates Pax1 expression and subsequent thymus development. This correlation may contribute to the phenotype in the combined *Hoxa3-, Hoxb3-,* and *Hoxd3*-knockout mice.[168,179] *HoxC* genes also are expressed in the lymphoid cell line, where they appear to be particularly important for regulation of these cells.[180]

The fact that erythroid cells express almost exclusively *HoxB* cluster genes, whereas other hematopoietic cells express *Hox* genes from the *HoxA* and *HoxC* clusters, suggests that *HoxB* genes are important for regulation of erythroid cell fate and cellular differentiation. *Hoxb4* appears to be important for maintaining a source of hematopoietic progenitor cells. In fact, unlike the overexpression of other *Hox* genes, which is associated with abnormal lymphoproliferative disorders, overexpression of *Hoxb4* in adult bone marrow enhances repopulation of bone marrow with hematopoietic cells without inducing leukemia. Additionally, the level of *Hoxb4* expression appears to have an impact on myeloid growth and differentiation while suppressing erythroid differentiation.[181-183]

HoxC genes are important for appropriate commitment of the lymphoid cell line. Studies on hematopoietic and leukemic cell lines show distinct changes in the expression levels of Hoxc4, Hoxc5, and Hoxc6 proteins, depending on level of cellular differentiation of lymphoid cells or on the neoplastic or lymphoblastic potential.[180] With progressive maturation of lymphoid cells, Hoxc4 and Hoxc6 levels are increased, but Hoxc5 levels are greater in lymphoma cell lines and in certain types of non-Hodgkin lymphoma.

Studies in hematopoietic cells and certain related cancers have contributed to understanding the downstream pathways that are regulated by *Hox* genes in general. Hox proteins regulate expression of several categories of genes, including those that code for other transcription factors (including other *Hox* genes), cell adhesion molecules, and growth factors (reviewed by van Oostveen and colleagues[168]). Among the cell adhesion molecules regulated by *Hox* genes are integrins, cadherin, and neural adhesion molecule (N-CAM) and extracellular matrix molecules such as tenascin-C and connectin. Changes in expression of some of these groups of molecules can have profound effects on cellular migration, cell

death, and cellular proliferation, as well as on cell differentiation. Changes in cell adhesion to surrounding stroma are imperative for appropriate migration of differentiating cells within the bone marrow, and shedding of certain cell adhesion molecules is necessary for release of cells from bone marrow into bloodstream. Certain *Hox* genes become down-regulated as blood cells mature and are released from bone marrow, whereas others become up-regulated, possibly reflecting changes in cell adhesion molecules leading to migration and homing of blood cells to new locations within the body. Additionally, auto- and cross-regulation clearly occurs in and between the *Hox* clusters, affecting downstream regulation by Hox proteins. Other transcriptional regulators regulated by Hox proteins include members of the homeobox families of *Pax* and *NKx* genes, and the forkhead family of winged helix transcription factors. Growth factors including members of the TGF-β and epidermal growth factor (EGF) families also have been shown to be regulated by Hox proteins.[20,134,136,184]

HUMAN DISEASES ASSOCIATED WITH HOMEOBOX GENE ANOMALIES

In the preceding sections, several human conditions associated with homeobox gene anomalies have already been mentioned. Table 8-1[185-194] summarizes human conditions in which homeobox gene anomalies are known. These represent three categories of findings. In the first category are conditions in which a genome mutation in a homeobox gene has been documented in humans and appears to be responsible for the disease. Many of these are specific syndromes presenting at or shortly after birth; others are diseases that arise in childhood or adulthood. The second category consists of diseases in which a homeobox gene appears to be normal but is misexpressed—either overexpressed or underexpressed. In these disorders it is not clear whether the abnormal expression is the main cause of the disease or whether another insult is the proximate cause leading to abnormal expression of homeobox gene(s). In the latter case, abnormal homeobox expression would help establish the phenotype of the disease. Finally, interest in homeobox gene mutations in cancer has been engendered by their role in determining cell fate. The third category comprises specific cancers in which a mutation of a homeobox gene has been identified. In these diseases, as well as some others in the table, other causes of the disease separate from homeobox gene abnormalities are likely.

SUMMARY

Several classes of genes are responsible for the development and differentiation of the embryo into the complex adult organ system. A major class of such genes is the group of homeobox genes, which control axial patterning and rostral-caudal definition, as

TABLE 8-1

Homeobox Genes and Human Diseases

Disease	Gene(s)	Relevant Study(ies)
Diseases Caused by or Associated With Homeobox Gene Mutations		
Birth Defects		
Hand-foot-genital syndrome (autosomal dominant)	*HOXA13*	Mortlock and Innis, 1997[125]
Synpolydactyly (autosomal dominant)	*HOXD13*	Muragaki et al, 1996[123]
Radioulnar synostosis	*HOXA11*	Thompson and Nguyen, 2000[126]
Schizencephaly	*EMX2*	Brunelli et al, 1996[185]
Aniridia, Peter's anomaly, anophthalmia; isolated foveal dysplasia	*PAX6*	*Respectively*: Glaser et al, 1994[186]; Azuma et al, 1996[187]
Waardenburg syndrome (type I, type III)	*PAX3*	Morell et al, 1997[98]; reviewed by Stuart et al, 1993[188]
Hypodontia	*MSX1*	Reviewed by Thesleff and Nieminen, 1996[189]
Craniosynostosis (Boston type)	*MSX2*	Reviewed by Richman, 1995[190]
Rieger syndrome	*RIEG*	Semina et al, 1996[158]
Combined pituitary hormone deficiency	*PIT1*	Reviewed by Rhodes et al, 1994[191]
Deafness with fixation of the stapes	*POU3F4*	Kok et al, 1995[192]
Congenital absence of pancreas	*PDX1*	Stoffers et al, 1997[93]
Hirschsprung disease; congenital central hypoventilation, neuroblastoma	*PHOX2B*	Gaultier et al, 2005[193]
Cardiac septal defects; hypoplastic left heart syndrome; tetralogy; Ebstein's anomaly; conduction defects	*NKX2.5*	*Respectively*: Kasahara and Benson, 2004[152]; Mittmann, 2004[153]; Konig et al, 2006[154]; Elliott et al, 2003[155]
DiGeorge/velocardiofacial syndrome	*HOXA3*	Reviewed by Goodman, 2003[136]
Currarino syndrome	*HLXB9*	Riebel et al, 2004[16]
Other Diseases		
Maturity-onset diabetes of the young type 4	*PDX1*	Stoffers et al, 1997[93]
Acute myelogenous leukemia	*HOXA9*	Reviewed by Lawrence et al, 1996[174]
Pre-B cell acute lymphocytic leukemia	*PBX1*	Reviewed by Nakamura et al, 1996[176]
T cell acute lymphocytic leukemia	*HOX11*	Reviewed by Nakamura et al, 1996[176]
Rhabdomyosarcoma	*PAX3, PAX7*	Reviewed by Sorensen and Triche, 1996[194]
Diseases Associated With Homeobox Gene Misexpression		
Congenital cystic adenomatoid malformation of the lung	*HOXB5*	Volpe et al, 2003[44]
Bronchopulmonary sequestration	*HOXB5*	Volpe et al, 2003[44]; Volpe et al, 2000[45]
Type 2 diabetes	*PDX1*	Del Guerra et al, 2005[86]
Hirschprung disease–associated enterocolitis	*CDX1, CDX2*	Lui et al, 2001[114]
Gastric intestinal metaplasia	*CDX2*	Silberg et al, 2002[108]
Bile duct carcinoma	*HOXB7*	Feldmann et al, 2006[75]

well as influencing the patterning of specific organs and the differentiation and function of specialized cells. Although it is yet too early to predict treatments of diseases based on homeobox gene functions, it is reasonable to hope that understanding the varied and complex function of these genes will provide unique insights that will translate into novel approaches to a variety of human disease conditions.

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9

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Apoptotic Cell Death

Cell death can proceed by different routes, either apoptotic or necrotic, which have been classified on the basis of biochemical and morphologic criteria. *Apoptosis* has come to be used synonymously with the phrase *programmed cell death* (PCD), because it is a cell-intrinsic mechanism for suicide that is regulated by a variety of cellular signaling pathways. Apoptosis is an energy-requiring process associated with nuclear condensation and fragmentation, cleavage of chromosomal DNA into internucleosomal fragments, and packaging of the deceased cell into apoptotic bodies without breakdown of the plasma membrane. Apoptotic bodies are recognized and removed by phagocytic cells, so inflammation is notably absent around the dying cell. Apoptotic cell death plays a crucial role in the development of the mammalian nervous system and also is a prominent feature of injury or disease of the brain.

In contrast with apoptosis, *necrosis* is a passive form of cell death and often is the result of a severe or overwhelming insult that damages cellular organelles such as mitochondria. It is characterized by cellular swelling, disruption of membrane integrity, and leaking of cytoplasmic contents into the extracellular space, thereby activating an inflammatory response. As such, massive necrosis is a severe threat to tissue. More recently, the idea that cells can commit suicide by mechanisms other than apoptosis has been gaining momentum. Alternative, nonapoptotic forms of PCD have been described and classified as autophagic cell death. *Autophagy,* meaning "eating of oneself," has been shown in many different organisms. In this process, cells switch to a catabolic metabolic program in which cellular constituents are degraded for energy production as a survival mechanism during periods of nutrient stress.[1] In addition, autophagy plays a degradative role by providing a mechanism for the turnover of both damaged organelles and long-lived proteins. Autophagy may contribute to the pathogenesis of some neurodegenerative disorders, including Huntington disease and Alzheimer disease, by altering the processing of mutant forms of the huntington protein and amyloid precursor protein.[2] In the context of neurodegeneration, autophagy has been shown to contribute to cell death following cerebral hypoxia-ischemia in neonatal and adult mice.[3]

APOPTOSIS

Apoptosis is an active process leading to the ordered dismantling of cellular components such as proteins and DNA. Specialized cysteinyl aspartate–specific proteases termed caspases have been shown to be key components of the apoptotic pathway.[4] Synthesized in the cytoplasm as inactive proenzymes, caspases are activated during apoptosis cleaving key cellular proteins, including DNA repair enzymes, regulators of the cell cycle and structural components, resulting in disassembly of the cell. Studies in the nematode *Caenorhabditis elegans* identified a number of genes involved in apoptosis, including Ced-3, which now has been shown to be a member of the caspase family. Some caspases (e.g., caspase-8 and caspase-9) act either as upstream initiators in the apoptotic pathway by processing and activating other caspases (such as caspase-3), which then in turn act as downstream executioners, cleaving cellular proteins.

At least two pathways to apoptosis exist[5] (Figure 9-1). Initiator caspases most commonly are activated by an extrinsic death receptor pathway or by the intrinsic mitochondrial pathway. In the *extrinsic pathway,* ligand binding to death receptors such as Fas or tumor necrosis factor (TNF) receptor in the cell membrane leads to receptor oligomerization. The activated trimeric death receptors undergo specific conformational changes on the cytoplasmic face of the plasma membrane, allowing the subsequent recruitment of adaptor molecules and, ultimately, an initiator caspase. These sequential protein-protein interactions lead to the formation of a death-inducing signaling complex (DISC) and caspase activation.[6] By contrast, in the *intrinsic pathway* of apoptosis initiated by stress or damage to mitochondria, cytochrome *c* is released from the mitochondrial intermembrane space into the cytosol after membrane depolarization and forms a complex (termed the apoptosome) with apoptotic protease-activating factor-1 (Apaf-1) (the equivalent of Ced-4 in *C. elegans*), adenosine triphosphate (ATP), and procaspase-9, which activates caspase-9 activation. Apoptosis thus proceeds by an ordered cascade of protolysis whereby initiator caspases (i.e., active caspase-8 and possibly caspase-10 from the DISC or caspase-9 from the mitochondrial pathway) cleave and activate the executioner caspases (such as caspase-3, -6, and -7). These act as downstream executioners by processing cellular proteins in order to facilitate disassembly of the cell. In some instances, executioner caspases can inactivate proteins that would disrupt apoptosis. For example, the DNA repair enzyme, poly(ADP ribose) polymerase (PARP), which catalyzes attachment of ADP ribose units from nicotinamide adenine dinucleotide (NAD) to nuclear proteins, is inactivated by caspase-3–mediated cleavage, resulting in an increase in the ATP available for energy-demanding apoptosis. In other instances, active downstream caspases can trigger enzyme activation. Thus caspase-3 cleaves the inhibitor of the caspase-activated DNase (ICAD), thereby destroying its ability to bind and repress the apoptotic endonuclease CAD. The end result of ICAD cleavage is CAD activation, with consequent initiation of DNA

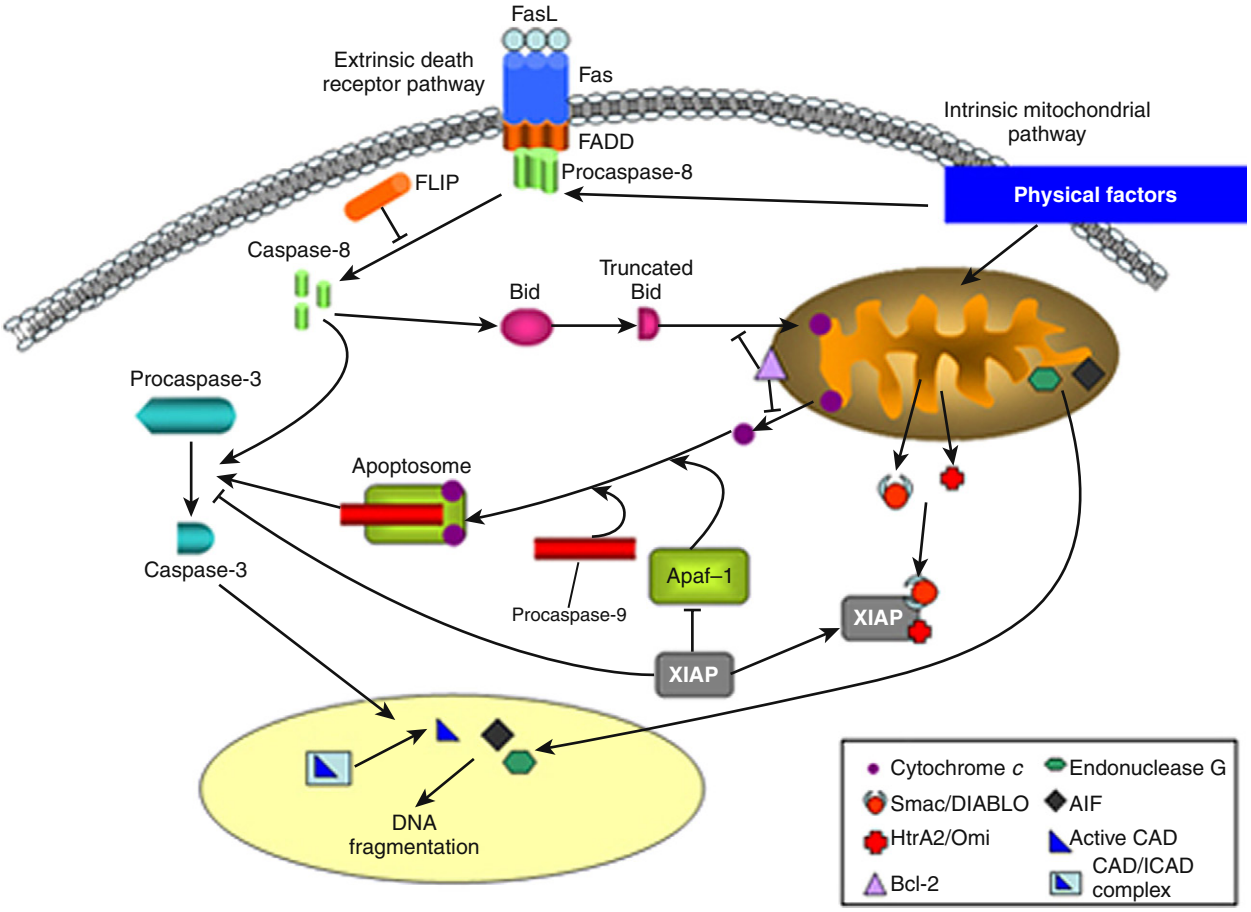


Figure 9-1 Schematic diagram of mitochondrial proteins involved in apoptosis. Stress signals such as DNA damage and accumulation of calcium ions (Ca^{2+}) and production of reactive oxygen species (ROS) in the mitochondria trigger the release of apoptogenic proteins including cytochrome *c (purple circle),* apoptosis-inducing factor (AIF) *(black triangle),* endonuclease G *(green hexagon),* and HtrA2/Omi *(red cross).* Release of at least some of these factors is inhibited by antiapoptotic Bcl-2 proteins. Once in the cytosol, cytochrome *c* can form a complex with Apaf-1 and caspase-9. The serine protease Omi/HtrA2 can sequester the X-linked inhibitor of apoptosis protein (XIAP), leading to enhanced caspase-9 activity. Both cytochrome *c* and Omi/HtrA2 in the cytosol enhance the activation of caspase-9, which in turn proteolytically activates caspase-3. Active caspase-3 then triggers apoptotic execution by activating downstream caspases and endonucleases. AIF translocation from mitochondria to nuclei in response to death stimuli triggers cellular changes, such as phosphatidyl serine exposure and chromatin condensation, that are independent of Apaf-1 and caspase-9. Endonuclease G is a mitochondrion-specific nuclease that translocates to the nucleus during apoptosis, where it cleaves chromatin-associated DNA into nucleosomal fragments independently of caspases. *CAD,* Caspase-activated DNase; *FLIP,* FLICE-inhibitory protein; *FADD,* Fas-associated protein with death domain; *FasL,* Fas ligand; *ICAD,* inhibitor of the caspase-activated DNase.

fragmentation, a key feature of apoptosis.[7] In the final stages, cell fragments are "shrink-wrapped" in the contracting plasma membrane and bud off as apoptotic bodies, which subsequently undergo phagocytosis.

Several regulators of apoptosis have now been identified as normal resident proteins in healthy cells. Bcl-2 originally was identified in follicular lymphoma as an antiapoptotic protein, supporting the concept that it is the combination of impaired apoptosis and increased proliferation in the same cell that ultimately leads to tumor formation. All members of the Bcl-2 family share at least one conserved region, termed the Bcl-2 homology (BH) domain. Other members of the Bcl-2 family that possess only the BH3 domain have now been shown to be proapoptotic (e.g., Egl-1, Bax). Some BH3-only proteins (e.g., Bid) are thought to act by binding to and preventing the action of antiapoptotic Bcl-2 family members, whereas others (possibly Bax) may integrate into the outer mitochondrial membrane and increase its permeability to proapoptotic messengers such as cytochrome *c*.[8]

Antiapoptotic family members also associate with the mitochondrial membrane as well as the endoplasmic reticulum and nuclear membranes. It is speculated that they act by maintaining membrane integrity and possibly preventing cytochrome *c* release from mitochondria. Inhibitors of apoptosis (IAP) family members act as endogenous caspase inhibitors.[9] They contain between one and three BIR (baculovirus IAP repeat) motifs that interact with capases, blocking their catalytic sites. Some IAPs also possess C-terminal RING domains that enable polyubiquitination of proteins and thus their destruction in the proteasome.[10] A number of other proteins have now been identified that are released from mitochondria after membrane depolarization and that activate apoptosis.[11] Among these, Smac/DIABLO and HtrA2/Omi each bind to and inhibit IAPs, thereby removing the block on caspase activation. Htr2A/Omi also is thought to activate caspase-independent cell death through its serine protease activity. It was recently reported, however, that the phenotype of mice lacking expression of HtrA2/Omi showed no evidence of reduced rates of cell death but in fact showed

a loss of a population of neurons in the striatum, resulting in a neurodegenerative disorder with a parkinsonian phenotype that led to death of the affected mouse approximately 30 days after birth.[12]

Early reports suggested that caspase-12, which localizes to the endoplasmic reticulum (ER) and is activated after ER stress including release of intracellular calcium stores, was the initiator caspase in ER st ress–induced apoptosis.[13] Recent reports have disproved this claim, however, and a sufficient body of evidence has accumulated to suggest an important role for caspase-12 in inflammation. Of interest, only 20% of people worldwide express the full length of caspase-12 protein; such persons have an increased risk for the development of sepsis.[14]

APOPTOSIS IN NEURAL DEVELOPMENT

The occurrence of PCD early in the development of the nervous system has been observed in a spectrum of organisms ranging from *C. elegans* to mice. A common feature of such PCD is that it takes place early during embryogenesis within the proliferative or newly postmitotic undifferentiated populations of cells in the nervous system and often is among the earliest cell death to be observed during development. It is now established that early neural PCD is essential for the normal development of the nervous system, and that the requirement for such early death is separate from that of the neurotrophic death occurring later. Although the triggers of early PCD are largely unknown, it is evident that much of developmental PCD occurs by apoptosis. Studies in mice have shown that the earliest PCD affecting the presumptive neural tissue is within the anterior distal epiblast, which is destined to become the neural plate during gastrulation at E6.5.[15] Apoptotic cells also are found in other regions including the hindbrain neural folds between embryonic days 8 and 9 (E8 and E9). Later, between E12 and E16, PCD occurs within the ventricular and intermediate zones of the cerebral cortex, which are composed of proliferating neural precursors and newly postmitotic neuroblasts.[16,17] Mutant mice defective in regulators of apoptosis (such as caspases) exhibit morphologic deformities in the central nervous system (CNS). Several mutants affected in the execution or regulation of apoptosis and abnormal closure of the neural tube show expansion of neural tissues before terminal neuronal differentiation, resulting in defects such as exencephaly and spina bifida.

A general principle of development in multicellular organisms is that excess numbers of cells are made, and then those surplus to requirement are selected to undergo PCD by apoptosis during the maturation of functional organs.[18] In the developing nervous system, neuronal and glial cell populations are shaped by apoptosis, which serves to adjust cell numbers, refine connections, and ultimately sculpt the sophisticated cytoarchitecture of the nervous system that supports its complex functionality. It is estimated that up to 50% of neuronal and glial cell types are lost during development as a result of limiting trophic support from target tissues that they innervate.[19] This is described by the *neurotrophic theory,* which suggests that it is the loss of trophic factor stimulation that initiates cell death. In other words, neurons that make connections with their target receive trophic support, which is required for active maintenance of the cells in a viable state, whereas neurons that do not make these connections die by apoptosis. Although this concept arose from studies of the nervous system, it is now recognized to be relevant to all tissues, including the innate immune system.

Finally, regardless of how a cell dies, the maintenance of normal tissue requires that cell to be removed; this removal is achieved by phagocytosis. In addition, neighboring cells become semiprofessional phagocytes to perform this function; however, the efficient recognition and ingestion of apoptotic cells, cell fragments, and necrotic cells in the brain are carried out by the microglial cells, the resident macrophages.

MODES OF CELL DEATH IN CEREBRAL INJURY

Accumulating evidence suggests that apoptosis can contribute to the pathogenesis of a number of human diseases, including cancer, viral infections, autoimmune diseases, neurodegenerative disorders, and AIDS. Although trophic factor withdrawal has a prominent role during nervous system development, it is not thought to be the primary pathogenic mechanism for cerebral injury or in neurodegenerative diseases, in which toxic insults are more likely to trigger the apoptotic pathway.

As mentioned previously, necrosis is triggered by stress or injury that damages cellular organelles, resulting in the loss of plasma membrane integrity, release of cytoplasmic contents, and inflammation. By contrast, cells dying by apoptosis do not lose membrane integrity, and the organelles remain largely intact. The apoptotic bodies formed are removed from the tissue by phagocytes. Because the apoptotic response to damage largely circumvents an inflammatory response, this mode of cell death would be particularly beneficial after injury to the brain, which has only a limited capacity for repair and regeneration. The initiation of inflammation is an important distinction between necrosis and apoptosis. In fact, evidence suggests that ingestion of apoptotic bodies can suppress an inflammatory response.[20]

Another key distinction between apoptosis and necrosis is the availability of intracellular energy. Apoptosis as a biochemically and genetically PCD requires time, energy, and, in some cases, new gene transcription and translation. As discussed later, however, the distinction between apoptosis and necrosis in vivo is not always clear-cut, and in many instances, these two modes of cell death can be regarded as a continuum of a single cell fate after injury.

APOPTOSIS AND NECROSIS SECONDARY TO HYPOXIA-ISCHEMIA

Although the precise mechanisms of brain damage in newborn infants secondary to birth asphyxia are multifactorial, an increasing body of evidence has accumulated showing that after cerebral hypoxia-ischemia, neuronal cell damage occurs not only through necrotic but also through apoptotic pathways. Necrosis can occur during hypoxia-ischemia or immediately after resuscitation, when blood flow is very low and when an acute deficit in substrate delivery results in a rapid reduction in cerebral energy production, membrane pump failure, and severe ionic imbalances with cell swelling and necrotic cell death.[21] These acute early changes may be particularly associated with an increase in excitotoxic amino acid levels and free radical production, the damaging effects of which can be blocked by specific receptor antagonists and scavengers.[22]

Apoptotic cells also are seen after hypoxic-ischemic injury in both the immature[23,24] and adult brain.[25,26] Administration of the antiapoptotic growth factor insulin-like growth factor-1 (IGF-1) after the primary insult ameliorates delayed injury; glutamate receptor blockade is only partially protective.[27,28] Apoptosis has been shown to be involved in human perinatal brain injury. In infants who die after intrauterine injury, either with or without evidence of hypoxia-ischemia, a significant number of cells in the brain show the morphologic characteristics of apoptosis.[29,30]

RELATIONSHIP BETWEEN APOPTOTIC AND NECROTIC DEATH IN CEREBRAL INJURY

It might be convenient to consider immediate cell death after hypoxic-ischemic injury as necrotic and delayed cell death as apoptotic. Published data do not entirely support such a simplifying concept, however. In experimental hypoxia-ischemia, apoptosis and necrosis can occur in adjacent populations of neurons and glial cells in the cingulate sulcus, whereas quantitative analysis of cell death show that the numbers of both apoptotic and

necrotic cells are linearly related to the severity of ATP depletion during hypoxia-ischemia.[31] Human infants who have suffered secondary energy failure have a preponderance of necrotic cells, whereas those dying in utero show a higher proportion of apoptotic cells.[29]

These complexities may arise in part from the fact that some necrotic cells represent the secondary degradation of apoptotic cells. In this context, primary cell necrosis can be regarded as an acute response to severe injury and is the common pattern of change in cerebral infarcts. Secondary cell necrosis can occur in cells triggered to begin apoptosis but unable to complete the program.[32] Cell necrosis thus should be distinguished from tissue necrosis, in which a large number of cells undergo necrosis together, which may be primary, secondary, or both.

Oxygen-glucose deprivation may induce apoptosis initially and then secondary necrosis in cerebellar granule cells.[33] Similarly, primary apoptosis precedes secondary necrosis after the injection of excitatory amino acid receptor agonists into the adult rat brain,[34] and in rat dorsal root ganglion cell cultures treated with oxidized low-density lipoprotein.[35] Studies with cultured neurons subjected to oxygen deprivation have confirmed that necrosis and apoptosis can occur in a single cell population.[36] In one such study, the administration of Trolox (6-hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid), which interferes with lipid peroxidation, prevented necrosis but allowed neurons to undergo apoptosis.[37] In a separate study, the induction of apoptosis in target cells by cytotoxic T lymphocytes was blocked by inhibitors of transcription or translation; instead, the target cells underwent necrosis.[38] Thus it may be possible to switch the fate of damaged cells between necrosis to apoptosis.

MOLECULAR EVIDENCE OF APOPTOSIS AFTER BRAIN INJURY

Although it is suggested that apoptosis occurring as a result of hypoxia-ischemia is morphologically different from developmental apoptosis, in that many hybrid necrotic-apoptotic phenotypes are seen, most biochemical studies agree that apoptotic processes are involved in hypoxia-ischemia. Indeed, key elements of apoptosis, such as caspase-3, Apaf-1, Bcl-2, and Bax, are up-regulated in the immature brain.[39] A number of molecular signals associated with apoptotic death are induced by impaired oxidative phosphorylation and oxidative injury.[40]

Caspases and Poly(ADP Ribose) Polymerase Cleavage

The nuclear enzyme PARP provides one of the most apparent links among oxidative stress, impaired energy metabolism, and cell damage. Free radicals such as NO and peroxynitrite can damage DNA, leading to activation of PARP, which catalyzes attachment of ADP ribose units from NAD to nuclear proteins. Excessive activation of PARP, however, can deplete NAD^+ and ATP (which is consumed in NAD regeneration), leading to an increase in the ratio of reduced (hydrogenated) NAD (NADH0 to NAD^+ and eventually cell death by energy depletion.[41] PARP is induced by hypoxia-ischemia, and genetic disruption of PARP provides profound protection against both glutamate-mediated excitotoxic insults in vitro and major decreases in infarct volume after reversible middle cerebral artery occlusion.[42] These results provide compelling evidence for primary involvement of PARP activation in neuronal damage. Genetic deletion of PARP or PARP inhibition by 3-aminobenzamide reduced infarct size after transient cerebral ischemia[43] but did not reduce the density of apoptotic cells.[44] The susceptibility of primary neurons toward apoptosis is unaffected in $PARP^{-/-}$ mice, suggesting that PARP activation is not necessary for apoptosis.[45,46] In cerebral ischemia, then, PARP may contribute to cell death by NAD^+ depletion and primary energy failure without direct involvement in apoptotic responses. However, PARP also is involved in apoptosis in another way: Specific proteolytic cleavage of PARP by caspase-3 is a mechanism to maintain the ATP needed for successful completion of the apoptotic program.[47] Both caspase activity[48] and PARP cleavage[49] have been shown to increase immediately after ischemic injury in vivo, followed several hours later by appearance of morphologic features of apoptosis.

Caspase inhibition can reduce neuronal loss from oxygen-glucose deprivation of cortical neurons[50] and hypoxic-ischemic injury in vivo.[51,52] Some results, however, suggest that although caspase inhibition may prevent the cellular manifestations of apoptosis, it does not prevent cell death.[53] For example, it was demonstrated that other caspase-independent mechanisms can contribute to neuronal cell death such as a translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus.[54] The precise roles of PARP cleavage and caspase activity in cell death after hypoxia-ischemia need to be defined further.

Gene Expression and Activation

Apoptotic death often is preceded by the induction and activation of a considerable variety of genes, including immediate early genes,[55,56] zinc finger genes, heat-shock proteins (HSPs), and nuclear factor-kappaB (NF-κB).[57] A number of these genes have been shown to possess either proapoptotic or antiapoptotic functions. For example, NF-κB[58] and HSPs[59] are thought to be protective against oxidative stress, whereas c-Jun, which is phosphorylated by stress-activated protein kinases (SAPKs), is strongly implicated in triggering neuronal apoptosis.[55]

In vivo studies of ischemic injury have shown that several SAPKs are activated by injury. A sufficient body of evidence has shown that Jun N-terminal kinase (JNK) plays a critical role neuronal cell death.[60,61] Studies using in vivo models of JNK-knockout animals or pharmacologic inhibition of JNK signaling have shown significant neuroprotection against hypoxic-ischemic stroke in neonatal and adult brain.[61,62] Moreover, the inhibition of excitotoxic pathways that involve glutamate receptor and JNK signaling also has been shown to be protective against kainite-induced neuronal toxicity.[63] It seems that different JNK isoforms have preferential function in neuronal cell death, because targeted disruption of the *jnk3* gene, but not *jnk1* or *jnk2,* protected mice from excitotoxic injury.[64] By contrast, disruption of *jnk1* or *jnk2* genes is associated with embryonic lethality, whereas *jnk3*-null mice are normal.[65,66] Furthermore, both neonatal[61] and adult[60] mice with deletions for *Jnk3* are protected against experimental hypoxia-ischemia, suggesting a critical role for *jnk3* in neuronal cell death.

Unlike with JNK, the involvement of p38 in neuronal cell death has been controversial. Although early activation of p38 has been shown to protect cells from apoptotic death induced by TNF treatment,[67] studies of CD40 and B cell receptor signaling have shown that p38 activation does not precede apoptosis triggered by the engagement of these receptors.[68] Conversely, the p38 inhibitor SB203580 is able to prevent glutamate-induced apoptosis in cerebellar granule neurons.[69] In addition, in vivo studies have shown that inhibition of p38 provides neuroprotection in cerebral ischemia, and that mitogen kinase-2 (MK-2)-null mice (MK-2 being directly regulated by p38) are partially protected from ischemic brain injury.[70,71]

The third subclass of MAPK, known as extracellular regulated kinases (ERKs), also is activated in response to neurotoxic stress factors. Although it has been documented that inhibition of ERK signaling can significantly protect against ischemic brain injury,[72] recent studies have demonstrated the important role of ERK in traumatic brain injury.[73] Taken together, the data show that all three subclasses of MAPK are selectively activated in a brain injury, depending on the nature of the neurotoxic stimuli.

Death Receptor Activation

The activation of SAPKs as one of the earliest signals downstream of death receptor activation provides evidence that intercellular signaling may be involved in triggering cell death during tissue injury. Receptors for proapoptotic signals including TNF and interleukin-1β (IL-1β) are expressed acutely in the injured brain and may contribute to the progression of neuronal damage following hypoxia-ischemia.[74]

One of the most extensively studied cytokine-responsive receptors is the Fas receptor. It was first described to play a role in death of immune cells but has now been found to function in other tissues, including the brain, and provides an example of the ontologic importance of the apoptotic mechanism in homeostasis and development. Fas is expressed in developing brain tissues, where it may be involved in signaling development. Moreover, a role in cell death following hypoxia-ischemia is suggested, because lack of functional Fas death receptor is neuroprotective in adult models of hypoxia-ischemia.[75,76] Fas expression also is up-regulated after hypoxia-ischemia in the neonatal brain where it functions as a death receptor.[77,78]Asphyxiated infants with pontosubicular neuronal necrosis secondary to severe perinatal asphyxia show marked increase in the expression of Fas receptor protein in degenerating neurons.[79] Fas expression is paralleled by the expression of several factors of the extrinsic apopotic pathway, including FLIP (FLICE-inhibitory protein), FADD (Fas-associated protein with death domain), and caspase-8 and -3, and by the translocation of cytochrome oxidase from the mitochondria.[80] In addition to the stimulation of the extrinsic death receptor pathway, abundant evidence shows that the intrinsic or mitochondrial cascade is activated after Fas death receptor signaling and functions to amplify Fas-mediated death.[81]

Other death receptors are present in the developing brain and have the potential to respond to their ligand with cell death signaling. For example,TNF receptor–null mice are more susceptible to hypoxic-ischemic injury,[82] whereas intracerebroventricular injection of recombinant IL-6 significantly reduces ischemic brain damage, suggesting that the large increases in TNF and IL-6 secondary to cerebral ischemia are endogenous neuroprotective responses.[83] Mice lacking the survival factor NF-κB are more susceptible to TNF-induced damage.[84] The marked disparity in up-regulation of the Fas receptor and that of other TNF-related receptors suggests fundamental differences in the role of these closely related receptors in brain development and in response to injury in the newborn period.

FACTORS DETERMINING THE CHOICE BETWEEN APOPTOSIS AND NECROSIS

A number of different factors influence whether a cell will undergo apoptosis or necrosis, including developmental stage, cell type, severity of injury, and availability of ATP.

Developmental Age, Differentiation, and Cell Type

The stage of cell development may be a particular determinant of apoptosis versus necrosis. For example, cerebellar Purkinje cells differentiate early in brain development. In the porcine model of hypoxic-ischemic injury, these cells are very sensitive indicators of the degree of injury but undergo only necrosis, never apoptosis. By contrast, cerebellar granule cells, that continue to divide and migrate after birth, undergo apoptosis on a large scale after hypoxic-ischemic injury.[85] Similarly, after excitotoxic injury to the newborn rat striatum, neuronal death occurs by apoptosis, whereas in the adult, the same insult produces rapid cytoplasmic disintegration, consistent with necrosis.[86]The mode of cell death also may depend on the degree of differentiation of the cell. Dividing progenitor cells are particularly sensitive to apoptotic stimuli, because the cell cycle machinery is intimately linked to apoptosis.[87] A number of separate studies also have indicated that specific cell types may be particularly sensitised to either necrosis or apoptosis.[88,89]

Severity of Injury

The same cell type can be triggered to undergo apoptosis after mild injury but necrosis if the damage is severe.[90]This dual possibility has been demonstrated in neuronal cells in a number of in vitro studies.[91,92] It is not clear precisely how the severity of injury dictates apoptosis or necrosis. One possibility is that the degree of damage to cell organelles influences the mode of cell death. Mitochondria are particularly sensitive to hypoxic injury and play a central role in both apoptosis and necrosis. For example, inhibitors of complex I trigger apoptosis at low concentrations and necrosis at high concentrations in neuronal cell lines in culture.[91] Indeed, it has been suggested that the severity of mitochondrial damage is the most important deciding factor between these two modes of cell death.[93] In this scenario, a small increase in mitochondrial membrane permeability can result in the controlled release of apoptogenic factors through the outer membrane, while severe mitochondrial damage releases a flood of Ca^{2+} and reactive oxygen species into the cytosol, leading to the disruption of plasma membrane integrity and cell necrosis. The importance of oxidative damage in this process is underlined by the finding that complex I inhibitor–dependent apoptosis is decreased by pretreatment with free radical scavengers,[94] although it remains to be determined whether free radical accumulation results directly from the block in the mitochondrial respiratory chain or indirectly from the apoptotic process itself.

Availability of Adenosine Triphosphate

As already mentioned, apoptosis requires energy, whereas complete ATP depletion causes necrosis.Thus ATP availability is likely to be a significant determinant of apoptotic versus necrotic death. If cells sustain a lethal insult, they will be able to execute the apoptotic program if ATP levels are sufficient, whereas in situations of limited ATP availability, the same degree of cellular injury can result only in necrosis. Consistent with this possibility, glutamate excitotoxicity has been shown to induce either necrosis or apoptosis, depending on mitochondrial function. During and shortly after exposure to glutamate, neurons that died by necrosis had reduced mitochondrial membrane potential ($\Delta\psi m$) and swollen nuclei. By contrast, neurons that recovered $\Delta\psi m$ and ATP levels subsequently underwent apoptosis.[95] Consumption of ATP by cell repair enzymes also may thus influence the mode of cell death. Inhibition of PARP prevents necrosis and triggers apoptosis in cells exposed to hydrogen peroxide.[96] Perhaps the most direct demonstration of the importance of ATP is in cultured mouse proximal tubular cells, where ATP depletion itself caused apoptosis or necrosis depending on the levels: Cells subjected to antimycin to deplete ATP to below 15% of levels in control cells died of necrosis, whereas cells with ATP levels between 25% and 70% of those in control cells all died by apoptosis.[97] Extending these observations, it also is possible to alter the mode of cell death by manipulating cellular ATP levels.[98,99]

Preserving ATP levels can thus reduce the proportion of necrotic cells after hypoxic-ischemic injury and increase the relative amount of apoptosis. Choi and colleagues demonstrated that primary cell necrosis secondary to severe ischemia is reduced by the addition of the *N*-methyl-D-aspartate antagonist MK-801. In their study, residual neurons underwent a form of cell death with the hallmarks of apoptosis.[100] On the other hand, protection from apoptosis can condemn cells to a necrotic death.Thus cells treated with agents that reduce $\Delta\psi m$ alone underwent apoptosis,

whereas those kept in identical conditions in the presence of the caspase inhibitor Z-VADfmk died from necrosis.[101]

Oxidative Stress

The newborn infant, especially the premature neonate, is thought to be particularly vulnerable to tissue damage from oxidative stress because of reduced total antioxidant capacity, and several studies have looked at the possibility of reducing morbidity in this age group by ameliorating the effects of oxidative stress.[102] Oxidative stress is considered to be a major mediator of apoptosis in several cellular systems, including neurons. Stimuli that cause oxidative stress, including culture in high oxygen,[103] exposure to β-amyloid,[104] and transient hypoxia-ischemia,[24,105,106] all trigger apoptosis. Both H_2O_2 and nitric oxide species (NO) can induce apoptosis when applied exogenously and are produced in toxic concentrations by cells that are undergoing apoptosis precipitated by other physiologic stimuli.

Oxidative signals for apoptosis can originate both extracellularly and intracellularly and also can be generated by reduced concentrations of antioxidants. Indeed, a number of cerebroprotective strategies are directed at specifically reducing oxidative stress.[107,108] A neuroprotective role for antioxidants has been documented using in vivo models of hypoxic-ischemic brain injury.[109,110] Cyclosporine, an inhibitor of prooxidant-induced Ca^{2+} release from mitochondria, has demonstrated a role for mitochondrial alterations in apoptotic processes. Cyclosporine can protect against loss of cell viability induced by prooxidants or by NO, can favorably alter mitochondrial function after ischemia,[111] and protects neuronal cells against apoptosis induced by Fe^{2+}, β-amyloid peptide, or NO-generating compounds.[112]

THE ROLE OF MITOCHONDRIA IN APOPTOSIS AND NECROSIS

Mitochondria as primary generators of cell energy are vital for cell homeostasis and viability. Disruptions or alterations in mitochondrial function can lead to cell death. Main events that may play a critical role in mitochondrial dysfunction, as discussed next, include ATP deprivation, elevation of intracellular calcium concentration and ROS production, and mitochondrial permeability transition and cell death, among others.

Adenosine Triphosphate Deprivation

One of the most important events is mitochondrial failure and falling ATP levels that can cause necrosis; direct inhibition of mitochondrial metabolism also can trigger apoptosis.[91,113] ATP is, however, required for apoptotic signaling, such as caspase activation and up-regulation of apoptotic genes. ATP regeneration during reperfusion of the ischemic brain is likely to promote neuronal apoptosis. Bioenergetic defecits also can result from failure in the electron transport chain, a compromised mitochondrial potential and reduced ATP levels. Such defects characterize many neurodegenerative diseases.[114]

Elevation of Intracellular Calcium Concentration and Reactive Oxygen Species Production

Ischemic injury causes a calcium influx into cells (through glutamate receptors), which then mediate excitotoxic cell death. Mitochondria become overloaded with calcium, leading to generation of reactive oxygen species (ROS), ATP synthesis reduction, depolarization of mitochondrial potential, and increased permeability of mitochondrial membrane. High concentrations of calcium also can contribute to uncontrolled activation of calcium-dependent proteases, calpains, and endonucleases, causing degradation of cellular proteins and DNA and cell death. Generation of ROS is an important event in cell signal transduction maintenance. However, a disruption of the respiratory chains results in elevated levels of ROS, causing a lipid and protein peroxidation that disrupts mitochondrial and other cellular membranes. Precise mechanisms are beginning to be elucidated, and much current interest is focused on the mitochondrial membrane permeability transition.

The Mitochondrial Permeability Transition Pore

One of the possible effects of free radical damage on mitochondria was first put forward in a hypothesis by Sulachev[115] and confirmed later by direct experimentation. The hypothesis suggested that the mitochondrial megachannel or permeability transition pore is involved in preventing ROS accumulation. High concentrations of ROS in the mitochondria would trigger opening of the pore, allowing release of free radicals into the cytosol. The resulting decrease in mitochondrial ROS would subsequently allow pore closure. Conversely, persistent free radical accumulation in mitochondria would prevent pore closure, leading eventually to the release of the apoptosis-inducing proteins. In this way, cells producing excess amounts of free radicals would be eliminated by apoptosis. In favor of this hypothesis, it is clear that the mitochondrial permeability transition pore is activated by ROS.[116]

The opening of the megachannel also can be triggered independently of ROS, although the result in all cases is a sudden increase in the permeability of the inner mitochondrial membrane to small molecules.[117] Although transient opening of the permeability transition pore may be involved in calcium homeostasis or free radical release, sustained permeability transition causes uncoupling of the respiratory chain enzymes, failure of ATP generation, and the release of specific apoptosis-initiating proteins such as cytochrome *c* into the cytosol (see further on). Although the exact composition of the permeability transition pore is not known, experiments using specific inhibitors of mitochondrial protein function have identified key components. For example, bongkrekic acid, a ligand of the mitochondrial adenine nucleotide translocator (ANT), abolishes mitochondrial permeability transition and inhibits p53-dependent thymocyte apoptosis induced by DNA damage.[118] Similarly, the protective effects of cyclosporine implicate cyclophilin D as a key component of the permeability transition pore. Other members include cytosolic hexokinase, porin (the voltage-dependent anion channel), creatine kinase, and proapoptotic members of the Bcl-2 family of proteins.[119] Although the regulation of Bax and Bak by antiapoptotic members of the Bcl-2 family is well known, more recent evidence suggests that additional proteins participate in the process, as both inhibitors and activators. Moreover, the rearrangements in mitochondrial lipids during apoptosis play a role in the permeabilization process that probably is more important than was first envisioned.[120]

Bcl-2 Family Members and Mitochondria

Bcl-2 is a mitochondrial membrane protein that blocks the apoptotic death of many cell types.[121] The precise mechanism of Bcl-2 protection still remains unclear; however, some evidence indicates that Bcl-2 regulates an antioxidant pathway at sites of free radical generation.[122] Moreover, the proapoptotic proteins Bax and truncated Bid interact with ANT and VDAC (voltage-dependent anion-selective channel protein) to cause alterations in mitochondrial permeability transition.[123] In addition, proapoptotic Bax and Bak may form channels that permit release of proapoptotic proteins from mitochondria.[124] However, new data indicating that Bcl-2 family members prevent the mitochondrial permeability transition[125] suggest that the inhibition of free radical generation may be a secondary effect.

Consistent with this role, cytochrome *c* release can be initiated by the proapoptotic protein Bax.[126] Furthermore, Bcl-xL

can physically interact with another mitochondria-derived protein Apaf-1, to inhibit its association (and subsequent activation) of caspase-9.[127] On the other hand, in some cell types, Bcl-2 cannot prevent or delay the decrease of the cellular ATP level subsequent to metabolic inhibition, suggesting that it blocks apoptosis at a point downstream of the collapse of cellular energy homeostasis.[128]

Although the precise mechanism of Bcl-2 protection is a matter of some debate, it has been shown to protect neurons from apoptosis secondary to cerebral ischemia. Overexpression of the human Bcl-2 protein under the control of neuron-specific promoters can reduce neuronal loss after permanent middle cerebral artery occlusion.[129] In addition, mice with deletions for the genes for proapoptotic Bcl-2 proteins Bim and Bad have been shown to be protected from neonatal hypoxic-ischemic brain injury.[130]

Mitochondrial Permeability Transition and Cell Death

In intact cells, an early consequence of mitochondrial permeability transition is a reduction in mitochondrial membrane potential, and at later time points a disruption of outer membrane integrity leads to the release of proteins that are involved in apoptotic execution.[131] Support for this proposal comes from the finding that supernatants from mitochondria undergoing the permeability transition can cause apoptotic changes in isolated nuclei.[132]

Much of the information regarding the identity of mitochondrial factors involved in apoptosis has been obtained from cell-free systems established to reproduce the apoptotic program in vitro.[133] In one such model, apoptosis could be initiated by addition of deoxyadenosine triphosphate (dATP) but also required cytochrome *c*. Intact cells undergoing apoptosis showed a translocation of cytochrome *c* (which normally functions as an electron carrier in the respiratory chain) from mitochondria to the cytosol,[134] which in turn results in the activation of specific caspases involved in apoptotic execution. Among these, caspase-3 is thought to be pivotal to apoptotic execution, because cells that lack detectable levels of this enzyme fail to undergo cytochrome *c*–dependent apoptosis.[135] Although caspase inhibitors substantially reduce mitochondrial membrane potential and cell death, they do not prevent the passage of cytochrome *c* from mitochondria to the cytosol, which must occur upstream of caspase activation.

Along with cytochrome *c* and dATP, Apaf-1 has been identified as an important protein that participates in the activation of caspase-3.[136] Apaf-1 shares significant homology with *C. elegans* Ced-4, a protein that is required for apoptosis in the nematode. A third protein factor, Apaf-3, also is required for caspase-3 activation in vitro. Apaf-3 was identified as a member of the caspase family, caspase-9.[137] Genetic studies suggest that caspase-9 is the most upstream member of the apoptotic protease cascade, and the formation of an Apaf-1/caspase-9 complex activates caspase-3 in a cytochrome *c*– and dATP-dependent manner.

The effects of cytochrome *c* translocation to the cytosol are paradoxical. Although ATP is required for apoptotic execution, the mitochondrial cytochrome *c* deficit eventually will result in shutdown of the respiratory chain, and consequently, ATP synthesis will cease. Thus Fas-driven apoptosis in Jurkat cells results in the inactivation of cytochrome *c* with cessation of oxygen consumption.[138] The converse is not always true, however: Other reports have confirmed that apoptosis can proceed in the absence of cytosolic translocation of cytochrome *c*.[139,140]

Nevertheless, cytochrome *c* release is an important link between mitochondria and apoptosis, in at least some systems. Consistent with this concept of the mitochondria as a controller of apoptosis, mitochondrial changes precede the activation of cytosolic enzymes involved in apoptotic execution, and inhibitors of the permeability transition pore can prevent apoptosis.[141]

Opening of the mitochondrial pore permits a free diffusion of proapoptotic molecules that are involved in both caspase-dependent and caspase-independent cell death. Although the involvement of AIF in apoptosis is still somewhat controversial, it has been shown that AIF is a major contributor to neuronal loss after neonatal cerebral hypoxia-ischemia.[142] Apoptosis is regulated on multiple levels by several mechanisms involving a number of proteins including X-linked inhibitor of apoptosis protein (XIAP), c-IAP1, c-IAP2, Smac/DIABLO, HtrA/Omi, and XIAP-associated factor-1 (XAF1). A recent report has demonstrated that the increased Smac/DIABLO levels are associated with the alteration of XIAP levels after forebrain ischemia in adults.[143] Nevertheless, the expression and function of these regulatory proteins in neonatal brain injury are not well understood.

FUTURE DIRECTIONS

It is now widely accepted that both apoptotic and necrotic cell death are important components of neuronal loss following injury to the developing brain. The distinction between apoptosis and necrosis in tissue injury is not always clear-cut, however. The mode of cell death is determined by a number of cellular factors, including developmental status, the severity of injury, and mitochondrial function. The complexity of the events involved in oxidative damage to the brain underlines the need for therapeutic approaches that will combat multiple mechanisms of damage and interrupt both apoptotic and necrotic processes. It is likely that antiapoptotic approaches will yield the most clinical benefit, because necrotic cell death is more likely to occur as an early consequence of brain injury. In this regard, it is perhaps encouraging that so many players in the apoptotic signaling pathway have been implicated in neural cell loss after hypoxic-ischemic injury, because researchers are now presented with a plethora of therapeutic targets. Over the next few years it will be important to determine which of these will be most effective in reducing apoptotic cell death resulting from neonatal brain injury.

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10 Angiogenesis

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Development of any tissue or organ requires the formation of a vascular network within the tissue, so vascular network formation within any tissue or organ can be viewed as a developmental requisite. Formation of tissue vasculature requires two distinct but related processes: vasculogenesis and angiogenesis. *Vasculogenesis* involves the formation of vessel tubes from the association of endothelial cell (EC) precursors or angioblasts, occurs solely in the embryo, and generally involves tissue of endodermal origin, including the heart tube and dorsal aorta, lung, pancreas, and spleen.[1] *Angiogenesis* is defined as the formation of new blood vessels from existing vessels,[2,3] is not limited to embryonic development, and is induced by inflammation, immune reactions, neoplasia, and wound healing.[4,5] Although these two processes are distinct, both are critical to embryonic development and the successful outcome of pregnancy, and they rely on many of the same molecular regulators.

A multitude of activators and inhibitors are involved in the regulation of vasculogenesis and angiogenesis.[6] During vasculogenesis, these factors induce differentiation of angioblasts to ECs, within embryonic and extraembryonic mesoderm, to form the initial vascular network[7] (Figure 10-1). The addition of new vessel segments to this vascular mesh, that is, angiogenesis, is required for the formation of a functional vascular system.[8] Formation of new capillaries, by both sprouting and nonsprouting (intussusception) angiogenesis, coupled with the remodeling and pruning of existing vessels,[8] provides for the extension and modification of the primitive vascular network (see Figure 10-1). At the same time, recruitment of perivascular cells (pericytes and smooth muscle cells) and deposition of the basement membrane provide stability and maturation of the developing vascular system (see Figure 10-1).

Although angiogenesis and the factors regulating it are important in postnatal tissues, the focus of this chapter is on the fetus and placenta and on the principal regulators of endothelium growth and morphogenesis.

EMBRYONIC/FETAL ANGIOGENESIS

The fetal circulatory system, consisting of the heart, blood components, and intraembryonic and extraembryonic vascular networks, develops out of necessity as the exponential growth of the embryo quickly outstrips the capacity of simple diffusion to meet the ever-increasing metabolic demands of the growing fetus. Definitive studies regarding the initiation of vasculogenesis and the development of extra- and intraembryonic vascular networks in the human fetus are essentially prohibitive, because yolk sac vasculogenesis begins at 16 days after conception and a

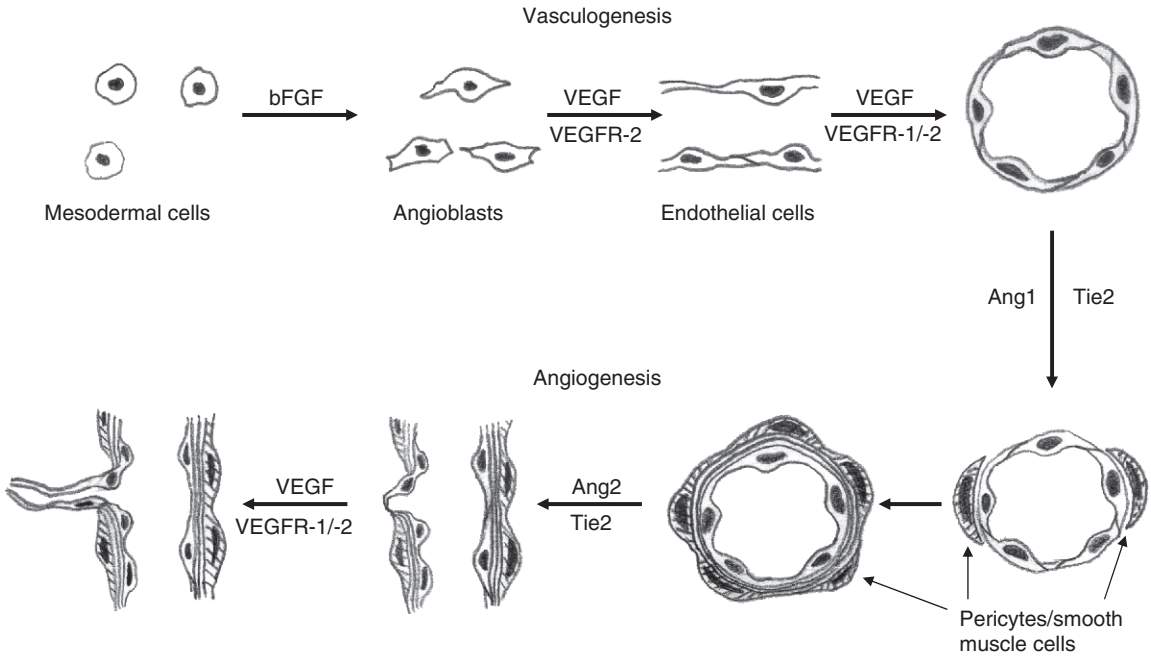


Figure 10-1 Schematic of blood vessel formation (vasculogenesis) and remodeling (angiogenesis). Embryonic mesodermal cells are stimulated by basic fibroblast growth factor (bFGF) to form angioblasts, which, under the influence of vascular endothelial growth factor (VEGF), form endothelial cells and vascular tubes. VEGF acts first through the VEGFR-2 receptor and then through VEGFR-1 and VEGFR-2. Angiopoietin-1 (Ang1), acting through Tie2, attracts pericytes around the primitive vessel, thereby stabilizing the vessel. The combined effects of VEGF and angiopoietin-2 (Ang2) provide for disruption of the vessel wall and for new vessel outgrowth or angiogenesis.

functional circulatory system between the two regions is present by day 21.[9] Therefore current understanding of human embryonic or fetal vasculogenesis and angiogenesis is derived from the conservative extrapolation and synthesis of in vitro observations, from clonogenic assays, embryonic stem cells, and embryonic explant cultures, as well as in vivo data derived from chimeric, transgenic, and knockin avian and mouse models.

De novo vasculogenesis is independently initiated in the extraembryonic (yolk sac and allantois) and intraembryonic (embryo proper) regions of the fetus.[10] Extraembryonic vasculogenesis precedes intraembryonic vasculogenesis as epiblasts, destined to become extraembryonic mesodermal progenitor cells, migrate to and localize at the proximal portion of the embryo and subsequently make ingress at the posterior region of the early primitive streak.[11] These early mesodermal cells migrate to and coalesce in the yolk sac, subsequently differentiating into either endothelial or primitive erythroid cells, and assemble into morphologically distinct structures called *blood islands.* Within the blood islands, the nascent ECs (i.e., angioblasts) surround the large, nucleated, primitive erythroblasts.[12] It has long been hypothesized that the concomitant appearance and close apposition of ECs and erythroid cells in the yolk sac are the result of the differentiation of these two cell lineages from a common progenitor, the hemangioblast. In vitro evidence demonstrates that the yolk sac blood islands are composed of cell populations that include individual enthothelial and hematopoietic progenitors, as well as bipotential progenitor cells from which both lineages may arise.[13-18] Definitive in vivo verification, however, is lacking. Support for the premise that blood islands are composed primarily of mixed progenitor cells (i.e., angioblasts, erythroblasts, and rare hemangioblasts) is provided by evidence that the extraembryonic vascular plexus is devoid of erythroid cells,[19] that differentiation of angioblasts directly from mesoderm cells occurs before the emergence of hemangioblasts,[18] that mapping studies indicate that the delineation of endothelial and hematopoietic lineages occurs during gastrulation,[20] and that multiprogenitor blood islands are present in tetrachimeric mice.[21]

Nascent yolk sac ECs assemble into strands around the erythroblasts, which remain in contact with the ECs and then coalesce with neighboring blood island ECs to form tubes and subsequently, a capillary plexus.[12] Inherent in this process is the delineation of the capillary bed into arterial and venous segments,[22-24] which then undergo rapid remodeling by sprouting angiogenesis and intussusception.[25,26] In the human, primitive, nucleated erythrocytes appear in the embryo proper concomitant with the onset of cardiac activity, thus establishing a functional vascular connection between the yolk sac and primitive heart by the end of the third week of gestation.[27] Yolk sac erythroblasts constitute a transient cell population which stop circulating from the yolk sac to the embryo by day 60 of human gestation[27] and gradually are replaced beginning at day 23 as hematopoietic cells emerge from the ventral floor of the dorsal aorta (hemogenic endothelium), the liver, and the bone marrow.[13,27-29] Unlike with the yolk sac, convincing evidence demonstrates that hematopoietic progenitors with the capacity to differentiate into multiple blood cell lineages, arise from ECs that make up the ventral aspect of the dorsal aorta.[30]

As the yolk sac regresses, the allantois emerges from the hindgut. The vascularization of the allantois, culminating in the formation and integration of the umbilical vessels into a functional placenta, is imperative for the survival of the fetus. The formation of the umbilicus proceeds by de novo vasculogenesis,[31] and much as with localization of the mesodermal cells to the yolk sac, the endothelial progenitors of the allantoic vessels cluster at the proximal segment of the embryo and exit the posterior primitive streak during the mid-streak stage to form the allantoic bud.[20,32] Once through the primitive streak, the mesodermal cells that make up the outer layer of the allantoic bud differentiate into mesothelial and chorioadhesive cells, proliferate, and subsequently fuse with the chorion. Next, the mesodermal cells in the core of the allantoic bud differentiate into angioblasts, followed by their assembly into nascent allantoic vessels—a process initiated at the distal (i.e., placental) end of the allantois and proceeding toward the developing embryo.[31,33] Much as in vasculogenesis in the yolk sac and in the embryo proper, the cells of the fledgling allantois, chorion, and placenta also can differentate into definitive hematopoietic cells.[34,35]

Development, integration, expansion and remodeling of intraembryonic vessels into a functional arterial-venous circulatory loop emanating from the heart and perfusing all tissues of the fetus, is a precisely coordinated, complex process incorporating both angiogenic and vasculogenic mechanisms.[10,25,26,36,37] Concurrent with the formation of the primary capillary plexus of the yolk sac, the heart, dorsal aorta, cardinal vein, and a sparse number of capillaries form within the embryo via de novo vasculogenesis.[10,38] Fate mapping indicates that unlike the epiblast progenitors of the yolk sac and allantois, the epiblast progenitors of the intraembryonic vasculature are medially localized and ingress at the anterior segment of the primitive streak during the mid-streak stage.[39] The newly formed mesodermal cells then migrate laterally to form the somatopleural or dorsal portion of the lateral plate mesoderm and the splanchnopleural or ventral layer of the lateral plate mesoderm.[40] Angioblasts, arising from the differentiating mesodermal somites, are dispersed and vascularize the body wall (i.e., limb buds), neural tube, and kidney.[41] By contrast, angioblasts arising from the differentiation of splanchnopleural mesoderm vascularize visceral tissues such as the lung, gut, pancreas, spleen, and liver.[29,42] Of interest, the aorta is composed primarily of ECs of somite origin, whereas the hemogenic wall of the aorta is composed primarily of ECs of splanchnopleural origin.[43,44] As the formation of hematopoietic cells from the ventral wall of the aorta ceases, the hemogenic endothelium is replaced by migrating somite ECs.[45]

PLACENTAL ANGIOGENESIS

Vasculogenesis and angiogenesis within the placenta probably are as important as embryonic angiogenesis for successful pregnancy outcome. Placental development begins early on and continues throughout gestation, as increased blood flow to the placenta is necessary to support the needs of the growing fetus. Beginning at the time of implantation, lacunae develop within the syncytiotrophoblast, coalescing to form the intervillous space, which will eventually fill with maternal blood. During this time frame, days 8 to 13 post coitus, the lacunae are separated by trabeculae composed of syncytiotrophoblasts. On approximately day 13 post coitus, maternal blood cells can be observed in the lacunae, and concomitantly the trabeculae are invaded by proliferating cytotrophoblasts, giving rise to the primary villi. Primary villi are quickly transformed into secondary villi as a result of mesenchyme cell invasion from the primary chorionic plate. Between days 18 and 20 post coitus, the first fetal capillaries are present within the mesenchyme, marking the development of the first tertiary villi.[46]

Early growth of the capillaries forms a primitive capillary network, and by 6 weeks post coitus, the basal lamina begins to form around the capillaries.[47] This primitive network is constantly being modified; the larger villi develop endothelial tubes serving as precursors of arterioles and venules. These tubes become surrounded by cells expressing α and γ smooth muscle actins, vimentin, and desmin.[48] Tertiary villi are transformed into immature intermediate villi, and after 15 weeks, the immature intermediate villi are transformed into stem villi. As the loose stroma becomes fibrotic and condensed, the peripheral capillary network condenses while the central capillaries differentiate into arterioles and venules.[49] The villous tree expands from its peripheral ends and gives rise to new villous outgrowth with new capillary networks.[47]

Increasing blood flow and pressure within the primitive vessels aid in stimulating further vessel development.[47] The first half

of gestation is characterized by branching angiogenesis, whereas from 26 weeks until full-term birth, angiogenesis within the placenta is predominantly nonbranching. Mature intermediate villi are produced in conjunction with increasing endothelial proliferation,[47] to form the main unit for nutrient exchange within the placenta. When the length of the intermediate villi vessels begins to exceed the length of the villi, formation of terminal villi occurs. Within the terminal villi, placental vessels lie very close to the intervillous space, such that placental and maternal blood supplies are separated by only a thin layer of syncytiotrophoblast. This arrangement provides a thin diffusion barrier, aiding in the transport of nutrients to the fetus.

ANGIOGENIC GROWTH FACTORS AND RECEPTORS

Angiogenesis is regulated through a complex balance between angiogenic and angiostatic substances exerting their influence at numerous levels. Promoters of angiogenesis include but are not limited to acidic and basic fibroblast growth factors, angiopoietin-1 and -2 (Ang1 and Ang2), placental growth factor, platelet-derived growth factor, transforming growth factor-α, tumor necrosis factor-α, and vascular endothelial growth factor (VEGF). Inhibitors of angiogenesis include but are not limited to angiostatin, endostatin, interferons, and platelet factor-4. For the purposes of this chapter, the focus of the following discussion is on the VEGF family and their receptors.

VASCULAR ENDOTHELIAL GROWTH FACTOR FAMILY

VEGF is a disulfide-linked homodimeric glycoprotein with a molecular weight of 40,000- to 45,000 that acts on ECs as a chemoattractant, mitogen, and morphogen.[50] VEGF is not a single protein, however, and comprises an entire family of gene products, including VEGF-A,[51] VEGF-B,[52] VEGF-C,[53] VEGF-D,[54] and VEGF-E.[55] As a result of differential splicing of the primary VEGF-A gene transcript, five isoforms of human VEGF-A are produced: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$. The larger isoforms, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, contain heparin-binding domains,[56] and although $VEGF_{121}$ and $VEGF_{165}$ are readily diffusible and are the predominant secreted isoforms, $VEGF_{189}$ and $VEGF_{206}$ remain membrane-associated[57] but retain their biologic activity. $VEGF_{145}$ appears to be specific to human endometrium.[58] An additional member of this gene family is placental-growth factor (PlGF), and through alternative splicing, three isoforms are generated: $PlGF_{149}$ (PlGF-1), $PlGF_{170}$ (PlGF-2), and $PlGF_{219}$ (PlGF-3).[59]

Adding to the functional complexity of this family are the ligand-receptor interactions that have been identified. Receptors for this growth factor family include three kinase domain receptors: fms-like tyrosine kinase 1 (Flt-1 or VEGFR-1),[60] kinase insert domain–containing receptor (KDR) (Flk-1 or VEGFR-2)[60] and fms-like tyrosine kinase 4 (Flt-4 or VEGFR-3).[53] The receptor VEGFR-1 binds homodimers of VEGF-A, VEGF-B, PlGF, or VEGF/PlGF heterodimers,[60] whereas VEGFR-2 appears to be specific for homodimers of VEGF. The third receptor, VEGFR-3, acts as the receptor for VEGF-C and VEGF-D, but it does not interact with VEGF-A.[61] Beyond the three classic receptors for the VEGF family, some tissues produce a soluble form of VEGFR-1, which could act as a functional antagonist.[62] Furthermore, the neuropilins (NP-1 and NP-2), previously described as receptors for semaphorins,[63] exist in human umbilical vein ECs and act as receptors for VEGF-A and PlGF.[64] NP-1 also acts as a receptor for VEGF-B[65] and VEGF-E.[66] The neuropilins could add another layer of regulation to the actions of VEGF and PlGF, because NP-1 and NP-2 act as receptors for $VEGF_{165}$ and PlGF-2, but only NP-2 acts as a receptor for $VEGF_{145}$.[64, 67] NP-1 not only binds $VEGF_{165}$ but also complexes with VEGFR-1,[68] a finding that led to the suggestion that VEGFR-1 acts as a negative regulator of angiogenesis by competing for NP-1.

Regulation

Various cytokines and hormones have been shown to stimulate production of VEGF, but the primary regulator of this family of angiogenic growth factors may well be oxygen tension. Low oxygen tension results in the up-regulation of VEGF-A, the down-regulation of PlGF,[69] and the up-regulation of VEGFR-1 and VEGFR-2.[70] Although this regulation has not been entirely characterized for all four of these proteins, hypoxic regulation of VEGF-A expression is mediated at both transcriptional and posttranscriptional levels. In the presence of low oxygen tension, VEGF-A mRNA is stabilized by the interaction of heterogenous nuclear ribonucleoprotein L with the 3′-untranslated region of VEGF-A mRNA,[71] thereby increasing the mRNA half-life.

At the transcriptional level, low oxygen tension allows hypoxia-inducible factor-1 (HIF-1), a basic helix-loop-helix transcription factor, to interact with a hypoxia response element (HRE) in hypoxia-induced genes such as the VEGF-A gene, thereby stimulating transcription.[72] HIF-1 is a heterodimeric protein composed of HIF-1α *(HF1A)* or HIF-2α *(EPAS1)* combined with HIF-1β *(ARNT).* The HIF-1β subunit is constitutively expressed, whereas HIF-1α and HIF-2α are regulated by oxygen tension. Under low oxygen tension, HIF-1 is stabilized, allowing it to interact with HREs in the promoters of responsive genes. Under normal oxygen tension, however, conserved proline residues in HIF-1α and HIF-2α are hydroxylated.[73] Prolyl hydroxylation allows targeting of HIF-1α and HIF-2α by the von Hippel-Landau tumor suppressor gene product (pVHL).[74] The HIF-1α and HIF-2α chains are captured by a pVHL-ubiquitin E3 ligase complex, are ubiquitylated, and are destroyed by cellular proteasomes.[75]

The importance of this regulatory system is borne out by targeted mutations in mice, in which inactivation of both alleles of *EPAS1, ARNT,* or *VHL* is associated with embryonic death,[76-80] with defects in cardiac function, angiogenesis, vascular remodeling, and placental vascularization. Additional regulation of HIF-1 activity, however, also may result from increases in HIF-1α or HIF-2α mRNA concentrations, from translocation of HIF-1 to the nucleus, and from recruitment of transactivators.[75]

Disruption of the genes encoding HIF-1α, HIF-2α, HIF-1β, or VHL resulted in phenotypes that infer the necessity of these genes in vasculogenesis, as well as in angiogenesis. Because these genes or their respective gene products are involved in regulating VEGF-A and its receptors, similar phenotypes could be predicted with disruption of the genes encoding VEGF-A, VEGFR-1, or VEGFR-2. Indeed, disruption of a single VEGF-A allele impairs early-stage vascular development, resulting in embryonic death around day 10.[81,82] Included in the processes impaired by VEGF-A deficiency are vasculogenesis, large vessel formation, capillary sprouting, and remodeling of the yolk sac vascular network. The severity of this phenotype[81,82] indicates that VEGF-A acts in a dose-dependent manner to maintain angioblast differentiation and survival. When expression of the primary receptors for VEGF-A was disrupted,[83,84] mice lacking functional VEGFR-2[83] exhibited the more severe phenotype. VEGFR-2–deficient mice exhibited a complete failure of vasculogenesis, EC differentiation, and hematopoiesis,[83] whereas disruption of VEGR-1[84] resulted in enlarged blood vessels as well as embryonic death. Disruption of the PlGF gene[85] resulted in normal development, potentially discrediting a critical role of PlGF in embryonic angiogenesis.

Mode of Action

Biologic activity of VEGF-A is mediated by binding VEGFR-1 or VEGFR-2, ligand-mediated dimerization of the receptor, and initiation of signals leading to EC migration and growth. VEGFR-2 is viewed as the predominant receptor in regards to aniogenic signaling and is associated with endothelial mitogenesis, migration,

cytoskeletal organization, vessel permeability, and cell survival. Within the intracellular domain, seven putative phosphorylation sites exist, with Tyr1175 being the major site of activity.[86] Phosphorylation results in phospholipase Cγ-1, protein kinase C, and mitogen-activated protein (MAP) kinase activation.[87,88] The latter pathway acts on MAP kinases such as ERK-1 and ERK-2 and stimulates mitogenesis.[89,90] The available evidence on the ability of VEGFR-2 to stimulate phosphotidylinositol-3-kinase activity is conflicting,[88,91] but c-Src and nitric oxide appear to play a role in VEGF signaling.[92] Src activation has been shown to play a role in stress-activated protein kinase-2–mediated cell migration,[93] and VEGF-stimulated endothelial nitric oxide synthase activity is abolished by the VEGFR-2 antagonist SU1498.[90] Finally, activation of VEGFR-2 induces the activation of Rho-dependent kinase (ROCK), and ROCK directly phosphorylates focal adhesion kinase, promoting cell migration.[94]

The intracellular domain of VEGFR-1 contains several tyrosine residues that can be autophosphorylated, although it generally is felt that these provide weak signals for cell growth and survival in postnatal life.[87] As with VEGFR-2, the major portion of VEGFR-2 activity occurs after phosphorylation of Tyr1169. This leads to MAP kinase and phospholipase Cγ-1 activation and EC proliferation.[95] The exact role VEGFR-1 plays in this pathway remains uncertain[89,96]; however, this and other work indicate that the pattern of VEGFR-1 autophosphorylation differs depending on whether VEGF-A or PlGF is the ligand. In contrast with VEGFR-2, the extracellular domain on VEGFR-1 can be secreted as a binding protein, as a result of alternative mRNA splicing.[97] The soluble form of VEGFR-1 is deficient in signaling and appears to act as a binding protein for VEGF,[87,98] the existence of which has been associated with preeclampsia.[99,100] The third receptor, VEGFR-3, contains five internal phosphorylation sites and is a primary modulator of lymphatic endothelial proliferation and survival.[87,101] VEGFR-3 activity is mediated through activation of protein kinase C–dependent MAP kinase, and also through the activation of phosphatidyl inositol-3-kinase and AKT (protein kinase B).[94]

Angiopoietins and Their Receptor

Ang1 and Ang2, although not part of the VEGF family, act to potentiate the actions of VEGF-A; Ang2 expression is induced by hypoxia and VEGF-A.[102] Ang1 acts synergistically with VEGF-A to stimulate angiogenesis,[103] whereas Ang2 is an antagonist to Ang1 and, in the absence of VEGF, is thought to destabilize or regress blood vessels.[104] It is believed that Ang1 is important in angiogenesis (rather than vasculogenesis[105]), during the remodeling of the vasculature into a hierarchical network of mature vessels composed of endothelial and adventitial cells, by recruiting and sustaining perivascular cells.[106] Both Ang1 and Ang2 interact with the receptor Tie2, with equal affinity[104]; whereas Ang1 induces maturation and stabilization of developing neovasculature,[107] Ang2 causes destabilization required for additional sprout formation[104] and branching angiogenesis. Ang1 and Ang2 are true antagonists, competing with equal affinity for Tie2,[104] and Ang1 is thought to be the natural agonist for Tie2. This concept is supported by the similar phenotypes exhibited by targeted inactivation of either the Ang1[107] or Tie2[108] genes, both resulting in defective vascular development and subsequent embryonic death. By contrast, transgenic mice overexpressing Ang1 appeared to be generally healthy; however, their skin exhibited greater numbers of large blood vessels.[107] Further studies with mice overexpressing Ang1 showed a greater incidence of leakage-resistant blood vessels in these animals.[109] Transgenic mice overexpressing Ang2[104] die during embryogenesis, with defects similar to those in mice harboring inactivated Ang1 and Tie2 genes[107,108]—a finding further supporting the concept that Ang2 is a natural antagonist to Ang1.

SUMMARY

In the overview of angiogenesis within the fetus and placenta presented in this chapter, the focus of the discussion was on a few of the many factors that may affect fetal and placental vascular development. Later chapters deal with angiogenesis within specific organs. Although the discussion was limited to the VEGF family and the angiopoietins, their receptors, and some of the regulatory mechanisms, it should still be obvious that the regulation of vasculogenesis and angiogenesis is extremely complex. Studies in mice harboring functionally disrupted genes encoding these growth factors, receptors, or regulators have provided invaluable insight into the absolute necessity for some of the vascular growth factors and their receptors in embryonic development and have underlined the importance of vasculogenesis and angiogenesis in embryonic development and survival. Finally, not only are VEGF-A and the angiopoietins important in vascular development of the embryo and placenta, but they also are produced and act on non-ECs within the placenta. Associated with these activities are alterations in vascular growth factor expression in placenta compromised by preeclampsia and intrauterine growth restriction, again highlighting the importance of these factors for successful pregnancy outcome. Although angiogenesis during fetal and placental development is a complex topic and is difficult to examine experimentally, it holds great potential as an area for development of therapies aimed at improving pregnancy outcome.

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SECTION II

Placenta and Intrauterine Environment

11

Hans-Georg Frank

Placental Development

EARLY DEVELOPMENT

As fetal membranes and uterine mucosa come into close contact, this contact zone defines the new organ placenta. Structurally, the constituting layers can just be apposed to each other or even be fused with each other; their functional connection always serves the purpose of maternofetal exchange of nutrients, gases, and waste substances. According to this definition, placental development starts at the time of implantation around 6 to 7 days after conception, as soon as the blastocyst starts invasion of the endometrium. At this stage, the blastocyst consists of an outer vesicular cellular cover, called the *trophoblast,* and of an inner cell mass, called the *embryoblast* (Figure 11-1, *A*). The placenta and the membranes are derived mainly from the trophoblast, whereas the embryoblast develops into the umbilical cord and the fetus and adds the mesenchyme to the placenta.[1]

Implantation and Lacunar Period

Before adhesion to the uterine mucosa, the blastocystic wall consists of a single layer of trophoblast cells (termed the cytotrophoblast). Those parts of the trophoblast that adhere to the mucosa and later invade it show an increased proliferation, resulting in a trophoblastic wall that is mostly double-layered. At the same time, the outer of the two layers shows disintegration of the lateral intercellular membranes. Thus it is transformed into a superficial layer of syncytiotrophoblast[2] (see Figure 11-1, *A*). Continuous proliferation of the inner cellular trophoblastic layer, together with subsequent syncytial fusion of some of the daughter cells with the covering syncytium, is responsible for an enormous increase in volume of syncytiotrophoblastic mass (prelacunar period) (see Figure 11-1, *B*). Those parts of the blastocystic wall that are not yet implanted consist of only a single layer of cytotrophoblast.[3]

At day 8 after conception, a system of confluent vacuoles appears in the syncytiotrophoblast at the implantation pole.[2] Their appearance marks the beginning of the lacunar period, which lasts from day 8 to day 13 after conception. These lacunae are formed only in the more central parts of the syncytiotrophoblast. The surrounding and separating lamellae and pillars of syncytiotrophoblast are called *trabeculae* (see Figure 11-1, *C*). This system of trabeculae and lacunae is covered by two uninterrupted syncytial layers: The basal layer, facing the endometrium, is called the *trophoblastic shell;* the superficial layer, facing the blastocystic cavity, is called the *primary chorionic plate.*

Starting at the primary chorionic plate at day 12 after conception, the cytotrophoblast invades the syncytial trabeculae and finally reaches the trophoblastic shell (see Figure 11-1, *D*). Trophoblastic proliferation inside the trabeculae is responsible for considerable longitudinal growth and for branching of the trabeculae. The branches that end blindly and protrude into the lacunae are the primary villi (see Figure 11-1, *D*). The trabeculae, from which they are derived, are called the *anchoring villi* because they connect the villous system with the trophoblastic shell. With the appearance of the first primary villi, the still-expanding lacunar system is called the *intervillous space.*

In parallel with these events, the trophoblast of the trophoblastic shell erodes maternal endometrial vessels as early as at day 12 after conception. Maternal blood leaving the eroded vessels enters the lacunar system through holes in the shell, thereby establishing a primitive maternal placental circulation (see Figure 11-1, *D*).

Meanwhile, the blastocyst is completely embedded in the endometrium and surrounded by endometrial stroma from all sides. The parts of the blastocyst surface that are implanted later pass through the same developmental steps as for the implantation pole. All data given in the following description are valid only for development at the implantation pole.

Villous Development

At day 14 after conception, mesenchymal cells from the embryonic disk spread along the inner trophoblastic surface of the blastocystic cavity, and there they transform into a loose network of branching cells, the extraembryonic mesenchyme.[1,4] From this date onward, the primary chorionic plate consists of three layers: the mesenchyme (the future connective tissue); a middle, discontinuous layer of cytotrophoblast; and a layer of syncytiotrophoblast facing the intervillous space (see Figure 11-1, *E*). Between days 15 and 20 after conception, cells of the extraembryonic mesenchyme invade the primary villi, transforming them into secondary villi. This mesenchyme never reaches the trophoblastic shell, so the segments of the anchoring villi that connect them to the trophoblastic shell remain merely trophoblastic. These trophoblastic segments are called the *cell columns* and consist of a voluminous cytotrophoblastic core and a thin, incomplete syncytial cover (see Figure 11-1, *E*). Their large share of cytotrophoblast is the main source for longitudinal growth of the anchoring villi. Some of the cytotrophoblast cells achieve an invasive phenotype and then migrate across the trophoblastic shell to invade deeply into the endometrium, forming an admixture with maternal tissue components—the so-called junctional zone (see later section, "Trophoblast Invasion").

Within a few hours of the mesenchymal invasion of the villi, some of the mesenchymal cells are transformed into macrophages that start secreting angiogenetic growth factors (see further on). Other mesenchymal cells differentiate into angiogenetic and hematopoietic precursor cells, the hemangioblastic cell cords. Around day 20 after conception, the first fetal capillaries are derived from the latter (see Figure 11-1, *F*).[5] The fetally vascularized villi are henceforth named the *tertiary villi.* Hematopoietic stem cells are derived from the same hemangiogenetic cell cords.

At the same time, the fetally vascularized allantois reaches the chorionic plate.[6] Allantoic vessels extend into the chorionic plate and even into the larger villi. There they fuse with the spreading

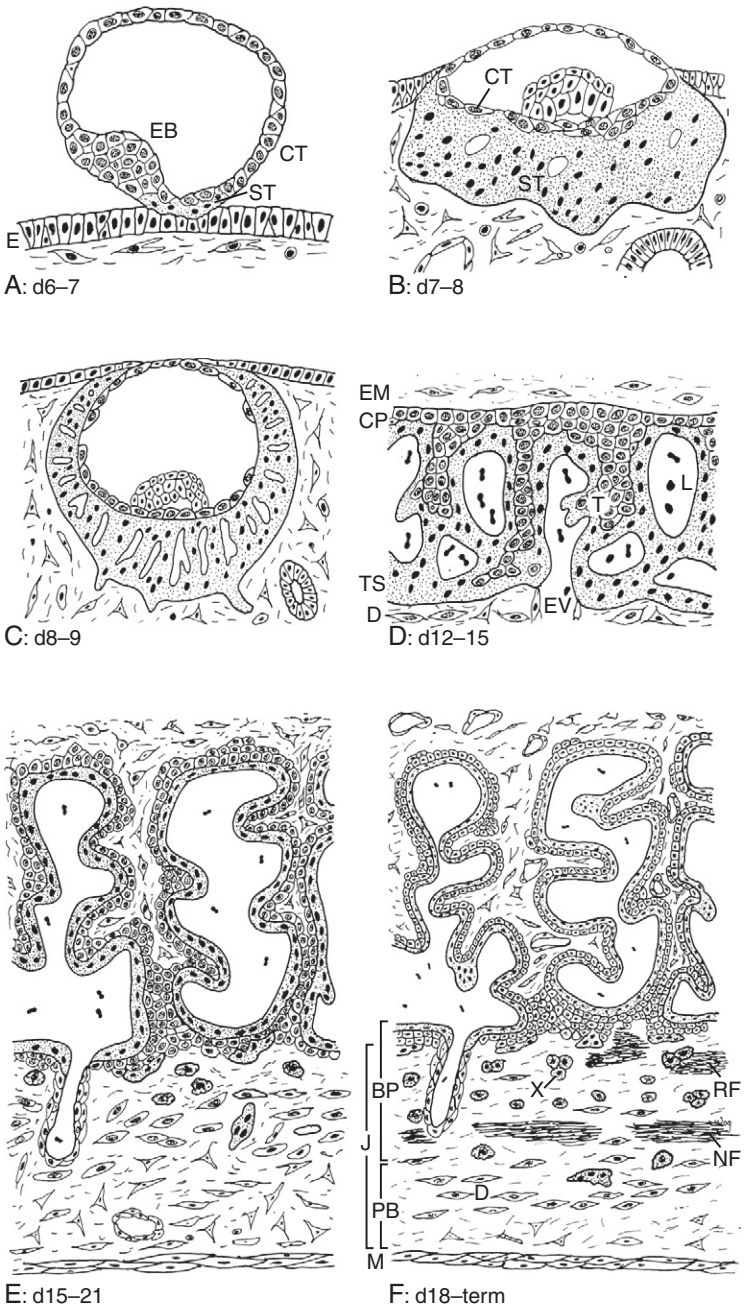


Figure 11-1 Typical stages of placental development. A, Implantation at 6 to 7 days after conception; **B,** prelacunar period (7 to 8 days); **C,** beginning of lacunar period (8 to 9 days); **D,** transition from lacunar period to primary villus stage (12 to 15 days); **E,** secondary villus stage (15 to 21 days); and **F,** tertiary villus stage (18 days to term). *BP,* Basal plate; *CP,* primary chorionic plate; *CT,* cytotrophoblast; *D,* decidua; *E,* endometrial epithelium; *EB,* embryoblast; *EM,* extraembryonic mesoderm; *EV,* endometrial vessel; *J,* junctional zone; *L,* maternal blood lacunae; *M,* myometrium; *NF,* Nitabuch fibrinoid; *PB,* placental bed; *RF,* Rohr fibrinoid; *ST,* syncytiotrophoblast; *T,* trabeculae and primary villi; *TS,* trophoblastic shell; *X,* extravillous trophoblast (X cells). *(Modified from Kaufmann P: Entwicklung der Plazenta. In Becker V, Schiebler TH, Kubli F (eds):* Die Plazenta des Menschen*, Stuttgart, 1981, Thieme Verlag, pp 13-50.)*

intravillous capillary bed. A complete fetoplacental circulation is established around the end of the fifth week after conception.[6] In newly formed villous capillaries, hematopoiesis can be observed even after this date.

Development of the Maternofetal Barrier

As soon as an intraplacental fetal circulation is established in the tertiary villi, fetal blood in the villi and maternal blood in the intervillous space come into close contact with each other (Figure 11-2). They are always separated by the placental barrier, which is made up of the following layers: (1) a continuous layer of syncytiotrophoblast, covering the villi and thus lining the intervillous space; (2) an initially complete (in the first trimester) but later discontinuous (in the second and third trimesters) layer of cytotrophoblast; (3) a trophoblastic basal lamina; (4) connective tissue derived from the extraembryonic mesoderm; and (5) fetal endothelium, which is surrounded by an endothelial basal lamina only in the last trimester (Figure 11-3). Throughout the following months of pregnancy, this barrier undergoes quantitative rather than qualitative changes; the thickness of the syncytiotrophoblast layer decreases is from more than 15 μm to a mean of 4.1 μm[6] (Table 11-1). Owing to the rapid expansion of the villous surfaces, the cytotrophoblast is relatively rarified and, at term, can be found on only 20% of the villous surface. The mean villous diameter decreases because the newly formed generations of villi generally are smaller than those of the previous generation. Accordingly, with advanced maturation, the intravillous position of the fetal capillaries is nearer to the villous surface. All of these factors result in a reduction of the mean maternofetal diffusion distance from between 50 and 100 μm in the second month down to between 4 and 5 μm at term gestation.[6]

PLACENTAL VILLI

The basic morphology of the placental villi can be seen in Figures 11-2 and 11-3. The villous core consists of fixed connective tissue cells, connective tissue fibers, macrophages (Hofbauer cells), some occasional mast cells, and fetal vessels. The stromal core is surrounded by the trophoblastic basal lamina, on which can be found variable numbers of cytotrophoblast cells (Langhans cells) and an outer, complete layer of syncytiotrophoblast.

Fetal Endothelium

Maternofetal exchange across the placental villi is focused on those villi (terminal villi and mature intermediate villi, see below) that are richly supplied with fetal capillaries. The capillaries are lined by a continuous endothelium without pores or fenestrations[6]; it acts as a passive filter, limiting macromolecular transfer across the vessel wall to a certain molecular size (below 20,000 Da).[7] In addition, permeability seems to be influenced by molecular charge.[8,9] The cells that surround the walls of the larger vessels (i.e., the pericytes and smooth muscle cells) are active in vasoregulation. Because nerves have never been observed in the placenta, vasoregulation must be accomplished by humoral means or local mechanisms, or both.[10]

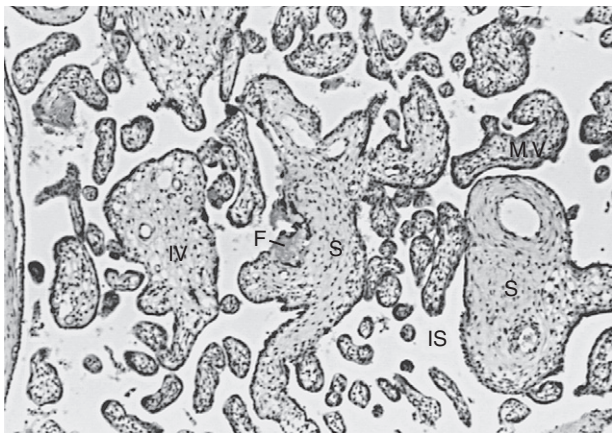


Figure 11-2 Paraffin section of placental villi, week 28 after last menstruation (×70). The assortment of villi of varied caliber is typical for the last trimester. Larger villi as stem villi *(S),* immature intermediate villi *(IV),* and mature intermediate villi *(MV)* are easily recognizable. The group of smaller villi in between, too small for identification in this figure, is composed of terminal villi, mesenchymal villi, and villous sprouts. *F,* Fibrinoid (attached to the villous surface, resulting from trophoblastic degeneration); *IS,* intervillous space.

Villous Stroma

The connective tissue cells of the vessel adventitia shade into the components of the surrounding villous stroma without any sharp demarcation line. Depending on villous type and age, mesenchymal cells, fibroblasts, or myofibroblasts will be the prevailing cell type.[11] These cells produce connective tissue fibers, which increase the mechanical stability of the villous core. In addition, macrophages (Hofbauer cells) can be observed in variable numbers. In addition to their phagocytotic activity, these are active paracrine cells that produce growth factors that regulate growth and differentiation of all villous components.[6]

Trophoblast

The trophoblastic cover of the villi is the main site for maternofetal transfer and for secretory functions. Most of these actions take place in the syncytiotrophoblast. The villous cytotrophoblast (Langhans cell) serves as a kind of stem cell, proliferating, differentiating, and afterward fusing with the syncytiotrophoblast.[12] Quantitative assessments of trophoblast proliferation and syncytial fusion have shown that it exceeds the needs for growth of syncytiotrophoblast by a factor of 5 to 6.[13] Throughout the last month of pregnancy, approximately 3.6 g of cytotrophoblast fuse syncytially per day. In this stage of pregnancy, however, the syncytiotrophoblast grows only by approximately 0.6 g per day. The daily excess production of 3 g syncytiotrophoblast is shed into the maternal circulation as syncytial knots that contain mainly aged syncytial nuclei and organelles. The reasons for this enormous turnover of trophoblast are not yet fully understood. Probably they are closely related to the apoptotic events underlying the process of syncytial fusion.[13-18]

TABLE 11-1

Summary of Mean Data on Placental Development*

Pregnancy week (postconception)	1, 2	3-6	7-10	11-14	15-18	19-22	23-26	27-30	31-34	35-38
Pregnancy month (postmenstruation)	1	2	3	4	5	6	7	8	9	10
Diameter of chorionic sac (mm)		8, 11, 17, 25	32, 40, 47, 55	62, 75, /, /						
Placental diameter (mm)				70	95	120	145	170	195	220
Placental weight (g) postpartum without clamping of cord		6	26	65	115	185	250	315	390	470
Placental wall thickness (mm) postpartum (mm)				12	15	18	20	22	24	25
Placental thickness, including uterine wall, measured by ultrasound in vivo (mm)					28	34	38	43	45	45
Length of the umbilical cord (mm)				180	300	350	400	450	490	520
Fetal weight (g) per g of placental weight		0.18	0.65	0.92	2.17	3.03	4.00	4.92	5.90	7.23
Villous volume (g) per placenta		5	18	28	63	102	135	191	234	273
Villous surface (cm^2) per placenta		830	3020	5440	14.800	28,100	42,200	72,200	101,000	125,000
Villous surface (cm^2) per g of villous tissue		166	168	194	235	275	313	377	432	458
Maternofetal diffusion distance (μm)		55.9		40.2	27.7	21.6		20.6	11.7	4.8
Villous trophoblastic thickness (μm)		15.4		9.6	9.9	7.4		6.9	5.2	4.1
Fetal vessel lumina per villous volume (%)		1.0	1.1		6.6		7.4		21.3	28.4

*All data refer to the end of the corresponding week after conception on to the end of the corrresponding month of the pregnancy.
All data from reference 6.
/, No data available for that week.

For syncytial fusion of cells, at least two contemporaneous signals are needed:

- To provide tissue specificity of fusion, the plasmalemmal exposition of fusogenic proteins is required; in the case of trophoblast the retroviral envelope protein HERV-W, also called syncytin-1,[19] was identified as one such fusogenic protein. It is expressed in syncytiotrophoblast.[20] Its receptor is the neutral amino acid transporter RDR that is expressed in villous cytotrophoblast.[21,22] Of interest, evidence for regulation of the expression of ligand and receptor is lacking; rather, both molecules seem to be constitutively expressed. It is, however, known, that trophoblast fusion is strictly regulated and depends on two prerequisites: (1) The cytotrophoblast must have reached a certain level of differentiation that makes it ready for fusion, and (2) the neighboring syncytiotrophoblast must show advanced stages of apoptosis, necessitating the incorporation of fresh cytotrophoblast for its survival.[6] These facts underline the need for an additional, immediate fusion signal.
- To act as such an acute fusion signal, phosphatidylserine flips from the inner to the outer plasmalemmal leaflet.[13,22] The outward flip of phosphatidylserine is provided by intracytotrophoblastic activation of initiator caspases that inactivate flippases and activate floppases.[14,23] The initiator caspases are intracellular proteases belonging to the molecular machinery of the early, still-reversible stages of the apoptosis cascade. Further progression of the apoptosis cascade in the freshly formed syncytiotrophoblast is blocked by the mitochondrial proteins Mcl-1 (myeloid cell leukemia-1) and Bcl-2, both of which inhibit cleavage and thus activation of execution caspases by the active initiator caspases. Both inhibitory proteins are transferred in large quantities from the cytotrophoblast into syncytiotrophoblast by the same fusion event.[13,24]

All cells entering the apoptosis cascade irreversibly leave the cell cycle, with consequent down-regulation of transcription to very low levels. Both kinds of apoptotic events have been demonstrated for syncytiotrophoblast. The nuclei of the latter do not incorporate ^{3}H-thymidine[25] and take up only very low quantities of ^{3}H-uridine.[13,26] Consequently, the syncytiotrophoblast depends on continuous incorporation of cytotrophoblast both for its growth and to ensure a continuous supply of mRNA for all translation processes. To enable functional mRNA transfer by a process of intercellular fusion, an unusual stability of mRNA

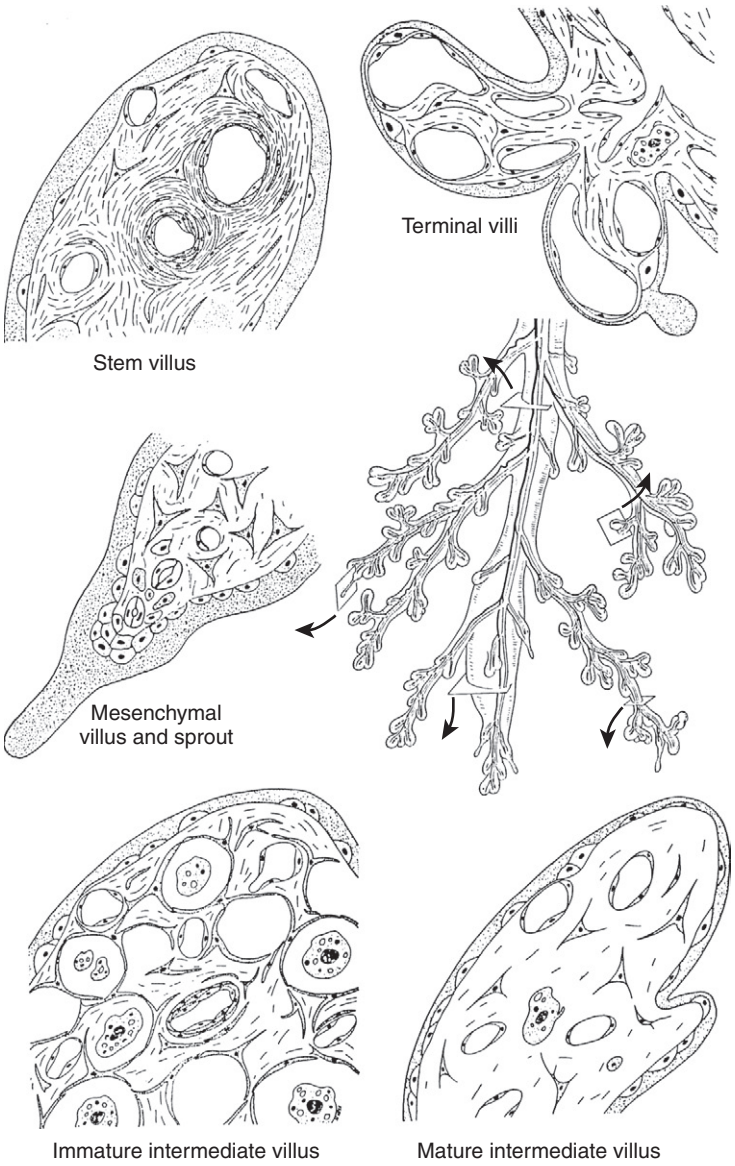


Figure 11-3 A peripheral part of the mature placental villous tree and typical cross-sections of the various villous types. *(Modified from Kaufmann P: Influence of ischemia and artificial perfusion on placental ultrastructure and morphometry,* Contrib Gynecol Obstet *13:517, S Karger AG, 1985, Basel.)*

levels is required. Such stability may be ensured by high placental concentrations of the RNAse inhibitor RNAsin.[27] Moreover, syncytial incorporation by fusion results in a kind of transplantation of fresh organelles and enzyme systems into the syncytium to replace the aged ones (Figure 11-4).

Removal of aged syncytiotrophoblast also involves apoptosis.[28] The nuclei incorporated by syncytial fusion into the syncytiotrophoblast become aggregated (see Figure 11-4). Parallel to this event, the apoptosis cascade is restarted and now passes through all execution stages until apoptotic death occurs, including activation of execution caspases, local degradation of the cytoskeleton, formation of membrane-protecting protein scaffolds by activation of transglutaminase II, activation of endonucleases with subsequent DNA degradation, and nuclear collapse.[13] The final stage of this cascade is the formation of the syncytial knots. Each of these knots is composed of 10 to 50 aggregated apoptotic nuclei, surrounded by some cytoplasm and an intact plasmalemma. The knots are then pinched off and shed into the maternal circulation (see Figure 11-4). At term, approximately 3 g of this kind of apoptotic trophoblast enters the maternal blood, from where these nuclei are removed by phagocytosis in the maternal lung. The mean survival time of a nucleus after syncytial fusion until shedding has been calculated to be approximately 3 to 4 weeks.[13]

Apoptosis is an energy-consuming process. In vitro and clinical studies[6,29-31] suggest that insufficiency of energy supply to the placenta results in abortion of the trophoblastic apoptosis cascade, to the benefit of necrotic trophoblast shedding. Necrotic trophoblast shedding products such as fetal DNA, fetal globular actin, and others have been shown to activate maternal macrophages and to damage maternal endothelium. These events are thought to be involved in the pathogenesis of preeclampsia.

As a consequence of the various stages of trophoblast turnover as described, the syncytiotrophoblast is composed of mosaic-like patches with variable ultrastructure, enzyme patterns, and functional specialization (see Figure 11-4)[6]:

1. The freshly fused, thick syncytial segments with predominant rough endoplasmic reticulum specialize in the secretion of proteohormones and placental proteins as well as in maternofetal protein transfer, including catabolism and resynthesis.
2. After degradation of ribosomes, the structurally similar segments, with predominant smooth endoplasmic reticulum, are involved in the metabolism of steroid hormones.
3. The epithelial plates or vasculosyncytial membranes are thin syncytial lamellae (1 to 2 μm thick) resulting from nuclear movement and removal. These are the main sites of diffusional transfer of gases and water and the carrier transfer of glucose.
4. True syncytial knots are a late to final stage of syncytial apoptosis, directly preceding shedding of the apoptotic material. Of note, however, not all syncytial protrusions in a histologic section of a mature placenta containing nuclear aggregates represent such true syncytial knots. Rather, many of these structures noted on histologic examination are tangential sections of the villous surfaces and are thus an unavoidable consequence of histologic sectioning.[32-34] Such artifical "trophoblast protrusions" are not only misleading mimics of apoptotic syncytial knots. In a similar way they are able to mimic trophoblastic

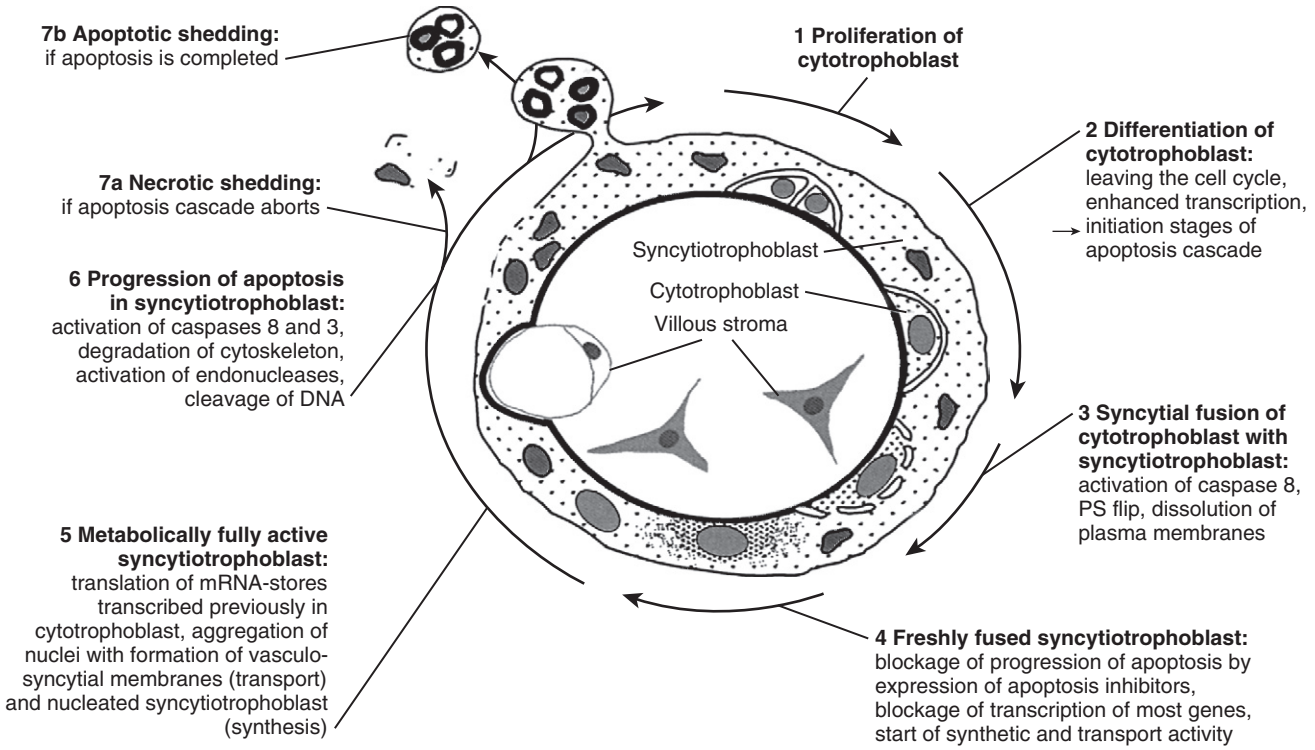


Figure 11-4 Villous trophoblast turnover. *Clockwise:* Proliferation *(1)* takes place exclusively in the cytotrophoblast cells, which act as stem cells. After differentiation of cytotrophoblast *(2),* syncytial fusion *(3)* is triggered by molecular mechanisms of early stages of the apoptosis cascade. On syncytial integration of the former cytotrophoblast into the syncytiotrophoblast, progression of the apoptosis cascade is blocked *(4).* As is typical for early apoptotic tissues, transcription is down-regulated to low levels. Therefore survival of syncytiotrophoblast depends on translation of mRNA, which is transferred into the syncytium by continuous syncytial fusion of differentiating cytotrophoblast *(5).* After more than 3 weeks of aging and restart of apoptosis *(6),* the accumulating apoptotic syncytial nuclei are shed into the maternal circulation. When the apoptosis cascade cannot be completed (e.g., in severe hypoxia), shedding is completed by necrosis *(7a/7b).* The complete cycle takes approximately 4 weeks.

(proliferative) sprouts in the early placenta. As a rule, true knots or sprouts are rare and section artifacts occur frequently.[33]

Integrity of the Maternofetal Barrier

The syncytiotrophoblast is a continuous, generally uninterrupted syncytial layer that extends over the surfaces of all villous trees; as such, it completely lines the intervillous space. In this context, therefore, use of plural terms such as *syncytial cells* or *syncytiotrophoblasts* is inappropriate. Because of the syncytial nature of this layer, it generally is believed that every substance passes from the maternal to the fetal circulation under the control of the trophoblast. Despite this view, physiologic evidence supports the existence of two different routes of transfer across the placental barrier, a transcellular and a paracellular route.[35] The transcellular route involves transfer across the plasma membranes and the cytosol of the syncytiotrophoblast. The paracellular route is thought to be an extracellular, water-filled pathway. Two different structural correlates for the latter route have been described:

- Integrity and completeness of the syncytiotrophoblastic barrier are not absolute. Gaps in the syncytiotrophoblast caused by degeneration or by mechanical forces are filled by fibrin as a result of blood clotting. Approximately 7% of the villous surfaces in normal term human placentas show corresponding fibrin spots as replacement of the syncytiotrophoblast.[36,37] Such areas may serve as relevant paratrophoblastic routes for maternofetal macromolecule transfer, thereby bypassing the syncytiotrophoblast; in vitro, maternally injected horseradish peroxidase (molecular weight, 48,000; molecular radius, 3.0 nm) was shown to pass through the fibrin spots into the fetal villous stroma.[36]
- Transtrophoblastic channels provide a paracellular transfer route for smaller molecules.[38-42] These channels, with a diameter of approximately 20 nm, pass through the syncytiotrophoblast as winding and branching, membrane-lined tubules from the apical to basal surface. Morphologic evidence for a basoapical membrane flux along these channels suggests that they are likely to be routes for membrane recycling from the basal to the apical syncytiotrophoblastic plasmalemma.[41] Functionally the transtrophoblastic channels are possible sites of transfer for water-soluble, lipid-insoluble molecules with an effective molecular diameter of less than 1.5 nm.[35,43,44] Moreover, they have been shown to be the sites of fetomaternal fluid shift, which is mediated by hydrostatic and colloid osmotic forces. In the guinea pig and in the isolated human placenta, an elevation of the fetal venous pressure of 5 to 17 mm Hg was sufficient to shift 30% to 50% of the fetal arterial perfusion volumes into the maternal circulation. Under the conditions of fetomaternal fluid shift, the channels dilate to several micrometers in diameter.[38,42] Pressure-dependent dilation and closure of the channels may act as important factors in fetal osmoregulation and water balance. Excessive fetal hydration results in an increase in fetal venous pressure and a decrease in osmotic pressure. Both factors have been experimentally proved to dilate initially narrow channels,[38,42] thereby allowing fetomaternal fluid shift and equilibration of the surplus water.

Villous Types

Human placental villi can be classified according to their position within the villous tree, the type of fetal vessels they contain, and their connective tissue structure.[45-48] Figure 11-3 illustrates a peripheral part of the villous tree containing all villous types described so far. The centrally positioned *stem villi* are characterized by centrally located arteries and veins or larger arterioles and venules surrounded by a stroma that is rich in connective tissue fibers.[49] Fetal capillaries are poorly developed. Most of the stromal cells are highly differentiated myofibroblasts that are oriented in parallel to the longitudinal villous axis.[50-52] They may be involved in longitudinal contraction of the stem villi (anchoring villi), thus regulating the width of the intervillous space and maternal blood flow impedance.[11] Such a fetal contractile system regulating the intervillous circulation is needed because the uteroplacental arteries are set free from vasomotor influences of the mother by physiologic changes in their walls[53] (see later). Approximately one third of the total villous volume of the mature placenta consists of this villous type.

The stem villi ramify into bundles of slender, slightly curved villi (see Figure 11-3) called the *mature intermediate villi.* Most of their vessels are fetal capillaries, in between which are small arterioles and venules.[54,55] The vessels are embedded in a loose connective tissue, with scant fibers and cells. These villi are the main sites of growth and differentiation of terminal villi.[48,49] Highly active enzyme patterns indicate their metabolic and endocrine activities. Approximately 25% of the villous branches are of this type.

The *terminal villi* are the final branches of the mature intermediate villi, with a morphologic resemblance to a cluster of grapes. Their bulbous peripheral parts are characterized by numerous dilated capillaries, so-called sinusoids, some of them with diameters up to 40 μm[54,55] (Figure 11-5; see also Figure 11-3). The extremely high degree of fetal vascularization and

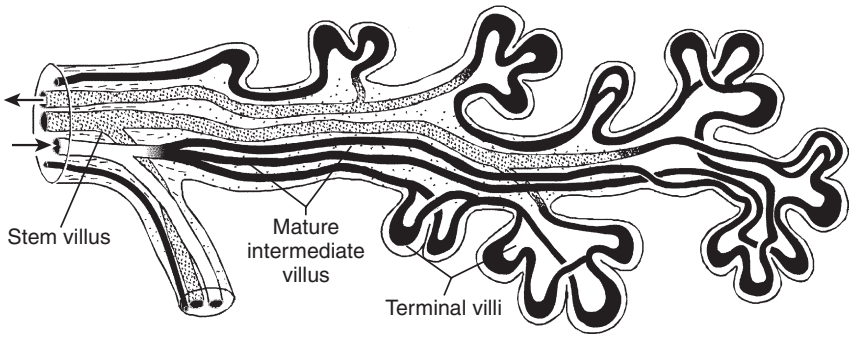


Figure 11-5 Arrangement of fetal vessels in a group of terminal villi *(no shading)* derived from one mature intermediate villus *(point-shaded).* Note the highly complex loop formation of the terminal fetal capillaries *(black).* Branching usually is followed shortly by refusioning of the two capillary branches. The branching pattern avoids basal shortcuts in such a way that each erythrocyte has to pass the terminal capillaries of several terminal villi in full length. Local dilatations of the capillaries in the terminal villi, so-called sinusoids, reduce blood flow resistance. Arterioles are *unshaded.* Venules are *dotted. (Modified from Benirschke K, Kaufmann P:* Pathology of the Human Placenta, *New York, 2006, Springer.)*

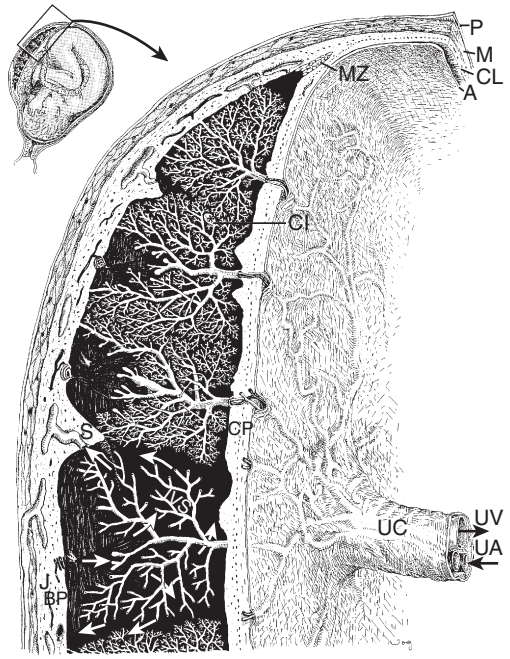


Figure 11-6 Survey diagram of the nearly mature human placenta in situ. Loose centers of the villous trees arranged around the maternal arterial inflow area are a frequent feature. These placentome centers usually exhibit immature patterns of villous branching and differentiation. *A,* Amnion; *BP,* basal plate; *CI,* cell island connected to the villous tree; *CL,* chorion laeve; *CP,* chorionic plate; *IVS,* intervillous space; *J,* junctional zone; *M,* myometrium; *MZ,* marginal zone between placenta and fetal membranes, with obliterated intervillous space and ghost villi; *P,* perimetrium; *S,* placental septum; *UA,* umbilical artery; *UC,* umbilical cord; *UV,* umbilical vein.

the minimal maternofetal diffusion distance (less than 4 μm) point to this villous type as the most likely place for diffusional exchange. The terminal villus volume amounts to 30% to 40% of the villous tree.

In some of these villous trees, stem villi are continuous with thicker bulbous villi; these are termed *immature intermediate villi*[47,48] (Figure 11-6; see also Figure 11-3). The typical structural features of immature intermediate villi include arterioles and venules, accompanied by numerous superficially located slender capillaries, and a voluminous, reticularly structured connective tissue that is rich in Hofbauer cells and poor in fibers. These villi can be found in the mature placenta only in small, restricted groups located in the centers of the villous trees[56] (see Figure 11-6). In immature placentas, they constitute the prevailing villous type. The main roles of immature intermediate villi are to act as precursors of stem villi (into which they are transformed continuously) and to provide a surface for sprouting of new villous side branches.[47,48]

The rarest and most inconspicuous villous type in the mature placenta is that of the *mesenchymal villi.* These villi represent a transient stage of villous development, derived from villous sprouts (see Figure 11-3). They differentiate via immature intermediate villi into stem villi or into mature intermediate villi. Because new sprouts are formed at the surfaces of mesenchymal and immature intermediate villi, they normally are found grouped in locally restricted sites. Structurally the mesenchymal villi can be identified by their slender shape, numerous Langhans cells, and poorly developed fetal capillaries. Some of the last-mentioned do not show lumina and are still developing and unperfused.[47,48]

Fetoplacental Angioarchitecture

Fetal arteries and veins are restricted to the stem villi, whereas arterioles and venules are mainly located in the smaller stem villi as well as in mature and immature intermediate villi.[48,54,55] All of the larger fetal vessels are accompanied by a system of long, slender capillaries, the so-called paravascular net.

In the mature intermediate villi, the arterioles and venules turn into long, coiled terminal capillary loops (see Figure 11-5).[55] At sites of maximum coiling, the capillaries stretch the surface of the mature intermediate villi, producing bulges—the terminal villi. Within the terminal villi, capillaries may dilate considerably, forming sinusoids (see Figures 11-3 and 11-5).[54] One capillary loop may supply several terminal villi in series,[54] dilating and narrowing several times for each single terminal villus. The probable function of the sinusoids is to reduce blood flow resistance and thereby guarantee an even blood supply to the long terminal capillary loops (mean length, 4000 μm) as well as to the shorter paravascular capillaries (mean length, 1000 μm).

Fetoplacental Vasculogenesis and Angiogenesis

Development of the fetoplacental vascular system entails two biologic steps, namely, *vasculogenesis*, which involves de novo formation of blood vessels from mesodermally derived precursor cells, and *angiogenesis*, which describes branching of new vessels from preexisting vessels.[57] Fetoplacental vasculogenesis starts at days 20 to 21 after conception by formation of hemangioblastic cords that shortly after develop into blind ending primitive capillaries containing blood precursor cells.[5,58] In the following phase of branching angiogenesis (day 32 to week 25 after conception), the primitive capillaries develop into a richly branched villous capillary bed that becomes connected to the allantoic vascular system of fetus and umbilical cord around the end of the fifth week after conception.[5,6] This highly branched early villous capillary bed has a low fetoplacental blood flow impedance.[58] This period is characterized by high placental levels of vascular endothelial growth factor (VEGF) but moderate placental growth factor (PlGF) expression.[57] During week 15, large centrally located villi show regression of peripheral capillary nets. In parallel, some remaining central capillaries acquire a tunica media and transform into arteries and veins.[6] Starting at approximately week 25, in the newly formed peripheral villi, angiogenesis switches from branching to nonbranching; this period is accompanied by a steep drop in VEGF and a slower decline in PlGF expression.[57,58] As a consequence of this switch, long, poorly branched capillary loops are formed in the periphery of the fetoplacental vascular trees. These loops increase fetoplacental impedance, but blood flow still increases as a result of rising fetal blood pressure. Both the biphasic changes in VEGF and PlGF levels and the resulting biphasic development of the villous vascular system are possibly due to a rise in intraplacental oxygen tension: Values are low in early pregnancy but steeply increase in the second trimester.[6,57] Abnormal placental oxygenation, resulting in abnormal levels of angiogenic growth factors, abnormal villous capillarization, and abnormal fetoplacental arterial impedance, are thought to be important factors in the pathogenesis of intrauterine growth restriction.[6,58,59]

Cotyledons and Villous Trees

The basal surface of the term placenta is characterized by 10 to 40 slightly protruding areas called *maternal cotyledons, lobes,* or *lobules*[57] (Figure 11-7, *B*). They are minimally separated from each other by the so-called placental septa[56] (see Figure 11-6). From the chorionic plate at term, 60 to 70 villous stems (trunci chorii) arise. Each of these trunks branches into a villous tree.[61] Each maternal cotyledon is occupied by at least one villous tree

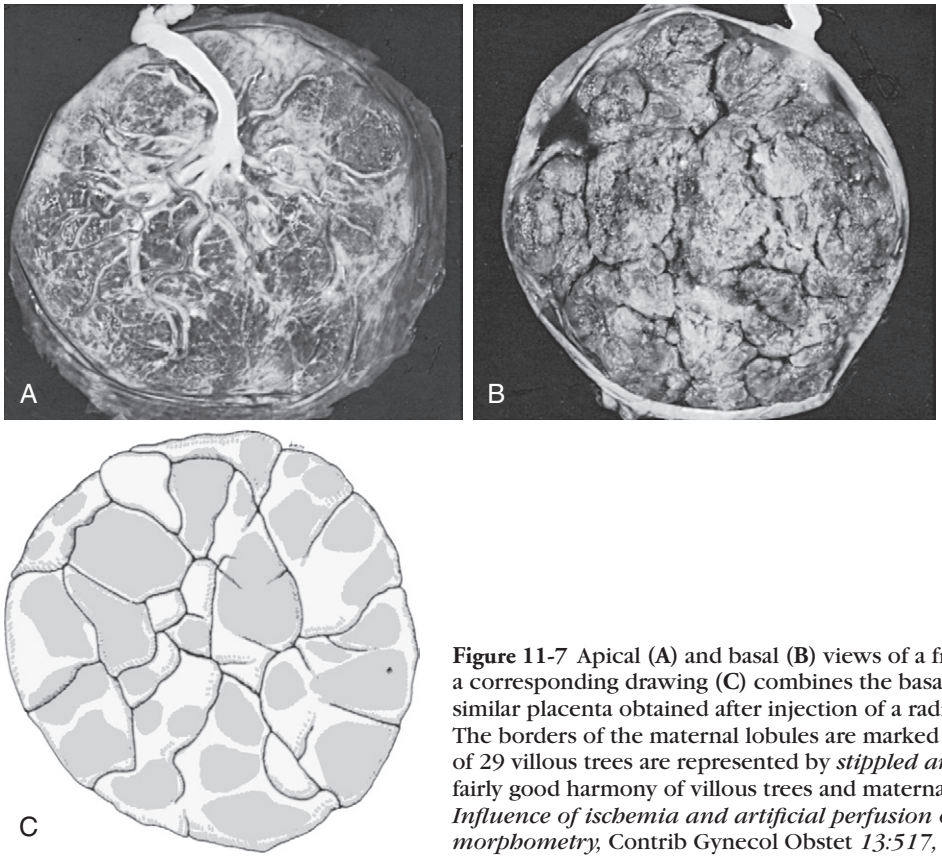


Figure 11-7 Apical **(A)** and basal **(B)** views of a freshly delivered mature human placenta; a corresponding drawing **(C)** combines the basal view with an x-ray photograph of a similar placenta obtained after injection of a radiopaque medium into the fetal vessels. The borders of the maternal lobules are marked by *lines.* The radiographic projections of 29 villous trees are represented by *stippled areas.* This combination demonstrates a fairly good harmony of villous trees and maternal lobes. *(Modified from Kaufmann P: Influence of ischemia and artificial perfusion on placental ultrastructure and morphometry,* Contrib Gynecol Obstet *13:517, Basel, 1985, Karger, AG.)*

(see Figures 11-6 and 11-7). As shown in Figure 11-7, *C*, the superficial cotyledonary borderlines are adjacent, to a large extent, to those of a corresponding group of villous trees.[56]

The human placenta belongs to the villous, hemochorial type of placentation: After leaving the spiral arteries, the maternal blood circulates through the diffuse intervillous space, flowing directly around the villous surfaces without any maternal vessel wall (see Figure 11-6). According to Schuhmann,[61] the arterial inlets normally are situated close to the centers of the villous trees. The venous openings in the basal plate are arranged around the periphery of the villous trees.

The fetomaternal circulatory unit, called the *placentone,* consists of a villous tree and the corresponding part of the intervillous space, which is centrifugally perfused with maternal blood[61] (see Figure 11-6). As depicted in Figure 11-7, *C,* every maternal lobe or cotyledon possesses at least one placentome. Owing to the insufficiency of placental septa (see further on), a majority of the maternal circulatory units belonging to the 40 to 60 placentomes overlap considerably.

The centers of typical placentomes exhibit loosely arranged villi, predominantly of the immature intermediate and mesenchymal types,[56,62] providing a large intervillous space for maternal arterial inflow. Occasionally the loose villous arrangement results in a central cavity (see Figure 11-6),[61] which may rapidly collapse after the placenta is delivered because it is a pressure-dependent in vivo structure. In the periphery of the placentomes, most villi are mature intermediate and terminal in type. They are separated by narrow intervillous clefts. Here, the maternal blood comes into intimate contact with the villous surface. Near the chorionic plate and at the border of neighboring placentomes, the villous arrangement becomes looser again, thus providing space for the venous backflow toward the venous openings in the basal plate.

The radioangiographic studies of Borell and co-workers[63] in the human placenta and of Ramsey and associates[65] in the rhesus monkey placenta support this placentome concept. Both studies have revealed a rapid filling of the centers of the maternal circulatory units, called *jet* or *spurt,* and subsequently a slow, centrifugal spreading of the blood toward the subchorial, lateral, and basal outflow area of the placentomes. In many places, the placentomes were seen to overlap so much that the typical differences between inflow and outflow areas vanished.[56]

As evident from the description of villous types, the immature centers of the placentomes act as growth zones where new formation and differentiation of villi take place. By contrast, the placentomes' periphery represents the metabolically active area and the fetomaternal exchange zone. The placentome centers are the remainders of completely immature villous trees from earlier stages of pregnancy. In normal pregnancy, they can be observed in at least some placentomes until term. They may disappear completely only in rare cases of preterm hypermaturity of the placenta.[6]

The heterogeneity of the villous trees poses problems for the histopathologic evaluation: Neighboring zones of the villous trees differ not only by the presence of different types of villi but also by different degrees of villous maturation. As a consequence, one histologic section never is representative for all parts of a villous tree or for all villous trees of a placenta.[65] This problem is greater still when small tissue samples are used, as for semithin histologic analysis and for electron microscopy. Neither technique allows a reliable diagnosis of the degree of maturation. The same holds true for histologic evaluation of samples obtained by chorionic villus sampling.

With regard to their structural arrangement and effectiveness, the fetomaternal blood flow interrelations are described as a multivillous flow system.[66] This designation implies that the fetal flow in hairpin-like capillary loops is crossed at various angles by the intervillous maternal bloodstream. Vessel arrangement and effectiveness, as defined physiologically,[66] ensure that the human

placenta does not exhibit a countercurrent flow system, as was proposed in earlier studies. The effectiveness of the system is further reduced by the fact that considerable amounts of maternal venous blood are recirculated by the arterial jet before leaving the intervillous space.

Intervillous Space

The usual appearance of the intervillous space of the delivered placenta is that of a system of narrow capillary-like clefts.[3,67] Boyd and Hamilton[3] questioned these interpretations. According to their findings on in vivo radioangiography, the rapid filling of the intervillous space is not compatible with a cleft system of capillary dimensions. The apparent width of the intervillous space (blood volume divided by one half of the villous surface area) ranges interindividually from 16.4 to 32 μm (assuming a postpartal intervillous blood volume of 23.3% to 37.9% and a villous surface area of 11.0 to 13.3 m^2).[6,28,68] In this context, an important consideration is the presence of a relatively large subchorial lake, as well as wide spaces in the arterial inflow area (central cavity). Thus the real "intervillous" volume in the exchange area is likely to be less and the intervillous clefts are likely to be narrower. With the addition of the considerable loss of maternal blood during labor,[69] however, these calculations may yield an approximation reflecting reality.

TROPHOBLAST INVASION

Trophoblast invasion is a peculiarity of all species showing interstitial implantation. This phenomenon is required for

- Anchorage of the placenta in the uterine wall
- Adaptation of the uteroplacental circulation to meet the embryonic/fetal requirements

Interstitial Trophoblast Invasion

Trophoblast invasion starts from the trophoblastic shell that is the base of the lacunar system at day 8 after conception (see Figure 11-1, *C* and *D*). In the beginning, it is a purely syncytiotrophoblastic layer. From day 13 after conception onward, cytotrophoblast enter the trophoblastic trabeculae and villi. Many of these cells remain in the villi, where they constitute the proliferating basis for later development and expansion of the villous trees. Others, which will become the extravillous trophoblast, reach and focally penetrate the trophoblastic shell (see Figure 11-1, *E*), from where they start invading endometrium and myometrium. The resulting mixture of maternal and fetal tissues stretches from the intervillous space down to the superficial third of the myometrium. It is described as the *junctional zone* (see Figure 11-1, *F*). Its superficial part, adhering to the placenta after placental separation, is the *basal plate*. Those parts that remain in the uterus after delivery are called the *placental bed* and consist mainly of intact and necrotic endometrial tissue, with intermingled trophoblastic cells.

With expansion of the placenta, within in few days the trophoblastic shell is disrupted into multiple clusters of proliferating extravillous trophoblast. Each of these clusters, a *trophoblastic cell column,* is located at the base of an *anchoring villus,* a stem villus that connects the villous trees to the basal plate.[70-72]

Trophoblast invasion is a truly invasive process during which the trophoblast cells penetrate their host tissue using a combination of migratory and proteolytic activities. The latter among others are based on the secretion of various matrix metalloproteinases[73] and serine proteases,[74] as well as urokinase and tissue-type plasminogen activators.[75] Unlike tumor invasion, however, trophoblast invasion in the placental bed stops within few days after delivery of the placenta. This raises the question as to the control of the process. Figure 11-8 summarizes the major differences between tumor and trophoblast invasion, accounting for local and time limitation of trophoblast invasiveness:

- Although tumor cells *proliferate* and invade at the same time, extravillous trophoblast cells achieve an invasive phenotype only after leaving the cell cycle. They proliferate only for as long as they are resting on the basal lamina of the anchoring villus. This separation of proliferation and invasiveness limits invasive depth to the life span of each single extravillous trophoblast cell.[76]
- Another regulatory factor may be provided by *integrin-matrix interactions.* Shortly after leaving the cell cycle, a majority of trophoblast cells start secreting ample extracellular matrix containing, among others, laminins, collagen type IV, heparan sulfate, vitronectin, cellular and oncofetal fibronectins, and fibrillin.[77-81] Of interest, extravillous trophoblast cells in late pregnancy with low invasiveness show a polygonal shape and express only those integrins that are receptors for the respective extracellular membrane (ECM) molecules (α6β4, α5β1, α1β1, and αVss integrins).[84] By contrast, in early pregnancy associated with highly invasive trophoblast, a majority of cells show a spindle-shaped body and a mismatch of integrins and surrounding ECM.[82]
- It is well known that normal extravillous trophoblast cells in vitro exhibit little invasiveness. This observation has elicited the question of whether cytokines up-regulate and control invasiveness in vivo. According to recent in vitro data, insulin-like growth factor-I (IGF-I), secreted by placental villous mesenchyme, is a very likely candidate stimulator of trophoblast invasiveness. This paracrine signal presumably is self-limiting because its concentration decreases with increasing invasive depth.[83]
- Another probable mechanism limiting trophoblast invasion is *syncytial fusion* (reviewed by Kaufmann and Castellucci[76]). The depth of the invasive zone close to the endometrial-myometrial border usually is marked by a layer of trophoblastic multinucleate giant cells that are derived from syncytial fusion and show no signs of further invasiveness.
- Finally, *apoptosis* of invasive extravillous trophoblast is induced by maternal macrophages by either of two mechanisms: (1) secretion of tumor necrosis factor-α (TNF-α) (the trophoblast cells express the respective TNF receptor I) and (2) tryptophan depletion (the maternal macrophages secrete tryptophan-catabolizing indoleamine 2,3-dioxygenase [IDO]) (see Figure 11-8).[84]
- The interaction between *maternal lymphocytes* and invasive trophoblast is still being investigated (as reviewed by Loke and King[85]). The relatively low number of T cells in conjunction with the absence of B cells at the site of implantation makes a classic allorecognition reaction of the trophoblast seem unlikely. This view is in agreement with the observation that the invasive trophoblast cells do not express classic polymorphic major histocompatibility complex (MHC) I but rather express an unusual combination of human leukocyte antigen (HLA)-C, HLA-E, and HLA-G. The absence of classic semiallogeneic MHC-I (HLA-A) molecules prevents attack by T lymphocytes, whereas the presence of the nonpolymorphic HLA-G precludes aggression by the uterine natural killer (NK) cells (large granular lymphocytes) (see Figure 11-8).
- Uterine NK cells are particularly numerous in the decidua basalis at the implantation site, where they come into close contact with invading trophoblast cells. Even if they do not interact with trophoblast invasion by direct killing activity, they may control the process by secretion of transforming growth factor-β (TGF-β), the receptor of which is expressed by trophoblast cells.[85] TGF-β has been implicated as an immunosuppressive factor in decidua by modulating the response of maternal leukocytes to trophoblast (reviewed by Loke and

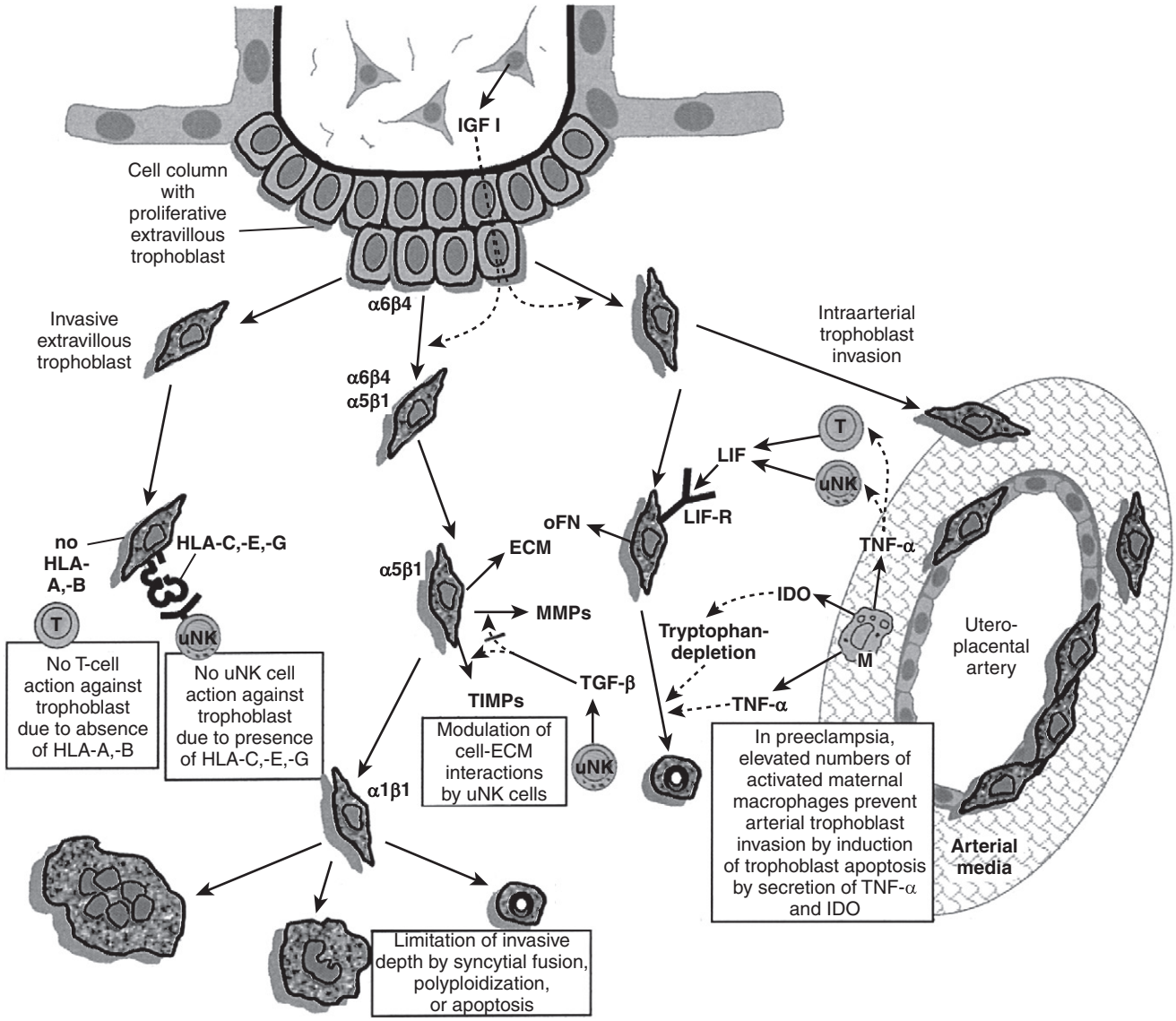


Figure 11-8 Maternofetal interactions among invasive extravillous trophoblast, maternal T cells *(T),* maternal uterine natural killer cells *(uNK),* and maternal macrophages *(M).* *Left invasion route:* Interaction between trophoblastic HLA exposition and maternal lymphocytes. *Central invasion route:* Gradual modification of integrin expression as well as modulation of ECM secretion. *Bottom:* Modes of limitation of invasive depth. *Right invasion route:* Some paracrine interactions between trophoblast and maternal immune cells; also shown: intraarterial trophoblast invasion and its inhibition by macrophages. See text for details. *ECM,* Extracellular matrix; *LIF/LIF-R,* leukemia inhibitory factor/leukemia inhibitory factor receptor; *HLA,* human leukocyte antigen; *IDO,* indoleamine 2,3-dioxygenase; *IGF-I,* insulin-like growth factor-I; *MMPs,* matrix metalloproteinases; *oFN,* oncofetal fibronectin; *TIMPs,* tissue inhibitors of metalloproteinases; *TNF,* tumor necrosis factor.

King[85]). This cytokine also restricts trophoblast invasion by down-regulation of collagenase secretion and induction of tissue inhibitors of metalloproteinases (TIMPs).[85] The inhibitory effect of TGF-β on trophoblast invasion is mediated by endoglin, a member of the TGF-β receptor complex. Moreover, TGF-β has been reported to enhance the secretion of fibronectins by trophoblast in vitro (reviewed by Benirschke and colleagues[6]). All of these observations suggest that TGF-β is a modulator of trophoblast invasion (see Figure 11-8).

Arterial Trophoblast Invasion

After erosion of the first endometrial capillaries at day 12 after conception, larger endometrial arteries (spiral arteries) and veins also erode and become connected with the intervillous space. General agreement exists that the number of corresponding maternal vessels that supply the placenta, although originally high, is considerably reduced by obliteration toward term gestation.[3] The final number of spiral arteries present in the term placenta is between 50 and 100.[3,86] The number of venous openings varies, ranging from 50 to 200.[3,86]

Pregnancy-induced changes in the uteroplacental vessels start in the second month of pregnancy. These affect primarily the uteroplacental arteries and only to a lesser extent the respective veins.[87-89] These changes obviously are induced by invasive extravillous trophoblast approaching the vessel walls. They start with funnel-like dilatation of the arteries (up to a diameter of 2000 μm), followed by dedifferentiation of the media smooth muscle cells, loss of all elastic elements, infiltration of the media with invasive extravillous trophoblast, and replacement of arterial

endothelium by so-called intraarterial trophoblast cells.[53,76,89-94] Earlier interpretations that these media changes are degenerative in nature[94] are no longer accepted[90-92]; rather, it was shown that the dedifferentiated arterial walls redifferentiate into intact media layers within a few days after delivery, with loss of extravillous trophoblast cells.[89]

The trophoblast cells infiltrating the arterial walls and replacing the arterial endothelium locally form huge intraarterial plugs, which were believed to inhibit uteroplacental blood flow. Whether plugging in the first trimester can completely block intervillous blood flow remains subject to further discussion.[6]

Moll and colleagues[53] and Brosens and co-workers[94] have pointed out that because of the degree of physiologic changes in uteroplacental arteries, these vessels must be set free from the vasomotor influences of the mother. This functional separation ensures unrestricted maternal blood supply to the placenta, regardless of maternal attempts to regulate the blood flow distribution within her own body; the mother cannot reduce the nutrient supply to the placenta without decreasing the nutrient supply to her own tissues.

Reduced physiologic vessel alterations, including reduced arterial dilatation and reduced intraarterial trophoblast invasion, are regularly observed in pregnancies complicated by preeclampsia, hypertension of pregnancy, and fetal intrauterine growth restriction.[86,95] By general agreement, failure of adaptation of the uteroplacental arteries to pregnancy conditions and thus preeclampsia and IUGR are thought to be closely related to impaired trophoblast invasion. However, several different hypotheses concerning the nature of this impairment have been proposed: The classic view is that of a general defect of trophoblast invasion,[96,97] possibly resulting from impaired integrin expression.[81] The inability of invasive trophoblast cells to express endothelial adhesion receptors, such as vascular endothelial cadherin (VE-cadherin), platelet endothelial adhesion molecule-1, vascular endothelial adhesion molecule-1, α4 integrins, and αvβ3 integrin, has been suggested as another possible mechanism.[98,99] Furthermore, recent data have shown that interstitial trophoblast invasion may be normal, whereas only the invasion of vessel walls is inhibited by activated maternal macrophages that secrete TNF-α and IDO, both inducing apoptosis in those trophoblast cells approaching the arterial walls[84,100] (see Figure 11-8).

PLACENTAL SEPTA AND CELL ISLANDS

Placental septa and cell islands are oddly shaped conglomerations of fibrinoid, intermingled with groups of trophoblastic and decidual cells. These structures are not vascularized. If they are connected to the basal plate, they are called *septa.* These septa are columnar or sail-like structures rather than real septa.[101] They cannot divide the intervillous space into separate maternally perfused chambers (see Figure 11-6). They are interpreted as dislocations of basal plate tissue into the intervillous space, caused by lateral movement and folding of the uterine wall and basal plate over each other. Parts of such septa detached from the basal plate and then attached to neighboring villi are called *cell islands.*

Similar islands, without decidual contribution, may be formed from villous tips that have not been opened up by connective tissue and fetal vessels during transition from primary to tertiary villi. In such cases, the cytotrophoblastic core proliferates and subsequently becomes largely transformed into fibrinoid. These cell islands are growth zones for the attached villous stems comparable to cell columns.[102,103]

CHORIONIC PLATE AND UMBILICAL CORD

At day 14 after conception, the primary chorionic plate consists of three layers: extraembryonic mesenchyme, cytotrophoblast, and syncytiotrophoblast (see Figure 11-1, *E* and *F*). These layers separate the intervillous space from the blastocystic cavity. Between 8 and 10 weeks after conception, the amniotic sac has expanded to such a degree that the amniotic mesenchyme comes into close contact with the mesenchymal surface of the chorionic plate and the chorion laeve (smooth chorion). As soon as amniotic and chorionic plate mesenchyme fuse with each other, the definitive chorionic plate is formed (see Figure 11-6).[104,105]

As part of the same process, the expanding amniotic sac surrounds the connective stalk and the allantois and joins them to form the umbilical cord. The allantoic vessels—two arteries and one vein—grow in thickness and length and convert into the umbilical vessels. The allantoic epithelium gradually disappears; small vesicular remnants of the allantois, however, may persist until term. The allantoic mesenchyme differentiates into a complex system of myofibroblasts that likely help regulating turgor of the cord and avoid bending of the latter with fatal consequences for the fetus.[106,107] Fusion of the umbilical vessels with the intravillous vessel system establishes a complete fetoplacental circulation at the end of the fifth week after conception. The cord is characterized by a spiral twisting, the number of spiral turns increasing as pregnancy progresses, up to a maximum of 380 turns. In most cases, the twist is leftward, or counterclockwise. The twists have been interpreted to originate with rotary movements of the fetus resulting from asymmetric uterine contractions.[6,108]

FETAL MEMBRANES

All developmental steps described so far are valid only for the implantation pole, which describes the part of the blastocystic circumference that is attached to the endometrium and implanted first. The other parts of the blastocystic circumference, implanted a few days later, undergo a corresponding although delayed development that is quickly followed by regressive changes. Collectively, these parts are called the *capsular chorion frondosum.* The first regressive changes can be observed as early as in the fourth week. The newly formed villi degenerate, and the surrounding intervillous space is obliterated. As a consequence of obliteration of the intervillous space, the chorionic plate, villous remnants, and the basal plate fuse, forming a multilayered compact lamella, the smooth chorion (chorion laeve). Formation of the smooth chorion starts opposite to the implantation pole at the so-called antiimplantation pole. From there it spreads over approximately 70% of the surface of the chorionic sac until the fourth month of gestation, when this process stops.[3,6] Between weeks 7 and 10 of pregnancy, the expanding amniotic sac comes into contact with the smooth chorion. In most places, the mesenchymal layers of both membranes fuse.

With complete implantation, the decidua recloses over the blastocyst, bulging into the uterine lumen, and is called the *capsular decidua.* With the increasing diameter of the chorionic sac, the capsular decidua locally touches the parietal decidua of the opposing uterine wall. Between weeks 15 and 20 after conception, both decidual layers fuse with each other, thereby obstructing the uterine cavity. From this point onward, the smooth chorion has contact over nearly its entire surface with the decidual surface of the uterine wall and may function as a paraplacental exchange organ. Owing to the absence of fetal vascularization in the smooth chorion and the amnion, all paraplacental material exchanged between fetal membranes and fetus has to pass through the amniotic fluid.

At term, the fetal membranes are structured as follows[6,109] (see Figure 11-6). The mean thickness of the membranes, after separation from the uterine wall during labor, is approximately 200 to 300 μm. The innermost layer, the *amniotic epithelium,* encloses the amniotic fluid. Amniotic epithelium may be involved in the production of the latter and even partially responsible for its resorption. Moreover, it seems to be involved in pH regulation

of the amniotic fluid.[110] Together with the *lamina propria,* it measures 30 to 60 μm in thickness.[111]

The next layer consists of *chorionic connective tissue,* which is directly adherent to a *cytotrophoblastic layer* of variable thickness.[112,113] Connective tissue and cytotrophoblast have a combined thickness of 80 to 120 μm. Near the placental margin, the thickness increases because persisting ghost villi, embedded in fibrinoid, split the cytotrophoblast into two layers. At the placental margin, these two layers completely disjoin by interposition of the intervillous space, and they become the chorionic and the basal plates (see Figure 11-6). Attached to the outer surface of the cytotrophoblast is a *decidual layer* approximately 50 μm thick. The latter finding indicates that the separation of membranes does not take place along the maternofetal interface but instead occurs at a somewhat deeper level.

Apart from recognition of the amniotic involvement in the production, resorption, and acid-base balance of the amniotic fluid,[110,113] definite and detailed knowledge about exchange and metabolic functions of the smooth chorion is lacking.

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12 Regulation of the Placental Circulation

Charles R. Rosenfeld

Pregnancy is associated with several alterations to the maternal cardiovascular system; however, it is the development of the low-resistance, high-flow placental vascular bed that characterizes this physiologic state. Moreover, this vascular bed is unique in that it is composed of maternal and fetal components separated by several cell layers that differ in structure and function among the mammalian species studied.[1] Although these two vascular beds are intimately associated with each other in all species, clearly they function independently of each other.[2-4] An understanding of the mechanisms that control their vascular tone and the magnitude of blood flow is important in that alterations in either the maternal or the fetal aspect of placental blood flow may potentially modify the delivery of oxygen or nutrients, or both, to the fetus, as well as affecting the removal of carbon dioxide and other fetal metabolic wastes.[5] Thus the integrity of the maternal uteroplacental and fetal umbilicoplacental vascular beds is essential to fetal growth and development, minute-to-minute fetal well-being, and possibly alterations in fetal programming and the subsequent occurrence of adult-onset cardiovascular disease.[6,7]

In view of the importance of the placental vascular beds to fetal well-being and survival of the species, research in this area has been intense since the time of Barcroft's research.[8] Because detailed studies in humans remain problematic and are generally descriptive, current understanding reflects in large part experimental findings in various animal models and, in particular, the pregnant ewe. Thus conclusions about the relative importance of the mechanisms responsible for the control of blood flow to these vascular beds in the human continue to require careful interpretation. Nonetheless, recent knowledge obtained from these models has been replicated in humans, providing an excellent basis for characterizing this important aspect of pregnancy and enabling improved interpretation of clinical data obtained in Doppler flow velocity studies in women.[9]

DEVELOPMENT OF THE UTEROPLACENTAL AND UMBILICOPLACENTAL VASCULAR BEDS

Although the placenta is of fetal origin and comprises solely fetal cells, its blood supply is derived from the maternal uterine and fetal umbilical vascular beds, the former serving as the source of placental and fetal oxygen and nutrients. Notably, both vascular beds undergo substantial independent modifications throughout pregnancy. For example, the maternal uterine vasculature undergoes remodeling.[10] and the fetal vasculature exhibits vasculogenesis, angiogenesis, and branching morphogenesis.[11] These changes are essential, occur in parallel, and contribute to the increasing requirements for oxygen and nutrient delivery necessary for the logarithmic fetal growth in the last third of pregnancy and the endocrine functions of the placenta. Understanding these independent yet parallel modifications will facilitate our knowledge of fetal growth and possibly the origins of some pregnancy-related diseases, for example, preeclampsia.

In women, the uteroplacental vascular bed is composed of cotyledons, lobes, or placentomes similar to those seen in many species.[1] In contrast with their counterparts in the sheep or cow, these structures adhere to each other, rather than existing as separate entities. Nonetheless, they can be identified as individual structures when viewed from the basal or maternal side of the placenta after its delivery. These placentomes receive their maternal arterial blood by way of the spiral arteries, and alterations in the tone of these or more proximal uterine arteries may be responsible for modifying the magnitude and distribution of maternal uteroplacental blood flow.[10,12] As in pregnant sheep, a fixed number of placentomes are ultimately available during normal human pregnancy. Therefore the increase in uteroplacental blood flow that occurs during pregnancy is initially due to the development of the placenta (i.e., implantation and growth) and vasodilation of the spiral arteries, allowing an increase in perfusion of the intravillous space.[13]

When maternal uteroplacental blood flow is examined throughout ovine gestation,[14] a complex scenario is observed.[12,13] Total uterine blood flow (Figure 12-1) increases approximately 40-fold during the course of a normal singleton pregnancy, and levels are even higher in multiple gestations. This increase in uteroplacental blood flow is coincident with a doubling of maternal cardiac output and an increase in the proportion of cardiac output directed to the uterus, increasing from less than 1% of cardiac output in nonpregnant sheep to 16% to 25% at term.[15] The pattern of this increase in total uterine blood flow (mL/minute) occurs in three phases (see Figure 12-1, *B*). The first is associated with relatively low absolute blood flows, which on the basis of uterine plus conceptus wet weight is actually high, achieving values of 0.8 mL/minute per gram, compared with 0.3 mL/minute per gram for the nonpregnant uterus (see Figure 12-1, *C*). This increase in uterine blood flow is thought to reflect vasodilation secondary to the increased production of maternal ovarian and possibly fetal hormones (e.g., estrogen, progesterone), which may be required for survival of the conceptus before implantation and the initial phase of implantation and placentation.[16,17] In primates and women, this occurs in the first days or weeks of pregnancy. The second phase is associated with the development of the placentomes and, in the primate, with the development of the intravillous spaces or the maternal placental vascular bed. At this time, the maternal placental vascular bed achieves its maximum size. If this does not occur, placentation may be restricted, resulting in a small placenta and a fetus characterized by proportionate growth restriction. In the ewe, total uterine blood flow plateaus at this time, averaging approximately 500 mL/minute; however, blood flow expressed on a weight basis at the same point in gestation, which takes into account metabolically active tissues, has fallen 50% to 0.3 to 0.4 mL/minute per gram, reflecting the ever-increasing weight of the fetus and placenta (see Figure 12-1, *C*). The final phase in the "growth" of uterine blood flow is exponential and associated with a threefold increase in fetal weight that occurs in the last third of pregnancy or after 110 days in sheep (75% of gestation) and beyond 30 weeks in humans (75%).[14] Because neither the total number of spiral arteries nor that of placentomes changes, this increase in uterine blood flow must be due to vasodilation.[13,14,17]

In more recent studies, this final vasodilation has been shown to involve increased activation of large-conductance

calcium-sensitive potassium channels (BK_{Ca}) in the uterine vascular smooth muscle and membrane hyperpolarization[18,19]; Although, the initiating events are unclear recent evidence suggests signaling involves nitric oxide synthase (NOS), nitric oxide (NO), cyclic guanosine monophosphate (cGMP), and cGMP-dependent protein kinase.[20] Moreover, similar mechanisms may also exist in women.[21] Of importance, the rise in blood flow in milliliters per minute is proportionate to and parallels the rise in wet weight of the uterus and its metabolically active tissues, which includes the growing fetus, membranes, and placenta. Thus blood flow per gram of wet weight remains low (0.2 to 0.3 mL/minute per gram) during the remainder of pregnancy. In the fetus with late-onset growth restriction (i.e., after 70% of gestation), blood flow per gram of fetoplacental mass is lower and nutrient and oxygen delivery is decreased. Fetal growth is attenuated until adaptation has occurred and this ratio is reestablished, at which time a new growth curve is established that lies below the curve for normally growing fetuses.

Although total uterine blood flow increases dramatically during pregnancy, this increase reflects changes in three separate vascular beds: the placentomes, endometrium, and myometrium. The change in distribution of blood flow to these tissues cannot be determined in women but has been studied in sheep and other species.[11,22] This limitation is an important issue because measurement of changes in total uterine blood flow in women will not reflect the issue of primary importance—that is, placental perfusion. As illustrated in Figure 12-2, blood flow to these three tissues is evenly distributed in the nonpregnant uterus, each receiving approximately one third. As pregnancy progresses, a gradual redistribution of uterine blood flow occurs, so that at term the placentomes (cotyledons) receive nearly 90% of total uterine blood flow. Thus, in the last third of pregnancy, the placentomes account for most of the observed increase in uterine blood flow.

Because the placentome is the site of nutrient and gas exchange, fetal well-being is determined by changes in placental growth and perfusion. Placental weight is relatively unchanged after the middle third of ovine gestation, yet maternal placental blood flow continues to increase (Figure 12-3). This discordance in placental growth and blood flow occurs earlier in pregnancy in women and other primates compared to sheep. The pattern of change in absolute blood flow (see Figure 12-3, *B*) resembles that seen for total uterine blood flow (see Figure 12-1, *B*). Because placental blood flow accounts for more than 65% of total uterine blood flow in the last third of pregnancy (see Figure 12-2), it is not surprising that the "patterns" are similar. The principal difference between the two patterns is obvious when blood flow per gram of metabolically active tissue is examined (see Figure 12-3, *C*). In contrast with the high value for total uterine blood flow early in pregnancy followed by a fall and leveling off, placental blood flow falls in midpregnancy and then increases until term. Thus placental blood flow rises from 0.5 to 1.0 mL/minute per gram to nearly 4 mL/minute per gram—a value five times greater than that observed for the total uterus. This increase in placental blood flow must reflect vasodilation, because no change occurs in either maternal arterial blood pressure (i.e., perfusion pressure) or in the number of maternal placental arteries.[11,13] The rise in maternal placental blood flow parallels a more general process of functional maturation of the placenta, which involves the simultaneous increase in fetoplacental perfusion[23] and permeability[24]; these physiologic changes are necessary to provide nutrients

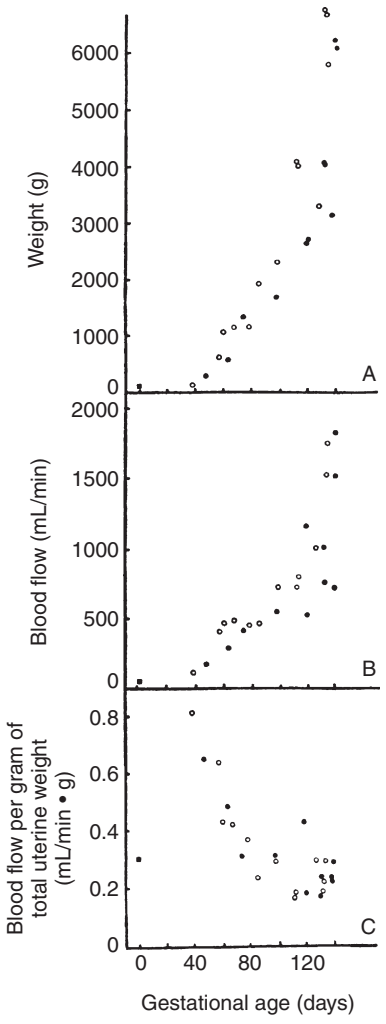


Figure 12-1 Changes in uterine weight **(A),** blood flow **(B),** and blood flow per gram of total uterine weight **(C)** during ovine pregnancy. Total weight is the sum of all metabolically active tissues. *Square,* nonpregnant; *open circle,* singleton pregnancy; *closed circle,* twin pregnancy. *(From Rosenfeld CR, Morriss FH Jr, Makowski EL, et al: Circulatory changes in the reproductive tissues of ewes during pregnancy,* Gynecol Invest *5:252, 1974.)*

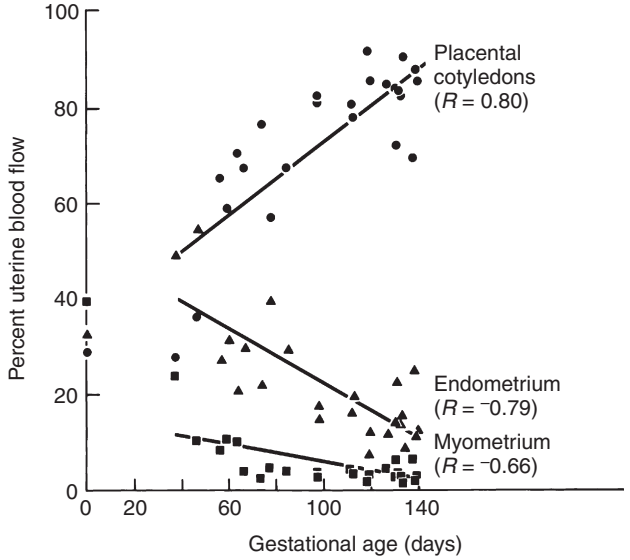


Figure 12-2 The distribution of uterine blood flow during ovine pregnancy (term = 145 days). Blood flow was determined using the microsphere technique. Correlation coefficients are significant at $p < .01$. *R,* Relative risk. *(From Rosenfeld CR: Consideration of the uteroplacental circulation in intrauterine growth,* Semin Perinatol *8:42, 1984.)*

and oxygen for the rapidly growing fetus. Of note, the volume of placental blood flow exceeds that required for normal fetal growth,[4,5] thereby providing a "margin" of safety that protects the fetus from episodic decreases in uteroplacental blood flow associated with transient increases in maternal plasma levels of vasoconstrictors (e.g., angiotensin II [ANG II]) or occurring at the time of parturition, when increases in myometrial tone may alter placental perfusion.[13]

Although much has been learned about the functional and anatomic aspects of the maternal uteroplacental circulation, few investigators have tried to describe in detail the pattern of change in umbilicoplacental blood flow over the entire course of pregnancy.This limited effort reflects the difficulty in performing such studies even in an animal model. Nonetheless, many similarities exist between species. For example, a villous stem originating from the fetal side of the placenta contains a distal branch of the umbilical artery and vein.As pregnancy progresses, a villous tree (in the human) or vascular bed originating from this villous stem undergoes angiogenesis and branching morphogenesis throughout pregnancy, providing an ever-increasing fetal placental vascular bed with an enormous surface area for nutrient and gas exchange. Although the sheep has no villous tree, continued growth of the fetal placental vascular bed occurs as in the human.[1,11] In both species, therefore the size of the fetal placental vascular bed and the magnitude of umbilicoplacental blood flow increase, the latter increasing exponentially in the last third of normal pregnancy.[23] In contrast with the rise in uteroplacental blood flow, which reflects vasodilation, the rise in umbilicoplacental blood flow is due primarily to vascular growth and, to a lesser extent, vasodilation. Thus umbilicoplacental blood flow remains approximately 200 mL/minute per kg of fetal weight throughout gestation in most species. Conditions that attenuate the growth of the fetal placental vascular bed in the last trimester (e.g., pregnancy-induced hypertension) or increase fetal placental resistance may result in fetal growth restriction.Although this correlation is not well studied, Doppler technology has shown increases in umbilical artery resistance associated with decreased fetal growth.[22]

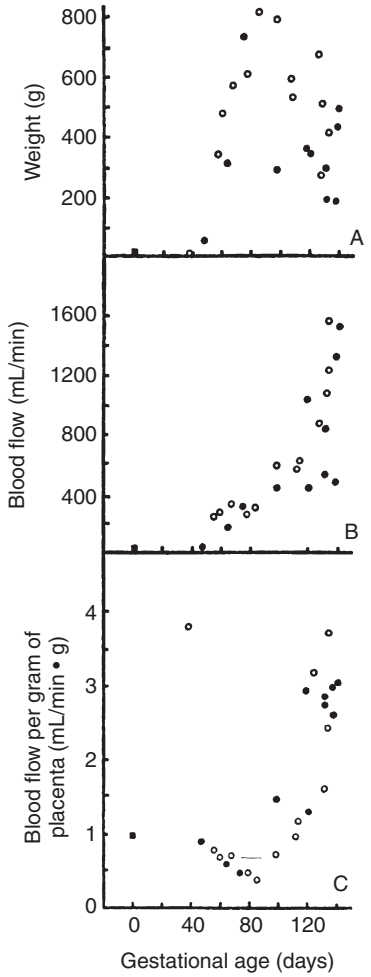


Figure 12-3 Changes in placental weight **(A),** blood flow **(B),** and blood flow per gram of placenta **(C)**. Observations on the nonpregnant animals represent the caruncles or future sites of implantation. Placental weight and blood flow in the twin gestations are the sum of the two placentas. Symbols are as noted in Figure 12-1. *(From Rosenfeld CR, Morriss FH Jr, Makowski EL, et al: Circulatory changes in the reproductive tissues of ewes during pregnancy,* Gynecol Invest *5:252, 1974.)*

VASOCONSTRICTORS

Studies of the factors controlling blood flow through the uteroplacental and umbilicoplacental vascular beds have centered on the effects of endogenous vasoconstrictors, including ANG II, catecholamines, and arginine vasopressin (AVP), a hypothalamic-pituitary peptide secreted during episodes of so-called fetal stress.This section reviews how these agents may affect placental perfusion and fetal well-being.

Normotensive pregnant women develop attenuated pressor responses to systemic infusions of ANG II early in the midtrimester, and this refractoriness is no longer evident in women in whom hypertension develops.[26,27] Pregnant sheep and several other species also exhibit refractoriness to this vasoconstrictor during pregnancy.[28,29] In pregnant women and sheep, the uteroplacental vascular bed is more refractory to Ang II than the systemic vasculature as a whole.[29-31] As in the peripheral vasculature, this uterine refractoriness also is absent in pregnant women in whom hypertension develops, possibly resulting in placental hypoperfusion.[31] This difference in sensitivity is evident when the dose-response curves depicting the relative changes in uterine and systemic vascular resistance are compared. As illustrated in Figure 12-4, at doses resulting in physiologic blood levels (less than 1.0 μg/minute), infusions of ANG II result in greater relative increases in systemic than in uterine vascular resistance. The rise in perfusion pressure also exceeds that of uterine vascular resistance; therefore uteroplacental blood flow increases throughout this dosage range.These observations suggest that the uteroplacental vascular bed is in a sense partially pressure-passive and, importantly, is protected from the vasoconstrictor effects of ANG II.[29] This protective mechanism is important because plasma ANG II levels rise in normal pregnancy and may intermittently increase further when pregnant women experience episodes of orthostatic hypotension. Notably, maternal placental blood flow is minimally affected, and the reproductive tissues as a group are more refractory than nonreproductive tissues.[32] The similarity in these responses to Ang II in women and sheep further validates the animal model as do the studies of BK_{Ca} function.[19-21]

This refractoriness of the uteroplacental circulation to Ang II may reflect several mechanisms. In earlier studies, uterine arteries from pregnant ewes demonstrated a three-fold rise in the basal synthesis of prostacyclin (i.e., prostaglandin I_2 [PGI_2]), a potent vasodilator and ANG II antagonist, and a further 1.5-fold increase in endothelium-derived PGI_2 synthesis when exposed to ANG II in vivo or in vitro by activating type 1 ANG II receptors.[29,33-35] Uterine arteries from normotensive pregnant women appear to respond similarly, whereas arteries from women with hypertensive disease appear to be unable to enhance PGI_2 synthesis in the presence of Ang II.[36] Because inhibition of cyclooxygenase activity

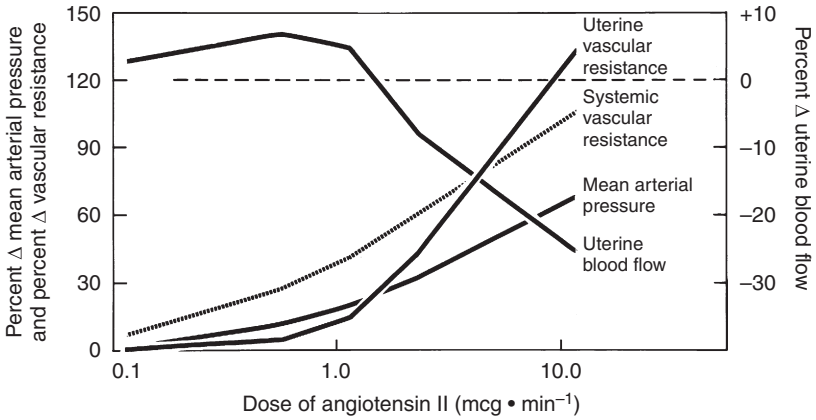


Figure 12-4 The relative changes in uterine blood flow, mean arterial pressure, systemic vascular resistance, and uterine vascular resistance during the systemic infusion of angiotensin II in pregnant sheep. *(From Rosenfeld CR: Consideration of the uteroplacental circulation in intrauterine growth,* Semin Perinatol *8:42, 1984.)*

in the uterine vascular bed of pregnant ewes decreased basal PGI_2 synthesis and enhanced constrictor responses to systemic ANG II infusions, it was believed this mechanism accounted for the attenuated uterine vasoconstrictor responses to ANG II.[35] This differential sensitivity to ANG II was not associated with down-regulation of ANG II receptors in uterine vascular smooth muscle. Of interest, however, it was observed that type 2 ANG II receptors, which do not mediate vasoconstriction, are predominant in uterine arteries from women and sheep.[29,34,37] Furthermore, uterine intraarterial infusions of ANG II did not increase vascular resistance except at doses that initiated pressor responses before uterine responses, the pattern seen with systemic infusions.[38] It now appears that the attenuated uteroplacental responses to ANG II reflect primarily type 2 receptor expression in the uterine vascular smooth muscle. Moreover, ANG II may exert its effect on uterine blood flow by activating local, systemic, or central adrenergic mechanisms and by the release of an α-agonist, which is antagonized by local PGI_2 synthesis.[29,38,39] In addition, the type 2 receptor may directly attenuate type 1 receptor signaling and activation.[40] Uteroplacental synthesis of endothelium-derived NO, which increases in pregnancy and increases further with ANG II, provides another route for attenuating responses to ANG II.[29,41] Thus several local mechanisms may contribute to the maintenance of uteroplacental blood flow while permitting adjustments in the peripheral vascular beds during increases in plasma levels of endogenous ANG II in pregnant women. Modification of these agonist-antagonist interactions may occur in hypertensive pregnancies, resulting in uteroplacental hypoperfusion and eventually fetal growth restriction.

The pressor responses to infused catecholamines, epinephrine, and norepinephrine, also are attenuated during normal pregnancy[42] and are increased in hypertensive pregnant women.[26] The uteroplacental vascular responses to α-agonists, in contrast with such responses to ANG II, exceed those seen in the systemic vasculature[42]; that is, increases in uterine vascular resistance exceed simultaneous increases in systemic vascular resistance and perfusion pressure at all doses of infused α-agonists (Figure 12-5). These changes result in a fall in uterine blood flow at doses of α-agonists that minimally alter maternal blood pressure (see Figure 12-6) and are paralleled by decreases in placental blood flow. Thus the maternal uteroplacental vascular bed is less protected from endogenous α-agonists than from ANG II. These differences are physiologically relevant. For example, it would be senseless to put the fetus at risk for placental hypoperfusion and decreased uterine oxygen delivery each time the pregnant woman rises from the supine position to standing and increases her plasma levels of Ang II. By contrast, in circumstances in which the mother's survival is at risk (e.g., from hemorrhage) and adrenal production and secretion of catecholamines are increased, it is physiologically sound to divert a significant proportion of the

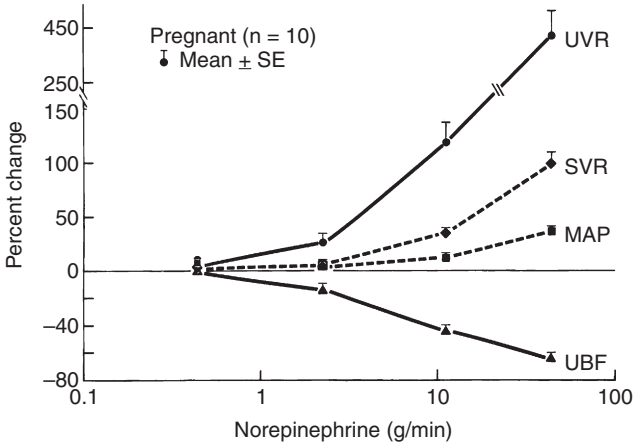


Figure 12-5 The relative changes in uterine blood flow *(UBF),* mean arterial pressure *(MAP),* systemic vascular resistance *(SVR),* and uterine vascular resistance *(UVR)* during the systemic infusion of norepinephrine in pregnant sheep. *(From Magness RR, Rosenfeld CR: Systemic and uterine responses to α-adrenergic stimulation in pregnant and nonpregnant ewes,* Am J Obstet Gynecol *155:897, 1986.)*

excessive volume of uteroplacental blood flow to tissues more relevant to maternal survival. During labor, however, circulating catecholamine levels are elevated, and this enhanced responsiveness of the placental arteries to catecholamines can potentially endanger the growth-restricted fetus or a normally grown fetus with a borderline "margin of safety" by decreasing uterine oxygen delivery to dangerous levels, resulting in fetal heart rate decelerations. This phenomenon has been noted in Doppler ultrasound studies.[9] In studies of human uterine arteries[21] and preliminary studies in intact pregnant sheep, the BK_{Ca} channel that modulates basal uterine blood flow also modifies uterine responses to α-agonists. Thus abnormalities of BK_{Ca} function or expression may contribute to abnormal placental perfusion and increased sensitivity to α-agonists as seen in hypertensive women.[43,44]

ANG II is a potent vasoconstrictor in the umbilicoplacental circulation.[45,46] It was previously thought that the fetus was more refractory than the pregnant mother to the effects of Ang II, resulting in the greater circulating levels in the fetus.[47] Of note, however, fetal clearance of ANG II is 10-fold greater than that in the mother, 95% of fetal clearance occurs across the placental vascular bed, and the umbilicoplacental vasculature is more sensitive to the vasoconstricting effects of this peptide than the uteroplacental circulation at similar plasma levels of ANG II

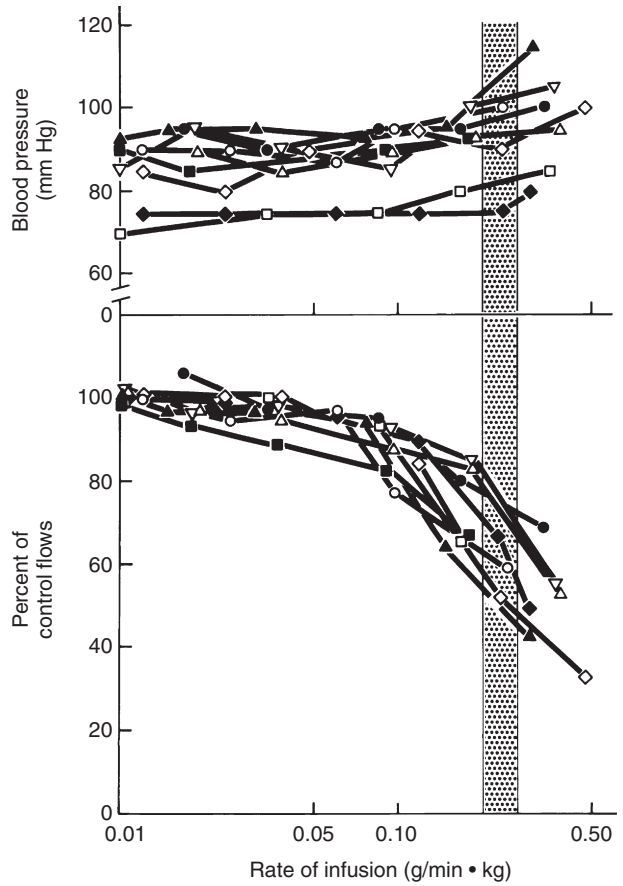


Figure 12-6 Responses of uterine blood flow and mean arterial pressure to increasing doses of norepinephrine. Uterine blood flow is represented as percentage of control flows. Different symbols represent responses of separate animals. *(From Rosenfeld CR, West J: Circulatory response to systemic infusion of norepinephrine in the pregnant ewe,* Am J Obstet Gynecol *127:376, 1977.)*

(Figure 12-7).[47] This difference between the umbilicoplacental and uteroplacental responses is explained by the predominance of type 1 ANG II receptors in the former and type 2 receptors in the latter in humans and sheep.[34,37,48-51] Furthermore, the umbilical artery smooth muscle demonstrates precocious functional maturation compared with fetal systemic arteries,[52] which express primarily the type 2 receptor.[48] By contrast, catecholamines minimally alter umbilicoplacental blood flow except at nonphysiologic doses,[53,54] and these decreases in placental perfusion may reflect a fall in fetal cardiac output, rather than increases in placental vascular resistance. Thus fetal responses to catecholamines are associated with the maintenance of umbilicoplacental perfusion as well as cerebral and myocardial blood flow. In fetal sheep, ANG II–induced modifications in arterial pressure are due primarily to increases in umbilicoplacental resistance mediated by type 1 receptors.[48,55,56]

AVP is a hypothalamic-pituitary hormone that exhibits increased secretion during episodes of intrauterine hypoxia and asphyxia.[57,58] As with adrenal catecholamines, which mediate the primary fetal responses to intrauterine "stress," umbilicoplacental perfusion is not affected substantially by AVP.[57] Thus the maternal and the fetal placental vascular beds respond differently to endogenous vasoconstrictors, probably reflecting important mechanisms necessary to ensure adequate fetal oxygen delivery and the survival of the fetus or the mother.

PROSTAGLANDINS

Interest has been considerable in the role of prostanoids in the regulation of uteroplacental and umbilicoplacental blood flows. This interest stems from observations of increased circulating levels of prostanoids, especially vasodilating prostanoids, in normotensive pregnant women and animals.[59,60] In addition, systemic and local infusion of cyclooxygenase inhibitors in pregnant sheep increases systemic and uterine responses to infused ANG II.[35] Furthermore, women with pregnancy-induced hypertension exhibit plasma levels of thromboxane (a potent vasoconstrictor) that exceed those of PGI_2, potentially accounting for the increased vascular reactivity seen in these women.[59] Because circulating PGI_2 and thromboxane may be of placental origin, these and other prostanoids could

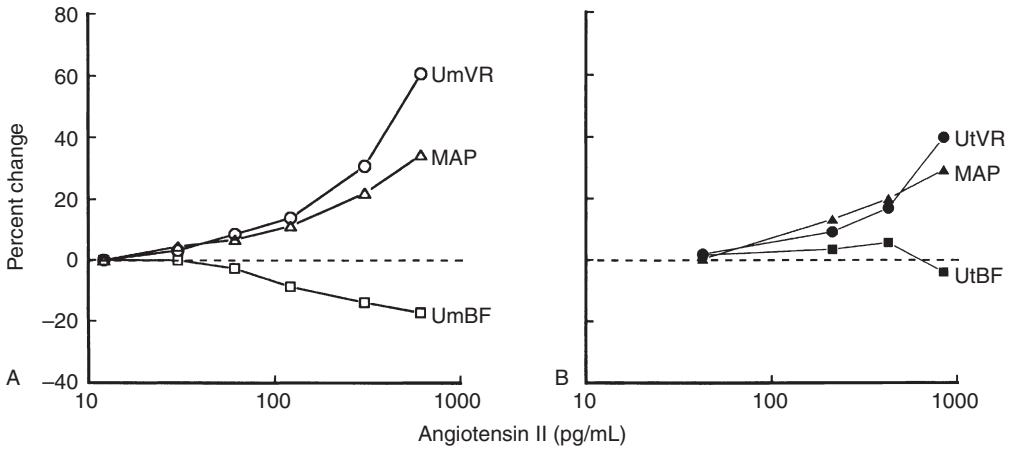


Figure 12-7 Comparison of the relative changes in blood flow, mean arterial pressure *(MAP),* and vascular resistance versus plasma levels of angiotensin II in the umbilicoplacental **(A)** and maternal uteroplacental **(B)** vascular beds during systemic infusions of angiotensin II. *UmBF,* Umbilical blood flow; *UmVR,* umbilical vascular resistence; *UtBF,* uterine blood flow, *UtVR,* uterine vascular resistence. *(From Rosenfeld CR, Gresores A, Roy TA, Magness RR: Comparison of ANG II in fetal and pregnant sheep: Metabolic clearance and vascular reactivity,* Am J Physiol *268:E237, 1995.)*

modulate the peripheral and uterine circulations in normotensive and hypertensive pregnant women. This view remains controversial, however. First, prostanoids may not be circulating hormones but rather mediate their effects through paracrine mechanisms. Thus plasma levels probably provide little physiologic information. Second, increases in vessel perfusion or shear stress increase vascular production of PGI_2.[61] The rise in placental blood flow seen in normal pregnancy, therefore, may be due to another mechanism, and the increase in prostanoid synthesis may be secondary. Third, although cyclooxygenase inhibition enhances systemic and uterine vascular responsiveness in pregnant women and sheep, this effect does not alter basal uteroplacental blood flow commensurate with the fall in plasma prostaglandin levels.[35,62] Finally, pregnancy-induced hypertension is not ameliorated or prevented by low-dose aspirin therapy.[63] This evidence suggests that prostanoids may be a class of stress hormones that antagonize the vascular responses to circulating placental vasoconstrictors, thereby protecting maternal uterine blood flow.

Similar controversy exists regarding the role of prostaglandins in the fetal compartment. PGI_2 is a potent placental vasodilator, whereas prostaglandin E_2 (PGE_2) is a vasoconstrictor (versus a vasodilator in the maternal placental circulation). The physiologic role of these two prostanoids, however, is unclear. It has been suggested that the differential effects of PGE_2 in the two placental vascular beds may act to autoregulate the two beds so that when umbilicoplacental blood flow falls, maternal placental perfusion increases to maintain fetal oxygen uptake and delivery.[64] More recently, it has been suggested that PGE_2 may potentiate the vasoconstriction seen with Ang II, whereas PGI_2 antagonizes these effects. These conclusions require further study.

NITRIC OXIDE

An endothelium-derived relaxing factor (i.e., not a prostanoid) has been identified and shown to induce vascular relaxation by activating smooth muscle guanylate cyclase and the synthesis of cGMP. This substance, NO, is the product of at least three isoforms of NOS expressed in endothelium and vascular smooth muscle. NO synthesis is increased in normotensive pregnancies and may contribute to normal cardiovascular adaptation and increased cGMP synthesis.[64] Furthermore, prolonged administration of an NOS inhibitor to the mother causes hypertension and leads to decreased cGMP levels. Marked increases in uterine venous cGMP and uterine cGMP synthesis occur in pregnant sheep, suggesting that NO may be essential to the regulation of uteroplacental blood flow.[65] Although uterine artery synthesis of NO increases in pregnancy, it is unclear what proportion of uterine cGMP synthesis is vascular versus placental or nonvascular.[64,66] Furthermore, the role of NO in the regulation of uteroplacental blood flow is unclear in view of the fact that NOS inhibition decreases uteroplacental perfusion no more than 25%.[65,67] Whereas studies of NO have focused on the role of endothelium-derived type 3 NOS, recent studies demonstrate type 1 NOS expression in uterine artery smooth muscle.[68] Its role in pregnancy is unstudied to date.

Studies of the role of NO in the regulation of umbilicoplacental blood flow are extensive, but it also is unclear to what extent NO is responsible.[64] Perfused human umbilical arteries and veins release more NO than PGI_2.[69] Inhibition of NOS increases umbilicoplacental vascular resistance in vitro and, in intact fetal sheep, results in a nearly 50% fall in umbilical blood flow.[70] Increases in NO generation also attenuate constrictor responses to several agonists. Thus NO may play an important role in both maintaining and modulating umbilicoplacental blood flow. Because inhibition of NOS does not decrease blood flow completely, NO contributes to but is not totally responsible for maintaining umbilicoplacental perfusion. Of note, potassium channels also are expressed in umbilical arteries.[71] At present, their role in umbilicoplacental blood flow is under study.

STEROID HORMONES

The placenta is a major site of synthesis of maternal and fetal steroid hormones, in particular, estrogen and progesterone. These hormones have profound vascular effects and may play important roles in promoting implantation and placentation[17] and modulating placental blood flow and other aspects of maternal cardiovascular adaptation during pregnancy. Unfortunately, few investigators have examined the role of these hormones on umbilicoplacental blood flow. In sheep, estrogen vasodilates the nonpregnant (increasing blood flow 10- to 15-fold) and pregnant uterus (increasing blood flow by 40% to 50%).[72] Furthermore, infusion of dehydroepiandrosterone (the fetal adrenal androgen precursor necessary for placental estrogen synthesis) to the fetus or the ewe increases placental estrogen production and uteroplacental blood flow.[72] No other naturally occurring substance has an effect of this magnitude on uterine or uteroplacental perfusion. Because the rise in uterine blood flow occurs in the absence of a change in perfusion pressure,[73] this phenomenon represents true vasodilation. Progesterone may antagonize and modulate these effects of estrogen. Thus the two steroids may coregulate the magnitude of maternal uteroplacental blood flow. The effects of estrogen on uterine blood flow are receptor-mediated and involve activation and transcriptional regulation of type 1 and type 3 NOS,[68,74] resulting in increased NO synthesis, increases in smooth muscle cGMP, activation of a cGMP-dependent protein kinase, and finally, phosphorylation and activation of BK_{Ca} in uterine artery smooth muscle in nonpregnant and pregnant sheep.[18,75] This potassium channel also modulates basal uteroplacental blood flow and may attenuate vasoconstrictor responses during pregnancy.[18,19] Of note, estrogen also regulates BK_{Ca} subunit expression in the uterine artery and can directly activate the channel.[76] Studies are under way to assess these effects in human uterine arteries, which may lead to the development of strategies for treating placental hypoperfusion in the future.

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13 Pathophysiology of Preterm Birth

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EPIDEMIOLOGY

Preterm birth refers to birth before 37 weeks of gestation. It accounts for 6% to 10% of all births in Western countries and more than two thirds of all perinatal deaths. Preterm birth may result from preterm labor, spontaneous or induced, or may be by planned cesarean section for maternal or fetal complications.[1] In recent estimates, up to 30% to 40% of all cases are induced or elective, and the remaining 60% to 70% occur spontaneously.[2] Epidemiologic studies of preterm labor vary in terms of categorization, but generally, labor before 24 weeks is considered previable; before 28 weeks, extremely preterm; at 28 to 31 weeks, very preterm; and at 32 to 36 weeks, mildly preterm.[3] Gross outcomes after 34 weeks generally are considered to be as good as those after 37 weeks gestation, although the risk of minor morbidity remains.[4] It is the 3% to 7% of preterm births that occur before 34 weeks gestation that account for approximately 75% of neonatal deaths and 50% of cases of long-term neurologic impairment in children.[2]

Risk factors for preterm labor include race, age, socioeconomic status, and body mass index (BMI).[1] In the United Kingdom, the risk of preterm birth is 6% in white Europeans but 10% in Africans or Afro-Caribbeans.[5] Similarly, African American women are overrepresented in the population of women experiencing preterm birth in the United States. Lower socioeconomic status in such groups and other minority groups adds environmental causes to the plausible genetic causes of preterm birth (e.g., polymorphisms in tumor necrosis factor-α [TNF-α] a proinflammatory cytokine).[6,7] Other environmental causes include inadequate nutrition, substance abuse, and psychosocial factors.[1]

A strong association with preterm birth with a low BMI, poor weight gain, or excess weight gain and obesity was reported in eight primary studies of 122,647 women.[8] Alcohol consumption, smoking, and cocaine exposure all increase the risk of preterm birth. Dose-response effects have been described with alcohol consumption in various European studies, as well as increased risk from low to moderate and heavy smoking.[9,10] Studies from the United States confirm increased risk with cocaine exposure and other psychiatric and substance use disorders.[11,12] Maternal stress may increase susceptibility to preterm labor. Maternal stress may act by way of a neuroendocrine pathway that activates the maternal-placental-fetal endocrine systems that promote parturition by way of an immune or inflammatory pathway.[13] Elucidation of the complex interactions among maternal demographics, environmental exposures, and genetic susceptibility of mother and fetus requires a coordinated research approach that brings together the disciplines of epidemiology, laboratory-based science, and clinical trials for the best hope of significant advances in understanding preterm birth.[1]

Endocrinology and Biochemistry of Labor

Predicting, diagnosing, and managing preterm labor all remain challenging, because despite extensive research, the mechanisms that control the duration of human pregnancy and that signal the onset of labor have not yet been fully determined. Throughout pregnancy, the uterine cervix needs to remain firm and closed while the body of the uterus grows by hypertrophy and hyperplasia yet remaining quiescent.[5] During pregnancy, "propregnancy" factors such as progesterone and prostacyclin (i.e., prostaglandin I_2 [PGI_2]) inhibit myometrial contractility. Parturition involves a synchronization of changes in the structure of the cervix, myometrial activity manifesting as painful uterine contractions, and ruptured fetal membranes leading to cervical dilation and effacement. At the end of the pregnancy, "prolabor" factors begin to mediate remodeling of the cervix, and the uterus is stimulated to begin coordinated contractions.[14] It has been suggested that labor results from the activation of a so-called cassette of contraction-associated proteins (CAPs), which include gap junction proteins, oxytocin and prostanoid receptors, enzymes for prostaglandin synthesis, and cell signaling proteins. The term *labor-associated proteins* may be more appropriate, because these also are responsible for activating fetal membrane prostaglandin and cytokine production, as well as for cervical remodeling and ripening.[15]

Inflammation

Inflammation is now considered to be integral to the pathophysiologic processes underlying preterm labor. Microarray and suppression-subtractive hybridization studies have demonstrated an up-regulation of a vast number of proinflammatory genes in fetal membranes, myometrium, and the cervix with the onset of labor.[16] Such up-regulation is demonstrated by an influx of inflammatory cells into the uterus and increased expression of proinflammatory cytokines.[17] The transcription factor nuclear factor kappa B (NF-κB), which is activated within the uterus at the time of labor, regulates a number of inflammatory factors, including the interleukins IL-6 and IL-8 and cyclooxygenase-2 (COX-2), which leads to prostaglandin synthesis.[18] At term, this inflammatory-type response may be triggered by the maturing fetal lung, which produces increased surfactant lipid and protein (i.e., surfactant protein A [SP-A]). This response is associated with the activation of macrophages in the amniotic fluid and an increased inflammatory response in the uterus.[19]

Corticotropin-Releasing Hormone

Corticotropin-releasing hormone (CRH) is a peptide produced by the hypothalamus in response to stress. CRH also is expressed abundantly in the placenta, and in maternal serum, CRH has been shown to rise in the second half of pregnancy, peak during labor, and rapidly decline post partum. In patients who have gone on to deliver prematurely, elevated levels of CRH in the maternal circulation have been identified as early as 18 weeks of gestation.[20] The curve depicting the CRH increase is shifted to the left in women who subsequently deliver preterm and to the right in women who deliver post dates. This finding suggests that CRH production is linked to a placental "clock" that determines the length of gestation.[21] Presumably, then, maternal plasma CRH concentrations could help identify women at high risk for preterm delivery, and CRH antagonists may be useful in preventing preterm labor.[22]

Progesterone

Progesterone is thought to act during pregnancy to inhibit labor-associated gene expression and inhibit contractions. Unlike those in mammalian counterparts, levels of human circulating progesterone and progesterone receptors (PRs) in the myometrium do not fall with the onset of labor. In clinical practice, however, the administration of a PR antagonist RU486 can be used to ripen the cervix and induce labor. In the absence of a demonstrable fall in progesterone concentrations, a functional progesterone withdrawal may precede the onset of labor. This diminished activity could be due to alterations in the different PR levels or isoforms[23]—specifically, a reduction in myometrial responsiveness to progesterone by an increased expression of PR-A relative to PR-B.[24] It also may be due to the interaction between NF-κB and PR or changes in PR coactivator and corepressor expression.

Nuclear Factor-κB

NF-κB is a cytokine-inducible transcription factor, activated in response to infection and proinflammatory cytokines, that regulates many proinflammatory and labor-associated genes.[25] In most cells, NF-κB exists in an inactive form in the cytoplasm, bound to an inhibitory protein, such as IκBα.

Three distinct NF-κB signaling pathways have been identified to date.[14] Labor is associated with an increase in the baseline level of NF-κB DNA binding and transcriptional activity in the human amnion, and because NF-κB down-regulates progesterone receptor function, it seems to be related to a "functional progesterone withdrawal" in the human myometrium.[26]

Prostaglandins and Cyclooxygenases

Prostaglandins are derived from arachidonic acid (AA) by means of a pathway that starts with the mobilization of AA from membrane phospholipids by phospholipase enzymes. The next step is a reaction catalyzed by the cyclooxygenase (COX) enzyme, whereby free AA is converted to the prostaglandin intermediate PGH_2. Finally, PGH_2 is converted to PGE_2, $PGF_{2\alpha}$, PGD_2, prostacyclin, and thromboxane by specific synthase enzymes. Sufficient AA is present in the amniotic fluid and intrauterine tissues for prostaglandin synthesis to occur before the onset of labor. It is the COX enzyme, however, that mediates the committing and rate-limiting step of prostaglandin synthesis.[27,28]

The three known isoforms of the COX enzyme are COX-1, COX-2, and COX-3. COX-1 is constitutively expressed in various tissue types, and its expression generally is not regulated. COX-2, however, is up-regulated only after stimulation by cytokines, growth factors, and phorbol esters. Similarly, in intrauterine tissues at term (before and during labor), COX-2 messenger RNA (mRNA) and protein levels have been shown to increase. Thus the current evidence more strongly supports the involvement of COX-2, rather than COX-1, in the onset of labor.[29]

Interleukin-8

IL-8 is a chemokine that is produced by human endometrium, choriodecidua, and myometrium. IL-8 plays an important role in cervical ripening, probably aids in the formation of the lower segment of the uterus in late pregnancy, and mediates the influx of inflammatory mediators, specifically leukocytes, seen in the myometrium during labor.[27]

Oxytocin

Oxytocin (OT) is a nonapeptide, produced by the posterior pituitary, that is known to have a potent contractile activity in the pregnant uterus. It does not seem to be essential for normal parturition, however, because no increase in circulating oxytocin in the mother or fetus occurs with the onset of or during labor. Moreover, normal parturition has been observed in cases of clinical pituitary gland dysfunction.[30] The expression of the oxytocin receptor (OTR), however, does increase in the pregnant uterus. An up-regulation by two orders of magnitude has been demonstrated, leading to a strong increase in sensitivity to OT.[31] Comparable increases in OTR mRNA concentrations in the myometrium are associated with this up-regulation of OT-binding sites.[32] After delivery, myometrial OT-binding sites rapidly decrease, whereas OTR expression remains high in the mammary gland throughout lactation.[31]

In many animal models, OTR is regulated by changes in steroid hormone concentrations.[33] In humans, the lack of marked changes in estrogen concentrations or with progesterone withdrawal suggests that OTR has a more complex regulation.[34] Additional relationships in the OTR system have been demonstrated, in particular with prostaglandins. Treatment with naproxen, a prostaglandin synthesis inhibitor, resulted in a dramatic decrease in OTR in the rat uterus at term. Administration of $PGF_{2\alpha}$ restored the increase in OTR, implying the possibility of a positive feedback loop between prostaglandins and OTR.[35]

Myometrial Contractility

The basic functional unit of the myometrium is the smooth muscle cell, which is in turn arranged into bundles approximately 300 ± 100 μm in diameter. Each bundle is surrounded by connective tissue interspersed with microvasculature. The bundles are further organized into fasciculi, which are covered by a dense collagen matrix and the major vasculature of the myometrium.[36] This arrangement enables the uterus to generate an expulsive force dependent on the orientation of these fibers relative to the cervix. Uterine contractions are then initiated by the propagation of action potentials within these defined vectors of muscle bundles.[37] The intracellular basis for contraction is via the cyclic attachment and detachment of crossbridges between myosin and actin filaments, triggered by activation of the regulatory 20-kDa chain of myosin molecules. Myosin light chain is activated by phosphorylation by myosin light chain kinase, which in turn is activated by calmodulin.[38] It is through the actions of calmodulin that during an action potential, the calcium influx can be connected with a contraction—a process known as excitation-contraction coupling.[37] Once the uterus has been activated, the myometrium is susceptible to stimulation by a number of different agonists, primarily prostaglandins and oxytocin.

CAUSES OF PRETERM LABOR

Common associations with the onset of premature labor in the human are intrauterine infection, placental abruption, cervical incompetence, premature membrane rupture, multiple pregnancy, intrauterine death, fetal and uterine abnormalities, and chorioamnionitis. In many cases, however, no discernible underlying pathologic process can be identified.[37] Although current antenatal screening relies on overt clinical and environmental signs and symptoms, the clustering of instances of preterm labor within families and recurrence in susceptible women together present the case for investigating a possible underlying genetic predisposition.[39]

Infection

A strong correlation has been found between infection within the uterus and the onset of spontaneous preterm labor. Infection has the potential to activate all of the biochemical pathways, ultimately leading to cervical ripening and uterine contractions—even more so in a cervix that may be shortened or weakened.

The bacterial species identified in a majority of cases of congenital infections usually is identical to that in the maternal lower genital tract. After twin preterm delivery, chorioamnionitis is more common and severe in the presenting twin than in the second twin. These factors all suggest that ascending infection from the lower genital tract is the most common pathomechanism for chorioamnionitis.[5]

A more recent hypothesis implicates chronic periodontal disease, with the infected gum tissue acting as a reservoir for bacterial products or inflammatory mediators that play a role in the development of preterm labor.[40] Moderate to severe periodontal disease identified in the antenatal period is associated with an increased risk for preterm delivery, as is periodontal disease progression.[40] More than six causative pathogens have been identified, including *Prevotella* and *Porphyromomas* species.[41]

Chorioamnionitis

Chorioamnionitis usually starts with overgrowth of potential pathogens in the maternal lower genital tract, followed by the presence of the organisms in the uterine cavity, particularly in the decidua of the lower segment. A localized inflammatory reaction then occurs, leading to deciduitis, chorionitis, and extension through the amnion into the amniotic cavity, before eventuating in infection of the fetus itself from aspiration and swallowing of infected amniotic fluid.[42] The most common microbial isolates from the amniotic cavity of women in preterm labor are *Ureaplasma urealyticum, Fusobacterium,* and *Mycoplasma hominis.* Bacterial vaginosis and *Trichomonas vaginalis* are the two most-studied organisms associated with preterm birth.[43] Of note, screening for and diagnosis of bacterial vaginosis do not seem to mitigate the increased risk of preterm birth.[44]

Abruption

Placental abruption may lead to the onset of preterm labor through release of thrombin, which stimulates myometrial contractions by protease-activated receptors. This effect may explain the clinical impression that preterm labor associated with chorioamnionitis often is rapid, whereas that associated with placental abruption is less so, because with placental abruption, no preripening of the uterine cervix has time to occur. Generation of thrombin also may play a role in preterm labor associated with chorioamnionitis, when it is released as a consequence of decidual hemorrhage.[5]

Cervical Incompetence

The cervix maintains the fetus in situ during pregnancy and dilates during labor to allow delivery of the baby. This distal component of the uterus is composed primarily of connective tissue, of which a major portion is collagen arranged in fibers and embedded in proteoglycans. Other components include a small amount of smooth muscle (10%) and some fibroblast cells.[45] Before onset of and during early labor, the biochemical makeup of the cervix changes, so that it becomes softer (i.e., "ripens"), allowing for dilation. These biochemical changes include a decrease in collagen associated with an increase in cervical prostaglandins, proinflammatory cytokines, leukocytes, cell adhesion molecules, and nitric oxide synthase production.[46]

Classic cervical incompetence may be diagnosed in women who have an inherent weakness in the cervix or those in whom the cervix has been compromised as a result of previous medical or surgical treatments. Such treatments include mechanical cervical dilation at the time of a termination of pregnancy and surgical evacuation of products of conception. During treatment for cervical neoplasia, destruction of cervical tissue can result from laser treatment or biopsy; in the case of cervical carcinoma, the entire cervix may be removed (i.e., trachelectomy). It is now recognized that "cervical competence" represents a continuum. In a woman with a shortened or damaged cervix, bacteria may ascend into the lower segment of the uterus and stimulate an inflammatory response, which itself leads to further cervical shortening and dilation in a vicious circle, ending with additional cervical dilation and then labor. The same syndrome may be seen when the cervix is of normal length but the microbiologic burden is heavy. Variations in microbiologic burden may lead to delivery at different gestational ages in different pregnancies in the same woman.

Multiple Pregnancies

The rate of preterm labor is high in clinical conditions such as multiple pregnancies and polyhydramnios that are associated with uterine stretch. Overdistention of the uterus leads to premature up-regulation of contraction-associated proteins that mediate cervical ripening. Studies in sheep have shown that stretch increases myometrial expression of OTR and COX-2,[47] whereas in human models, stretch of myometrial and amnion epithelial cells has been shown to increase the expression of COX-2 and PGE_2 synthesis.[48] In addition, multiple pregnancies are associated with multiple placentas and a consequent earlier rise in placental CRH concentrations in the circulation and, subsequently, higher rates of preterm birth.[5]

Genetics

The most consistent risk factor identified in women who experience preterm labor is a previous pregnancy resulting in preterm birth. Most studies quote a risk of 15% to 18% with one previous preterm birth and 28% to 32% with two previous preterm deliveries.[49,50] On the basis of these data, a previous preterm birth has been suggested as evidence of a genetic basis for preterm labor. This explanation is problematic, however, because preterm labor is a complex syndrome involving various etiologic disorders and masked by multiple confounding environmental risk factors. To date, a single-gene determinant for preterm labor inherited in a conventional mendelian manner has not been identified. Instead, it is generally accepted that susceptibility to preterm labor is governed by multigene effects[39] These effects would regulate inflammatory, uteroplacental, endocrine, uterine contraction and remodeling, and metabolic pathways.[51]

Matrix metalloproteinases (MMPs) are proteolytic enzymes that cause extracellular matrix degredation and are associated with tissue destruction and remodeling. In preterm labor, activation is a cytokine-mediated inflammatory process, resulting in the rupture of fetal membranes and cervical ripening and dilation. Single-nucleotide polymorphisms (SNPs), which may regulate MMP expression and activity, have been studied. At present, a vast number of polymorphisms have been identified in these proteases and their tissue inhibitors.[39,52] In addition to SNPs, complementary analytic approaches such as microarray technology and chip arrays have been used to investigate the susceptibility to preterm labor.[53] Once these genetic polymorphisms can be clearly related to physiologic effects, recognition of individual predisposition to preterm labor may enable a more targeted approach to preventive care.

PREDICTION OF PRETERM LABOR

Because precise disease mechanisms underlying preterm birth are not well understood, clinical predictive tests and preventive treatments remain limited. Nevertheless, if women who are at high risk for spontaneous preterm birth in early pregnancy can be identified, they may be targeted for more intensive antenatal care and prophylactic interventions. Determination of a history of previous preterm birth, second-trimester loss, or induced

abortion is a noninvasive, simple, cost-effective method to identify women who may benefit from more and intensive screening. Further assessment can be accomplished using ultrasonographic cervical length measurement, cervical-vaginal fetal fibronectin screening, and the detection of uterine contractions.

Fetal Fibronectin

Fetal fibronectin is an extracellular matrix found in the deciduas basalis next to the placental intervillous space. It helps to attach the fetal membranes to the uterine decidua and, when mechanical or inflammatory changes occur, is leaked into the cervicovaginal fluid. It can be measured quantitatively in cervicovaginal secretions obtained from the posterior fornix during a speculum-assisted internal examination.

A commercial kit for use in the clinic, which gives a positive result when concentrations are greater than 50 ng/mL, is now available. At present, it is widely accepted as a good test for excluding preterm birth in those presenting with threatened preterm labor on the basis of its strong negative predictive value: 0.5% deliver within 7 to 10 days after a negative test result.[54] Of note, however, this is the test for which a positive result is associated with the greatest odds ratio for preterm delivery before 35 weeks gestation.[55]

Cervical Length Measurement

Ultrasonographic cervical length measurement is a reproducible method of assessing the cervix, with little inter- and intraobserver error.[56] A transvaginal probe is used to determine the length between the internal and the external os. The risk of preterm delivery increases exponentially with decreasing length, from less than 1% at 30 mm to 80% at 5 mm.[57] Among women with symptoms of preterm labor, it is estimated that 50% of those with a cervical length less than 15 mm deliver within 7 days.[58]

PREVENTION OF PRETERM LABOR

Cerclage

If preterm labor does occur secondary to cervical incompetence, inserting a suture around the cervix is commonly used to increase mechanical strength to the cervix, thereby preventing premature opening in a subsequent pregnancy. Two approaches to the cerclage procedure are used: abdominal and vaginal. In the *vaginal* approach, the McDonald or Shirodkar method may be employed. The McDonald approach involves a suture or tape placed around the cervix at the junction between the vagina and the cervix. In the Shirodkar suture, the supravaginal cervix is incised and the bladder is reflected upward, allowing the suture to be placed at the cervicoisthmic junction. The cervical epithelium is then repaired.[46]

The most common indication for an *abdominal* cerclage is a failed vaginal cerclage. This more extensive procedure requires a transverse incision in the lower abdomen and the peritoneum over the cervix and bladder, to be deflected down, to allow placement of a suture above the cardinal and uterosacral ligaments.[59] A cesarean section is required to deliver the baby, and the suture often is not removed, unlike with vaginal cerclage, in which removal at 37 to 38 weeks gestation is needed to enable vaginal delivery of the baby. Abdominal cervical cerclage also may be performed laparoscopically, although published data currently are limited to isolated case reports.

Although cervical cerclage is widely used in clinical practice, evidence on its efficacy is still limited—regarding whether it is beneficial once cervical shortening has occurred and the amount or degree of shortening warranting intervention.[59] An international multicenter trial conducted by the Medical Research Council and the Royal College of Obstetricians and Gynaecologists recruited 1292 women with a history of early delivery or previous cervical surgery. In this study, 25 women needed to be treated to prevent one preterm delivery, with no benefit to the neonate. In women with three or more second-trimester losses, however, placement of a cervical suture halved the incidence of preterm delivery before 33 weeks, and in such cases a cervical cerclage was recommended.[60]

Progesterone

Progesterone is a propregnancy hormone, and withdrawal in many animal models leads to onset of labor. In humans, a functional withdrawal is more likely, but supplementation of progesterone may nevertheless be beneficial in preventing preterm labor. Progesterone has a negative effect on many contraction-associated proteins and on prostaglandin activity.[61] In addition, it decreases uterine sensitivity to oxytocin.[62] Placebo-controlled studies using 100 mg of progesterone or placebo as a vaginal suppository between 24 and 34 weeks found a significant reduction in preterm delivery rates before 37 weeks and before 34 weeks in high-risk populations.[63] A study that also examined neonatal outcomes showed a reduction in the incidence of birth weight less than 2500 g, necrotizing enterocolitis, intraventricular hemorrhage, and the need for supplemental oxygen, but not perinatal death or respiratory distress syndrome.[64] Studies of the clinical use of progesterone are ongoing. On the available evidence, it is estimated that 1 in 7 high-risk women need to be treated with progesterone prophylactically to prevent one case of spontaneous preterm birth before 34 weeks gestation.[2]

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14

Rebecca L. Jones, Robert D.H. Boyd, and Colin P. Sibley

Mechanisms of Transfer Across the Human Placenta

It has been known since the 18th century that the circulation of the human fetus is kept separate from that of the mother by intervening placental tissue.[1] This chapter reviews transport across the placenta, but current understanding of placental transfer is less clear and coherent than for more extensively researched transport organs, such as the intestine or the kidney. Also reviewed is the evidence linking altered placental transporter activities with anomalous fetal growth.

Placentas can be classified broadly according to morphologic structure into *hemochorial* types, found in the human, rat, rabbit, and guinea pig, in which maternal blood washes directly against fetal chorionic (trophoblastic) tissue, and *epitheliochorial* types, found in the sheep and pig, in which the maternal uterine epithelium is maintained[2] (Figure 14-1). Placentas of the dog and some other species are intermediate in structure between these two types (see Figure 13-1; a more detailed account of placental morphology is given in the textbook by Mossman[3]).

Corresponding functional differences are recognized in the mechanisms and degree of transfer of certain solutes, notably γ immunoglobulin G (IgG) (transferred by yolk sac in rabbits and by placenta in women, and untransported in sheep) and iron (transferred by red blood cells in sheep and cats, and receptor-mediated from plasma transferrin in women). Most other transport systems have been observed in a majority of species studied, but few extensive cross-species sets of data are available. An additional major interspecies difference is in the balance between diffusional and carrier-mediated transplacental transport. Hemochorial placentas are considerably more permeable than epitheliochorial placentas to lipid-insoluble molecules for which no specific transport systems exist—such molecules are presumed to leak across the placenta by diffusion through "pores."[4,5] The much more restricted leak pathway in sheep suggests that endogenous polypeptides and other lipid-insoluble molecules, in the absence of specific transport systems, cross the sheep placenta much less readily than the human placenta; the same may be predicted for hydrophilic drugs. These differences must be borne in mind in extrapolating conclusions drawn from one species to another.

Examples of the main mechanisms of transplacental transfer are presented next. A systematic listing of all transport studies has not been prepared since that of Schultz,[6] although many are considered in the more recent review by Atkinson and colleagues.[7] The focus of this chapter is predominantly on data obtained in humans and on those substances with net transfer toward the fetus. In the case of urea, carbon dioxide, and bilirubin, for example, net transfer is in the opposite direction. The methodologic approaches used in humans on which the results discussed hereafter are based are summarized in Table 14-1.[8-33] Placental nomenclature sometimes is confusing to persons from fields of study other than fetal-neonatal physiology. Accordingly, definitions of commonly used anatomic descriptions and terms are provided in Table 14-2.

SPECIFIC MECHANISMS OF TRANSFER

This section describes the mechanisms available for transfer across the placenta, summarized diagrammatically in Figures 14-2 and 14-3, and presents examples of each process.

Diffusional Transfer

Several specific transport systems are operational in the placenta, as discussed in subsequent sections, but any molecule for which a maternofetal concentration difference exists also tends to diffuse across the placenta. The net rate (J_{net}) of diffusional transfer of a given solute from maternal to fetal blood is influenced by the surface area available for transfer, the permeability of the placenta, and the maternofetal concentration difference. These relationships are defined by Fick's law[6]:

$$J_{net} = PS(\bar{c}_m - \bar{c}_f)\text{mol} \bullet \text{sec}^{-1} \bullet \text{g}^{-1} \quad (1)$$

where P is a proportionality constant (for placental permeability) ($cm^2 \bullet sec^{-1}$), S is surface area available for diffusion between

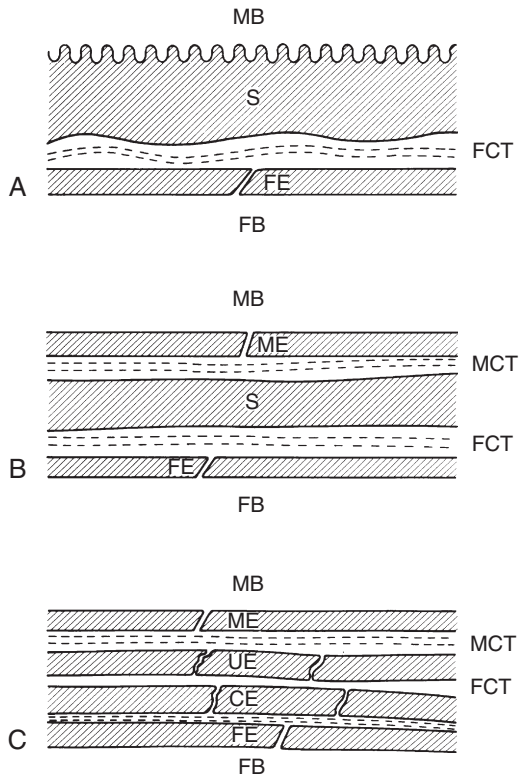


Figure 14-1 Diagrammatic representation of the three main types of placenta classified histologically. A, Hemochorial placenta, which has fetal endothelium and syncytiotrophoblast but no maternal endothelium (e.g., human). **B,** Endotheliochorial placenta, which has syncytiotrophoblast and both maternal and fetal endothelium (e.g., dog). **C,** Epitheliochorial placenta, which retains uterine epithelium in close proximity to chorionic epithelium (trophoblast) as well as both maternal and fetal endothelium (e.g., pig). *CE,* Chorionic epithelium; *FB,* fetal blood; *FCT,* fetal connective tissue; *FE,* fetal endothelium; *MB,* maternal blood; *MCT,* maternal connective tissue; *ME,* maternal endothelium; *S,* syncytiotrophoblast; *UE,* uterine epithelium.

TABLE 14-1

Some Methods of Placental Transfer Study

Method	Variable Measured	Details	Comment
Carcass analysis[8]	J_{net}*	Analysis of conceptus content at different gestational ages	Reliable only for nonmetabolized solutes, e.g., Ca^{2+}; includes all maternofetal routes; poor time resolution
Accumulation of exogenous tracer[9-12]	J_{mf}†	Fetal tracer content estimated after delivery	If fetal plasma concentrations are not low, a hybrid of J_{net} and J_{mf} is measured
Umbilical arteriovenous difference at delivery[13]	J_{mf} or J_{net}	Cord clamped at cesarean section	Non–steady-state artifact likely
Cordocentesis[14-16]	Maternofetal plasma concentration difference		Midgestation; minimal disturbance; usually only venous sample
Cotyledonary perfusion[17]	J_{mf} J_{fm}	Steady-state transfer	Perfusion may alter passive permeability, with consequent incorrect estimation of proportion of specific transport
Cotyledonary perfusion[18]	Kinetics of uptake and backflux from maternal and fetal circulations	Single pass, indicator dilution	Can demonstrate transport asymmetry; may not reflect rate-limiting element of transfer
Slices, fragments[19-21]	Kinetics of individual systems	Uptake or efflux studies	May be "housekeeping" rather than transplacental transport systems
Membrane vesicles[22-24]	Kinetics of individual systems	Microvillous (maternal facing); basal (fetal facing)	May be "housekeeping" rather than transplacental transport systems
Cultured trophoblast[25,26]	Uptake		Care needs to be taken that placental cells rather than other lineages are being studied; cells may never terminally differentiate
Receptor-binding studies[27]	Affinity/number	Many described	May have "housekeeping," endocrine, transport, or other function
Microelectrode and patch clamp studies[28-33]	Transmembrane potential difference and ion channel characteristics		Has implications for ion movements
Gene knock-out/over-expression[10]	Variety	Mainly mouse	Identify likely mechanisms/regulatory pathways

*See Equations 3 and 4 in the text.
†The permeability–surface area product PS can be derived from J for diffusional transport when microcirculation plasma water concentrations are known or inferred.[5,27]
Data from cited references.

TABLE 14-2

Some Anatomic Terms Common in Placental Transport Studies

Term	Meaning
Trophoblast	A fetally derived tissue that comprises a number of different lineages. Within the absorptive part of human placenta, these comprise syncytiotrophoblast, the syncytial layer in immediate contact with maternal blood, and, beneath it, a cytotrophoblast layer from which syncytiotrophoblast is derived. The cytotrophoblast layer is incomplete in later gestation. Other categories of trophoblast outside the exchange area include free cells in the endometrium, trophoblast invading spiral arteries, and trophoblast in nonplacental membranes. Trophoblast also may be free in the maternal circulation. The outer surface membrane of syncytiotrophoblast facing maternal blood is microvillous, and vesicular preparations made from this membrane are microvillous membrane (brush border membrane) vesicles. The fetal surface of syncytial trophoblast also is lined by a cell membrane, which can be isolated and formed into basal membrane vesicles.
Umbilical and uterine circulations	The intervillous space (maternal circulation flowing between the placental villi) is supplied by spiral branches of the (maternal) uterine artery. Circulation within the villi is supplied by the (fetal) umbilical artery and vein.
Chorion	The outermost layer of the conceptus in early gestation—hence, *chorion frondosum*, the chorionic surface that surrounds all sides of the conceptus in early gestation. As the definitive placenta develops, that part of the frondosum contributing to it becomes more villous; the remainder loses its villi to become *chorion laeve.*
Allantois	A fluid-filled compartment adjacent to the umbilical cord, connected to the fetal bladder by a tubular uracus running from the cephalic end of the bladder through the umbilicus. Of considerable size in the sheep, it is rudimentary in the human and disappears in early gestation. However, its tissue contributes to placental formation—hence the description of human placenta as chorioallantoic.
Endometrium	The lining of the uterus. In the human, it is shed at menstruation and hence is classified as deciduate. The shed tissue is the decidua.
Amnion	A fluid-filled compartment that is bounded internally by the fetal skin and externally by the amniotic membrane closely apposed to overlying chorion—hence, amniochorion. It is rupture of this membrane that allows fetal delivery through the cervix at term. The fetus drinks its contained amniotic fluid and passes lung liquid and urine into it.
Fetal membranes	These strictly comprise placenta and amniochorion, the latter being *extraplacental membranes.* In common usage, *fetal membranes* may refer to amniochorion only.
Villus	The fetal component of human placenta arborizes into a large number of small, fingerlike projections or villi; each is bathed by maternal blood flowing through the intervillous space.
Cotyledons	The human placenta is partially divided into cotyledons by incomplete septa. In some species, placental elements are quite distinct, as in the sheep, and each element is known as a cotyledon. In human placental perfusion, roughly one cotyledon is perfused. The human placenta as delivered includes some adherent maternal tissue, and the shiny fetal surface from which the umbilical cord projects is known as the chorionic plate, and the maternal tissue surface is known as the basal plate, not to be confused with thebasal vesicles (see "Trophoblast," above).
Yolk sac placenta	An important route of immunoglobulin transfer in rodents. The human yolk sac is functional only in the first trimester, and its contribution, if any, to transfer is uncertain.

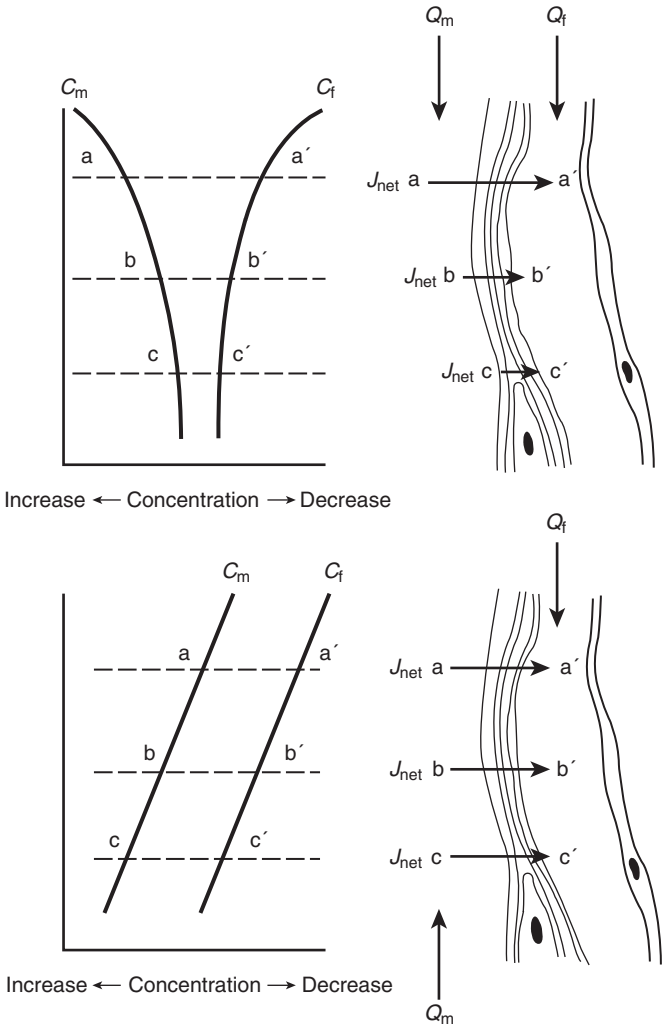


Figure 14-2 Effect of directional relationships of maternal (Q_m) and fetal (Q_f) blood flows on relative concentration differences and net fluxes of a substance crossing the placenta by diffusion under flow-limited conditions. *Top,* Concurrent maternal and fetal flows. *Left,* The maternal (C_m) and fetal (C_f) plasma concentration profiles are shown along the length of the exchange vessel that includes points a, b, and c on the maternal side of the vessel and corresponding points a′, b′, and c′ within the lumen of the fetal vessel. *Right,* This results in net fluxes (J_{net}). The net flux decreases from point a-a′ to point b-b′ to point c-c′ along the length of the exchange vessels. C_m declines along the length of the exchange vessel, and C_f increases along the length of the vessel until the concentration difference that drives net diffusion decreases to almost zero. *Bottom,* Countercurrent maternal and fetal blood flows. *Left,* C_m declines along the length of the exchange vessel, and C_f increases in the exchange vessel along its length. The maternofetal concentration difference is constant along the exchange vessel. *Right,* The net fluxes remain uniform along the length of the capillary. *Not illustrated* is the membrane-limited situation, in which the maternofetal concentration difference and the individual maternal and fetal concentration profiles remain approximately uniform along the length of the capillary; this is true regardless of the directional relationship of maternal and fetal blood flows. *(From Morriss FH, Boyd RDH, Mahendran D: Placental transport. In Knobil E, Neill JD [eds]:* The Physiology of Reproduction*, New York, 1994, Raven Press, pp 813-863.)*

the circulations within 1 g of placenta ($cm^2 \cdot g^{-1}$), and are mean plasma solute concentrations of the unbound solute in plasma in maternal and fetal blood, respectively, flowing past the exchange area.

Two pathways are available for diffusion: a *hydrophilic* route for lipid-insoluble molecules and a *lipophilic* route. Their relative importance in determining J_{net} is determined by the degree of solubility in lipid of the molecule of interest.

J_{net} may be considered as having two components:

$$J_{net} = J_{mf} - J_{fm} \tag{2}$$

where J_{mf} and J_{fm} are unidirectional transfer rates within 1 g of placenta in the maternofetal and fetomaternal directions, respectively. This general formula applies to any mechanism of transfer. For molecules crossing by diffusion only,

$$J_{net} = PS(\bar{c}_m)\text{mol} \bullet \text{sec}^{-1} \bullet \text{g}^{-1} \tag{3}$$

$$J_{net} = PS(\bar{c}_f)\text{mol} \bullet \text{sec}^{-1} \bullet \text{g}^{-1} \tag{4}$$

Hydrophilic Permeability

In the absence of specific transport mechanisms, lipid-insoluble (hydrophilic) molecules do not permeate the trophoblast cell membrane, and therefore are either excluded from the intracellular space of the placenta or cross the placental barrier by an extracellular porous route of molecular dimensions. Their transfer is slow, owing to the small surface area of this pathway, and is limited by both the size of the molecule and the membrane characteristics (permeability and pore size); thus it is said to be *membrane-limited*.[4,27,34,35] Contributions from any specific transport mechanisms for other hydrophilic solutes are additive to this baseline.

Nonphysiologic hydrophobic permeants (including mannitol, sucrose, inulin, chromium-labeled ethylenediaminetetraacetic acid, and high-dose vitamin B_{12}) have been used to assess placental permeability, because they do not possess specific transporters (or because, as in the case of vitamin B_{12}, the transport systems were saturated during studies). Table 14-3 lists the permeabilities, reported as permeability–surface area products (PSs), for some such hydrophilic molecules, across the term human placenta in vivo and, for comparison, across the term rat placenta, in various studies.[9,12,36-38] As is evident in the table, the permeability of hydrophilic substances is proportional to the diffusion coefficient in water (although the values obtained in different laboratories differ).

Such a relationship is most easily explained by diffusion through paracellular channels or pores sufficiently wide to allow free diffusion of low-molecular-weight substances (smaller than proteins). The epitheliochorial sheep placenta has reduced permeability to larger hydrophilic molecules,[39] possibly because the channels have a radius some 10 to 20 times smaller in sheep than in human and other hemochorial placentas.[5]

The morphologic nature of the paracellular channels or pores in the syncytiotrophoblast of hemochorial placentas is uncertain, but two possible pathways (not mutually exclusive) have been identified. Using electron-dense tracers, Kaufmann[40,41] and Kertschanska[42] and their colleagues visualized transtrophoblastic channels lined with tracer after imposing a hydrostatic pressure gradient across perfused guinea pig and human placenta. These workers hypothesized that under normal conditions of hydrostatic pressure, the channels are too narrow to be resolved by electron microscopy. They failed, however, to show channels that are simultaneously open to both the maternal and the fetal sides of the syncytiotrophoblast; the absence of such channels may not surprising in view of the small number of channels overall required to explain the permeability data.[43] A paracellular pathway in the human placenta also may be

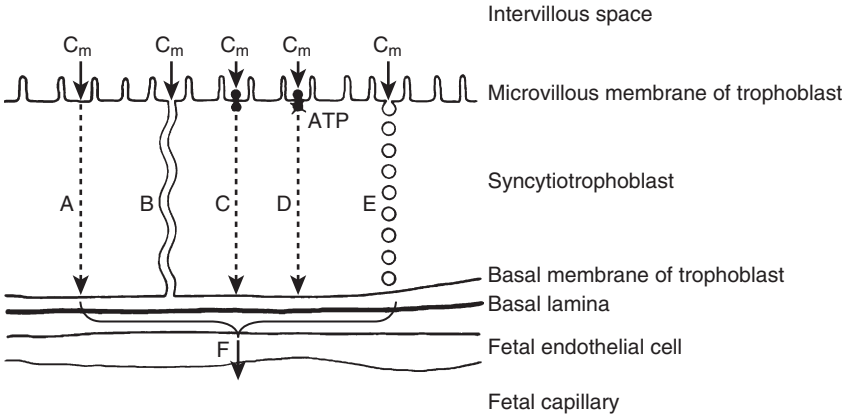


Figure 14-3 Transfer mechanisms within the placental tissue. *A,* Simple diffusion of relatively lipophilic substances (e.g., oxygen, anesthetic gases). *B,* Restricted diffusion of hydrophilic substances through hypothesized water-filled transmembrane channels of submicroscopic dimensions (e.g., mannitol). *C,* Facilitated diffusion (e.g., D-glucose). *D,* Active transport (e.g., amino acids). *E,* Receptor-mediated endocytosis (e.g., IgG—fluid-phase endocytosis). *F,* Exit mechanisms into fetal circulation. *Not indicated* are solvent drag, larger trophoblastic leaks, and the complicating factor of placental metabolism. *ATP,* Adenosine triphosphate; C_m, maternal plasma concentration; *IgG,* immunoglobulin G. *(From Morriss FH, Boyd RDH, Mahendran D: Placental transport. In Knobil E, Neill JD [eds]:* The Physiology of Reproduction*, New York, Raven Press, 1994, pp 813-863.)*

TABLE 14-3

Permeability of the Human and Rat Placenta Near Term

Substance	Diffusion Coefficient in Water at 37° C[36] ($cm^2 \cdot sec^{-1}$)	PERMEABILITY–SURFACE AREA PRODUCT* ($\mu L \cdot sec^{-1} \cdot g^{-1}$)	
		Human	*Rat*[36]
Sodium	17.0×10^{-6}	0.56[37]	0.32
Mannitol	9.9×10^{-6}	0.19[9]	0.07
Sucrose	7.5×10^{-6}		0.07
Chromium-labeled ethylenediaminetetraacetic acid	7.0×10^{-6}	0.11[9]	0.05
Cyanocobalamin	4.8×10^{-6} [12]	0.24[12]	
Inulin	2.6×10^{-6}	0.02[9] 0.15[38]	0.02

Data from cited references.

*For a discussion of the relationship of permeability surface with clearance, see Sibley CP, Boyd RDH: Control of transfer across the mature placenta. In Clarke JR (ed): Oxford Reviews of Reproductive Biology. Oxford, Oxford University Press, 1988, pp 382-435.

provided by areas of denudation of the syncytiotrophoblast.[44-47] These are normal features of the human placenta at all stages of gestation, and they are either open or filled with fibrin-containing fibrinoid deposits.[45]

Sodium

The relatively high permeability of hemochorial placentas toward hydrophilic solutes suggests that sodium transfer across the human placenta occurs primarily by membrane-limited diffusion.[4] However, the PS for sodium across human placenta is unexpectedly higher than for extracellular tracers (see Table 13-3). Several possible explanations have been proposed:

1. Placental structure may include a population of extracellular channels small enough to exclude mannitol (the smallest extracellular tracer so far studied in humans) but large enough to let through some excess sodium (indicating heterogeneity of pore size).[48]
2. Paracellular diffusion of sodium may be accelerated by a transplacental potential difference between the fetal capillary and the intervillous space (with the fetal side negative). Some evidence suggests a potential difference in certain species, but other data are contradictory.[35,48-50] An in vitro study using microelectrodes measured a transtrophoblast potential difference in mature intermediate villi of human placenta ranging from 2.5 to 10 mV, fetal side negative.[51] This finding is supported by some measurements in vivo.[52]
3. Maternofetal sodium transport may be partly transcellular, effected by the various sodium-linked transport systems demonstrable in trophoblast membrane vesicle preparations. This transport mechanism is of interest because it suggests potential endocrine control of active sodium transport to the fetus and thus of fetal hydration (see the later discussion of water transfer). In the rat, in which the sodium PS–diffusion coefficient ratio is similarly higher than that for extracellular tracers (see Table 13-3), good evidence supports active transport, involving sodium–amino acid cotransport and sodium-potassium–adenosine triphosphatase (Na^+,K^+-ATPase).[53] Other evidence points to a similar system in the pig,[54] but as yet such a mechanism has not been proved to operate in humans, even though sodium–amino acid cotransport and Na^+,K^+-ATPase activity have been convincingly demonstrated.[55,56]

Other Ions

Although the paracellular route appears to be large enough in the human to permit some diffusional permeability for all the unbound inorganic ions of plasma, the proportion of their movement that is transcellular generally is unknown. For calcium, in vitro data suggest that the paracellular route contributes approximately 50% of total maternofetal transfer,[57] with the remainder due to active transcellular transfer (see Chapter 33). Chloride, phosphate, and sulfate are maintained at higher concentrations in fetal than in maternal plasma,[58] and therefore transcellular active transport is likely to be involved in the translocation of these ions; the components of such routes certainly exist.[28,59-62]

Lipophilic Permeability

Lipid-soluble substances diffuse through the entire trophoblast cell surface. Because of the large surface area involved, P in Equation 1 is large, and the transport rate depends predominantly on which, in turn, depend on the geometric relationships of the maternal and fetal bloodstreams and their flow rates. Therefore

relatively lipophilic molecules, such as respiratory or anesthetic gases, are said to demonstrate *flow limitation* in their permeability pattern.[5,34,35] The hypothetical effect of two different geometric patterns of flow, concurrent and countercurrent, on the profile of c_m and c_f (and thus on) is illustrated in Figure 14-2.

The human placenta probably belongs to an anatomically intermediate type of exchanger[5] in that, on the fetal side, there are hairpin-like capillary loops, with opposite flow directions in each limb.[41] The maternal circulation in the intervillous space exhibits a *multivillous pool* geometry and lies at approximately 90 degrees to the fetal circulation. This arrangement is intermediate in transfer efficiency.[5]

Oxygen

It seems probable that oxygen transfer across the human placenta is flow-limited as it is in sheep[63] and rabbits.[64] For gases such as oxygen, the terms are replaced in the Fick equation by the equivalent partial pressures, and the driving force for net oxygen diffusion is the partial pressure difference between maternal intervillous space and fetal capillary. Values for partial oxygen pressure (Po_2) in blood taken from large vessels in sheep, monkeys, and humans and from the intervillous space in humans are shown in Table 14-4.

In an ideal concurrent blood flow system, the umbilical venous Po_2 should be identical to the uterine venous Po_2. In sheep and monkey, however, these two values are not identical[65] because of shunting (some maternal and fetal blood does not pass close to the site of exchange) or uneven perfusion (maternal-fetal blood flow ratios that are different between different segments of the placenta). In addition, the placenta and membranes can consume considerable amounts of oxygen from the uterine circulation. In the human, the situation is somewhat uncertain. The Po_2 of placental intervillous space samples is slightly higher than that in umbilical samples (see Table 14-4). Because the intervillous space samples are likely to be a mixture of arterial and venous blood, resulting in overestimation of the true venous Po_2, the human placenta does not fit the concurrent system model and is most consistent with the proposed multivillous pool arrangement.

It is well known that in most species, fetal hemoglobin has a higher affinity for oxygen than maternal hemoglobin (the oxyhemoglobin dissociation curve is shifted to the left).[5] In sheep direct experimental evidence demonstrates that the left shift is important, because replacement of fetal blood with maternal blood results in a halving of the umbilical venous oxygen saturation and a drop in net transplacental flux of oxygen to the fetus.[66] Similarly, fetal acidosis develops in human fetuses transfused with adult blood for Rhesus hemolytic disease.[67] Because of the left shift in the fetal oxygen-hemoglobin dissociation curve, fetal tissue Po_2 is lower than it would be if adult blood at the same Po_2 flowed through it at the same rate.

TABLE 14-4

Oxygen Tension of Maternal and Fetal Blood in Sheep, Monkey, and Human

	Po_2 (mm Hg)		
Sample	***Sheep****	***Monkey****	***Human**†*
Maternal artery	90	108	100
Uterine vein	50	37	55 (intervillous space sample)
Umbilical artery	22	15	—
Umbilical vein	35	22	51

*Data from Meschia G: Placental respiratory gas exchange and fetal oxygenation. In Creasy RK, Resnik R (eds): *Maternal Fetal Medicine: Principles and Practice*, Philadephia, 1984, WB Saunders, pp 274-285.

†Data from Soothill PW, Nicolaides KH, Rodeck CH, Gamsu H: Blood gases and acid-base status of the human second-trimester fetus, *Obstet Gynecol* 68:173-176, 1986; and Nicolaides KH, Soothill PW, Rodeck CH, Campbell S: Ultrasound-guided sampling of umbilical cord and placental blood to assess fetal wellbeing, *Lancet* 1:1065-1067, 1986. Umbilical blood was obtained by percutaneous blood sampling.

Endocytosis and Exocytosis

Endocytosis is an invagination of the cell surface to form an intracellular membrane-bound vesicle containing extracellular fluid[68]; *exocytosis* may be considered the opposite process, that is, fusion of a plasma membrane-bound vesicle to the cell surface, followed by release of its contents. Endocytosis is classically divided into two types: *phagocytosis,* the process by which large particles are ingested; and *pinocytosis,* the process by which small solutes and water are absorbed. Iron transport may use the former process in the sheep,[69] but in the human the latter process is relevant to placental transport. Low-density lipoprotein, the transferrin-iron complex, and IgG all probably gain entry to the human placental syncytiotrophoblast by endocytosis.[70-72] Only IgG seems to be transported intact across the cell and exocytosed. Transfer of iron to the fetus may involve an exocytotic process as well, but not until after some intracellular processing has occurred that results in the return of transferrin to the maternal plasma.[72] The latter also is probably true for the transfer of retinol across the subhuman primate placenta.[73]

Asymmetric transport by endocytosis and exocytosis has three recognized phases, as described next.

Pinocytosis

The pinocytosis phase of the transport process may be fluid phase (nonselective),[68,74,75] adsorptive with nonspecific solute binding (e.g., that between cationized proteins and anionic sites in the plasma membrane of human and guinea pig placenta),[76,77] or mediated by a specific receptor. In receptor-mediated transport, the specific receptors always become associated with specialized regions of the plasma membrane called *coated pits,* which on electron microscopy can be seen to be covered with a "coat" of the protein clathrin.[78,79] Invaginated coated pits with their associated receptors and receptor-ligand complexes form coated vesicles within the cell.

Transsyncytial Movement of Vesicles

The mechanism by which transsyncytial movement of vesicles occurs remains obscure. In other tissues, many vesicles fuse with lysosomes, and for some macromolecules, such as low-density lipoproteins in fibroblasts, this is the last step of the process.[78] For directed transplacental transport of intact IgG to occur, however, either some vesicles must avoid lysosomal fusion or the transported molecule must be protected from digestion. Transsyncytial movement of vesicles may occur by diffusion down a concentration gradient from the site of endocytosis to the other side. Alternatively, as in secretory cells, microtubules and other components of the cytoskeletal system may direct vesicle shuttling.[80-82] In this regard, certainly a well-developed cytoskeletal system is known to be present in human placental syncytium.[83]

Exocytosis

In the process of exocytosis, vesicles fuse with the plasma membrane at the opposite pole of the syncytium followed by release of the contents by fission. It is unknown how exocytosis occurs in the placenta. Generally, endocytosis cannot occur without exocytosis, as areas of plasma membrane that are continuously lost from a cell surface must be continuously replaced to maintain the polarity of the epithelium.[68,75] It is unclear how this process takes place in an asymmetric syncytial transporting epithelium such as human placenta.

Although investigations of the role of endocytosis and exocytosis in the placental transfer of proteins and other macromolecules generally have focused on the syncytiotrophoblast, the fetal capillary endothelium also provides a considerable barrier to the exchange of such molecules.[84,85] Endocytosis and exocytosis also could be important mechanisms in this layer,[86] but this area of investigation requires a considerable amount of additional work.

Immunoglobulin G Transfer

The selective transfer of IgG across the placenta confers passive immunity to the fetus. In other species, particularly rabbits, guinea pigs, and rodents, this function is served by the yolk sac placenta, rather than by the chorioallantoic placenta.[74,87] The mechanism of selective IgG transfer across the yolk sac placenta has been studied in depth. The pioneer of these studies, Brambell,[88] suggested that uptake of IgG is by a nonselective process but subsequent binding to receptors selectively prevents its degradation in phagolysosomes, allowing release of the intact molecule on the fetal side. The identification of specific Fc receptors by placental cells,[74] however, suggests that IgG transfer is accomplished through receptor-mediated endocytosis (reviewed by Wild[87]). This proposed mechanism is supported by the fact that maternofetal immunoglobulin transfer is highly selective (IgG, but not IgA or IgM, crosses the placenta, and transfer of IgG1 is greater than that of other IgG subclasses). FcRn (neonatal FcR) and FcγRIII are expressed by the syncytiotrophoblast[86] and function to transport IgG through the cytoplasm in microvesicles, avoiding fusion with lysosomes. Interference with this pathway by inactivating mutations of FcRn reduces IgG transfer to the fetus.[89] Transfer across the fetal capillary endothelium is less well understood; FcγRII is expressed by endothelial cells; however, it is not clear whether this functions to block immune complex transfer to the fetus or to facilitate IgG transport.[90]

Water Transfer

The human conceptus at term gestation contains 4 L of water (80% by weight of the intrauterine contents[91]). As Barcroft[92] pointed out, the net molar flux of water into the fetus is greater than that of any other substance, including oxygen. From kinetic analysis of plasma and amniotic fluid deuterium oxide concentrations, Hutchinson and colleagues[93] concluded that at 40 weeks of gestation, the unidirectional flux of water across the human placenta is approximately 60 mL/minute in both directions, some 10,000 times the net flux.[94] Unidirectional fluxes from amniotic fluid to fetal plasma and amniotic fluid to maternal plasma (and in the reverse directions) represent only approximately 5% of the transplacental fluxes.

The main reasons for asymmetric unidirectional fluxes and thus for net water flux across the placenta are likely to be hydrostatic and osmotic pressure differences between fetal and maternal placental microcirculations. For example, acutely increasing the osmotic pressure of either maternal or fetal plasma by injection of hypertonic solutions leads to net flux of water out of the fetus or mother in primates,[95] rabbits,[96] and sheep.[97-99] In less acute experiments in the guinea pig, increasing fetal plasma colloid but not crystalloid osmotic pressure increased water acquisition over 20 hours.[100] Similarly, altering the hydrostatic pressure difference changes flow across both perfused guinea pig[101] and sheep placenta.[102] Despite these observations, the size and direction of osmotic and hydrostatic pressure differences between the microcirculations in vivo are unknown.

The factors regulating water acquisition remain somewhat obscure for at least two reasons. First, the reflection coefficients across placenta for the many solutes present in plasma are unknown. Accordingly, calculation of the total effect of osmotic pressure of fetal and maternal plasma from knowledge of their individual solute concentrations,[4] or from freezing point depression or colloid osmometry data, cannot be accomplished.[103] The relatively large apparent pore radius in the hemochorial placenta led Faber and Thornburg[4] to suggest that hydrostatic pressure, together with colloid osmotic pressure, rather than small solute osmotic pressures, governs net water flux across these in contrast with epitheliochorial placentas. Second, solute concentrations in plasma of the microcirculation may be different from those in the large vessels from which samples have been collected. Similar problems apply to the measurement of hydrostatic pressure. In women, it is not even clear whether fetal plasma total osmolarity is higher than[104] or equal to[4] maternal plasma osmolarity, whereas hydrostatic pressure in the intervillous space (insofar as it can be estimated) is lower than in the umbilical vein.[91,105] Both of these latter observations, if they reflect the steady state in vivo, are, in the absence of other forces, incompatible with the net transplacental acquisition of water by the fetus.

A further area of uncertainty is the extent to which transplacental water flow is transcellular as opposed to paracellular. Both Faber and Hart,[106] using the fetal side–perfused rabbit placenta, and Meschia and colleagues,[35] using an in vivo sheep preparation, showed that the clearance of ^3H_2O was much greater than would have been predicted on the basis of molecular size alone if water had been restricted to the same paracellular route as, for example, for sodium. Illsley and Verkman[107] measured the osmotic water permeability of a microvillous membrane vesicle preparation from which the paracellular route was excluded. Although uptake was found to be low compared with other plasma membranes, these investigators calculated that it was sufficiently high to account for the entire transplacental water flux[94] without the need to invoke a paracellular route for water. Despite the many probable inaccuracies in such a calculation, it does at least appear that a proportion of transplacental water movement in the human placenta is likely to be transcellular. Such transcellular fluxes may be mediated by aquaporins, a family of membrane proteins functioning as water channels and found in the syncytiotrophoblast.[108]

One report suggests that the transfer across the rat placenta of inert hydrophilic tracers is asymmetric, with fetomaternal transfer greater than maternofetal transfer.[109] Such asymmetric transfer is impossible to explain by paracellular diffusion alone, and the study investigators suggested that it could result from bulk flow of water, creating solvent drag in the fetomaternal direction. On this basis, these workers further suggested that solute-free water is driven transcellularly across the placenta, driven by osmotic gradients set up by active transport of solute. Water and dissolved solute then return from fetus to mother by paracellular filtration across the placenta, the whole system runs to ensure that the net flux of water to the fetus is exactly correct. Reports of asymmetric transfer across the perfused human placental cotyledon,[110] together with the hydrostatic gradient as noted previously, suggest that a similar model could apply in humans. Despite this and several other attempts to model fetal water acquisition,[94,111] the nature and control of water transfer across the placenta are poorly understood at present. It also remains possible that an important fraction of net water transfer between mother and fetus occurs extraplacentally by way of the amniochorion.

TRANSPORTER PROTEIN–MEDIATED TRANSPORT

The flux of small hydrophilic solutes across the plasma membrane is mediated by transporter proteins. These include *carriers,* which translocate solute by a saturable mechanism and use energy either from prevailing electrochemical gradients (facilitated diffusion—e.g., glucose) or from the hydrolysis of ATP (active transport—e.g., amino acids, calcium), and *channels* through which ions diffuse along electrochemical gradients.[112]

The transporter protein itself may hydrolyze ATP, or it can cotransport with another solute, a diffusion gradient for which is set up by the hydrolysis of ATP by a separate transporter protein (secondary active transport). Many such transporter proteins in the microvillous (maternal-facing) and basal (fetal-facing) plasma membranes of the human syncytiotrophoblast have been characterized. These transporter proteins are important for transplacental transfer or are involved in solute supply to the trophoblast for its own metabolism. For transplacental transfer to occur by way of this transcellular route, both an entry and an exit step would be required (see Figure 13-3).

Transplacental active transport can occur by means of a pump-leak or leak-pump mechanism. In the former process, the active step, localized to the microvillous membrane, generates a higher intracellular concentration of the substance than in maternal plasma, followed by a leak across the opposite plasma membrane (as is probably the case for many amino acids). In a leak-pump system, the substance is pumped out of the trophoblast at the basal plasma membrane, thereby lowering its intracellular concentration so that a leak from maternal plasma into the syncytium occurs (as is perhaps the case for calcium). Two solutes for which transporter proteins are recognized to play an essential role in maternofetal transfer are glucose and amino acids.

Glucose Transfer

The fetus has high glucose demands, particularly in the third trimester, so a rapid, high-capacity transfer system is necessary. Glucose transfer is membrane-limited, and paracellular diffusion alone is not sufficient to meet the needs of the developing fetus.[9] Instead, glucose is primarily transferred across the placenta by facilitated diffusion, in a saturable, stereospecific and competitive manner, which is independent of energy sources.[113-118] This transfer is achieved through the activity of facilitative glucose transporter (GLUT) proteins on both the microvillous and basal plasma membranes of the syncytiotrophoblast[22,119] and is driven by a concentration gradient from maternal to fetal blood. This mechanism is highly efficient, and it has been shown, in the isolated perfused human placental cotyledon, that transplacental glucose flux is directly proportional to the maternal arterial concentration.[113]

Glucose transporter protein-1 (GLUT1) is one of a family of transporter proteins that translocate glucose by facilitated diffusion.[120] GLUT1 is abundantly expressed by the placenta and is present at three-fold higher levels on microvillous than on basal plasma membrane, thereby prioritizing transport into the syncytiotrophoblast from maternal blood.[22] This fulfills the glucose requirements of the placenta (up to 60% of the total glucose uptake is believed to be metabolized intracellularly) and establishes a steep concentration gradient from syncytiotrophoblast to fetal blood, facilitating transsyncytial transfer. Other GLUT family members also have been detected in the placenta, including GLUT3, GLUT4, and GLUT12,[121-123] although discrepancies regarding their exact messenger RNA (mRNA) and protein expression patterns are apparent in the literature (as reviewed by Atkinson and colleagues[124]).

Amino Acids

Transplacental transfer of amino acids is essential to meet the developmental and metabolic requirements of both fetus and placenta.[125] Amino acids are actively transported across the syncytiotrophoblast by a variety of transporter molecules, which are saturable, stereospecific. and energy-dependent (reviewed by Jansson and Powell[126] and by Regnault and associates[127]). The transporter systems are characterized on the basis of competitive interactions and are analogous to those described for other epithelia.[128] The expression of at least 13 amino acid transporter systems by the placenta has been described; findings of relevant studies are summarized and referenced in Table 14-5. These are members of the solute carrier family (SLC) and can be classified into monomeric transporters and heterodimeric systems, whereby a specific light chain functions in conjunction with shared heavy chains (CD98/4F2hc or rBAT) and, further, according to sodium dependence or independence. Expression patterns across gestation indicates highly specific regulation of placental amino acid transfer; however, despite differences in substrate specificity, localization (microvillous membrane or basal plasma membrane) and predominant direction of flux, the transporters have overlapping actions.

Uptake of amino acids by vesicles prepared from either the microvillous membrane or the basal plasma membrane of the syncytiotrophoblast of the human placenta has been extensively investigated.[55,149] Recently a method of quantifying uptake of ^{14}C-methyl aminoisobutyric acid (MeAIB) into intact placental villous fragments has been developed, resulting in a more physiologic system with multiple cell types and enabling longer-term in vitro treatments to investigate transporter regulation.[150]

Human umbilical venous plasma concentrations of most amino acids are significantly higher than maternal concentrations at midgestation[14,151] and at term.[152] Placental tissue concentrations of free amino acids appear to be higher than in either maternal or fetal plasma,[153,154] presumably because of a high syncytiotrophoblast concentration. This observation suggests active transport into the syncytiotrophoblast from maternal blood, followed by a passive leak down the concentration gradient to fetal plasma. However, no intrinsic reason exists to suggest that a passive leak should not also occur from syncytiotrophoblast into maternal plasma. The observed asymmetric transcellular transport (maternofetal flux) is explained by the differential expression or activity of carriers on the microvillous and basal plasma membranes of the syncytiotrophoblast.

Studies with perfused placental cotyledons have confirmed preferential maternofetal transfer of the neutral amino acids L-leucine and L-alanine in the human placenta.[155] In addition, a faster rate of maternofetal transfer has been demonstrated for AIB (a nonmetabolizable amino acid analogue), L-leucine, and the basic amino acid L-lysine.[156] The fetomaternal transfer rates of AIB, L-leucine, and L-lysine are all nearly half the maternofetal transfer rate. Transport of these amino acids in the fetomaternal direction is thought to occur by the paracellular route, because transfer rates are not different from those for the extracellular marker L-glucose. These experiments have been carried out with equal maternal and fetal perfusate amino acid concentrations, and the importance of paracellular transfer may be even greater in vivo because fetal plasma amino acid concentrations are higher than maternal levels.

The sodium-dependent uptake of many amino acids across the microvillous plasma membrane suggests that uptake from the maternal circulation is driven by a low sodium concentration within the trophoblast compared with that in the intervillous space. The sodium gradient is maintained by trophoblastic Na^+,K^+-ATPase activity, and inhibition of the sodium-potassium (Na^+-K^+) pump with ouabain inhibits AIB accumulation by the perfused cotyledon.[157] More puzzling, however, is how the accumulation of amino acids such as leucine is energized, when they appear to have only a sodium-independent pathway for uptake.[158] One possibility is that this uptake is energized by exchange transport with amino acids that are concentrated in the syncytiotrophoblast by sodium-dependent cotransport.[159] In fact, despite a high concentration of leucine in placental tissue[133] and fetal plasma,[152] ^{14}C-leucine is not concentrated by human placental slices, apparently because of a rapid efflux of leucine from the slices.[160] By contrast, ^{14}C-alanine is concentrated eightfold. It could be that the pump-leak hypothesis represents an oversimplification and is not always applicable.

Neutral amino acids are transported by sodium-dependent transporters system A and ASC, and by sodium independent

TABLE 14-5

Placental Amino Acid Transport Systems and Their Distribution in the Syncytiotrophoblast

System	Protein(s)	Gene(s)	Na^+ Dep	Substrates	Excludes	Location
Monomeric						
A[129-131]	SNAT1 SNAT2 SNAT4	SLC38a1 SLC38a2 SLC38a4	Yes	Alanine, serine, proline, glycine, MeAIB	Anionic and cationic amino acids, leucine	MVM, BM
ASC[25,131]	ASCT1	SLC1a4	Yes	Alanine, serine, cysteine	MeAIB and cationic amino acids	BM
N[132]	SN1	SLC38a3	Yes	Histidine, asparagine, glutamine	Cysteine, MeAIB	MVM
y^+ [133-135]	CAT1 CAT2 CAT4	SLC7a1 Slc7a2 Slc7a4	No	Major cationic amino acid transporter	Neutral and ionic amino acids	MVM, BM
B^0 [136]	ASCT2	SLC1a5	Yes	Neutral amino acids	Cationic and anionic amino acids, MeAIB	BM
B^{0+} [137]	ATB^{0+}	SLC6a14	Yes	Cationic and neutral amino acids	Anionic amino acids	Not known
β[138-140]	TauT	SLC6a6	Yes Also Cl^-	Taurine, β-amino acids	α amino acids	MVM, BM
Gly[141]	GLYT1	SLC6a9	Yes also Cl^-	Glycine		MVM
X^-_{AG}[142]	EAAT1 EAAT2 EAAT3	SLC1a3	Yes Also Cl^-	Glutamate, aspartate	Nonanionic amino acids	MVM, BM
Heterodimeric						
L[143,144]	LAT1/CD98 LAT2/CD98	SLC7a5/Slc3a2 Slc7a8/ SLC3a2	No	Neutral amino acids, including leucine	MeAIB	MVM, BM
y^+L[134,145,146]	y^+LAT1/CD98	SLC7a7/Slc3a2	No	Cationic amino acids and neutral amino acids (in the presence of Na^+)	Anionic amino acids	MVM, BM
b^{0+} [133,145]	B^{0+}AT/rBAT	SLC7a9/SLC3a1	No	Cationic and neutral amino acids	Anionic amino acids	MVM, BM
asc[147,148]	asc1/CD98 asc2/unknown	SLC7a10/SLC3a2 SLC7a12/unknown	No	Small neutral amino acids	Cationic and anionic amino acids	Not known

Data from cited references.
BM, Basal plasma membrane; *MeAIB,* methylaminoisobutyric acid; *MVM,* microvillous plasma membrane.

transporter system L. System A has been widely studied in placenta, particularly in relation to fetal growth control, and exists in three isoforms—SNAT1, SNAT2, and SNAT4—expressed by both microvillous and basal plasma membranes.[119,158,161-164] ASC is present only on the basal plasma membrane and is distinguished from system A by its relative pH insensitivity, higher stereospecificity, and intolerance of *N*-methylated amino acids.[25,161] Both isoforms (LAT1 and LAT2) of system L are expressed by microvillous membrane and basal plasma membranes[158,161] but differ in their affinities for leucine and alanine. System L also is present in the basal membrane. Tryptophan, which may have an important role in immune evasion by placenta and fetus,[165] also is transported by system L.[143,158,166]

Taurine is an essential amino acid for fetal growth and is transported by a β-amino acid–specific sodium- and chloride-dependent transporter, TauT.[138,139,167] Cationic amino acids are taken up by syncytiotrophoblast membranes using the sodium-independent transporters system y^+, system b^{0+}, and system y^+L.[133,134,168] However, in the term placenta, transport of cationic amino acids across the microvillous plasma membrane is predominantly by systems y^+ and y^+L, and that across the basal membrane is predominantly by system y^+L.[134]

Only limited maternofetal transfer of the anionic amino acid glutamate across the perfused cotyledon has been described.[155] An additional finding is a negative umbilical artery–umbilical vein concentration difference, suggesting uptake from the fetal circulation into placenta.[169] The reasons for the lack of transfer of anionic amino acids are incompletely understood. A sodium-dependent, potassium-coupled, anionic amino acid transporter, system X^-_{AG}, is present in both microvillous and basal plasma membranes.[129,170] System X^-_{AG} seems to be efficient, because aspartate and glutamate are concentrated 23- and 34-fold, respectively, by human placental slices, whereas other amino acids tested are concentrated no more than 12-fold.[160]

GESTATIONAL CHANGES

A majority of early studies examining the mechanisms of solute transfer across the human placenta have focused on the organ at term. The fetus clearly has varying nutrient demands across pregnancy, however, and because fetal growth in the third trimester far exceeds that of the placenta, increased expression or activity of transporters must account for the more efficient uptake of nutrients. Recent studies on villous fragments or membrane vesicles from first-trimester placentas reveal changes in the expression or activity of many transporter systems across gestation; however, the patterns are system-specific, illustrating the tightly regulated and selective nature of placental nutrient transfer. For example, the activity of system A and system y^+ in microvillous membrane vesicles from term placentas is elevated compared with that in preparations from first-trimester placenta,[171,172] whereas system y^+L is down-regulated across gestation,[172] and system β is unchanged.[173]

These changes in activity do not necessarily represent differences in gene expression or protein synthesis, because discrepancies exist among mRNA, protein, and activity levels for most transporter systems examined. Such discrepancies may be due to post-translational modification or regulation at the level of membrane trafficking or may reflect differential expression of transporter isoforms, as illustrated by system A, in which mRNA for SNAT1 and SNAT2 remain constant over gestation, whereas SNAT4 is elevated in first-trimester placenta.[174] Of interest, the activity of the the glucose transporter and the sodium-hydrogen (Na^+-H^+) exchanger in the microvillous membrane is similar in

second-trimester and in term placentas,[22,175,176] suggesting that the first trimester is the key time of change. Furthermore, some evidence indicates differences in mRNA expression for system A isoforms between early and late first trimester, potentially corresponding to the onset of maternal blood perfusion of the placental villi.[174]

Glucose transporters also exhibit changes in expression over gestation, with recent studies demonstrating up-regulation of GLUT1 expression and activity (in basal plasma membrane only) at term, coincident with down-regulation of GLUT-3 and -4.[122] This pattern is accompanied by changes in the cellular localization of GLUT12, from syncytiotrophoblast in first trimester to vascular endothelial at term, reinforcing that GLUT1 is the predominant syncytial glucose transporter at term.[22]

Studies of the microvillous membrane of the syncytiotrophoblast suggest that the activity of the Na^+-H^+ exchanger and Ca^{2+}-ATPase is higher at term than in the first trimester[142,171,175]; however, its total electrogenic ion transport appears to be reduced with advancing gestation.[29] More detailed studies are needed to examine the transfer capacity of the placenta as a whole, to delineate temporal shifts in the expression and activity of the different transporter systems and to elucidate how the gestational changes are regulated.

TABLE 14-6

Activity of Transporters in Syncytiotrophoblast Microvillous and Basal Plasma Membranes in Intrauterine Growth Restriction

Transporter*	MVM Change	BM Change
System A (alanine-glycine)[119,176,181-184]	dec	nc
Leucine[159]	dec	dec
Lysine[159,172]	nc	dec
Sodium-dependent taurine[185]	dec	nc
Sodium-independent taurine[185]	nc	dec
GLUT1 (glucose)[119]	nc	nc
Monocarboxylate transporter (lactate)[186]	nc	dec
Calcium (ATP-dependent)[187]	not present	inc
Sodium-hydrogen exchanger[176,188]	dec	not measured

Data from cited references.

*In all studies, isolated MVM or BM vesicles were used for measurement of transport activity or expression.

ATP, Adenosine triphosphate; *BM,* basal plasma membrane; *dec,* decreased activity; *inc,* increased activity; *MVM,* microvillous plasma membrane; *nc,* no change in activity in comparison with gestation-matched pregnancies with normally grown babies.

PLACENTAL TRANSFER AND FETAL GROWTH

Net placental transfer over the course of gestation must exactly equal the solute and water composition of the baby at term. Placental transfer is thus intimately involved in determining fetal growth. Idiopathic intrauterine growth restriction (IUGR) has therefore been ascribed for many years to placental insufficiency. Because of the importance of oxygen delivery, the tendency of babies with IUGR to be hypoxic, and the flow limitation of oxygen transfer (see earlier), placental insufficiency has become almost synonymous in the literature with a reduction in uterine or fetoplacental blood flow. This concept also is consistent with the association of IUGR with preeclampsia and abnormal uterine Doppler measurements.[177] However, although placental oxygen delivery and blood flow are undoubtedly important in determining fetal growth, more recent data show that this is not the complete picture. Growth-restricted fetuses are hypoaminoacidemic and hyperlacticacidemic, and they also tend to be acidotic.[15,178-180] Placental transfer of amino acids, lactic acid, and protons is not predominantly flow-limited; therefore the barrier characteristics of the placenta in IUGR also need to be considered.

Such a focus has led to the demonstration that the activity and expression of certain transporter proteins in both the microvillous and basal plasma membranes of the syncytiotrophoblast are altered in IUGR. These data, showing that a syncytiotrophoblast transport anomaly is an important facet of the pathophysiology in IUGR resulting from placental insufficiency, are summarized and referenced in Table 14-6. Some transporter activities increase, some decrease, and some exhibit no change. This variability shows that the anomaly is complex and does not reflect a general down-regulation of metabolic activity, as might be expected.

Although no differences in glucose placental transport have been detected between normal and IUGR pregnancies, a reduction in lactate transfer has been demonstrated. Lactate is an end product of metabolism, but during pregnancy it is an important energy source for the fetus in late gestation.[189] It is supplied to the fetus by cotransport across the placenta with H^+, by the monocarboxylate transporters (MCTs).[190] Multiple MCTs are expressed by human syncytiotrophoblast price[191]; the best-characterized are MCT1 (found predominantly on the basal plasma membrane) and MCT4 (located on both basal and microvillous membranes).[192] Of interest, the reduced lactate transfer in IUGR is confined to the basal membrane, indicating that fetomaternal lactate transfer is diminished.[186] This observation is consistent with the elevated lactate levels measured in growth-restricted fetuses[193] and suggests that placental clearance of lactate is attenuated in IUGR.

The reduction in activity of the amino acid transporters is likely to be particularly important in determining fetal growth rate. Human in vivo studies with stable isotopes revealed that in normal pregnancy, the delivery of neutral amino acids is only just sufficient to meet fetal requirements,[125] and in IUGR, maternofetal ^{13}C-leucine transfer is reduced in comparison with normal findings.[194] In healthy pregnancies, the activity of the system A amino acid transporter in the microvillous plasma membrane, per milligram of membrane protein, is inversely proportional to fetal size at birth.[195] This finding may reflect a compensatory up-regulation of the transporter in the smaller placentas of small normal babies, a hypothesis that is supported by data from mice in which the placenta-specific insulin-like growth factor-II (IGF-II) gene has been knocked out.[10] In the absence of placental IGF-II, the fetus maintains a normal growth trajectory despite placental abnormalities until day 16 of embryonal life (E16) of pregnancy, as a result of an up-regulation of system A. Failure of this compensatory mechanism (by a subsequent reduction in system A activity) between E16 and term corresponds to the onset of fetal growth restriction.[196] The finding that the same transporter activity is lower than normal in the microvillous membrane vesicles of placentas of IUGR-affected babies[176,181] suggests that they are doubly disadvantaged compared with physiologically normal infants: They have a small placenta and less transporter activity per milligram of membrane protein.

The activity of other amino acid transporters (system L and system β) is reduced in membrane vesicles prepared from placentas in pregnancies complicated by IUGR,[172,185,197] together with certain ion transport systems, such as the Na^+-H^+ exchanger,[188,198] which could indirectly affect fetal development through altering trophoblast pH and electrochemical gradients necessary for optimal syncytiotrophoblast function.

Diabetes mellitus during pregnancy can result in fetal overgrowth (macrosomia), and data suggest that syncytiotrophoblast transporter activities also are affected in this condition.[199-202] Materanofetal glucose transfer is increased in women with diabetes, not solely as a result of elevated maternal glucose levels but also because of a two-fold increase in basal plasma membrane GLUT1 levels.[201] Microvillous membrane GLUT1

remains constant, further supporting basal plasma membrane transfer as the limiting step in glucose movement across the syncytiotrophoblast.

Certain amino acid transporters also are altered with macrosomia; however, significant differences have been noted in the responses of individual transporters to gestational diabetes and type 1 diabetes. System A activity is up-regulated in gestational diabetes and type 1 diabetes,[201,203] whereas system L is elevated only in the former.[204] Moreover, these changes were observed only in those placentas that were themselves overgrown, suggesting increased placental metabolic activity in addition to, or instead of, elevated maternofetal amino acid transfer.[199,204] Further evidence for highly selective and differential regulation of transporter systems comes from findings that both the Ca^{2+}-ATPase[205] and lipoprotein lipase[206] are dysregulated in type 1 diabetes but not in gestational diabetes. These observations further demonstrate the need for greater understanding of the regulation of placental transporter activity, across normal pregnancy and in patients affected by IUGR and diabetes.

REGULATION OF PLACENTAL TRANSPORTERS

Investigations into the regulation of placental transporter activity and gene expression have thus far focused mainly on the GLUTs and system A. Evidence from in vitro studies indicates that GLUT1 is regulated by substrate availability. Glucose uptake is enhanced in isolated trophoblast cells incubated in a low glucose environment, and is inhibited by hyperglycaemia.[207] These effects correlate with changes in GLUT1 mRNA expression; however, they are apparent only at extreme glucose concentrations and thus may be more relevant to pathologic states (e.g., diabetes) rather than to normal pregnancy. Longer-term nutritional manipulation also affects placental glucose transport: Undernourishing pregnant rats in late gestation results in IUGR, coincident with reduced expression of GLUT3 but not GLUT1 or GLUT4.[208]

The effects of insulin on placental glucose transport also have been investigated. Studies using term placenta, either perfused cotyledon[209] or microvillous membrane vesicles,[23] failed to show any effect of insulin on glucose uptake. However, insulin can acutely stimulate glucose uptake by first-trimester placental villous fragments,[122] suggesting differences in the regulation of glucose transport across gestation. This observation is consistent with a stimulation of glucose uptake by insulin by the early pregnant rat placenta. These acute effects may be due to changes in membrane localization of GLUTs, supported by the preferential up-regulation of basal plasma but not microvillous membrane GLUT1 protein by IGFs, hypoxia, and growth hormone (GH).[210] Longer exposure to insulin in isolated first-trimester trophoblast cells leads to a sustained elevation in glucose uptake, through the up-regulation of GLUT1 mRNA expression.[207] A similar effect was detected in response to insulin-like growth factors (IGF-I and IGF-II).[211]

Insulin also may affect glucose transport by enhancing the activity of GLUT4 and GLUT12. Both of these insulin-responsive GLUT isoforms are gestation regulated: GLUT4 is abundant in the syncytiotrophoblast in early pregnancy but absent at term,[122] whereas a shift in cellular localization of GLUT12, from syncytial to stromal-vascular, is apparent over the course of pregnancy.[123] Such shifts show remarkable similarity to the spatial and temporal expression patterns of insulin receptors in the placenta, which change from predominantly syncytial to endothelial[212,213] in the third trimester, suggesting that the syncytial uptake of glucose is more sensitive to changes in insulin levels in early pregnancy.

Similar experiments have been performed to examine the regulation of neutral amino acid uptake. System A activity is regulated by substrate availability in fibroblasts, with low concentrations of amino acid stimulating accelerated uptake of MeAIB[214,215]; a similar adaptive regulation is observed in placental cell lines.[216] Acute exposure to leptin and insulin stimulates ^{14}C-MeAIB uptake by villous fragments from term placentas[217] but has no effect on first-trimester placentas. By contrast, growth hormone appears to inhibit system A activity in first-trimester but not term villous fragments.[217,218] More chronic effects of hormones—insulin, IGF-1, and cortisol—on MeAIB uptake have been investigated in cultured trophoblasts derived from first-trimester placenta or choriocarcinoma cell lines (BeWo). In each case, system A activity was stimulated in a dose- and time-dependent manner[219,220]; as supported by some evidence, cortisol may mediate its effects through changes in ATA2 mRNA and its trafficking to the plasma membrane.[220]

The actions of these hormones on placental transport are of interest because the endocrine and metabolic environment may be altered in pregnancy complications. Dietary manipulation during pregnancy in animals is a powerful model for studying the chronic effects of hormones on nutrient transport. Pregnant rats fed a low-protein diet have low circulating levels of insulin, IGF-1, and leptin, and fetuses are growth-restricted at term.[221,222] Also of interest, changes in the expression and activity of system A appear to coincide with the onset of IUGR, with a down-regulation of SNAT2 mRNA and protein, and MeAIB uptake, from E16 only. Similarly, subjecting rats to a brief perioid of hyperglycemia in early pregnancy results in decreased system A activity at term, again through effects on the SNAT2 isoform.[223] These studies further support amino acid transport as an important mechanism for modulating fetal growth and provide in vivo models to elucidate how placental transport is regulated across gestation and in normal and abnormal pregnancies.

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15 Endocrine and Paracrine Function of the Human Placenta

Theresa M. Siler-Khodr

By the early 1900s the placenta was recognized as an endocrine organ. Reports by Zondek and Aschheim[1] and Philipp[2] described placental hormone activities similar to those of both ovarian and hypophyseal hormones, and the significance of placental hormone products for the maintenance of pregnancy was recognized. Since that time the role of placental hormones, which act as endocrine, paracrine, and autocrine regulators of pregnancy, has been defined not only for hypophyseal and gonadal hormones but also for multiple hypothalamic hormones, cytokines, growth factors, other proteins, peptides, and eicosanoids. The regulation and function of each of these factors as related to the placenta are discussed in this chapter.

HYPOPHYSEAL- LIKE HORMONES

Possibly the best-known hormone of pregnancy is human chorionic gonadotropin (hCG). Its activity was first reported in the early 1900s.[1,2] Wislocki and Bennett[3] originally proposed that hCG was synthesized by the cytotrophoblast because its concentrations in pregnancy were parallel to the number and density of cytotrophoblast in the placenta. Since then, it was shown that hCG is synthesized primarily by the syncytiotrophoblastic cells[4] but is regulated by a releasing factor, gonadotropin-releasing hormone (GnRH I and GnRH II isoforms), which is produced largely in the cytotrophoblasts.[5-7] Human CG is a glycoprotein composed of α and β subunits that are translated from separate messenger RNAs (mRNAs).[8] The α subunit is produced in excess of the β subunit, and this ratio increases as gestation progresses. As a result, little free β-hCG is secreted, and the hCG in maternal circulation is mostly intact hCG or free α-hCG. Intact hCG (i.e., having both α and β subunits) is required for its known biologic activities, which are similar to those of human luteinizing hormone (hLH). In addition, the degree of glycosylation of the hCG molecule decreases as pregnancy advances and a decreased level of glycosylation in very early pregnancy has been correlated with early pregnancy loss.[9,10]

Human CG can be immunologically distinguished from hLH using antisera directed against the C-terminal amino acids of its β subunit, which are not present in hLH. Use of β-subunit hCG–specific antisera is the basis for most current pregnancy

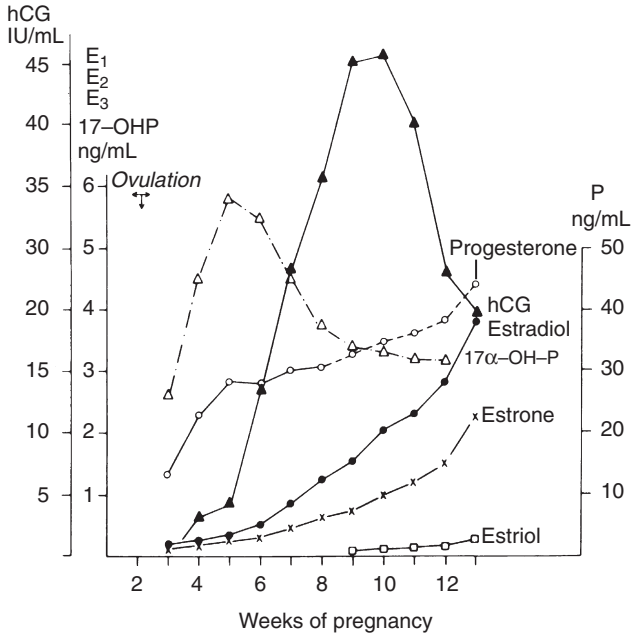


Figure 15-1 Maternal plasma levels of human chorionic gonadotropin (hCG) and steroids during human pregnancy. *E_1,* Estrone; *E_2,* estradiol; *E_3,* estriol; *17α-OH-P,* 17α-hydroxyprogesterone; *17-OHP,* 17-hydroxyprogesterone; *P,* progesterone. *(From Tulchinsky D, Hobel CJ: Plasma human chorionic gonadotropin, estrone, estradiol, estriol, progesterone, and 17 alpha-hydroxyprogesterone in human pregnancy. 3. Early normal pregnancy,* Am J Obstet Gynecol *117:884, 1973.)*

tests. Increasing hCG secretion can be detected in the maternal circulation by 24 hours after implantation and increases with a doubling time averaging 2.11 days. It reaches peak levels of approximately 50 international units/mL at 9 to 10 weeks from the date of the last menstrual period, declining thereafter to 1 international unit/mL by midgestation[11] (Figure 15-1). In early gestation, the hCG doubling time is an effective way to predict the outcome of a pregnancy. An abnormally slow doubling time of hCG is considered to be a sign of a poor prognosis for pregnancy outcome.[12]

In early pregnancy, hCG has both endocrine and paracrine functions: (1) to stimulate formation of the corpus luteum of pregnancy, which in turn produces progesterone, and (2) to stimulate placental steroidogenesis and uterine activities allowing for implantation. In very early pregnancy hCG maintains corpus luteal progestone production. As the placenta develops, an ovarian-placental shift occurs whereby the placenta takes over the production of progesterone. This shift is completed at least 2 weeks before peak placental hCG production is attained (35 to 47 days after ovulation) (see Figure 15-1). Another hormonal function ascribed to hCG is that of a thyrotropic activity, which may affect increased thyroid function. Other data, however, suggest that a distinct thyrotropic factor is synthesized by trophoblastic tissue. In addition, hCG may have immunosuppressive functions.[11,12]

Multiple factors can influence hCG production, such as GnRH,[14] neurotransmitters,[15] cyclic adenosine monophosphate (cAMP),[16] epidermal growth factor,[17] activin,[18] cytokine,[19] prostaglandins,[20] and hCG itself.[20] Each of these is produced by the placenta as well as other trophoblastic tissues. Human CG is known to affect placental steroidogenesis by stimulating both progesterone and estrogen formation. Estrogens can inhibit GnRH stimulation of hCG,[22] thereby completing a feedback axis in the paracrine placenta. Other hormones such as inhibin[23] also have been shown to modulate this axis.

Proteins with lactogenic and growth hormone–like activity were first extracted from human placenta by Higashi in 1961.[24] Shortly thereafter, Josimovich and MacLaren[25] isolated the protein originally named human placental lactogen (hPL). This designation was later changed to *human chorionic somatomammotropin* (hCS), to reflect its growth hormone–like activity as well as its lactogenic activity. This peptide chain of 192 amino acids has considerable homology with human growth hormone and prolactin (PRL). It is synthesized by the syncytiotrophoblasts of the placenta, attaining maximal production in late gestation. At that time accounts for 10% of the total protein synthesis of the placenta. It is the product of two genes that flank the human growth hormone V (hGH) gene expressed in the human placenta.[26,27]

Placental concentration of hCS during pregnancy parallels the mass of the placenta throughout gestation. As a result, maternal circulating hCS levels increase throughout gestation and appear to reflect placental function. Human CS is secreted into the maternal circulation, where it induces metabolic changes such as mobilization of fatty acids, insulin resistance, decreased utilization of glucose, and increased availability of amino acids through decreased maternal use of protein.[28] In addition, the lactogenic activity of hCS suggests a synergistic role with that of PRL and steroids in preparation of the breast for lactation.[29] In the placenta, both hCS and hGH-V may regulate IGF-I[30] and affect fetal growth by direct action on placental nutrient transport. The absence of hCS and hGH-V expression is reported to result in severe fetal growth restriction.[28]

Factors that control the release of hCS are poorly defined. Human CS is stimulated by high-density lipoproteins, apolipoproteins,[27,30] angiotensin,[31] cyclic adenosine monophosphate (cAMP),[32] and arachidonic acid (AA) and are inhibited by the prostaglandins PGE_2 and $PGF_{2\alpha}$,[33] catecholamines, phorbol esters, and diacylglycerols.[34] In addition, dopaminergic agents have been shown to inhibit its release.[35] The releasing and inhibiting factors found in the placenta, however, have not yet been shown to affect hCS release.

PRL also is known to increase during pregnancy both in maternal blood and in amniotic fluid.[36] The PRL in maternal blood is primarily of maternal pituitary origin, whereas that in amniotic fluid PRL is synthesized by the decidua. The role of these high amniotic fluid PRL levels (up to 3 μg/mL) in pregnancy is not understood. Decidual PRL can be stimulated by α-hCG.[37]

Other pituitary-like peptides such as adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (MSH), β-endorphins, and β-lipoproteins have been demonstrated in the human placenta by immunologic and biologic assays,[38] although chemical identity of these placental peptides has not been completely defined. Prostaglandins have been found to increase β-lipotropin and β-endorphin levels in amniotic fluid.[39] Chorionic corticotropin-releasing hormone (CRH) is produced by the placenta and stimulates the release of chorionic ACTH.[40] The physiologic role of chorionic ACTH has not been defined, but it may affect placental cortisol production or fetal adrenal function or both.

STEROIDOGENESIS AND THE MATERNAL-FETAL-PLACENTAL UNIT

Although the placenta was known to be the source of steroid hormones since the early 1900s, it was not until the late 1950s that their biosynthetic pathyways were understood. In 1959, Ryan[41] demonstrated that the placenta could convert dihydroepiandrosterone sulfate (DHEA) to estrogens; five years later, Diczfalusy[42] proposed and subsequently demonstrated the existence of a fetal-placental unit in which fetal enzymes and placental enzymes work in concert to produce estrogens. Although

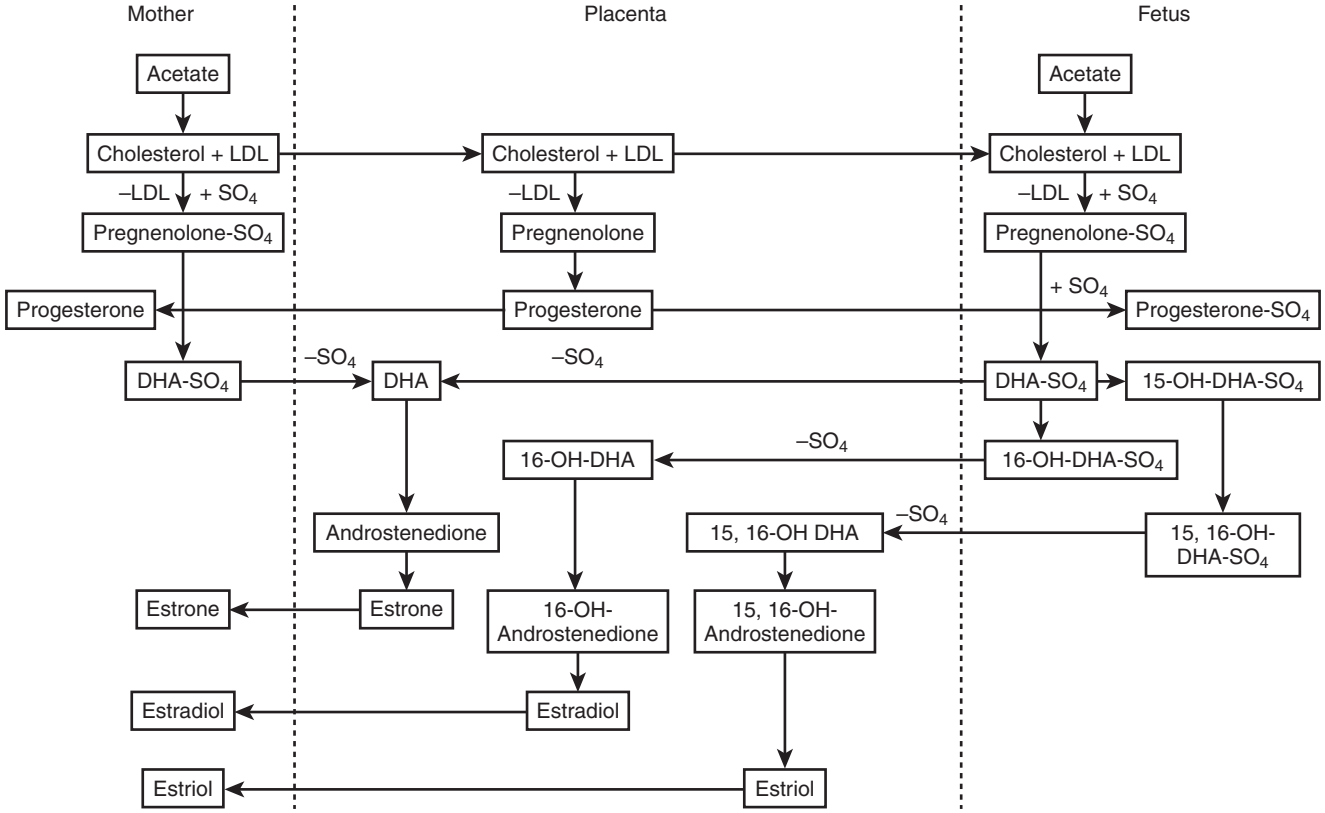


Figure 15-2 Steroidogenic pathways in the human maternal placental-fetal unit. *DHA-SO_4,* Dihydroepiandrosterone sulfate; *LDL,* low-density lipoprotein; *OH,* hydroxy.

the use of maternal cholesterol for placental progesterone synthesis was suggested by Bloch as early as 1945,[43] further studies that unquestionably confirmed these findings and established that maternal cholesterol was the principal precursor of progesterone production were not completed until 1970.[44] The fetal-placental unit was then expanded to include the mother, because maternal cholesterol is needed for placental production of progesterone and for the precursors used for fetal androgen production.[45] Fetal DHEA-SO_4, in turn, is used as a substrate for placental synthesis of estrogens. Thus the maternal-fetal-placental unit functions to produce progesterone and estrogens. These steroids are found largely in the maternal circulation during pregnancy. A comprehensive review of the literature defining their synthesis and functions is available.[45]

Figure 15-2 diagrams the tissues and enzymes that participate in the biosynthesis of progesterone and estrogens.[46] Maternal cholesterol, derived from low-density lipoprotein (LDL), is transported to the placenta and bound to specific LDL receptors on the syncytiotrophoblast, where it is incorporated by endocytosis and hydrolyzed to free cholesterol in the lysosomes.[8] In the syncytiotrophoblastic cells of the placenta, the cholesterol is converted to pregnenolone. Mitochondrial enzymes—the 20, 22-desmolase complex—catalyze this metabolic transformation. Some of the pregnenolone is sent to the fetus, but much is converted to progesterone by the 3β-hydroxysteroid dehydrogenase-Δ^5,Δ^4-isomerase complex.[10,11] Approximately 90% of this progesterone goes to the mother and 10% to the fetus. The fetus has the former enzyme activity needed for pregnenolone synthesis but is severely lacking in the latter enzymatic complex, which is needed for progesterone production. Thus the progesterone that circulates in the fetus at high levels is of placental origin and may be used as a precursor of Δ^4-3-ketosteroids. Because progesterone is not dependent on fetal enzymes, it is a poor indicator of fetal well-being and more related to placental function.

In the fetus, steroids are rapidly sulfated by an adrenal sulfatase, resulting in steroid sulfates, which are steroids biologically inactive. This sulfation step may serve as a protective mechanism to ensure that the fetus is not exposed to high levels of active steroids. Pregnenolone sulfate is rapidly converted to DHEA-SO_4 by fetal adrenal zone enzymes, 17α-hydroxylase and 20,21-desmolase.[47] DHEA-SO_4 may be hydroxylated in the fetal liver at the 16α or 15α position or at both positions. These androgens are then transferred to the placenta.

In the placenta, the SO_4 must be cleaved before the 3β-hydroxysteroid dehydrogenase–isomerase complex can convert DHEA or the hydroxylated DHEAs to androstenedione or hydroxylated androstenediones. These androgens are then aromatized to estrone (E_1), 16α-OH estrone, or 15α-OH estrone and then converted to estradiol (E_2), estriol (E_3), or estetrol (E_4) by the action of the placental 17β-hydroxylation enzyme.[15-17] Although maternal DHEA-SO_4 serves as 40% of the precursor for E_2 synthesis, the latter hormones are formed predominantly from fetal precursors because the maternal liver has limited 5α- or 16α-hydroxylation capabilities.[48] Thus E_3 and E_4 levels are good indices of fetal well-being. Most of the estrogens are then sent to the mother.

Placental steroidogenic capacity is functional early in pregnancy, but not until 35 to 47 days after ovulation can placental progesterone production alone support the maintenance of pregnancy.[49] Before that time, human pregnancy is dependent on the progesterone produced by the corpus luteum of pregnancy or provided by exogenous supplementation. At 35 to 47 days after ovulation, the ovary may be removed and the pregnancy will continue. This change in control locus from ovary to placenta is the aforementioned ovarian-placental shift.

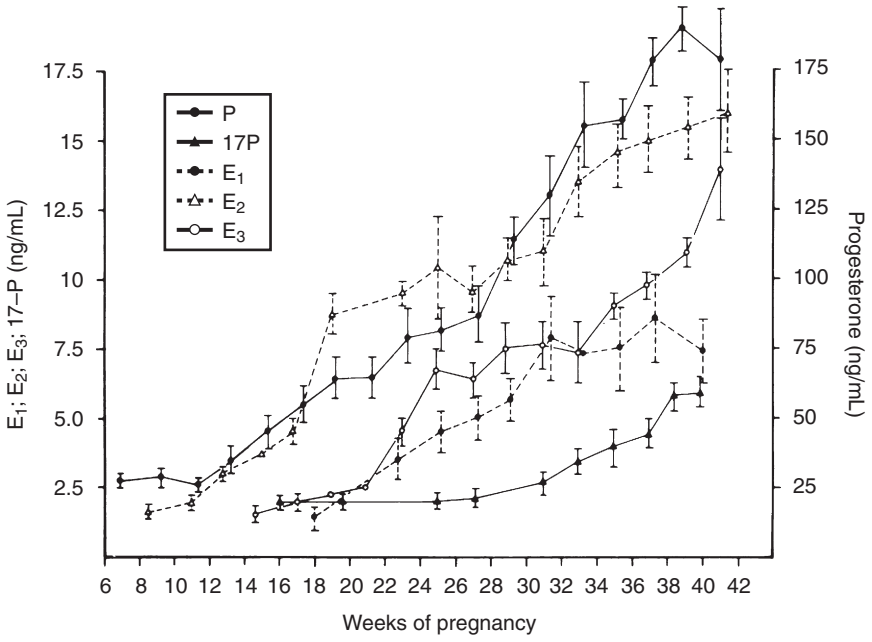


Figure 15-3 Maternal plasma levels of progesterone (P) and 17-hydroxyprogesterone (17P) and of estrone (E_1), estradiol (E_2), and estriol (E_3) during human pregnancy. *(From Tulchinsky D, Hobel CJ, Yeager E, Marshall JR: Plasma estrone, estradiol, estriol, progesterone and 17-hydroxyprogesterone in human pregnancy: I. Normal pregnancy,* Am J Obstet Gynecol *112:1095, 1972.)*

The concentration of steroid hormones in the maternal circulation increases dramatically throughout gestation[50] (Figure 15-3). Multiple functions of estrogens and progesterone in pregnancy are recognized. As discussed previously, progesterone is required for the maintenance of pregnancy by means of its suppressant effect on uterine contractions.[46,49,51] It also influences breast development while suppressing lactation by inhibiting lactose synthesis. Progesterone also affects glucose homeostasis and water metabolism. In addition, it has been shown to have immunosuppressive activity.[51] Estrogens influence uterine growth, blood flow, contractility, metabolism, and breast development.[22] Both estrogens and progesterone modulate the activity of other primary factors involved in the initiation of labor, such as prostaglandins and oxytocin. Before parturition, an increase in the estrogen to progesterone ratio (E/P) occurs within the intrauterine tissues. This altered E/P milieu may enhance and modulate prostaglandin and oxytocin production and action at the time of parturition. It is now known that placental steroid hormone production is influenced by protein trophic hormones and other factors such as hypothalamic-like releasing or inhibiting hormones, growth factors, cytokines, and prostaglandins.

Maternal circulating levels of glucocorticoids and mineralocorticoids are increased in pregnancy.[52] Although the placenta produces ACTH, its action is primarily in the placenta. However, the placenta has the ability to produce cortisol and to convert it to cortisone, as well as the reverse reaction. The activity of these enzymes differ with gestation stage and endocrine milieu.[53] A positive feed-forward axis of cortisol on placental CRH in late gestation (discussed later) may directly affect the fetal adrenal gland and can increase the production of DHEA-SO_4 (an estrogen precursor). CRH may therefore have an important role in parturition.

HYPOTHALAMIC-LIKE RELEASING AND INHIBITING ACTIVITIES

Although it was recognized since the early 1900s that the human placenta is an endocrine tissue, it was not until 1967, possibly both in protest and in prophecy, that Benirschke and Driscoll[54] stated, "Perhaps even more challenging at this time is the quest for an understanding of the regulatory phenomena which must be responsible for the predictability of levels of various hormones in the secretions of a normal pregnant woman, since it is difficult to conceive that no feedback mechanisms exist for those various rises and declines of hormone titers." In the 1970s, elucidation of such factors began.[5]

Since the mid-1970s, numerous studies have established that trophoblastic tissues contain and produce all the defined hypothalamic releasing and inhibiting activities.[5-7,14,55] These data have led to the general acceptance of the hypothesis that the classic hormones of the placenta are produced by means of a controlled paracrine-autocrine system. Human chorionic GnRH, the first-described placental hormone with hypothalamic-like activity, has been localized to the cytotrophoblast of the placenta,[5] and the synthesis of peptides having GnRH biologic and immunologic activity has been demonstrated.[6] The complementary DNA (cDNA) that encodes the GnRH I (mammalian GnRH) has been defined.[56] This same mRNA contains a 56–amino acid peptide, called gonadotropin-releasing hormone–associated peptide (GAP), that is thought to have prolactin-inhibiting factor (PIF) activity. Thus the placenta also can make PIF. Further chemical[57] and biologic data[58] suggest that another chorionic GnRH also may exist. The expression and action of a GnRH II (chicken II GnRH) by the placenta also have been demonstrated.[7]

GnRH I and GnRH II and their analogues bind to specific receptors on the syncytiotrophoblast. GnRH I stimulates cAMP[59] and hCG release without affecting hCS release.[13,14,60] The pattern and degree of hCG response are related to gestational age[13] and estrogen and progesterone levels[62,63] and can be modulated by inhibin.[24] A role for Ca^{2+} in the GnRH I stimulation of hCG has been indicated.[61] GnRH I and GnRH II also directly regulate placenta steroid release[63] and regulate prostanoid production from the term placenta.[7,64,65] In early gestation, GnRH I antagonists and GnRH I superagonists can inhibit hCG, steroid, and prostaglandin release from placental explants, and because this action can be partially reversed with exogenous GnRH I, a specific receptor-mediated action is suggested.[7,65] The release of GnRH

I by placenta is affected by cAMP, prostaglandins, epinephrine,[66] and inhibin.[24] Thus a paracrine axis for chorionic GnRH I and GnRH II–hCG–steroids–prostaglandins–inhibin–PRL has been proposed. In addition, a placental peptidase that can inactivate GnRH I and GnRH II has been isolated.[67] This peptidase may play an important role in the metabolism of GnRH and thus affect the homeostasis of this axis.

Numerous studies in which GnRH analogues or antisera have been administered to pregnant rats, monkeys, or baboons have indicated a possible function of chorionic GnRHs in the maintenance of pregnancy.[68] Administration of GnRH analogues in early pregnancy can induce pregnancy failure.[69-71] Maternal concentrations of GnRH I vary throughout pregnancy and parallel GnRH levels in the placenta and those of hCG.[14,72] Premature labor has been associated with low levels of circulating GnRH I in the first half of gestation. Thus these data support the hypothesis that chorionic GnRHs plays a significant role in the normal physiology of pregnancy.

Human placenta and membranes also produce a chorionic thyrotropin-releasing hormone (TRH). Attempts to define its chemical identity have led to conflicting findings. Shambaugh and colleagues identified chorionic TRH with bioactivity and immunoactivity coincident with TRH.[73] Maternal levels have not been studied; however, at least a part of the TRH administered to the mother can pass through the placenta and affect fetal thyroid function.[74] One study has reported that low levels of immunoreactive TRH in amniotic fluid were correlated with low Apgar scores.[75]

A chorionic somatostatin also has been localized to the cytotrophoblast of the human placenta.[76] Concentrations are highest in first-trimester placentas. Amniotic fluid has the capacity to rapidly degrade somatostatin. Somatostatin administered to the mother can cross the placenta to the fetus[77]; however, the high levels at term in the fetoplacental circulation appear to be of fetal origin.[78] Studies on somatostatin at other times of gestation have not been done. A role for somatostatin in the placental-decidual unit has been reported. Somatostatin is thought to acutely inhibit prolactin-releasing factor (PRL-RF).[79] PRL-RF is a 24,000-Da molecule that has been extracted from human placenta and is active in stimulating both the chronic and the acute release of decidual PRL. No effect of either somatostatin or PRL-RF on hCS release has been found. A chorionic growth hormone–releasing factor (GRF) has been identified in both the rat and porcine placenta, yet its chemical nature or activity remains to be determined.[80,81]

Chorionic CRH is another hormone with hypothalamic-like activity identified in the placenta.[82,83] It has been localized to both the syncytiotrophoblastic and cytotrophoblastic cells of the placenta and also is found in the cytotrophoblasts of the chorion.[84] Its chemical nature appears to be essentially identical to that of hypothalamic CRH, having a placental mRNA that hybridizes with the hypothalamic cDNA. Other studies, however, also have found some larger-molecular-weight chorionic CRHs that await further definition. Human chorionic CRH is transferred to both the maternal and the fetal circulations.[40,84,85] Concentrations in the maternal circulation are greater than those in the fetus and increase throughout pregnancy. A rapid increase is seen at term gestation, and the higher levels are maintained during labor (Figure 15-4). In pregnancies complicated by hypertension, the maternal circulating levels of CRH are already elevated by 28 weeks of pregnancy.[86] Chorionic CRH has been shown to be biologically active in stimulating the release of ACTH and β-endorphin.[40,84,87] Thus it is speculated that chorionic CRH may control placental, fetal, or maternal ACTH and endorphin levels. In late gestation, dexamethasone induces a paradoxical stimulation of mRNA for chorionic CRH.[88]

The increasing CRH released into the maternal circulation has been noted to correlate with the length of gestation and is sometimes referred to as a *placental clock*.[89] Ongoing studies suggest that CRH level may serve as a parameter that can predict preterm delivery.[89,90] Other studies have demonstrated that CRH can directly stimulate DHEAS production by the fetal adrenal.[91] Because DHEAS is the precursor for estrogen, this may be one mechanism by which a favorable endocrine milieu for parturition is effected. CRH is also thought to regulate the immune tolerance at implantation.[92]

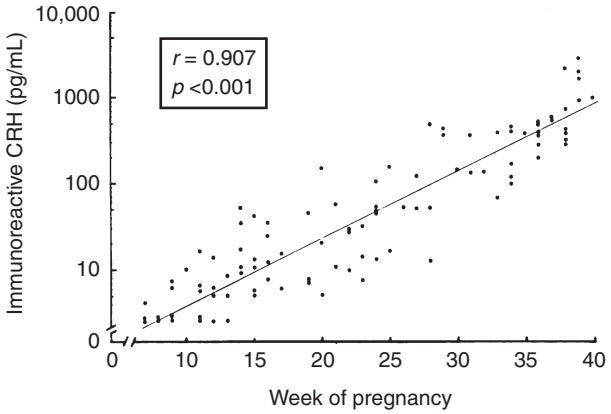


Figure 15-4 Maternal plasma levels of corticotropin-releasing hormone (CRH) during human pregnancy. *(From Sasaki A, Shinkawa O, Margioris AN, et al: Immunoreactive corticotropin-releasing hormone in human plasma during pregnancy, labor, and delivery,* J Clin Endocrinol Metab *64:224, 1987.)*

Another hypothalamic hormone thought to play a role in parturition is oxytocin. A review of this topic has been presented by Soloff.[93] A placental oxytocinase, cystine aminopeptidase, has been described and may play an important role in the regulation of oxytocin levels. Oxytocin has been shown to increase both prostaglandin[94] and ACTH[87] release from intrauterine tissues.

OTHER CHORIONIC CYTOKINES, GROWTH FACTORS, AND PEPTIDES

Cytokines, such as interferons, tumor necrosis factor-α (TNF-α), leukemia inhibitory factor (LIF), and interleukins,[95-99] also are produced by the placenta, and their receptors are expressed in the uteroplacental tissues. The cells of the placenta that express cytokines include the trophoblast, macrophage, and endothelial cells.[99-102] Cytokine expression varies with the stage of gestation but is now known to play an important role in implantation and immunosuppression during pregnancy,[103,104] as well as in regulation of trophoblastic and vascular functions in the placenta during gestation[105] and in the initiation and progrss of parturition.[100-102,106] Cytokines affect these activities by regulation of other cytokines, growth factors, hormones, and prostanoid production.[107-109]

At the time of implantation, the shift of the cytokine helper T cell ratio T_H1/T_H2 toward T_H2 is postulated to be of primary importance in suppressing the immune response at the uteroplacental interface.[106] The role of cytokines in parturition has been likened to an inflammatory response. Cytokine production is thought to be involved in preterm labor in pregnancies associated with intrauterine infection, as well as in preterm labor of other causes[101]; prostanoid production is increased, possibly through cytokine increases.

Another cytokine produced by the placenta is leptin.[110,111] It is proposed to play a role in the cytotrophoblast invasion at the time of implantation and to participate in regulating fetal growth and placental function.[112,113] Its increased production in preeclampsia and gestational diabetes has led to the hypothesis that this cytokine may play a role in these diseases of pregnancy.

Another class of hormones identified in the placenta and chorionic membranes is that of growth factors. The early blastocyst expresses platelet-derived growth factor A (PDGF-A), transforming growth factor (TGF)-α, and TGF-β.[114] These factors are thought to be involved in signaling implantation. At this early stage, other growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), nerve growth factor (NGF-β), and granulocyte colony-stimulating factor (G-CSF) are not detected in early gestation[17] but are expressed by the placenta at later gestation stages.[115] Receptors for EGFs, as well as many other growth factor receptors, have been identified in the placenta and membranes.[116] Most of the EGF receptors in the placenta are localized to the syncytiotrophoblast and correlate with induction of differentiation function of trophoblast rather than proliferation.[117-119] The finding that EGF stimulates hCG secretion supports this hypothesis.[17] EGF also stimulates prostaglandin synthesis.[119] Another growth factor, hepatocyte growth factor or scatter factor, appears to be essential for placental development.[120,121]

The production of insulin-like growth factors I and II (IGF-I and IGF-II) from human placental tissues also has been demonstrated.[122] Within the placenta, IGF-I has been localized predominantly to the syncytiotrophoblast of the fetal villi.[123] IGF-binding proteins (IGFBPs), which are carrier proteins protecting IGF from degradation while blocking its bioactivity, also have been localized to these cells.[122,123] In addition to placental IGFBP, IGFBP-1 is produced by the decidua in large amounts. Receptors that bind IGFs have been identified in the human placenta; IGF-I receptor is the most dominant.[122-124] Placental growth hormone concentrations correlate with IGF-I levels throughout pregnancy.[30] IGFs potentiate the action of EGF[125] and stimulate decidual PRL production.[126] The production of progesterone is enhanced, whereas that of estrogen is inhibited.[127] Placental transport of glucose and amino acids also is enhanced by IGFs, and IGFs also regulate both fetal and placental growth.[128-130] In addition, production of placental thromboxane, a potent vasoconstrictor, is specifically inhibited by IGF-I.[131] Thus the production of placental IGF-I and its binding proteins is thought to be of major importance for normal intrauterine fetal growth.[129]

Relaxin also is synthesized by the membranes, decidua, and placenta.[132] Receptors for relaxin are present in the syncytiotrophoblast of the placenta.[133] A biologic action for relaxin on the collagenase activity of the chorionic-amnionic membranes has been demonstrated. Relaxin also stimulates prostaglandin release and increases collagenase, the enzyme that degrades collagen. Thus an important role for relaxin in human parturition is likely.

Inhibin also is synthesized by the cytotrophoblast of the placenta.[134,135] Its release by the placenta can be stimulated by 8-Br-cAMP as well as α-hCG. Inhibin has been shown to inhibit GnRH stimulation of hCG and chorionic GnRH production.[135] Antiserum to inhibin increases hCG release, and this effect can be partially reversed by GnRH I antagonist. Activin potentiates the GnRH-stimulated hCG release.[23] Thus activin and inhibin are actively involved in the GnRH-hCG-steroid-prostaglandin axis.

Vasoactive peptides, such as vascular endothelial growth factor, endothelin, angiotensin, and arginine vasopressin and their receptors, also are expressed by the human placenta. The peptides endothelin and endothelin 3 are produced by the placenta, and both are active and potent vasocontrictors in the fetal-placental circulation.[136-139] Endothelins also inhibit decidual prolactin release.[139] The angiotensin-renin system has been described in the placenta and is thought to be a factor in the regulation of vascular tone in the placental bed mediated by angiotensin II production and action.[140] Other peptide hormones effecting vascular tone, such as atrial natriuretic peptide (ANP) and its receptors, have been demonstrated in the placenta.[141] ANP is a vasodilator hormone that is thought to be produced by the cytotrophoblast because secretion decreases as these cells differentiate in vitro to syncytiotrophoblast. ANP inhibits the vasoconstrictive action of endothelin and angiotensin and induces vasodilatation in the uterus and the placenta.

Neuropeptides such as enkephalins[142] and dynorphin[143] have been found in the placenta. Receptors for opiate-like substances are present on the syncytial membrane of the placenta. The stimulation of these receptors has been shown to modulate acetylcholine[144] and hCG release.[145,146] Adrenergic agonists and antagonists affect placental hCG and PRL release,[147] whereas dopaminergic receptor stimulation has been shown to lower hCS[35] and increase hCG release.[35] A parathyroid hormone–related protein has been extracted from placental tissues[148] and localized to the cytotrophoblastic cells.[149] Parathyroid hormone–related protein has been shown to have potent vasodilator activity in the fetal-placental circulation.[150]

EICOSANOIDS

An important role for eicosanoids, such as thromboxanes, prostacyclins, and leukotrienes, during pregnancy is well recognized. In general, prostanoids and eicosanoids are thought of as products of the decidua and chorionic membranes, which are active in their production. Prostanoids and leukotrienes, however, also are produced in large quantities by the placenta.[151] In the placenta, the chorionic membranes, and decidua, AA must be mobilized (i.e., deesterified from lipoproteins) before it can be used for production of eicosanoids and prostanoids.[152] Mobilization of AA is induced through two enzymatic pathways. In the first pathway, the hydrolysis of AA from phosphatidylethanolamine is catalyzed by phospholipase A_2. The other pathway releases AA from phosphatidylinositol by the enzymatic activities of phospholipase C, monoacylglycerol lipase, and diacylglycerol lipase. AA is converted to prostaglandin G_2 (PGG_2) by cyclooxygenase and then by the enzyme prostaglandin hydroperoxidase to PGH_2. PGH_2 is the common precursor for the prostaglandins PGD_2, PGE_2, and $PGF_{2\alpha}$, which are formed by the enzymatic action of specific endoperoxides isomerases D and E, and reductase F. The levels of PGD_2, $PGF_{2\alpha}$, and PGE_2 can be measured directly. Thromboxanes and prostacyclin (i.e., prostaglandin I_2 [PGI_2]) are synthesized from PGH_2 by thromboxane and prostacyclin synthetases. Owing to the rapid nonenzymatic conversion of thromboxane A_2 (TxA_2) to TxB_2, TxB_2 levels are used as a measure of TxA_2 synthesis. Similarly, prostacyclin is enzymatically metabolized rapidly to 6-keto-$PGF_{1\alpha}$, which is measured directly.

The level of some prostanoids in term amnion, chorion, decidua, and placenta has been determined.[58,151] Human term placentas convert AA primarily to thromboxane, prostacyclins, PGE_2, $PGF_{2\alpha}$, and PGD_2. Production of $PGF_{2\alpha}$ is approximately half that of PGE_2; and prostacyclin production is one fifth.[153] Prostaglandins, thromboxanes, and prostacyclin have been implicated as important hormones during pregnancy, at the time of implantation, in the regulation of fetal growth and in the initiation and progress of labor. Their role in implantation has been demonstrated by the inhibitory effect of indomethacin (a cyclooxygenase inhibitor) on implantation. Other studies suggest that production of prostaglandins, thromboxane, and, particularly, prostacyclin may enable the trophoblast to invade and colonize the maternal spiral blood vessels of the myometrium.[154] Prostacyclin is known to be a potent vasodilator and inhibitor of platelet aggregation.

After implantation, during the period of fetal growth and development, prostaglandins, thromboxane, and prostacyclin play an important role as vasoregulators of the fetal-placental unit.[155] PGE_2 is known to be a vasodilator of uterine blood flow while constricting the umbilical circulation. $PGF_{2\alpha}$ vasoconstricts both the maternal and the umbilical circulations. In pregnancies associated with hypertension, increased production of thromboxane and reduced prostacyclin have been demonstrated.[156] Amniotic, chorionic, decidual, and placental tissue levels of PGE_2 and $PGF_{2\alpha}$ also are decreased in pregnancies complicated by hypertension.

Similarly, prostacyclin levels in the amnion are diminished in hypertensive pregnancies. Thromboxane production was not found to be increased in pregnancies associated with normotensive intrauterine growth restriction and in some cases was reduced.[157]

Other pregnancy-related functions of prostaglandins are the ability to affect protein synthesis in the placenta and modulation of the production of certain other hormones.[158-160] One study found that $PGF_{2\alpha}$ suppresses hCS production. Other studies in vitro have shown a direct stimulatory action of AA but not $PGF_{2\alpha}$ or PGE_2 on hCS. The ability of $PGF_{2\alpha}$ to stimulate hCG release in pregnant women has been reported. In addition, both PGE_2 and prostacyclin have been shown to increase cAMP levels.

An important function of prostaglandins, thromboxanes, and prostacyclin in pregnancy is their role in the initiation and progress of labor. Prostaglandins of the E and F series are elevated 6- to 12-fold in the amniotic fluid of women in labor.[156,161] Metabolites of thromboxane and prostacyclins (i.e., TxB_2, 6-keto-$PGF_{1\alpha}$) also are increased in amniotic fluid (two- to four-fold). It has been shown that infusion of PGE_2 and $PGF_{2\alpha}$ can be used to induce labor. These prostaglandins in larger doses also are effective in inducing midtrimester abortions. Intraamniotic administration of AA, the precursor of prostanoids, has resulted in the initiation of labor and delivery.[162] Chronic ingestion of aspirin, a cyclooxygenase inhibitor (of production of prostanoids from AA), can override this action of AA.[163]

As discussed previously, factors that are known to stimulate prostanoid production include CRH, cytokines, and growth factors. Both CRH and cytokines are thought to play a primary role in parturition, and increased prostanoid production is proposed to be one of the mechanisms of their action. In addition, it has been demonstrated that glucocorticoids, progesterone, and estrogens have an effect on prostaglandin production.[164] Studies have shown that GnRH can affect prostaglandin release from the placenta.[58,59,65] Placental prostaglandin release is highest when chorionic GnRH is highest; however, the response of prostaglandins to GnRH varies throughout gestation, as does the basal release of prostaglandins.[88] Of interest, basal prostaglandin release can be inhibited by a GnRH antagonist.[65]

As noted earlier, EGF can promote prostaglandin release from amniotic membranes. Another factor known to stimulate prostanoid release is platelet-activating factor (PAF).[165] The mechanism of action of PAF may involve mobilization of Ca^{2+}. PAF is present in amniotic fluid during active labor but not when labor is not occurring. Thus a role for PAF in the regulation of prostaglandins at parturition has been hypothesized.[166] A role for PAF at the time of implantation also has been suggested.[167]

ENZYMES THAT REGULATE PLACENTAL ENDOCRINE AND PARACRINE ACTIVITIES

Placenta enymatic activities also regulate autocrine, paracrine, and endocrine activities of the placenta by affecting their metabolism and thus their concentrations. In a number of cases these enzymes have been well demonstrated. In the case of GnRH, a highly active post–proline peptidase is known to regulate its concentration.[67] The placenta, having both endocrine and enzymatic activity, produces large quantities of this enzyme, providing a means for compartmentalization and localization of this specific activity. A role for protease in the degradation of angiotensin II during pregnancy has been described.[168] In the case of cortisol, a dual oxidative and reductive enzymatic activity regulates the balance between cortisol and cortisone.[53] In the placenta, oxidation of cortisol to cortisone predominates, whereas in the decidua, the reverse reaction dominates. Furthermore, differential regulation of these enzymatic systems throughout pregnancy has been demonstrated.[53] For prostaglandins, regulation of expression of the prostaglandin dehydrogenase has been proposed to participate in the initiation of labor.[169,170] In addition, oxytocinase and aminopeptidase may be involved in the regulation of parturition.[171,172]

INTEGRATION OF THE PLACENTA WITH THE INTRAUTERINE TISSUES

The human placenta has multiple physiologic functions that can be appreciated from the long list of regulatory factors it produces that act on the various cell types. Of note, however, the placenta does not act in isolation. It functions are integrated with those of other intrauterine tissues, such as the maternal uterus, chorion, amnion, decidua, and amniotic fluid and the fetus. These other intrauterine tissues produce or extract some of the same hormones and carrier proteins that regulate placental hormone activity. Metabolic signals and precursors as well as hormones are carried by maternal and fetal blood or transported from cell to cell from the uterus, decidua, chorion, amnion, and amniotic fluid to and from the placenta. Integration of the intrauterine tissues provides for the normal physiology of pregnancy, allowing for its maintenance and timely parturition. In addition, recent studies have demonstrated that fetal experience in utero results in the programming of adult functions and beyond—that is, in utero experience can affect epigenetic phenomena in generations to come.[173,174] Thus aberrations within these intrauterine autocrine, paracrine, and endocrine systems may be responsible for many of the disorders that occur during pregnancy and in adult life. Understanding of this system is rapidly developing and promises to lead to an enhanced ability to regulate these systems and thus to better maintain pregnancy, control the timing of parturition, and ensure the well-being of offspring in future years and generations.

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Fetal and Maternal Responses to Intrauterine Infection

In the last 20 years, infection has emerged as a significant cause of preterm parturition.[1-8] It is the only pathologic process for which a firm causal link with prematurity has been established and a defined molecular pathophysiology is known.[9] Moreover, fetal infection and inflammation have been implicated in the genesis of fetal and neonatal injury,[10,11] leading to cerebral palsy[12-16] and chronic lung disease.[17-20] This chapter reviews the evidence showing the association of infection with spontaneous preterm birth, the role of cytokines and other inflammatory mediators in the host response to intrauterine infection, and finally, the fetal and maternal inflammatory response to intrauterine infection.

Systemic maternal infection (e.g., pneumonia, pyelonephritis, malaria, typhoid fever) has been associated with preterm labor and delivery.[21-31] Yet many of these conditions are rare in developed countries; thus the attributable risk of systemic maternal infection for prematurity is considered to be low. In addition, periodontal diasease was proposed as a risk factor to preterm labor.[32-34] Evidence suggests that some microorganisms isolated in amniotic fluid may have an oral origin,[32] and preterm neonates have evidence of a humoral immune response to microorganisms normally present in the oral cavity.[33,34] However, after adjustment for other factors, periodontal disease associated with

preterm birth was not related to abnormal intrauterine bacterial colonization or histologic chorioamnionitis.[35]

Intrauterine infection and inflammation frequently are associated with preterm labor and delivery. It has been estimated that at least 40% of preterm births occur to mothers with evidence of intrauterine infection or inflammation, which is largely subclinical in nature.[36-38] Moreover, the lower the gestational age at delivery, the greater the frequency of intrauterine infection,[39] and thus the higher the burden of disease attributable to intrauterine infection and inflammation. Discussed next are the pathways of intrauterine infection, its frequency and microbiology, and the rate of fetal infection.

PATHWAYS OF ASCENDING INTRAUTERINE INFECTION

Microorganisms may gain access to the amniotic cavity through several pathways: ascending from the vagina and cervix; hematogenous dissemination through the placenta (transplacental infection); retrograde seeding from the peritoneal cavity through the fallopian tubes; and accidental introduction at the time of invasive procedures, such as amniocentesis, percutaneous fetal blood sampling, chorionic villus sampling, or shunting.[40-44] The most common pathway of intrauterine infection is the ascending route.[3,40,43,44] Supporting evidence includes the following: (1) Histologic chorioamnionitis is more common and severe at the site of membrane rupture than in other locations, such as the placental chorionic plate or umbilical cord[45]; (2) in virtually all cases of congenital pneumonia, inflammation of the chorioamniotic membranes is present[40-42]; (3) bacteria identified in cases of congenital infections are similar to those found in the lower genital tract[43]; and (4) in twin gestations, histologic chorioamnionitis is more common in the firstborn and has not been demonstrated in the second twin alone. Moreover, when intraamniotic infection is detected, the presenting sac is nearly always involved.[46] Indeed, the membranes of the first twin are generally apposed to the cervix, which is taken as evidence in favor of an ascending infection.[43]

STAGES OF ASCENDING INTRAUTERINE INFECTION

Ascending intrauterine infection is believed to occur in four stages[3] (Figure 16-1). Stage 1 corresponds to a change in the vaginal/cervical microbial flora or the presence of pathologic organisms (e.g., *Neisseria gonorrhoeae*) in the cervix. Some forms of bacterial vaginosis may be an early manifestation of this initial stage. In stage 2, the microorganisms gain access to the intrauterine cavity, reside in the decidua, and a localized inflammatory reaction leads to deciduitis. Microorganisms may then be located in the chorioamniotic membranes. In stage 3, the pathogens may invade the fetal vessels (choriovasculitis) or proceed through the amnion (amnionitis) into the amniotic cavity, leading to microbial invasion of the amniotic cavity. Rupture of the membranes is not a prerequisite for intraamniotic infection because microorganisms are capable of crossing intact membranes.[47] Finally, in stage 4, bacteria may gain access to the fetus by different ports of entry. Aspiration of the infected fluid by the fetus may lead to congenital pneumonia. Otitis, conjunctivitis, and omphalitis may occur by direct spreading of microorganisms from infected amniotic fluid. Seeding from any of these sites to the fetal circulation may result in fetal bacteremia and sepsis.

MICROBIOLOGY OF INTRAAMNIOTIC INFECTION

The most common microbial isolates from the amniotic cavity of women with preterm labor and intact membranes are *Ureaplasma urealyticum, Fusobacterium* spp., and *Mycoplasma hominis.*[3,37,48-50] Other microorganisms that have been found in the amniotic fluid include *Streptococcus agalactiae, Peptostreptococcus* spp., *Staphylococcus aureus, Gardnerella vaginalis, Streptococcus viridans,* and *Bacteroides* spp. Occasionally, *Lactobacillus* spp., *Escherichia coli, Enterococcus faecalis, Neisseria gonorrhoeae,* and *Peptococcus* spp. have been encountered. *Haemophilus influenzae, Capnocytophaga* spp., *Stomatococcus* spp., and *Clostridium* spp. are rarely identified.[51,52] In 50% of patients with microbial invasion, more than one microorganism is isolated from the amniotic cavity. The inoculum size varies considerably, and in 71% of cases, more than 10^5 colony-forming units per milliliter are found.[45]

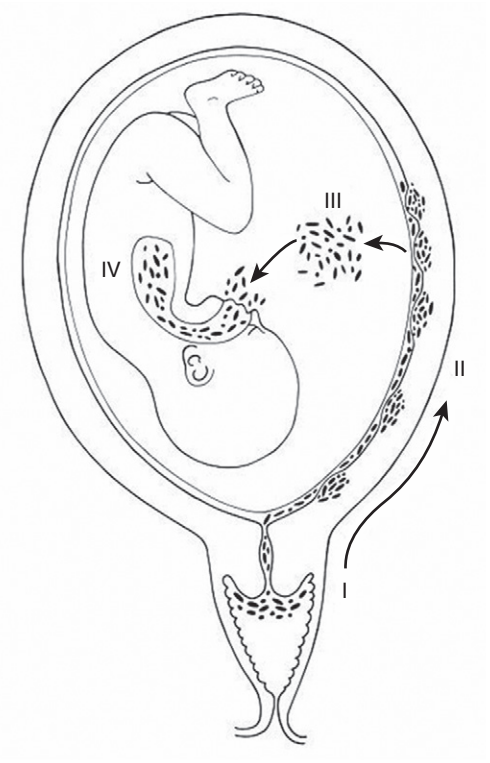


Figure 16-1 The most common pathway of intrauterine infection is the ascending route. *(Reproduced with permission from Romero R, Mazor M. Infection and preterm labor.* Clin Obstet Gynecol *31:553, 1988.)*

The role of *Chlamydia trachomatis* as an intrauterine pathogen has not been clearly defined. This microorganism is an important cause of cervicitis and has been isolated from amniotic fluid.[53,54] Ville and colleagues[55] tested 89 patients with preterm prelabor rupture of membranes (PROM) for the presence of *C. trachomatis* DNA in the uterine cervix (collected by cervical swabs) and amniotic fluid (collected by transabdominal amniocentesis). Cervical samples tested positive for *C. trachomatis* DNA in 23% (20 of 89) of the cases, whereas amniotic fluid samples were positive in 30% (6 of 23). The presence of *C. trachomatis* DNA was not associated with a shorter amniorrexis-to-delivery interval or with an increase in perinatal morbidity or mortality. Thus, *C. trachomatis* DNA is present in the amniotic fluid of symptomatic and asymptomatic patients; however, the clinical implication of its presence is not yet clear.

Viral genome has been recovered from the amniotic fluid of both high- and low-risk pregnancies (Table 16-1).[56-61] In high-risk pregnancies, especially those complicated by nonimmune hydrops, viral genome may be recovered in a substantial proportion of cases.[57,61,62] In contrast, the prevalence of viral genome in the amniotic fluid of asymptomatic pregnancies may range from 0% to 15%, with no apparent change in perinatal outcome.[56-58] Adenovirus is the most common viral DNA isolate, in either symptomatic or asymptomatic pregnancies, followed by cytomegalovirus (CMV) and enterovirus.[56-61] The pathways by which

TABLE 16-1

Summary of Studies Reporting Recovery of Viral Genome in the Amniotic Fluid

Study (Year)	Population	*n*	Viral DNA, *n* (%)	Viral Genome Recovered	Complications
McLean et al. (1995)	Low-risk	277	0	—	—
Wenstrom et al. (1998)	Low-risk: normal outcome	60	9 (15)	Adenovirus (5), CMV (3), parvovirus B19 (1)	—
	Low-risk: poor outcome†	62	5 (8)	Adenovirus (4), HSV (1)	—
Van den Veyver et al. (1998)	Low-risk	154	4 (2.6)	Adenovirus (3), CMV (1)	—
	High-risk	303*	124 (41)	Adenovirus (74), CMV (30), enterovirus (22), HSV (9), parvovirus (8), EBV (4), RSV (2)	Nonimmune hydrops, ventriculomegaly, growth restriction, stuck-twin syndrome, hepatic calcifications, ascites, myocarditis, pericardial effusion, hydramnios
Baschat et al. (2000)	Low-risk	240	20 (8.9)	Adenovirus was more commonly isolated from complicated pregnancies (16/240 versus 23/138, $p < .005$) Among all pregnancies: adenovirus (39), enterovirus (5), CMV (3), RSV (1), EBV (1), parvovirus (1)	
	Fetal complications‡	138	27 (19.6)		CNS malformations (7), SGA (4), multiple malformations (4)
Reddy et al. (2002)	High-risk	138	25 (18)	Tested for CMV, parvovirus, adenovirus, enterovirus, HSV, EBV, RSV (not discriminated)	Increased incidence of preterm labor and delivery, PPROM, IUFD, SGA
Baschat et al. (2003)	Low-risk	686	44 (69)	Adenovirus (37), CMV (5), EBV (2), enterovirus (2), RSV (1)	—

*371 specimen samples: amniotic fluid ($n = 253$); fetal blood at cordocentesis ($n = 42$), cord blood at the time of delivery ($n = 18$), fetal ascites ($n = 8$), pleural effusion ($n = 10$), and other tissue samples ($n = 40$).

†Pregnancies lost within 30 days of genetic amniocentesis.

‡Fetal anomalies, SGA, proven maternal infection; samples obtained from amniotic fluid, fetal blood or pleural fluid.

CMV, Cytomegalovirus; *EBV,* Epstein-Barr virus; *HSV,* herpes simplex virus; *IUFD,* intrauterine fetal demise; *PPROM,* preterm premature rupture of the membranes; *RSV,* respiratory syncitial virus; *SGA,* small for gestational age.

viruses gain access to the amniotic cavity in asymptomatic low-risk pregnancies have not been clearly elucidated.

Congenital infection by CMV, rubella virus, parvovirus, and herpes simplex virus has been extensively described; however other viruses are emerging as potential fetal pathogens, including adenovirus, coxsackievirus, human papilloma virus, influenza virus, lymphocytic choriomeningitis virus, and respiratory syncytial virus. In addition to the well-known teratogenic effects, viral infections during pregnancy have been proposed as being involved in the pathogenesis of placental implantation[63-65] and neuropsychiatric disorders.[66] Reddy and collegues[61] studied the association of positive viral genome in the amniotic fluid and abnormal sonographic findings at the time of amniocentesis that was performed for clinical indications.The presence of viral DNA was studied by polymerase chain reaction (PCR) assay for CMV, parvovirus B19, adenovirus, enterovirus, herpes simplex virus, Epstein-Barr virus, and respiratory syncytial virus in 423 pregnancies with a normal fetal karyotype. Amniotic fluid positive for vital PCR was found in 13.5% (57 of 423). Among patients with abnormal sonographic findings, a higher rate of positive viral PCR in the amniotic fluid was reported in patients with structural malformations (20.3%), fetal growth restriction (29.6%), hydrops (50%), and other abnormalities (36%) than those with normal findings on ultrasound examination (8.4%).[61]

FREQUENCY OF INTRAAMNIOTIC INFECTION IN PRETERM GESTATION

Microbial Invasion of the Amniotic Cavity and Preterm Delivery

Preterm labor and preterm PROM account for approximately two thirds (one third each) of preterm deliveries, and the remaining third are the result of indicated delivery because of maternal or fetal indications (i.e., preeclampsia, growth restriction).[67-70] Microbiologic studies suggest that intrauterine infection is the underlying cause for 25% to 40% of preterm births[8]; however, this is probably an underestimation because some pathogens are difficult to identify by conventional cultivation techniques.[71,72] Indeed, using molecular microbiologic techniques, additional bacterial recombinant DNAs have been identified in culture-negative amniotic fluid from women in preterm labor.[51,73,74] In addition, microbial colonization of the chorioamnion was shown to be twice that of the amniotic cavity; thus rates of intrauterine infection based on amniotic fluid cultures further underestimate its true incidence.[75] A review of the evidence supporting the association between intrauterine infection and spontaneous preterm labor (with or without intact membranes) follows.

Microbial Invasion of the Amniotic Cavity in Patients With Preterm Labor and Intact Membranes

Patients with preterm labor and intact membranes have a mean rate of positive amniotic fluid cultures for microorganisms, of approximately 12.8% (379 of 2963), based upon the review of 33 studies.[36,37] Women with positive amniotic fluid cultures generally do not have clinical evidence of infection at presentation. However, they are more likely to develop clinical chorioamnionitis (37.5% [60 of 160] versus 9% [27 of 301]), to be refractory to tocolysis (85.3% [110 of 129] versus 16.3% [8 of 49]), and to rupture their membranes spontaneously (40% [6 of 15] versus 3.8% [2 of 52]) than patients with negative amniotic fluid cultures. Moreover, the earlier the gestational age at preterm birth, the more likely that microbial invasion of the amniotic cavity is present,[39] and at 21 to 24 weeks gestation, most spontaneous births are associated with histologic chorioamnionitis, compared with approximately 10% at 35 to 36 weeks.[39] Several investigators

have demonstrated that the rate of neonatal complications is higher in neonates born to women with microbial invasion of the amniotic cavity than in those without infection.[76]

Microbial Invasion of the Amniotic Cavity in Patients With Preterm PROM

In patients with preterm PROM, the rate of positive amniotic fluid cultures for microorganisms at admission is approximately 32.4% (473 of 1462)[37]; however, clinical chorioamnionitis is present in only 29.7% (49 of 165) of cases with proven microbial invasion.[9,37] The rate of microbial invasion in preterm PROM reported by these studies is probably an underestimation of the true prevalence of intrauterine infection. Indeed, available evidence indicates that the incidence of intraamniotic infection is higher among women with preterm PROM and a severely reduced volume of amniotic fluid than among those without oligohydramnios.[77,78] Because women with oligohydramnios are less likely to undergo an amniocentesis, the bias in these studies is to underestimate the prevalence of infection. In addition, women with preterm PROM admitted in labor generally do not undergo amniocentesis. These patients have a higher rate of microbial invasion of the amniotic cavity than those admitted without labor (39% [24 of 61] versus 25% [41 of 160]; $p < .05$).[79] Furthermore, of patients who are not in labor at admission, 75% have a positive amniotic fluid culture at the time of the onset of labor.[79] Therefore studies restricted to women not in labor provide a lower rate of microbial invasion of the amniotic cavity than those that include patients in labor.

Microbial Invasion of the Amniotic Cavity in Patients Presenting with Acute Cervical Incompetence or a Sonographic Short Cervix

Women presenting with a dilated cervix, intact membranes, and few, if any, contractions before 24 weeks gestation are considered to have clinical cervical incompetence. Of these patients, 51.1% have a positive amniotic fluid culture for microorganisms.[80] A sonographic short cervix in the midtrimester, defined as a cervical length of 25 mm or less, is a powerful predictor of spontaneous preterm delivery.[81,82] Recently subclinical microbial invasion of the amniotic cavity was detected in 9% (5 of 57) of asymptomatic patients with a sonographic cervical length of less than 25 mm.[83] Thus intraamniotic infection is frequently associated with an acute cervical incompetence.[84] Whether intraamniotic infection is a cause or a consequence of cervical dilatation or a short cervix has not been determined. It is possible that shortening of the cervical canal or a silent cervical dilatation with protrusion of the membranes into the vagina may lead to a secondary intrauterine infection. The outcome for patients with microbial invasion of the amniotic cavity is uniformly poor because they develop subsequent complications such as rupture of membranes, clinical chorioamnionitis, or pregnancy loss.

Microbial Invasion of the Amniotic Cavity in Patients with Twin Gestations and Preterm Labor

Microbial invasion of the amniotic cavity occurs in 11.9% of twin gestations.[46] This finding is in contrast to the 21.6% of abnormal amniotic fluid cultures observed in singleton gestations with preterm labor and delivery.[45] These data suggest that intraamniotic infection is a possible cause of preterm labor and delivery in twin gestations, but they do not support the hypothesis that intraamniotic infection is responsible for the high rate of preterm delivery observed in these patients.[85,86] Moreover, when intraamniotic infection is detected, the presenting sac is nearly always involved.[46]

Chorioamniotic Infection, Histologic Chorioamnionitis, and Preterm Birth

Inflammation of the placenta and chorioamniotic membranes is a nonspecific host-response to a variety of stimuli, including infection. Traditionally, acute inflammation of the chorioamniotic membranes has been believed to be an indicator of amniotic fluid infection,[40-44,87-89] a view based upon indirect evidence. Several studies have demonstrated an association between acute inflammatory lesions of the placenta and the recovery of microorganisms from the subchorionic plate[90,91] and the chorioamniotic space.[87] Bacteria have been recovered from the subchorionic plate of 72% of placentas with histologic evidence of chorioamnionitis.[49,87,90] Furthermore, a strong correlation is seen between positive amniotic fluid cultures for microorganisms and histologic chorioamnionitis.[88,89] Moreover, Cassell and associates[75,92] have reported an association between positive microbial cultures from material obtained from the chorioamniotic interface and histologic chorioamnionitis. The presence of inflammation in the umbilical cord (funisitis) represents evidence of a fetal inflammatory response.[93,94] In contrast, histologic chorioamnionitis reflects a maternal inflammatory response.

Several studies have examined the prevalence of inflammation in placentas from women delivering preterm infants. Collectively, the evidence shows an association between preterm birth and the occurrence of acute chorioamnionitis, and that the lower the gestational age at birth, the higher the frequency of histologic chorioamnionitis and funisitis.[49]

Fetal Infection

The most advanced and serious stage of ascending intrauterine infection is fetal infection (stage IV). The overall mortality rate of neonates with congenital neonatal sepsis ranges from 25% to 90%.[95-102] The wide array of results may reflect the effect of gestational age on the likelihood of survival. One study, which focused on infants born before 33 weeks gestation, found that the mortality rate was 33% for those infected and 17% for noninfected fetuses.[100] Carroll and colleagues[103] have reported that fetal bacteremia is found in 33% of fetuses with positive amniotic fluid culture and in 4% of those with negative amniotic fluid culture. Furthermore, Goldenberg and colleagues[104] reported for the Alabama Preterm Birth Study that 23% of neonates born at 23 to 32 weeks gestation have positive umbilical blood cultures for *U. urealyticum* and *M. hominis.* Newborns with positive blood cultures had a higher frequency of a neonatal systemic inflammatory response syndrome, higher umbilical cord serum concentrations of interleukin (IL)-6, and more frequent histologic evidence of placental inflammation than those with negative cultures.[104] Therefore subclinical fetal infection is far more common than traditionally recognized.

INTRAUTERINE INFECTION AND INFLAMMATION AS A CHRONIC PROCESS

Although intrauterine infection has been traditionally considered an acute complication of pregnancy, accumulating evidence suggests that this may be a chronic condition. The evidence in support of this view is derived from studies of the microbiologic state of the amniotic fluid and the concentration of inflammatory mediators at the time of genetic amniocentesis.

Microbial Invasion of the Amniotic Cavity at the Time of Genetic Amniocentesis

Compelling evidence from studies of the microbiologic state of the amniotic cavity, using traditional cultivation techniques at the time of genetic amniocentesis, suggests that intraamniotic

infection may be chronic in nature. Cassell and associates[92] reported the recovery of genital mycoplasmas from 6.6% (4 of 61) of amniotic fluid samples collected by amniocentesis between 16 and 21 weeks. Two patients had positive cultures for *M. hominis* and two for *U. urealyticum.* The patients with *M. hominis* were delivered at 34 and 40 weeks without neonatal complications, whereas those with *U. urealyticum* had premature delivery, neonatal sepsis, and neonatal death at 24 and 29 weeks. Subsequently, Gray and colleagues[105] reported a prevalence of positive cultures for *U. urealyticum* of 0.4% (9 of 2461) in amniotic fluid samples obtained during second-trimester genetic amniocentesis. After excluding one patient who had a therapeutic abortion, all patients (8 of 8) with positive amniotic fluid cultures had either a fetal loss within 4 weeks of amniocentesis ($n = 6$) or preterm delivery ($n = 2$). Furthermore, all had histologic evidence of chorioamnionitis. A similar finding was reported by Horowitz and associates,[106] who detected *U. urealyticum* in 2.8% (6 of 214) of amniotic fluid samples obtained between 16 and 20 weeks of gestation. The rate of adverse pregnancy outcome (fetal loss, preterm delivery, and low birth weight) was significantly higher in patients with a positive amniotic fluid culture than in those with a negative one (50% versus 12%; $p = .04$). Of interest, patients with a positive amniotic fluid culture were more likely to have an obstetric history that included more than three previous spontaneous abortions than those with a negative culture (33% versus 4%; $p = .03$).[106] In a retrospective study of 2718 women undergoing genetic amniocentesis, 1.8% (49 of 2718) of samples were positive for *Ureaplasma/Mycoplasma;* 34 patients were treated with oral erythromycin.[107] Midtrimester pregnancy loss was significantly higher in the untreated than the treated group (44.4% versus 11.4%; $p = .04$). In contrast, no significant differences were observed between the untreated and the treated group in the rate of preterm delivery (20% versus 19.4%; $p = .7$) and adverse pregnancy outcome (55.6% versus 28.6%; $p = .1$).[107] These observations suggest that microbial invasion of the amniotic cavity could be subclinical in the midtrimester of pregnancy and that pregnancy loss or preterm delivery may take weeks to occur.

Chronic Intraamniotic Inflammation and Preterm Birth

IL-6 concentrations in amniotic fluid are considered to be a marker of intraamniotic inflammation frequently associated with microbiologic infection in the amniotic fluid or the chorioamnionitic space.[108-111] Romero and colleagues[112] reported the results of a case-control study in which IL-6 determinations were conducted in the stored fluid of patients who had a pregnancy loss after a midtrimester amniocentesis and a control group who delivered at term. Patients who had a pregnancy loss had a significantly higher median amniotic fluid IL-6 concentration than those with a normal outcome.[112] Similar findings were reported by Wenstrom and associates.[113] Of note, the maternal plasma concentrations of IL-6 were not associated with adverse pregnancy outcome.

The same approach was subsequently used to test the association between markers of inflammation in midtrimester amniotic fluid of asymptomatic women and preterm delivery. The concentrations of matrix metalloproteinase (MMP)-8,[114] IL-6,[115,116] IL-10,[117] C-reactive protein,[118] tumor necrosis factor-alpha (TNF-α),[119] and angiogenin[120] in amniotic fluid obtained at midtrimester amniocentesis were significantly higher in patients who subsequently delivered preterm than in those who delivered at term.

Collectively this evidence suggests that a chronic intraamniotic inflammatory process is associated with both spontaneous abortion and spontaneous preterm delivery. Whether intraamniotic inflammation can be detected noninvasively remains to be determined. Goldenberg and associates[121] have demonstrated that the maternal plasma concentration of granulocyte colony-stimulating factor at 24 and 28 weeks gestation is associated with early preterm birth. To the extent that granulocyte colony-stimulating factor may reflect an inflammatory process, this finding suggests that a chronic inflammatory process identifiable in the maternal compartment is associated with early preterm birth.

Is the Relationship Between Intrauterine Infection and Spontaneous Preterm Birth Causal?

The existence of an association does not mean that infection *causes* preterm delivery. Indeed, it has been argued that microbial invasion of the amniotic cavity is merely the consequence of labor.[122-125] Determining whether or not this relationship is causal is critical because it has major clinical and therapeutic implications. The evidence supporting a causal role for infection follows the criteria outlined by Sir Branford Hill during his presidential address before the Section of Occupational Medicine of the Royal Society of Medicine in 1965[126] and includes (1) biologic plausibility, (2) temporal relationship, (3) consistency and strength of association, (4) dose-response gradient, (5) specificity, and (6) human experimentation.

Although animal experimentation indicates that the administration of bacterial products or microorganisms to pregnant animals can lead to preterm labor and delivery,[127-138] three observations in humans also suggest that infection precedes the spontaneous onset of preterm labor and delivery: (1) subclinical microbial invasion of the amniotic cavity or intrauterine inflammation in the midtrimester of pregnancy leads either to spontaneous abortion or preterm delivery; (2) patients with preterm PROM who on admission had a positive amniotic fluid culture for mycoplasmas (*U. urealyticum* or *M. hominis*) had a significantly shorter amniocentesis-to-delivery interval than those with sterile amniotic fluid[139] (suggesting that patients with preterm PROM and microbial invasion of the amniotic cavity are more likely to initiate preterm labor than those with a negative amniotic fluid culture)[139]; and (3) colonization of the lower genitourinary tract with microorganisms is a risk factor for preterm delivery. These conditions include asymptomatic bacteriuria, bacterial vaginosis, and infection with *N. gonorrhoeae.*[140-151]

The consistency and strength of association between infection and preterm delivery has been demonstrated by the many studies in which amniotic fluid was cultured for microorganisms in patients with preterm PROM[152] and preterm labor with intact membranes.[45] Moreover, the relative risk is higher (greater than 2) for preterm delivery in patients with preterm labor and intact membranes and microbial invasion of the amniotic cavity than in those without microbial invasion of the amniotic cavity. The hazards ratio is also high for the duration of pregnancy in women with preterm PROM with intrauterine infection.

The likelihood of a causal relationship is increased if a dose-response gradient can be demonstrated. Evidence supporting such a gradient includes the following: (1) the median concentration of bacterial endotoxin is higher in patients in preterm labor than in patients not in labor[153]; (2) the microbial inoculum is significantly greater in patients with preterm PROM admitted with preterm labor than in those admitted with preterm PROM but not in labor[79]; and (3) the rate of abortion or preterm delivery after the administration of *Escherichia coli* bacterial endotoxin to pregnant mice exhibits a clear dose-response gradient.[154]

One of the criteria for causality that is not met is specificity. Although intrauterine infection appears to be sufficient to induce preterm labor and delivery, it is not specific because many patients have a preterm delivery in the absence of evidence of intrauterine infection and inflammation. However, a high degree of specificity is rare in biologic systems. Although the causal relationship between smoking and lung cancer is widely accepted,

it is also nonspecific. Lung cancer occurs in nonsmokers and, of course, smoking can cause diseases other than lung cancer, such as emphysema and chronic bronchitis. Moreover, the formulation of "the necessary and sufficient cause" can inappropriately restrict the conceptualization of causality. In the case of preterm labor, microbiologic, cytologic, biochemic, immunologic, and pathologic data indicate that preterm labor is a syndrome and that infection is only one of its possible causes.[9,155]

An important criterion for causation is whether eradication of the agent can decrease the frequency of outcome or illness. Many trials of antimicrobial treatment for the prevention of preterm birth have been conducted. Evidence shows that treatment of patients with asymptomatic bacteriuria will reduce the rate of prematurity and low birth weight[150] and that antibiotic treatment of patients with preterm PROM prolongs the latency period and reduces the rate of maternal and neonatal infection.[156-158] However, treatment of patients with preterm labor and intact membranes has not been effective in most trials.[159-162] The reasons for this are likely related to the syndromic nature of preterm labor with intact membranes, the chronic nature of the process, and the inclusion in the clinical trials of many patients who do not have intrauterine infection and thus cannot benefit from antimicrobial treatment. This applies to patients presenting with preterm labor and those with bacterial vaginosis.[163,164]

Detection of Microbial Footprints in Amniotic Fluid with Sequence-Based Techniques

Estimates of the frequency and type of microorganisms in intrauterine infections are based on standard microbiologic techniques (i.e., culture). A positive amniotic fluid culture can only be obtained if the culture conditions in the laboratory are able to support the growth of a particular microorganism. Thus, although a positive culture is indicative of microbial invasion of the amniotic cavity, a negative culture indicates that the laboratory was not able to grow bacteria from the specimen, either because bacteria was absent (a true negative result) or because the laboratory conditions did not support the growth of a specific microorganism (a false negative result). For example, surveys of terrestrial and aquatic ecosystems indicate that more than 99% of existing microorganisms cannot be cultivated in the laboratory and that their identification can be performed only with molecular methods.[165] Two strategies have been used to detect microorganisms in the amniotic fluid with PCR. The first, also known as broad-range PCR, utilizes primer pairs designed to anneal with highly conserved DNA regions of all bacteria, such as the 16S ribosomal DNA. A positive result indicates the presence of bacteria, but identification of the specific organism requires sequencing of the PCR products. Another approach to molecular diagnosis of infectious diseases is to use specific primers for a particular microorganism. For example, three studies have used primers to the conserved sequence of microorganisms,[51,73,172] and others have favored specific primers to recover bacterial DNA from the amniotic fluid.[173,174]

Blanchard and colleagues[174] were the first to report the recovery of *U. urealyticum* in amniotic fluid samples using specific primers for the urease structural genes. In their study, 293 amniotic fluid samples collected by amniocentesis at cesarean delivery were cultured for bacteria, mycoplasmas, and chlamydiae and had PCR assay performed for *U. urealyticum*. Among the ten PCR-positive amniotic fluid samples, only four were also culture-positive. Subsequently, Jalava,[73] Hitti,[51] and Markenson[172] and their colleagues applied broad-spectrum bacterial 16S recombinant DNA PCR for the detection of bacteria in the amniotic fluid. These studies indicate that PCR assay is a more sensitive method to detect the presence of microorganisms. For example, Jalava and associates[73] studied 20 amniotic fluid samples from patients with PROM and 16 control samples: PCR assay detected five microorganisms (*U. urealyticum* [n = 2], *H. influenzae* [n = 1], *Streptococcus oralis* [n = 1], and *Fusobacterium* spp. [n = 1]); however, only two were positive by routine bacterial culture. Furthermore, the two patients who developed infectious complications were correctly identified by PCR assay, and amniotic fluid glucose concentrations were lower in the PCR-positive samples than in PCR-negative patients. Hitti and associates[51] studied 69 women in preterm labor with intact membranes. PCR assay results were positive in 94% (15 of 16) of the culture-positive amniotic fluid samples and 36% of the patients with a negative culture result. Of the women with elevated IL-6 concentrations, 36% (5 of 14) had bacteria detected by PCR assay. In the study by Markenson and colleagues,[172] 55.5% of the amniotic fluid samples from women in preterm labor (with no clinical evidence of infection) were PCR-positive; microorganisms were recovered in only 9.29% of the samples ($p < .05$). Of the amniotic fluid samples with elevated IL-6 concentrations, 67% (6 of 9) were also PCR-positive.

Oyarzun and colleagues[173] described a PCR amplification technique aimed at detecting the 16 microorganisms most commonly cultured from the amniotic fluid of patients in preterm labor (*U. urealyticum, M. hominis, G. vaginalis, E. coli, Fusobacterium* spp., *Peptostreptococcus anaerobius, Bacteroides fragilis, C. trachomatis, H. influenzae, N. Gohorrhoeae* and *Streptococcus* spp., among others). In 50 patients with preterm labor and intact membranes and 23 patients not in labor, amniotic fluid samples were studied using bacterial culture and PCR assay. All control samples were both culture-negative and PCR-negative. A higher proportion of samples were positive by PCR when compared with culture (46% [23 of 50] versus 12% [6 of 50]). The sensitivity of PCR for the prediction of preterm labor before 34 weeks was better than culture (64% [7 of 11] versus 18% [4 of 11], $p < .05$). However, some patients with positive results on PCR assay delivered at term without maternal or neonatal complications.

The clinical significance of the detection of *U. urealyticum* by PCR assay with specific primers in the amniotic fluid of women in preterm PROM has been investigated.[175] In this study, patients were divided into three groups according to the results of amniotic fluid culture and PCR assay: culture-negative and PCR-negative (n = 99), culture-negative and PCR-positive (n = 18), and culture-positive regardless of PCR results (n = 37). PCR analysis was more sensitive than culture to detect *U. urealyticum* (28% versus 16%). Moreover, patients with a positive PCR assay of amniotic fluid but a negative culture had a stronger inflammatory reaction in amniotic fluid, white blood cell count, and amniotic fluid IL-6 concentration, a shorter interval to delivery, and a higher rate of histologic chorioamnionitis, funisitis, and neonatal morbid events than those with a negative amniotic fluid culture and a negative result on amniotic fluid PCR assay for *U. urealyticum*.[175] This study demonstrated that patients with preterm PROM and a positive result on PCR assay for *U. urealyticum* (but a negative culture) had a worse pregnancy outcome than those with a sterile culture and a negative PCR. Similar results have been observed in the context of preterm labor with intact membranes.[176]

Compelling evidence from studies using PCR at the time of genetic amniocentesis suggests that asymptomatic intraamniotic infection may be more frequent than previously reported using traditional microbiologic techniques. Gerber and colleagues[177] identified *U. urealyticum* using PCR in 11.4% (29 of 254) amniotic fluid samples obtained between 15 to 17 weeks gestation. Patients that were positive for *U. urealyticum* had a significantly higher rate of preterm labor in a previous pregnancy (20.7% versus 2.7%; $p = .0008$), a subsequent episode of preterm labor in the current pregnancy (58.6% versus 4.4%; $p < .0001$), preterm birth before 37 weeks gestation (24.1% versus 0.4%; $p < .0001$), and preterm PROM (20.7% versus 0.4%; $p < .001$) than those who had a negative result. Similar findings were reported by Nguyen and colleagues,[178] who identified *M. hominis* in 6.4% (29 of 456)

of amniotic fluid samples from 456 women obtained between 15 to 17 weeks gestation. The rate of preterm labor and spontaneous preterm delivery before 37 weeks gestation was higher in women with a positive result on PCR assay for *M. hominis* than in those with negative PCR (14.3% versus 3.3%; $p = .01$; 10.7% versus 1.9%; $p = .02$, respectively). Perni and colleagues[179] performed genetic amniocentesis in 179 asymptomatic women between 15 and 19 weeks gestation. *M. hominis* and *U. urealyticum* were detected by PCR assay in 6.1% (11 of 179) and 12.8% (22 of 172) of cases, respectively. Preterm PROM occurred in 2.8% (5 of 179) of women, and the same proportion of patients had a spontaneous preterm birth with intact membranes. All women with preterm PROM had a positive amniotic fluid culture for either *M. hominis* or *U. urealyticum,* whereas none with spontaneous preterm labor were positive for these microorganisms. Finally, Wenstrom and colleagues[58] compared amniotic fluid samples from a group of 62 pregnancy losses after midtrimester amniocentesis with 60 control subjects from a population of 11,971 women. PCR assay was performed for the presence of adenovirus, parvovirus, CMV, Epstein-Barr virus, herpes simplex virus, β-actin DNA, enterovirus, and influenza A virus. No difference in the prevalence of viruses in the amniotic fluid samples was observed between cases and controls (8% versus 15%; $p = .3$). Of interest, adenovirus was identified in 6% of cases and in 8% of controls ($p = .7$).

Collectively, these studies suggest that PCR assay is more sensitive than culture for the detection of microorganisms in the amniotic fluid, particularly in patients with evidence of intraamniotic inflammation. We predict that the application of sequence-based methodology for the identification of microorganisms will lead to increased recognition of the importance of infection in perinatal disease.

Are Microorganisms in the Chorioamniotic Membranes Always Indicative of Pathology?

Intrauterine infection can also be present without a positive amniotic fluid culture or a positive PCR assay result. Specifically, if the infection is localized to the decidua or the space between the amnion and chorion, microorganisms may not be detected in the amniotic cavity. Evidence shows that the rate of microbial colonization in the chorioamniotic space is higher than that observed in the amniotic cavity.[180] Patients with positive amniotic fluid cultures in the membranes but negative cultures in the amniotic fluid often have elevated amniotic fluid concentrations of inflammation indicators, such as IL-6.[180] Therefore some patients with intraamniotic inflammation but negative cultures in the amniotic fluid may have infection in the extraamniotic space. Indeed, fluorescent *in situ* hybridization with a DNA probe specific for conserved regions of bacterial DNA (the 16S ribosomal RNA) has detected bacteria in the fetal membranes of up to 70% of women undergoing elective cesarean delivery at term.[181] Organisms were also detected in fetal membranes after preterm delivery without labor.[181] Inflammatory cells were frequently found in the amnion or chorion of preterm fetal membranes but not in term tissues. These findings suggest that the presence of bacteria alone is not sufficient to cause preterm labor and delivery and that microbial colonization of the chorioamniotic membranes may not always elicit a fetal or maternal inflammatory response. However, preterm labor is more frequent when a fetal inflammatory response is elicited.[181]

MOLECULAR MECHANISMS FOR PRETERM PARTURITION IN THE SETTING OF INTRAUTERINE INFECTION

A solid body of evidence supports a role for inflammatory mediators in the mechanisms of preterm parturition associated with infection. These factors may also play a part in spontaneous labor at term, although the evidence for this is less compelling. Attention has focused on the role of proinflammatory cytokines, such as IL-1β, TNF-α, and chemokines, such as IL-8. However, other proinflammatory platelet-activating factors, prostaglandins (PGs), and antiinflammatory cytokines may also play a role.[9] The current understanding of the proposed pathophysiology of preterm parturition in the context of intrauterine infection is summarized. The interested reader is referred to the references for a comprehensive discussion.[9]

During the course of ascending intrauterine infection, microorganisms may reach the decidua, where they can stimulate a local inflammatory reaction and the production of proinflammatory cytokines and inflammatory mediators (e.g., platelet-activating factor, PGs, leukotrienes, reactive oxygen species, nitric oxide).[3,9] If this inflammatory process is not sufficient to signal the onset of labor, microorganisms can still cross intact membranes into the amniotic cavity where they can stimulate the production of inflammatory mediators by resident macrophages and other host cells. Finally, microorganisms that gain access to the fetus may elicit a systemic inflammatory response syndrome, characterized by increased cord blood concentrations of IL-6[10,11] and other cytokines,[182] as well as cellular evidence of neutrophil and monocyte activation.[183]

The Importance of Toll-Like Receptors in Preterm Parturition

The first line of defense against infection is provided by the innate immune system. One of the mechanisms by which the innate immune system recognizes microorganisms is by using pattern recognition receptors, which bind to molecular structures present on the surfaces of microorganisms[184] and are classified according to their function and subcellular localization.

Toll-like receptors (TLRs) are a group of transmembrane pattern recognition receptors,[185] and ten different TLRs have been identified in humans.[184] TLR-2 recognizes peptidoglycans, lipoproteins, and zymosan (gram-positive bacteria, mycoplasmas, and fungi); TLR-4 recognizes the presence of lipopolysaccharide, which is produced by gram-negative bacteria; and TLR-3 recognizes double-stranded RNA (viruses).[184,186,187] Ligation of TLRs results in activation of nuclear factor (NF)-κB which, in turn, leads to the production of cytokines, chemokines, and antimicrobial peptides.[184] Moreover, activation of the toll pathway also induces surface expression of costimulatory molecules, required for the induction of adaptive immune responses, such as CD80 and CD86. In combination with antigenic microbial peptides, these molecules, presented by major histocompatability complex class II proteins in dendritic cells and macrophages, can activate naïve $CD4^+$ T cells that initiate most adaptive immune responses.[184]

In pregnant women, TLR-2 and TLR-4 are expressed in the amniotic epithelium.[188] Because TLRs are crucial for the recognition of microorganisms, it could be anticipated that defective signalling through these pattern recognition "receptors" will impair bacteria-induced preterm labor. Indeed, a strain of mice that has a spontaneous mutation for TLR-4 is less likely to deliver preterm after intrauterine inoculation of heat-killed bacteria or lipopolysaccharide administration than wild-type mice.[189,190] Moreover, in humans, spontaneous labor at term or preterm with histologic chorioamnionitis, regardless of the membrane status (intact or ruptured), is associated with an increased messenger RNA and protein expression of TLR-2 and TLR-4 in the chorioamniotic membranes.[188] These observations suggest that the innate immune system plays a role in parturition.

Prostaglandins and Lipoxygenase Products

Intrauterine PGs have been considered to be the key mediators in the biochemic mechanisms regulating the onset of labor. They can induce myometrial contractility[191-194] and changes in the

extracellular matrix metabolism associated with cervical ripening,[195-199] and they are thought to participate in decidual and fetal membrane activation.[9]

Supporting the role for PGs in the initiation of human labor includes evidence that (1) administration of PGs can induce early or late termination of pregnancy (abortion or labor)[200-208]; (2) treatment with indomethacin or aspirin (PG inhibitors) can delay spontaneous onset of parturition in animals[209-211]; (3) PG concentrations in amniotic fluid abruptly increase before the onset of spontaneous labor at term,[212] and concentrations of PGs in plasma and amniotic fluid increase during labor[213-219]; and (4) intraamniotic injection of arachidonic acid can induce abortion.[220]

Infection is thought to increase PG production by amnion, chorion, or decidua through the activity of bacterial products, cytokines, growth factors, and other inflammatory mediators. Indeed, amniotic fluid concentrations of PGE2 and PGF2α, and their stable metabolites are significantly higher in women with preterm labor and microbial invasion of the amniotic cavity than in women with preterm labor alone.[218,221] Similar observations have been reported on patients in labor with high concentrations of proinflammatory mediators in the amniotic cavity (e.g., IL-1β, TNF, IL-6). Moreover, amnion obtained from patients with histologic chorioamnionitis produces higher amounts of PGs than that obtained from patients without documented chorioamnionitis. In mice, quantitative real-time reverse transcriptase (RT)-PCR measurements demonstrated that bacteria-induced preterm labor substantially increased the expression of genes involved in PG synthesis.[222]

Metabolites of arachidonic acid derived through the lipoxygenase pathway, including leukotrienes (LTs) and hydroxyeicosatetraenoic acid (HETEs), have also been implicated in the causation of spontaneous preterm and term parturition. Concentrations of 5-HETE, LTB_4, and 15-HETE are increased in the amniotic fluid of women with preterm labor and microbial invasion of the amniotic fluid cavity.[223,224] Similarly, amnion from patients with histologic chorioamnionitis release more LTB_4 in vitro than amnion from women who delivered preterm without inflammation.[225] Moreover, ovariectomy-induced preterm labor increased the expression of genes involved in lipoxin, LTs, and HETEs synthesis.[222] However, the precise role of arachidonate lipoxygenase metabolites in human parturition remains to be determined. 5-HETE and LTC_4 can stimulate uterine contractility, and LTB_4 is thought to play a role in the recruitment of neutrophils to the site of infection and the regulation of arachidonic acid metabolites of the cyclooxygenase (COX) pathway.[226,227] Additionally, LTB_4 has been shown to act as a calcium ionophore (i.e., increases phospholipase activity and enhances the rate of PG biosynthesis in human intrauterine tissues).[228]

Proinflammatory Cytokines and Preterm Labor

A substantial body of evidence supports the participation of the proinflammatory cytokines IL-1 and TNF-α in preterm parturition: (1) IL-1β and TNF-α stimulate PG production by amnion, decidua, and myometrium[229-231]; (2) human decidua can produce IL-1β and TNF-β in response to bacterial products[230,232,233]; (3) the concentrations and bioactivity of IL-1β and TNF-α in amniotic fluid are elevated in women with preterm labor and intraamniotic infection[234-237]; (4) women with preterm PROM and intraamniotic infection have higher IL-1β and TNF-α concentrations in the presence of labor[228-231]; (5) IL-1β and TNF-α can induce preterm parturition when systemically administered to pregnant animals[127;238]; (6) TNF-α is involved in the mechanism of bacterial-induced preterm parturition in animal models[239;240]; (7) pretreatment with the natural IL-1 receptor antagonist before the administration of IL-1 to pregnant animals prevents preterm parturition[241]; (8) the concentrations of IL-1β in fetal plasma is elevated in the context of preterm labor with intrauterine infection[242]; and (9) placental tissue obtained from patients in labor, particularly those with chorioamnionitis, produces greater amounts of IL-1β than that obtained from women not in labor.[243]

Considerable redundancy exists, however, in the cytokine network, and blockade of a single factor is insufficient to prevent preterm delivery in the context of infection and inflammation. Indeed, the administration of anti-TNF and the natural IL-1 receptor antagonist to pregnant animals with intrauterine infection did not prevent preterm delivery.[244] Moreover, results of knockout animal experiments suggest that infection-induced preterm labor and delivery occur in animals that lack a particular cytokine.[245]

The precise mechanisms by which IL-1 and TNF participate in the activation of the myometrium has been the subject of intensive research, and evidence shows that they involve the participation of cytosolic phospholipase A-2, COX-2,[246,247] mitogen-activated protein (MAP) kinases,[248] and NF-κB.The latter transcription factor is responsible for many of the actions of IL-1 and TNF in amnion and may affect uterine function by blocking factors that promote uterine quiescence (e.g., progesterone).[249]

Antiinflammatory Cytokines and Preterm Labor

IL-10 is thought to be a key cytokine for the maintenance of pregnancy[250] and has been implicated in the control of preterm parturition associated with inflammation.[251] Indeed, IL-10 expression is reduced in the placental tissues of pregnancies complicated by preterm labor and chorioamnionitis compared with that in placental tissue from normal controls.[251] IL-10 inhibits COX-2 messenger RNA expression in cultured placental explants from women after preterm labor and preterm birth but not in those from women in labor at term, indicating that the mechanisms involved in the regulation of the inflammatory response during term and preterm parturition may be different.[251] Further evidence for the role of IL-10 in down-regulation of the inflammatory response during preterm labor derives from a study where the administration of dexamethasone or IL-10 to pregnant rhesus monkeys, significantly reduced the IL-1B–induced uterine contractility. IL-10 treatment also attenuated the concentrations of TNF-α and white blood cells in amniotic fluid.[252] Moreover, the administration of IL-10 to animal models of infection has been associated with improved pregnancy outcome.[253,254]

Matrix-Degrading Enzymes

The causal mechanisms of preterm PROM are poorly understood. Because the tensile strength and elasticity of the chorioamniotic membranes have been attributed to extracellular matrix proteins, matrix-degrading enzymes have been implicated in preterm PROM. Compelling evidence shows that preterm PROM is associated with increased availability of MMP-1,[249] MMP-8,[255-257] MMP-9,[258-260] and neutrophil elastase,[261] but not MMP-2,[260,262] MMP-3,[263] MMP-7,[264] and MMP-13.[264a]

Vaginal bleeding may predispose to membrane rupture by causing a separation between the chorioamnion and the decidua, which weakens the fetal membranes.[265] Alternatively, during the formation of a retroplacental clot, thrombin is generated.[266] This enzyme can stimulate the production of MMP-1[267] and MMP-3[266] by decidual cells and MMP-9 by amniochorionic membranes.[268] These MMPs can degrade fibrillar collagen (type I and III) and other components of the extracellular matrix of the chorioamniotic membranes.[269] The mechanisms responsible for defective endometrial hemostasis during pregnancy have not been identified. However, in some cases, the only manifestation of intrauterine infection may be vaginal bleeding.[265] This observation would link vaginal bleeding in the first and second trimesters of pregnancy, endometrial bleeding/deficient hemostasis, and intrauterine infection. This association is important because women with preterm PROM often have clinical or subclinical abruptions,

and histologic examination of the membranes in patients with abruption indicates that acute chorioamnionitis is a frequent occurrence.[270]

Because intrauterine infection is present in 30% of patients with preterm PROM and proinflammatory cytokines can stimulate the production of MMP-1, MMP-9, and MMP-8, a genetic predisposition to overproduction of MMPs in response to microorganisms may lead to preterm PROM. Indeed, fetal carriage of a 14 CA repeat in the promoter of the gene encoding for MMP-9 is associated with preterm PROM,[271] and a polymorphism in the promoter region of the gene for MMP-1 (nt-1607) also conferred an increased risk for preterm PROM (OR 2.29; 95% CI 1.09 to 4.82).[272] Additionally, single nucleotide polymorphisms in the MMP-8 promoter in neonates of African American women modified the risk for preterm PROM.[273] The combination of three minor allele haplotypes (−799T/−381G/+17G) was associated with a significantly higher risk for preterm PROM (OR 4.63; 95% CI 2.01 to 11.94). In contrast the combination of the three major allele haplotypes (−799C/−381A/+17C) had a protective effect against preterm PROM (OR 0.52; 95% CI 0.36 to 0.75).[273]

FETAL INFLAMMATORY RESPONSE SYNDROME

What is Fetal Inflammatory Response Syndrome?

Fetal microbial invasion is the last stage of ascending intrauterine infection. Clinical and experimental evidence suggests that the fetus can be exposed to microorganisms and their products (i.e., bacterial endotoxin)[153,274] that are present in the amniotic cavity and that the fetus can respond by mounting both cellular and humoral immunoresponses.[275-277] Gomez and colleagues proposed that fetal microbial invasion can result in a systemic fetal inflammatory response syndrome (FIRS) that can progress to multiple organ dysfunction, septic shock, and death in the absence of a timely delivery.[10] FIRS represents the fetal counterpart of the systemic inflammatory response syndrome (SIRS),[10] described in adults.[278] However, FIRS is a peculiar condition because of the unique circumstances of the patient (fetus) and of its environment (uterus); thus its diagnosis, management, treatment, and consequences are sui generis.[279-281]

The term *fetal inflammatory response syndrome* was coined to define a subclinical condition originally described in fetuses of women presenting with preterm labor and intact membranes as well as preterm PROM. The operational definition was an elevation of fetal plasma IL-6 concentration greater than 11 pg/mL.[10] This cytokine was selected as the marker for the definition of FIRS[242] for the following reasons: (1) IL-6 is a major mediator of the host response to infection and tissue damage; it is capable of eliciting biochemic, physiologic, and immunologic changes in the host, including stimulation of the production of C-reactive protein by liver cells; and (2) because many of the diagnostic criteria proposed for SIRS in adults[278] cannot be applied to the human fetus because the vital signs (with the exception of heart rate) cannot be readily determined before birth or in the intrapartum period. In contrast, the proinflammatory cytokine IL-6 is easy to measure and readily detectable in peripheral blood. IL-6 is also elevated in adults with SIRS.[282]

The original work describing FIRS was conducted in samples obtained by cordocentesis (Figure 16-2).[10,11] In patients with preterm PROM, fetal plasma IL-6 concentrations greater than 11 pg/mL are associated with a shorter cordocentesis-to-delivery interval than that observed with plasma IL-6 concentrations of 11 pg/mL or less (median, 0.8 day [range, 0.1 to 5 days] versus 6 days [range, 0.2 to 33.6 days], respectively; $p < .05$).[11] One approach to determine whether fetal inflammation was present before birth is to examine the umbilical cord. Funisitis and chorionic vasculitis (inflammation of the umbilical cord or of the fetal vessels on the chorionic plate, respectively) are the histopathologic landmarks of FIRS.[94] Neonates with funisitis are at increased risk for neonatal sepsis,[93] as well as pathologic conditions with long-term consequences such as bronchopulmonary dysplasia[20] and cerebral palsy.[16] The amniotic fluid white blood cell count is another indirect marker of fetal inflammation. Indeed, neutrophils are not normally present in amniotic fluid; these cells are recruited into the amniotic compartment in the course of intrauterine infection, and their origin is predominantly fetal.[283]

		n	Procedure-to-delivery interval (median, range, days)
I	AF IL-6 ≤7.9 ng/mL FP IL-6 ≤11 pg/mL	14	5 (0.2-33.6)
II	AF IL-6 >7.9 ng/mL FP IL-6 ≤11 pg/mL	5	7 (1.5-32)
III	AF IL-6 >7.9 ng/mL FP IL-6 >11 pg/mL	6	1.2 (0.25-2)
IV	AF IL-6 ≤7.9 ng/mL FP IL-6 >11 pg/mL	5	0.75 (0.13-10)

Figure 16-2 Classification and procedure-to-delivery interval of women according to amniotic fluid (AF) and fetal plasma (FP) IL-6 concentrations. Analysis was restricted to 30 women with preterm prelabor rupture of membranes (PROM) and available AF. White in the fetal or AF compartment represents a low FP or AF IL-6 concentration, respectively, whereas black/gray in the fetal or AF compartment denotes elevated fetal plasma or AF IL-6 concentration, respectively. In the absence of a fetal systemic inflammatory response, no differences in the procedure-to-delivery interval were observed between patients with and those without inflammatory response in the amniotic fluid (median 5 days versus median 7 days, respectively; groups I and II; $p > .05$). Among patients with an elevated fetal plasma IL-6 concentration (groups III and IV), the presence or absence of an inflammatory response in the amniotic fluid did not alter the procedure-to-delivery interval *(Reproduced with permission from Romero R, Gomez R, Ghezzi F, et al. A fetal systemic inflammatory response is followed by the spontaneous onset of preterm parturition,* Am J Obstet Gynecol *179:186, 1998.)*

Fetal Target Organs in the Fetal Inflammatory Response Syndrome

After adjustment for gestational age, FIRS is associated with a higher rate of neonatal complications,[16,20] and mothers of affected fetuses frequently have subclinical microbial invasion of the amniotic cavity.[10] Evidence of multiorgan involvement is characteristic (Figure 16-3), with a higher rate of severe neonatal morbidity (respiratory distress syndrome, suspected or proven neonatal sepsis, pneumonia, bronchopulmonary dysplasia, intraventricular hemorrhage, periventricular leukomalacia, and necrotizing enterocolitis).[10,11]

Hematopoietic System

In FIRS, the fetal hematologic profile is characterized by significant changes in the granulocyte and red blood cell lineages.[284] Neutrophilia is present in two-thirds of affected fetuses; neutropenia occurs in 7%.[284] The median plasma concentration of granulocyte colony-stimulating factor is significantly higher than that in the absence of FIRS, which may contribute to the fetal neutrophilia.[182] FIRS has also been associated with changes in markers of monocyte and neutrophil activation.[183] Fetuses delivered within 72 hours of cordocentesis had a higher expression of CD11c, CD13, CD15, and CD67 than those delivered at term.

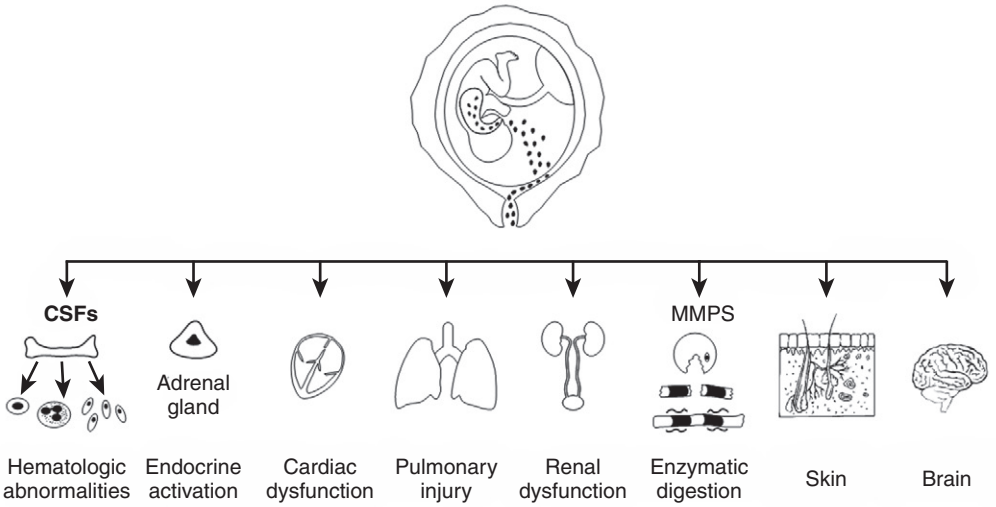


Figure 16-3 Fetal target organs during the fetal inflammatory response syndrome (FIRS). Several fetal organs, including the brain, lungs, heart, and kidneys, as well as the hematopoietic system, have been proposed to be targets during FIRS. *CSFs,* Colony-stimulating factors; *MMPs,* matrix metalloproteinases.

The median nucleated red blood cell count is higher in fetuses with FIRS than in those without FIRS.[284] It was proposed that IL-6 may have a unique, short-term role in elevating fetal nucleated red blood cell counts.[285] Collectively, these results indicate that fetuses destined to deliver prematurely have phenotypic evidence of activation of the monocyte-neutrophil system.[183]

Fetal Thymus

Considerable evidence indicates an association between thymus involution and infection in both fetuses and neonates.[286-288,288a,288b] Indeed, subclinical chorioamnionitis has been associated with the finding of a small thymus in very-low-birth-weight infants,[286] possibly because of an acute fetal[287] and neonatal[287,288] involution. In neonates, thymic involution has been reported to correlate with the duration of acute illness and with the percentage of lymphocytes in the peripheral blood smear.[288] Thymic involution has been attributed to both stress (through steroid production) and proinflammatory cytokines.[289-291] Di Naro and colleagues[292] reported that in the presence of intraamniotic infection and inflammation, fetuses from mothers with preterm labor and intact membranes had a sonographically small thymus. In another study, infants born preterm (less than 28 weeks gestation) with sonographic signs of cerebral white matter damage (white matter echolucencies in postnatal cranial ultrasound scans) were more likely to have undergone thymic involution (interval between birth and thymus involution) before the median interval for the gestational age-group.[291]

Adrenal Glands

Similar to adult patients admitted to intensive care units with burns[294] or pancreatitis,[294] fetuses with FIRS have endocrine evidence of "stress," which is expressed by an elevation of the cortisol and dehydroepiandrosterone sulfate ratio.[293] This endocrine milieu may contribute to the onset of spontaneous preterm labor. Indeed, Yoon and colleagues[293] reported that in patients with preterm PROM there is a significant association between the fetal plasma cortisol and dehydroepiandrosterone sulfate ratio and a shorter interval from cordocentesis to delivery (hazards ratio, 2.9; 95% CI, 1 to 8.4). Patients with preterm PROM who went into spontaneous labor and delivered within 7 days of cordocentesis had a significantly higher median fetal plasma concentration of cortisol than those who delivered after 7 days ($p < .0001$). Fetal plasma cortisol, but not maternal cortisol, was an independent predictor of the duration of pregnancy after adjusting for gestational age and the results of amniotic fluid cultures (hazards ratio, 2.9; 95% CI, 1.3 to 6.7). Importantly, a significant correlation was seen between fetal plasma cortisol and fetal plasma IL-6 ($r = 0.3$; $p < .05$).[293] These endocrine changes may have short and long-term implications given the observations about the effects of glucocorticoids in fetal programming of several metabolic functions.[295-299]

Fetal Skin

Fetal dermatitis can occur during FIRS. Skin samples from fetuses between 21 and 24 weeks gestation expressed both TLR-2 and TLR-4 in the fetal epidermis[300]; TLR-2 expression is dramatically increased in fetuses born from mothers with chorioamnionitis. Moreover, TLR-2 and TLR-4 were expressed in the mononuclear inflammatory infiltrate of the dermal-epidermal junction. The investigators in the study reporting these findings proposed that microorganisms are recognized by the fetal skin through "pattern-recognition receptors," and thus participates in the fetal inflammatory response to microbial products.[300]

Fetal Kidneys

Yoon and colleagues[78] reported an association between oligohydramnios and a higher rate of fetal infection and inflammation in patients with preterm PROM. In view of the antimicrobial properties of the amniotic fluid,[301,302] oligohydramnios may reduce the protective effect of this component of innate immunity. Alternatively, oligohydramnios may be the result of a redistribution of blood flow away from the kidneys occurring during the host response to microbial products.[78]

Fetal Heart

Echocardiographic studies have shown that fetuses with preterm PROM and intraamniotic infection undergo changes in cardiac function that are consistent with a high left ventricular compliance.[303] These changes may reflect a compensatory mechanism similar to that observed in adults with sepsis. In cases of overwhelming fetal sepsis, myocardial depression may lead to fetal death. In the context of FIRS, it is possible that soluble factors that are elevated in the circulation of patients with septic shock, such as bacterial products and cytokines, contribute to the myocardial depression.[304-306] Fetuses that are unable to modify their cardiac compliance to maintain ventricular stroke volume and cardiac output may suffer inadequate brain perfusion, predisposing them

to hypotension and brain ischemia *in utero*. This could create conditions predisposing to periventricular leukomalacia and brain injury.[307,308]

Fetal Lung

Amniotic fluid and its contents can be inhaled by the fetus and reach the distal parts of the airways and the alveoli. Color Doppler ultrasound studies demonstrated influx and efflux of amniotic fluid from the fetal upper respiratory tract.[309,310] Intrauterine inflammation has been associated with the development of bronchopulmonary dysplasia.[311-314]

Watterberg and colleagues[17] reported that low-birth-weight infants exposed to chorioamnionitis had higher concentrations of IL-1β in tracheal lavage samples and were more likely to develop bronchopulmonary dysplasia, but had lower incidence of respiratory distress syndrome in comparison with control infants. Ghezzi and colleagues[19] reported that IL-8 concentrations in amniotic fluid were higher in women with preterm labor (with intact membranes) and preterm PROM, delivering infants at 24 to 28 weeks gestation in whom bronchopulmonary dysplasia developed. In a subsequent study,[18] preterm neonates born at 33 weeks gestation or earlier in whom bronchopulmonary dysplasia developed had significantly higher median amniotic fluid IL-1β and IL-8 concentrations than those who did not. Similarly, Yoon and colleagues[20] reported that neonates born between 25 and 34 weeks gestation who developed bronchopulmonary dysplasia had a significantly higher median umbilical cord plasma IL-6 concentration than that of preterm infants without bronchopulmonary dysplasia, further supporting the association between FIRS and bronchopulmonary dysplasia.

Models of intrauterine inflammation, such as intraamniotic administration of endotoxin or the inoculation of bacteria, have supported the effects of microbial products and inflammation on lung development.[315-317] Bry and colleagues[318] conducted the first experimental study on the effects of antenatal inflammation on the fetal lung. Intraamniotic injection of IL-1α to pregnant rabbits induced an increase in the messenger RNA and protein expression of surfactant proteins A and B as well as surfactant lipids, resulting in an improvement of neonatal lung function. Injection of IL-1α also induced preterm delivery. These observations suggested that fetal lung inflammation accelerates fetal lung maturation in preparation for preterm birth. However, the biologic consequences include a reduction in the number of alveoli (that are increased in size), an inhibition of the microvascular development, and a thickening of the arteriolar walls, which collectively resemble the changes observed in infants with bronchopulmonary dysplasia.[319] Therefore the short-term gain in lung maturity can result in a predisposition to the development of chronic lung disease.

Fetal Brain

A link between exposure to perinatal infection and inflammation and brain injury is well recognized.[12-15,320] The first observations of this association date back to 1955 when Eastman and DeLeon[321] reported that intrapartum maternal fever conferred a seven-fold increased risk for cerebral palsy. Subsequently, Nelson and Ellenberg,[322] using data from the Collaborative Perinatal Project, showed that among low-birth-weight infants, chorioamnionitis was associated with an increased incidence of cerebral palsy from 12 to 39 per 1000 live births.

Dammann and Leviton[323] proposed that intrauterine infection leads to a fetal inflammatory response, which, in turn, contributes to adverse outcomes such as preterm labor and delivery, intraventricular hemorrhage, white matter damage, and neurodevelopment disability (mainly cerebral palsy). Several lines of evidence support this concept: (1) A fetal inflammatory response precedes spontaneous preterm delivery in the context of infection[10,11]; (2) clinical and histologic chorioamnionitis are associated with an increased risk of cerebral palsy[12-14,16,320,324-337] (this association has also been reported in near-term infants)[13,338]; (3) a large body of experimental evidence indicates that intrauterine infection results in white matter damage and neuronal lesions[339-344]; (4) white matter lesions are associated with intrauterine inflammation and infection in women with spontaneous preterm labor[331,345-347]; (5) elevated concentrations of cytokines in amniotic fluid and fetal plasma,[14,16,348-352] and fetal vasculitis (chorionic and umbilical cord vessel inflammation),[16,353-356] are associated with the development of intraventricular hemorrhage, white matter damage, and cerebral palsy.

Leviton[357] proposed that inflammatory cytokines (TNF-α) released during the course of intrauterine infection could participate in the pathogenesis of periventricular leukomalacia by four different mechanisms: (1) induction of fetal hypotension and brain ischemia[358]; (2) stimulation of production and release of tissue factor, which, in turn, can activate the hemostatic system and contribute to coagulation necrosis of white matter[359]; (3) induction of the release of platelet activating factor, which could act as a membrane detergent resulting in brain damage[360]; and (4) a cytotoxic effect of TNF-α on oligodendrocytes and myelin.[361,362] Yoon and colleagues[14] have suggested another mechanism: microbial invasion of the amniotic cavity (which occurs in approximately 25% of preterm births) results in congenital fetal infection and inflammation that stimulates fetal mononuclear cells to produce IL-1β and TNF-α. These cytokines promote the passage of microbial products and other cytokines into the brain by increasing the permeability of the blood-brain barrier.[363,364] Interferon-γ and lipopolysaccharide also increase the permeability of the blood-brain barrier. This increase in permeability is, at least in part, dependent on cyclic guanosine monophosphate and nitric oxide.[364] Microbial products activate human fetal microglial cells to produce IL-1 and TNF-α, which in turn stimulate astrocyte proliferation and production of TNF-α. TNF-α damages oligodendrocytes, which are the cells responsible for the deposition of myelin.

A strong association is recognized between prematurity and cerebral palsy.[365] Approximately one-third of the neonates who develop cerebral palsy have birth weights less than 2500 g.[366] Cerebral palsy rates are 25 to 31 times higher in newborns with a birth weight less than 1500 g compared with normal birth weight neonates.[366] Extremely premature neonates, in addition to being exposed to an exaggerated fetal systemic inflammatory response, display a limited ability to buffer the effects of the proinflammatory cytokines[15,367-373] generated by intrauterine infection and inflammation. It is important to stress that some studies did not demonstrate an association between inflammation and brain damage in the preterm infant.[374-377] Hypoxic and ischemic damage and inflammation are proposed to have a synergistic role in causing fetal brain injury.[378-380] Recently, the administration of *N*-acetylcysteine has been demonstrated to restrict brain damage in an animal model, which combined inflammation induced by lipopolysaccharide and ischemia. This has potential therapeutic effects in humans, given that *N*-acetylcysteine crosses the placenta.[381-383]

CONCLUSION

The evidence reviewed in this chapter indicates that intraamniotic infection and inflammation is causally linked to preterm parturition and the development of FIRS in a subset of patients. FIRS can affect multiple organ systems and predispose to short-term and long-term complications. Two of the long-term complications include bronchopulmonary dysplasia and cerebral palsy. Possible modulation of the maternal and fetal inflammatory response during pregnancy may have considerable importance for the understanding of the mechanisms of disease and preterm birth, and for limiting fetal injury.

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17

Douglas A. Woelkers

Maternal Cardiovascular Disease and Fetal Growth and Development

Within the womb, fetal growth and development depend on inherited genetic programs, the nutritive support of the maternal host, and interactions between the two. The importance of the fetal environment is evident in the long-term consequences of suboptimal *in utero* conditions on the future health of the offspring.[1-3] In crafting the fetal environment, both the placenta and the mother play critical roles. The placenta must initiate maternal adaptations to support the developing pregnancy, and then must successfully distribute substrates and wastes across the uterine interface. For her part, the mother must respond successfully to placental demands for resources by undertaking dramatic cardiovascular adaptations without simultaneously jeopardizing her own health. Disturbances at any of these steps, from implantation to maternal adaptation, may have an impact on the uterine environment and trigger adaptive or pathologic alterations in fetal growth and development.

Although essentially all maternal systems undergo some degree of adaptation during pregnancy, the maternal cardiovascular system experiences the most dramatic changes, indicating its critical role in determining the adequacy of the intrauterine environment. By augmenting cardiac output and blood volume, reducing peripheral vascular resistance, and remodeling uterine vasculature, pregnancy triggers a cardiovascular response that increases blood flow to the uterus by more than 10-fold, generating a

dependable vascular supply designed to withstand moment-to-moment fluctuations in substrate supply and removal.[4-6] In most women, these fetal and placental demands for adaptation are met without compromise of the mother's well-being, and the fetus is well served by successful maternal cardiovascular adaptation. But in cases of abnormal placental development combined with latent or overt maternal cardiovascular disease, these essential physiologic demands may jeopardize maternal health, or fail to occur altogether, thereby compromising fetal growth, development and survival. In this regard, both fetal growth and maternal cardiovascular adaptation to pregnancy are intimately linked by their dependence on proper placental function.

This chapter examines the associations between maternal cardiovascular disease (CVD) and aberrant fetal growth and development. Although much has been learned about the metabolic and endocrine regulation of fetal growth, its associations with maternal cardiovascular disease are still largely descriptive. Beyond these clinical associations, this chapter addresses several unique mechanisms by which maternal cardiovascular disease alters the fetal environment and, alternatively, mechanisms by which fetal or placental maldevelopment may trigger maternal vascular disease. One key to understanding the maternal-fetal-placental relationship is an appreciation of the essential cardiovascular adaptations to normal pregnancy. This chapter therefore begins with an overview of the changes that pregnancy induces in a woman's cardiovascular physiology. Thereafter follows a summary of some common vascular diseases of pregnancy, including special mention of preeclampsia, an important condition unique to human gestation. The proposed pathophysiology of preeclampsia is considered in detail in order to highlight mechanisms by which maternal, fetal, and placental maladaptation may be related to one another. The chapter then addresses the associations of disturbed fetal growth with maternal disease and proposes potential mechanisms for this interaction. Finally, mention is given to several potential long-term consequences of cardiovascular disease in pregnancy for the offspring.

TABLE 17-1

Maternal Cardiovascular Adaptation in Normal Pregnancy

Parameter	Modification	Magnitude	Timing
Oxygen consumption (Vo_2)	⇈	+20%-60%	Term
Oxygen delivery (Do_2) blood volume	↔ to ↑	700-1400 mL/min	Term
Plasma	⇈	+45%-60%	32 weeks
Red blood cells	⇈	+25%-32%	30-32 weeks
Total body water	⇈	+6-8 L	Term
resistance changes			
Systemic (SVR)	⇊	−20%	16-34 weeks
Pulmonary (PVR)	⇊	−34%	34 weeks
blood pressure (SVR × cardiac output)			
Systolic	↔ to ↓		
Diastolic	↓	10-15 mm Hg	24-32 weeks
Myocardial contractility			
Chronotropism (HR)	⇈	+20%-30%	28-32 weeks
Inotropism (SV)	↑	+11-32%	Term
Cardiac output (HR × SV)	⇈	+30%-50%	28-32 weeks
Uteroplacental circulation	⇈⇈	>10-fold	Term
Decidual	⇈	10%-15% of total	
Myometrial	↑	5% of total	
Placental	⇈⇈	80%-85% of total	

Data from Resnik R: Anatomic alterations in the reproductive tract. In Creasy RK, Resnik R, editors: *Maternal Fetal Medicine,* Philadelphia, 1999, WB Saunders; Ramsey PS, Ramin KD, Ramin SM: Cardiac disease in pregnancy, *Am J Perinatol* 18:245, 2001; and Gei AF, Hankins GD: Cardiac disease and pregnancy, *Obstet Gynecol Clin North Am* 28:465-512, 2001.

HR, Heart rate; *PVR,* pulmonary vascular resistance; *SV,* stroke volume; *SVR,* systemic vascular resistance.

MATERNAL CARDIOVASCULAR ADAPTATIONS TO PREGNANCY

Maternal cardiovascular adaptations in pregnancy serve to augment uterine blood flow by more than 10-fold over the course of pregnancy and are essential for supporting a nutritive intrauterine environment.[6] Most of these vascular adaptations are induced early and progressively by signals from placental hormones, mechanical forces, and physiologic demands placed by other maternal organ systems. The adaptive changes of pregnancy include increases in cardiac output, stroke volume, heart rate, myocardial contractility, plasma volume, blood volume, and total body water and decreases in systemic vascular resistance and blood pressure. A summary of the major cardiovascular changes of pregnancy is presented in Table 17-1.

Cardiac Output

Cardiac output begins to rise as early as 5 weeks of gestation.[7-9] By 8 weeks, it is already elevated some 22% above nonpregnant values, increasing more slowly thereafter to a maximum of 30% to 50% by the third trimester.[7,8,10,11] The timing of these cardic output peaks in pregnancy has not been established, although a review by van Oppen and co-workers[12] of 33 cross-sectional and 19 longitudinal studies suggests a range of 25 to 30 weeks of gestation. A majority of latent maternal cardiac diseases will become clinically apparent at this same time, potentially impairing uteroplacental blood flow and fetal growth. An exaggerated increase in cardiac output is noted in pregnancies complicated by multiple gestation,[8,13] anemia,[14,15] high altitude,[16] hyperthyroidism,[17] and other coincident risk factors for cardiovascular disease. Such pregnancies are associated with increased risk for failed maternal adaptation, fetal growth restriction, and preeclampsia.

Heart Rate and Stroke Volume

Cardiac output is the product of heart rate and stroke volume, both of which increase in pregnancy. The maternal heart rate varies widely in pregnancy but generally rises 10% to 20% above normal values by 32 weeks of gestation.[8,18] The tachycardia of pregnancy (100 beats per minute or greater) usually is well tolerated but does result in an elevated myocardial oxygen demand and decreased diastolic filling time—changes that may be debilitating for women with preexistent cardiac disease. Stroke volume, which increases rapidly to its maximum of 20% to 30% above baseline by 20 weeks of gestation, accounts for much of the early gestational increase in cardiac output.[11,19,20]

Contractility and Blood Volume

The hemodynamic factors determining stroke volume include contractility, preload and afterload. An increase of maternal myocardial contractility in pregnancy is suggested by some studies, but is difficult to distinguish from concomitant alterations in preload (i.e., venous return).[20,21] It is clear, however, that venous return to the heart increases dramatically as total body water, blood volume, and possibly venous tone are augmented within the first few weeks of pregnancy.[11,22,23] Water gain is mediated by the nonosmotic release of arginine vasopressin (AVP), activation of the renin-angiotensin-aldosterone system (RAS), and resetting of the osmolar set point early in pregnancy.[24] It is proposed that rapid vasodilatation in the first weeks of pregnancy induces

relative arterial underfilling, which triggers the water- and sodium-sparing response.[25] A woman's total blood volume may increase by 30% to 50% (for an additional 1.5 to 1.6 L), depending on her response to early placental signals (the so-called hypervolemia of pregnancy).[8,26,27] Both plasma and red cell compartments contribute to the increase in total blood volume, although a more rapid and robust increase in plasma volume (40% to 60% by 28 to 32 weeks) compared with red cell volume (25% to 33% at term) results in a physiologic anemia of pregnancy.[28] As with cardiac output, multiple gestations (e.g., twins) induce an earlier and greater blood volume expansion, which may exceed 65% of normal.[29,30] In the setting of latent or overt pregestational cardiac disease, the hypervolemia of pregnancy may overwhelm right ventricular pump function and lead to heart failure as blood volume approaches its maximum values at 28 weeks. Conversely, failure to achieve this critical augmentation of blood volume and cardiac output early in pregnancy is strongly associated with pregnancy loss and the ultimate development of preeclampsia and fetal growth restriction.[31-38]

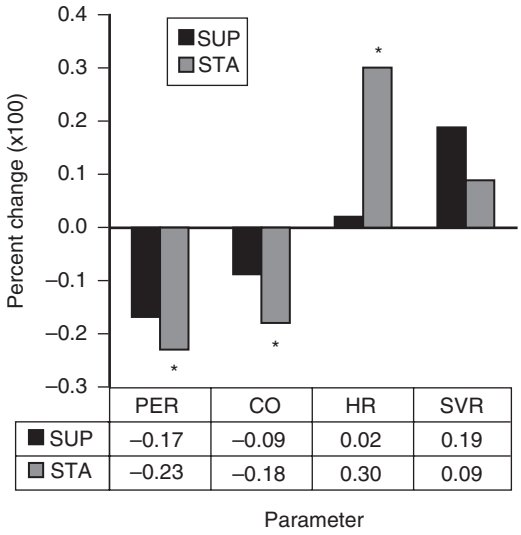
Blood Pressure and Systemic Vascular Resistance

Despite substantial increases in blood volume and cardiac output, maternal blood pressure manages to fall gradually to its nadir at 24 to 32 weeks before returning to prepregnancy values at term.[18,26] This phenomenon is due to an early and sustained fall in the systemic vascular resistance, reaching values 25% to 35% below normal by midpregnancy.[10,21,39] The exact physiology of this response is unknown but probably includes peripheral vasodilation by estrogens, progesterone, atrial natriuretic peptide (ANP), prostacyclin (i.e., prostaglandin I_2 [PGI_2]), and nitric oxide, and the arteriovenous shunt effect of the developing uteroplacental circulation.[21,22,25,40] The peripheral vasodilation is further enhanced by maternal refractoriness to arterial vasopressors, most notably angiotensin II (Ang-II) and norepinephrine. A relative loss of this refractoriness to vasopressors in early pregnancy identifies women at risk for the subsequent development of preeclampsia and fetal growth restriction.[41,42]

Regional Blood Flow and the Uteroplacental Circulation

The enhanced cardiac output of pregnancy is not evenly distributed among all organs. Disproportionate increases in blood flow benefit the uterus (and placenta), breasts, kidneys, and skin. The requirement for substantially increased uterine blood flow vis-à-vis nutritional support of the growing fetus and placenta is obvious, and its importance cannot be overstated. Moreover, it has become clear that the uteroplacental circuit is the common factor linking maternal cardiovascular function to fetal growth.

Data from studies in humans suggest that uterine blood flow must increase from a baseline of 40 mL/minute before pregnancy to greater than 500 mL/minute[4,5,43] at term. By the end of gestation, nearly 90% of flow is directed to the placenta, with approximately 10% reserved for the decidua and 5% for the myometrium.[6] Blood flow through the uterine circulation is critically dependent on maternal cardiac output, as evidence suggests that at baseline, this circuit is maximally dilated and unable to further increase its flow by autoregulation.[44] This point is well illustrated by the 20% decrease of intervillous blood flow induced by simple changes in maternal posture (such as upright standing or supine recumbency) which can reduce maternal venous return and cardiac output[45-48] (Figure 17-1). Conditions that decrease maternal cardiac output, and therefore uterine blood flow, are not well tolerated by the fetus, and lead to transient or sustained metabolic adjustments. Moreover, the uterine circulation remains sensitive to certain vasoconstrictors during pregnancy (e.g., nicotine) which may impose periodic limitations on placental perfusion and lead to disturbances of fetal growth.[49]



	PER	CO	HR	SVR
SUP	–0.17	–0.09	0.02	0.19
STA	–0.23	–0.18	0.30	0.09

Figure 17-1 Effect of posture on placental and maternal hemodynamics. Percent change in selected parameters with postural change from left lateral recumbent position to supine recumbency (SUP) or standing (STA). *CO,* Cardiac output; *HR,* heart rate; *PER,* placental perfusion; *SVR,* systemic vascular resistance. *$p < .05$. *(Modified from Clark SL, Cotton DB, Pivarnik JM, et al: Position change and central hemodynamic profile during normal third-trimester pregnancy and post partum.* Am J Obstet Gynecol *164:883-887, 1991; and Suonio S, Simpanen AL, Olkkonen H, Haring P: Effect of the left lateral recumbent position compared with the supine and upright positions on placental blood flow in normal late pregnancy,* Ann Clin Res *8:22-26, 1976.)*

Anatomic Changes in Maternal Uterine Blood Flow in Pregnancy

Anatomically, blood is directed to the uterus primarily through the uterine arteries, which arise from the internal iliac arteries in the pelvis, and secondarily through the ovarian arteries, which arise from the abdominal aorta. The uterine arteries arborize in an arcuate pattern (arcuate arteries) to supply the myometrium, and then descend in a radial pattern (radial arteries) to the endometrium (decidua) before terminating as the spiral arterioles. The intervillous spaces (IVSs) of the term placenta are supplied by approximately 100 to 200 spiral arterioles and drained by 75 to 175 uterine venules[50] (Figure 17-2) (see Chapter 11 for details of placental implantation and villous structure) At the level of the spiral arterioles, pulsatile blood pressure has been largely dissipated (25 mm Hg in spiral arteries, 15 to 20 mm Hg in the intervillous space, and 5 to 10 mm Hg in the uterine veins), allowing a high volume of slow-moving maternal blood to bathe the placental villi floating in the IVS.

Of all the vascular adaptations of the uterus, none is more remarkable than the "physiologic transformation" of the spiral arterioles, which converts the highly resistive, muscular vessels of the decidua and myometrium into widely dilated, high-flow "uteroplacental arteries" that supply the intervillous spaces of the placenta[51] (Figure 17-3). The remodeling of these arterioles takes place in three phases under the primary influence of extravillous cytotrophoblast cells which migrate distally from the anchoring villi of the placenta deep into the maternal decidua and myometrium. In the initial phase (beginning at approximately 5 weeks of gestation), the terminal ends of the spiral arterioles are plugged by clumps of extravillous trophoblasts which prevent maternal perfusion of the developing lacunar spaces, thus maintaining a local hypoxic environment essential for the proliferation of placental cells.[52-54] Over the ensuing 5 weeks, a true vascular

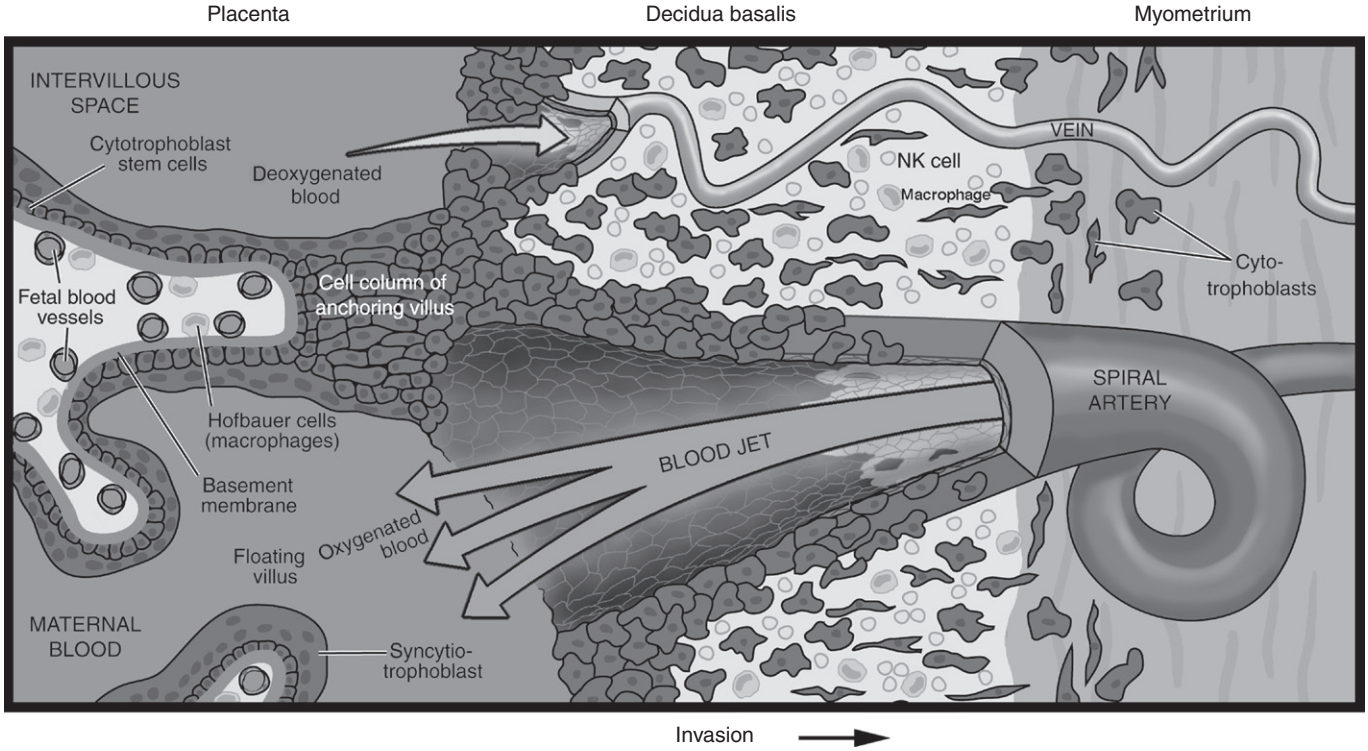


Figure 17-2 Blood supply to the intravillous space. The spiral arteries penetrate the uterine myometrium and decidua basalis beneath the placenta. Invasive trophoblast from columns of anchoring villi migrate toward these vessels and invade their lumens, replacing the normal intima and media. Blood is directed into the intravillous space, where it percolates over the floating villi and is removed by the decidual veins. *(Modified from Zhou Y, McMaster M, Woo K, et al: Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome,* Am J Pathol *160:1405-1423, 2002.)*

connection to the placenta is delayed while a gradual loss of the medial smooth muscle fibers and decidual basalis occurs in the distal portion of the spiral arterioles.[54] The second phase of remodeling at 11 to 14 weeks is characterized by the dissolution of the endovascular plugs within the spiral arterioles, the rapid increase in oxygen tension within the placenta, and the proximal infiltration and replacement of arteriolar endothelium with endovascular cytotrophoblast cells.[55-57] During this phase, the oxygen tension within the placenta increases from 20 to greater than 50 mm Hg, which triggers the conversion of placental villous trophoblast from a state of proliferation to one of differentiation and maturation.[58,59] Finally, a second "wave" of arterial transformation is proposed to occur from 14 to 17 weeks[60] as endovascular trophoblast cells complete their replacement of the maternal endothelium, eroding the elastic lamina and muscular intima of the spiral arterioles to the level of the inner third of the myometrium, to form the mature so-called uteroplacental arteries.[61] The endovascular trophoblast lining the transformed vessels embeds itself in a fibrinoid extracellular matrix composed of secreted proteins and maternal fibrin, thereby creating large, dilated conduits connecting the radial arteries of the myometrium to the IVSs. These low-resistance uteroplacental arteries are essentially unresponsive to local vasoconstrictors.[62,63]

Trophoblast cell differentiation and invasion are regulated by physicochemical signals at the decidua-placenta interface.[64-66] Low oxygen tension in the early placenta enhances the activity of hypoxia-inducible factor (HIF), which facilitates the expression of transforming growth factor-β3 (TGF-β3), a cytokine that helps maintain the proliferative, "noninvasive phenotype" of the epithelial villous cytotrophoblast.[67,68] This phenotype is characterized by the cell surface expression of E-cadherins (the epithelial class) and integrins αvβ6 and α6β4, which interact with the superficial extracellular matrix proteins of the decidua.[64] The "invasive phenotype" of the extravillous trophoblast is induced in a carefully choreographed manner by local paracrine factors, cell-to-cell contact, extracellular matrix, and oxygen tension.[66,67,69,70] The access of oxygenated blood to the villous spaces after 10 weeks of gestation and consequent increase in Pa_{O_2} down-regulates HIF activity and TGF-β3 expression, thereby enabling expression of stage-specific invasive cell surface markers—integrins αvβ3 and α1β1, vascular endothelial (VE)-cadherin, PECAM-1—on the trophoblast surface.[52,71] Additionally, it is proposed that the oxygen tension gradient at the tips of the anchoring villi regulates the stage and site-specific expression of the vascular endothelial growth factor (VEGF) family members VEGF-A to -C and of their tyrosine kinase receptors, VEGFR-1 and VEGFR-2.[72,73] During normal placentation, deeply invasive cytotrophoblasts down-regulate expression of VEGFR-2 and up-regulate expression of VEGFR-1 and secretion of VEGF-A and soluble VEGFR-1 (i.e., sFlt-1 [soluble FMS-like tyrosine kinase-1]), which has the autocrine and paracrine effects of promoting α1 integrin and promoting the invasive endovascular phenotype. Simultaneously, the invading trophoblast will begin to express matrix metalloproteinases (MMPs) to facilitate migration through the decidua and into the spiral arterioles.[74,75] On reaching their destination in the lumen of the spiral arteriole, these cells assume an endothelial cell phenotype through the expression of typical surface adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1), a process termed *pseudovasculogenesis.*[64,76,77] Disruption of this process leads to shallow implantation and absent or incomplete remodeling of the spiral arterioles. These pathologic findings,

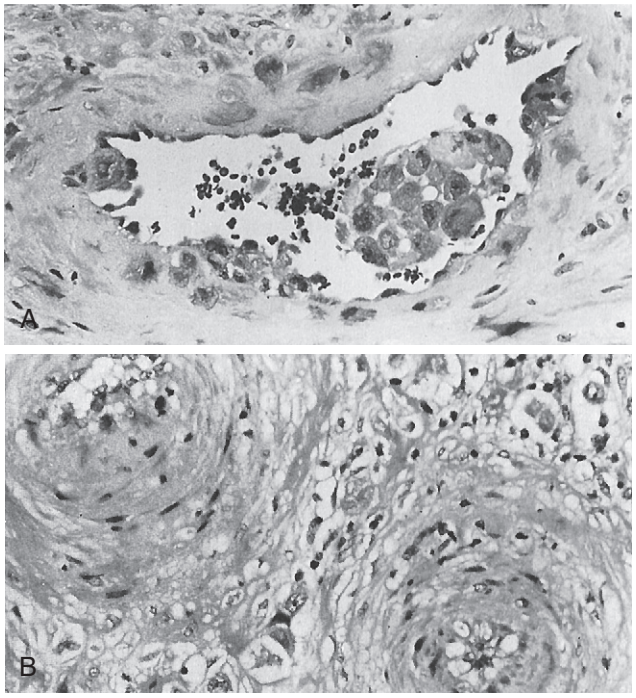


Figure 17-3 Photomicrographs of placental bed spiral arteries. **A,** Normal transformed spiral artery with irregular, dilated shape, attenuated media and intima, and intravascular trophoblasts. **B,** Untransformed basal arteries with medial hyperplasia and restricted lumen. *(Modified from Rushton DI: Placenta as a reflection of maternal disease. In Perrin E, Roth LM [eds]: Pathology of the Placenta.* Contemporary Issues in Surgical Pathology. *New York, Churchill Livingstone, 1984, p 57.)*

which may lead to vascular insufficiency and chronic hypoxia, are described in placental bed biopsy material obtained in pregnancies complicated by both preeclampsia and idiopathic intrauterine fetal growth restriction.[50,51,63,78]

Functional and Molecular Changes in Maternal Uterine Blood Flow in Pregnancy

Augmented uteroplacental blood flow in pregnancy requires more than just physiologic transformation of the spiral arterioles. Equally important is the functional transformation of the uterine arteries to a low-resistance shunt capable of accepting up to 20% of maternal cardiac output. This is accomplished via two mechanisms: (1) the growth and remodeling of uterine vessels (angiogenesis) throughout pregnancy, and (2) the development of uterine vascular refractoriness to vasoconstrictors. With these adaptations, the relative fall in uterine vascular resistance exceeds even the fall in systemic vascular resistance.[79] Data from pregnant animals demonstrate elevations of circulating metabolites for nitric oxide and prostacyclin in the uterine circulation, suggesting that an increase in the endogenous production of these endothelium-derived vasodilators may be acting on the uterine vasculature.[80-83] Similar to the maternal systemic circulation, the uterine arteries are resistant to vasoconstrictor actions of angiotensin II (Ang-II), and likewise show enhancement of the vasodilator effects of prostacyclin and nitric oxide. Consequently, it can be shown that direct uterine artery infusion of inhibitors of prostacyclin or nitric oxide synthesis (L-NAME or indomethacin) will decrease ovine uterine blood flow and enhance the local constrictor response to Ang-II).[83,84] In humans, systemic circulating prostacyclin metabolites are elevated,[85,86] whereas conflicting data suggest the same for nitric oxide metabolites (nitrite and nitrate).[40,87,88] Significant arteriovenous gradients of cyclic guanosine monophosphate (cGMP), the second messenger of nitric oxide, have been documented across the uterine circulation suggesting, at the least, a functional role for NO-mediated vasodilatation in this circuit.[88]

Adaptive Changes That Mimic Cardiovascular Disease

From the preceding discussion it is apparent that virtually all aspects of maternal cardiovascular physiology are altered during the course of a normal pregnancy, some to quite an extreme degree. These changes lead to a number of signs and symptoms that mimic cardiac disease. Dyspnea is experienced by nearly 70% of pregnant women by the third trimester, although it usually is of a mild and nondebilitating nature.[80] Other symptoms include decreased exercise tolerance, occasional orthopnea, fatigue, and syncope. Physical findings that mimic disease include tachycardia, jugular venous distention, and altered heart sounds such as systolic and diastolic murmurs or ancillary heart sounds. Edema is common to 70% of pregnancies,[90] and results from increased venous hydrostatic pressure coupled with decreased colloid oncotic pressure. It is important to recognize that these signs and symptoms are secondary to normal cardiovascular adaptations in pregnancy and do not necessarily indicate maternal disease.

CARDIOVASCULAR DISEASES IN PREGNANCY

Overview and Definitions

Cardiovascular disease in pregnancy is not rare—despite the fact that reproductive aged women are among the healthiest members of society. This speaks to the physiologic stresses placed on the cardiovascular system by fetal and placental demands for maternal adaptation. Overall, greater than 10% of pregnancies in developed countries will manifest some form of pregestational or de novo cardiovascular impairment; an even greater percentage suffer these diseases in underdeveloped regions.[91-94] In total, cardiovascular disorders in pregnancy are associated with nearly 15% of perinatal deaths and more than 15% of preterm deliveries.[93,95] These disorders are important risk factors for intrauterine growth restriction, stillbirth, and in some instances, congenital heart disease. Furthermore, growing evidence suggests a link between maternal cardiovascular disease, a suboptimal intrauterine environment, and long-term adverse health outcomes in the offspring.[96-100]

Hypertensive disorders constitute the largest class of cardiovascular disease in pregnancy, affecting 5% to 8% of gestations and having the greatest overall impact on fetal outcomes.[95,101] The hypertensive disorders of pregnancy include chronic hypertension and four unique pregnancy-associated conditions: eclampsia, preeclampsia, superimposed preeclampsia, and gestational hypertension.[27] These conditions have in common the element of hypertension—defined most recently as sustained systolic and diastolic blood pressures of 140 mm Hg or greater and higher than 90 mm Hg, respectively[27]—but they differ dramatically in their pathophysiology. *Preeclampsia* is a syndrome diagnosed clinically as *de novo* hypertension *and* proteinuria after 20 weeks of pregnancy, with associated pathologic activation and dysfunction of the maternal vascular endothelium injury leading to diverse manifestations of tissue hypoxia and end-organ injury.[102-104] *Eclampsia* is the occurrence of seizures in the setting of preeclampsia, and superimposed preeclampsia refers to preeclampsia in the setting of chronic hypertension.[27] A new diagnosis of hypertension in pregnancy without accompanying evidence of preeclampsia is termed *gestational hypertension*, which may be found to be transient or may be the first presentation of chronic hypertension (Table 17-2).

TABLE 17-2

Classification of the Hypertensive Disorders of Pregnancy

Classification	Definition and Diagnostic Criteria
Preeclampsia	Hypertension with proteinuria *Hypertension*: Blood pressure >140 mm Hg systolic or >90 mm Hg diastolic after 20 weeks in a previously normotensive woman *Proteinuria*: >300 mg/L in a random specimen or >300 mg/day in a 24-hr timed collection (de novo hypertension with systemic symptoms of *preeclampsia* in the absence of proteinuria is highly suspicious)
Severe preeclampsia	Includes any of the following: *Blood pressure* >160/110 mm Hg *Proteinuria*—excretion >5 g/day, *or* 3+ or greater on dipstick testing CNS symptoms or seizures Liver injury Thrombocytopenia Pulmonary edema Renal failure/oliguria Fetal growth restriction
HELLP syndrome	Variant syndrome of preeclampsia manifesting as hemolysis, elevated liver enzymes, low platelets
Chronic hypertension	Prepregnancy hypertension >140/90 mm Hg or newly diagnosed hypertension before 20 weeks gestation that persists longer than 6 weeks after conclusion of pregnancy (new-onset chronic hypertension); may or may not include chronic proteinuria or other chronic end-organ injury
Superimposed preeclampsia	Diagnosis of preeclampsia in setting of chronic hypertension; suspected when acute worsening of hypertension is accompanied by evidence of accelerating end-organ injury (e.g., proteinuria)
Gestational hypertension	Development of hypertension without evidence or symptoms of preeclampsia; resolves shortly after conclusion of pregnancy

CNS, Central nervous system.

Preexistent acquired or congenital cardiac disease affects a much smaller percentage of pregnancies, especially in developed countries. In the United Kingdom, the incidence of congenital or acquired heart disease in pregnancy has declined from 3% to 1% and would have fallen further if it were not for the greater percentage of females with previously lethal congenital cardiac defects now surviving until the reproductive years.[91,105] Cardiac lesions may adversely affect fetal growth by (1) restricting pregnancy-associated increases in cardiac output, or (2) enabling right-to-left shunting of deoxygenated blood (cyanotic heart disease). Examples of restrictive lesions are valvular stenosis, coarctation, and cardiomyopathy. Cyanotic lesions include Eisenmenger syndrome, corrected transpositions, and uncorrected patent ductus arteriosis. Fetal growth restriction accompanies 6% of pregnancies with restrictive congenital lesions, but 25% of corrected, and 67% of uncorrected cyanotic lesions.[106,107] Owing to the multifactorial inheritance pattern of congenital heart disease, infants of women with these lesions also carry an 8% to 14% risk of having a heart defect themselves,[106,108] although the specific lesion usually is not recapitulated in the offspring. Marfan syndrome, however, is a genetic condition that can affect mother and fetus similarly. Marfan syndrome is an autosomal dominant inherited defect of the fibrillin-1 gene leading to defective collagen biosynthesis, which may manifest clinically as aortic valve insufficiency and aortic root dissection in pregnancy.[109] As a genetic model of heart disease, Marfan syndrome illustrates the potential for a maternal cardiac condition to signify a developmental risk in the offspring. Indeed, affected fetuses have been diagnosed prenatally with Marfan syndrome on the basis of the typical ultrasound findings of aortic regurgitation and heart failure.[108,110]

Preexistent maternal renovascular disease also may affect fetal growth and development. Nearly 40% of infants in a cohort of pregnancies complicated by moderate or severe renal disease were born small for gestational age (SGA); 20% of the cases also were associated with preeclampsia.[111] Autoimmune conditions such as systemic lupus erythematosus are associated with high rates of fetal growth and developmental aberrations arising through at least two mechanisms. First, maternal rheumatalogic disorders associated with vascular or renal involvement may impair normal cardiovascular adaptation and predispose to inadequate uteroplacental circulation, resulting in fetal loss, growth restriction, and preeclampsia.[112,113] Indeed, rates of fetal growth restriction approach 40% in women with active lupus erythematosus.[114] Placental examination in these cases reveals a high incidence of decidual vasculopathy and villous infarction.[115-117] The exact mechanisms of placental vasculopathy are still unclear but may involve prothrombotic (i.e., thrombophilic) actions of antiphospholipid autoantibodies such as anticardiolipin antibody or the lupus anticoagulant. These autoantibodies are proposed to promote inappropriate intravascular and intravillous thrombosis by disturbing phospholipid–annexin-V association on the surface of endovascular membranes.[118] Furthermore, evidence from murine studies suggests that antiphospholipid antibodies recognizing epitopes on the surface of trophoblast cells induce activation of the classic complement cascade, resulting in placental injury and subsequent fetal loss or growth restriction.[119-121] As a second mechanism, autoimmune disease in pregnancy has the potential to induce fetal and neonatal rheumatologic disorders through the transplacental passage of self-reactive IgG autoantibodies (e.g., anti-Ro/SSA, anti-La/SSB).[122] The neonatal lupus syndrome is a recognized complication of active maternal disease and may include several transient manifestations such as lupus dermatitis, anemia, and thrombocytopenia but also permanent congenital heart block secondary to autoimmune-mediated injury of cardiac conduction fibers.[123,124]

Preeclampsia and Eclampsia

Preeclampsia deserves special attention because of its strong association with aberrant fetal growth and development. Preeclampsia is a pregnancy-specific and uniquely human disorder, affecting from 2.6% to 7.3% of gestations, and having a predilection for first conceptions, African-American race, multiple gestations, and those pregnancies complicated by medical conditions such as chronic hypertension, diabetes, or collagen vascular disease (for which rates may approach 25%).[93,125] The exact etiologic mechanism of preeclampsia is unknown, although the multitude of documented risk factors and predispositions point to a multifactorial origin that often involves placental or vascular susceptibility (Box 17-1). More than any other cardiovascular disease of pregnancy, preeclampsia is associated with disruptions of fetal growth and development because of the common involvement of the placenta. Known as the "disease of theories," preeclampsia is perhaps best regarded as a two-stage process in which relative or absolute placental ischemia initiates—by design or accident—a maternal inflammatory response and endothelial dysfunction, whose manifestations include hypertension, proteinuria, and various derangements of end-organ performance.[103,126,127] It is not known exactly how placental dysfunction translates into endothelial dysfunction, although many proposed factors have experimental support, including, for example, oxidative stress, cytokine and immune cell activation, prostaglandin imbalance, trophoblast deportation, VEGF antagonism, and endocrine disturbances of leptin and insulin.[128-135] In this context, alterations of fetal growth in preeclampsia may arise from (1) primary or acquired placental insufficiency, (2) deficient maternal hemodynamic adaptation and uterine hypoperfusion, (3) pathologic disturbances of fetal-placental growth signaling,

Box 17-1

Risk Factors for Preeclampsia

- Nulliparity
- History of preeclampsia
- History of fetal growth restriction
- Preexisting medical conditions
  - Chronic hypertension
  - Diabetes
  - Renal disease
  - Thrombophilia
- Family history of preeclampsia
- Obesity
- Multiple gestation
- Molar pregnancy
- Dietary deficiencies or poverty
- Genetic predisposition
  - Trisomy 13
  - Lipoprotein lipase variant
  - Angiotensin receptor polymorphism
  - Factor V Leiden mutation
- New sexual partner or long interpregnancy interval

or (4) shared genetic and environmental constraints. The following summary of the pathophysiology of preeclampsia illustrates mechanisms by which fetal growth and developmental are likely to be impaired.

Placental Origin

A placental origin of preeclampsia is suggested by the many marked pathologic and biochemical abnormalities identified in comparison with pregnancies without preeclampsia. The vascular lesions characteristic of severe or early preterm preeclampsia clearly lead to impairment of uteroplacental blood flow, offering an immediate explanation for the association with fetal growth disturbances.[135] Indeed, many similar lesions are apparent in placentas from nonpreeclamptic pregnancies associated with such growth restriction.[51,137] An important point, however, is that investigation of the placenta often is limited to cases of severe and early-onset disease—the variant of preeclampsia most closely associated with fetal growth restriction. Cases of mild preeclampsia at term are underrepresented in many reports and may show no histologic, biochemical, or molecular disturbances.

Failed Trophoblast Invasion

Among the pathologic hallmarks of preeclampsia, several placental findings warrant mention. These include decidual atherosis, placental infarction, and failed trophoblast invasion and transformation of the spiral arterioles. Examination of placental bed biopsy material obtained in women with preeclampsia reveals shallow decidual penetration of the interstitial trophoblast and absent or severely restricted invasion and transformation of the distal third of the spiral arterioles that feed the placenta.[63,138]

On the basis of a number of elegant histologic in situ and in vitro experiments, Zhou and associates[64,66,77] have proposed that in preeclampsia, cytotrophoblast fails to switch its cell surface adhesion molecules from the proliferating but noninvasive phenotype to the invasive phenotype and, finally, to the endovascular phenotype. Their studies demonstrate that superficially invading cytotrophoblasts in preeclamptic pregnancies continue to express markers of the early, undifferentiated trophoblast lineage including E-cadherin and αvβ6 and α6β4 integrins, and that these cells fail to express markers of the invasive or endovascular phenotype such as αvβ3, α1β1, VE-cadherin, VCAM-1, and PECAM-1.[77,139] TGF-β3, whose transcription is activated by hypoxia-inducible factor-1α (HIF-1α), is known to suppress expression of the invasive phenotype in trophoblast.[67,140] It has been proposed, although not universally accepted, that impaired oxygen sensing in the nascent placenta maintains activation of HIF-1α and, consequently, overexpression of TGF-β3, thereby preventing the cells from assuming their invasive phenotype.[68,141,142]

Another line of research has demonstrated dysregulation of the VEGF family of ligands and receptors in preeclampsia, leading to altered angiogenic control of trophoblast invasion and spiral artery remodeling.[72,73,143] Specific findings include decreased expression of VEGF-A and VEGFR-1 with increased secretion of soluble VEGFR-1 (sFlt-1). The resulting failure of trophoblast pseudovascularization and endovascular invasion allows persistence of the native muscular and intimal layer of the spiral arterioles, thereby impeding placental blood flow; this pathophysiologic mechanism is supported by numerous clinicopathologic studies[144-146] (Figure 17-4). Indeed, failure of transformation of the spiral arterioles also is noted in cases of pure intrauterine growth restriction, even without a diagnosis of preeclampsia.[138]

Infarction, Atherosis, Thrombosis

Other vasculopathic findings of the preeclamptic placenta having relevance for fetal growth include decidual atherosis, villous infarction and thrombosis (see Figure 17-4).[136] Placental vascular resistance may be markedly increased, to the point at which uterine artery blood ceases to flow, or even reverses direction, during cardiac diastole[147,148] (Figure 17-5). In vivo estimates of *intervillous* blood flow obtained by Doppler ultrasound investigations in severe preeclampsia suggest a reduction in blood flow approaching 40%.[148,149] At the subcellular level, preeclamptic placentas may demonstrate ultrastructural abnormalities including increased syncytial knotting, decreased microvillous surface area, and decreased villous arborization—all of which serve to further reduce the effective placental surface area and nutrient exchange.[150,151] Shedding of syncytiotrophoblast microvillous membranes and cell fragments is increased in preeclampsia and can be measured in the maternal circulation.[131,152] Villous thrombosis occasionally is noted in the preeclamptic placenta, especially in the setting of growth restriction or with early and severe disease.[153] Maternal thrombophilias, such as antiphospholipid antibody syndrome or factor V Leiden mutation, are associated with increased placental thrombosis, and with both preeclampsia and fetal growth restriction.[154,155] Of importance, certain of the thrombophilias have a genetic origin and may consequently be expressed in the fetus and placenta, thereby affecting fetal growth and development in a direct fashion.[156,157]

The primary cause of placental maldevelopment in preeclampsia is unknown but may include constitutional defects in the fetal trophoblast or interaction with a "hostile" maternal decidual environment.[158] Regardless of the primary etiologic process, local imbalances of growth factors, cytokines, prostanoids, and nitric oxide will arise in underperfused decidua and placenta, further contributing to vasospasm, shunting, apoptosis, and defective vascularization.[130,158] Pathologic activation of these signaling pathways in the placenta is suspected to contribute to disturbances of fetal growth and development already initiated by deficient uteroplacental blood flow. Angiogenic factors that mediate hypoxic signaling and that are dysregulated in preeclamptic placentas include platelet-derived growth factor (PDGF), VEGFs and their receptors (Flt-1 and KDR), placental growth factor (PlGF), and angiopoietin-1 and -2 (Ang-1 and Ang-2).[73,133,159-162] Altered expression of inflammatory cytokines including tumor necrosis factor-α (TNF-α) and the interleukins IL-1β and IL-6 has been demonstrated in the placenta of women with preeclampsia by some[163,164] but not all[165,166] investigators. Changes in levels of placental factors reported to be associated with preeclampsia are summarized in Table 17-3.

Vascular reactivity is abnormally regulated in the preeclamptic placenta, and abundant evidence implicates alterations in the

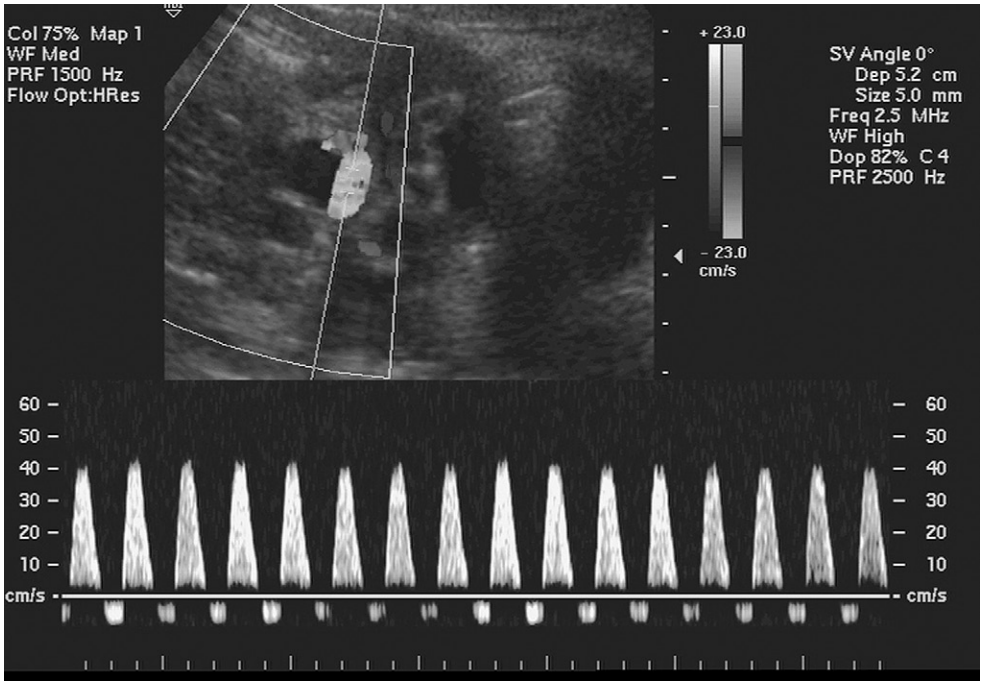


Figure 17-4 Doppler velocimetry of umbilical artery. The Doppler waveform demonstrates reversal of flow in the umbilical artery of this infant whose mother developed severe preeclampsia at 28 weeks. *(Modified from Tekay A, Campbell S: Doppler ultrasonography in obstetrics. In Callen PW [ed]:* Ultrasound in Obstetrics and Gynecology. *Philadelphia, Saunders, 2000, p 677.)*

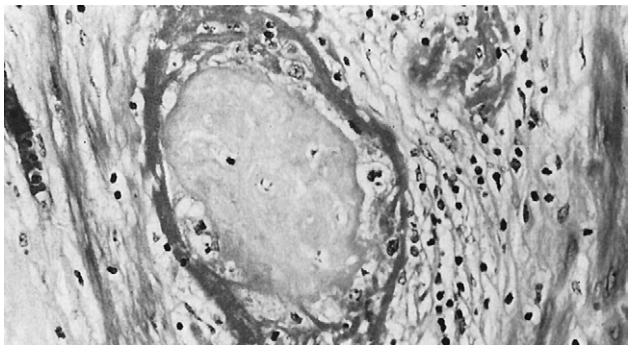


Figure 17-5 Early endothelial atherosis and fibrinoid deposition in a spiral artery from the placental bed. Vacuolated foam cells are seen lining the vessel lumen inside a dark band of fibrinoid deposits. *(From Rushton DI: Placenta as a reflection of maternal disease. In Perrin E, Roth LM [eds]:* Pathology of the Placenta. Contemporary Issues in Surgical Pathology. *New York, Churchill Livingstone, 1984, p 57.)*

TABLE 17-3

Placental Factors in Preeclampsia

Class	Agent	Change
Growth factors	TGF-β3[52,67,167]	Increased
	VEGF[160,168,169]	Increased *or* decreased *or* no change
	VEGF receptor	
	Flt-1[170]	Increased
	sFlt-1[73]	Increased
	KDR	
	IGF-I, IGF-II[171]	Increased
	IGFBPs[172]	Increased
	PlGF[170]	Decreased
	Angiopoietin-2[162]	Decreased
Apoptotic factors	Bcl-2[173,174]	Decreased
	Fas[175]	Increased
	Fas ligand[175]	Decreased
Cytokines	TNF-α[163,176]	Increased
	IL-6[166]	Decreased
	IL-1[164,177]	Increased
Oxidative markers	Isoprostanes[178]	Increased
	Malondialdehyde[179]	Increased
	Lipid peroxides[180,181]	Increased
	Nitrotyrosine[182,183]	Increased
	Xanthine oxidase[184]	Increased
	Vitamin E[185]	Decreased
	Superoxide dismutase[185]	Decreased

Data from cited references.

Flt-1, FMS-like tyrosine kinase 1 [i.e., VEGF receptor-1]; *IGF*, insulin-like growth factor; *IGFBPs*, insulin-like growth factor–binding proteins; *IL*, interleukin; *PlGF*, placental growth factor; *sFlt-1*, soluble FMS-like tyrosine kinase 1; *TGF-β3*, transforming growth factor-β3; *TNF-α*, tumor necrosis factor-α; *VEGF*, vascular endothelial growth factor.

production and response of local vasoregulators such as the renin-angiotensin system,[186,187] endothelin-1,[188-190] nitric oxide,[191-194] prostacyclin and thromboxane,[178,195,196] and peptide hormones such as adrenomedullin and atrial natriuretic peptide.[197,198] Oxidative stress, which is proposed to arise in the preeclamptic placenta secondary to hypoxia and ischemia-reperfusion injury, may have many consequences for placental, maternal, and even fetal well-being.[184] The isoprostane 8-iso-$PGF_2\alpha$, a marker of oxidative injury, is elevated in preeclamptic placentas, potentially blocking the invasive capacity of trophoblast by reducing their metalloproteinase and collagenase expression, with further exacerbation of placental insufficiency.[199] Other markers of oxidation found in the placenta include lipid peroxides, nitrotyrosine, and protein carbonyls. ROS-generating enzymes such as NADPH and xanthine oxidase are expressed at high levels in preeclamptic placentas, accompanied by down-regulation of antioxidant systems.[158] To a variable degree, disturbances of these same vasoregulatory systems may be demonstrated in the cord blood of newborn infants whose mothers have preeclampsia. For example, the concentration of soluble IL-8 and endothelin and the degree of monocyte

and neutrophil activation were elevated, while the concentration of IL-6 and the expression of cell adhesion molecules were decreased, in the cord blood of these infants versus control subjects.[200-202] Although these usually are thought of as potential mediators of maternal endothelial and inflammatory activation, studies are just now beginning to focus on the potential for placental inflammation to disrupt fetal growth and development.

Maternal Pathophysiology of Preeclampsia

As previously mentioned, the maternal syndrome of preeclampsia is characterized by endothelial dysfunction and intravascular inflammation that is thought to originate in the placenta.[102,103,127,203] Examination of maternal serum at the time of severe or early disease may reveal alterations of numerous factors and homeostatic systems potentially involved in the primary or secondary pathophysiology of this process. The metabolism of nitric oxide, prostacyclin, thromboxane, endothelin, angiotensin, ANP, and other vasoactive substances is disturbed.[204] Lipid hydroperoxides and other reactive oxygen species may be elevated, while antioxidant systems depleted.[205] Simultaneous activation of the coagulation and fibrinolytic cascades are sometimes evident. Circulating markers of endothelial, platelet, and monocyte activation may be elevated, along with inflammatory cytokines that mediate these responses.[204] The breadth and degree of activation of these systems generally are not seen in other hemodynamic disorders of pregnancy such as chronic or gestational hypertension, or congenital or acquired heart disease.

Of particular relevance to fetal growth, however, are maternal metabolic alterations which may precede or accompany preeclampsia. For years it has been known that diabetes is a significant risk factor for preeclampsia. Infants of diabetic mothers display numerous aberrations in growth and development including macrosomia, growth restriction, and congenital anomalies. More recent work confirms that insulin resistance and hyperlipidemia—two components of the heritable metabolic syndrome—also predispose affected women to preeclampsia and, as metabolic markers, tend to worsen during the disease process and for up to 3 months afterward[205,206] (Table 17-4). As part of this syndrome, preeclamptic women are found to have elevated triglycerides, free fatty acids, and small dense low-density lipoprotein (LDL), all of which are susceptible to oxidative modifications that impair vascular endothelial function.[207,209] It is not known what effect these metabolic changes would have on the developing fetus, although experimental maternal hyperinsulinemia in late pregnancy is associated with intrauterine growth restriction and altered carbohydrate metabolism in rat pups.[209,210]

Cardiovascular Disease and Fetal Growth and Development

It is now apparent that fetal growth disturbances are associated with maternal cardiovascular diseases in pregnancy. Many clinical studies have estimated the size and prevalence of this effect.[211] Clinical outcome studies categorize birth weights as SGA, defined as less than 10% of normal for gestational age; large for gestational age (LGA), defined as greater than 90% of normal for gestational age; or appropriate for gestational age (AGA). In general, cardiovascular disorders of pregnancy are associated with reduced intrauterine growth, although this is not always the case. Depending on the clinical situation, anywhere from 4% to 40% of infants will be SGA.[211] A mixture of growth disturbances that varies with gestational age and associated pregnancy complications has been described for the infants of preeclamptic women.[212,213] It is important to remember that birth weight is but one crude marker of fetal growth and development. Body composition parameters such as body mass index (BMI) (calculated as weight/height^2) or ponderal index (PI) (calculated as weight/length^3) are proposed to be more sensitive markers for overgrowth or undergrowth than is crude birth weight, although these measurements are not universally reported.[213]

TABLE 17-4

Correlates of Insulin Resistance Associated With Preeclampsia

Condition	Biomarker(s)
Gestational diabetes	Hyperglycemia
Polycystic ovary syndrome	Hyperinsulinemia
Obesity/excessive weight gain	Hyperlipidemia
	Increased TNF-α
	Elevated PAI-1
	Hyperleptinemia

PAI-1, Plasminogen activator inhibitor-1; *TNF-α,* tumor necrosis factor-α.

Growth Restriction

Because preeclampsia is a heterogeneous syndrome with multiple potential inciting factors, it is not surprising that the fetal growth effects are also heterogeneous. In a review of the epidemiologic literature, Misra[211] concluded that the best evidence suggests a consistent negative association of preeclampsia with birth weight only when the disease occurs preterm, or is of a severe nature. Data from other investigators confirmed this conclusion, reporting that the rate of SGA was 5.8% in pregnancies complicated by mild preeclampsia (not different from controls), but increased to 20.5% in pregnancies with severe preeclampsia.[214] Gestational hypertension without preeclampsia, however, does not appear to be associated with fetal growth restriction.[215] Therefore, when proteinuria is included as a covariate, the association of gestational hypertension with SGA increases two- to four-fold.[215] In a large prospective cohort study at McMaster University Medical Centre, 1948 hypertensive pregnancies were studied.[217] Compared with newborns of women with gestational hypertension only, infants of women with preeclampsia or superimposed preeclampsia were 2.2 and 2.1 times more likely to be SGA, whereas infants of women with chronic hypertension only were not at risk for being SGA. The overall likelihood of SGA in preeclamptic offspring was 25% in this study. The fact that chronic and gestational hypertensive women delivered infants without growth restriction suggests that maternal hypertension per se is not the primary determinant of fetal growth in hypertensive pregnancies. Rather, it is likely that the placental defects uniquely associated with preeclampsia determine the fetal outcome, and that low birth weight is more closely related to iatrogenic preterm delivery. Illustrating this point, a controlled retrospective study reported that 26% of infants born to mothers with preeclampsia were of "low birth weight" (<2500 g), which represented a four-fold increased risk above that in control pregnancies.[218] The adjusted odds for SGA, on the other hand, were only 2.5, suggesting that much of the excess in low birth weight was due to earlier iatrogenic delivery. A more recent study, by Xiong and co-workers,[219] that controlled for multiple demographic and obstetric factors including race, age, parity, obesity, medical history, anemia, and smoking reported an increased likelihood of being SGA for infants of preeclamptic mothers only when born before 37 weeks of gestation. Those born after 37 weeks did not manifest growth restriction, and as the pregnancy passed 40 weeks, the infants from preeclamptic mothers were actually larger than the control infants (Figure 17-6).

The fetal growth decrements attributed to preeclampsia affect body composition as well as birth weight. A large population-based study of 672,130 births in Norway demonstrated that infants born to preeclamptic mothers were lighter, shorter, and leaner than control infants (born to nonpreeclamptic mothers).[213] The fetal growth decrements were accentuated in infants born before

37 weeks but persisted even in the term neonates. As would be expected for infants of preeclamptic mothers, the rate of asymmetric SGA was twice that of symmetric SGA, although rates of symmetric SGA were still elevated two-fold over those in control subjects at both early and late gestational ages, suggesting that a significant proportion of infants began to slip from their growth potential earlier than the second trimester.

On careful evaluation, it is possible to detect abnormal maternal cardiovascular function as soon as fetal growth delay becomes evident. Vasapollo and associates used echocardiography to study 21 otherwise healthy pregnant women newly diagnosed as carrying SGA fetuses.[220] Compared with matched control subjects (with AGA fetuses), these women demonstrated higher average blood pressure and peripheral vascular resistance and lower cardiac output, stroke volume, and left ventricular mass. Similarly, a failure to increase cardiac output in the first weeks of pregnancy was prospectively associated with SGA[36] Very commonly, the failure of maternal cardiovascular adaptation also is associated with abnormal placentation. Of women with Doppler evidence of shallow placentation, 45% also had evidence of suboptimal maternal cardiovascular adaptation, and 80% of these patients ultimately developed preeclampsia or fetal growth restriction.[221] These findings lend support to Roberts' two-compartment hypothesis, which predicts the highest rates of preeclampsia in pregnancies with both an underlying maternal vascular impairment and shallow placentation.[126]

Growth Excess

Contrary to obstetric lore, not all infants born of a hypertensive pregnancy will suffer from growth restriction. Xiong and coworkers[218] examined a perinatal database from Alberta, Canada, for the years 1991 to 1996 to determine the impact of preeclampsia and gestational hypertension on birth weight. After controlling for multiple factors, these investigators found that, in addition to the expected association with SGA and low birth weight, the offspring of women with gestational hypertension and preeclampsia were more likely to be LGA than infants of nonhypertensive mothers. Even though the effect is not large (odds ratio [OR], 1.1 to 1.5), Rasmussen and Irgens were able to demonstrate that the rates of birth weight, length, and ponderal index above the 97.5th percentile are significantly higher in births with preeclampsia than in those without preeclampsia.[213] This finding has been replicated by other investigators[211,219] and lends support to the idea that near term, some infants of preeclamptic and hypertensive women may have enough placental reserve to benefit from increased cardiac output and metabolic manipulations of the mother.

Mechanisms of Growth Disturbance

Reduced Placental Perfusion

At first glance, it appears obvious that abnormalities of fetal growth and development in preeclampsia may be immediately related to decreased placental size and perfusion. Direct tracer technique measurements of blood flow suggest a two- to three-fold decrease in uteroplacental perfusion in hypertensive women from that in normotensive gravidas.[222,223] These studies, performed at the time of diagnosis, however, do not fully address the variety of growth patterns that may be associated with preeclampsia. More recent studies controlling for gestational age and birth weight, however, suggest that placental mass may be the same, or even greater, in some pregnancies complicated by preeclampsia and growth restriction.[224,225]

Substrate Delivery

Very little information exists to describe the transport of substrates across the placenta in preeclampsia. Growth-restricted fetuses without preeclampsia were noted to have lower serum alpha aminonitrogenous amino acids such as valine, leucine, isoleucine, lysine, and serine weeks before delivery,[226] and placental homogenates contained significantly more free L-arginine and phenylalanine.[227] Only tyrosine was decreased in free amino acid preparations from preeclamptic placentas.[227] More recently, Powers and co-workers[228] measured amino acid concentrations in maternal and neonatal blood from pregnancies complicated by preeclampsia. Maternal serum amino acids were elevated in the setting of preeclampsia, as were the fetal blood concentrations. Surprisingly, among the preeclamptic pregnancies, SGA fetuses had significantly higher cord amino acid concentrations than those in non-SGA fetuses (321 ± 32 μmol/dL versus 251 ± 17 μmol/dL), suggesting that in contrast with conditions in pure intrauterine growth restriction, placental transport of amino acids may be enhanced or their utilization may be reduced, or both, in preeclampsia, especially among those fetuses with growth restriction. Likewise, cord venous concentrations of total cholesterol and triglycerides and the total cholesterol to high-density lipoprotein cholesterol (HDL-C) ratio were reported to be elevated in offspring born after preeclamptic pregnancies compared with control infants, whereas only the level of triglycerides

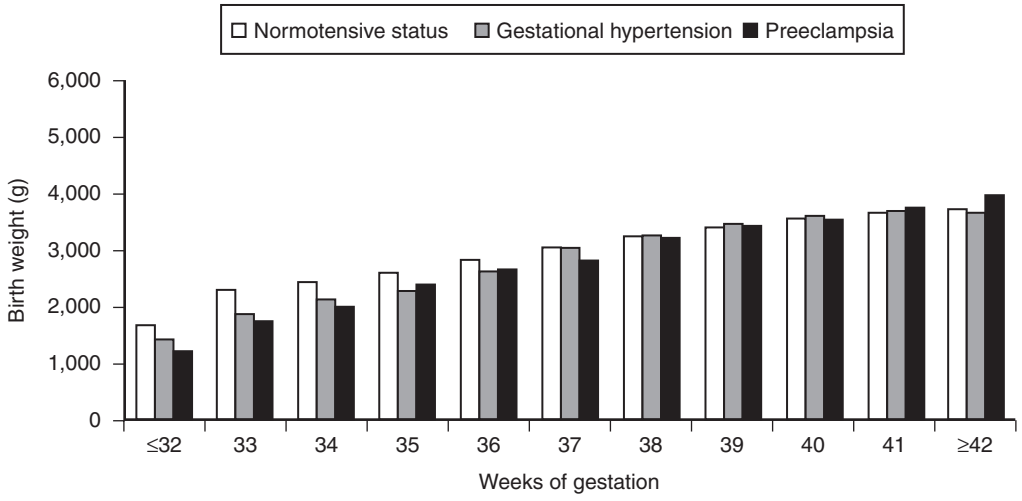


Figure 17-6 Impact of preeclampsia and gestational hypertension on birth weight (n = 87,798), Alberta, Canada, 1991 to 1996. *(From Xiong X, Demianczuk NN, Saunders LD, et al: Impact of preeclampsia and gestational hypertension on birth weight by gestational age,* Am J Epidemiol *155:203-209, 2002.)*

was elevated in SGA infants of women without preeclampsia.[229] These findings also implicate selective enhancement of transplacental substrate delivery, rather than the maternal dyslipidemia of pregnancy; no significant correlation has been made between newborn and maternal concentrations of lipids or cholesterol.

Cytokines

Transplacental transfer of cytokines has been implicated as a means of altering fetal development in settings of infection and prematurity. Low-dose endotoxin infusion into the pregnant rat has been used as a model for glomerular injury of preeclampsia.[230] The endotoxin induces a generalized Shwartzman reaction in the pregnant rat with fibrin and platelet thrombi in the kidneys, which can be reduced with prostacyclin infusion.[231] This protocol also has been suggested as a good model for preeclampsia because it results in proteinuria, thrombocytopenia, and hypertension.[232,233] Infusion of low-dose endotoxin in pregnant rats may lead to fetal death, resorption, and fetal anomalies, presumably through reduced uterine blood flow or induction of other humoral factors.[233,234] The placentas of endotoxin-treated rats display more apoptosis and higher peroxynitrite levels, suggesting an overall reduction in effective placental surface area in association with oxidative stress, which may explain some facets of growth restriction.[235] This finding also has been reported in rats infused with the nitric oxide synthase inhibitor L-NAME (nitro-L-arginine methyl ester).[235] Endotoxin may or may not cross the placenta from mother to fetus,[236,237] but a fetal inflammatory response has been documented. Fetal and placental growth restriction demonstrates a dose response to maternal endotoxin administration[233,235] but not necessarily at low doses. At sub-fetotoxic levels, an increase in central nervous system (CNS) anomalies of the offspring is seen, including periventricular leukomalacia and neuronal necrosis.[238] Expression of proinflammatory cytokines TNF-α and IL-1β is increased within a few hours of maternal lipopolysaccharide administration.[237] More subtle testing of the offspring disclosed no impairment in the maze test but revealed aberrations in male sexual behavior.[239]

Human studies of fetal cytokine concentrations in preeclampsia show conflicting results. Levels of TNF-α, IL-6, and IL-1β have been variously reported to be lower[200,240,241] or unchanged.[242,243] In the largest study reported, Odegård and colleagues[200] measured cord IL-6 in 271 preeclamptic pregnancies and 611 control pregnancies and demonstrated a significant decrease, especially in infants with concomitant growth restriction. Although human studies have suggested that inflammatory cytokines are elevated in umbilical cord blood of infants born to preeclamptic mothers, no direct association with neurodevelopmental injury has been documented.

Growth Factors

The TGF-β family of cytokines plays a role in both growth and immunology of pregnancy.[244] The three members of the family—TGF-β1 to TGF-β3—and their receptors are expressed in the placenta both early in its development and at term.[167,2456] In normal pregnancies, umbilical cord TGF-β correlates well with birth weight, presumably owing to its regulation by IGF-1, which also correlates with birth weight.[244] Ostlund and associates demonstrated that cord TGF-β1 levels were significantly lower in the cord blood from fetuses with intrauterine growth restriction with or without preeclampsia, but levels were not decreased with preeclampsia alone.[244] This finding suggests that preeclampsia does not a priori alter fetal TGB-β1 production, unless other disturbances in growth also are present.

Glucocorticoids

Glucocorticoids may be powerful effectors of fetal growth and development. High fetal concentrations of glucocorticoids are associated with low birth weight and subsequent hypertension in adulthood.[246-248] The fetus has an independent and functioning HPA axis to regulate its glucocorticoid level that becomes active at 16 weeks of gestation. The fetal HPA axis is shielded from excess maternal glucocorticoids and exogenous negative feedback by the placenta, which possesses high levels of the enzyme 11β-hydroxysteroid dehydrogenase types 1 and 2.[249,250] The type 2 isoform efficiently catalyzes the conversion of active corticosterone and cortisol to their inactive metabolite, cortisone, thus preventing high maternal glucocorticoid concentrations from overwhelming the fetal system.[251-253] Maternal cortisol levels normally are several times higher than fetal concentrations, and increase near term.[254] Placental 11β-HSD2 activity increases gradually with gestational age in parallel with placental size and maternal cortisol levels, thereby protecting the fetus from excess glucocorticoid exposure.[255] Growth-restricted newborns demonstrate lower placental 11β-HSD2 activity and mRNA expression and higher circulating cortisol–cortisone ratio, which may result from deficient placental inactivation of maternal compounds.[255-257] Among preterm newborns with growth restriction, placental 11β-HSD2 activity does not appear to increase appropriately with gestational age, and its inactivity is strongly correlated with low relative birth weight, or length and infant head circumference[257] (Figure 17-7).

Other important findings include a significant decrease in placental 11β-HSD type 2 activity and an increase in fetal cord cortisol in preeclamptic pregnancies compared with levels in normal control subjects.[258-260] McCalla and co-workers[258] demonstrated

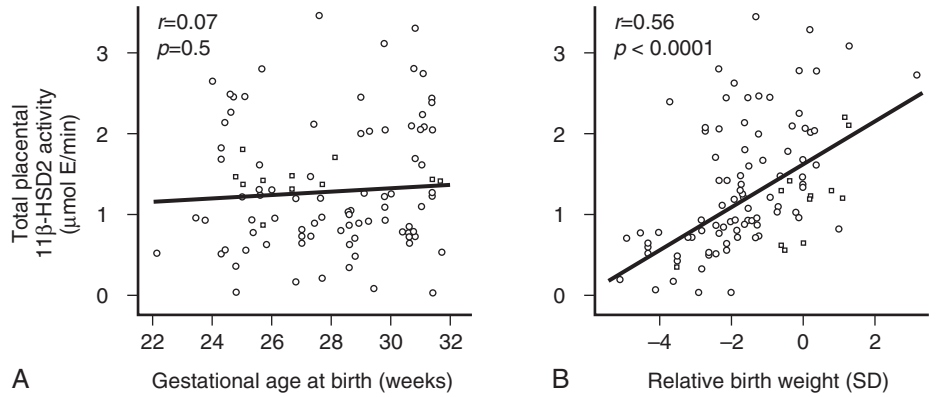


Figure 17-7 Correlation of total placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) activity with gestational age **(A)** and relative birth weight **(B)** in preterm SGA pregnancies. *E,* cortisone; *SGA,* small for gestational age. *(From Kajantie E, Dunkel L, Turpeinen U, et al: Placental 11beta-hydroxysteroid dehydrogenase-2 and fetal cortisol/cortisone shuttle in small preterm infants,* J Clin Endocrinol Metab *88:493-500, 2003.)*

that placental 11β-HSD2 activity was reduced approximately 25% in cases of preeclampsia. Fetal cord cortisol was more than doubled, and infants of these preeclamptic pregnancies were significantly smaller even when controlled for gestational age. Kajantie and associates also demonstrated reduced total placental 11β-HSD activity and fetal cord cortisone in preterm pregnancies with preeclampsia from levels measured in uncomplicated preterm pregnancies.[257] Other investigators have demonstrated a three-fold reduction in the level of placental 11β-HSD2 mRNA in hypertensive pregnancies compared with matched control pregnancies[259] (Figure 17-8). The infants in this study were all born near term (37.0 versus 36.9 weeks) and did not manifest fetal growth restriction (2462 versus 2702 g), suggesting that preeclampsia inhibits 11β-HSD expression independently of growth restriction. Why placental 11β-HSD2 function is decreased in preeclampsia is not known, although subsequent work suggests that hypoxia down-regulates the gene expression and enzyme activity in both early and late placenta, consistent with theories of the pathogenesis of preeclampsia.[260]

Insulin and Insulin-Like Growth Factors

The insulin-like growth factors, IGF-1 and IGF-2, are potent regulators of cell division, differentiation, and apoptosis and also influence fetal and placental growth.[261-264] Their concentrations in the fetus are strongly correlated with birth weight, and the absence of either factor in genetically deficient mice is associated with up to a 40% reduction in fetal size.[265] The actions of IGFs are mediated by IGF receptors and are stringently regulated by specific binding proteins (IGFBPs), six of which have been identified.[262,266] IGFBP-3 is the major circulating binding protein, sequestering approximately 80% of IGFs in the plasma. IGFBP-1, on the other hand, has a high affinity for IGF, and acts to modulate acute plasma changes or local concentrations of IGF-1 by reducing its bioavailability. Consistent with this role, circulating fetal IGFBP-1 concentrations are inversely related to birth weight.[261,263] IGF-2 is important for fetal embryonic development.[267] IGF-2 influences pancreatic beta cell proliferation and thus may regulate fetal growth and metabolism by enabling insulin secretion.[268,269] IGF-1, which predominates in late gestation, is produced mainly in the fetal liver under the influence of insulin and nutritional supply.[268]

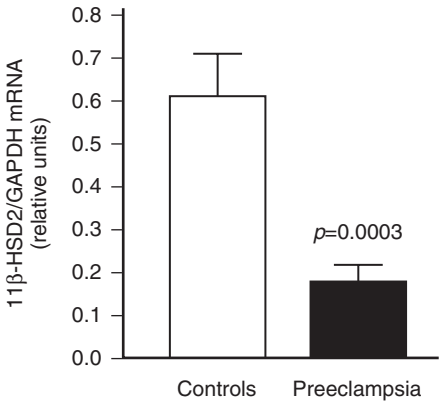


Figure 17-8 Relative expression of placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) messenger RNA (mRNA) in women with preeclampsia (n = 18) and in control subjects (n = 20). *GAPDH,* Glyceraldehyde 3-phosphate dehydrogenase. *(From Schoof E, Girstl M, Frobenius W, et al: Decreased gene expression of 11beta-hydroxysteroid dehydrogenase type 2 and 15-hydroxyprostaglandin dehydrogenase in human placenta of patients with preeclampsia,* J Clin Endocrinol Metab *86:1313-1317, 2001.)*

IGF signaling is important for decidual development, and appears to be critical for proper trophoblast differentiation and invasion in the first trimester, and subsequent placental growth in the second and third trimesters.[172,270,271] Cytotrophoblasts express IGF (especially IGF-2) and high levels of IGF receptors which promote invasive growth in an autocrine and paracrine fashion.[272] In a coordinated manner, maternal decidua express large quantities of IGFBP-1, which potentially acts as a counter-regulatory component but also may induce trophoblast proliferation. Indeed, overexpression of human decidual IGFBP-1 in transgenic mice leads to modest fetal growth restriction and placentomegaly with abnormal, shallow decidual invasion.[273]

Normal IGF-IGFBP relationships are disrupted in the maternal, placental, and fetal compartments in preeclampsia and may contribute to fetal growth abnormalities.[172,271,274-277] IGFBP-1 immunostaining is reportedly increased in the syncytiotrophoblast and anchoring villi of placentas from women with severe preeclampsia compared with controls.[172] Likewise, maternal plasma IGFBP-1, the main source of which is the placenta, is significantly increased in women with severe or early preeclampsia at the time of diagnosis.[172,278-280] It has been proposed that increased maternal IGFBP-1 production at the decidual interface impairs normal trophoblast invasion, predisposing the mother to the future development of preeclampsia.[172] It is not known why IGFBP-1 expression would be up-regulated in preeclamptic placentas, although hypoxia, which stimulates IGFBP-1 expression in other cell types, has been implicated.[281] By contrast, at 16 to 24 weeks of gestation, maternal plasma IGFBP-1 concentrations are significantly *decreased* in women destined to develop preeclampsia at term, suggesting that the hypoxic trigger does not develop until later in pregnancy.[277,282,283]

In contrast with IGFBP-1, maternal plasma IGF-1, which normally increases in pregnancy and is derived from hepatic synthesis, is reduced in preeclamptic women.[172,284,285] The significance of this decrease for fetal growth is uncertain, because although maternal IGF-1 concentrations correlate with fetal size at birth, fetal and maternal IGF systems appear to function independently of one another.[286] Maternal IGF-2 does not change significantly in either normal or preeclamptic placentas.

Most pertinent for fetal growth, of course, are fetal concentrations of the IGFs and their IGFBPs. In a rigorous study of more than 800 infants, Vatten and colleagues demonstrated that cord plasma IGF-1 was significantly lower in the setting of severe or early preeclampsia, independent of fetal weight.[287] Conversely, cord IGFBP-1 was elevated in neonates from a pregnancy with severe preeclampsia, replicating many other investigators' findings. After adjustment for birth weight, however, the association of IGFBP-1 with preeclampsia was lost. Thus it appears that cord IGFBP-1 is regulated primarily by fetal weight, and not by processes related to preeclampsia.

Apoptosis

Another potential link between preeclampsia and fetal growth restriction is the process of trophoblast apoptosis, which appears to be up-regulated in both conditions.[288,289] Ishihara and co-workers[289] demonstrated a significant down-regulation of the antiapoptotic protein Bcl-2 in the syncytiotrophoblast of placentas from pregnancies complicated by either SGA or preeclampsia, whereas other investigators[288] have reported increased evidence of apoptotic activity in pathologic specimens of cytotrophoblast. Fas ligand expression was found to be elevated in trophoblast and in paired maternal-cord blood samples from hypertensive pregnancies.[290,291] Isolated trophoblast from pregnancies complicated by either intrauterine growth restriction or preeclampsia demonstrates increased susceptibility to induction of apoptosis by hypoxia or TNF-α.[292,293] It is hypothesized that dysregulated trophoblast apoptosis during the process of endovascular invasion would impair placental perfusion, contributing to both SGA

and preeclampsia.[292,293] Similarly, inappropriate loss of syncytiotrophoblast from the floating villi nearer to term would reduce the effective villous surface area, thereby restricting transplacental passage of nutrients and oxygen.

Leptin

The disruption of metabolic pathways in preeclampsia may have particular relevance for alterations of fetal growth. Leptin, a 16-kDa hormone secreted by adipocytes, placenta, and select other tissues, is involved in the neuroendocrine regulation of body fat, feeding behavior, energy homeostasis, and reproduction.[294,295] Leptin levels correlate with body mass index in the nonpregnant state, and its production by adipocytes is stimulated by estrogens, progesterone, insulin, and inflammatory cytokines (e.g., TNF-α, IL-1).[294,296,297] Leptin actions include alteration in food intake, sympathetic nervous system activity, fatty acid synthesis, and triglyceride oxidation.[294] In pregnancy, leptin has dual functions in both the maternal and fetal compartments. In the mother, serum leptin levels increase two- to four-fold in normal pregnancy, presumably owing to placental production.[298-300] The increase of maternal leptin in pregnancy is related not to maternal body mass index but rather to placental size.[301,302] Leptin is actively produced in the syncytiotrophoblast and secreted into the maternal circulation. Leptin and its receptor also are expressed in the early placenta and may play a role in regulating the phenotype of invasive trophoblast.[303,304] The high levels of leptin in pregnancy reflect some degree of leptin resistance at the level of the hypothalamus and serve to augment mobilization of fatty acids in the periphery. Levels of circulating leptin are significantly higher in pregnancies complicated by preeclampsia.[305,306] The increase predates the clinical diagnosis, but levels rise still further once the diagnosis is made.[277] The source is likely to be placental, and secretion of leptin by cultured trophoblast is increased by hypoxia and cytokines, both of which are present in the preeclamptic placenta.[307-309]

It is widely suspected that leptin plays a role in the regulation of fetal growth.[310,311] In the fetus, similar to the nonpregnant adult state, leptin reflects adiposity and body composition. Cord leptin levels correlate with placental weight, birth weight, ponderal index, infant length, head circumference, gestational age, and cord insulin levels.[312-318] Cord leptin does not correlate with maternal leptin in normal pregnancies, suggesting a fetal-placental mode of regulation independent from the mother's compartment.[314,319,320] With only 1.6% of placental leptin being secreted into the fetal circulation, it is suspected that fetal adipose tissue is the major source of cord leptin.[321,322] Cord leptin levels are elevated in LGA infants and decreased in SGA infants[323-325] in proportion to body mass. Alternatively, the lower leptin concentration in SGA infants may be due to placental insufficiency or to lower levels of IGF-1 and insulin.[294,320,326]

Severe or early preeclampsia appears to disturb fetal leptin regulation and may be one mechanism of impairing fetal growth.[294] In contrast with uncomplicated pregnancy, a positive correlation between maternal and fetal leptin levels is observed in cases of preeclampsia, suggesting either enhanced placental permeability or basolateral secretion.[305] In the setting of preeclampsia, cord leptin has been reported to be increased, decreased or unchanged.[305,327-329] Odegård and associates[329] demonstrated that among hypertensive pregnancies, cord leptin was elevated at each gestational age. These workers suggest that leptinemia may be secondary to uteroplacental hypoxia or a disturbed fetal-placental interface and speculate that a resetting of leptin signaling may result in future insulin resistance and cardiovascular disease in the offspring.

Inherited Thrombophilias

Fetal growth restriction also may be linked to preeclampsia by its association with inherited and acquired thrombophilic conditions.[330,331] Multiple studies have reported an association between thrombophilic tendencies in women and adverse pregnancy outcomes, including both preeclampsia and intrauterine growth restriction,[330-335] but not all.[336,337] It is hypothesized that a maternal or fetal predisposition to clotting would lead to thromboses in the placental vasculature, thereby restricting oxygen and nutrient exchange, resulting in fetal growth impairment. Alternatively, fetal thrombophilia may lead to vascular disruption and potential developmental anomalies.[338] Histopathologic examination of placentas from preeclamptic women with thrombophilic conditions does not always disclose a greater frequency of thrombotic or inflammatory lesions,[339] thus casting doubt on the potential etiologic role of thrombophilia in the pathogenesis of preeclampsia.

Long-Chain Fatty Acid Metabolism

One particularly intriguing genetic association between maternal vascular disease and in utero growth restriction concerns a fetal inborn error of metabolism. Long-chain 3-hydroxyacyl-coenzyme A (CoA) (LCHAD) deficiency is a recessive condition resulting in the interruption of fatty acid β-oxidation and the accumulation of 3-hydroxy fatty acid metabolites.[340,341] Fetal manifestations of the disease include premature delivery, asphyxia, growth restriction, and potentially stillbirth.[342] Mothers of affected infants have an 80% likelihood of developing HELLP syndrome (*h*emolysis, *e*levated *l*iver enzymes, *l*ow *p*latelets) or acute fatty liver of pregnancy (AFLOP), two severe pregnancy complications with similarities to preeclampsia.[341] One possible explanation of this preeclampsia-like response is fetal-to-maternal transmission of accumulated oxidized 3-hydroxy fatty acids and their metabolites. As with preeclampsia, oxidative stress at the placental interface and within the maternal vasculature may lead to both fetal and maternal manifestations.

Therapeutics

In some instances, fetal growth disturbances may be associated with the treatment for cardiovascular disease, and not the disease itself. Pregnant women with vascular disease are subjected to numerous therapies, including seizure prophylaxis, antihypertesive treatment, immunosuppression, and others.

Magnesium

Magnesium sulfate is administered to nearly all preeclamptic women in the United States as a means of preventing eclamptic seizures in labor, and is commonly used as an inhibitor of preterm labor. Maternal magnesium administration has been associated with decreased rates of cerebral palsy when used in preterm pregnancies to prevent seizures or labor.[343-347] Other investigators, however, have not found a decrease in frequency of this disorder among preterm infants treated with magnesium whose mothers did not have preeclampsia.[100,348-351] Although preeclampsia and prematurity may be associated with fetal intracranial hemorrhage and subsequent neurodevelopmental delay, magnesium does not reduce the incidence of objective ultrasound evidence of CNS abnormalities.[350,351] It is, however, associated with lowered cerebral perfusion in preterm infants on the first day of life.[352,353] Although one small trial for the prevention of neurologic injury in preterm infants showed that magnesium exposure tended to *increase* the overall risk for neonatal death, a recent review of data for more than 6000 subjects concluded that administration of magnesium in doses used to prevent eclamptic seizure had no effect on pediatric mortality rates but decreased the risk for cerebral palsy by 30%.[354,355]

Antihypertensives

Substantial evidence accumulated from interventional trials in pregnancy indicates that the β-blocker atenolol increases the risk of intrauterine growth restriction, whether used to treat chronic hypertension or to prevent preeclampsia.[356] Other β-blockers, such as labetalol, do not share this effect on fetal growth.

Angiotensin-converting enzyme (ACE) inhibitors generally are not used in pregnancy because of deleterious effects on fetal and neonatal renal function and the associated potential for teratogenic effects.[357] The other commonly used antihypertensives, such as diuretics, calcium channel blockers, and direct vasodilators, have not been associated with disturbances of fetal growth or development.

Future Risks for Offspring of Preeclamptic Mothers

Neurodevelopmental Outcomes

In view of the "hostile" intrauterine environment supposed by current models of preeclampsia, offspring of mothers with preeclampsia or other hypertensive disorders of pregnancy might be expected to have increased long-term risk for neurodevelopmental disorders such as neurodevelopmental delay or impaired cognition. Few well-designed studies have been conducted to answer this question, however, and conflicting results have been obtained from retrospective cohort and case-control studies. Oftentimes, adequate controls for confounding factors such as gestational age at delivery, the presence or absence of growth restriction, and the peripartum use of medications such as glucocorticoids or magnesium sulfate seizure prophylaxis are lacking.

Cerebral Palsy

Cerebral palsy is a chronic, nonprogressive disorder of the white matter causing motor function impairment that becomes detectable within months to years after birth.[358,359] The inciting etiologic event in cerebral palsy is still elusive but traditionally has been thought to be asphyxial injury associated with labor and delivery.[358] More recent studies, however, suggest that in a majority of cases, predelivery antecedents related to early developmental anomalies or infections can be identified.[343,358] The association of cerebral palsy with preeclampsia has been studied in a number of investigations. A recent meta-analysis of 10 observational trials from 1966 to 1999 reported that the risk for cerebral palsy in low-birth-weight and growth-restricted infants was 50% lower in cases complicated by maternal preeclampsia.[359] This protective benefit appeared to persist even after controlling for the use of magnesium sulfate. Preeclampsia seemed to reduce the risk for cerebral palsy in low-birth-weight infants even when magnesium was not used.[360-362] It is unclear how preeclampsia, a disorder characterized by placental hypoxia-ischemia and inflammatory activation, could be protective against cerebral palsy. One potential explanation is that the early compensatory signals initiated by placental insufficiency (such as increased cardiac output[363] or altered uteroplacental blood flow[364]) more than make up for the reduced perfusion later on in pregnancy and prepare the infant for subsequent deprivation.[359] It is illustrative that in term preeclamptic pregnancies, the negative association with cerebral palsy and preeclampsia is *not* seen, suggesting that longer in utero exposure to placental hypoxia may eventually override initial protective effects.[365,366] Recent studies support this possibility by suggesting that most infants born to preeclamptic mothers are either AGA or LGA in comparison with gestational age–matched control infants.[212,218,219] Alternatively, it is proposed that rather than preeclampsia's conferring a protective effect, perse, there is a comparative excess of cerebral palsy in the preterm control groups who are disproportionately exposed to chorioamnionitis and other risk factors.[100,367]

Cognition

Several observational studies have noted an association between hypertension or preeclampsia in pregnancy and developmental delay.[368] These studies found a greater frequency of mild neurodevelopmental abnormalities in SGA infants born of hypertensive mothers[369,370] compared with nonhypertensive mothers. Of note, birth weight and SGA were more strongly correlated with future deficits than was hypertension per se. Some researchers have even suggested that preeclampsia may help prevent cognitive delay because germinal matrix hemorrhage is less common in very-low-birth-weight infants when preeclampsia was present.[371-373] Reduced placental blood flow by itself does not seem to increase the risk of neurodevelopmental or cognition delay, because no difference in behavior was observed between preterm preeclamptic children with and those without ultrasound evidence of placental blood flow restriction.[374] Thus other factors associated with early embryonic or fetal development are likely to influence the cognitive outcome.

Hypertension

Within multiple populations, long-term, prospective studies have identified a weak association between hypertension in pregnancy and hypertension in the offspring.[375-377] Seidman and co-workers[375] demonstrated a 2.3-fold increased risk of systolic hypertension at the age of 17 years in female (but not male) offspring born after a preeclamptic pregnancy. However, it appears that growth restriction accompanying a hypertensive pregnancy, rather than the hypertension itself, better predicts future cardiovascular risks. In a large epidemiologic survey of the Danish Perinatal Cohort Study, Klebanoff and colleagues[378] reported that pregnant women who were themselves SGA at birth had a 1.8-fold increased risk of developing hypertension in pregnancy over that in with women who were AGA at birth. Of interest, no association was found between hypertension in these womens' mothers' pregnancies and that in their own pregnancies. Prematurity by itself also predicted an increased risk of hypertension in pregnancy, although this relationship was not statistically significant.

Tenhola and co-workers[379] studied the vascular and metabolic outcomes in a group of 12-year-old children born after either a normal or a preeclamptic pregnancy. These investigators noted that the offspring of the preeclamptic women had significantly elevated systolic and diastolic blood pressure even after adjustment for current weight and height. The preeclamptic pregnancy offspring also had higher circulating epinephrine levels. The blood pressure at 12 years of age was inversely correlated with birth weight only in the children in the preeclampsia group. No difference was observed in serum lipids, insulin, blood glucose, or adrenocortical hormones, or in current weight or height. These data (and other findings[377]) suggest that preeclampsia may be related to future hypertension, but that birth weight and adolescent obesity may better predict the future risk for metabolic syndrome.

CONCLUSION AND OUTLOOK

It is apparent that cardiovascular diseases are common in pregnancy and that they are associated with considerable morbidity in both mother and fetus. The importance of cardiovascular function in pregnancy relates not only to maternal well-being but also to normal fetal growth and development. The intimate association between fetal growth and maternal cardiovascular function hinges on the adaptive and maladaptive potential of the placenta, and future investigation of the links between maternal disease and the fetal environment must focus on this interface. Increased understanding of the compensatory potential of the placenta can be expected to drive the development of therapies aimed at ameliorating diseases that remain dormant for nearly a lifetime.

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18 Fetal Origins of Adult Disease

David J. P. Barker

Hitherto the search for the causes of chronic disease, and for ways to prevent them, has been guided by a "destructive" model. The principal causes to be identified were believed to act in adult life by accelerating destructive processes associated with normal aging—for example, the formation of atheroma, rise in blood pressure, and loss of glucose tolerance. This model, however, has had limited success. Obesity, cigarette smoking, and psychosocial stress have been implicated, and evidence on dietary fat has accumulated to the point at which a public health policy of reduced intake is prudent, if not proven. The effects of modifying adult lifestyle, when formally tested in randomized trials have, however, been disappointingly small.[1] The model has proved incapable of answering important questions. For example, in Western countries the steep increase in coronary heart disease has been associated with rising prosperity, so why do the poorest people in the poorest parts of these countries have the highest rates?

PROGRAMMING

The recent discovery that people who develop coronary heart disease grew differently than other people during fetal life and childhood has led to a new "developmental" model for the disease.[2-5] Like other living creatures in early life, human beings are "plastic" and able to adapt to their environment. Phenotypic plasticity is the phenomenon by which one genotype can give rise to a range of different physiologic or morphologic states in response to different environmental conditions during development.[6] Gene-environment interactions are ubiquitous in development. Their evolutionary benefit is that, in a changing environment, they enable the production of phenotypes that are better matched to their environment than would be possible if one genotype produced the same phenotype in all environments.[6]

The development of the sweat glands provides a simple example of what is often referred to as "programming."[4] All human beings have similar numbers of sweat glands at birth, but none of them function. In the first 3 years after birth, a proportion of the glands become functional, depending on the temperature to which the child is exposed. The hotter the conditions, the greater the number of sweat glands that are programmed to function. After 3 years, programming is complete and the number of sweat glands is fixed. Thereafter the child who has experienced hot conditions will be better equipped to adapt to similar conditions in later life because people with more functioning sweat glands cool down faster.

The sweat glands encapsulate the essence of programming: a critical period during which a system is plastic and sensitive to the environment, followed by loss of plasticity and development of a fixed functional capacity. It may be advantageous, in evolutionary terms, for the body to remain plastic during development. Why this plasticity is ultimately lost in most tissues and systems is a question for which only speculative answers have emerged. Some tissues, the trabeculas of bone, for example, do remain plastic throughout life. It has been suggested that plasticity during intrauterine life enables animals, and humans, to receive a "weather forecast" from their mothers that prepares them for the type of world in which they will have to live.[7] If the mother is poorly nourished, she signals to her unborn baby that the environment it is about to enter is likely to be harsh. The baby responds to these signals by adaptations, such as reduced body size and altered metabolism, which help it to survive a shortage of food after birth. In this way programming gives a species the ability to make short-term adaptations, within one generation, in addition to the long-term genetic adaptations that come from natural selection.

TABLE 18-1

Hazard Ratios (95% Confidence Intervals) for Death From Coronary Heart Disease (CHD) According to Weight at Birth and at Age 1 Year in 10,636 Men in Hertfordshire

Birth Weight (pounds)	DEATH FROM CHD	
	Before 65 Years	All Ages
At birth		
≤5.5	1.50 (0.98-2.31)	1.37 (1.00-1.86)
6.5	1.27 (0.89-1.83)	1.29 (1.01-1.66)
7.5	1.17 (0.84-1.63)	1.14 (0.91-1.44)
8.5	1.07 (0.77-1.49)	1.12 (0.89-1.40)
9.5	0.96 (0.66-1.39)	0.97 (0.75-1.25)
> 9.5	1.00	1.00
***p* for trend**	.001	.005
Age 1 year		
≤18	2.22 (1.33-3.73)	1.89 (1.34-2.66)
20	1.80 (1.11-2.93)	1.58 (1.15-2.16)
22	1.96 (1.23-3.12)	1.66 (1.23-2.25)
24	1.52 (0.95-2.45)	1.36 (1.00-1.85)
26	1.36 (0.82-2.26)	1.29 (0.93-1.78)
≥27	1.00	1.00
***p* for trend**	< .001	< .001

TABLE 18-2

Percentage of Men Aged 64 Years With Impaired Glucose Tolerance or Type 2 Diabetes According to Birth Weight

Birth Weight (pounds)	Men With 2-hr Glucose of ≥7.8 mmol/L (%)	Odds Ratio (95% Confidence Intervals)*
≤5.5	40	6.6 (1.5-28)
6.5	34	4.8 (1.3-17)
7.5	31	4.6 (1.4-16)
8.5	22	2.6 (0.8-8.9)
9.5	13	1.4 (0.3-5.6)
>9.5	14	1.0
***p* for trend**	< .001	

*Adjusted for current body mass index

Overlooked until recently, a growing body of evidence has shown that systems of the body that are closely related to adult disease, such as the regulation of blood pressure, are also plastic during early development. In animals it is surprisingly easy to produce lifelong changes in the blood pressure and metabolism of a fetus by minor modifications to the diet of the mother before and during pregnancy.[8] When undernutrition during development is followed by improved nutrition, many animals stage accelerated or "compensatory" growth in weight or length. This restores the animal's body size but has long-term costs that include reduced life span.[9] This chapter sets out the evidence that chronic disease is initiated through two basic biologic phenomena: developmental plasticity and compensatory weight gain in childhood.

BIRTH WEIGHT AND CHRONIC DISEASE

Exploring the developmental origins of chronic disease has required studies of a kind that had not previously been carried out. It was necessary to identify groups of men and women now in middle or late life whose size at birth had been recorded. Their birth weights could then be related to later occurrences of chronic diseases. In Hertfordshire, United Kingdom, starting in 1911, when women had babies they were attended by a midwife, who recorded the birth weight.[2,10] A health visitor went to the babys' homes at intervals throughout infancy, and weights at 1 year were recorded. Table 18-1 lists the findings for 10,636 men born between 1911 and 1930. Hazard ratios for coronary heart disease decrease with increasing birth weight. Stronger trends with weight are seen at 1 year. Table 18-2 shows findings for a sample of men who had glucose tolerance tests.[11] The percentage with impaired glucose tolerance or type 2 diabetes decreased steeply with increasing birth weight. The association between low birth weight and coronary heart disease has now been replicated among men and women in Europe, North America, and India.[12-17] Low birth weight has been shown to predict altered glucose tolerance in studies of men and women around the world.[18-22] These associations with low birth weight and later disease depend on slow fetal growth rather than premature birth. The association between coronary heart disease and poor weight gain in infancy, first shown in Hertfordshire,[2,10] has been confirmed in the Helsinki Birth Cohort.[5]

Figure 18-1 shows the growth of men and women who either were hospitalized for management of coronary heart disease or died of it.[5] They belonged to a cohort of 8760 people who were born in Helsinki during 1934 to 1944. Growth is expressed as Z-scores. The Z-score for the cohort is set at zero, and a child maintaining a steady position as large or small in relation to other children would follow a horizontal path on the figure. Boys and girls in whom coronary heart disease developed later in life, however, were small at birth and remained small in infancy but experienced accelerated gain in weight and body mass index (weight/height^2) after the age of 2 years. Their heights, however, remained below average, which is consistent with the known association between the disease and short adult stature.[23]

Table 18-3 is based on 2997 patients treated for hypertension and 698 patients treated for type 2 diabetes in the Helsinki Birth Cohort.[24] These two disorders are associated with the same general pattern of growth as for coronary heart disease. Small size at birth is followed by accelerated weight gain in childhood. The highest risk for each disease occurs among men and women who had low birth weight but were in the highest body mass index group at 11 years. As with coronary heart disease, the risk for disease is determined not only by the absolute value of body mass index in childhood but also by the combination of body size at birth and during childhood.[25,26] It is the tempo of weight gain in childhood as well as the attained body size that determines risk.

BIOLOGIC MECHANISMS

There are a number of mechanisms by which reduced fetal and infant growth followed by accelerated weight gain in childhood may lead to later disease. Babies who are thin at birth lack muscle, a deficiency that will persist, because the critical period for muscle growth is around 30 weeks in utero, and little cell replication occurs after birth.[27] If thin babies develop a high body mass index in childhood, fat mass may be disproportionately high. This is associated with the development of insulin resistance.[28]

Small babies have reduced numbers of nephrons.[29] It has been suggested that this leads to hyperperfusion of each nephron and resulting glomerular sclerosis, further nephron death, and a cycle of increasing blood pressure and nephron death. This may be exacerbated if accelerated weight gain increases the degree of hyperperfusion. This framework fits with the hypothesis that essential hypertension is a disorder of growth with two separate mechanisms: a growth-promoting process in childhood and a self-perpetuating mechanism in adult life.[30]

One of the mechanisms linking poor weight gain in infancy with coronary heart disease is altered liver function, reflected in elevated serum concentrations of total and low density lipoprotein cholesterol and high plasma fibrinogen concentrations.[31]

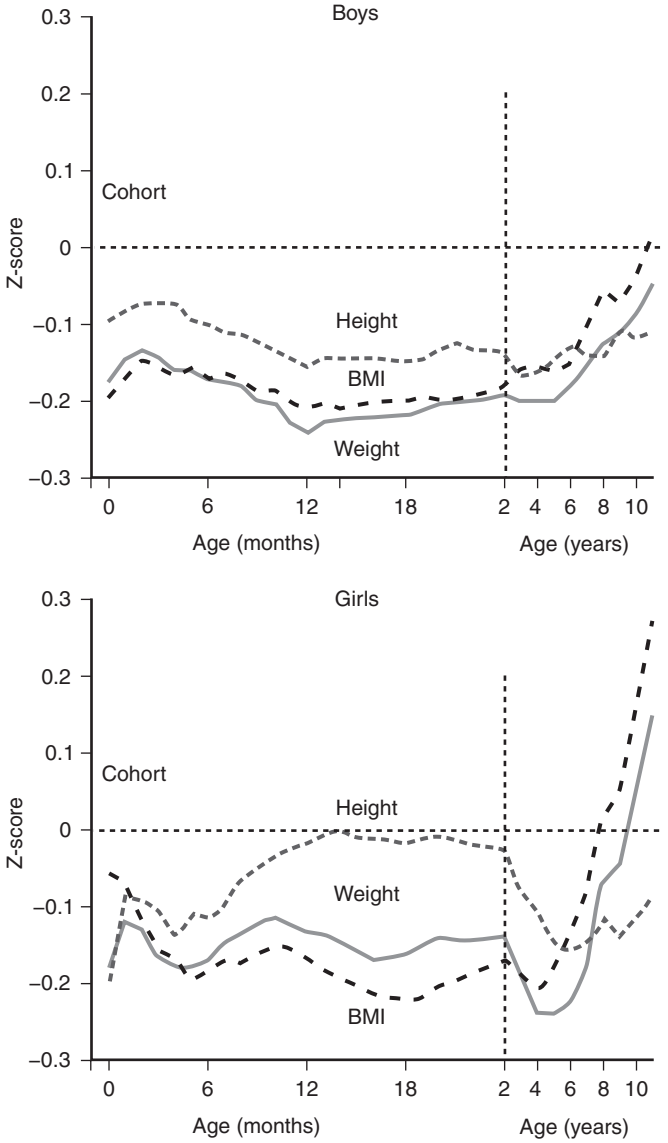


Figure 18-1 Mean Z-scores for height, weight, and body mass index in the first 11 years after birth among boys and girls in whom coronary heart disease developed in adulthood. The mean values for all boys and all girls are set at zero, with deviations from the mean expressed as standard deviations (Z-scores).

Unlike organs such as the kidney, the liver remains plastic during its development until the age of approximately 5 years. Its function may be permanently changed by influences that affect its early growth.[32-33] Liver development may play an important role in the early pathogenesis of coronary heart disease. Studies of men and women born in Sheffield, England[34] showed that a smaller abdominal circumference at birth (a measure that reflects reduced liver size) was a stronger predictor of later elevations of serum cholesterol and plasma fibrinogen than any other measure of body size at birth.

Observations on animals show that the environment during development permanently changes not only the body's structure and function but also its responses to environmental influences encountered in later life.[7] Table 18-4 shows the association of low income in adult life with coronary heart disease among men in Helsinki.[35] As expected from numerous studies in the Western world, men who had low incomes had higher rates of the disease. The association of low income and heart disease is, however, limited to men who exhibited slow fetal growth and were thin at birth, defined as a ponderal index (birth weight/length^3) of 26 kg/m^3 or less. Approximately one fourth of all boys born in Britain today are as thin as this. Men who were not thin at birth were resilient to the effects of low income on coronary heart disease.

One explanation of these findings emphasizes the psychosocial consequences of a low position in the social hierarchy, as indicated by low income, and suggests that perceptions of low social status and lack of success lead to changes in neuroendocrine pathways and hence to disease.[36] The findings in Helsinki seem consistent with this. People who are small at birth are known to have persisting alterations in responses to stress, including raised serum cortisol concentrations.[37]

Table 18-3 shows that the effect of a high body mass in childhood is conditioned by size at birth. Table 18-4 shows that the effect of low income in adult life is conditioned by size at birth. Therefore the effect of any single influence on the burden of disease cannot usefully be quantified because its effect will depend on the path of development that preceded it. The pathogenesis of coronary heart disease and other chronic diseases cannot be understood within a model in which risks associated with adverse influences at different stages of life add to each other.[38] Rather, the consequences of adverse influences depend on events at earlier critical stages of development. This embodies the concept of developmental "switches" triggered by the environment.[7] The effects of any particular birth weight on disease will depend not only on the subsequent path of development but also on the path of growth that led to the birth weight. The same weight can be attained by many different paths of fetal growth, and each is likely to be accompanied by different gene-environment interactions, although this remains to be demonstrated.[39]

TABLE 18-3

Odds Ratios (95% Confidence Intervals) for Hypertension and Type 2 Diabetes According to Birth Weight and Body Mass Index at 11 Years

	BODY MASS INDEX AT 11 YEARS (kg/m^2)			
Birth Weight (kg)	**15.7**	**16.6**	**17.6**	**>17.6**
Hypertension				
≤3.0	2.0 (1.3-3.2)	1.9 (1.2-3.1)	1.9 (1.2-3.0)	2.3 (1.5-3.8)
3.5	1.7 (1.1-2.6)	1.9 (1.2-2.9)	1.9 (1.2-3.0)	2.2 (1.4-3.4)
4.0	1.7 (1.0-2.6)	1.7 (1.1-2.6)	1.5 (1.0-2.4)	1.9 (1.2-2.9)
> 4.0	1.0	1.9 (1.1-3.1)	1.0 (0.6-1.7)	1.7 (1.1-2.8)
Type 2 diabetes				
≤3.0	1.3 (0.6-2.8)	1.3 (0.6-2.8)	1.5 (0.7-3.4)	2.5 (1.2-5.5)
3.5	1.0 (0.5-2.1)	1.0 (0.5-2.1)	1.5 (0.7-3.2)	1.7 (0.8-3.5)
4.0	1.0 (0.5-2.2)	0.9 (0.4-1.9)	0.9 (0.4-2.0)	1.7 (0.8-3.6)
>4.0	1.0	1.1 (0.4-2.7)	0.7 (0.3-1.7)	1.2 (0.5-2.7)

TABLE 18-4

Hazard Ratios (95% Confidence Intervals) for Coronary Heart Disease According to Ponderal Index at Birth and Taxable Income in Adult Life

Household Income 1000 Marks/yr (£)	Ponderal Index ≤26.0 (kg/m^3) (n = 1475)	Ponderal Index >26.0 (kg/m^3) (n = 2154)
>140 (15,700)	1.00	1.19 (0.65-2.19)
111-140 (15,700)	1.54 (0.83-2.87)	1.42 (0.78-2.57)
96-110 (12,400)	1.07 (0.51-2.22)	1.66 (0.90-3.07)
76-95 (10,700)	2.07 (1.13-3.79)	1.44 (0.79-2.62)
75 (8,400)	2.58 (1.45-4.60)	1.37 (0.75-2.51)
***p* for trend**	< .001	0.75

THE PLACENTA'S ROLE IN PROGRAMMING

To date, most epidemiologic observations that have demonstrated the link between prenatal life and later chronic disease have used birth weight as the marker of early life in utero. A baby's birth weight reflects its success in obtaining nutrients from its mother. The source of these nutrients is not only the mother's daily diet but her nutritional stores and metabolism, which are the product of her lifetime's nutrition.[40] A baby's nutrition also depends on the placenta's ability to transport nutrients to it from the mother.[39] This ability is reflected in its size because large babies usually have large placentas.[41] New data from the Helsinki Birth Cohort allow researchers to address three questions: (1) What measures of placental size can be used as markers of its function for long-term studies linking placental size with later disease? (2) Is a large placenta always beneficial? (3) Are there sex differences in the maternal-placental phenotypes that predict chronic disease?

Measurements of Placental Size That Reflect Its Function

Low placental weight at birth has been shown to predict hypertension and coronary heart disease in later life.[25,42] Placental weight, however, does not distinguish the thickness of the placenta from its surface area. To increase the surface for nutrient and oxygen exchange, the placenta can increase the surface area of its villi, expand its invasion across the surface of the uterine lining, or invade the maternal spiral arteries more deeply. The long-term consequences may be different. Textbooks of midwifery written early in the last century describe the placental surface as being either oval or round.[43] To describe the extent to which the surface was more oval than round, in the past two so-called diameters of the surface were routinely recorded in some hospitals—a maximal diameter and a lesser one bisecting it at right angles. These measurements are available for the Helsinki Birth Cohort comprising 20,436 men and women born in the city in 1924 to 1944. The mean lengths of the two diameters were 19.5 and 16.9 cm, and the lengths were highly correlated (correlation coefficient = 0.63).[44] The diameters were used to estimate the surface area. How closely this area reflects the total surface area for maternal-fetal exchange through the gestational period is not known, but it is reasonable to suppose that they correlate. The two diameters and placental weight were combined to estimate thickness.

Placentas from pregnancies complicated by preeclampsia had a more oval surface than those from normotensive pregnancies because of a disproportionate reduction in the lesser diameter.[45] When the two diameters were analyzed together, preeclampsia was not associated with the length of the maximal diameter, but was strongly associated with a short lesser diameter. This is shown in Figure 18-2. The relation with the lesser diameter was graded: The shorter the lesser diameter, the greater the risk for, and severity of, preeclampsia. This led to the suggestion that placental growth is polarized from the time of implantation.[45] Growth along the major axis, reflected in the major diameter, may align with the rostrocaudal growth of the fetus; growth along the minor axis may be nutritionally sensitive. Because preeclampsia is only related to growth of the minor axis, tissue along this axis may be functionally different to tissue on the major axis. Alternatively growth along the minor axis may be a more sensitive marker of the impaired implantation that initiates preeclampsia.

Large Placentas

Although low placental weight at birth has been shown to predict hypertension and coronary heart disease in later life,[25] a study of men and women born in a maternity hospital in Preston, United Kingdom, showed that high placental weight in relation to birth weight is also associated with hypertension.[46] Table 18-5 shows that whereas people with both low placental weight and low birth weight had higher mean systolic blood pressures than people with low placental weight but high birth weight, the highest mean systolic pressures occurred in people who had high placental weight but low birth weight. This observation has been replicated, and high placental weight in relation to birth weight has also been shown to predict coronary heart disease.[47] These associations with large placental size suggest that cardiovascular disease can be initiated through placental expansion as well as through restricted placental growth. Observations in sheep show that in response to undernutrition a fetus is able to extend the area of the placenta by expanding the individual cotyledons.[48,49] This increases the area available for nutrient and oxygen exchange and results in a larger lamb than otherwise would have been. This is profitable for the farmer, and manipulation of placental size by changing the pasture of pregnant ewes is standard practice in sheep farming.

Placental expansion in response to undernutrition in midgestation can only occur, however, if the ewe was well nourished before conception.[49] A similar phenomenon may occur in humans. In the Helsinki Birth Cohort, the effects of the placental area on hypertension depended on the mother's socioeconomic status, a marker of her current nutrition, and on her height, which reflects her nutrition and metabolism in childhood and adolescence.[44] In people whose mothers were of low socioeconomic status or below average height, hypertension was associated with low placental weight and area. In people whose mothers were likely to have been the best nourished and who were tall and middle class, hypertension was predicted by large placental size in relation to birth weight. Figure 18-3 shows the trends in the

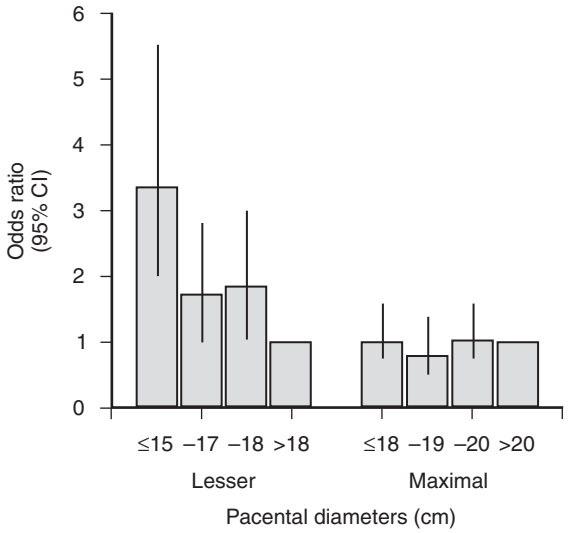


Figure 18-2 Odds ratios for preeclampsia according to the diameters of the placental surface.

TABLE 18-5

Mean Systolic Blood Pressure (mm Hg) of Men and Women, Aged 50, Born at Term

Birth Weight (pounds)	PLACENTAL WEIGHT (pounds)			
	≤1.0	1.25	1.5	>1.5
6.5	149	152	151	167
7.5	139	148	146	159
>7.5	131	143	148	153

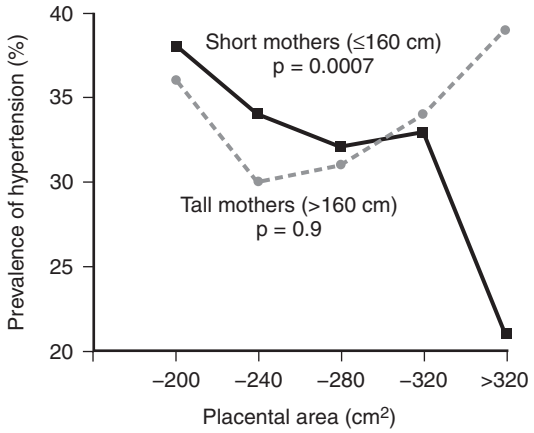


Figure 18-3 Prevalence of hypertension according to placental area in the offspring of short and tall mothers.

prevalence of hypertension with placental area in people divided according to whether their mother's height was above or below the median of 160 cm. In people whose mothers were short, the prevalence of hypertension fell progressively with increasing placental area, whereas in people with tall mothers, the association was U-shaped: If the mother was of lower social class, hypertension was associated with small placental area, but if she was middle class, it was associated with large area. Figure 18-3 illustrates the important point that the effects of placental size in programming later disease are conditioned by the mother's nutrition.

Of the two diameters of the surface, the lesser diameter was more closely associated with hypertension, suggesting that expansion of the surface along its minor axis may be a response to fetal undernutrition.[44] Compensatory placental growth may be beneficial in some circumstances, but if the compensation is inadequate and the fetus continues to be undernourished, the need to share its nutrients with an enlarged placenta may become an added metabolic burden. A long-term cost of this added burden is hypertension, possibly as a result of impaired development of low-priority organs like the kidney.[29]

Sex Differences in Placental Programming

Boys grow faster than girls from an early stage of gestation, even before implantation, and this makes them more vulnerable if nutrition is compromised.[50] In the Helsinki Birth Cohort, small differences in average body measurements at birth concealed large differences in body proportions. At any placental weight, boys tended to be longer than girls; the placentas of boys were smaller than the placentas of girls when related to the weight of the baby.[17,51] This suggests that the placentas of boys are more efficient but may have less reserve capacity, which therefore increases the babies' vulnerability to undernutrition. Boys tend to have larger head circumferences at birth but are thinner than girls.[17] These differences suggest that they may more readily trade visceral development to protect brain growth. The growth of every human fetus is constrained by the limited capacity of the mother and placenta to deliver nutrients to it.[52] The male fetus, by growing more rapidly and investing in brain growth rather than placental growth, is adopting a more dangerous strategy that puts it at greater risk for becoming undernourished.

The relation between the placental diameters and later hypertension was different in the two sexes.[51] In women, hypertension was associated with a short lesser diameter and a small placental area. These trends were stronger in women whose mothers were short but did not differ with the mother's social class. In men, hypertension was associated with a large lesser diameter in relation to birth weight. This trend was confined to men whose mothers were middle class. An interpretation of these findings is that during development in the womb girls respond more to their mother's lifetime nutrition and metabolism than to their current diets, whereas boys are more responsive to their mother's current diet. The greater effect of the Dutch famine on boys supports this,[53] as do the greater effects of experimental maternal malnutrition on male animals.[54,55] Although boys' responsiveness to their mothers' current diet enables them to capitalize on an improving food supply and promotes the agenda of rapid growth, it makes them vulnerable to food shortages. An ultimate manifestation of boys' dangerous growth strategies in the womb may be that men have higher blood pressures and shorter lives than women.

MOTHERS AND BABIES TODAY

In view of the considerable evidence showing that coronary heart disease and the related disorders stroke, hypertension, and type 2 diabetes originate through undernutrition and other adverse influences in utero, followed by accelerated weight gain in childhood, protecting the nutrition and health of young women and their babies must be part of any effective strategy for preventing chronic disease. Evidence that certain cancers are initiated in fetal life adds to the urgency of this.[56-58] The so-called fetal origins hypothesis resulted from studies of the geographic association between coronary heart disease and low income in England and Wales and the recognition that a poor intrauterine environment played a major role in this association.[59] Areas of the country with high coronary mortality rates are characterized historically by poor maternal nutrition and health, reflected in high maternal and neonatal mortality.[60] It is becoming clear that the concept of maternal nutrition must be extended beyond a mother's diet in pregnancy to include her lifetime nutrition and metabolism. The Helsinki studies allow estimation of the magnitude of the effects of early growth on later disease. If, for example, each child in the cohort had been in the highest third of birth weight and had reduced his or her standard deviation score for body mass index between age 3 and 11 (see Table 18-3), the incidence of type 2 diabetes would have been reduced by 57%.[24] Thus early development establishes a substantial part of the risk for chronic disease in later life.

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Jane Cleary-Goldman and Mary E. D'Alton

Physiologic Effects of Multiple Pregnancy on Mother and Fetus

BACKGROUND INFORMATION

Multiple pregnancy, including twins and higher-order multifetal gestations, presents a challenge to obstetricians, perinatologists, and neonatologists. Besides the social, economic, and psychologic effects on individual patients and families, multiple pregnancy has a significant physiologic impact on both the mother and each individual fetus. Multiple births account for a disproportionate share of adverse obstetric and neonatal outcomes. Parturients are at increased risk for gestational hypertension, gestational diabetes, abnormal placentation, placental abruption, preterm labor, and cesarean delivery, whereas fetuses are at increased risk for genetic and anatomic anomalies, growth abnormalities, intrauterine fetal demise (IUFD), prematurity, and abnormalities specific to monochorionicity, such as the twin-to-twin transfusion syndrome (TTTS).

Maternal and neonatal outcomes may be optimized by use of management protocols based on an appreciation of the physiologic effects of multiple pregnancy on both the mother and the fetus. A multidisciplinary approach to the care of these patients has been recommended.[1] This chapter reviews important physiologic and pathophysiologic aspects of multifetal gestations.

Incidence

As of 1973, the reported incidence of twins worldwide was 1 in 80 pregnancies, whereas higher-order multiples were significantly less common.[2] In the United States, however, the incidence of multiple gestations has increased since the late 1960s because of the use of infertility treatments, such as superovulation and assisted reproductive technology (ART).[3] In the more recent past, some countries have reported a doubling of twin pregnancy rates.[4] The incidence of multiple pregnancies has been increasing at a greater rate than that for singleton pregnancies. In the United States, between 1973 and 1990, twin births increased by 65% and higher-order births increased by 221%, whereas singleton births increased by only 32%.[3]

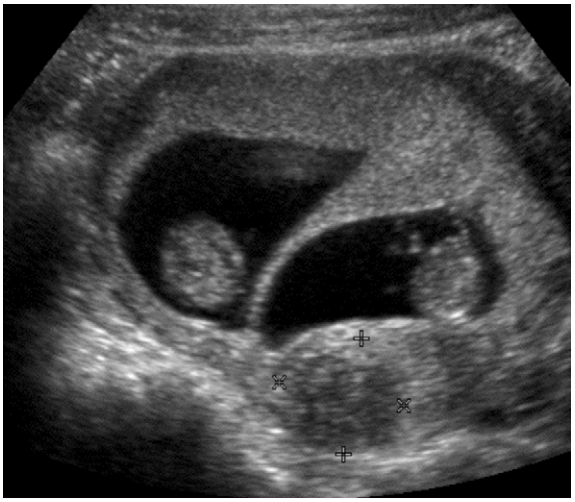


Figure 19-1 First-trimester twin peak sign in a dichorionic twin pregnancy.

Zygosity and Chorionicity

Zygosity refers to the genetic make-up of a pregnancy. Multiple gestations can be monozygotic or dizygotic. *Monozygotic* twins result from the division of a zygote arising from the fertilization of one ovum by one sperm. *Dizygotic* twins result from the fertilization of separate ova by separate sperm. Approximately two thirds of twins are dizygotic, with "identical twins" accounting for the remaining third.[5]

Chorionicity refers to the membrane composition of the pregnancy—the chorion and amnion. It is determined by the mechanism of fertilization and by the occurrence and timing of embryo division. All dizygotic twins have separate dichorionic diamniotic placentas; that is, each fetus has its own separate placenta and amniotic sac. The reason is that dizygotic twins result from the fertilization of two separate ova by two separate sperm. Monozygous gestations can be dichorionic diamniotic, monochorionic diamniotic, or monochorionic monoamniotic, depending on the timing of division. If the zygote splits during the first 3 days after fertilization (at the morula stage), a *dichorionic diamniotic* placenta results. Approximately one third of monozygotic twins have this type of placenta.[5] If the zygote splits between days 3 and 8 (at the developmental stage), the pregnancy will be *monochorionic diamniotic,* accounting for approximately two thirds of monozygous twins.[5] These twins share one placenta but coexist in separate amniotic sacs. A *monochorionic monoamniotic* pregnancy results if the zygote splits after the amnion has formed (the embryonic disk stage) at approximately 9 to 12 days; the twins share the same placenta and amniotic sac. This type accounts for approximately 3% of all monozygotic twins.[5] If the yolk sac has already formed and division occurs, the monozygotic pair will be conjoined. Conjoined twins are rare, occurring in 1 in 50,000 births.[6] With zygote division after day 17, a singleton gestation will result.[7] Higher-order multiples may be composed of any combination of monozygotic and dizygotic fetuses.[8]

The precise mechanisms causing spontaneous monozygotic and dizygotic twins are not well understood. Dizygotic twins and high-order multiples probably derive from multiple ovulations resulting from simultaneous growth of multiple follicles.[9] Factors well known to be associated with an increased incidence of dizygotic twins include advanced maternal age, family history of such twinning, multiparity, and certain ethnicities.[10-12] Dizygotic twinning may be related to higher baseline levels of gonadotropins in certain women. Fertility treatment also is associated with increased rates of dizygotic twins. Under all of these conditions, the ovary is exposed to high levels of gonadotropic hormones, resulting in multiple ovulation.[9]

Monozygotic twinning, however, is less understood than dizygotic twinning. Before the use of ART, the frequency of monozygotic twins was constant throughout the world. The incidence was approximately 4 per 1000 births.[2] With infertility treatment, however, the incidence of monozygotic twin gestations may be increasing.[13] The mechanism is unknown, but twinning may be secondary to trauma during the procedure or associated with increased levels of gonadotropins.[14,15]

Determination of Chorionicity

The determination of chorionicity is important in the management of multiple gestations because monochorionic twins are at increased risk for adverse outcomes.[16] Prenatally, ultrasound imaging is the best diagnostic method. Chorionicity is most accurately determined in the first trimester (from 6 to 10 weeks) by counting the number of gestational sacs. The presence of two separate gestational sacs, each with its own heartbeat, suggests a dichorionic diamniotic pregnancy, whereas one gestational sac with two separate heartbeats suggests a monochorionic pregnancy.[17] The amnion can be visualized by transvaginal ultrasound imaging at approximately 7 to 8 weeks of gestation. Before this time, amnionicity may be difficult to determine. When two separate amniotic sacs, each with a single heartbeat, are visualized, the pregnancy is diamniotic. Conversely, when one amniotic sac with two heartbeats is visualized, the pregnancy is monoamniotic.[18] In addition, determination of the number of yolk sacs can be used as an indirect method to indicate the number of amniotic sacs and consequently the amnionicity.[19] At early gestational ages, chorionicity and amnionicity should be confirmed by another ultrasound scan in 2 to 3 weeks, because misdiagnosis is a possibility[20] (Figures 19-1 to 19-3).

After the first trimester, determination of chorionicity and amnionicity becomes less accurate. As a result, different techniques are needed to determine amnionicity and chorionicity in multiple gestations in the second and third trimesters. These methods include assessing fetal gender, evaluating the placenta, and examining the intertwin membrane.

If the fetuses are of different genders, the pregnancy is almost certainly dichorionic. If the gender is unknown, however, or is the same for all fetuses, chorionicity is still unknown. When placentas are clearly separate, the pregnancy is likely to be dichorionic. If

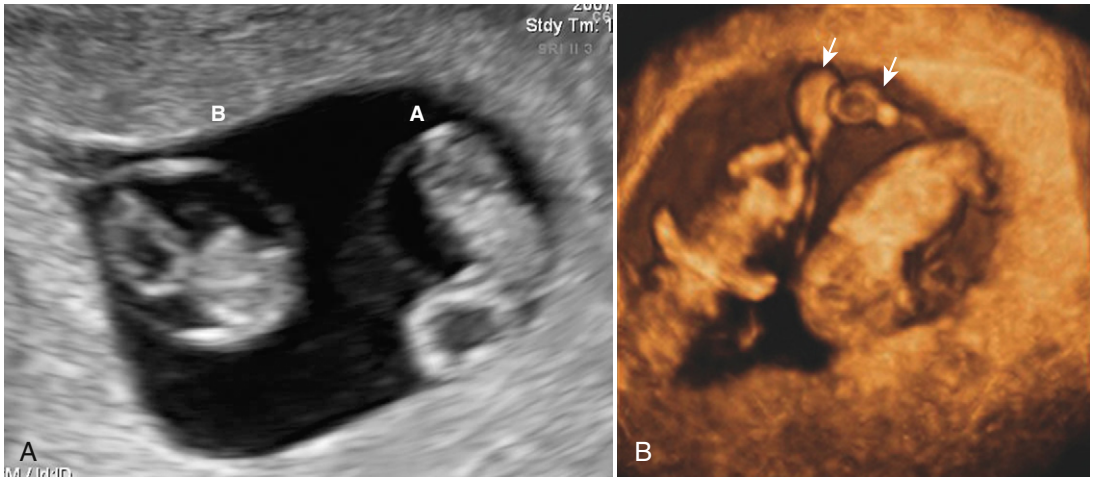


Figure 19-2 **A,** Two-dimensional ultrasound image of first-trimester monochorionic diamniotic twins. A single gestational sac with two yolk sacs and two embryos (*A* and *B*) can be seen. **B,** Three-dimensional ultrasound image from the same patient. *(Courtesy Carnegie Hill Imaging for Women, New York.)*

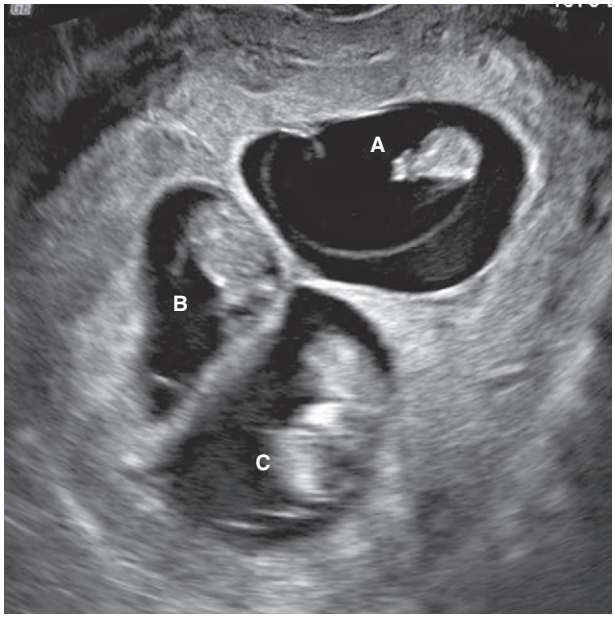


Figure 19-3 First-trimester image of a trichorionic triamniotic triplet pregnancy (the three embryos are labeled *A* to *C*). *(Courtesy Carnegie Hill Imaging for Women, New York.)*

the placentas are adjacent or fused, however, no diagnostic information is gained.

Valuable information may be obtained by assessing the dividing membrane. In dichorionic pregnancies, the membrane contains four layers: two amnions and two chorions. In monochorionic diamniotic pregnancies, the membrane is composed of two layers: two amnions. In dichorionic placentas, a *twin peak sign* has been described in all three trimesters.[21] Placental tissue forms a triangular projection from the chorionic plate into a cleft between the membrane layers. By contrast, a T-shaped junction is present in monochorionic placentas.[22]

Membrane thickness also can be evaluated to help with the diagnosis of chorionicity.[23, 24] Because a dichorionic membrane is composed of four layers, it should appear thicker than a monochorionic membrane. Winn and associates[25] designated a 2-mm thickness as indicative of a thickened, dichorionic membrane. In 32 pregnancies, these workers found a 95% positive predictive rate for dichorionic pregnancies and an 82% predictive rate for monochorionic pregnancies.[25] Other studies have evaluated both membrane thickness and the number of membrane layers.[26] Examining membranes by ultrasound imaging in the second and third trimesters may be difficult and less accurate. Monochorionic membranes may appear thicker, depending on the ultrasound technique used, and dichorionic membranes can thin out as the pregnancy progresses.[20]

If a dividing membrane is not noted, careful evaluation must be performed to determine amnionicity. The differential diagnosis includes a monochorionic monoamniotic pregnancy versus a monochorionic diamniotic pregnancy, in which one twin has oligohydramnios, with an adherent membrane giving the illusion of monoamnionicity. Rare cases have been reported in which monochorionic diamniotic membranes rupture.[27] Nonetheless, in most cases, the pregnancy is a normal monochorionic diamniotic pregnancy, and the intertwin membrane is difficult to visualize.

Although early determination of chorionicity is a primary objective in the management of multifetal pregnancies, extenuating circumstances, such as late diagnosis of a multiple gestation or lack of conclusive findings on early ultrasound studies, may make this goal unattainable. The optimal management of certain obstetric situations, such as discordant anomalies, requires knowledge of chorionicity. DNA zygosity studies on amniocytes have been used successfully in complex cases requiring definitive diagnosis of chorionicity.[28]

MATERNAL PHYSIOLOGY AND MULTIPLE PREGNANCY

The maternal physiologic adaptation to singleton pregnancy is exaggerated in multiple gestations and involves every organ system. Although most current teachings on maternal physiology in multiple gestations derive from actual studies in the pregnant patient, some concepts have been extrapolated from knowledge regarding maternal physiology in singleton pregnancies.[29]

Uterine Changes

In both singleton and multiple pregnancies, uterine growth and change begin soon after conception. By 18 weeks, the intrauterine volume of a twin pregnancy is approximately twice that of a singleton pregnancy.[30] This difference in intrauterine volume

between singleton and multiple gestations continues until term. As should be appreciated, higher-order multiples have even greater intrauterine volumes.[31]

In singleton pregnancies at term, blood flow to the uterus is approximately 500 to 700 mL/minute. The increased blood flow is directly related to the increasing uterine size and results from a combination of the increased cardiac output of pregnancy and uterine demand. In multiple pregnancies, the increase in uterine blood flow is thought to be even greater.[31] A portion of the increased blood flow may be secondary to the decreased uterine and placental vascular resistance associated with multiple gestations. Doppler studies have indicated that the resistance in the uterine arteries is lower in twins than in singletons.[32]

Uterine activity appears to be increased in multiple pregnancies. After 23 weeks, women with twins report more contractions than those experienced by women with singleton pregnancies.[33] Uterine distention is one of many factors that may contribute to the onset of parturition.[34]

Cardiovascular Adaptation

The cardiovascular changes in multiple gestations are similar to those in singletons but are expressed to a greater degree.[35] The maternal heart size in twin pregnancies is the same as in singleton pregnancies. The cardiac output is increased, however, to compensate for increased uterine blood flow. As a result, contractility and heart rate also are increased.[35] An even greater drop in diastolic blood pressure in the second trimester and a greater rise during the third trimester have been documented in women pregnant with twins independent of age, race, or body habitus.[36] Blood pressure is dependent on maternal position, and postural changes can lead to hypotension. Because patients pregnant with twins have a greater decrease in mean arterial pressure, they seem to have be at greater risk for postural hypotension.[31]

Intravascular Volume

Pregnancy is known to be a hypervolemic state, and the increase in plasma volume at term averages approximately 45% to 50% in singletons.[37] Development of hypervolemia is thought to be a protective mechanism against excessive blood loss.[37] Plasma volume is increased even further in a multiple pregnancy.[38] Depending on the number of fetuses, plasma volume can increase by 50% to 100%.[39] As a result of this increased volume, total concentrations of serum proteins and electrolytes are reduced when compared with those in singleton pregnancies. Total intravascular protein mass is unchanged, however, as are serum sodium, potassium, and chloride concentrations and osmolality.[40]

These physiologic changes are of particular importance in addressing preterm labor and preeclampsia. Because of the slight drop in colloid oncotic pressure associated with β-mimetic medications, women with multiple gestations are at increased risk for volume overload and pulmonary edema when they are given this type of tocolysis.[37,41] Likewise, magnesium sulfate has been associated with fluid overload.[42] Other tocolytic agents (especially when used simultaneously) and antenatal steroids also have been associated with an increased risk for development of pulmonary edema.[43]

Although most young healthy women adapt to the cardiac and volume changes associated with multiple pregnancy, those with underlying medical problems and older women may be at increased risk for adverse outcomes such as myocardial infarction, particularly when they are treated for preterm labor and for preeclampsia.[35] For example, a woman with a multiple gestation and an underlying heart condition may be at increased risk for myocardial infarction because of the increased volume status combined with use of β-mimetic tocolytics or magnesium sulfate therapy.[31]

Pulmonary Adaptation

The changes in pulmonary function are similar in singleton and in multiple gestations. Such changes bring about a state of compensated respiratory alkalosis. The diaphragm level rises approximately 4 cm during a singleton pregnancy, the subcostal angle widens, and the thoracic circumference increases. Consequently, the residual volume decreases, and tidal volume increases. No increase in respiratory rate is noted, although the mother may have a sensation of tachypnea from increased respiratory effort.[44] In multiple gestations, the level of the diaphragm rises higher than in singleton gestations, a change that further reduces residual volume and functional residual capacity while increasing tidal volume.[40] Because of increased tidal volume and oxygen consumption, it is likely that women with multiple gestations have higher pH than women with singleton gestations.[39]

Hematologic Adaptation

Pregnancy is associated with dilutional anemia. Although placental production of chorionic somatomammotropin, progesterone, and prolactin stimulates erythropoiesis, only a small increase in red blood cell mass is seen.[37] The increased plasma volume of pregnancy, combined with the smaller increase in red blood cell production, results in maternal dilutional anemia despite adequate iron stores.[45] Anemia (characterized as a hemoglobin concentration lower than 10 g/dL or a hematocrit less than 30%) occurs 2.4 times more frequently in mothers of twins than in mothers of singletons.[46] Malone and partners[47] prospectively studied 55 women with triplet pregnancies at a tertiary care center and found a 27% incidence of anemia in these patients. Multiple gestations have been shown to increase erythropoietin production, perhaps as a physiologic mechanism to improve the anemia associated with pregnancy.[48]

Certain components of the coagulation system are elevated in pregnancy. Factor VII, VIII, IX, and X levels increase in pregnancy, whereas plasma fibrinolytic activity decreases. These alterations result in a hypercoagulable state until after the postpartum period. The changes are similar in both women with singleton pregnancies and women with multiple gestations.[44] Levels of plasminogen, fibrin split products, antithrombin III, and α_2-macroglobulin also are similar in both types of pregnancies.[49] Women with twin gestations, however, generally demonstrate a greater increase in plasma fibrinogen than that typical for women with singleton pregnancies.[31]

Women with multiple gestations are at increased risk for postpartum hemorrhage, which is defined as blood loss greater than 500 mL during the first 24 postpartum hours.[50] The risk for such hemorrhage is increased after vaginal and cesarean deliveries. The average blood loss at a twin delivery is approximately 1000 mL.[38] Uterine atony, secondary to overdistention of the uterus, is thought to be the main contributory pathophysiologic change.[8] In addition, women with multiple gestations also are at increased risk for placental abruption, which may result in increased blood loss at delivery.[46]

Endocrine Adaptation

Serum levels of progesterone, estradiol, estriol, human placental lactogen, human chorionic gonadotropin (hCG), α-fetoprotein, cortisol, aldosterone, and free thyroxine are increased in women with multiple gestations compared with levels in singleton pregnancies.[39] To date, it is not known whether carbohydrate metabolism is affected by multiple pregnancy.[46] It seems intuitive that women with multiple gestations would be at increased risk for gestational diabetes because of increased placental lactogen production; however, published studies have yielded conflicting findings. Spellacy and associates[46] evaluated 1253 women with twins

and did not find an increased risk for gestational diabetes. More recently, Malone and co-workers[47] found a 7% incidence of gestational diabetes in women pregnant with triplets compared with the 2% incidence in singleton pregnancies. These investigators suggested that the increased risk for gestational diabetes could be secondary to maternal age–related changes (many patients were older gravidas, with a mean age of 32 ± 2.9 years), the weight gain associated with multiple gestations, or the increased serum levels of human placental lactogen.[47]

Renal Adaptation

The normal increase in glomerular filtration rate and size of the renal collecting system is more marked in women with multiple gestations.[39] It is well known that kidney size is increased during pregnancy. Higher levels of progesterone or compression at the pelvic brim, secondary to overdistention of the uterus, may lead to increased stasis and an increased incidence of urinary tract infections.[44]

Gastrointestinal Adaptation

The enlarging uterus displaces the stomach and intestines during both singleton and multifetal pregnancies. Increased progesterone levels decrease gastrointestinal tract motility and tone while delaying gastric emptying time. As a result, pregnant women commonly suffer from constipation. The esophageal sphincter has decreased tone, resulting in acid reflux. Hemorrhoids also are common, being secondary to the venous compression and pressure from the enlarged uterus.[44] In addition, nausea and vomiting complicate almost 50% of multiple gestations, probably the result of the higher levels of β-hCG and steroid hormones.[51]

Patients with multiples are at increased risk for acute fatty liver of pregnancy, a rare condition of unknown origin resulting in elevated values on liver function tests, nausea, and vomiting.[52] If undiagnosed, this condition is associated with poor outcomes including coma, liver failure, disseminated intravascular coagulopathy, renal failure, metabolic acidosis, and fetal death. Even if it is diagnosed early, maternal mortality rates are increased (8% to 10%).[53] Treatment includes supportive care and delivery.[39]

The incidence of acute fatty liver is 1 per 13,000 in the general obstetric population.[54] Four series of cases have shown a 16.7% occurrence in twins.[31] Meanwhile, in the series of 55 triplet pregnancies evaluated by Malone and co-workers,[47] the disease was present in 7% of patients. An association between this disease and an autosomal recessive order of mitochondrial fatty acid oxidation in the children of affected women has been postulated.[55-58] When patients with multiple gestations demonstrate elevated values on liver function testing in combination with vague complaints of nausea and vomiting, a high level of clinical suspicion is waranted in order to diagnose this potentially catastrophic disease.[52]

Nutrition

Weight gain is a physiologic response to pregnancy. The average woman carrying twins gains between 40 and 45 pounds.[59] Few studies have examined weight gain in triplet and higher-order multiples.[3] The pattern of weight and the amount of weight gain appear to be different in twin pregnancies from those in singleton pregnancies. For example, weight gain occurs faster and earlier in women with multiple gestations.[3] Weight gain and pattern of gain seem to affect pregnancy outcomes.[60] Poor weight gain in early pregnancy (less than 0.85 lb/week) has been associated with poor intrauterine growth.[3] By contrast, large weight gains have been associated with greater birth weights.[3] Nutritional counseling may lead to improved weight gain in women pregnant with twins.[60] Whether better weight gain translates into better perinatal outcomes is yet to be determined.

It is recommended that women with multifetal pregnancies increase their daily caloric intake approximately 300 kcal more than that of women with singleton pregnancies.[61] Iron and folic acid supplementation also is recommended.[62]

Gestational Hypertension and Preeclampsia

The risk for developing pregnancy-induced hypertension or preeclampsia is between 10% and 20% in twin pregnancies and between 25% and 60% in triplet pregnancies, and it may be as high as 90% in quadruplet pregnancies.[39] The cause of these increased rates is unknown, but the disorder may be secondary to high levels of circulating β-hCG.[63] The rates of preeclampsia seem to be the same for monozygous and for dizygous twins.[64] Eclampsia frequency also seems to be increased in multiple pregnancies.[63] In addition, when preeclampsia occurs in higher-order multiple gestations, it seems to occur earlier and is more severe, and the clinical presentation often is atypical.[63] Because of the increased risk for preeclampsia, women with multiple gestations require frequent monitoring for early signs and symptoms.[50]

FETAL PHYSIOLOGY AND MULTIPLE PREGNANCIES

Multiple gestations contribute disproportionately to overall perinatal morbidity and mortality. These pregnancies carry an increased risk for both preterm birth and low birth weight.

Perinatal outcome is influenced by chorionicity, because dichorionic pregnancies have substantially better outcomes than those documented for monochorionic ones.[39] The effect of zygosity on outcomes, however, is less clear.[66]

The offspring of multiple births constitute less than 3% of the U.S. population but are highly represented among infants of low birth weight (less than 2500 g) or very low birth weight (less than 1500 g).[3] Ninety percent of triplets and 50% of twins are of low birth weight, compared with 6% of singletons. Preterm birth (before 37 weeks of gestation) occurs in 88% of triplets and in 48% of twins, compared with 11% of singletons. Birth before 32 weeks of gestation occurs in 31% of triplets, 11% of twins, and less than 2% of singletons.[3] More important, the incidence of extreme prematurity in multiple gestations is significant. For triplets, the risk for delivery before 28 weeks may be as high as 14%.[67]

Various interventions have been attempted to decrease the rates of preterm delivery in women with multiple gestations, such as home uterine monitoring, voluntary bed rest, prophylactic tocolytics, and elective cerclage.[1,68-70] Nevertheless, no intervention has proved to be successful.[1] Predictors for preterm delivery have been identified. At 24 weeks, a cervical length of 25 mm or less is a predictor of spontaneous preterm delivery before 32 weeks. Likewise, a positive fetal fibronectin study at 28 weeks also is indicative of spontaneous preterm birth (at less than 32 weeks).[71] If delivery between 24 and 34 weeks is anticipated, maternal corticosteroids are recommended (Figure 19-4).

Fetal Growth in Multiple Pregnancy

Normal Growth

As suggested earlier, multiple gestations are associated with increased risk for low birth weight.[72] Part of this increased risk results from preterm labor and delivery, and a portion of this risk is secondary to complications inherent to multiple gestations. Serial ultrasound imaging is the best method for evaluating fetal growth in a multiple pregnancy, and the biometric parameters used to assess singletons are accurate in multiples.[39] Although individual growth curves for twins and triplets have been described, singleton weight standards generally are used for assessing weight in twins and higher-order multiple births.[39,73]

In twins, each fetus grows at the same rate as for singletons up to approximately 32 weeks of gestation. After this gestational age,

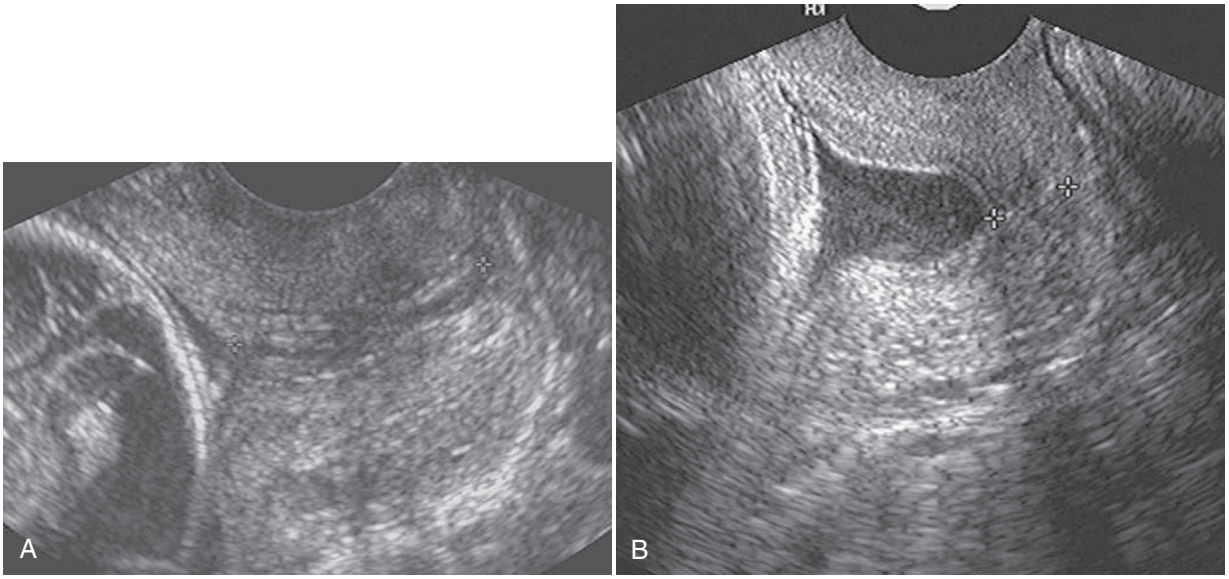


Figure 19-4 A, Normal cervix. **B,** Shortened cervix.

twins do not gain weight as rapidly as singletons.[74] As a result, multiple gestations are at increased risk for poorer growth compared with age-matched singletons. After approximately 32 weeks, the combined weight gain in twins is the same as in a singleton at the same gestational age.[75] Nonetheless, values for head circumference and body length generally are similar. Ultrasound imaging has demonstrated that twin femur length growth patterns are similar to those of singletons throughout gestation, but a reduction in abdominal circumference occurs at approximately 32 weeks.[76-79] In higher-order multiple pregnancies, this weight difference seems to become evident at even earlier gestational ages.[80]

Discordant Growth

Multiple gestations exhibit a significant increase in fetal growth abnormalities directly proportional to the number of fetuses.[81] *Growth discordancy* is the difference in sonographic estimated fetal weight expressed as a percentage in relation to the larger twin.[81] Various definitions of fetal growth discordancy have been proposed, with specific weight differences ranging between 15% and 40%.[81] However, adverse outcomes generally are related to discrepancies of 25% and greater. Discordancy has been associated with a 6.5-fold increased risk for fetal death and a 2.5-fold increased risk for perinatal death.[82] The more severe the discordance, the greater the likelihood of fetal compromise such as perinatal asphyxia.[81]

Growth discordancy has different implications depending on chorionicity.[83] Dizygotic twins are distinct individuals that exhibit genetically different growth patterns. It is not surprising, therefore, that these twins may have divergent birth weights. Nonetheless, several pathologic conditions result in discordant weight gain in these fetuses, including in utero crowding, unequal sharing of the placenta, and the combination of a normal fetus with an anomalous fetus. Situations unique to monochorionic twinning, such as TTTS, also can result in growth discordancy.[81]

Two of the most frequent findings in severely discordant twins include small placental weight and umbilical cord abnormalities.[84] For example, velamentous cord insertion—umbilical cord inserting directly into the membranes—occurs 9 to 10 times more frequently in twin gestations than in singletons and 25 to 50 times more frequently in triplets than in singletons.[85] A 13-fold increase in birth weight discordancy has been noted in monoamniotic twins with a velamentous umbilical cord insertion[83] (Figure 19-5).

In addition to growth discordancy, multiple gestations are associated with increased risk for intrauterine growth restriction. Uteroplacental insufficiency is thought to be the main cause of growth restriction in multiple gestations. Concordant growth in twins does not rule out intrauterine growth restriction, because in certain situations, both twins may exhibit growth restriction.[80] Management of both growth discordancy and intrauterine growth restriction includes intensive antepartum surveillance with delivery at term, provided that results of fetal testing are reassuring. Antenatal corticosteroids should be administered to the mother if delivery is anticipated between 24 and 34 weeks, to decrease complications of prematurity.

Multifetal Reduction

The purpose of first trimester multifetal reduction is to improve outcomes by decreasing maternal complications secondary to multiple gestations and by decreasing adverse fetal outcomes associated with preterm delivery.[39] Intracardiac injection with potassium chloride is performed between 10 and 13 weeks of gestation in dichorionic pregnancies.[86] This procedure is contraindicated in monochorionic pregnancies because vascular communications between the fetuses can result in long-term adverse sequelae for the surviving fetus.[87] Adverse outcomes, such as pregnancy loss, are thought to be decreased with provider experience.[88] In addition, it is controversial whether or not the procedure is associated with adverse outcomes, such as intrauterine growth restriction.[89]

Stone and colleagues[90] reported on a series of 1000 cases of multifetal reduction. In each case, multifetal reduction was performed transabdominally at 10 to 13 weeks of gestation. This series demonstrated a loss rate of 6.2%. Although the loss rate was highest among patients who originally were carrying six or more fetuses, the rate did not differ for starting numbers of three, four, or five fetuses. Birth weights demonstrated a linear decline with increasing starting and finishing numbers of fetuses. Gestational ages at delivery for finishing numbers of one, two, and three fetuses were similar to those for spontaneous singletons, twins, and triplets. The same group demonstrated improved outcomes when twin pregnancies were reduced to singleton pregnancies.

Stone and colleagues[91] compared 101 women with twin pregnancies reduced to singleton pregnancies with 365 patients with triplet reduced to twin pregnancies. These investigators found

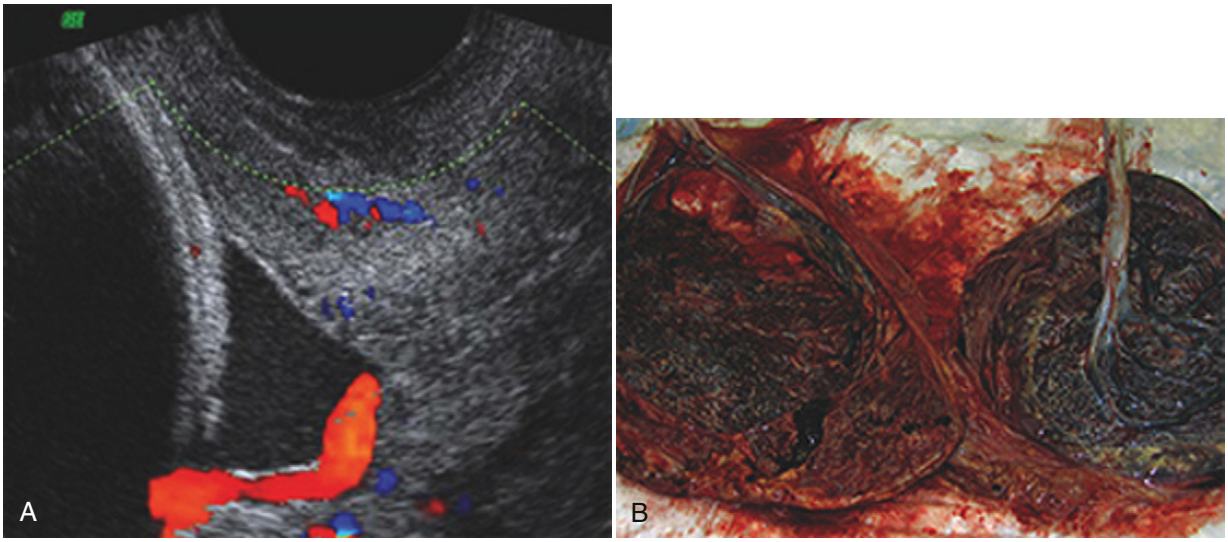


Figure 19-5 Increased risk for placental abnormalities associated with multiple pregnancies. A, Velamentous cord insertion with vasa previa as demonstrated by color Doppler ultrasound imaging at 26 weeks. **B,** Twin placenta at 34 weeks with velamentous cord insertion and vasa previa. *(Courtesy Carnegie Hill Imaging for Women, New York.)*

a threefold decrease in the spontaneous loss rate in the group reduced to singleton pregnancies compared with the group reduced to twin pregnancies. Reduction to singleton pregnancy also was associated with statistically significant increased birth weight and gestational age at delivery. In addition, cesarean delivery rates were decreased in the patients undergoing reduction to singleton pregnancies.

Multifetal pregnancy reduction is an ethical dilemma.[92] The starting number of fetuses needed to justify the procedure is controversial. Various studies have yielded conflicting findings regarding whether multifetal reduction from triplets to twins results in improved perinatal outcomes over those achieved with expectant management.[93-95] Triplet and higher-order multiple pregnancies have been associated with increased costs derived from antepartum hospital stays, missed work hours, and neonatal intensive care in the postpartum period. Multifetal pregnancy reduction has the potential to reduce such costs.[96] Nonetheless, some women will experience grief after their decision to reduce.[97]

Fetal Lung Maturity in Multiple Gestation

At this point, consensus is lacking on whether pulmonary maturation differs between singleton and multiple pregnancies. In one study of singleton and twin pregnancies, after 31 weeks of gestation, twins exhibited higher values on the TDx-FLM ("twin diagnosis"–fetal lung maturity) fluorescence polarization assay (Abbott Laboratories, Abbott Park, Illinois) than those measured for singletons.[98] The reason for the apparent acceleration of changes in pulmonary maturity indices in twin gestations is unknown. Likewise, it is not known whether this difference may reflect an increased risk for respiratory distress syndrome associated with multiple pregnancies. If age-specific risks of developing respiratory distress syndrome are similar for twins and for singletons, new guidelines will be needed to avoid false-positive prediction of adequate lung maturity in twins.[98]

For assessing lung maturity in multiple gestations, a debate is ongoing regarding whether all amniotic sacs or just one sac should be tapped. Some evidence indicates that in certain situations, one fetus may attain pulmonary maturity before the others.[80] Two series found lecithin-sphingomyelin ratios to be similar in 34 sets of twins.[99,100] In another study in 8 sets of twins (6 of 8 twins were delivered vaginally), a significant difference was noted in phospholipid profiles for tracheal aspirates obtained in the postpartum period.[101] Other studies have shown a significant discrepancy between lecithin-sphingomyelin ratios in the presenting fetus of a multiple pregnancy and the other fetuses irrespective of delivery mode.[102,103]

Fetal Pathophysiology and Multiple Pregnancy

Pathophysiology Specific to Multifetal Gestations

Certain pathophysiologic situations are unique to multiple pregnancies. These include early vanishing twin, fetus papyraceus, monochorionic monoamniotic twins, conjoined twins, TTTS, and twin reversed arterial perfusion (TRAP).

Vanishing Twin

The incidence of early pregnancy loss in multiple gestations is higher than was initially thought.[44] The routine use of ultrasound monitoring has shown that early fetal wastage is common in multiple gestations. In patients with twin gestations scanned in the first trimester, rates of demise range from 13% to 78%.[104] This early disappearance of a twin has been termed the *vanishing twin phenomenon.* Explanations include physiologic resorption, misinterpretation of artifact, and sonographic error.[44] Although this phenomenon may be associated with first-trimester bleeding and spotting, it has not been associated with adverse pregnancy outcomes.[80]

Fetus Papyraceus

Fetus papyraceus, also known as fetus compressus, results when one twin dies at approximately 15 to 20 weeks. The dead fetus loses all water content and becomes compressed with continued development of the surviving twin. Such pregnancies rarely enter birth statistics. Thus, along with the vanishing twin phenomenon, fetus papyraceus may contribute to underestimation of the incidence of twinning.[7]

Monoamniotic Twins

Monoamniotic twins are a rare form of monozygous twins in which both fetuses occupy the same sac. The diagnosis is made by ultrasound imaging. No amniotic membrane is visualized in a same-sex twin pregnancy with one placental mass

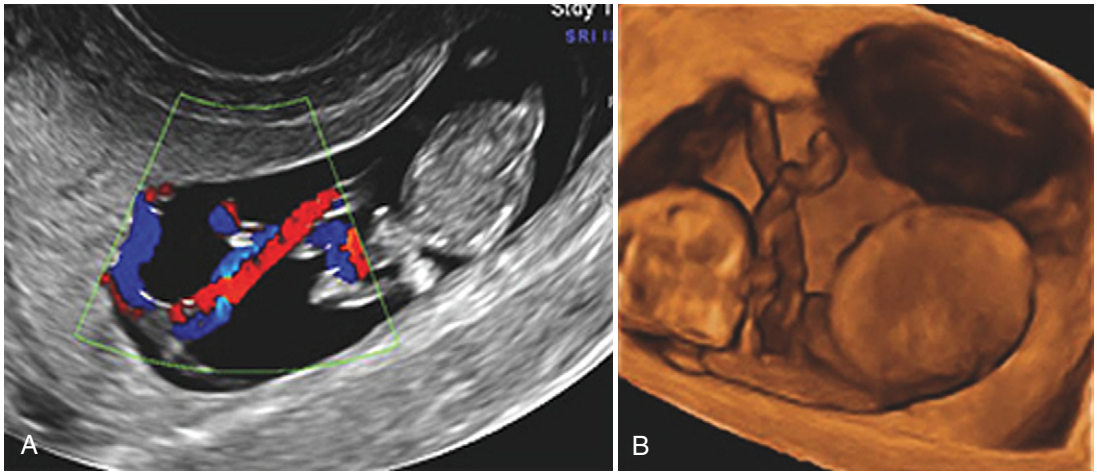


Figure 19-6 **A,** Two-dimensional ultrasound imaging with color Doppler demonstrating cord entanglement in a monoamniotic twin pair at 10 weeks. **B,** Three-dimensional image from the same patient. *(Courtesy Carnegie Hill Imaging for Women, New York.)*

(see Figure 19-8). Unexplained rupture of a dividing membrane also has been observed. However, these pregnancies should be managed as for true monoamniotic twins, because they have been found to have similar outcomes.[27] The diagnosis of monoamniotic twins has been confirmed by observing cord entanglement[105] (Figures 19-6 and 19-7).

Monoamniotic twins are associated with a high perinatal mortality rate. Past studies indicated fetal mortality rates ranging from 30% to 68%.[106-108] Monoamniotic pregnancies are associated with preterm delivery, growth restriction, vascular anastomosis, congenital anomalies, cord entanglement, and cord accidents.[109] The management of these pregnancies has been controversial, particularly regarding the optimal timing of delivery and protocol for antenatal surveillance.[108,110,111] Delivery generally is performed by cesarean section. Although vaginal delivery is not contraindicated, it should be undertaken with caution.[112] Inadvertent severing of the second twin's umbilical cord at delivery of the presenting twin has been reported.[113]

Data suggest better outcomes with intensive surveillance. In a study by House and colleagues,[114] 22 patients were managed at a tertiary care center over a 10-year period. Management included close fetal surveillance with daily testing starting at 24 to 26 weeks of gestation. Antenatal corticosteroids were routinely used, and elective delivery was performed at 34 to 35 weeks, provided that the results of testing were reassuring. No fetal deaths occurred in this group of patients. Nevertheless, monoamniotic twins were more likely to be born prematurely and had more respiratory problems compared with monochorionic diamniotic and dichorionic diamniotic pregnancies.

Conjoined Twins

Conjoined twins are an extremely rare form of monoamniotic twins, occurring in 1 in 50,000 pregnancies.[6] The diagnosis is made ultrasonographically. If the diagnosis is suspected early in the first trimester, a follow-up scan is needed for confirmation, because an incorrect diagnosis is possible at early gestational ages.[115]

Conjoined twins may be joined at various anatomic locations. Five distinct types have been recognized.[39] The first type is thoracopagus, accounting for 75% of conjoined twins. The fetuses face each other and usually have a common sternum, diaphragm, upper abdominal wall, liver, pericardium, and gastrointestinal tract.[116] The second type is omphalopagus, also known as xiphopagus, which is a rare form of thoracophagus, in which the abdominal wall is connected and the fetuses have a common liver. The third type, pygopagus, accounts for 20% of the cases

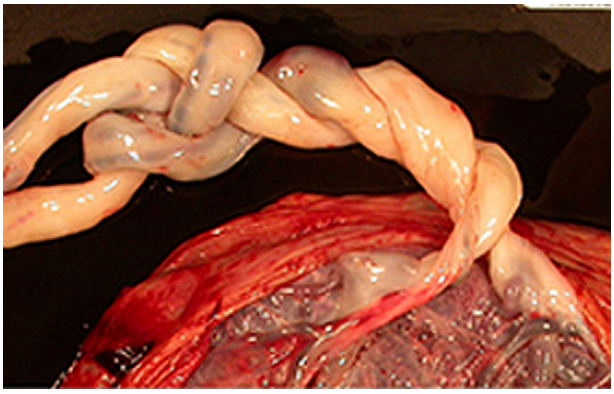


Figure 19-7 Entangled cords in a monoamniotic twin pregnancy at delivery. *(Courtesy Carnegie Hill Imaging for Women, New York.)*

of conjoined twins. The fetuses face away from one another and share a sacrum, bladder, and rectum.[116] In the fourth type, ischiopagus, the twins share a bony pelvis. This type accounts for 5% of the cases.[116] Finally, the fifth type, craniopagus (cephalopagus), is classified by partial or complete fusion of the skull, meninges, and vascular structures and accounts for 1% to 2% of the cases.[115]

If conjoined twins are diagnosed early, parents should be counseled about the option for termination of the pregnancy. Depending on the type of conjoined twin and the gestational age, this procedure can be performed by dilatation and curettage, dilatation and evacuation, induction, or hysterotomy.[115] If expectant management is desired, care should be transferred to a referral center. Generally, delivery should be performed by cesarean section as near to term as possible, with a multidisciplinary team approach.[115] Vaginal delivery is possible in selected cases of extreme prematurity or if survival is improbable. Successful separation of the twins depends on the degree of organ and vascular sharing between the two fetuses.[117]

Twin-to-Twin Transfusion Syndrome

TTTS is a complication of monochorionic pregnancies characterized by an imbalance in the blood flow across a shared placenta of two fetuses. The net effect of this imbalance in fetal support is marked developmental discrepancy, with a large, hyperperfused recipient twin and a small, hypoperfused and anemic donor twin. If the condition goes untreated, the prognosis is poor, with a 60%

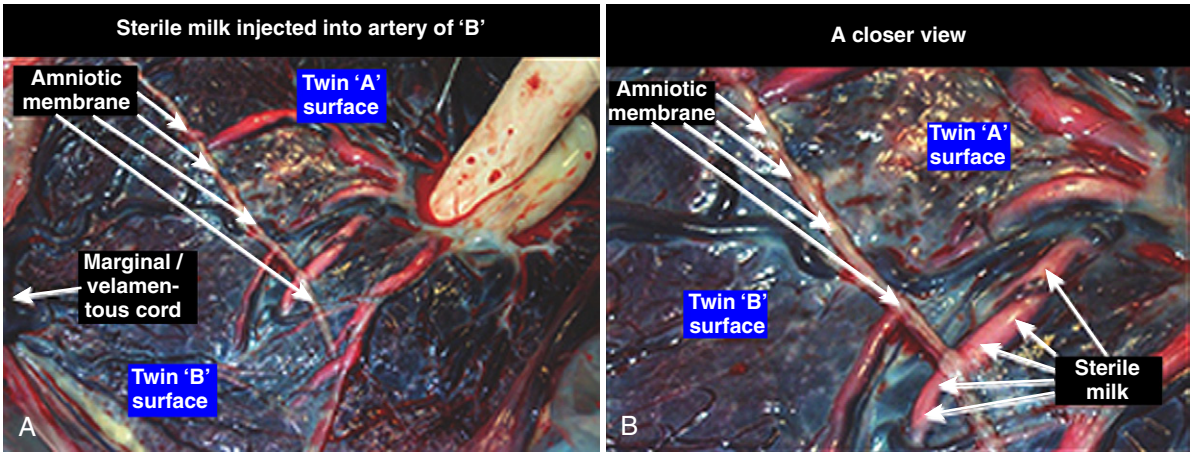


Figure 19-8 **A** and **B,** Placental anastomoses in twin-to-twin transfusion syndrome (TTTS). *(Courtesy Carnegie Hill Imaging for Women, New York.)*

to 100% mortality rate for both twins.[118-120] When one twin dies in utero, the surviving twin is at risk for severe neurologic damage. The true incidence of TTTS is unknown, because many of these pregnancies result in early loss.[116]

Although all monochorionic twins share a portion of their vasculature, TTTS will develop in only 5% to 15%.[2] TTTS also has been described in dichorionic pregnancies in a few case reports.[121,122] Although the exact origin of the condition is not known, Bajoria and colleagues[123] proposed the following mechanism. In monochorionic placentas, three types of vascular communications are possible: arteriovenous communications, arterioarterial communications, and venovenous communications. One cotyledon near the dividing membrane may receive arterial blood supply from one fetus yet may drain directly by a vein to the other side. The arteriovenous shunt is usually deep in the placenta and is unidirectional. Superficial arterioarterial and venovenous connections are crucial for maintaining the bidirectional flow.[115] According to the theory proposed by Bajoria's group,[123] TTTS results from absence of the superficial anastomoses that maintain balanced blood flow (Figure 19-8).

Diagnosis of TTTS is made by ultrasound imaging. The four requirements for the diagnosis of TTTS are (1) the presence of a single placenta, (2) same-gender fetuses, (3) weight discordance of greater than 20%, and (4) significant amniotic fluid discordance, often with a "stuck twin."[115] The recipient twin may exhibit signs of heart failure and hydrops. On echocardiography, the recipient twin may have decreased ventricular function, tricuspid regurgitation, and cardiomegaly.[124] Doppler studies may show abnormalities in both twins. A staging system has evolved for the purpose of describing this disease progression: In stage I, the bladder of the donor twin is still visible; in stage II, the donor twin bladder is no longer visible; stage III is characterized by abnormalities on Doppler studies; stage IV, by presence of hydrops; and stage V, by IUFD[125] (Figure 19-9).

Weight and hematologic discordances are no longer considered diagnostic of TTTS.[116] Likewise, a stuck twin is not pathognomonic for TTTS because it has been observed in cases of structural fetal anomalies, congenital infection, chromosomal abnormalities, and premature rupture of membranes.[116] The differential diagnosis for TTTS includes abnormal cord insertion or uteroplacental insufficiency with poor fetal growth and decreased urine output in one twin and fetal anomalies, such as bladder obstruction and aneuploidy.[115]

Because of the poor outcomes associated with this disorder, expectant management is rarely employed. Successful medical management with use of digoxin has been described.[126] Use of indomethacin is no longer recommended after reports of associated fetal demise.[127,128]

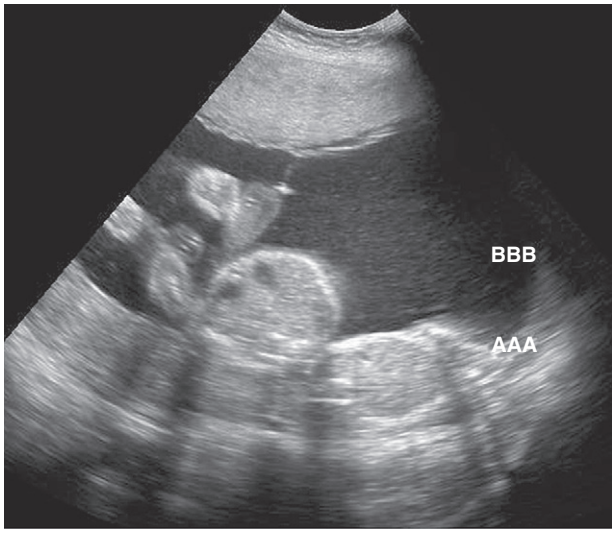


Figure 19-9 "Stuck twin" in multiple gestation complicated by twin-to-twin transfusion syndrome (TTTS). The two fetuses are labeled *AAA* and *BBB*. *(Courtesy Carnegie Hill Imaging for Women, New York.)*

One management option for TTTS is serial reduction amniocentesis.[129-136] Under ultrasound guidance, an 18-gauge spinal needle is placed into the sac with polyhydramnios. Fluid is removed until the amniotic fluid level returns to normal (provided that the patient can tolerate the procedure). This procedure is repeated as often as necessary to maintain a normal amniotic fluid volume. The mechanism by which this procedure restores the amniotic fluid balance is unknown. It is possible that removing fluid from the sac with polyhydramnios results in decreased pressure on the sac with oligohydramnios, which may result in increased perfusion to the stuck twin, with consequent improved amniotic fluid volume for that twin.[115]

Amniotic septostomy is another management option.[137] In this procedure, a 20-gauge spinal needle is inserted through the dividing membrane under ultrasound guidance. The amniotic fluid equilibrates across the two sacs. As in serial amnioreduction, it is uncertain how this technique improves outcomes.[115]

According to one proposed mechanism, the donor swallows a large fluid volume, resulting in restoration of circulating blood volume and better perfusion. Studies regarding the effectiveness of this treatment technique have been conflicting. In one report of 12 cases, pregnancy was prolonged by 8 weeks, with an 83% survival rate.[138] In a second study comprising 3 cases, all of the pregnancies were lost in 5 days as a result of preterm premature rupture of membranes.[139]

Serial reduction amniocentesis and amniotic septostomy do not affect the placental anastomoses. As a result, neither of these techniques is thought to prevent the neurologic complications associated with this disease entity.[115] Laser ablation of placental anastomoses is a treatment that may correct TTTS and reduce the neurologic complications associated with these pregnancies.[126,132,140-143] In this procedure, a fetoscope is placed in the sac with polyhydramnios under ultrasound guidance, and the anatomy of the placental vessels is studied. An arteriovenous shunt is diagnosed by identifying an artery supplying a cotyledon without a vein returning on the same side of the dividing membrane. A vein may be seen on the other side of the dividing membrane draining the cotyledon of the second fetus. A 0.4-mm neodymium:yttrium-aluminum-garnet laser fiber is then placed in the fetoscope and used to ablate these vessels. A reduction amniocentesis is simultaneously performed. A multicenter trial is currently in progress.

Until this trial is completed, antenatal management includes obtaining serial ultrasound scans with assessment of cardiac function, amniotic fluid, and weight discordancy. Treatment should be tailored to each clinical situation; however, reduction amniocentesis may be performed as the first therapeutic intervention. For patients with severe and early-onset TTTS, referral to a center performing laser therapy may be warranted. Antenatal corticosteroids should be administered to the mother if delivery is anticipated between 24 and 34 weeks. If either fetus shows compromise between 32 to 34 weeks, immediate delivery should be considered.[115]

Twin Reversed Arterial Perfusion Syndrome

TRAP, also known as acardia, is defined by the absence of a normally functioning heart in one fetus of a multiple pregnancy. The incidence is estimated to be 1% of monozygotic twin pregnancies.[109] The true incidence is unknown, because many cases of TRAP may result in early pregnancy loss.[116] The normal fetus perfuses the acardiac twin by an umbilical artery–to–umbilical artery anastomosis at the placental surface. Reversal of blood flow in the umbilical artery of the recipient twin is characteristic, and deoxygenated blood is brought from the pump twin to the acardiac twin. As a result, the gestation includes a normal twin and an amorphous twin. A range of anomalies can be seen in the acardiac twin, including anencephaly, absent limbs, intestinal atresia, abdominal wall defects, and absent organs.[144] As a consequence of the increased cardiac workload, heart failure can develop in the normal twin.[39]

Ultrasound imaging is used for diagnosis, and the differential diagnosis of acardia includes IUFD and anencephaly in one twin.[116] The cause is thought to be the development of arterial-to-arterial vascular anastomoses between the umbilical arteries of twins in early embryogenesis.[116] Seventy-four percent of affected gestations are monochorionic diamniotic, and 24% are monochorionic monoamniotic. The pump twin generally is morphologically normal, but approximately 9% of these twins are found to have trisomy. Thirty-three percent of acardiac twins have an abnormal karyotype, including monosomy, trisomy, deletions, mosaicism, and polyploidy.[145]

The goal of management is to optimize the outcome for the normal twin. Poor prognosis has been associated with polyhydramnios and congestive heart failure in the pump twin. Prognosis for the pump twin depends on the ratio of the weight of the perfused twin to the weight of the pump twin. Standard biometric measurements cannot be used to measure the acardiac twin. As a result, a second-order regression equation has been devised[146]:

$$\text{Weight in grams} = (-1.66 \times \text{length}) + (1.21 \times \text{length}^2)$$

When the twin weight ratio is greater than 0.70, the risk for congestive heart failure is 30% for the pump twin.[39]

In the absence of indicators of poor prognosis (congestive heart failure, polyhydramnios, and a twin weight ratio greater than 0.70), expectant management with serial ultrasound imaging is suggested.[109] Antenatal corticosteroids for the mother are advised if delivery is anticipated between 24 and 34 weeks, and tocolysis is suggested to stop preterm labor.[116] Medical management with indomethacin and digoxin has been used.[147,148] More invasive management has included hysterotomy of the acardiac twin followed by interval delivery of the pump twin.[149,150]

Percutaneous interruption of the circulation in the acardiac twin also has been described. Procedures have included insertion of a thrombogenic coil into the recipient twin's umbilical cord, endoscopic ligation of the cord, injection of silk soaked in alcohol into the cord, injection of absolute alcohol into the cord, and radiofrequency cord ablation.[151-160] These methods usually are reserved for when cardiac failure has already developed in the pump twin before a viable age has been attained.[39]

Pathophysiology With Special Considerations in Multiple Pregnancy

Intrauterine Fetal Demise of One Twin

Early loss of one fetus during the first trimester is common and appears to have no adverse impact on the surviving fetus or fetuses. IUFD of one fetus in the second or third trimester is less common, occurring in 2.6% of twin pregnancies and in 4.3% of triplet pregnancies.[161] This phenomenon can result in morbidity for the surviving cotwin resulting from preterm delivery.[109] The mean gestational age at delivery is 30.6 weeks for monochorionic pregnancies and 32.9 weeks for dichorionic pregnancies.[109,162]

Neurologic morbidity may be increased in monochorionic twins after IUFD of one twin.[109] Studies report a 12% to 20% risk for multicystic encephalomalacia in the twin survivor of a monochorionic pregnancy.[109,163] The proposed mechanism is a hypotensive event at the time of death resulting in acute blood loss into the dying twin, rather than thromboembolic arterial occlusion.[164-167] Neurologic morbidity has been reported after demise of the twin as early as the end of the first trimester.[109]

Management of IUFD in one twin depends on the gestational age and the chorionicity of the pregnancy. In a monochorionic pregnancy, the risk of neurologic handicap may be present from the moment of the twin's demise. As a result, immediate delivery after the demise of one twin may not prevent an adverse outcome in the surviving twin. Management recommendations include close fetal surveillance and delivery at 37 weeks (or when lung maturity has been established). Of note, multicystic encephalomalacia may not be evident on ultrasound imaging or from fetal heart rate tracing.[39] Antenatal corticosteroids should be given to the mother if delivery is expected to occur between 24 and 34 weeks. For maternal or fetal indications, delivery should occur before 37 weeks.[39]

A theoretical risk for maternal coagulopathy arises when IUFD occurs in one twin and delivery of the surviving twin is delayed.[168] In two series of twin gestations complicated by a single intrauterine demise, coagulopathy did not develop in any of the mothers; only two patients demonstrated transient biochemical evidence of coagulopathy.[162,169]

Congenital Anomalies

Congenital anomalies appear to be more common in multiple than in singleton gestations.[170] Discordancy of structural anomalies can occur in both monochorionic and dichorionic pregnancies.[39]

When a multiple gestation is complicated by a fetal anomaly in one fetus, the pregnant patient has three options: (1) expectant management and, depending on the gestational age at diagnosis, either (2) termination of the entire pregnancy or (3) selective termination of the anomalous fetus.[171] Counseling must take into account the preferences of the patient and her family, as well as the chorionicity of the pregnancy, the severity of the anomaly, and the effect the anomaly will have on the normal cotwin.[172]

If a patient elects expectant management, the patient and the family should be counseled about possible outcomes. Malone and colleagues[171] studied 14 twin pregnancies complicated by an anomalous fetus over a 5-year period at one tertiary care center and compared their findings with data on 78 normal twin pregnancies from the same center during a 2-year period. The obstetric management approach was essentially the same for all patients. Gestational age at delivery and birth weight were significantly lower in the study group. The mean gestational age at delivery for the study group was 33.6 weeks, whereas it was 35.6 weeks for the control subjects. The risk for delivering at 36 weeks or less was 78.6% for the anomalous twin group, compared with 59% for the normal twin group. Likewise, the mean birth weight for the study group was 1864 g, versus 2318 g. The lower birth weights were the result of the higher incidence of prematurity in the study group. The study infants also appeared to be at increased risk for cesarean delivery. No difference in perinatal mortality between the two groups was noted.[171] Thus it is important to counsel patients that conservative management can result in an adverse outcome for the healthy twin.[172]

Some patients may opt for selective termination of the pregnancy after counseling. In dichorionic pregnancies, ultrasound-guided intracardiac injection of potassium chloride is used. Eddelman and colleagues[173] reported favorable outcomes in 200 cases, including 164 twins, 32 triplets, and 4 quadruplets. The average gestational age at time of the procedure was 19⅔ weeks, with a range of 12 to 23⅘ weeks. The reasons for selective reduction included chromosomal abnormalities, structural abnormalities, mendelian disorders, placental insufficiency, and cervical incompetence. The unintended pregnancy loss rate was 4%. The loss rate was almost five times higher in triplets than in twins. The average gestational age at delivery was 36⅕ weeks. In approximately 84% of the cases, delivery was after 32 weeks of gestation.[173] According to this study, selective termination may result in good outcomes for the surviving twin.

In monochorionic twins, selective termination is more challenging. Complete ablation of the umbilical cord of the anomalous fetus is needed to avoid neurologic morbidity in the remaining normal fetus. Techniques used have included fetoscopic cord ligation, fetoscopic laser ablation of umbilical vessels, percutaneous injection of alcohol or occlusive materials into the umbilical cord or into the umbilical vein, and radiofrequency cord ablation.[154,155,157-159,174,175]

Chromosomal Anomalies

Most known chromosomal anomalies have been reported in multiple gestations.[39] Both monozygotic and dizygotic twins can be discordant for these anomalies. In dizygotic pregnancies, the maternal age–related risk for chromosomal abnormalities for each twin may be the same as in a singleton pregnancy.[39] It has been suggested that the chance that at least one fetus will be affected by a chromosomal defect is twice as high as in a singleton. In monozygotic twins, the risk for a chromosomal abnormality is the same as for a singleton, and in most cases, both fetuses are affected. These patients are offered genetic testing if they are 35 years of age or older. As previously stated, cases of monozygotic twins discordant for abnormalities of autosomal or sex chromosomes resulting from postzygotic nondisjunction have been reported. In the most common situation, one fetus has Turner syndrome and the other fetus has either a normal male or a normal female phenotype with a mosaic karyotype.[80]

In light of the increased risk for both genetic and structural abnormalities, patients with multiple gestations should be offered both genetic screening and prenatal diagnosis. In the first trimester, ultrasound nuchal translucency can be used to help determine whether a patient's offspring is at risk for having a genetic or structural abnormality.[39] At our institution, second-trimester screening for Down syndrome and for open neural tube defects with maternal serum α-fetoprotein, hCG, and unconjugated estriol generally is not performed, because these tests are not as reliable for multiple gestations.[39] If invasive testing such as amniocentesis or chorionic villus sampling is used, all fetuses should be tested, because of the possibility of discordancy for genetic abnormalities despite monozygosity.[39]

SUMMARY

Multiple pregnancy has a physiologic impact on both the mother and the fetus. A multidisciplinary approach may be needed for the management of these patients. The mother is at increased risk for adverse outcomes such as anemia, gestational diabetes, gestational hypertension, placental abnormalities, preterm delivery, need for cesarean delivery, and postpartum hemorrhage. The fetus is at increased risk for anatomic and genetic anomalies, growth abnormalities, and prematurity and for several problems related to monochoronicity.

Fetal outcomes are dependent on gestational age at delivery and on chorionicity. Adverse outcomes are more commonly associated with monochorionicity. Ultrasound imaging is the cornerstone of management in these patients. It is critical for diagnosis of chorionicity and structural abnormalities, as well as for surveillance of fetal growth and well-being. To date, no strategies have proved useful to prevent adverse outcomes in these pregnancies. However, antenatal corticosteroids should be administered if preterm delivery is anticipated. Furthermore, patients with complex issues unique to multiple gestations should be referred to a tertiary care center with perinatologists, neonatalogists, and pediatric specialists proficient at caring for such patients.

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SECTION III

Developmental Pharmacology and Pharmacokinetics

20

Sylvain Chemtob

Basic Pharmacologic Principles

PRINCIPLES OF DRUG ABSORPTION, BIOAVAILABILITY, AND DISTRIBUTION

Drug Absorption

A majority of drugs administered to the premature newborn are injected using the intravenous route and therefore are not affected by factors that govern systemic absorption. Some agents are administered intramuscularly (e.g., vitamin K), given by an enteral route (e.g., thiazides, caffeine), or applied topically (e.g., topical antiseptics and anesthetics, nitric oxide, bronchodilators). Regardless of the route of administration, drugs often must cross cell membranes to reach their sites of action. Therefore the mechanisms governing the passage of drugs across cell membranes and the physicochemical properties of molecules and membranes are important to consider in drug transfer. Among the most important physicochemical properties of the drug molecule are lipid solubility, degree of ionization (pK_a), molecular weight, and protein binding.

Although some drugs (those with molecular size smaller than 200 Da) cross the cellular lipid membranes by diffusion,[1] it is now well known that many require transporters.[2] The greater the lipid solubility and the lower the degree of ionization, the more easily a drug will transfer across the cell membrane. Furthermore, because the active molecule is believed to be the non–protein-bound portion, drugs with lower protein binding have greater access to their site of action.

Transport Mechanisms

Other than the physicochemical properties of the drug molecules, a number of physiologic transport processes influence the mechanism by which a drug traverses the cell membrane.[3] Such processes include passive diffusion, active transport, facilitated diffusion, and pinocytosis.

Passive diffusion is the principal transmembrane process for a number of small-molecule drugs. According to Fick's law of diffusion, drug molecules diffuse from a region of high concentration to a region of low concentration, at a rate given by the following equation:

$$\frac{dQ}{dt} = \frac{DAK}{h(C_1 - C_2)}$$

where dQ/dt = rate of diffusion, D = diffusion coefficient, K = partition coefficient, A = surface area of membrane, h = membrane thickness, and $C_1 - C_2$ = concentration gradient across the membrane. Because $D, A, K,$ and h are constants under usual conditions, a combined constant P, or permeability coefficient, may be defined: $P = DAK/h$. Therefore the previous equation can be simplified to

$$\frac{dQ}{dt} = P(C_1 - C_2)$$

This equation does not take into account the ionization state of the drug molecule, the effect of regional blood flow, or the influence of tissue affinity on drug partitioning. The ionization state is affected by the pH on both sides of the membrane, according to the Henderson-Hasselbalch equation:

$$pH = pK_A + \log([base]/[acid])$$

Therefore acidic compounds, such as salicylic acid, diffuse across cell membranes more readily when the environmental pH is low because they are less ionized at low pH. Regional blood flow also influences the rate of diffusion because it affects the delivery and consequently the local concentration of drug. Finally, some drugs demonstrate increased affinity for a particular tissue component, which influences the concentration of drug on either side of the membrane. For example, tetracycline forms a complex with calcium in the bones and teeth.

Active transport is a carrier-mediated transmembrane process that plays an important role in the renal, intestinal and biliary secretion and absorption of many drugs and metabolites. Active transport is characterized by a transfer of molecules against a concentration gradient. Thus energy must be consumed to achieve this process. An example of this transport system is contributed by the adenosine triphosphate (ATP)-dependent secretion of organic acids in the renal tubule, permitting secretion of indomethacin.[4] Of note, this process, in contrast with diffusion, is saturable and therefore follows Michaelis-Menten kinetics. The Michaelis-Menten equation describes the rates of irreversible enzymatic reactions by relating the rate of substrate conversion or uptake by a protein (enzyme, transporter, receptor) to the concentration of the substrate.

Facilitated diffusion also is a carrier-mediated transport system, in which, in contrast with active transport, molecules move along a concentration gradient. This system does not require energy input but also is saturable and selective. A major class of transporters involved in facilitated diffusion includes the organic cation transporters, the organic anion transporters, and the dipeptide transporters including PepT1[2,5,6]; examples of drugs transported by these transporters are corticosterone, ethacrynic acid, and captopril, respectively.

Pinocytosis, the process of engulfing large molecules such as immunoglobins, is another important physiologic transport mode.[7]

Membrane Transporters

Transporters are membrane-integrated proteins that facilitate movement of nutrients such as amino acids, di- and tripeptides, sugars, nucleosides, vitamins, and bile acids into cells. In the intestine, it has now become increasingly clear that several of these transporters play a significant role in drug absorption. Cellular import of numerous nutrients and drugs depends upon transporters which utilize voltage and ion gradients generated by primary transporters and exchangers. Sodium and protons

frequently are cotransported with the compound of interest. Because the activity of a given transporter is a function of the electrochemical gradient driving force, the efficiency of the transport is influenced by the activity of the Na^+-H^+ antiporter and of the Na^+-HCO_3^- symporter.

A number of membrane transporters play key roles in drug import. These include the H^+-dipeptide symporters, facilitative glucose transporter-related proteins, Na^+-glucose and Na^+-nucleoside cotransporters, amino acid transporters, Na^+-neurotransmitter symporters, and the organic cation and anion transporters.[2,5,6,8] The H^+-dipeptide symporters carry cephalosporins and angiotensin-converting enzyme inhibitors, the organic cation transporters antihistaminics and β-blockers, and the organic anion transporters aspirin, indomethacin, and methotrexate. These ports of entry, along with their structural polymorphisms, may pose an additional challenge to pharmacokinetics and drug delivery.

Net absorption also is affected by the activity of exporters on the brush border membrane. The multidrug resistance-associated protein (MRP) and the P-glycoprotein families are the prototypes of these transporters. These proteins are ATP-dependent transporters (part of the large family of ATP-binding cassette [ABC] transporters) that contribute significantly to extrusion of drugs such as steroids, cyclosporines, and digoxin.[9]

The distribution of drugs requires transport across compartments, which affects their concentration at the site of drug action. Along these lines, P-glycoproteins are important efflux transporters in the blood-brain barrier and consequently influence drug concentration in brain. This transporter role, however, may not be their only function: Of interest, P-glycoproteins have been found on intracellular vesicles and, more important, at the inner membrane of the nuclear envelope,[10] suggesting that P-glycoprotein expression at the nuclear envelope may play a role in subcellular distribution of various endogenous substrates and drugs.[11] This intracellular localization is particularly relevant in the context of the recently recognized expression of cognate transmembrane receptors on subcellular organelles, notably on the nuclear envelope.[12,13]

Kinetics of Absorption

Most pharmacokinetic models assume that drug absorption and elimination follow first-order kinetics (i.e., the rate of change in the concentration of a drug depends on the amount of drug present at that particular time). This process is described in the following equation:

$$\frac{dD_B}{dt} = FK_aD_{si} - K_{el}D_B$$

where dD_B/dt = rate of change of drug in the body (D_B), F = fraction absorbed (bioavailability term), K_a and K_{el} = absorption and elimination constants, and D_{si} = drug concentration at the site of absorption. This equation applies to a single-compartment pharmacokinetic model. In a single-compartment model, the drug rapidly equilibrates with tissues of the body. Accordingly, changes in the concentration of the drug in serum or plasma mirror those in the tissues. Although many drugs follow multiple-compartment kinetics, K_a may still be calculated from a one-compartment model.[14] The importance of K_a lies in the design of a multiple dosage regimen. Knowledge of K_a and K_{el} allows for the prediction of the peak and trough steady-state plasma drug concentrations ($C_{ss\ max}$ and $C_{ss\ min}$) following multiple dosing (see section on clinical pharmacokinetics and Figure 20-3):[15]

$$C_{ss\ max} = (FD_{si} / V_d) / (1 - e^{-Kel\tau})$$
$$C_{ss\ min} = C_{ss\ max} \cdot e^{-Kel\tau}$$

where τ = dosing interval. From these equations, it is evident that peak and trough concentrations depend on the absorption rate, the volume of distribution, the elimination rate constant, and the dosing interval.

Factors Affecting Absorption of Drugs

The systemic absorption of a drug from its site of administration depends on the variables discussed previously, which constitute the physicochemical properties of the agent and of the membrane. Other factors also can influence the efficiency of drug absorption, including the disintegration, dissolution, and solubility of the compound; the blood flow to the site of absorption; the surface area available for absorption; transit time of the drug through the gastrointestinal tract; export of drugs by P-glycoproteins in enterocytes; and in situ metabolism of the agent, including the first-pass effect. A *first-pass effect* is defined as the rapid uptake and metabolism of an agent into inactive compounds by the liver, immediately after enteric absorption and before it reaches the systemic circulation. Drugs that exhibit a first-pass effect include morphine, isoproterenol, propranolol, and hydralazine. Each of the factors affecting absorption, taken separately or in conjunction, may have profound effects on the efficacy and toxicity of a drug.

In evaluating absorption of drugs through the gastrointestinal tract of the newborn, the developmental stages of this organ must be taken into account.[16] Gastric acid secretion is low in premature infants.[17] The increased pH results in a reduced absorption of weak acids and bases. By contrast, lipid-soluble drugs (such as methylxanthines) are more easily absorbed in newborns than in older children.[18] Bile salt secretion also is diminished in the newborn infant,[19] which can secondarily reduce the absorption of fats and lipid-soluble vitamins, such as vitamins D and E[20,21]; on the other hand, vitamin E is adequately absorbed in premature infants,[22] probably owing to a lower intake of iron.[23]

Enzymatic development of the gastrointestinal tract also may alter drug absorption. The elevated activity of β-glucuronidase in the brush border of newborn intestine may cleave drug-glucuronide conjugates,[24] resulting in enhanced reabsorption of free unconjugated drug into the systemic circulation; as a result, the pharmacologic activity of certain agents, such as indomethacin, may be prolonged.[25] By contrast, the presence of P-glycoproteins, highly distributed in the gastrointestinal tract epithelium apical brush border as well as in the bile canalicular face of hepatocytes, can reduce drug absorption and bioavailability[26]; decreased expression of P-glycoproteins in the immature subject may contribute to variable bioavailability.[27] Although modulators of P-glycoproteins, primarily cyclosporine and verapamil, have been used with marginal effectiveness to enhance drug action, such as in cancer treatment,[28,29] these drugs exert their own toxicity; selective inhibitors of P-glycoproteins and related multidrug resistance proteins (MRPs) have yet to be developed. Another factor that influences drug absorption and access to the target organ is the presence of metabolizing enzymes in intestinal epithelium, especially the cytochrome P-450 enzymes.[30] Depending on their activity, limited or excess bioavailability may be observed.

In comparison with adults, newborn infants also exhibit qualitative and quantitative differences in the bacterial colonization of their gastrointestinal tracts. The development of the intestinal flora has clearly been shown to affect the absorption of vitamin K.[31] Thus maturation of the gastrointestinal tract also may explain some of the characteristics of intestinal absorption of drugs in the growing child.

Finally, in addition to the physiologic changes in the gastrointestinal tract that occur during development, drug absorption also can be altered by disease processes. Diseases of genetic (e.g., cystic fibrosis) and microbial or circulatory (e.g., necrotizing enterocolitis) origin may alter the intestinal mucosa, resulting in a reduced absorptive surface.

Bioavailability

Drug *bioavailability* is the fraction of the administered dose that reaches the systemic circulation. For the clinician, the most relevant consideration is the percentage of active drug that reaches the central compartment. Bioavailability does not take into account the rate at which the drug is absorbed. Bioavailability is affected by factors that influence absorption. The absolute availability of a drug may be determined by comparing the respective areas under the plasma concentration curve (AUC) after oral and intravenous administration:

$$\text{Absolute availability} = (\text{AUC}_{\text{PO}} / \text{dose}_{\text{PO}}) / (\text{AUC}_{\text{IV}} / \text{dose}_{\text{IV}})$$

This measurement may be performed so long as the volume of distribution (V_d) and the elimination rate constant (K_{el}) are independent of the route of administration.

Distribution

The *disposition* of a drug refers to its passage in the body from absorption to excretion. After absorption, a drug is distributed to various body compartments. This distribution determines its efficacy as well as its toxicity. The *distribution* of drugs is influenced by several factors, including the size of the body water and lipid compartments, regional hemodynamic features, the degree of binding of drugs to plasma and tissue proteins, at the molecular level the tissue expression of transporter proteins (importers and exporters).

The initial phase of distribution reflects regional blood flow. Organs that are well perfused, such as the brain, heart, and kidneys, are the first exposed to the drug. The second phase of distribution involves a large fraction of the body mass, including the muscle and adipose mass. Thus the various distribution compartments form the apparent volume of distribution (V_d), which is expressed by the following equation:

$$V_d = \text{total drug in the body} / [\text{drug in plasma}]$$

Assuming instant equilibration of the drug after administration, V_d can be determined by extrapolating the drug concentration to time zero (C_0) and dividing the dose of drug administered by the concentration of drug at time zero (C_0). This equation, however, can be applied only to a single-compartment model. V_d also may be calculated using the following equation, which is independent of the model used:

$$V_d = \text{Dose}/K_{el}\,[\text{AUC}]_0^{\infty}$$

Physiologic and Pathologic Factors Affecting Distribution of Drugs

The factors that influence the distribution of drugs in the body are subject to developmental changes. The amount and distribution of total body water undergo marked changes in the perinatal period.[32] Total body water and extracellular fluid volume decrease with increasing gestational age. Consequently, the volume of distribution of many drugs has been observed to increase in preterm neonates.[33] After birth, total body water decreases, and the volume of intracellular fluid increases relative to that of the extracellular fluid. In the term newborn, as in the older child,[34] the degree of insensible water loss is linked to the metabolic rate of the infant. By contrast, in the preterm newborn, no fixed relationship exists between metabolic rate and insensible water loss, and in the very-low-birth-weight infant, evaporative heat loss is substantially greater than heat produced by basal metabolic rate.[22,35]

Many disorders of the newborn as well as drugs administered to critically ill newborn infants (such as diuretics[36,37]) can affect total body water and, secondarily, the distribution of drugs. For instance, renal and hepatic dysfunction may have important consequences on both elimination and distribution of xenobiotics. Similarly, diseases that lead to increased total body water (e.g., congestive heart failure, syndrome of inappropriate secretion of antidiuretic hormone) can have profound effects on drug pharmacokinetics and pharmacodynamics. Therefore any change in either total body water content or the relationship between extracellular and intracellular fluid volume may have significant effects on the distribution of drugs within the body.

The extent and disposition of the lipid mass in the body also contribute to the distribution of drugs. The adipose tissue mass changes markedly during development. Between 28 and 40 weeks of gestation, the amount of adipose tissue (expressed as a percentage of total body mass) increases from 1% to 15%,[38] and by 1 year of age, it represents 25% of body mass.

The nervous system contains a high proportion of lipids. Normally the maximal increment in weight of the human brain occurs in the few weeks preceding term gestation; however, a substantial part of myelination (and lipid deposition) occurs postnatally.[39] The entry of drugs into the central nervous system generally is restricted. In contrast with capillaries elsewhere in the body, endothelial cells of brain capillaries exhibit a predominance of tight junctions producing nonfenestrated capillaries, which restrict the penetration of hydrophilic substances into the brain. Consequently, ionized molecules, such as quaternary amines (e.g., neostigmine), exhibit limited capacity to diffuse into the central nervous system, whereas lipid-soluble compounds, such as cefotaxime and pentobarbital, traverse the blood-brain barrier more readily.

The distribution of drugs into brain and other organs is also dependent upon specific transporters of nutrients and endogenous compounds, as described earlier. Furthermore, efflux carriers present in brain endothelium and glia, primarily P-glycoproteins, limit drug concentration in brain. This effect is well described for a number of drugs including human immunodeficiency virus (HIV) protease inhibitors, vinca alkaloids, and anthracyclines.[40] Of interest, P-glycoproteins also are present in placenta, where they function as export transporters to limit fetal exposure to potentially toxic agents.

A major determinant of drug distribution is the cardiac output and blood flow to various organs.[41] Marked changes in the neonatal circulation take place during the perinatal period.[42,43] In addition, regional blood flow also may change acutely as a result of congestive heart failure (secondary to patent ductus arteriosus or other congenital heart diseases),[41] as a result of sudden changes in acid-base balance (especially respiratory acidosis), or secondary to the limited ability of the stressed preterm neonate to autoregulate regional blood flow.[44]

The affinity of a drug for plasma proteins is another important variable affecting drug distribution. The degree of binding is inversely related to the volume of distribution, such that increased protein binding tends to maintain the drug within the vascular space. Protein binding affects renal and plasma clearance, the half-life, and the efficacy of the agent at its site of action. Table 20-1 lists percent protein-binding values for some commonly used agents in the neonate.

Several factors modify the binding of drugs to plasma proteins:

- The amount of plasma-binding proteins
- The number of binding sites
- The affinity of the drug for the protein
- Pathophysiologic conditions that alter drug-protein binding, including blood pH, free fatty acids, bilirubin, other drugs, and disease states (e.g. renal failure, liver failure)

Albumin binds principally acidic drugs, whereas basic agents are bound to lipoproteins, β-globulins, and α_1-acid glycoproteins.[45] Albumin contains a few high-affinity and several low-affinity binding sites.[46] In the preterm newborn, both albumin

TABLE 20-1

Plasma Protein Binding of Commonly Used Drugs in the Newborn

Drug	Percent Protein-Bound
Ampicillin	~10
Atropine	25
Caffeine	25
Cefotaxime	25-50
Dexamethasone	65
Digoxin	20
Ethacrynic acid	95
Furosemide	95
Gentamicin	45
Hydrochlorothiazide	40
Indomethacin	95
Morphine	30
Phenobarbital	~20
Phenytoin	~80
Theophylline	35

Data from Nordberg A, Winblad B: Cholinergic receptors in human hippocampus: regional distribution and variance with age, *Life Sci* 29:1937, 1981; Kolterman OG, Insel J, Saekow M, Olefsky JM: Mechanisms of insulin resistance in human obesity: Evidence for receptor and postreceptor defects, *J Clin Invest* 65:1272, 1980; Levine MA, Eil C, Downs RW Jr, Spiegel AM: Deficient guanine nucleotide regulatory unit activity in cultured fibroblast membranes from patients with pseudohypoparathyroidism type I: A cause of impaired synthesis of 3′,5′-cyclic AMP by intact and broken cells, *J Clin Invest* 72:316, 1983; and Schilling EE, Rechler MM, Grunfeld C, Rosenberg AM: Primary defect of insulin receptors in skin fibroblasts cultured from an infant with leprechaunism and insulin resistance, *Proc Natl Acad Sci U S A* 76:5877, 1979.

BOX 20-1

Drugs That Cause Significant Displacement of Bilirubin from Albumin in Vitro

Sulfonamides
Moxalactam
Fusidic acid
Radiographic contrast media for cholangiography (sodium iodipamide, sodium ipodate, iopanoic acid, meglumine ioglycamate)
Aspirin
Apazone
Tolbutamide
Albumin preservatives (sodium caprylate and *N*-acetyl tryptophan: rapid infusions in vivo)
High concentrations of ampicillin (rapid infusions in vivo)
Long-chain free fatty acids (FFA) at high molar ratios of FFA to albumin

and α_1-acid glycoprotein concentrations and binding affinities are deficient, resulting in an increased fraction of free drug[47] and increased distribution of free drug outside the vascular compartment.[48] Numerous conditions may further reduce the binding of drugs to proteins. For example, a decrease in pH may enhance the dissociation of weak acids from their albumin-binding sites. Thus the frequent occurrence of acidosis in premature infants may significantly change the binding of drugs to plasma proteins. The elevated plasma free fatty acid content of the newborn also may alter drug binding to plasma proteins[49,50]; this effect, however, has been questioned.[51] In a similar fashion, maternal drugs that have crossed the placenta or other agents concomitantly administered to the infant also may compete for the same plasma protein–binding sites of drugs given to the newborn.

The potential interference of endogenous compounds, particularly unconjugated bilirubin, with drug-protein binding has been well addressed.[52] A displacement of bilirubin from its albumin binding site may result in free circulating unconjugated bilirubin, which can penetrate into the brain and ultimately cause injury. Of interest, however, bilirubin itself is tightly bound to albumin and may displace drugs from their protein-binding sites.[52] In addition, free bilirubin is only sparingly lipophilic. Thus the drug-induced displacement of bilirubin from albumin binding sites possibly plays a minor role in the development of bilirubin-induced encephalopathy. Nonetheless, a few drugs can alter the binding affinity of albumin for bilirubin[52] (Box 20-1).

The volume of distribution of certain compounds also is affected by their binding to proteins outside the vascular space. For instance, digoxin exhibits a higher degree of binding to myocardial and skeletal proteins in the newborn than in the adult.[53,54] This increased binding results in an increase in the volume of distribution of digoxin.

Numerous factors influence the distribution of drugs in the body. These factors are themselves affected by development and disease conditions of the newborn infant. Major changes in the distribution of fluids and fat and their proportion relative to body mass occur at the end of gestation and during the neonatal period. Perinatal and neonatal alterations in cardiac output and regional blood flow, secondary to physiologic and pathophysiologic changes, also occur. Furthermore, the degree of drug binding to plasma proteins between the newborn and adult varies for several drugs. These variables should be taken into account in deciding on the appropriate drug dosage for a newborn.

PRINCIPLES OF DRUG ELIMINATION

The relatively high lipophilicity of many drugs does not permit their rapid elimination. After filtration through the glomerulus or passage into the bile, these agents are readily absorbed by the renal tubule or gastrointestinal mucosa. Consequently, the elimination of most drugs from the body involves the process of biotransformation followed by excretion. This section reviews the different biotransformational processes that take place in the human body and the mechanisms of renal drug excretion, with particular reference to developmental aspects.

Drug Biotransformation

Drug *biotransformation* converts drug molecules into more polar derivatives that are less able to diffuse across cell membranes. As a consequence of biotransformation, these converted molecules do not reach their receptors and in addition are not reabsorbed by the renal tubule. Thus biotransformation of drugs not only facilitates their excretion from the body but also may diminish their pharmacologic activity.

The metabolism of drugs does not always produce inactive compounds. Initial biotransformation of certain agents results in the formation of active metabolites. For instance, codeine is demethylated to morphine, acetylsalicylic acid is hydrolyzed to salicylic acid, and theophylline is methylated to caffeine. Furthermore, oxidation of certain aromatic compounds produces highly reactive electrophiles (compounds that serve as electron acceptors). This latter reaction may be primary (aromatic hydrocarbons) or may be an increasingly active secondary reaction resulting from an inhibited or overwhelmed primary metabolic pathway (as, for example, with an excessive dose of the agent, such as with acetaminophen overdose). Therefore biotransformation can produce relatively innocuous metabolites or highly toxic compounds.

The mechanisms affecting the biotransformation of drugs are usually the same as those that metabolize endogenous products (e.g., hormones). Most biotransformation takes place in the liver, but some may occur at other sites, such as the kidneys, intestinal

TABLE 20-2

Biotransformation Reactions

Reaction	Examples of Drug Substrates
Phase I (Nonsynthetic Reactions)	
Oxidation	
Aromatic ring hydroxylation	Phenytoin, phenobarbital
Aliphatic hydroxylation	Ibuprofen
N-hydroxylation	Acetaminophen
N-, *O*-, *S*-dealkylation	Morphine, codeine
Deamination	Diazepam
Sulfoxidation, *N*-oxidation	Cimetidine
Reduction	
Azoreduction	Sulfonamidochrysoidine (Prontosil)
Nitroreduction	Chloramphenicol
Alcohol dehydrogenase	Ethanol
Hydrolysis	
Ester hydrolysis	Acetylsalicylic acid
Amide hydrolysis	Indomethacin
Phase I Enzymes	
CYP1A2	Caffeine, theophylline
CYP2C	Warfarin, ibuprofen, phenytoin, omeprazole, diazepam
CYP2D6	Codeine, imipramine, propranolol, timolol, tamoxifen
CYP2E1	Acetaminophen, tamoxifen, caffeine, ethanol
CYP3A	Erythromycin, midazolam, 6β-hydroxycortisol
Phase II (Synthetic Reactions: Conjugations)	
Glucuronide conjugation	Morphine, acetaminophen
Glycine conjugation	Salicylic acid
Sulfate conjugation	Acetaminophen, α-methyldopa
Glutathione conjugation	Ethacrynic acid
Methylation	Dopamine, epinephrine
Acetylation	Sulfonamides, clonazepam

Modified from Turki J, Pak J, Green SA, et al: Genetic polymorphisms of the beta 2-adrenergic receptor in nocturnal and nonnocturnal asthma. Evidence that Gly16 correlates with the nocturnal phenotype, *J Clin Invest* 95:1635, 1995.

mucosa, and lungs. Biotransformation reactions are classically divided into two phases: phase I, the nonsynthetic reactions, and phase II, the synthetic or conjugation reactions (Table 20-2). Each phase has reactions that can take place in the microsomes or outside of the microsomal system. The great majority of phase I reactions (oxidation, reduction, and hydrolysis) are catalyzed by microsomal enzymes; other than glucuronidation, phase II reactions are extramicrosomal.

Phase I Reactions (Nonsynthetic Reactions)

Microsomal

The portion of microsomes containing the enzymes that metabolize drugs are found in the smooth endoplasmic reticulum. The oxidative enzymes of this system have attracted particular attention in research. This group of enzymes has been called mixed-function oxidases or monooxygenases. They have three principal components: an electron transporter, nicotinamide adenine dinucleotide phosphate (NADPH)–cytochrome P-450 reductase (a flavoprotein), and one of the many recently discovered cytochrome P-450 isozymes, which are oxidase hemoproteins.[55] The system requires both a reducing agent, NADPH, and molecular oxygen. The end result of the reactions catalyzed by cytochrome P-450 is the incorporation of one oxygen atom into the endogenous or exogenous compound being metabolized (hence the name *monooxygenase*) and the formation of water after reduction of the second oxygen atom.

The reactions catalyzed by the microsomal monooxygenases include aromatic ring and aliphatic side chain hydroxylation, *N*-, *O*-, *S*-dealkylation, deamination, dehalogenation, sulfoxidation, *N*-oxidation, *N*-hydroxylation, nitroreduction, and azoreduction. Epoxides also are formed by monooxygenases, converting aromatic moieties of agents to arene and alkene oxides, which are in turn detoxified by epoxide hydrolases present in endoplasmic reticulum. These electrophilic compounds react avidly with proteins and nucleic acids, exerting potential mutagenic and carcinogenic effects; polychlorinated and polybrominated biphenyls exert their toxicity by way of their metabolites.[55]

Several drugs, including cimetidine, spironolactone, and propylthiouracil, can inhibit cytochrome P-450 enzyme activity. This inhibition reduces the metabolism of potential substrates and secondarily retards their elimination. By contrast, other substrates can act as inducers of the cytochrome P-450 system. Prototypes of the most extensively studied inducers of separate cytochrome P-450 isozymes are phenobarbital (CYP3A), rifampin (CYP1A, CYP2C), and the polycyclic aromatic hydrocarbon 3-methylcholanthrene (CYP1A).

Approximately 1000 cytochrome P-450s are known; only 50 or so are functionally active in humans. Seventeen families and many subfamilies have been sequenced. CYP1, CYP2, and CYP3 families are involved in a majority of all drug metabolism reactions; members of the other families are important in the synthesis and degradation of steroids, fatty acids, vitamins, and other endogenous compounds. Individual CYP isoforms tend to have substrate specificities, but overlap is common. CYP3A4 and CYP3A5 are similar isoforms, which together are involved in metabolism of approximately 50% of all drugs. CYP2C and CYP2D6 also are involved in metabolism of many drugs. CYP1A1/2, CYP2A6, CYP2B1, and CYP2E1 are not extensively involved in drug metabolism but rather are important in activation of procarcinogenic agents including aromatic amines and aromatic hydrocarbons.

The cytochrome P-450–dependent monooxygenase system develops in fetal life and progresses through a rapid and significant increase in activity during the perinatal period[56]; nonetheless, in general, its activity in the fetus and newborn remains considerably lower than that in adult liver.[30,57] The diminished enzyme activity may be clinically important because drugs that are oxygenated slowly by these enzymes (e.g., phenobarbital, phenytoin) can exhibit a prolonged half-life in the young infant[58]; this is especially the case for CYP2A, CYP2B6, CYP2C, and CYP3A4/5 (Table 20-3). However, significant differences have been found in the developmental expression of the three important gene families implicated in xenobiotic metabolism; accordingly, drugs converted by CYP2D6 and CYP3A7, such as carbamazepine, dextromethorphan, and tricyclic antidepressants, are eliminated in the newborn nearly as rapidly as in the adult.[30] Of interest, CYP2E1 is induced and metabolized by ethanol in the fetus and has been proposed to be implicated in the development of fetal alcohol syndrome.[59]

Extramicrosomal

A few of the oxidative and reductive reactions are mediated by enzymes in the mitochondria and cytosol of the liver and other tissues. These enzymes include those involved in oxidation of alcohols and aldehydes; alcohol and aldehyde dehydrogenases; and enzymes that partake in the metabolism of catecholamines, namely tyrosine hydroxylase and monoamine oxidase. Although the activity of some of these enzymes can be detected early in gestation, their full activity is reached only in early childhood.[30] Once again, however, marked ontogenic differences between

TABLE 20-3

Ontogeny of Human Phase I and Phase II Metabolizing Enzymes

Enzyme	Fetus	Newborn	Infancy	Adult
Phase I				
CYP1A1	+	−	−	−
CYP1A2	−	−	+	+
CYP2C	−	+	+	+
CYP2D6	±	+	+	+
CYP2E1	±	+	+	+
CYP3A7	+	+	−	−
CYP3A4/5	−	+	+	+
Phase II				
UGT1A1	−	+	+	+
UGT1A3	±	±	+	+
UGT2B7	±	±	+	+
GSTA	±	±	+	+
SULT1A1	±	±	+	+
SULT1A3	+	+	+	±

GST, Glutathione *S*-transferase; *MGT,* UDP-glucuronosyltransferase; *SULT,* sulfotransferase.

Modified from Morselli PL, Franco-Morselli R, Bossi L: Clinical pharmacokinetics in newborns and infants: Age-related differences and therapeutic implications, *Clin Pharmacokinet* 5:485, 1980; and Lang D, Hofstetter R, von Bernuth G: Postmortem tissue and plasma concentrations of digoxin in newborns and infants, *Eur J Pediatr* 128:151, 1978.

TABLE 20-4

Plasma Half-Life of Miscellaneous Drugs in Newborns and Adults

	PLASMA HALF-LIFE (hr)	
Drug	**Newborn**	**Adult**
Acetaminophen	3.5	2.2
Phenylbutazone	21-34	12-30
Indomethacin	7.5-51.0	6
Meperidine	22	3.5
Phenytoin	21	11-29
Carbamazepine	8-28	21-36
Phenobarbital	82-199	24-140
Caffeine	100	6
Theophylline	30	6
Chloramphenicol	14-24	2.5
Salicylates	4.5-11.5	2.7
Digoxin	52	31-40

Data from Hillman LS, Martin LA, Haddad JG: Absorption and maintenance dosage of 25-hydroxycholecalciferol (25-HCC) in premature infants [abstract], *Pediatr Res* 13:400, 1979; Kolterman OG, Insel J, Saekow M, Olefsky JM: Mechanisms of insulin resistance in human obesity: Evidence for receptor and postreceptor defects, *J Clin Invest* 65:1272, 1980; and Ruffolo RR Jr: Important concepts of receptor theory, *J Auton Pharmacol* 2:277, 1982.

enzymes are observed. For instance, class I alcohol dehydrogenase, the major ethanol-metabolizing enzyme, tends to be well expressed in fetal liver, whereas class III alcohol dehydrogenase is relatively deficient in the fetus.[30]

Phase II Reactions (Synthetic Reactions)

In phase II reactions, molecules that are naturally present in the body are conjugated or combined with the drug or other endogenous compounds. The drug may have first undergone a phase I reaction, or the original drug may be directly conjugated. Conjugation converts drugs into more polar compounds, which are pharmacologically less active and are more readily excreted; an exception applies to acetylation, whereby the metabolite often is less water-soluble. Although it was previously thought that conjugation reactions represented true inactivation and detoxification reactions, current evidence indicates that certain conjugation reactions may lead to the formation of reactive species responsible for hepatotoxicity (*N*-acetylation of isoniazid). The major conjugation reactions are listed in Table 20-4.

Glucuronidation

The formation of glucuronides is the principal conjugation reaction in the body. Natural substrates of this pathway include bilirubin and thyroxine. Many drugs containing hydroxyl, amino, carboxyl, thiol, and phenolic groups, however, also use the same pathway (e.g., morphine, acetaminophen, phenytoin, sulfonamides, chloramphenicol, salicylic acid, and indomethacin).

The conjugation of a compound with glucuronic acid results in the production of a strongly acidic substance that is more water-soluble at physiologic pH than the parent compound. This heightened solubility reduces its transfer across membranes, facilitates its dissociation with its receptor, and enhances its elimination in urine and bile. The fate of the glucuronidated drugs in urine or bile depends on their molecular size. Compounds of relatively low molecular weight are almost completely excreted in urine, whereas those of high molecular weight (greater than 500) are eliminated almost entirely in bile.

Glucuronides are eliminated by the kidneys predominantly through glomerular filtration; however, tubular secretion and tubular reabsorption followed by secretion represent alternative pathways. Biliary excretion of drugs conjugated to glucuronic acid occurs by simple diffusion or by active secretion. Once in the intestine, these drugs may be reabsorbed after being deconjugated (hydrolyzed) by glucuronidase, which exhibits an elevated activity in the fetus and newborn.[24]

Drugs or conditions that inhibit formation of glucuronides are likely to prolong the pharmacologic activity of these agents. Inhibition may occur at the level of synthesis of glucuronic acid (e.g., by certain steroid hormones) or at the level of uridine diphosphate (UDP)-glucuronyl transferase activity itself, which consists of 16 different isoforms in humans. In the human fetus and newborn, glucuronide conjugation reactions exhibit reduced activity, and fetal underexpression of the 2B isoform is responsible for the gray baby syndrome associated with chloramphenicol intake. Postnatal development proceeds relatively rapidly, as occurs with the metabolism of bilirubin.[60,61]

Other Synthetic Conjugation Reactions

Other kinds of conjugation reactions that occur in the body are listed in Table 20-4. As is the case with glucuronyl transferase, the activity of the other various transferases also is lower in the fetus and newborn than in the adult.[60,61]

Overall, available data permit the following generalizations regarding drug metabolism in the fetus and newborn:

1. The rates of drug biotransformation and overall elimination are low.
2. The rate of drug elimination from the body exhibits marked interpatient variability.
3. The maturational changes in drug metabolism and disposition occurring as a function of postnatal age are extremely variable and depend on the substrate (or drug) being used.
4. Neonatal drug biotransformation and elimination are vulnerable to pathophysiologic states.
5. Neonates may exhibit activation of alternate biotransformation pathways.

Factors Affecting Biotransformation in the Liver

Several factors may alter the rate, extent, and type of biotransformation reactions in the liver. The issues of enzyme activity that may be modified by development and by endogenous or

exogenous compounds have been addressed. Environmental influences also significantly affect drug metabolism, to include the inducing or inhibitory effects of drugs per se on enzyme activities. For instance, calcium channel blockers, antifungal agents, and macrolide antibiotics are potent inhibitors of CYP3A enzymes; quinidine inhibits CYP2D6; and other compounds such as cimetidine, amiodarone, and fluoxetine reduce activity of many cytochrome P-450 enzymes. Conversely, a number of agents, including anticonvulsants and aromatic hydrocarbons, can stimulate induction of certain CYP subfamilies and isoforms.

Metabolic enzyme activity also is affected by genetic factors, which is the rule rather than the exception; however, the interplay between ontogeny and genetics remains largely unknown in drug metabolism. Approximately 70 single-nucleotide polymorphisms and other genetic variants of the CYP2D6 gene, many of which diminish enzyme activity, have been recognized; this decreased activity may be of particular importance with drugs metabolized by CYP2D6, such as β-blockers and certain opiates. In the case of CYP3A enzymes, no significant functional polymorphisms have yet been identified; hence factors regulating gene expression are more important to explain the interindividual variability (greater than 10-fold). Because genetic factors and environmental influences exert greater effects on drug metabolism than on renal excretion, drugs that are metabolized are considerably more affected by interpersonal differences than those not requiring biotransformation.

Other factors that affect biotransformation include blood flow to the liver, gender, and disease states. For certain drugs (e.g., morphine, meperidine, propranolol, verapamil), blood flow may be the limiting factor controlling drug elimination. These drugs (often termed *flow-limited* drugs) are so readily metabolized by the liver that hepatic clearance is essentially equal to liver blood flow. By contrast, the biotransformation of *capacity-limited* drugs (e.g., phenytoin, theophylline, diazepam, chloramphenicol) is determined by the liver's metabolizing capacity, rather than by hepatic blood flow.[62] The effect of gender in newborn infants remains unclear but may not be as important as in the adult.

Renal Excretion of Drugs

The renal excretion of drugs and their metabolites occurs by means of three major processes: glomerular filtration, active tubular secretion, and passive tubular reabsorption. After the entry of a drug into a renal tubule, its elimination is dependent on its lipophilicity and ionization state.

Glomerular Filtration of Drugs

The amount of drug filtered through the glomerulus depends on a molecular size less than that of albumin (molecular weight 69,000) and degree of protein binding. Hence nearly all non–protein-bound drugs are filtered. Glomerular filtration rate increases during fetal development, but in the newborn it remains far below that observed in the adult.[63-66] Furthermore, conditions that lead to decreased glomerular filtration rate, such as asphyxia, severe respiratory insufficiency, renal failure, and patent ductus arteriosus, are associated with reduced drug excretion. Certain agents (e.g., indomethacin, tolazoline) may hamper their own excretion by reducing glomerular filtration rate.[67-69]

Active Tubular Transport of Drugs

A number of transport systems found in the proximal tubule are involved in the energy-dependent secretion of organic compounds (endogenous or xenobiotic).[70] These carrier systems are relatively nonselective. One such system transports organic anions, including drug conjugates with glucuronic acid, glycine, and sulfates; examples of drugs that use this excretory system are penicillins, furosemide, and chlorothiazide. The other transport system secretes organic cations, such as histamine and choline. Multidrug resistance proteins 1, 3, 5, and 6 have been localized to the basolateral membrane, whereas multidrug resistance proteins 2 and 4 are found at the brush border membrane of tubular epithelium. Generally, these transporters operate as efflux pumps. A counteracting mechanism involves the peptide transporters PEPT1 and especially PEPT2 localized at the proximal tubular epithelium apex, which operate as influx pumps of filtered drugs. All transporters described are energy-dependent. In the case of organic cation, organic anion, and peptide transporters, cellular import requires an electrochemical gradient especially of Na^+, which is maintained largely by the Na^+,K^+-ATPase pump. By contrast, multidrug resistance proteins are directly ATP-dependent.[70] Membrane transporters, located mainly in the distal tubule, operate to actively reabsorb drugs from the tubular lumen back into the systemic circulation. Most such reabsorption occurs by nonionic diffusion. Although polymorphisms of transporters are likely to significantly affect drug disposition, knowledge regarding such polymorphisms remains limited.

Tubular secretion of organic anions and cations also is lower in newborns than in adults. This developmental characteristic explains the prolonged half-life of certain agents that use this system of elimination, as is the case with furosemide, penicillins, and glucuronidated drugs, in the newborn.

Passive Tubular Reabsorption of Drugs

In both proximal and distal tubules, nonionized forms of weak acids and bases undergo net passive reabsorption. Accordingly, this form of renal reabsorption of drugs is regulated by three factors: the concentration gradient across the tubular membrane, the ionization state of the compound in the tubular fluid (which depends on the drug's p*K*a and the tubular fluid pH), and the lipid solubility of the drug. Manipulation of these physicochemical properties can be used to enhance renal excretion. For instance, alkalinization of the urine can increase the elimination of salicylic acid up to six-fold. As inferred earlier, the term *passive diffusion* does not exclude specific transporters or exchangers involved in movement across cell membranes and into compartments.

Overall, patterns of renal excretion of drugs can be summarized as follows: (1) Renal excretion of lipid-soluble drugs depends largely on urine volume; (2) elimination of polar drugs depends on glomerular filtration rate; and (3) the renal elimination of drugs that ionize readily is principally dependent on the activity of the tubular secretory systems and the urine pH.

Effect of Disease on Drug Elimination

Most pharmacokinetic parameters are determined in healthy or moderately ill persons. In neonatal intensive care units, as in other similar environments, drugs often are administered to very sick patients. Life-threatening illnesses produce remarkable variation in pharmacokinetic behavior. Adjustment in drug dosage in the face of a changing disease is critical to avoid toxicity.[16,71] For this reason, an understanding of the pathophysiology of a disease and its pharmacologic consequences becomes of utmost importance in decisions regarding appropriate pharmacologic therapy. This section addresses the issue of disease as it applies to drug disposition in the newborn.

Cardiovascular Disease

Cardiac output is a major determinant of drug elimination. Heart failure produces a decrease in cardiac output that alters the regional distribution of blood flow. Significant decreases in cardiac output or oxygen delivery can result in adverse effects on liver function, with consequent impairment of drug clearance.[72]

Congestive heart failure also is associated with a reduction in renal blood flow and glomerular filtration rate. This compromise in renal function contributes to a decrease in drug elimination. In newborns with congestive heart failure, elevated plasma levels

of digoxin have been observed, suggesting a decreased clearance of the drug.

Renal Disease

Because the kidneys are a major route of drug elimination, alterations in their function can significantly influence drug pharmacokinetics.[64] The pharmacologic consequences of renal dysfunction depend on the fraction of drug cleared by the kidneys and on the degree of renal failure. Under these circumstances, drugs for which the kidneys represent the primary route of elimination (e.g., aminoglycosides, cephalosporins) accumulate in the body; this is especially true when renal clearance is greater than 90% of total drug clearance.

As glomerular filtration rate falls, drug elimination via glomerular filtration decreases; such is the case for digoxin, aminoglycosides, and cephalosporins. Reduction in tubular function can significantly affect the elimination of drugs that depend on tubular reabsorption or secretion, such as penicillins and furosemide. In addition, changes in plasma and urine pH alter the excretion of ionized drugs. The development of uremia may be associated with changes in cardiac output, liver function, and blood-brain barrier permeability, all of which can further disturb drug disposition.

The clinical significance of drug accumulation depends on whether the unexcreted products are pharmacologically active or toxic. Nomograms have been developed to allow modification of drug dosage in patients with renal failure. Most such nomograms, however, apply primarily to adult rather than infant patients and therefore must be used with caution.

Liver Disease

Because the liver is a major site of drug disposition, hepatic insufficiency is associated with defects in multiple liver functions, many of which have the potential to alter drug excretion. Hepatic insufficiency can affect drug elimination by (1) reducing plasma protein binding, (2) decreasing liver blood flow, and (3) disturbing intrinsic biotransformation reactions.

A decrease in plasma protein concentration influences the disposition of drugs extensively bound to proteins. Drugs with liver uptake and metabolism can be categorized into two groups: one dependent on liver blood flow (flow limited) and the other dependent on the liver's metabolic capacity (capacity limited). Drugs belonging to the first group exhibit a more uniform change in drug elimination during hepatic failure. By contrast, drugs dependent on the metabolic activity of the liver exhibit variable changes in biodisposition. This variability results from inconsistent qualitative and quantitative changes in liver enzyme activity in the presence of liver disease of variable severity.

Because of the marked variability in degree of liver dysfunction encountered clinically, it is difficult to formulate rules for dosage modification. Marked changes in pharmacokinetics (as noted with renal insufficiency) generally are not observed in liver failure.[73] Nonetheless, dosages of drugs eliminated mainly by hepatic biotransformation should be reduced in infants with severe liver disease.

The consequences of intercurrent disease on the pharmacokinetics and pharmacodynamics of drugs are irrefutably complex. An accurate prediction of drug disposition in the face of interacting intricate individual variables is virtually impossible. The relative paucity of information on pharmacokinetics of drugs in infants with cardiac, renal, and hepatic dysfunction and the often changing and transient nature of these disorders render careful clinical observations and appropriate therapeutic monitoring imperative under these circumstances.

CLINICAL PHARMACOKINETICS

The fundamental assumption of clinical pharmacokinetics is that a relationship exists between the concentration of a drug at its site of action and its serum or plasma level. Measurement of the concentration of a drug in the blood can be used to monitor the dose-response relationship and to predict its pharmacokinetics. Thus drug levels in blood permit appropriate individual dosing adjustments.

Pharmacokinetic principles can be understood only with respect to the various compartments into which a drug is distributed (Figure 20-1). A compartment is not necessarily a defined physiologic or anatomic site but is considered to consist of one or many tissues that exhibit similar affinity for a drug.

The factors that determine the movement of a drug into and out of compartments are those that affect distribution. Therefore the lipophilicity, ionization state, transporter systems (of both influx and efflux carriers), and protein binding of a drug, as well as regional blood flow, regulate the extent and rate of passage of an agent into and out of a compartment.

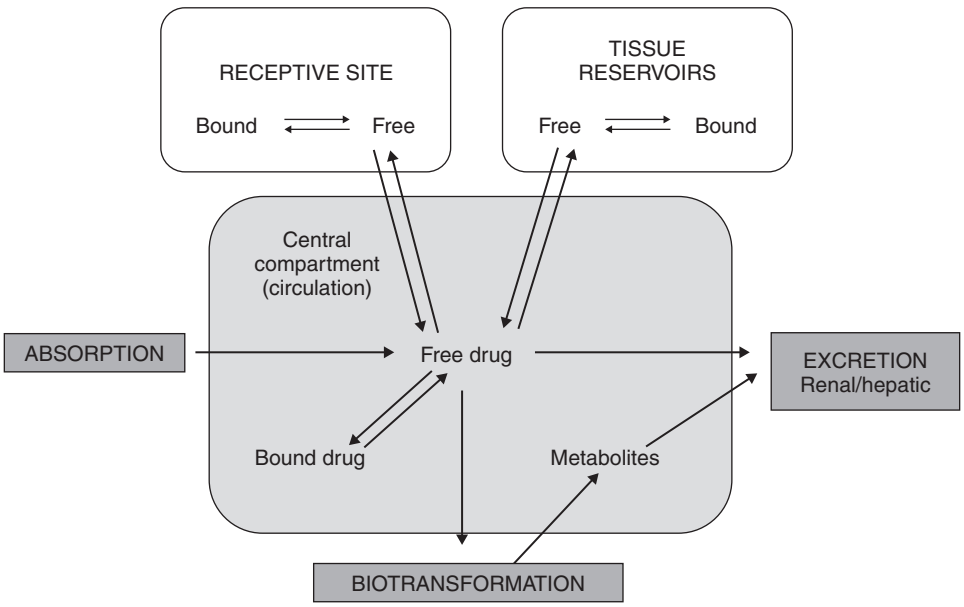


Figure 20-1 Schematic of possible drug disposition to various compartments in the body.

Single-Compartment Distribution

The simplest pharmacokinetic model is the single-compartment model. In this model, it is assumed that after administration of the drug, immediate equilibration of drug concentration is achieved in all major tissues. First-order kinetics apply with this model, meaning that the rate of change in the amount of drug in the body is a constant fraction of the amount of drug present at the time. The higher the dose, therefore, the greater the elimination rate from the compartment. The rate of disappearance of a drug concentration using first-order kinetics can be expressed as

$$\frac{dC}{dt} = -kC$$

where C = concentration of the drug, and k = first-order elimination rate constant, expressed in units of time^{-1} (e.g., h^{-1}). Integration of this equation yields the drug concentration at time t:

$$\log C = -kt/2.3 + \log C_0 \text{ or } C = C_0\, e^{-kt}$$

where C_0 = concentration at time $t = 0$. Thus the slope of the log of the concentration-time curve = $-k/2.3$.

Drug Half-Life

From the previous equation ($\log C = -kt/2.3 + \log C_0$), the first-order half-life can be obtained:

$$t_{1/2} = 0.693/k$$

Regardless of the initial concentration of drug, the time required for the concentration to decrease by half is a constant determined by k. Thus the time it takes to achieve a steady-state plasma concentration or the time it takes to eliminate the near-totality of the drug is equal to approximately five half-lives (Figure 20-2). In newborns, because the elimination rate is decreased for many drugs, the half-life often is prolonged compared with that in the adult (see Table 20-4).

To minimize the time a drug takes to achieve average steady-state plasma concentrations ($C_{ss\ av}$), a loading dose (D_L) can be given. This dose is equal to

$$D_L = C_{ss\ av} \times V_d$$

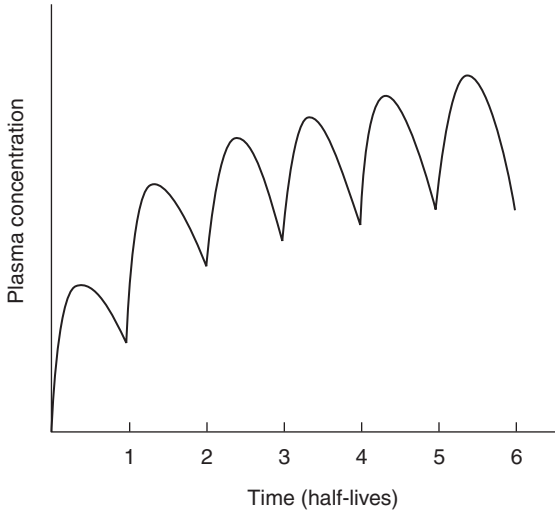


Figure 20-2 Pharmacokinetic relationships of a multiple dosing regimen. It can be readily appreciated from the graph that the time required to attain steady-state drug concentrations is equal to approximately five half-lives, when first-order rates of absorption, distribution, and elimination are in process.

Maintenance Dose

After steady-state concentration is achieved (following a loading dose), the dose necessary to maintain this concentration (maintenance dose, D_{ss}) can be calculated from the following equation:

$$D_{ss} = (0.693 \times C_{ss\ av} \times V_d \times \tau)/t_{1/2}$$

where τ = dosing interval. Thus D_{ss} also is equal to the rate of drug elimination during the dosing interval.

Optimal Dosing Schedule

Knowledge of the half-life permits the clinician to determine an optimal dosing schedule, having set the maximum and minimum effective steady-state concentrations during a multiple dosing regimen (see Figure 20-2). Using the same equation to calculate the concentration at time t ($\log C = -kt/2.3 + \log C_0$), an optimal dosing interval, τ, also can be determined. By substituting C with $C_{ss\ min}$ and C_0 with $C_{ss\ max}$, the following equation is obtained:

$$\log C_{ss\ min} - \log C_{ss\ max} = -kt/2.3$$

By further substituting k by $0.693/t_{1/2}$, the dosing interval, τ, which is the time it takes for the drug concentration to decrease from $C_{ss\ max}$ to $C_{ss\ min}$, can be determined using the following equation:

$$\tau = \log(C_{ss\ max}/C_{ss\ min}) \times 3.3 \times t_{1/2}$$

Drug Clearance

Drug clearance can be defined as the plasma volume in the vascular compartment that is cleared of drug per unit of time. Total clearance gives an indication of drug elimination from the central compartment without reference to the mechanism of this process. For drugs that are eliminated by first-order kinetics, clearance is constant.

Clearance by the kidneys is called *renal clearance,* and that by all other organs is referred to as *nonrenal clearance.* The latter most often represents clearance by the liver. Total clearance is the sum of all body clearances. The same factors that determine renal and hepatic elimination of drugs affect drug clearance.

Clearance (Cl) is mathematically defined as excretion rate/plasma concentration. Over a drug excretion time, clearance can be expressed by the following equation:

$$\text{Cl} = D_0/[\text{AUC}]_0^t$$

Rearrangement of this formula according to the equation expressing V_d (see under "Distribution" earlier) yields

$$\text{Cl} = V_d \times k$$

Multicompartment Distribution

Many drugs distribute in the body according to the kinetics of a multicompartment model. Consequently, after intravenous administration of the drug, the plasma concentration does not decline linearly as a first-order rate process but exhibits nonlinear elimination (Figure 20-3). The first part of the curve with its sharper slope, the α phase, represents the distribution of drug to highly perfused areas, including the blood. This area consists of the central compartment. With time and depending on the affinity of the drug for certain tissues, the agent distributes to the peripheral compartment or compartments. After equilibration, the tissues are saturated with the drug, and its decline in blood usually occurs by a first-order elimination process, the β phase (see Figure 20-3). Usually the half-life of drugs that distribute according to a two-compartment model is determined during the β phase.

To apply kinetic analysis of a multicompartment model, it is assumed that all rate processes for the passage of drug from one

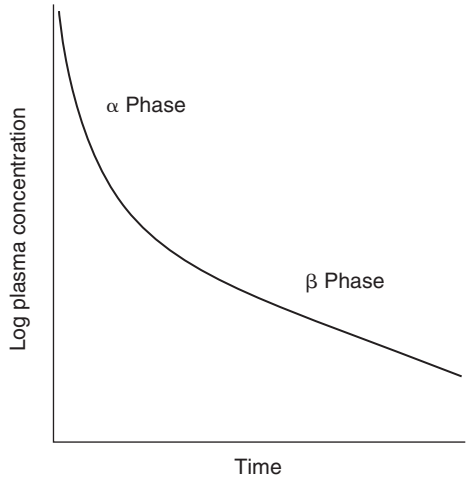


Figure 20-3 Two-compartment model for serum drug disappearance curve. The α phase represents the distribution phase, and the β phase, the elimination phase.

compartment to another exhibit first-order kinetics. Therefore, the plasma level–time curve for a drug that follows a multicompartment model is described by the summation of several first-order rate processes.

Zero-Order Kinetics

When elimination processes become saturated, disposition of certain drugs occurs according to zero-order kinetics. In contrast with a first-order process, in which the fraction of drug eliminated is constant, in zero-order kinetics, the elimination rate itself is constant. Thus the drug is eliminated at a constant rate. Consequently the zero-order half-life is not constant but is proportional to the initial amount or concentration of the drug:

$$t_{1/2} = 0.5C_0 / k_0$$

where k_0 = zero-order rate constant. Many drugs exhibit zero-order kinetics with elevated concentrations, and as these decline, first-order kinetics prevails. Examples of drugs that exhibit saturation kinetics are salicylates, phenylbutazone, phenytoin, diazepam, and chloramphenicol.

The determination of drug pharmacokinetics has not been widely accepted as an essential part of newborn intensive care. The amount of reliable pharmacokinetic data for drugs used in the ill neonate has lagged considerably behind knowledge of pathophysiology. Little doubt exists that this information as well as monitoring of serum levels can optimize drug dosage. In view of the risks of therapeutic failure and toxicity, the rationale for drug monitoring is obvious. In addition, the many complex developmental and disease-related dynamic processes that take place in the sick newborn provide sufficient justification to measure drug blood levels to obtain an indication of its kinetics.

PRINCIPLES OF PHARMACODYNAMICS: DRUG-RECEPTOR INTERACTION

Pharmacodynamics is defined as the biochemical and physiologic effects of drugs. This aspect of pharmacology is the raison d'être of pharmacotherapeutics. The mechanism of action of drugs at the receptive site has received much attention over the past 4 decades. This section reviews some of the issues regarding drug-receptor interaction; the term *receptive site* refers to the molecular entity with which the agent is presumed to interact.

Sites of Drug Action

Drugs may act outside the cell, at the cell membrane, or inside the cell. Regardless of cell localization, the drug action may be mediated by receptors or can be independent of receptors. Certain agents do not act on cellular sites but instead act on extracellular products of cells. A majority of drugs bind to specific receptor sites, whereas the action of others does not seem to be mediated by receptors. For instance, chelating agents, such as dimercaprol, penicillamine, and desferrioxamine, bind circulating metals. The action of these agents can be considered truly extracellular.

Drugs that bind to physiologic receptors and mimic the regulatory effects of the endogenous ligands are termed *agonists.* Compounds that block the effects of endogenous ligands are termed *antagonists,* which may be partial or inverse; the latter applies to drugs that stabilize the receptor in its inactive conformation. Localization of drug action refers both to drug distribution and to the specificity of the drug action. Such specificity implies the existence of receptors. Receptors consist of macromolecules that recognize and bind specific ligands and translate this binding into propagation of an intracellular message, either directly (e.g., nicotinic receptor: ion transport) or indirectly by virtue of a second messenger (e.g., protein kinase C: angiotensin II). This concept of receptor characteristics has led some investigators to suggest the existence of functional domains on the receptor molecule: a ligand-binding domain and an effector domain. Such conceptualization of the receptor is generally consistent with the mode of action of agonists and antagonists but appears to be a simplified version of complex processes (see later discussion).

Receptor Classification

Receptors traditionally have been classified pharmacologically by their response to specific antagonists. The precise classification of receptors with respect to structure-activity relationship, however, relies on multiple approaches, which include physiologic, biochemical, biophysical, and immunologic techniques. These broadened characteristics delineate more precisely the action of drugs, facilitating the development of therapeutic agents having selectivity for specific receptors. This broader scope also provides the clinician with an appropriate basis for therapeutics, by ameliorating efficacy and limiting toxicity.

Receptor Regulation

The concentration and affinity of receptors are physiologically regulated by ligand-receptor binding and activation. Receptor down-regulation is the process by which the concentration and affinity of receptors are decreased. This regulation of receptor function can be classified on the basis of the time course, short- and long-term. Short-term regulation occurs on the order of seconds to minutes, and long-term regulation takes place over hours to days. The mechanisms responsible for short-term regulation appear to involve conformational changes, transient intracellular receptor sequestration (e.g., nicotinic and α-adrenergic receptors), and phosphorylation of receptors. Protein phosphorylation is the mechanism by which most receptors are regulated, although myristoylation and palmitoylation also are involved in the regulation of expression of certain receptors. Long-term receptor down-regulation involves initial protein phosphorylation (myristoylation or palmitoylation) followed by internalization and degradation.

The term *receptor up-regulation* refers to the process of increasing receptor number. An example of up-regulation is the phenomenon of denervation supersensitivity of nicotinic receptors. The process of up-regulation is less clearly understood. Alterations in transcription of messenger RNA (mRNA), translation, or posttranslational modifications seem to contribute to receptor

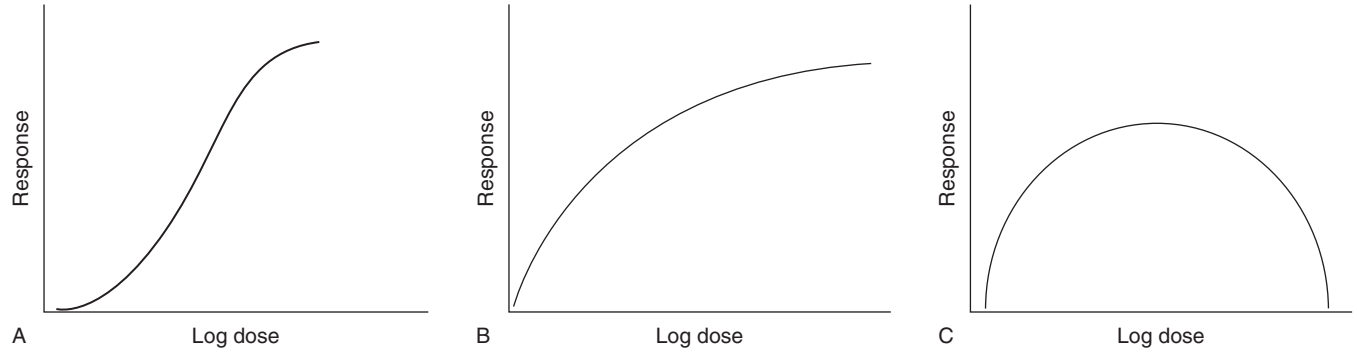


Figure 20-4 The dose-response relationship. A, "Classical" hyperbolic dose-response relationship. **B** and **C,** Examples of complex dose-response relationships: sigmoidal **(B)** and bell-shaped **(C).** The slope and shape of the curve reflect the mechanism of action of the drug. The potency is reflected by the position of the curve along the abscissa. The efficacy is reflected by the maximum position of the curve along the ordinate.

up-regulation. Thus changes in receptor number and affinity may well explain certain forms of drug tolerance, tachyphylaxis, and desensitization.

Changes in receptor binding, density, and coupling events also can occur with development. For instance, in the brain of the rat and pig, marked ontogenic changes in cholinergic muscarinic, α-adrenergic, and prostaglandin receptor density have been observed.[74-76] In addition, the pathophysiology of diseases such as testicular feminization, pseudohypoparathyroidism, myasthenia gravis, and certain forms of diabetes seems to involve receptor-associated dysfunctions.[77-79] Similarly, mutations of receptors can increase vulnerability to certain conditions or accelerate desensitization to receptor agonists, as reported for β-adrenoceptor agonists.[80] Thus developmental and pathologic considerations must be accounted for by clinicians and the investigators evaluating a response.

Relationship Between Drug Dose and Response

According to the receptor occupancy theory, response is proportional to receptor binding.[81] Thus maximal response is achieved when all receptors are bound. Although this concept has a degree of practical validity, interpretation of receptor binding often is difficult, particularly when the coupling events encompass a complex sequence of reactions. Such is the case when further receptor occupancy does not produce greater response; these observations have led to the concept of *spare receptors,* whereby a maximal response is achieved when a relatively small proportion of the receptors is occupied. Other, more complex receptor-evoked functions involve allosteric modulations and protein interactions.

The Allosteric Nature of G Protein–Coupled Receptor Signaling

The theoretical framework associated with the study of ligand-receptor interactions adheres to the law of mass action—essentially a simple reversible and saturable one-to-one interaction between ligand and receptor (Figure 20-4, *A*). This classical view has served many pharmacologists. Insights obtained from research on enzymes, ion channels, and hemoglobin, however, have led to the inference of more complex interactions between ligands and receptors. Essentially, more than one ligand may potentially interact with a receptive unit, yielding the concept of "cooperativity." This concept assumes conformational changes in the protein of interest as well as its interaction with adjacent partners, forming a dynamic oligomeric complex. Accordingly, specific molecules can be envisioned with sites distinct from the "classical" ligand-binding site, and consequently to affect this conformational state; such molecules are termed allosteric modulators. This model will yield ligand-receptor interactions of much greater complexity than those observed for the typical simple hyperbolic profiles (see Figure 20-4, *A*), and may include those for which the graphed data form a sigmoidal or bell-shaped curve (see Figure 4, *B* and *C*). (The slope and the shape of the curve reflect the mechanism of action of a drug. The potency describes the location of the curve along the *x*-axis and is influenced by the inherent affinity of the drug for its receptor and the latter's ability to couple with the postreceptor mechanisms. The efficacy is characterized by the maximum response observed regardless of dose-response profile.)

In the following discussion, G protein–coupled receptors (GPCRs), which have been the most thoroughly studied thus far, are used for the purposes of illustration. GPCRs constitute the largest superfamily of surface receptors. These receptors mediate a plethora of responses. The basic transduction unit of GPCRs is governed by reciprocal allosteric interactions occurring among three elements: (1) a seven-transmembrane domain receptor polypeptide, which binds the transmitter; (2) a trimeric (αβγ) G protein; and (3) an effector component. Binding of a transmitter leads to the exchange of GDP for GTP on the Gα chain. The activated G protein, through either its α-GTP chain or the β γ dimer, or both, in turn modulates the effector components, leading to the response. Traditionally known effectors modulated by GPCRs include enzymes such as adenylyl cyclase and phospholipases C as well as ion channels and antiporters. Recently, additional effector systems linked to the activation of receptor tyrosine kinases also were shown to be modulated by GPCR. In particular, the Erk1/Erk2/p38 MAP/JNK kinase signaling pathways were shown to be activated by stimulation of G proteins of the Gq, Gi, and Gs family by way of many distinct signaling pathways.[82-84] In addition to their G protein–mediated effects, GPCRs also have been found to interact directly and modulate the activity of an increasing list of proteins including: β-arrestins, NHERF/EBP50, JAK, endothelial nitric oxide synthase (eNOS), *N*-ethylmaleimide–sensitive factor (NSF), Rho and Arf, spinophilin, 14-3-3, Nck and Grb2, endophilins, CREB and ATFx, the gamma-aminobutyric acid isoform $GABA_A$, and the glutamate *N*-methyl-D-aspartic acid (NMDA) receptors (reviewed by Bockaert and associates[85]). In addition, contrary to traditional views, increasing evidence indicates that GPCRs exist as homo- and heterodimers (or even larger oligomeric assemblies), which may have important consequences on their signaling properties.[86,87] Although the physiologic importance of many of these interactions needs to be investigated further, the large number of proteins that appear to be involved in GPCR signaling suggests a level of allosteric complexity that was not anticipated just a few years ago.

Classically, orthosteric ligands are thought to bind to the receptor in the same space that is occupied by the endogenous ligand and to promote or stabilize specific receptor conformations. In the traditional two-state receptor activation model,[88] they were believed to control the equilibrium between an active and an inactive GPCR conformer. Increasing evidence, however, indicates that separate ligands can differentially regulate various signaling cascades engaged by a given receptor, suggesting that numerous active receptor conformations may exist and in turn result in different signaling efficacy profiles towards distinct effector systems.

One of the most striking illustrations of this phenomenon, known as *agonist trafficking,*[89] is the observation that β-adrenergic ligands that act as inverse agonists for the adenylyl cyclase pathway behave as agonists for the MAPK pathway after binding to the β_2-adrenergic receptor.[90] Similar observations also have been reported for the arginine vasopressin receptor 2,[90] the histamine receptor,[91] and the angiotensin receptor.[92] These findings led to the concept that distinct receptor conformations are responsible for the differential allosteric regulation of distinct subsets of signaling partners opening the avenue for the development of compounds that could selectively target only one of the receptor signaling modes. In this context, allosteric regulation of receptor conformation through homo- or heterodimerization, or mediated through interactions with various effectors and accessory proteins, opens many potential target sites for pharmacologic modulation. Receptors can be seen as allosteric machines for which pharmacologic potential has been only superficially explored; examples of allosteric modulators of receptors, thus far few, are oxytremorine,[93] THG113.31,[94] and SCH-202676.[95] The study and exploitation of allosteric phenomena will become of progressively greater importance in drug discovery and development, to enhance pathway selectivity and diminish adverse effects.[96]

In Vivo Efficacy

In vivo, potency also is influenced by disposition of the drug. In the clinical setting, potency per se should not be a determining factor in the selection of an agent. By contrast, maximal efficacy must be taken into consideration in choosing drugs. In all instances, toxicity of the agent must be taken into account. Under all therapeutic circumstances, the drug chosen ideally should provide the greatest margin of safety. This selectivity has been quantified as the *therapeutic index,* which usually is defined as the ratio of the median toxic dose to the median effective dose (TD_{50}/ED_{50}) and in laboratory studies as the ratio of the median lethal dose to the median effective dose (LD_{50}/ED_{50}).

CONCLUSION

Marked differences in drug disposition and action between the newborn and the adult are well recognized. As might be expected, such differences are even greater for the ill preterm neonate. These differences must be taken into consideration in decisions regarding pharmacologic therapy for newborns. Appropriate application of basic principles in pharmacology as well as adequate drug monitoring allows individualization of drug dosage and will optimize clinical outcomes in neonates by reducing adverse drug effects.[71]

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21 Basic Pharmacokinetic Principles

Ralph A. Lugo and Robert M. Ward

Pharmacokinetics describes the absorption, distribution, metabolism, and excretion of drugs. The pharmacokinetic parameters of a drug are used to characterize the drug concentrations reached within the body after a dose and the changes in those concentrations over time.[1] Clinical pharmacokinetics is the discipline that applies pharmacokinetic principles to individualize dosage regimens, optimize the therapeutic effects of a medication, and minimize the chances of an adverse drug reaction. These goals are accomplished by achieving an effective concentration of unbound drug at the site of action. Clinically important sites of action include receptors, membrane transport systems, intracellular enzymes, interstitial tissues in which infections may occur, and many others. Correlations have been made between drug concentrations in the circulation and effective and toxic drug concentrations at various sites of action.[2] Depending on the strength of those correlations, ranges of effective, toxic, and ineffective circulating drug concentrations have been defined for many drugs. Pharmacokinetics serves as a guide to effective therapy, but achieving specific concentrations is not the goal of therapy. Effective therapy can be best judged by improvements in function, not just by reaching the desired peak and trough concentrations in the circulation.

Although the mathematical principles used to describe pharmacokinetics have become quite sophisticated, with advanced mathematical modeling, statistical arm theory,[3] and complex noncompartmental analysis,[4] the clinical issues in dosing are simple. Once a drug has been selected, three primary factors must be decided: what route to use for administration, how large a dose to administer, and how often to administer the dose. The size of the dose relates to its distribution within the body, and the frequency of administration relates to clearance or the rate of removal of active drug from the body. In this chapter, the factors needed to determine these parameters of drug therapy are presented mathematically and then are simplified for bedside application.

PHARMACOKINETIC MODELS

Mathematical principles may be used to describe pharmacokinetic processes to predict a drug's behavior in the body. To apply these principles using a compartmental approach requires selection of a compartmental model that best represents a drug's distribution and rate(s) of concentration change in the body.[1] Compartmental models may be classified as one-compartment, two-compartment, or multicompartment models, depending on the number of exponential equations required to describe the concentration changes. Seldom does a drug require more than three exponential equations to characterize its kinetics. Each theoretical "compartment" consists of a group of similar tissues, fluids, or organs and may be correlated with different anatomic fluids and tissues.

In a one-compartment model, all body tissues are included in the model, and an intravenously administered drug is assumed to distribute instantaneously and homogeneously throughout the compartment.[2] In general, drugs that do not distribute widely into extravascular tissues can be described by a one-compartment model, such as aminoglycosides or very large molecules. This approach simplifies the calculations necessary for therapeutic drug monitoring of these agents, because only two or three concentration determinations may be needed to characterize the pharmacokinetics for dosage adjustments adequately.

With many drugs, however, the pharmacokinetics do not fit a one-compartment model because of slow distribution from the blood into other fluids and tissues: The drug reaches the central compartment, made up of blood or plasma and highly perfused organs, such as the heart, liver, kidneys, and lungs, and diffuses out to peripheral sites such as adipose tissue, cerebral spinal fluid, and muscles.[2] The disposition of such drugs is best described using a multicompartmental approach. Examples of drugs with such behavior are vancomycin, digoxin, and lipophilic drugs (e.g., fentanyl, midazolam, propofol). For the purposes of therapeutic drug monitoring in which only two or three plasma concentrations are obtained around an administered dose, characterization of the multicompartmental pharmacokinetics is not feasible or accurate. Thus this multicompartment approach is reserved for more detailed pharmacokinetic study, when more frequent plasma sampling can be obtained.

FIRST-ORDER ELIMINATION

The change in drug amount within the body can be described with the following general equation, in which A is the amount of drug within the body, k is the rate constant of change for A within the body, and n defines the order (e.g., zero, first, second) of the process.[5]

$$\frac{dA}{dt} = -kA^n \tag{1}$$

Most drugs are eliminated by first-order exponential rates *(k)* in which a constant fraction of the drug is eliminated per unit of time. If the kinetics is characterized by first-order elimination, the rate of change of drug in the body (dA/dt) is proportional to the amount in the body. Thus, at high concentrations, a greater amount of drug is eliminated per hour than at low concentrations. This higher elimination rate results in an exponential decline in plasma concentration (Figure 21-1), which can be represented by the following equation:

$$C_t = C_0 \times e^{-kt} \tag{2}$$

where C_t is the concentration at some time t, C_0 is the initial concentration at time 0, and e^{-kt} represents the exponential decline in plasma concentration associated with first-order elimination. The plasma concentration versus time relationship may be made linear by taking the natural logarithm of each side of Equation 2:

$$\ln C_t = \ln C_0 - kt \tag{3}$$

The slope of this straight line is the elimination rate constant k (time^{-1}), which may be calculated by rearranging Equation 3:

$$k = (\ln C_1 - \ln C_2)/\Delta t \tag{4}$$

where C_1 is the higher concentration and C_2 is the lower concentration measured some time later. If the logarithm (base 10) of concentration is graphed versus time, the slope will be $k/2.303$. The half-life of a drug exhibiting first-order elimination is the time

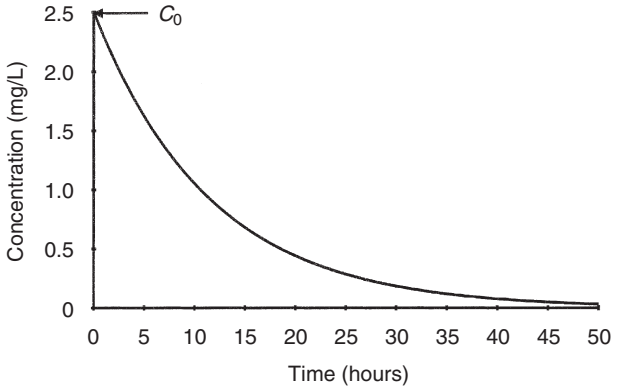


Figure 21-1 Exponential decrease in drug concentration after a rapid intravenous bolus injection graphed on linear-linear axes. The initial concentration (C_0) of 2.5 mg/L declines with a half-life of 8 hours.

necessary for the concentration in the plasma to decrease by one half. The relationship between the elimination rate constant and the half-life for a first-order process may be derived mathematically from Equation 3. For a drug whose initial concentration is 100 mg/L, the concentration at one half-life is 50 mg/L.

$$\ln 50 = \ln 100 - kt$$

$$kt = \ln (100/50)$$

which can be rearranged to

$$k = \ln 2 / t$$

where t represents one half-life; therefore

$$k = 0.693 / t_{1/2} \tag{5}$$

To illustrate the concept that the amount of drug removed per unit time decreases with declining amounts (or concentrations) of drug in the body, the amount of drug in the body at different times can be calculated with Equation 2 by substituting plasma concentration with the amount of drug in the body. For example, if a 100-mg dose of a drug with first-order elimination is administered by rapid intravenous bolus, and it has a 2-hour half-life (k = 0.693/2 h = 0.347 h^{-1}), a constant fraction of drug will be removed each hour: $e^{(0.347)(1)}$ = 29.3% of the amount of drug in the body. The amount of drug in the body each hour can be calculated to show the decreasing amount of drug removed each hour (Table 21-1).

First-Order Multicompartment Kinetics

If drug clearance from the circulation is studied thoroughly, with concentration measurements made multiple times soon after intravenous administration as well as during the terminal elimination phase, two or more exponential rates of clearance often are detected by a change in slope of a semilogarithmic graph of concentration versus time (Figure 21-2). The number and nature of the compartments for the clearance of a drug do not necessarily correspond to specific body fluids or tissues. When two first-order exponential equations are required to describe the clearance of drug from the circulation, the kinetics is designated first-order and two-compartment or biexponential.[6] Such kinetics commonly is observed for drugs that rapidly distribute from the central compartment (blood and highly perfused organs) to peripheral compartments after intravenous administration. Figure 21-2 illustrates the kinetics for such drugs. The initial rapid decrease in concentration is referred to as the α distribution phase and is primarily the result of drug distribution to tissues; however, elimination also is occurring. Accordingly, the slope of

TABLE 21-1

Amount of Drug Remaining in the Body Each Hour for a Drug Eliminated by First-Order Kinetics with a Half-Life of 2 Hours (k = 0.347 h^{-1})

Time (h)	Amount of Drug in Body (mg)	Drug Eliminated over Preceding Hour (mg)	Fraction of Drug Eliminated During Preceding Hour
0	100	0	0
1	70.7	29.3	0.293
2	50	20.7	0.293
3	35.4	14.6	0.293
4	25	10.4	0.293
5	17.7	7.3	0.293
6	12.5	5.2	0.293

this initial decrease in concentration is determined by both drug elimination and drug distribution. After the inflection point in the slope and during the terminal or β elimination phase of the curve, elimination accounts for most of the change in drug concentration and has a rate constant of β. The α distribution rate constant can be determined by subtracting the concentration during the initial period after drug administration from concentrations extrapolated to time zero from the β elimination phase (see Figure 21-2). The rate constant α is the slope of the natural logarithm of the concentration difference (Δ) versus time curve shown as the line of alternating dashes and dots in Figure 21-2. A more detailed mathematical discussion can be found elsewhere.[6,7] The equation that represents a first-order, two-compartment model is as follows:

$$C_t = Ae^{-\alpha}t + Be^{-\beta t} \tag{6}$$

where C_t is concentration at time t after the dose, A is the concentration at time 0 for the distribution rate represented by the broken line graph, α is the rate constant for the initial distribution phase, B is the concentration at time 0 for the terminal elimination phase, and β is the rate constant for terminal elimination. When concentration is graphed on semilogarithmic axes using logarithm (base 10) of concentration, the slope of the lines for α and β = α/2.303 and β/2.303, respectively.

Although many drugs demonstrate multicompartment kinetics, the intensive blood sampling required to fit data to more than one compartment usually is not clinically feasible, particularly in the newborn patient. Furthermore, the mathematical complexity of two-compartment models makes this kinetic approach clinically impracticable. To minimize cost and to simplify pharmacokinetic calculations, only two plasma concentrations—peak and trough—usually are obtained for therapeutic monitoring of commonly used drugs, such as gentamicin and vancomycin. Accordingly, a one-compartment model is assumed, and the elimination rate constant *(k)* is determined from the slope of these points plotted on a semilogarithmic scale. Because the elimination rate constant should be determined during the terminal elimination phase, it is important that blood samples for determining peak concentrations of multicompartment drugs not be drawn prematurely, that is, during the initial distribution phase. If samples are drawn too early, the high initial predistribution concentration will result in an overestimation of the slope and terminal elimination rate constant. Clinically, early sampling usually is not problematic with gentamicin because the initial distribution phase commonly occurs during the 30- to 60-minute infusion.[8] Thus a peak concentration obtained 30 to 60 minutes after the end of infusion usually reflects the terminal phase of elimination. With vancomycin, however, the half-life of the initial distribution phase is 0.05–0.45 hours.[9] Thus a peak vancomycin concentration determined using blood drawn prematurely may lead to error in estimating the pharmacokinetic parameters.

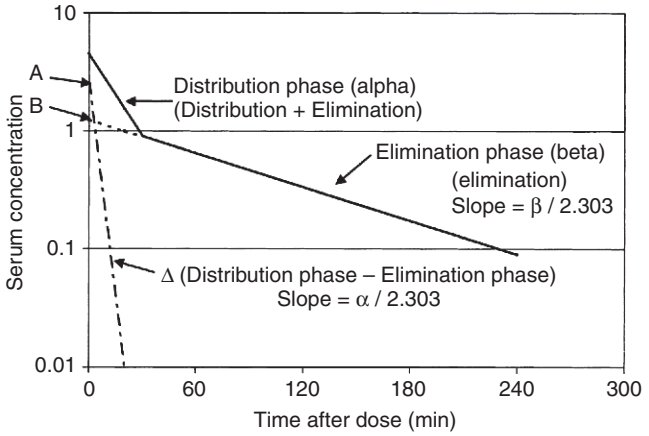


Figure 21-2 *A,* Two-compartment or biexponential kinetics graphed as a *solid line* on semilogarithmic axes with the initial rapid decrease in concentration resulting from distribution and elimination during the distribution phase (α) followed by the slower decrease in concentration during the elimination phase (β) with a slope of the elimination rate constant β/2.303. *B,* The intercept of the elimination phase extrapolated with a *dashed line* to time 0. The concentration difference (Δ) between the distribution and elimination phases is graphed with *dashes alternating with dots* with intercept A and a slope of the distribution rate constant α/2.303.

ZERO-ORDER ELIMINATION

Elimination of some drugs occurs with loss of a constant amount of drug per time, rather than a constant fraction per time as with first-order kinetics. When the rate of change in the amount or concentration of a drug is constant, these rates are described as zero-order rates, and the concentration can be calculated using the following equation:

$$C_t = C_0 - k_0 t \tag{7}$$

In this equation, C_t equals the concentration at time t, C_0 is the initial concentration, and k_0 (amount/time) is the rate constant for this process. Zero-order elimination may occur when the elimination process of a drug becomes saturated, that is, when the eliminating organ has reached the maximum capacity for drug removal. This may be referred to as *saturation kinetics.* Some drugs administered to newborns that are eliminated, at least in part, by zero-order kinetics include caffeine, chloramphenicol, diazepam, furosemide, indomethacin, and phenytoin.[10] Such elimination may occur for other drugs after excess doses or when the organ responsible for drug elimination becomes dysfunctional. With change from first-order to zero-order kinetics (dose-dependent kinetics), continuation of the same dosage usually leads to a rapid accumulation of drug, resulting in toxic drug concentrations.[10]

APPARENT VOLUME OF DISTRIBUTION

The *apparent volume of distribution* (V_d) is a mathematical term that relates the total amount of drug in the body to the drug concentration in the circulation. After a rapid intravenous bolus dose and assuming a one-compartment model, the following equation may be used to relate V_d, dose, and the change in circulating concentration.

$$\Delta \text{ Circulating drug concentration (mg/L)} = \tag{8}$$

V_d may be viewed as the volume of dilution into which the dose is added to produce the observed change in concentration. The larger the volume of distribution, the greater the dilution of a dose, and the smaller the increase in circulating concentration. Volume of distribution does not necessarily correspond to a true physiologic body fluid or tissue volume—hence the designation "apparent." For drugs that are extensively distributed to peripheral tissues, the V_d derived from changes in concentration within the circulation may be very large. For example, the V_d of digoxin in neonates and infants may reach 10 L/kg—an anatomic impossibility.[11] Large volumes of distribution indicate that tissue concentrations of drug may greatly exceed the concentration in the plasma, often because of protein binding in tissues or fat solubility. Drugs that distribute primarily into extracellular fluid (e.g., gentamicin) have small volumes of distribution (V_d of 0.25 to 0.35 L/kg in adults) that significantly increase in fluid overload states. In addition, because extracellular water makes up a larger percentage of body weight in premature and term infants, their volumes of distribution at birth will be larger for these drugs.

When administering a rapid intravenous bolus dose, Equation 8 serves as the basis for other pharmacokinetic calculations because it is easily rearranged to solve for V_d and dose. If a dose is infused over a longer period, a significant portion of the dose may be cleared from the body during the infusion, in particular for drugs with half-lives that are only three to four times longer than the infusion time. Accordingly, a more complex exponential equation that accounts for concurrent drug administration and drug elimination is required to describe the change in concentration. Such equations are needed, however, only when the drug is rapidly eliminated and the duration of infusion approaches the drug's half-life. In neonates, who often have relatively slow rates of drug elimination, only a small fraction of drug is eliminated during the time of infusion, and such adjustments often can be omitted. Accordingly, the simpler and more practical equation to estimate pharmacokinetic parameters may be used.

CLEARANCE

Clearance represents the capacity for drug removal by various organs and is defined as the volume of blood from which all drug is removed per unit of time. Both clearance and distribution volume are model-independent parameters, so plasma drug concentrations are determined by the rate at which drug is administered, its clearance, and the volume of distribution. Similarly, rate of elimination is determined from clearance (Cl) and V_d.

$$k = \text{Cl} / V_d \tag{9}$$

Drugs can be cleared through numerous pathways, although most drugs are cleared by some combination of renal clearance (Cl_R), hepatic clearance (Cl_H), and biliary clearance (Cl_B) (Figure 21-3). Thus the total systemic clearance of a drug is the sum of all the clearances by various mechanisms and can be calculated using the following equation:

$$Cl_S = Cl_R + Cl_H + Cl_B + Cl_{other} \tag{10}$$

Because it is seldom possible to calculate each organ's clearance of drug, total systemic clearance (Cl_S) is often determined by measuring the area under the plasma concentration–time curve (AUC) after a single dose:

$$Cl_S = \text{Dose} / \text{AUC} \tag{11}$$

During a continuous infusion, clearance may be determined easily by the relationship between the rate of infusion and the resultant steady-state concentration:

$$Cl_S = \text{rate of drug administration} / \text{steady-state drug concentration} \tag{12}$$

FIRST-PASS CLEARANCE

For some drugs, dramatic differences in concentrations and effects are seen for enteral versus parenteral administration, as a result of first-pass effect or presystemic drug clearance. During

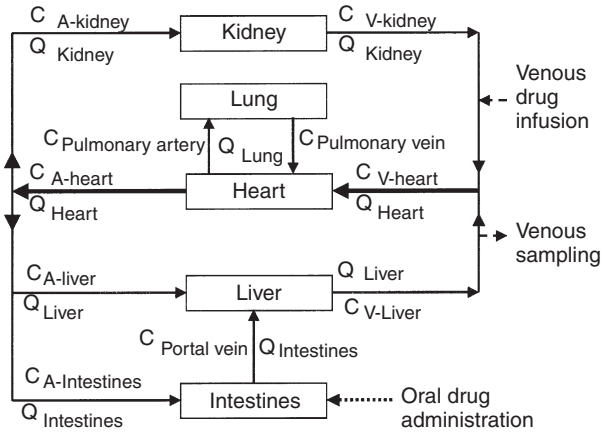


Figure 21-3 Components of drug clearance by several organs that combine to produce total body clearance in which C_A is arterial concentration, C_V is venous concentration, and Q is organ blood flow. First-pass clearance may occur during drug absorption from the intestines or during circulation of portal venous blood through the liver before it reaches the systemic circulation.

enteral absorption, drug passes through the intestinal wall, enters the portal venous circulation, and passes through the liver before reaching the systemic circulation (see Figure 21-3). Nearly complete metabolism may occur in the intestinal wall or the liver (especially for drugs metabolized by cytochrome P-450 3A4), so the amount of parent drug reaching the systemic circulation is only a small fraction of the dose administered.[12,13] The fraction *(F)* of the oral dose that reaches the systemic circulation is that which remains after hepatic or intestinal metabolism and is expressed as the extraction ratio (ER) in the following equation:

$$F = 1 - \text{ER} \tag{13}$$

F is determined from the ratio of the area under the plasma concentration curve after oral administration to that after intravenous administration. After an intravenous dose of medication, drug enters either the inferior or superior vena cava circulation, returns to the heart, and enters the systemic circulation before perfusing the liver. Drugs that undergo almost complete hepatic or intestinal metabolism before reaching the systemic circulation are described as having a high hepatic or intestinal intrinsic clearance. Drugs used in the care of newborns that exhibit moderate to significant first-pass, presystemic clearance in adults and infants include midazolam,[14] morphine,[15] and propranolol.[16]

STEADY STATE

Steady-state conditions prevail when plasma and tissue concentrations are in equilibrium and the amount of drug removed per unit time is equal to the amount administered per unit time. For drugs administered intermittently, peak and trough concentrations at steady state are unchanged after each dose. For drugs administered by continuous infusion, serum concentrations are constant. Constant serum concentrations, however, do not define steady state because distribution between tissues and the circulation may still be occurring.

The time to reach steady state is dependent only on the elimination rate constant and thus half-life. If drug is administered repeatedly at a fixed dosing interval, the time to reach greater than 90% of steady state is four or more half-lives (Table 21-2).

During long-term drug treatment, dose adjustments should be made when concentrations are close to steady state and generally not less than three half-lives after a previous dose adjustment, unless organ dysfunction is altering the half-life or volume of distribution.

TABLE 21-2

Percentage of Steady-State Concentration Reached After Drug Administration for One to Five Half-Lives

Number of Half-Lives Drug Is Administered	% of Steady-State Concentration
1	50
2	75
3	87.5
4	93.75
5	96.88

SINGLE-DOSE ADMINISTRATION BY SHORT-DURATION INFUSION

When drugs are infused over a period approaching 25% to 50% of the half-life, significant drug elimination occurs during the infusion process, and thus the standard intravenous bolus equation (e.g., Equation 8) is no longer accurate in calculating the change in concentration or the volume of distribution. A more complex mathematical model must be used to account for drug elimination during drug infusion.

To determine the concentration immediately at the end of the first infusion for a one-compartment model, Equation 14, which commonly is used to calculate concentration during a continuous infusion, may be modified:[17]

$$C_t = (R_0 / \text{Cl}) \times (1 - e^{-kt}) \tag{14}$$

where C_t is the concentration at any time (t) during the continuous infusion, R_0 is the rate of infusion, and k is the first-order elimination rate constant. During a continuous infusion, steady state (C_{ss}) is defined by the relationship between infusion rate (R_0) and clearance (Figure 21-4):

$$C_{ss} = R_0 / \text{Cl} \tag{15}$$

In Equation 14, it is intuitive to think of the factor $[(1 - e^{-kt})]$ as the percentage of steady state that is achieved at time t. Thus the concentration at any time t during the infusion process is the steady-state concentration (C_{ss}) multiplied by the percentage of steady state achieved at time t. As t becomes larger, the factor $[(1 - e^{-kt})]$ in Equation 14 approaches 1, resulting in steady-state Equation 15.

Equation 14 can be adapted easily for a single short-duration infusion. Accordingly, the duration of the infusion is represented by t, and C_t therefore represents the concentration immediately at the end of the infusion. Plasma concentrations anytime thereafter can be calculated by multiplying C_t by the decay factor ($e^{-kt'}$) for a first-order process (see Equation 2). Thus, the concentration measured at any time (t') after the completion of a single short-duration infusion can be calculated by the following equation[17]:

$$C_{t'} = R_0 / \text{Cl} \times [(1 - e^{-kt_{\text{inf}}})] \times e^{-kt'} \tag{16}$$

where C_t' is the concentration measured at some time t' after the end of infusion. The infusion time is represented by t_{inf} and often is 0.5 hour or 1 hour, and R_0 is the rate of infusion, defined by (dose/time of infusion). This single-dose equation is commonly used to calculate the measured peak and trough concentrations after administration of the first dose of an aminoglycoside or vancomycin.

Because $\text{Cl} = V_d \times k$ (derived from Equation 9), Equation 16 can be rearranged to solve for V_d after a single short-duration infusion.

$$V_d = [R_0 / (C_{t'} \times k)] \times (1 - e^{-kt_{\text{inf}}}) \times e^{-kt'} \tag{17}$$

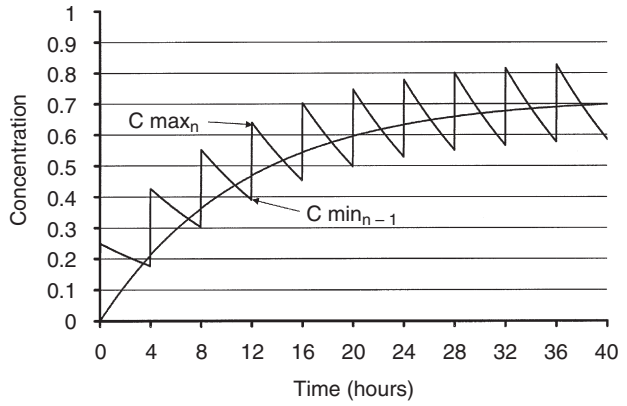


Figure 21-4 Plasma drug concentrations over time during a continuous intravenous infusion and during repeated dosing. For a continuous infusion, the rate of infusion and the clearance of the drug define the steady-state concentration, a plateau reached when the rate of elimination matches the rate of input. For multiple drug dosing using the same daily dose, the average steady-state concentration is the same as the steady-state concentration for a continuous infusion. During multiple drug dosing, the increase in concentration between the trough immediately before the nth dose, $C_{\min\,(n-1)}$, and the peak immediately after the nth dose, $C_{\max\,(n)}$, is the same increase noted after a single (i.e., first) dose of drug.

This equation represents the calculation of V_d after a single dose of drug. If pharmacokinetic parameters are calculated after multiple doses, the accumulation of drug must be taken into account.

MULTIPLE-DOSE ADMINISTRATION

In most clinical situations, pharmacokinetic parameters are calculated and dosage adjustments are made after multiple doses are administered. When multiple doses are administered at a fixed dosage interval (τ), concentrations will accumulate until steady state is achieved some five half-lives later (see Figure 21-4). The maximum concentration after the *nth* dose may be determined by adding the minimum concentration just before the nth dose (i.e., $n - 1$) and the maximum concentration achieved after the first dose is administered (see Figure 21-4). Mathematically, an *accumulation factor* may be derived that relates the maximum drug concentration after a single dose to drug concentration after n doses administered at a fixed dosage interval (τ). A full mathematical derivation is beyond the scope of this chapter and may be found elsewhere.[18] This accumulation factor *(R)* may be calculated using the following equation[17]:

$$R = (1 - e^{-nk\tau}) / (1 - e^{-k\tau}) \tag{18}$$

As n approaches ∞ (i.e., as steady state is achieved), the numerator of the accumulation factor approaches 1. Thus the steady-state accumulation factor is represented by the following equation:

$$\text{Steady-state accumulation factor} = 1 / (1 - e^{-k\tau}) \tag{19}$$

Accordingly, to calculate the maximum concentration after the nth dose of drug, the maximum concentration after a single dose ($C_{\max\,(1)}$) is multiplied by the accumulation factor:

$$C_{\max(n)} = C_{\max(1)} \times [(1 - e^{-nk\tau}) / (1 - e^{-k\tau})] \tag{20}$$

Similarly, to calculate the maximum concentration at steady state, the maximum concentration after the first dose is multiplied by the steady-state accumulation factor:

$$C_{\max\,(ss)} = C_{\max(1)} \times [1 / (1 - e^{-k\tau})] \tag{21}$$

The accumulation factor also can be used to calculate the minimum concentration after any number *(n)* of doses or at steady state by multiplying the minimum concentration after a single dose, $C_{\min\,(1)}$, by the appropriate accumulation factor.

$$C_{\min(n)} = C_{\min(1)} \times [(1 - e^{-nk\tau}) / (1 - e^{-k\tau})] \tag{22}$$

$$C_{\min(ss)} = C_{\min(1)} \times [1 / (1 - e^{-k\tau})] \tag{23}$$

From Equation 2, $C_{\min\,(1)} = C_{\max\,(1)} \times e^{-kt'}$, where $e^{-kt'}$ accounts for the first-order decline in concentration between the maximum concentration and the minimum concentration during time t'. Therefore, from Equations 22 and 23, the following equations are derived:

$$C_{\min(n)} = [C_{\max(1)} \times e^{-kt'}] \times [(1 - e^{-nkt}) / (1 - e^{-k\tau})] \tag{24}$$

$$C_{\min\,ss} = [C_{\max\,(1)} \times e^{-kt'}] \times [1 / (1 - e^{-k\tau})] \tag{25}$$

Accumulation After Multiple Intravenous Boluses

After a single, rapid intravenous bolus dose, $C_{\max\,(1)} = \text{dose}/V_d$ (derived from Equation 8); therefore the maximum concentration after the nth intravenous bolus dose—$C_{\max\,(n)}$—is determined by substituting $C_{\max\,(1)}$ into Equation 20, which is the product of the maximum concentration after a single dose (Dose/V_d) multiplied by the accumulation factor[17]:

$$C_{\max(n)} = \text{dose} / V_d \times [(1 - e^{-nk\tau}) / (1 - e^{-k\tau})] \tag{26}$$

To calculate the concentration at any time t' after administration of the nth dose, Equation 26 is multiplied by the exponential term for a first-order process:

$$C_{t'\,(n)} = \text{dose} / V_d \times [(1 - e^{-nk\tau}) / (1 - e^{-k\tau}] \times e^{-kt'} \tag{27}$$

At steady state, the accumulation factor term in the numerator ($1 - e^{-nkt}$) is equal to 1, which results in the following equation:

$$C_{t'\,ss} = \text{dose} / V_d \times [1 / (1 - e^{-k\tau})] \times e^{-kt'} \tag{28}$$

This equation may be rearranged to solve for dose and V_d so long as k and the concentration are known.

Accumulation After Multiple Administrations of Short-Duration Infusions

For a short-duration infusion, the $C_{\max\,(1)}$ after a single dose equals $R_0/Cl \times [(1 - e^{-kt_{inf}})]$, which adjusts for the amount of drug eliminated during the infusion. Thus the maximum concentration after the nth dose is the $C_{\max\,(1)}$ after a single dose multiplied by the accumulation factor; that is, $C_{\max\,(1)}$ is substituted into Equation 20[17]:

$$C_{\max\,(n)} = [R_0 / Cl] \times (1 - e^{-kt_{inf}}) \times [(1 - e^{-nk\tau}) / (1 - e^{-k\tau})] \tag{29}$$

To calculate the concentration at any time t' after completing the infusion of the nth dose, Equation 29 is multiplied by the exponential term for a first-order process:

$$C_{t'\,(n)} = [R_0 / Cl] \times (1 - e^{-k\tau_{inf}}) \times [(1 - e^{-nk\tau}) / (1 - e^{-k\tau})] \times e^{-kt'} \tag{30}$$

At steady state, the accumulation factor term in the numerator ($1 - e^{-nkt}$) is equal to 1, which results in the following equation:

$$C_{t'\,(ss)} = [R_0 / Cl] \times (1 - e^{-kt_{inf}}) \times [(1 - e^{-k\tau})] \times e^{-kt'} \tag{31}$$

In the hospital setting, pharmacokinetics often is calculated for aminoglycosides and vancomycin and usually is measured at steady state. Therefore Equation 31 represents the most important equation for the purposes of therapeutic drug monitoring. Equation 31 may be rearranged to solve for any desired parameter,

including V_d and dose. Recall that Cl = $V_d \times k$ (derived from Equation 9); thus

$$V_d = [R_0 / (C_{t'(ss)} \times k)] \times (1 - e^{-k\tau_{inf}}) \times [1 / (1 - e^{-k\tau})] \times e^{-kt'} \quad (32)$$

Similarly, because R_0 = dose/time of infusion (t_{inf}), Equation 31 can be rearranged to solve for dose:

$$\text{Dose} = [V_d \times t_{inf} \times C_{t'(ss)} \times k \times (1 - e^{-k\tau})] / [(1 - e^{-k\tau_{inf}}) \times e^{-kt'}] \quad (33)$$

where $C_{t'(ss)}$ is a steady-state concentration measured at time t' after the end of the infusion. In clinical practice, this is often the *measured peak concentration* (e.g., 1 hour after the end of infusion), a random concentration, or a *measured trough concentration.*

EXAMPLES OF MATHEMATICAL PHARMACOKINETIC DOSE ADJUSTMENTS

The foregoing equations may be used to adjust the dose and dosing intervals to attain desired peak and trough concentrations. We recommend a simple four-step approach: (1) calculate k (and half-life, $t_{1/2}$), (2) calculate V_d, (3) calculate a dosing interval and dose based on a desired peak and trough, and (4) check the peak and trough of the new dosage regimen.

Example 1

Use the four-step approach to solve the following: 100 mg of a drug is administered by rapid intravenous bolus every 8 hours. At steady state, the peak concentration measured 1 hour after the bolus is 10 mg/L, and the trough concentration measured immediately before the next dose is 2 mg/L. Calculate k, V_d, and the new dose to achieve a measured peak of 15 mg/L and a trough of 1 mg/L.

Step 1

Equations 4 and 5 are used to calculate the elimination rate constant and half-life, respectively.

$$k = [\ln(10) - \ln(2)] / 7\text{h} = 0.2299\ \text{h}^{-1}$$

Because the peak concentration was measured 1 hour after administration of the intravenous bolus, the Δt is 7 and not 8 hours.

$$t_{1/2} = 0.693 / 0.2299\text{h}^{-1} = 3.0\ \text{hours}$$

Step 2

Equation 28 may be rearranged to solve for V_d.

$$V_d = [\text{dose} / C_{t'ss}] \times [1 / (1 - e^{-k\tau})] \times e^{-k\tau'}$$

$C_{t'ss}$ is the measured peak concentration at steady state (10 mg/L), and $e^{-kt'}$ represents the decline in concentration between the end of the bolus and the measured peak, that is, 1 hour.

$$V_d = [100\text{mg} / (10\ \text{mg/L})] \times [1 / (1 - e^{-(0.2299)(8)})] \times e^{-(0.2299)(1)} = 9.45\ \text{L}$$

Step 3

Equation 4 can be rearranged to solve for Δt for a desired peak (15 mg/L) and desired trough (1 mg/L) concentration with a known k. This term is represented as the new dosing interval (τ).

$$\tau = [\ln(15) - \ln(1)] / 0.2299\text{h}^{-1} = 11.8\ \text{hours}$$

Because this desired peak concentration is measured 1 hour after the dose is administered, the dosing interval must include the additional hour. Thus the optimal τ = 12.8 hours, which can be rounded to a 12-hour dosing interval.

Equation 28 can be rearranged to solve for the new dose with a known V_d, k, and τ.

$$\text{Dose} = [C_{t'(ss)} \times V_d \times (1 - e^{-k\tau})] / e^{-kt'}$$

$C'_{t(ss)}$ is equal to the new desired measured peak concentration at steady state, that is, 15 mg/L; and $e^{-kt'}$ represents the decline in concentration between the end of the bolus and the measured peak, that is, 1 hour. Therefore

$$\text{Dose} = [15\text{mg/L} \times 9.45\ \text{L} \times (1 - e^{-(0.2299)(12)})] / e^{-(0.2299)(1)} = 167\ \text{mg every 12 hours}$$

Step 4

The new steady-state peak and trough can be measured using Equation 28.

$$C_{t'(\text{ss peak})} = 167\ \text{mg} / 9.45\ \text{L} \times [1 / (1 - e^{-(0.2299)(12)})] \times e^{-(0.2299)(1)} = 15.0\ \text{mg/L}$$

The trough is determined by changing t' from 1 to 12 hours.

$$C_{t'(\text{ss trough})} = 167\ \text{mg} / 9.45\ \text{L} \times [1 / (1 - e^{-(0.2299)(12)})] \times e^{-(0.2299)(12)} = 1.2\ \text{mg/L}$$

Example 2

In another patient, 100 mg of the same drug is infused over 60 minutes every 8 hours. The peak concentration measured 1 hour after the end of infusion is 10 mg/L, and the trough concentration measured immediately before the next dose is 2 mg/L. Calculate k, V_d, and the new dose to achieve a measured peak of 15 mg/L and a trough of 1 mg/L.

Step 1

Equations 4 and 5 are used to calculate the elimination rate constant and half-life, respectively.

$$k = [\ln(10) - \ln(2)] / 6\ \text{h} = 0.2682\text{h}^{-1}$$

$$t_{1/2} = 0.693 / 0.2682\text{h}^{-1} = 2.6\ \text{hours}$$

Step 2

Equation 32 is used to solve for V_d.

$$V_d = [R_0 / (C_{t'(ss)} \times k)] \times (1 - e^{-kt_{inf}}) \times [1 / (1 - e^{-k\tau})] \times e^{-kt'}$$

$$V_d = \frac{(100\ \text{mg} / 1\ \text{h}) \times [1 - e^{-(0.2682)}] \times e^{-(0.2682)(1)}}{[10\ \text{mg/L} \times 0.2682\ \text{h}^{-1}] \times [1 - e^{-(0.2682)(8)}]} = 7.6\ \text{L}$$

Step 3

Equation 4 can be rearranged to solve for Δt for a desired peak (15 mg/L) and desired trough (1 mg/L) concentration with a known k. This term is represented as the new dosing interval (τ).

$$\tau = [\ln(15) - \ln(1)] / 0.2682\ \text{h}^{-1} = 10.1\ \text{hours}$$

Because this desired peak concentration is measured 1 hour after a 1-hour infusion, the dosing interval must include the additional 2 hours. Thus the optimal τ = 12.1 hours, which can be rounded to a 12-hour dosing interval.

The new dose is calculated using Equation 33:

$$\text{Dose} = [V_d \times t_{inf} \times C_{t'ss} \times k \times (1 - e^{-kt})] / [(1 - e^{-kt_{inf}}) \times e^{-kt'}]$$

$$\text{Dose} = \frac{[7.6\ \text{L} \times 1\ \text{h} \times 15\ \text{mg/L} \times 0.2682\ \text{h}^{-1} \times (1 - e^{-(0.2682)(12)}]}{[(1 - e^{(0.2682)(1)}) \times e^{-(0.2682)(1)}]} = 164\ \text{mg every 12 hours}$$

Step 4

The new steady-state peak and trough can be measured by using Equation 31.

$$Ct'_{(ss)} = [R_0/\mathrm{Cl}] \times (1 - e^{-\mathrm{kt}_{\mathrm{inf}}}) \times [1/(1 - e^{-k\tau})] \times e^{-kt'}$$
$$= [(164\ \mathrm{mg}/1\ \mathrm{h})/(7.6\ \mathrm{L} \times 0.2682\ \mathrm{h}^{-1})] \times (1 - e^{-(0.2682)(1)}) \times [1/(1 - e^{-(0.2682)(12)})] \times e^{-(0.2682)(1)}$$
$$= 15.0\ \mathrm{mg/L}$$

The trough is determined by changing t' from 1 to 11 hours. This represents the Δt between the end of infusion and the measured trough.

$$C_{t'\,ss\ \mathrm{trough}} = 1.03\ \mathrm{mg/L}$$

EXAMPLES OF SIMPLIFIED BEDSIDE PHARMACOKINETIC DOSE ADJUSTMENTS

In the clinical setting, in which drugs are administered according to dosing intervals of 4, 6, 8, 12, 18, or 24 hours, approximations of pharmacokinetic values can be used to adjust dosages at the bedside using the simple equation for bolus doses (e.g., Equation 8) and calculators without linear regression or logarithmic functions. This approach only applies, however, to drugs that have half-lives that are severalfold longer than the time required for drug administration. Because drugs administered to neonates often have prolonged half-lives compared with the duration of administration, these bedside techniques are readily applicable, particularly for aminoglycosides and vancomycin. At steady state, volume of distribution can be calculated from the trough preceding a dose and the subsequent peak concentration as outlined earlier without adjusting for drug eliminated during administration. The half-life can be closely approximated by dividing the time between the peak and trough concentration (Δt) by the number of times the concentration decreased in half. When a fraction of a half-life has elapsed, the half-life can be approximated by assuming a linear process, whereas in fact it is exponential. If the exact fall in concentration is calculated, the concentration decreases during the first half of a half-life by approximately 59% of the total decrease during that half-life. This decrement introduces a small error, but it is seldom large enough to alter the outcome of the dosage adjustment, because times for drug administration are rounded off to fit fractions of the 24-hour clock.

EXAMPLE 1

Gentamicin, in a dose of 2.5 mg/kg, is infused over 30 minutes every 12 hours, resulting in a steady-state trough concentration of 1.9 mg/L and a peak of 4.5 mg/L, which was sampled 30 minutes after the end of the infusion. Recall that at steady state, the next trough (11 hours after the peak) also will be 1.9 mg/L.

Calculation of V_d

$$\Delta\ \mathrm{Concentration}(\mathrm{mg/L}) = \frac{\mathrm{Dose}\ (\mathrm{mg/kg})}{V_d(\mathrm{L/kg})}$$

$$C_{\mathrm{peak}} - C_{\mathrm{through}} = \frac{2.5\ \mathrm{mg/kg}}{V_d(\mathrm{L/kg})}$$

$$V_d(\mathrm{L/kg}) = \frac{(\mathrm{mg/kg})}{4.5 - 1.9(\mathrm{mg/L})} = 0.96\ \mathrm{L/kg}$$

Calculation of Half-Life

The half-life can be calculated from the time between the peak concentration of 4.5 mg/L and the next trough concentration of 1.9 mg/L.

$$t_{1/2}\#1: \text{Concentration decreases from 4.5 to 2.25 mg/L}$$

$$t_{1/2}\#2: \text{Concentration decreases from 2.25 to 1.12 mg/L}$$

In this example, the concentration decreases from 2.25 to 1.9 mg/L, which represents a decline of 0.35 mg/L during the second half-life, or 31% of the expected decline of 1.12 mg/L (0.35/1.12 = 31%). Thus an estimated 1.31 half-lives has elapsed.

$$t_{1/2} = 11\ \mathrm{h}/1.31\ \text{half-lives} = 8.4\ \text{hours}$$

This result is close to that obtained by the mathematically purer calculation of half-life using Equation 4:

$$t_{1/2} = 0.693/[(\ln 4.5 - \ln 1.9)/11\ \mathrm{h}] = 8.8\ \text{hours}$$

Dosage Adjustment

If the desired peak concentration is 6 mg/L for effective treatment of infection, the volume of distribution can be used to adjust the next dose after reaching the trough of 1.9 mg/L to achieve that concentration.

$$\Delta\ \mathrm{Concentration}\ (\mathrm{mg/L}) = \frac{\mathrm{Dose}\ (\mathrm{mg/kg})}{V_d(\mathrm{L/kg})}$$

$$C_{\mathrm{peak}} - C_{\mathrm{through}} = \frac{2.5\ \mathrm{mg/kg}}{V_d(\mathrm{L/kg})}$$

$$0.96\ \mathrm{L/kg} \times (6 - 1.9)\ (\mathrm{mg/L}) = \mathrm{dose}(\mathrm{mg/kg})$$
$$3.9\ \mathrm{mg/kg} = \mathrm{dose}\ (\mathrm{mg/kg})$$

If the half-life and the dosing interval remain constant, increasing the peak to 6 mg/L will raise the subsequent trough concentration to a level associated with nephrotoxicity (i.e., 2 mg/L). To maintain a trough of approximately 1.5 mg/L, the dosing interval must be increased to two half-lives or 16.8 hours (one half-life elapses as the concentration decreases from 6 to 3 mg/L, and the second as the concentration decreases from 3 to 1.5 mg/L). An additional hour must be added because the dose is infused over 30 minutes and the peak is obtained 30 minutes after the end of infusion. Thus the dosing interval of 17.8 hours is rounded to 18 hours. This regimen will maintain a trough of 1 to 2 mg/L and will achieve a peak of approximately 6 mg/L.

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22

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Physicochemical and Structural Properties Regulating Placental Drug Transfer

Evidence has accumulated that essentially all pharmacologic agents, and other exogenous substances as well, are transferred to the embryo and fetus, regardless of whether this transfer is wanted, as in intrauterine nutrition[1] or medical treatment of the fetus,[2-4] or unwanted, with possible teratogenic or toxic fetal effects.[4,5] Because of the increasing numbers of readily available over-the-counter drugs taken as self-medication by women who may be unaware of an early pregnancy or of possible adverse effects to a fetus, demands on public information are growing.[6,7] An appreciation of these considerations is important in designing meaningful toxicokinetic and drug disposition studies.[8] For such studies, it is desirable to identify parameters that determine the extent (total amount) and rate (amount per time) of placental transfer of a specific compound.

METHODOLOGIC APPROACHES

Although investigations of the pharmacokinetics of clinically indicated drugs in the maternal circulation can easily be performed, fetal sampling at earlier gestational stages is restricted to rare procedures (e.g., ultrasound-guided exchange transfusions, termination of pregnancy after maternal ingestion of the drug) and the moment of delivery, when cord blood samples can be obtained. Thus serial sampling usually is not possible, and the transplacental kinetics must be pieced together from many different subjects studied at various times.

Analysis of amniotic fluid leaking after premature rupture of membranes may allow an indirect approach to assessment of the distribution of xenobiotics from the mother to the fetus. As an alternative to fetal blood sampling, Jauniaux and Gulbis[9] introduced coelocentesis to study placental drug transfer in the first trimester, when coelomic and amniotic fluid are important compartments and the permeability of the placenta is greater than at term. Ascertaining the presence or absence of compounds or their metabolites in meconium samples gained from the newborn after birth also can be helpful to obtain qualitative data on intrauterine exposure; this approach already is used to detect maternal drug abuse in pregnancy.[10,11] These methods, however, do not allow use of kinetic models based on blood or plasma samples, which are necessary to allow extrapolating quantitative drug transfer to the fetus and subsequent risk assessment.

When more than one isolated sample is needed to establish a kinetic model (distribution between maternal and fetal circulations), the use of animal models is helpful. Their relevance with regard to human kinetics may be limited, however, because of difficulties in extrapolating the experimental results to humans.[12] Humans and guinea pigs have a hemomonochorial placenta; the rabbit has a hemodichorial placenta; and the rat has a hemotrichorial placenta.

In the rodent, the yolk sac placenta is of paramount importance during the entire period of organogenesis, as well as later in development; the hemoendothelial (hemotrichorial) placenta starts to function from the late organogenesis stage onward.[13-15] By contrast, in monkeys and humans, the yolk sac plays a much more limited and poorly understood role, and the hemomonochorial placenta starts to function at an early stage.[13-15] It is not completely understood how the species differences in placental morphology influence the transfer of exogenous compounds to the embryo and fetus.

If extrapolations from toxicologic in vitro models are planned, then the maternofetal interface must be taken into consideration.[16] The presence of membrane barriers around the developing embryo in the whole-embryo culture system could prevent the water-soluble all-*trans*-retinoyl-β-D-glucuronide from reaching the conceptus and there exerting its teratogenic potential, which, in other models without such lipophilic barrier (e.g., the limb bud organ culture), is equal to the lipid-soluble all-*trans*-retinoic acid.[17] Positron emission tomography–based study of placental transfer using radiolabeled drugs (e.g., ^{11}C-labeled heroin) may become a useful technique but probably will not be suitable for investigations in pregnant women.[18]

Human placenta obtained atraumatically at birth provides a way to investigate the human organ in in vitro perfusion studies.[19] Both maternofetal clearance and fetomaternal clearance can be standardized by comparison with antipyrine clearance. Limitations of these models include the following: The experimental period is limited to a few hours; the only available material is human placenta at term (versus that in early gestational stages, which may be more relevant with regard to toxic effects); and this type of model does not allow the observation of physiologically relevant parameters, such as blood flow (important for rapidly transferred compounds) or plasma protein binding.

PLACENTAL TRANSFER

Both rate and extent of transfer depend on the physicochemical and structural characteristics of drugs, as well as on physiologic characteristics of the maternal–placental–embryonic-fetal unit.[20-26] The concept of the placenta as lipoid membrane is useful to describe the influence of physicochemical characteristics of drugs on their placental transfer. Most drugs cross the placental membranes by diffusion, the rate of which is governed mainly by physicochemical factors according to Fick's law:[20-24,26]

$$\text{Rate of diffusion} = D \times \Delta c \times A / d \quad (1)$$

where A = area of exchange, d = membrane thickness, Δc = drug concentration gradient across the membrane (e.g., difference between maternal and fetal plasma drug concentrations), and D = diffusion constant for the drug.

From this equation, it may be predicted that a large area of placental exchange (A) consisting of membranes with limited thickness (d) favors placental transfer of drugs. A, d, and Δc can be determined in a model; D is far more difficult to predict, however, because it results from the interactions between the membrane and the molecule. The resistance within the tissue layers interposed between the maternal and fetal circulations (compartments) limits the diffusion, which is significant for hydrophilic molecules. In the human placenta, two

layers contribute to diffusional resistance: the trophoblast and the endothelium. Hydrophilic molecules either have to pass through these layers (i.e., the *membrane hypothesis*) or find their way through water-filled channels that extend through the trophoblast and communicate with the intracellular channels of the endothelial layer (i.e., the *aqueous pores hypothesis*). Such a process is known to be present, but it is not efficient in the intestinal mucosa.[27] Rapid placental transfer is therefore related to better lipid solubility and low ionization and protein binding of drugs with a molecular weight less than 500.

The permeability of lipid-soluble substances is much higher. For these substances, therefore placental flux rate is limited mainly by availability of the drug (resulting in the initial maternofetal concentration gradient, Δc) at the area of exchange, which is determined by uterine and umbilical blood flow.[28] In vivo, however, the placenta cannot be described by area and thickness. Indeed, earlier studies noted that as placental thickness and the number of placental layers decreased and the area of exchange increased during gestation, increased placental transfer occurred.[20,22-26] Thornburg and Faber[27] found that in rabbit placenta, the fetal endothelium, which is not markedly altered during pregnancy, is the layer defining the transfer rate of many drugs; area of exchange *(A)* and membrane thickness *(d)* apparently were of secondary importance. The diffusion of drugs across the hemotrichorial placenta (mouse, rat) often is faster than across hemomonochorial placenta (monkey, human) despite greater numbers of placental membranes. The sheep epitheliochorial placenta allows only small molecules to pass its "aqueous pores."

This chapter is concerned primarily with the influence of such physicochemical parameters of drugs on their placental transfer. The foregoing discussion summarizes useful concepts, but the outline of events presented in this section constitutes an oversimplification of a complex process. Thus structural characteristics of drugs must play an important role, although little is known about this important aspect.

PHARMACOKINETIC MODELS OF MATERNOFETAL DRUG EXCHANGE

The rate and the extent of drug transfer to the embryo and fetus are determined by numerous variables, listed in Table 22-1.[12] Several general types of pharmacokinetic concentration-time curves can therefore be observed in the maternofetal unit[12,29,30] (Figure 22-1). Furthermore, the rate of transfer may change during the course of treatment.[31] Model A in Figure 22-1 is applicable for a drug that crosses the placenta rapidly and distributes rapidly within a single fetal compartment, which quickly attains equilibrium with the maternal compartment. Fetal concentrations rapidly rise to reach maternal plasma levels; thereafter, the fetal and maternal curves overlap. When maternofetal exchange is rapid, two additional models are possible: After equilibrium between the maternal and fetal compartments has been attained, fetal concentrations may exceed maternal plasma levels (see curve in model C in Figure 22-1), or they may be less than corresponding maternal plasma levels (see curve in model E). As discussed later on, differential protein binding or a pH gradient in the maternofetal unit may be responsible for the relatively large (curve in model C) or small (curve in model E) extent of fetal drug exposure after equilibrium between the maternal and fetal compartments has been attained.

When the rate of placental transfer is low, model D in Figure 22-1 often is applicable in the maternofetal unit. The rise in drug concentration in the fetus is slow. However, because the transport of drug from the fetus back to the mother also is slow, fetal concentrations exceed maternal plasma levels after the crossover point of the two curves. Thus the fetus can be considered to represent a "deep" or "slow" compartment. A protein binding or pH gradient may affect the fetomaternal concentration gradient after postdistribution equilibrium in model D. In model F, the fetal concentrations never reach the corresponding maternal plasma values because the plasma protein binding or the pH gradient may favor higher maternal than fetal concentrations. Alternatively, efficient metabolism by the fetus or fetal excretion (e.g., into amniotic fluid) also may be responsible for the relatively low fetal drug levels. In model B, which is seldom observed, drug transport is slow from mother to fetus but rapid from fetus to mother. This situation implies an active or facilitated transport system, which typically is not a feature of therapeutic agents.

Such considerations in defining models may appear on first glance to be of theoretical interest only. These models are of

TABLE 22-1

Factors Determining Rate and Extent of Drug Transfer to the Embryo or Fetus

	CHARACTERISTICS	
Transfer Feature	***Drug***	***Maternal-Placental-Fetal Unit***
Rate	Lipid solubility	Placental structure and function
	Molecular weight	Placental blood flow
	Structural characteristics	Thickness of placental membranes
	Protein binding*	
	Type of transfer†	
Extent	Degree of ionization (pK_a)	Maternal-fetal pH gradient
	Protein binding	
	Type of transfer†	

*If transfer is relatively slow.
†Passive diffusion, facilitated or active transport, pinocytosis.

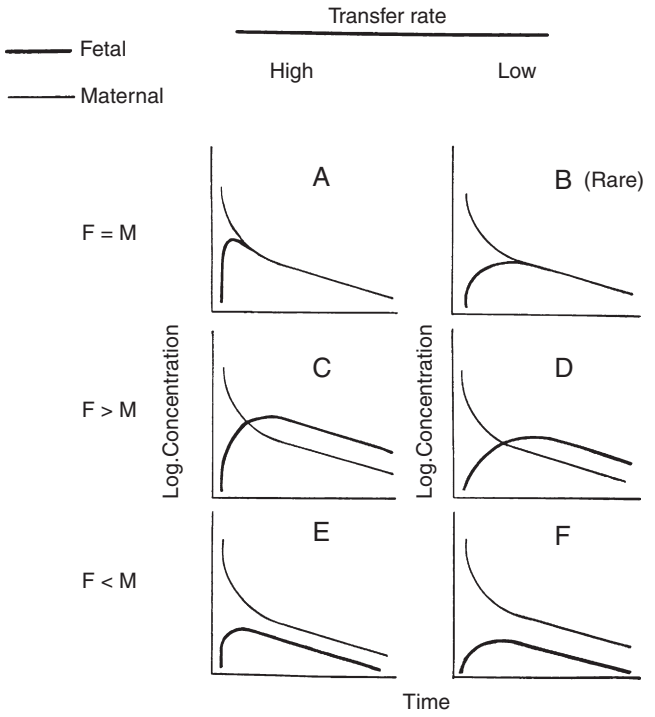


Figure 22-1 Simulation of maternal (M) and fetal (F) plasma concentration–time curves from various transplacental pharmacokinetic models. Example drugs are listed in Box 22-1.

extreme importance, however, in interpreting results from experimental and clinical studies. When kinetic curves are inadequately defined, results may be completely misinterpreted. Owing to ethical and practical limitations, often only one or a few fetomaternal sample pairs are available for analysis in clinical studies. If these samples were taken before crossover (see model C in Figure 22-1), for example, the wrong conclusion (i.e., relatively low fetal exposure) could be reached. For an adequate description of fetal exposure, both the maternal and the fetal concentration-time curves must be defined. Although such complete data on placental transfer are rare, attempts have been made to assign the kinetics of some drugs, on a tentative basis, to the models defined in Figure 22-1; the results of various studies are compiled in Box 22-1.

The foregoing discussion makes clear why it is difficult to identify parameters that determine the placental transfer of drugs. The selection of the correct model is a prerequisite for rational interpretation of the relative importance of such parameters, such as physicochemical properties of drugs.[32] Drugs have the greatest chance of crossing the placenta if they (1) are lipid-soluble, (2) are weak acids, and (3) are of a low molecular weight (less than 500). Another important factor is protein binding.[8]

LIPID SOLUBILITY OF DRUGS

According to the basic concept of drug diffusion through a lipoid barrier, lipophilic drugs (high lipid–water partition coefficient *K*—poor water solubility) should be rapidly transferred across the placenta (panels on the *left* in Figure 22-1); the reverse should be true for hydrophilic drugs (low lipid–water partition coefficient *K*—high water solubility).[20-24,27,33] Thus substances such as thiopental, secobarbital, propofol, and antipyrine cross the placenta extremely rapidly.[20,26,34]

In the pregnant rat, the parathion metabolite *p*-nitrophenol is rapidly transferred into the fetus, where its elimination half-time is the longest of all tissues examined.[35] Bisphenol A reaches peak levels in maternal blood and fetus 20 minutes after oral administration of a single dose to the rat, with the fetal concentration attaining approximately 60% of the level detected in maternal blood.[36] Cocaine, a weak base characterized by high lipid solubility, low molecular weight (305), and low plasma protein binding (8% to 10%), quickly equilibrates between the maternal and fetal compartments, as does its metabolite cocaethylene.[37-40] More polar compounds, such as the barbiturates butethal and phenobarbital or the more hydrophilic cocaine metabolite benzoylgonine, cross the placenta more slowly. The highly lipid-soluble benzodiazepine receptor agonist abecarnil (log $P = 4.6$) reaches higher concentrations in blood of the rabbit fetus than in maternal blood, whereas its highly polar and consequently hydrophilic metabolites cannot cross the placental barrier to the same extent.[40] Although no particular influence of lipophilia or other chemostructural properties determining the extent of maternofetal transfer of a group of opioids (fentanyl > alfentanil > sufentanil) was observed, especially in the event of fluctuation in maternal flow, lipophilicity was found to be an important factor when the opioid transfer is compared with that of antipyrine.[41] Another highly lipophilic opiate, buprenorphine (with a molecular weight of 504.1 and extensive binding to α- and β-globulins but barely detectable binding to albumin), shows marked sequestration to tissues including the placenta but only a low rate of transplacental transfer (less than 10%), in spite of its high lipid solubility. These properties could explain why neonates born to mothers treated with buprenorphine during pregnancy rarely demonstrate withdrawal signs and symptoms.[42]

Comparison of the in vitro maternal-fetal gradients of antibiotics used to treat mother and fetus for perinatal infections (e.g., chorioamnionitis) or conditions associated with an increased risk for infection (e.g., prolonged rupture of membranes) revealed extremely limited placental transfer of clavulanate and ticarcillin despite low plasma protein binding (9% and 45%, respectively) and molecular weight (236 and 428) for these drugs. The equally large but more lipophilic molecule ceftizoxime (with less than 30% protein binding and a molecular weight of 406) reaches higher concentrations in the fetus than in the mother.[43,44]

Exogenous bases such as 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine and the more lipophilic antiviral drug

BOX 22-1

Examples of Drugs with Transplacental Pharmacokinetics According to Models A to F in Figure 22-1*

Model A or B	Model C	Model D	Model E	Model F
Some barbiturates	**Some benzodiazepines**	Ascorbate	**Amide-type local anesthetic agents**	Heparin
Thiopental	Diazepam	Colistimethate†	Lidocaine	TCDD
Pentobarbital	Lorazepam	Furosemide	Bupivacaine	**Quaternary ammonium compounds**
Secobarbital	Desmethyldiazepam	Meperidine	**Some β-adrenoceptor blockers**	Tubocurarine
Antipyrine	Oxazepam	**Some cephalosporins**	Propranolol	Succinylcholine
Promethazine	Valproate	Cephalothin	Sotalol	Vecuronium
Ritodrine	Salicylate	Cefazolin	Labetalol	Pancuronium
Magnesium (sulfate)	Nalidixic acid	Cephapirin	Dexamethasone	Fazadinium
Thiamphenicol	Nicotine	Cephalexin	Cimetidine	Alcuronium
Digoxin	Urea	**Some aminoglycosides**	Ranitidine	**Elemental ions:** Cd^{2+}, Hg^{2+}
	Some penicillins	Gentamicin	Methadone	Fenoterol
	Ampicillin	Kanamycin	**Some sulfonamides**‡	Chlorthalidone
	Penicillin G			Etozolin (ozolinone)
	Methicillin			Dicloxacillin
	Azidocillin			Erythromycin
				Nitrofurantoin

Data from references 20-26, 30, 55, 60, 65, 71, 77, 82, 90, 111, 172, 174-176, 178-180, 196.

*Differentiation between models A and B, C and D, and E and F often is uncertain because of incomplete data on the initial phase of drug distribution across the placenta; model B is rarely applicable.

†Polypeptide antibiotic; molecular weight, 1200.

‡First and second trimesters.

TCDD, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin.

azidothymidine cross the placental barrier rapidly by simple diffusion.[45-47] The purine derivative 2′,3′-dideoxyinosine, however, crosses the placental barrier more slowly than the pyrimidines.[45,48] Again, a marked difference between their partition coefficients has been reported, with $P_{azidothymidine}$ = 0.86 to 1.26, as opposed to $P_{dideoxyinosine}$ = 0.07 to 0.32.[48] In vitro, and in women before termination of pregnancy, therapeutic maternal levels of azidothymidine resulted in fetal levels within the therapeutic range as well.[49,50] In one study, umbilical cord sample levels ranged as high as 113% to 127% of maternal levels, probably reflecting the prolonged elimination half-life in the newborn.[51] This finding raises the possibility of efficacious preventive treatment for the conceptus but also points to a high potential for adverse fetal effects. Within the human placenta, the drug is metabolized to more polar and still-undefined metabolites, which are not released into the perfusates, probably as a consequence of their reduced lipid solubility.[52] A more polar glucuronidated metabolite could cross the human perfused placenta at approximately half the rate observed for azidothymidine.[52] Tuntland and colleagues[53] proposed models that predict the extent of placental transfer of dioxynucleoside drugs. Predicted fetal-maternal steady-state plasma concentration ratios using their in vitro partition coefficient model deviated from observed values by only 3.9% ± 79% (mean % ± SD). In vivo and in vitro clearance indices were highly and significantly related to the drug octanol-water partition coefficient[53] (Figure 22-2, *A*).

Another drug in the same group of compounds is acyclovir, which is taken orally by women with recurrent genital herpes simplex infections during late pregnancy to reduce the risk of transmission of herpes simplex to the fetus. As a lipophilic compound with low plasma binding (22% to 33%), it also appears to be quickly transferred by simple diffusion.[54] The highest levels of this compound were measured in the amniotic fluid, probably secondary to unchanged renal excretion of the drug by the fetus, thus providing additional protection against ascending viral infection.[54] Placental transfer of hydrophilic substances is proportional to the coefficient of free diffusion in water, with some evidence of steric restriction of larger molecules (see Figure 22-2, *B*).[27]

The relationship between partition coefficients and placental transfer does not hold for drugs with a high or low value for the partition coefficient *K*. If *K* is large, the substance may be so tightly bound to membranes or other cell constituents (proteins, lipids) that it cannot readily cross into an aqueous phase from the lipid phase. Examples of drugs in this category are cyclosporine and chlorinated aromatics, such as 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD), which are extremely lipophilic but are transferred to the embryo and fetus in minute amounts only.[55,56] These drugs are stored in maternal liver and fat. Therefore the low fat content of the conceptus may be an additional factor limiting the placental transfer of these compounds. This concept is supported by the finding that TCDD is efficiently transferred by mother's milk, which contains a high level of fat.[57] Phylloquinone (vitamin K_1), supplemented in pregnant women whose infants are at risk for spontaneous hemorrhage, is another compound that despite its high lipophilicity is transferred through the human placenta (at

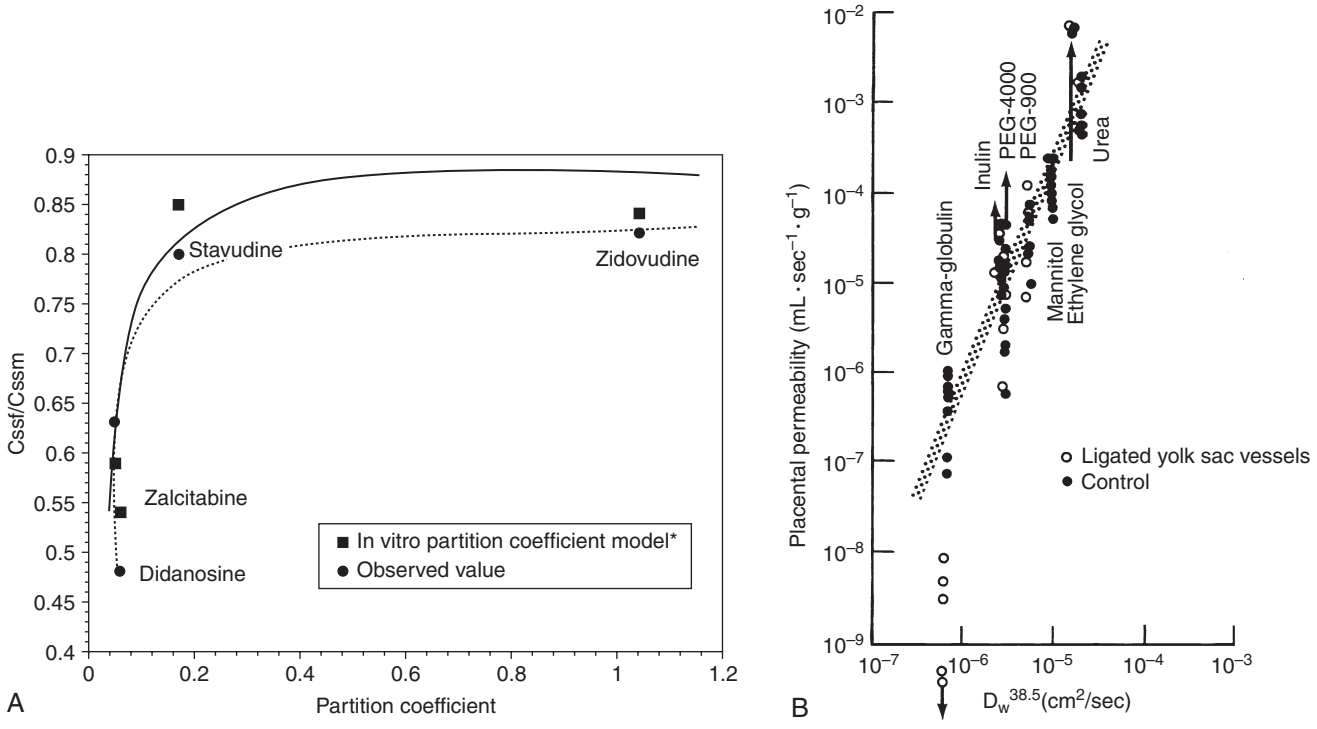


Figure 22-2 A, Comparison of an observed and in vitro partition coefficient model of predicted fetal-maternal steady-state plasma concentration ratios ($C_{ss\,f}/C_{ss\,m}$). Details regarding the model can be found in the original publication by Tuntland and associates.[53] The octanol-water partition coefficient is taken as the mean of reported values. **B,** Permeability of guinea pig placenta in fetuses with *(closed circles)* and without *(open circles)* intact vitelline circulations, as functions of coefficients of free diffusion in water at 38.5° C. The linear regression line is a least-squares fit of a logarithmically transformed datum, and the *shaded area* is the 95% confidence interval of means. γ-Globulin data are shown but were not used in computation of regression, because γ-globulin transfer is not a diffusional process in chorioallantoic placenta. *PEG,* Polyethylene glycol. *(**A,** Modified from Tuntland T, Odinecs A, Pereira CM, et al: In vitro models to predict the in vivo mechanism, rate and extent of placental transfer of dideoxynucleoside drugs against human immunodeficiency virus,* Am J Obstet Gynecol *180:198, 1999; **B,** from Thornburg KL, Faber JJ: Transfer of hydrophilic molecules by placenta and yolk sac of the guinea pig,* Am J Physiol *2:C111, 1977.)*

22 to 34 gestational weeks) only slowly and in minor quantities.[58] Data suggest, however, that if with an adequate lag period between maternal supplementation and delivery, maternofetal transport of the vitamin can be enhanced.[58]

In the perfused term rat placenta, hydrophilic *p*-aminobenzoic acid crosses to the fetal compartment more readily than does lipophilic antipyrine. The mechanism of its transplacental passage is not clear.[59]

IONIZATION OF DRUGS

Most drugs are weakly acidic or basic substances and are thus ionized at physiologic pH. Passive diffusion across lipophilic membranes depends on the degree of ionization. Only the nonionized (lipophilic) portion of the drug is assumed to pass readily through the lipoid barriers, whereas the ionized form carries a charge and is therefore polar, so it cannot pass through the membranes.

This generalization is true for quaternary ammonium compounds, such as neuromuscular blocking agents, which are fully ionized (e.g., dimethyltubocurarine, pancuronium, succinylcholine).[60-64] After maternal administration, fetal drug levels increase slowly up to levels that are only approximately 10% of corresponding maternal plasma concentrations (see model F in Figure 22-1 and Box 22-1), during both early[60] and late[61] gestation. Transfer of these compounds is confined to water-filled pores within the lipid membranes—a mechanism much less efficient than transfer across the lipid portions of the membranes.

This rule does not apply to all highly ionized substances. Weak acids, which constitute an important group of drugs, appear to be rapidly transferred across the placenta. Salicylate[65,66] and valproate (valproic acid [VPA]),[5,67-69] two drugs essentially fully ionized at physiologic pH, are rapidly transported to the conceptus. Fetal salicylate concentrations reach corresponding maternal levels approximately 1 hour after administration;[66] in mouse and rat, transplacental equilibrium of VPA between embryonic and maternal compartments was reached in less than 0.5 hour.[70-72] Similar observations have been made for the antibiotics ampicillin and methicillin.[4] It seems that the small portions of nonionized drug are responsible for the efficient transport across the membranes; the equilibrium between the ionized and the nonionized form is rapidly reestablished for further transfer of nonionized drug.

The importance of ionization is supported by studies using the perfused human placenta. One such investigation compared the maternal-fetal clearance ratios of VPA and its glucuronides—β-glucuronidase–susceptible (VPA-G) and non–β-glucuronidase–susceptible (VPA-GR, in which R denotes *resistant*). The glucuronidated acids characterized by partition coefficients of 0.0141 and 0.219 (compared with 1.6 for VPA) were expected to have negative log of dissociation constant (pK_a) values between 3 and 4, resulting in higher ionization compared with VPA. As expected, the placental transfer was found to be significantly lower for the glucuronides (13% and 17%, compared with 95% for VPA). Furthermore, although transport was positively correlated with log partition coefficient, no significant dependence on molecular weight was noted.[73] In vivo, similar observations were made concerning lower placental transfer of glucuronidated retinoids in the rabbit.[74] Barbiturates[20] and ascorbate[75] also are efficiently transferred to the fetus.

Similar reasons may underlie the efficient transfer of some basic substances, such as the tertiary phenothiazines, meperidine, nicotine,[39,76,77] and lysergic acid diethylamide (LSD).[78] As noted earlier, the nonionized portion (in rapid equilibrium with the ionized form) of a drug may cross to the conceptus. Lead and metallic mercury can be transferred from the blood of occupationally exposed mothers to the fetus.[79,80]

In late gestation, the pH of fetal blood is 0.1 to 0.15 unit lower than the pH in maternal blood.[20] This pH gradient between fetal and maternal blood influences the extent of transfer of acidic and basic drugs. As discussed earlier, only the nonionized form of a drug can readily pass the lipoid membranes. Because basic drugs are ionized at the relatively low (acidic) pH of fetal blood to a higher degree than in maternal blood, this class of drugs accumulates in fetal blood *(ion trapping)* (models C and D in Figure 22-1). The reverse is true for acidic drugs, which reach lower concentrations in fetal than in maternal blood in late gestation (models E and F in Figure 22-1). The ratio between the fetal and maternal concentrations ($C_{fetal}/C_{maternal}$) for a particular drug can be calculated using the Henderson-Hasselbalch equation from the pK_a of the drug and the fetal and maternal blood pH:

$$\frac{C_{fetal,\ free}}{C_{maternal,\ free}} = \frac{1 + 10^{(pk_a - pH,\ fetal)}}{1 + 10^{(pk_a - pH,\ maternal)}} \quad (2)$$

The pH gradient determines the concentration gradient of *free* concentrations, which are unbound to plasma proteins. The *total* concentrations (free and protein-bound) are determined by the protein-binding gradient across the placenta (see later). Basic drugs such as amide-type local anesthetic agents are ionized to a greater degree in fetal blood than in maternal blood. Therefore the free concentrations of drugs such as lidocaine, bupivacaine, 2-chlorprocaine, ropivacaine, and pethidine accumulate at considerably higher levels in fetal blood (by a factor of approximately 1.5)[81,82] (Table 22-2). An obvious influence of the fetal pH on maternofetal clearance of bupivacaine and ropivacaine has been demonstrated in vitro.[83] The total concentrations of these drugs are, however, much lower in fetal than in maternal blood because of low fetal protein binding (see later).

Low fetal-maternal total concentration ratios do not imply minor or no effects on the fetus. Both the pharmacologic effects and the toxicologic actions of a drug usually are associated with the free, but not the total, drug concentration. Indeed, fetal side effects of amide-type local anesthetic agents are related to the pH gradient across the placenta and not to the protein-binding gradient.[82] A fall in fetal pH (fetal acidosis as in fetal distress), particularly if accompanied by a rise in maternal pH (maternal alkalosis from hyperventilation), increases the fetomaternal pH gradient and increases fetal drug accumulation and the risk of fetal side effects. This correlation has been observed in experimental and clinical studies for both lidocaine[84-86] and bupivacaine.[87-89] The reverse would be expected with regard to the effects of the fetomaternal pH gradient on the extent of placental transfer of acidic drugs. In agreement with the pH partition hypothesis, the fetal free concentrations of VPA were lower than the corresponding maternal values (fetomaternal free concentration gradient was 0.82 ± 0.34 in one study).[90] Again, the concentration gradient of the total levels was quite different. Owing to decreased maternal plasma protein binding (see later), the total concentrations in fetal plasma exceeded those in maternal plasma by a factor of 1.7.[68,90]

The pH partition hypothesis is supported by data in experimental animals that indicate that the transplacental distribution of salicylate is affected by maternal acidosis. Treatment of pregnant rats with ammonium chloride decreases maternal pH and increases the fetomaternal concentration gradient.[91] Maternal acidosis during treatment with VPA can be expected during or after prolonged epileptic seizures. Acetylsalicylic acid often is taken by patients suffering from pyrexia, which under certain conditions also can be associated with acidosis. Low-dose aspirin is used to treat pregnancy-induced hypertension. Acetylsalicylic acid transfers rapidly and reaches a steady state in the perfused placental cotyledon model.[92] Maternal acidosis in the course of the illness may lead to a significant fetomaternal gradient.

Little is known of the plasma and embryonic tissue pH during early human gestational periods. The period between week 3 and weeks 6 to 8 after conception may be particularly important for the formation of major malformations, such as those found after

intake of thalidomide or retinoids. Studies during similar early periods of organogenesis in mouse, rat, and monkey have yielded surprising and potentially important results. The intracellular pH (pH_i), as determined by distribution of the weak acid dimethadione, is surprisingly high during early organogenesis.[70,93,94] Embryonic pH_i considerably exceeded corresponding maternal plasma pH at that period and decreased during later developmental periods to approach values similar to those for other tissues. By comparison, maternal muscle pH remained low (6.9 and 7.0) during all developmental periods.

An acidic drug such as VPA would be expected to be ionized to a greater degree in the relatively basic milieu of the early embryo. Based on the pK_a of VPA (4.7), the pH partition hypothesis predicts a concentration of the drug in the embryo twice as high as that in maternal plasma, and this has been confirmed experimentally[70,93] (Table 22-3). The pH_i of the embryo decreases with advancing gestation; thus, in agreement with the pH partition hypothesis, so does the embryonic-maternal concentration gradient. Salicylate, dimethadione, metabolites of halothane (trichloroacetic acid) and methoxyethanol (methoxyacetic acid), and acidic thalidomide metabolites also accumulate in the early rodent embryo (see Table 22-3).[70] It has been hypothesized that acidic metabolites formed by hydrolysis of thalidomide within the embryo may be trapped there because of their high polarity.[95-97] The pH hypothesis is just as likely to provide an explanation for the embryonic accumulation of these acidic metabolites.

Neutral drugs, such as the anticonvulsants valpromide and ethosuximide (see Table 22-3), should not be affected by transplacental pH gradient. Concentrations of these two drugs in the embryo are essentially the same as corresponding concentrations in maternal plasma.

Certain human teratogens or their metabolites are weakly acidic substances, such as VPA, trimethadione (metabolite: dimethadione), phenytoin, thalidomide (glutamic acid derivatives as metabolites), warfarin, isotretinoin, and etretinate (metabolite etretin). Basic drugs are not represented in lists of human teratogens. Perhaps the reason for the predominance of acidic drugs in the list of human teratogens stems is that these substances accumulate in the early embryo, resulting in unexpectedly high-level "exposure" relative to that incurred by the maternal organism. Some weak acids may be teratogenic because they can alter the pH_i of the embryonic cell. This concept is attractive as a general mechanism of teratogenesis because the pH controls numerous cellular functions, including proliferation and intercellular communication. Structure-activity studies are required to answer some of these questions. Certainly, the finding that some

TABLE 22-2

Extent of Transplacental Distribution of Amide-Type Local Anesthetic Agents

Drug	Measured F-M Total Concentration Ratio (Range)	% PROTEIN BINDING		F-M Protein-Binding Ratio	F-M Free Concentration Ratio*	F-M Total Concentration Ratio†
		Maternal	*Fetal*			
Lidocaine	0.4-0.7	63	34	0.54	1.4	0.80
		64	24		1.4	0.66
Bupivacaine	0.18-0.56	91	51	0.56	1.5	0.27
		92	72	0.78	1.5	0.42
Etidocaine	0.09-0.37	92	—	—	1.4	—
Mepivacaine	0.5-0.8	5	36	0.65	1.4	0.71
Prilocaine	1.0-1.3	55	—	—	1.4	—

Data from references 82 and 84-89 and literature citations therein.
*Predicted according to Equation 2.
†Predicted according to Equation 4.
F-M, Fetal-maternal.

TABLE 22-3

Teratogenicity and Embryo-Maternal Concentration Ratios for Acidic, Neutral, and Basic Substances

Type of Compound	Drug	Species	PLACENTAL TRANSFER		
			Period of Gestation	*E-M Ratio*	*Teratogenic*
Acids	Valproic acid	Mouse	Day 9	1.6-2.3	Yes
	Valproic acid	Rat	Day 11	1.5-1.7	Yes
	Salicylic acid	Rat	Day 10	1.3-1.7	Yes
	Thalidomide (acid metabolite)	Rabbit	Day 8 (blastocyst)	1.2	Yes
	Dimethadione	Mouse	Day 9	1.4-1.7	Yes
		Rat	Day 11		Yes
	Halothane (trichloroacetic acid)	Mouse	Day 11	Accumulation of radioactivity	Yes
	Methoxyethanol (methoxyacetic acid)	Rat	Day 12	2	Yes
		Monkey	Day 28	1	Yes
	Hydrochloric acid (acidified seawater)	Sea urchin embryo, in vitro	5 hours (32 cells)	—	Yes
	2-En-valproic acid	Mouse	Day 9	1.2-1.5	No
Neutral substances	Valpromide	Mouse	Day 9	1	No
	Ethosuximide	Mouse	Day 9	1	No
Basic substances	Nicotine	Mouse	Day 9	3	No
	Doxylamine	Mouse	Day 9	5	No

Data from references 70, 93, and 159 and literature citations therein.
E-M, Embryo-maternal.

related substances without an acid function do not accumulate in the embryo and exhibit low teratogenic potency supports the hypothesis of a special significance of the acid function in teratogenicity.

PROTEIN BINDING OF DRUGS

Protein binding of xenobiotics can occur in maternal blood plasma and other maternal tissues, in placental tissues, and in fetal blood plasma and other fetal tissues. Therefore not only may drugs from the maternal plasma cross the placenta, but also a portion that initially was bound to placental proteins, acting as a temporary sink, may diffuse to the conceptus later. In the human placenta, the transfer of some drugs, including vinblastine,[98] vincristine,[98] cyclosporine,[99] and digoxin,[98,100] appears to be regulated by an adenosine triphosphate (ATP)-dependent membrane protein, P-glycoprotein, presumably protecting the fetus from toxic substances.[98] Rifabutin, being more lipophilic than rifampin, has a greater clearance in the single-cotyledon perfusion system; however, because of its trend toward greater disposition in the placental tissue, accumulation in the fetal circulation may be proportionately less.[101] Placental binding and metabolism are not discussed in this chapter.

Plasma protein binding may affect both the rate and the extent of placental drug transfer (see Table 22-1). The plasma proteins have two functions: For the drugs bound to them they serve as *vehicles* allowing the drug to be transported, although, depending on the affinity of compound, they may impair transfer. Conversely, they offer binding sites on the other side of the placental barrier, *acceptors,* that potentially allow for additional transfer.

Maternal plasma protein binding is a limiting factor for drugs that are transferred through placental membranes relatively slowly. Only the portion of the drug unbound to proteins ("free drug") is available for placental transfer, and it takes considerable time to reach distribution equilibrium between the maternal and fetal compartments. Here the permeability across the placenta is rate-limiting, and the plasma protein-bound fraction can be regarded as a storage bin or reservoir of drug.

Many lipophilic drugs, which are highly protein-bound, actually permeate the placenta rapidly. Plasma protein binding is rapidly reversible and may even increase the amount transferred by presenting more drug to the placenta. Here, plasma protein binding plays a transport role, serving as a vehicle especially for drugs with poor water solubility, which otherwise would not reach the placenta in large amounts. Protein binding depends on the concentration of the protein (P) to which the drug is bound as well as on the binding affinity constant (K_a) and the number of binding sites available (n):

$$B/F = n \times K_a \times P \quad (3)$$

where B/F is the ratio of bound concentration to free concentration of drug.

The importance of protein binding, however, depends on the degree of binding; a clinically significant effect is expected only when it exceeds approximately 80%.[102] This may account for the failure to detect differences in transplacental transfer between methimazole (no binding to plasma proteins) and propylthiouracil (67% bound to albumin) in vitro.[103] Little information is available on the effects of maternal or fetal hypoproteinemia on placental drug transfer. In a study by Brown and colleagues,[104] fetal (umbilical cord) serum concentrations of the antibiotic cefazolin in human newborns suffering from hydrops fetalis were not different from those obtained from nonhydropic infants. Conversely, no transfer was observed in the presence of little albumin (1 mg/mL) in the perfused human placental cotyledon,[105] and indeed, low digoxin levels have been found in transplacentally treated human fetuses suffering from severe rhesus disease, resulting in hypoproteinemia.[106] In vitro, the transplacental transfer of olanzapine (plasma binding in vitro: albumin, 90%; α_1-acid glycoprotein, 77%) was largely dependent on the concentration of acceptor proteins on the opposite side of initial drug placement.[107]

Convincing evidence indicates that the binding affinity or the number of binding sites of fetal proteins is similar to that of maternal proteins (see later).[108-110] Therefore the extent of placental transfer is determined by the relative concentrations of the binding proteins in maternal and fetal blood.[111] Two major binding proteins are known: albumin and α_1-acid glycoprotein. The fetal-maternal concentration ratios of these two proteins differ greatly from each other (Table 22-4). At the end of the first trimester and the beginning of the second, fetal albumin concentrations are approximately 30% of corresponding maternal levels, increasing during gestation to reach maternal levels during the third trimester. At the time of birth, fetal albumin levels even exceed corresponding maternal values[112-115] (Figure 22-3). Fetal α_1-acid glycoprotein concentrations are low during early gestation (reaching only 10% of maternal values by the end of the first trimester and the beginning of the second trimester) and then slowly increase to reach approximately one third of corresponding maternal concentrations during late gestation (see Table 22-4).[112,114]

Therefore the development of a fetomaternal plasma protein-binding gradient (and thus the extent of the placental transfer) for a particular drug depends greatly on the nature of the binding protein. This correspondence is exemplified by a comparison of fetal and maternal protein binding of diazepam,[114] VPA,[113] and propranolol[114] (Table 22-5). The first two drugs are bound predominantly to albumin; consequently, protein binding is low in fetal plasma during early to midgestation but increases steadily to reach an extent that exceeds maternal binding at term (Figure 22-4; see also Figure 22-3). Propranolol is bound predominantly to α_1-acid glycoprotein; consequently, protein binding is low in fetal plasma during early and midgestation but does not significantly increase during later gestational periods.

Protein binding is defined either as the percentage of drug bound ($C_{bound}/C_{total} \times 100$) or as the unbound (free) fraction ($C_{unbound}/C_{total}$). The fetal-maternal concentration ratio of total drug at postdistributive equilibrium can therefore be predicted as follows:

TABLE 22-4

Human Maternal and Fetal Serum Albumin and α_1-Acid Glycoprotein Concentrations During Gestation

	ALBUMIN CONCENTRATION (g/L)			α_1-ACID GLYCOPROTEIN CONCENTRATION (g/L)		
Weeks of Gestation	*Maternal*	*Fetal*	*F-M Ratio*	*Maternal*	*Fetal*	*F-M Ratio*
12-15	28	11	0.28	0.57	0.05	0.09
16-25	34	19	0.66	0.73	0.08	0.11
26-35	28	26	0.97	0.53	0.16	0.24
35-41	29	34	1.20	0.60	0.21	0.37

Data from references 111-115.
F-M, Fetal-maternal.

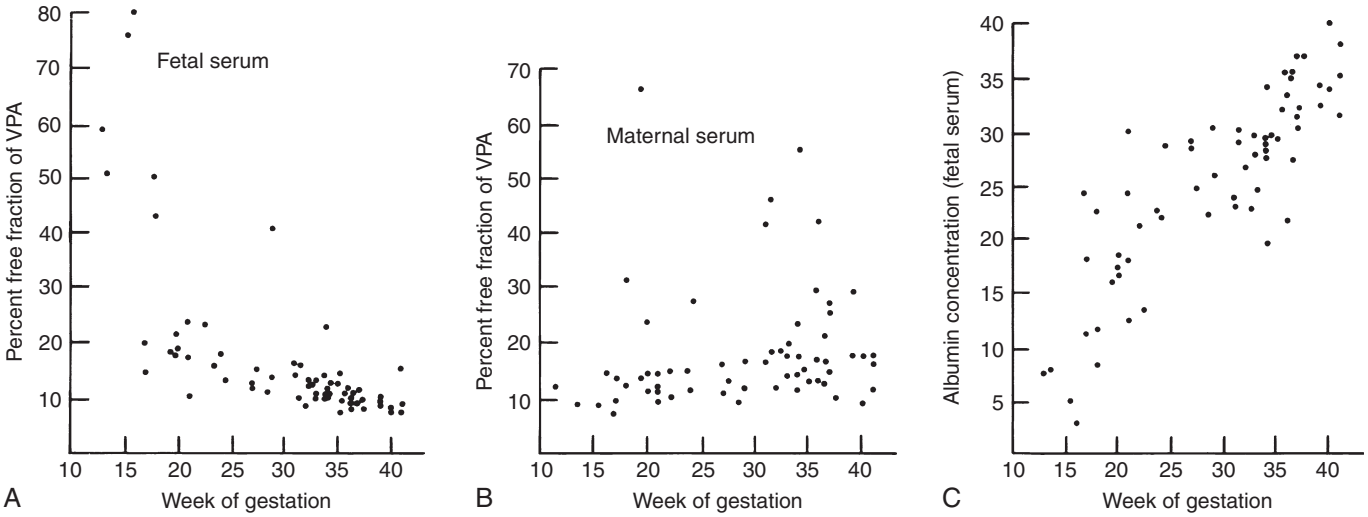


Figure 22-3 Percentage of free fraction of valproic acid (VPA) in fetal serum **(A)** and maternal serum **(B)** as well as albumin concentrations in fetal serum versus gestational age **(C).** *(From Nau H, Krauer B: Serum protein binding of valproic acid in fetus-mother pairs throughout pregnancy: Correlation with oxytocin administration and albumin and free fatty acid concentrations. J Clin Pharmacol 26:215, 1986.)*

TABLE 22-5

Dependence of Plasma Protein Binding of Some Drugs on Gestational Age in Humans

		% BOUND DURING WEEK OF GESTATION					
		16		*23*		*37*	
Drug	**Binding Protein**	***M***	***F***	***M***	***F***	***M***	***F***
Diazepam	Albumin	90	97	96	97	98.5	97
Valproic acid	Albumin	90	50	85	80	80	90
Propranolol	α_1-Acid glycoprotein	63	85	70	85	71	85

Data from references 69, 113, 114.
F, Fetal; *M,* maternal.

$$\frac{C_{fetal,\ total}}{C_{maternal,\ total}} = \frac{\text{unbound fraction (mother)}}{\text{unbound fraction (fetus)}} \quad (4)$$

If the drug is an electrolyte, the pH difference between fetal and maternal blood has to be taken into account:

$$\frac{C_{fetal,\ total}}{C_{maternal,\ total}} = \frac{1 + 10^{(pk_a,\ fetal)}}{1 + 10^{(pk_a - pH,\ maternal)}} \frac{\text{unbound fraction (mother)}}{\text{unbound fraction (fetus)}} \quad (5)$$

Such predicted fetal-maternal concentration ratios are indeed in gross agreement with those found in clinical studies for numerous drugs[111] (Table 22-6 and Figure 22-5; see also Figure 22-4).

Owing to the low fetal albumin concentrations during early pregnancy, fetal concentrations of drugs such as VPA are much lower in fetal blood than in maternal plasma during this period (see Figure 22-4).[113] The concentration pattern of albumin and free fatty acids in the maternofetal unit has important implications for the placental transfer of some drugs that are predominantly bound to albumin and the binding of which can be modified by free fatty acids. Free fatty acid concentrations in maternal plasma rise slowly during pregnancy. The free fractions of those drugs, which may be displaced from albumin-binding sites, would therefore also be expected to rise during pregnancy. Decreasing maternal albumin concentrations amplify this effect.[115-118] During birth, maternal plasma free fatty acid concentrations rise sharply to levels two to three times higher than normal concentrations; the free fractions of salicylate,[119-121] diazepam,[116-122] VPA,[69] and *N*-desmethyl diazepam[118] in maternal plasma also rise sharply to values approximately three times those observed for adult control subjects or in cord plasma samples.[115-118] Accordingly, the amount of "free" drug available for placental transfer also is increased. In the fetus, the fatty acid concentrations remain at low levels, and binding of these drugs is therefore not compromised by endogenous ligands.[123,124] Consequently, the fetus acts as a "sink" for these drugs, and fetomaternal plasma concentration gradients for VPA (see Figures 22-4 and 22-5), diazepam, *N*-desmethyl diazepam, and salicylate[125] are significantly higher than unity. Fetal and neonatal distress may be a result of this unexpectedly high total drug load and its partial displacement after birth. Such adverse effects have been observed in neonates of VPA-treated mothers,[126] as well as in neonates and older infants of mothers who received relatively high doses of diazepam *(floppy infant syndrome).*[127-130] This situation may be especially serious for the neonate because the increased free concentrations of these drugs may persist for a prolonged period as a result of the known deficiency of the neonatal drug elimination mechanisms. This effect may be pronounced in critically ill and preterm neonates.

The pattern of protein binding of warfarin[118] in fetal, maternal, and neonatal plasma mirrors that of VPA, diazepam, and salicylate. It may be that free fatty acids increase the albumin binding of warfarin by allosteric interaction. The free fractions of drugs such as phenytoin, phenobarbital, furosemide, and indomethacin

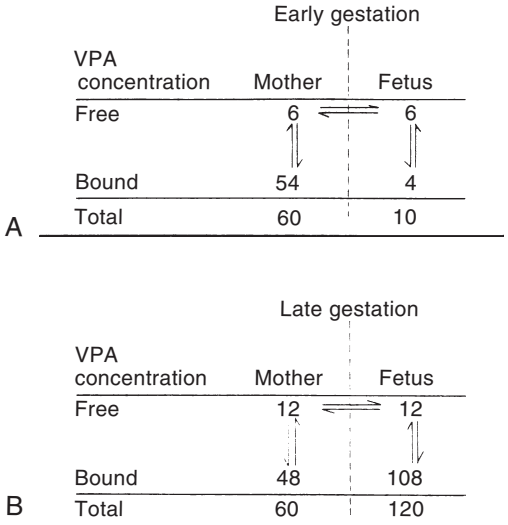


Figure 22-4 Valproic acid (VPA) free and bound concentration gradients in mother and fetus during early gestation **(A)** and late gestation **(B).** The *dashed line* represents the placental membranes. Concentrations are measured in milligrams per liter. *(From Nau H, Krauer B: Serum protein binding of valproic acid in fetus-mother pairs throughout pregnancy: Correlation with oxytocin administration and albumin and free fatty acid concentrations,* J Clin Pharmacol *26:215, 1986.)*

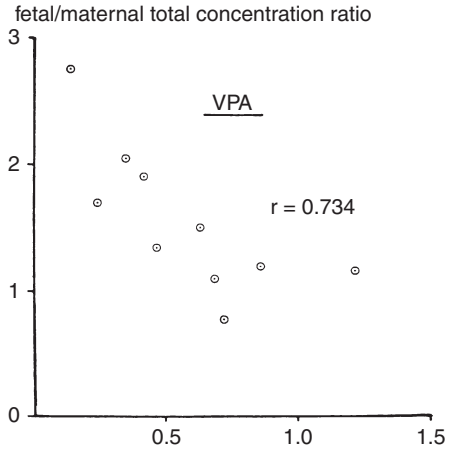


Figure 22-5 Correlation between valproic acid (VPA) total concentration ratio of cord serum to maternal serum at birth *(ordinate)* and VPA free fraction ratio of cord serum to maternal serum *(abscissa).* Accumulation of VPA in the fetus correlates with low free fraction (high protein binding) of this drug in the fetus as compared with the mother. *(From Nau H, Helge H, Luck W: Valproic acid in the neonatal period: Decreased maternal serum protein binding results in fetal accumulation and neonatal displacement of the drug and some metabolites,* J Pediatr *104:627, 1984.)*

TABLE 22-6

Fetal-Maternal Plasma Protein Binding and Total Concentration Ratios of Selected Drugs During Late Human Gestation

Drug	Primary Binding Protein	% BOUND *M*	% BOUND *F*	Fetal-Maternal Primary Ratio Total Plasma Concentration
Betamethasone	AGP	60	41	0.33
Bupivacaine	AGP	91	51	0.27
Diazepam	ALB	97	98.5	1.6
Lidocaine	AGP	64	24	0.66
Mepivacaine	AGP	55	36	0.71
N-desmethyldiazepam	ALB	95	97	1.7
Phenobarbital	ALB	41	36	1.0
Phenytoin	ALB	87	82	1.0
Salicylate	ALB	43	54	1.2
Valproic acid	ALB	73	88	1.7

Data from references 65, 69, 71, 82, 93, 108-122, 175.
AGP, α_1-Acid glycoprotein; *ALB,* albumin.

also are increased in neonatal and infant plasma[118]; the reasons for these effects are not clear, and other endogenous displacing agents, such as bilirubin, may play a role. Lethargy has been observed, particularly in those neonates with elevated plasma free fraction values of phenobarbital.[131]

MOLECULAR WEIGHT OF DRUGS

Placental transfer cannot be predicted on the basis of protein binding, lipid solubility (partition coefficient), or ionization (dissociation constant) alone. Although warfarin has a good placental passage, the lipophilic superwarfarin brodifacoum appears not to cross the human placenta, which may result from its large molecular structure with a long polycyclic hydrocarbon side chain.[132] Most investigators agree that for drugs of molecular weight greater than 500 to 600, transfer across the human placenta is limited.[2,4] Accordingly, the high molecular weight of erythropoietin is believed to be the reason for its poor placental transfer in the placental perfusion model ex vivo.[133] When four oral hypoglycemic agents were compared with regard to maternofetal transport in the recirculating single-cotyledon human placenta model, it was found that glyburide (with a molecular weight of 494) did not cross the human placenta in significant amounts. The glyburide-antipyrine transport ratio (0.11:0.21) was much lower than the tolbutamide-antipyrine transport ratio (0.74:1.05) (Figure 22-6). The investigators found a highly significant relationship between the mean drug (tolbutamide, chlorpropamide, glipizide, and glyburide) antipyrine transport ratios obtained in their experiment and the independent variables (molecular weight, log partition coefficient, and selected dissociation constants [$R^2 = 0.91$; $p = .0001$]). This relationship was described by the following formula:

$$T^{d} / T^{a} = -4.90 + 0,5 \log (P^{d}) + 1.26\ pK_{a} - 0.0073\ MW^{d} \qquad (6)$$

where T^{d} = placental transport of the drug, T^{a} = placental transport of antipyrine, and MW^{d} = molecular weight of the drug.

Molecular weight was the most important variable in this regression ($F = 61,75$; $p < .001$) and determined the cumulative percentage of transport of the drugs tested. Neither the log partition coefficient nor the dissociation constant individually provided significant associations by simple regression with either the drug-antipyrine ratio or the cumulative percentage of transport of these drugs. Furthermore, the high plasma protein binding of glyburide (99%) could account for this finding, as discussed by Koren.[134] Diazepam has similar physicochemical properties but crosses the placental barrier rapidly.[135,136] These results are consistent with the aqueous pore hypothesis noted previously, according to which higher molecular weights and "unsuitable" molecular structures result in steric inhibition. Frequently used drugs such as the nonsteroidal antiinflammatory agent diclofenac (with a molecular weight of 318.15) readily cross the placenta in the first trimester.[137]

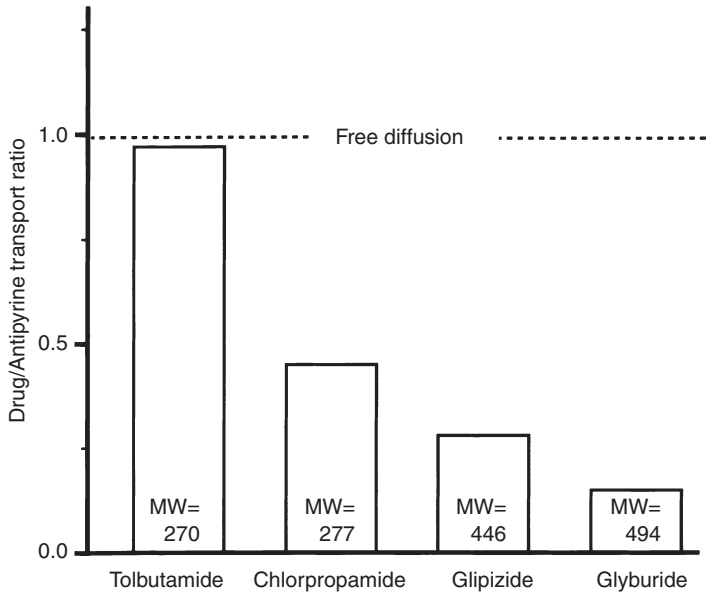


Figure 22-6 Placental transport of four oral hypoglycemic agents in comparison with the freely diffusible substance antipyrine. Values are means. *MW,* Molecular weight. *(Modified from Elliott BD, Schenker S, Langer O, et al: Comparative placental transport of oral hypoglycemic agents in humans: A model of human placental drug transfer,* Am J Obstet Gynecol *171:653, 1994.)*

In vitro heparin does not cross the human term placenta;[138] no biologic activity and very low fractions of radioactivity used for labeling were found in the fetal circulation in the human perfused placental cotyledon model for unfractionated heparin, low-molecular-weight heparin (Cy 216), and dermatan sulfate.[139] These data correspond with clinical observations in newborns whose mothers had been treated with unfractionated heparin or low-molecular-weight heparin.[140] The naturally occurring coagulation inhibitor dermatan sulfate (Desmin 370) (with a molecular weight of 370) cannot pass the ovine placenta at all. Although both molecular weight and charge density of the glycosaminoglycan may account for the placental barrier to heparin, these factors do not explain the failure of the low-molecular-weight dermatan sulfate, which is characterized not only by a smaller molecular size but also by a lower charge density, to cross the placenta.[141]

STEREOSELECTIVITY

Amino acids circulating in the maternal plasma are the primary source of material for protein synthesis by the fetus and also contribute to the fetal energy supply. Amino acids are transferred across the placenta from mother to fetus against a concentration gradient by active, stereospecific placental transport, which functions unidirectionally from the maternal to the fetal side.[142-144] The transfer of neutral branched-chain amino acids is faster than that of neutral straight chains or basic amino acids.[145,146] According to the results of in vitro studies, taurine, β-alanine, and hypotaurine share a specific putative membrane carrier. Histidine also is quickly transported, reaching a maternofetal gradient of 1:2 (1:3 to 1:4 in vivo). It has been suggested that intracellular partitioning or preferential release (transport asymmetry) at the fetal-facing surface of the syncytiotrophoblast may play a role in the placental transport of amino acids.[144] In the guinea pig, the transfer is stereoselective for L-alanine over D-alanine.[147] In the perfused human placenta, S(+)-γ-vinyl-γ-aminobutyric acid was cleared from the maternal circulation more effectively than the R(–)-enantiomer. This more complete clearance, even if it did not result in higher levels in the fetal circulation, suggests either stereoselective sodium-dependent placental uptake on the maternal side of the placenta (as it is known to exist in other tissues) or stereoselective binding to enzymes such as γ-aminobutyric acid transaminase.[148]

The chiral nonsteroidal antiinflammatory drug ketotifen is known to be transferred to the fetus with S-R plasma concentrations averaging 2.3 in premature neonates given a racemic formulation of ketoprofen as a tocolytic agent. Maternal S-R plasma concentrations are close to 1. Because the R(–)-enantiomer does not undergo substantial metabolic inversion in humans, another mechanism had to account for this finding; it appears that stereoselective protein binding results in a higher "free" S(+)-ketotifen concentration with a consequently increased transfer across the placenta (compared with R(–)-ketotifen).[149]

Placental transfer of the water-soluble antiepileptic drug vigabatrin (with a molecular weight of 129), which is unbound to plasma proteins, is low and occurs by simple diffusion. In a human case report, however, a slight difference was found between the kinetics of the entantiomers.[150] The anthelmintic drug albendazole sulfoxide has a chiral center, and the formulations used are racemic. After single-dose oral administration in the last trimester in the ewe, area-under-the-curve ratios of the (–) and (+) enantiomers were 0.36 in the dam) and 0.64 in the fetus, indicating a higher impairment for (+)-albendazole sulfoxide in its placental transfer to the fetus.[151]

The placental transport of retinol and related substances (retinoids) is highly structure-specific. Although in the mouse, rat, and rabbit, retinoids with a free carboxyl group in the 13-*cis* configuration are only poorly transferred early in pregnancy,[152-158] a significant increase in transfer has been demonstrated for later gestational periods in the mouse and in the rat.[159] The β-glucuronide conjugates also show very limited placental transfer (in mouse, rat, rabbit, and monkey).[160] The placental transfer of all-*trans*-retinoic acid (in rat) and all-*trans*-4-oxoretinoic acid (in rabbit) are efficient.[151,158,159,161] The high affinity of the retinoids with the all-*trans* configuration for certain plasma and embryonic cellular binding proteins may be the reason for the efficient transport of these compounds. Binding to plasma albumin cannot explain these differences in transport, because all retinoids with free carboxyl groups bind avidly to albumin and other plasma macromolecules. The increase in glucuronide

transfer in later gestational periods may result from the change in the rodent placental structure from a choriovitelline to a chorioallantoic placenta. In the cynomolgus monkey, a relatively efficient transport for 13-*cis*-retinoic acid has been observed after the chorioallantoic placenta has been fully established.[162] This placenta type is functional at earlier developmental stages in the monkey (and presumably in the human) than in the rodent species; this timing may explain the efficient placental transfer and high teratogenicity of 13-*cis*-retinoic acid when it is administered during early primate organogenesis. The racemic antiepileptic drug ethosuximide appears to be transferred by nonstereoselective diffusion.[163]

SELECTIVE TRANSFER MECHANISMS: ACTIVE TRANSPORT, FACILITATED DIFFUSION, AND PINOCYTOSIS

Available evidence is limited for transfer of therapeutic agents to the embryo or fetus by active transport. Such a transport mechanism would function against a concentration gradient and at the expense of metabolic energy; furthermore, it would be saturable at high substrate concentrations, and it would be competitively inhibited by similar compounds that use the same carrier. Active transport, however, is of great importance for the transfer of endogenous compounds and nutrients, such as amino acids, acetylcholine, vitamin B_{12}, vitamin H (biotin), creatine, and ions such as sodium, potassium, calcium, magnesium, and iron.[164] Folate is preferentially transferred to the fetus.[165] A folate receptor in the placenta may play a crucial role in the transfer of folate to the fetus.[166]

Even though many dideoxynucleosides are at least partially actively transported across cell membranes, this transport mechanism does not appear to be operative for antiviral drugs, such as acyclovir, azidothymidine, dideoxyinosine, and ganciclovir in the human placenta.[167-170] Some evidence, however, supports a carrier-dependent, nucleobase-type uptake in the placenta in addition to diffusional transfer of the drug.[171]

A number of studies suggest that dexamethasone is actively transported across the placenta. In experimental animals (rat, sheep), fetal levels of this steroid are much lower than corresponding maternal values, even after prolonged treatment.[143,172] This low fetal-maternal concentration ratio also has been observed after fetal administration of this glucocorticoid. In addition, sex differences mediated by differential placental transport from the mother have been documented.[173] This concentration gradient is not the result of differential protein binding or metabolism. Placental clearance of dexamethasone from the fetus to the mother is 8.5 times higher than in the reverse direction.[174] The fetomaternal concentration gradient of betamethasone also is low in the human[175]; the reasons for this effect have not been established. Pteridine also is actively transported from the sheep fetus to the mother at a rate that greatly exceeds transport in the reverse direction.[176] [1β-^{3}H]Calcitriol and its metabolites hardly cross the placenta of the rat.[177] The placenta also plays the role of a "metabolic barrier" for endogenous, but not synthetic, estrogens.[178-180] Maternal estradiol does not appear in the fetus but is present there as the less potent estrone and its sulfate conjugate. Placental metabolism may therefore protect the fetus from potent estradiol. By contrast, the synthetic estrogen diethylstilbestrol is transferred to the fetus as the potent parent compound.[178-180] The phytoestrogen daidzein is transferred across the placenta of the rat.[181]

The placental transfer of polypeptides is extremely limited, as has been demonstrated for thyroid hormones, growth hormones, corticotropin, chorionic gonadotropin, erythropoietin,[182] oxytocin,[183,184] interferon,[185] placental lactogen,[29] and protein C.[186] Maternal hormones therefore cannot necessarily influence the development of fetal organ systems when the fetus is deficient in synthesis of its own hormones. Thus fetal endocrine systems function autonomously, and the placenta protects the fetus from exposure to maternal hormones.[29]

The polypeptide atosiban, a synthetic oxytocin antagonist used as a tocolytic agent in women in preterm labor, is transferred minimally to the ovine fetus and under steady-state conditions reaches only 10% of maternal levels in the umbilical cord blood.[183,184] Insulin complexed to antibodies, however, can be transferred to the fetus.[187] This finding is of clinical significance because extremely high concentrations of animal (bovine or porcine) insulin-antibody complex levels were measured in samples of cord blood from infants with macrosomia whose mothers were treated with animal insulin. The transferred insulin must therefore have biologic activity, which may even account for harmful effects such as macrosomia, which is known to be associated with an increased risk for respiratory distress syndrome and hypoglycemia.[188] As the transport of insulin-antibody complexes suggests, proteins are transferred to the fetus to some extent.[189-191] Considerable selectivity, however, has been recognized in the placental transport of proteins. Molecular weight does not play a major role. As discussed previously, polypeptides do not enter the fetal circulation to a significant extent, whereas much larger macromolecules, such as γ-globulins (immunoglobulin G [IgG]) and polyvinylpyrrolidone, are transported. Relatively small fragments of IgG (with molecular weights of 20,000 to 50,000) are poorly transported, as compared with larger fragments (with molecular weights of 50,000 to 82,000) and intact IgG.[192] Therefore binding of IgG is high, resulting in relatively extensive transport of this protein as compared with other proteins such as albumin, transferrin, IgE, IgM, IgA, insulin, and growth hormone, all of which pass the placental membranes to a much lower extent.[190] The process by which antibodies are transported across the placenta appears to be highly selective.

Retinol (vitamin A) is transported to the embryo or fetus bound, at least in part, to retinol-binding protein.[192-194] This transport mechanism appears to be especially important during early gestation because the early embryo is not able to synthesize its own binding protein.[192] In addition, lipoproteins, as well as retinol-binding protein present in large amounts in the yolk sac, may play a role in placental transport of retinol.[193,194]

Water-soluble vitamins are present in higher concentrations in fetal blood than in maternal blood, and active transport mechanisms have been suggested for some of these substances.[195,196] The most extensive data are available for vitamin B_{12}, a polar molecule of high molecular weight (1355) that is not expected to transfer by simple diffusion. A receptor-mediated endocytosis process has been identified as a carrier mechanism for this compound.[164]

In the rabbit placenta, maternal iron is concentrated by a facilitated process as an iron-transferrin complex. This step is followed by a release of iron into the placental cells and return of the apoprotein to the maternal circulation. Thus, in the rabbit placenta, a carrier protein for iron transport may be involved in the process of facilitated diffusion.

In the term human placenta, an electroneutral anion exchange mechanism mediating the transfer of conjugated bile acids across the brush border of the syncytiotrophoblast has been identified.[197] This mechanism could be the basis for previous observations demonstrating higher serum bile acid levels in the umbilical artery (versus the umbilical vein) in human newborns at the time of delivery and suggests a transport of bile acids from the fetal to the maternal circulation.[198]

CLINICAL APPLICATIONS

In a group of drugs with similar pharmacodynamic properties, the efficiency of placental transfer can vary considerably. If no further information is available, comparison of lipid solubility,

protein binding, ionization, and molecular weight may help to select the drug with efficient placental transfer for fetal therapy, as well as the drug with limited placental transfer to prevent effects on the fetus.An example of these considerations has been published in the field of antihypertensive treatment in pregnancy.[199] Oral hypoglycemic agents and biologically active insulin-antibody complexes cross the placental barrier, whereupon they can induce harmful fetal effects such as macrosomia. Newer oral hypoglycemic agents (characterized by a higher molecular weight) cannot pass the placenta; use of such agents may therefore constitute a future alternative to the more invasive insulin therapy in some patients even in pregnancy.[135,200] This finding indicates that identification of structural properties responsible for pharmacodynamic and pharmacokinetic differences is helpful in estimating which drug will be the least hazardous for the fetus if delivery is imminent and maternal pain relief is unavoidable.

A successful reduction in placental transfer of the cytostatic cisplatin was achieved by coupling the drug to cholylglycinate (Bamet-R2)[201]; this finding suggests the possibility of treating certain maternal tumors during pregnancy with a lower risk to the fetus. Liposome encapsulation of VPA reduced placental transfer in the perfused placenta model by 30%, and microencapsulation of chloramphenicol reduced maternal transfer by 85%.[202]

To treat intrauterine infection, broad-spectrum antibiotics that cross the placenta and concentrate in the fetus (e.g., the lipophilic ceftizoxime) are desirable.[203,204] Some investigations have been performed to identify antibiotics with efficient placental transfer rates in the human perfused placenta.[205-211] If only maternal treatment is desired, antibiotics with relatively limited placental transfer, such as macrolides, are favored.[212] Another example is that of treatment for toxoplasmosis: In early pregnancy, spiramycin is preferred because it barely crosses the placenta, where it accumulates. Later, however, when treatment of the fetus is necessary, pyrimethamine is used, because this drug is efficiently transferred to the fetus.[2]

Another indication for intrauterine drug treatment is intentional or accidental drug poisoning. Once the ingested compound has been identified as one that is transferred to the fetus and the ingestion has been confirmed to involve potentially harmful quantities, it is essential that both mother and fetus be adequately treated with an available antidote. Acetaminophen and warfarin-like compounds frequently are used for intentional drug poisonings. Whether the antidotes—*N*-acetylcysteine and vitamin K, respectively—cross the placenta in sufficient degree to ensure effectiveness is still a matter of discussion.[58,132,213,214]

In the ex vivo placenta model, an inhalant (birch pollen major antigen Bet v1, a17-kDa protein) and a nutrient allergen (the cow's milk whey protein beta-lactoglobulin [BLG], with a molecular weight of 18.5 kDa) appear to be actively and selectively transferred across the human placenta.As discussed in this chapter, this phenomenon may be useful as a new preventive strategy for induction of tolerance to specific allergens in the fetus by controlled intrauterine exposure.[215] Estimations for the placental transfer of substances of abuse have been made by Little and VanBeveren using the chemical properties described in this chapter.[216]

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23 Pharmacogenetics

Denis M. Grant

Pharmacogenetic disorders constitute a special subclass of genetic disorders (see Chapter 1) that happen to involve proteins functioning in drug metabolism or action.[1] Accordingly, a key distinguishing feature of pharmacogenetic defects is that they often are phenotypically innocuous in the absence of a drug challenge. Pharmacogenetic variants, then, are not life-threatening per se, obviating the need for prenatal genetic diagnosis. For the perinatal pharmacologist, however, genetically based variation in drug disposition in the fetus, the mother, and the neonate must be included among many variables, such as developmental changes in fetal and neonatal gene expression, organ maturation, placental transfer and metabolism of drugs, and a host of hormonal and environmental influences that can combine to produce specific patterns of toxicity in mother and child on exposure to xenobiotics. Therefore knowledge of potential genetic contributions to drug-induced toxicity in pregnancy and neonatal life can be important in ascertaining drug involvement in observed pathophysiologic states and to prevent their further occurrence in patients.

Recent years have witnessed both an explosion of interest in the field of pharmacogenetics and the birth of the related field of "pharmacogenomics," as a result of technological advances in genetic analysis capability and the availability of the complete sequence of the human genome. The terms *pharmacogenetics* and *pharmacogenomics* often are used interchangeably, and indeed the distinction between the two is largely related simply to differences in experimental approach.[2,3] Classic pharmacogenetics—the study of the role of inheritance in producing interindividual differences in drug response—is a "phenotype to genotype" approach that starts with a clinical observation of unexpected drug response, employs a range of biochemical and molecular genetic methods to elucidate its underlying mechanisms, and uses this information in the development of predictive tests that can be applied to the individualization of therapy. Pharmacogenomics, on the other hand, is a "genotype to phenotype" approach that takes advantage of the availability of human genome sequences and modern genomic analysis methods to discover novel disease susceptibility genes and potential drug targets using whole-genome association and linkage methods or more focused candidate gene approaches.

A number of excellent reviews have summarized current knowledge of the molecular biology of pharmacogenetic defects and understanding of their clinical consequences, practical potential, and ethical implications.[3-12] Although many of the best-studied examples are of genetic alterations that affect the biotransformation of drugs, much recent research also has begun to focus on how genetic differences in intended drug targets can directly influence drug efficacy. This chapter provides an overview and examples of some selected pharmacogenetic traits that influence the metabolism of drugs and hence their *pharmacokinetics* (Table 23-1); briefly discussed are some that alter drug targets and hence directly affect their *pharmacodynamics* (Table 23-2). Polymorphic variation in drug transporters (see Table 23-1), although also an important determinant of drug distribution and disposition at sites of action, is not considered in detail here.

PHARMACOGENETIC DEFECTS AFFECTING DRUG PHARMACOKINETICS

Variation in the function of drug-metabolizing enzymes among human populations results in variable drug and metabolite concentrations, and thus has effects that are directly attributable to altered tissue drug levels. Genetic defects in a number of drug-metabolizing enzymes have been well characterized with respect to both their molecular mechanisms and clinical correlates, although evidence is now accumulating that altered drug transporter function also may produce significant phenotypic variation in drug response. In general, the clinical consequences associated with genetically polymorphic drug-metabolizing enzymes may be classified under four main categories:

1. Functional drug overdose in persons unable to eliminate an active drug efficiently
2. Lack of therapeutic effect either in persons who metabolize an active drug too quickly or in those unable to convert a prodrug to its pharmacologically active metabolite
3. Idiosyncratic drug toxicity unrelated to the intended effect of a therapeutic agent (e.g., hypersensitivity reactions, drug-induced lupus, drug-induced birth defects), which may occur more frequently in persons of a particular metabolizer phenotype
4. Apparently spontaneous disorders for which the cause is unknown but presumably is complex and multifactorial, and which may involve drug or chemical exposures

Polymorphisms of Phase I Drug Oxidation

Considering that a majority of lipid-soluble drugs are metabolized at least to some extent through oxidation, predominantly by members of the liver microsomal monooxygenase (cytochrome P-450 [CYP]) enzyme system,[13] it is somewhat surprising that until the mid-1970s, only a few isolated reports of inherited defects of drug oxidation had been published.[14] This scarcity of research may be due largely to the known multiplicity and overlapping substrate specificity of this enzyme superfamily, implying that for many drugs, multiple biotransformation pathways exist. Thus compensatory metabolism by alternate pathways may potentially prevent the clinical manifestations that would be associated with a defect in a particular metabolic reaction. Nonetheless, in the more than 3 decades since the first discovery of a CYP oxidation polymorphism, a large number of genetic defects of drug oxidation have been characterized in considerable detail[15] (see also Table 23-1). Selected examples drawn from known polymorphisms in the CYP superfamily are presented next.

CYP2D6 Oxidation Polymorphism

CYP2D6 oxidation polymorphism, previously reviewed in detail,[16] provided the first clear demonstration of polymorphic drug oxidation involving a CYP enzyme. It also illustrates the classic experimental "phenotype to genotype" paradigm of pharmacogenetic investigation, progressing from initial clinical observation of a variable drug response to an understanding of the biochemical and molecular mechanisms that produce the

TABLE 23-1

Some Genetically Polymorphic Enzymes Associated With Variable Drug Pharmacokinetics

Enzyme	Representative Chemical Affected
Cytochrome P-450 family	
CYP1A1	Benzo[a]pyrene
CYP1A2	Caffeine
CYP1B1	17β-Estradiol
CYP2A6	Nicotine
CYP2A13	Nicotine-derived nitrosamine ketone (NNK)
CYP2B6	Bupropion
CYP2C8	Paclitaxel
CYP2C9	Warfarin
CYP2C19	Omeprazole
CYP2D6	Codeine
CYP2E1	Halothane
CYP2J2	Arachidonic acid
CYP3A4	Cyclosporine
CYP3A5	Nifedipine
CYP3A7	
CYP4B1	2-Aminofluorene
Flavin-dependent monooxygenase (FMO3)	Trimethylamine
Dihydropyrimidine dehydrogenase (DPD)	5-Fluorouracil
NAD(P)H:quinone oxidoreductase (NQO1)	Benzene
Myeloperoxidase (MPO)	Benzo[a]pyrene
Microsomal epoxide hydrolase (EPHX1)	Benzo[a]pyrene-4,5-oxide
Endothelial nitric oxide synthase (eNOS)	Nitric oxide
Butyrylcholinesterase (BCHE)	Succinylcholine
Paraoxonase (PON1)	Paraoxon
Glutathione *S*-transferases	
GSTM1	Nitrogen mustard
GSTT1	Dichloromethane
GSTM3	
GSTP1	
GSTA3	
Arylamine *N*-acetyltransferases	
NAT1	Sulfamethoxazole
NAT2	Isoniazid
Thiopurine *S*-methyltransferase	
TPMT	6-Mercaptopurine
UDP-glucuronsyltransferases	
UGT1A1	Irinotecan
UGT1A6	
UGT1A7	Benzo[a]pyrene
UGT2B4	
UGT2B7	Oxazepam
UGT2B15	
Histamine *N*-methyltransferase (HNMT)	Histamine
Sulfotransferases	
SULT1A1	Troglitazone
SULT1A2	Hydroxy-2-acetylaminofluorene
SULT1C1	
Catechol *O*-methyltransferase (COMT)	Levodopa
Transporters	
MDR1	
OCT2	
MRP1	

clinically distinct phenotypes. Such fundamental knowledge can now be applied back to the development and clinical use of predictive tests that may be of value to the clinician in choosing the appropriate type and dose of drug to prescribe to individual patients.

TABLE 23-2

Some Genetically Polymorphic Drug Targets Associated with Variable Pharmacodynamics

Target	Representative Drug Class/Example Drug(s)
Mu (μ) opioid receptor	Opiates, e.g., morphine-6-glucuronide
β_2-Adrenergic receptor	β-Agonists, e.g., albuterol; antagonists, e.g., metoprolol
5-Lipoxygenase (5-LO)	5-LO inhibitors, e.g., montelukast
Bradykinin B2 receptor	ACE inhibitors, e.g., enalapril
Dopamine receptors	Dopamine antagonists, e.g., clozapine
Serotonin transporter	SSRIs, e.g., fluoxetine, paroxetine
Estrogen receptor-α	Conjugated estrogens

ACE, Angiotensin-converting enzyme; *SSRIs,* selective serotonin reuptake inhibitors.

The first clinical observations leading to this discovery were provided during independent studies of the oxytocic agent sparteine[17] and the antihypertensive drug debrisoquine.[18] Wide interpatient variations in the dose of debrisoquine required to achieve a hypotensive response were shown to result from genetic differences in the extent to which the drug is hydroxylated to 4-hydroxydebrisoquine. Using a metabolic ratio of the parent drug to its hydroxylated metabolite excreted in urine after a single oral dose, a bimodal population frequency histogram was observed that divided subjects into extensive metabolizers (EMs) and poor metabolizers (PMs), with PM phenotype frequencies ranging from 3% to 10% in various white populations. At the same time, studies of variations in the response to sparteine showed that a similar percentage of German subjects were almost entirely unable to metabolize the compound to its dehydro metabolites.[17] Correlation studies subsequently established that defective metabolism of debrisoquine and sparteine is under identical genetic control. Because these initial observations, the polymorphism has been shown to control, either fully or partially, the rate of oxidation of a number of other clinically useful drugs as well[16] (Box 23-1). The fact that the debrisoquine-sparteine polymorphism is not a generalized drug oxidation defect, however, is supported by numerous reports showing unimpaired metabolism of a wide variety of other drugs whose metabolic fate depends in general on cytochrome P-450 oxidation.

The clinical consequences of the debrisoquine drug oxidation defect have been thoroughly investigated for a number of the affected drugs in Box 23-1.[16,19] One interesting example is that of codeine, which exerts its analgesic effect predominantly through its conversion to morphine—a reaction mediated by the debrisoquine-oxidizing cytochrome P-450. Thus, in PMs of debrisoquine, codeine is ineffective in the relief of pain.[20] An important point is that although the biotransformation of each of the compounds listed in Box 23-1 is measurably affected by the debrisoquine oxidase polymorphism, not all result in the occurrence of altered drug responses. For example, PMs with a genetic oxidation defect experience a greater incidence of excessive β-blockade and loss of cardioselectivity because of elevated plasma drug concentrations after administration of the β-adrenergic antagonist metoprolol[21] but not after administration of the closely related propranolol,[22] even though both are linked to the oxidation defect. The reason for this difference is that propranolol follows several additional metabolic pathways, which, along with renal elimination, can compensate for defective biotransformation by the debrisoquine oxidizing enzyme. In addition, certain drugs such as quinidine and certain neuroleptic agents are potent inhibitors of the polymorphic enzyme without necessarily being significantly metabolized by it. Coadministration of these with any of the drugs listed in Box 23-1 could then lead to potentially significant drug-drug interactions or even misclassification (phenocopying) of normal persons as genetic PMs.

BOX 23-1

Some Drugs With Oxidation Affected by the Debrisoquine Oxidation (CYP2D6) Polymorphism

Antiarrhythmics
- Encainide
- Flecainide
- *N*-propylajmaline
- Propafenone
- Sparteine
- Aprindine
- Mexiletine

β-Adrenergic Receptor Blockers
- Alprenolol
- Bufuralol
- Carvedilol
- Metoprolol
- Propranolol
- Timolol
- Bunitrolol

Neuroleptics
- Haloperidol
- Perphenazine
- Sertindole
- Thioridazine
- Zuclopenthixol
- Risperidone

Antidepressants
- Amitriptyline
- Brofaromine
- Citalopram
- Clomipramine
- Desipramine
- Fluoxetine
- Ifoxetine
- Imipramine
- Maprotiline
- Mianserin
- Nortriptyline
- Paroxetine
- Selegiline
- Tomoxetine

Other Drugs
- Cinnarizine
- Codeine
- Debrisoquine
- Dexfenfluramine
- Dextromethorphan
- Dihydrocodeine
- Guanoxon
- Hydrocodone
- Phenformin
- Indoramin
- Methoxyamphetamine
- Methoxyphenamine
- Perhexiline
- Terodiline
- Tropisetron
- Tramadol
- Orphenadrine
- Tolterodine
- Tamoxifen
- Dolasteron
- Flunarizine
- MDMA ("ecstasy")
- Mequitazine
- MPTP
- Nicergoline

MDMA, 3,4-Methylenedioxymethamphetamine; *MPTP,* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

Much progress has been made in determining the biochemical and molecular mechanisms leading to the occurrence of the PM-of-debrisoquine phenotype in human populations. The CYP isoform that is now known to be responsible for the biotransformation of debrisoquine and other affected compounds is designated CYP2D6. Thus far a total of 104 different alleles at the gene locus (*CYP2D6*) encoding the CYP2D6 protein have been detected in human populations, many of which contain sequence alterations that contribute to the occurrence of EM and PM phenotypes.[23] Of note, the underlying basis of a clinical phenotype that generally is considered by clinicians to be rather homogeneous—the poor metabolizer of debrisoquine—is based on a large and heterogeneous set of molecular defects ranging from single-nucleotide substitutions (SNPs), insertions or deletions that change single amino acids and alter enzyme stability or kinetics, to entire gene deletions or duplications that produce either completely absent or elevated function, respectively. As a result, it also is now clear that significant phenotypic differences may exist within as well as between the so-called metabolizer phenotypes. Although such a high degree of allelic multiplicity poses practical problems in devising simple yet reliable molecular tests to predict defective drug oxidation by CYP2D6 and its severity, the rarity of many of the known variants coupled with ongoing improvements to high-throughput genotyping technology may allow for the development of relatively robust and predictive tests for large-scale population studies.[24,25]

CYP2C9 Oxidation Polymorphism

Polymorphic variants of CYP2C9[26] may also be of considerable clinical importance because of the role of this enzyme in metabolizing a number of therapeutically useful drugs, including warfarin, phenytoin, glipizide, and tolbutamide.[27,28] A total of 37 allelic variants are known to exist at the *CYP2C9* gene locus.[29]

The most common of the activity-impaired enzymes arise from the *CYP2C9*2*[30] and *CYP2C9*3*[31] variants, each possessing one SNP that alters a single amino acid and impairs enzyme function significantly. Because of the narrow therapeutic index of the anticoagulant warfarin, significant hypercoagulation-related toxicity from this drug has been observed in persons possessing CYP2C9-defective alleles,[32,33] although it is evident that other environmental factors also play significant roles in determining warfarin plasma concentrations and therapeutic efficacy.[34,35] Similar observations have been made for the anticonvulsant phenytoin, a drug with saturation kinetics and significant dose-related cognitive side effects that are amplified in PMs of CYP2C9.[36] On the other hand, the antihypertensive response to the angiotensin II type 1 receptor antagonist irbesartan is actually improved in persons possessing one defective CYP2C9 allele over that in persons with only wild-type alleles.[37]

CYP2C19 Oxidation Polymorphism

A well-studied cytochrome P-450 polymorphism affects the function of another member of the CYP2 family, CYP2C19. Originally discovered during studies with the seldom-used anticonvulsant

mephenytoin, the defect is now known to affect the function of a small number of commonly prescribed drugs, including the proton pump inhibitors omeprazole, lansoprazole, and pantoprazole and the anxiolytic diazepam.[38] A total of 25 allelic forms of the *CYP2C19* gene have been characterized to date,[39] with two defective alleles making up the bulk of those associated with PM status. The frequency of defective alleles (specifically the *CYP2C19*3* variant) is higher in Asian than in other populations,[40] resulting in an incidence of PM status approaching 20% in many Asian groups compared with approximately 3% in white populations. The efficacy of omeprazole in increasing intragastric pH and in successfully treating peptic ulcer has been shown to be significantly higher in persons possessing defective CYP2C19 alleles,[41] suggesting that dosing regimens for this drug in normal extensive metabolizers in fact may be inadequate. On the other hand, although the clearance of diazepam[42] and the bioactivation of the antimalarial proguanil[43] are altered in PMs of CYP2C19, the clinical consequences of these findings are modest at best.

Polymorphisms of Phase II Drug-Conjugating Enzymes

Arylamine N-Acetyltransferase 2 Polymorphism

The arylamine *N*-acetyltransferase 2 (NAT2) polymorphism (reviewed by Boukouvala and Fakis[44]) lends a special historical perspective to the field of pharmacogenetics. In 1953, high interindividual variation was observed in the urinary excretion of the new tuberculostatic drug isoniazid. This observation was followed by the finding that frequency histograms of plasma isoniazid concentrations after a single oral dose in a normal population were distinctly bimodal, allowing for classification of subjects as "rapid" or "slow" eliminators of the drug. It was soon established that the basis of the observed population variations was related to differences in the rate of arylamine and hydrazine *N*-acetylation taking place to a large extent in the liver.[45] The enzymatic reaction is now known to be catalyzed by a cytosolic arylamine *N*-acetyltransferase (NAT), which uses the essential cofactor acetyl coenzyme A as acetyl group donor to conjugate primary amine and hydrazine nitrogen atoms, or hydroxylamine oxygen atoms with acetate, producing amides, hydrazides, and acetoxy esters.

The clinical and toxicologic consequences of the acetylation polymorphism have been studied in considerable detail. For instance, slow acetylators are more prone to develop a drug-induced systemic lupus erythematosus–like syndrome during prolonged therapy with procainamide or hydralazine, hematologic toxicity from dapsone, or polyneuropathy after isoniazid treatment. The slow acetylator phenotype also appears to be a predisposing factor in the etiology of sulfonamide-induced idiosyncratic adverse drug reactions. In addition, epidemiologic studies suggest an increased risk for bladder cancer in slow acetylators who are exposed to carcinogenic arylamines, which are substrates for NAT, whereas rapid acetylators may be at higher risk for colon cancer. Rapid acetylators also encounter therapeutic failure more often on treatment with once-weekly isoniazid dosage regimens and require higher doses of hydralazine to control hypertension or of dapsone for dermatitis herpetiformis.

Molecular cloning experiments established that two functional human NAT enzymes exist, NAT1 and NAT2, and that genetically based variation in NAT2 is associated with the isoniazid acetylation polymorphism. Work over the past 2 decades has provided direct evidence for the existence of, at last count, 36 variant alleles at the human *NAT2* gene locus that are correlated with the acetylator phenotype.[46] Each of the quantitatively significant human allelic variants possesses a diagnostic combination of nucleotide substitutions (i.e., SNPs) at positions 191, 282, 341, 481, 590, 803, and 857 within the intronless 870–base pair *NAT2* gene coding region. At the level of protein function, the single I114T amino acid change produced by a T341C mutation common to the nine *NAT2*5* alleles drastically reduces V_{max} values for substrate acetylation without altering enzyme affinity or stability, possibly by preventing the proper folding of the nascent polypeptide into its enzymatically active conformation. On the other hand, the mutations causing R197Q (*NAT2*6*), G286E (*NAT2*7*) and R64Q (*NAT2*14*) amino acid substitutions produce proteins with significantly reduced stabilities.[47] The population frequencies of certain of the *NAT2* alleles also show marked interethnic differences.[47] For instance, *NAT2*5B* (I114T) is the most common allele in white populations but is almost entirely absent in Asians, accounting for most of the known interethnic difference in the frequency of the slow acetylator phenotype between these populations. *NAT2*7B* (G286E), on the other hand, is more prevalent in Asians than in white persons.

NAT1 function also is variable in human populations, and at least some of this variation may be genetically determined. The use of the NAT1-selective substrate *p*-aminosalicylic acid as a probe drug has allowed for the identification of probands from whom variant alleles at the *NAT1* gene locus have been isolated and characterized. [48] A total of 26 *NAT1* allelic variants are known,[49] although those recognized to significantly alter function are rare in most human populations. Association studies have attempted to correlate variation in the more common *NAT1* alleles, particularly *NAT1*10*, with susceptibility to a variety of cancers, with equivocal results; the functional consequences of these variants for enzyme activity in vivo are also still unclear.

Thiopurine S-Methyltransferase Polymorphism

Clinical relevance has been clearly demonstrated for a polymorphism that occurs in the drug methylating enzyme thiopurine *S*-methyltransferase (TPMT).[50-52] This enzyme catalyzes the detoxifying methylation of the cytotoxic agent 6-mercaptopurine, a drug commonly used in cancer chemotherapeutic regimens for the treatment of childhood acute lymphoblastic leukemia. The TPMT-catalyzed reaction competes with a reaction that produces cytotoxic thioguanine nucleotides, which constitute the pharmacologically active principle in producing leukemic cell death. Thus, in persons with impaired TPMT function, excessive production of thioguanine nucleotides leads to a higher incidence of adverse reactions to the drug, often necessitating cessation of therapy and drastic dose reduction.[53] To date, 8 variant TPMT alleles have been identified[54]; three of these, *TPMT*2, TPMT*3A,* and *TPMT*3C,* account for 95% of the cases of defective function. Although the frequency of each of the 7 known activity-impairing variants is quite low, such that persons possessing two defective copies number only approximately 1 in 300, functional impairment is observed even in heterozygotes, who make up approximately 11% of a typical white population. Because of the relatively small number of relevant mutations known to impair function, simple genetic diagnostic assays can be devised as guides to dosing regimens in initiating chemotherapy. On the other hand, TPMT expression in blood cells is an accurate reflection of tissue enzyme activity, so a direct blood enzyme assay also is feasible.[55]

PHARMACOGENETIC DEFECTS AFFECTING DRUG PHARMACODYNAMICS

Table 23-2 provides some selected instances in which genetic variation in specific drug targets may influence drug efficacy. Although a detailed discussion of these examples is beyond the scope of this review, a brief summary of one selected instance is presented next. Of note, however, because of the multifactorial nature of many drug responses and physiologic processes, the clinical significance of many of the genetic variants in drug targets observed so far has yet to be conclusively established.[56] In addition, because most drug targets, such as receptors, have important endogenous physiologic roles, the likelihood of

encountering severely function-impairing mutations arising from single-gene defects for many of these proteins is quite low.

β-Adrenergic Receptor Polymorphisms

Variations in the genes encoding β-adrenergic receptors recently have been shown to correlate with a variety of responses to β-agonists as well as to physiologic and pathologic stimuli.[57-60] For instance, recent experimental observations have suggested that allelic variants possessing an arginine at position 16 of the β_2-adrenergic receptor protein impart increased agonist-mediated vascular desensitization, whereas variants with glutamic acid at position 27 have increased agonist-mediated responsiveness.[61] Asthmatic patients who were homozygous for arginine at position 16 of the β_2-adrenergic receptor also were shown to have greater therapeutic response to albuterol for the relief of their airway constriction.[62] Polymorphisms in the β_1-adrenergic receptor have been shown to affect exercise capacity in patients with congestive heart failure[63] and resting hemodynamics in patients undergoing diagnostic testing for ischemia.[64] Finally, it has been suggested that polymorphic variants of the β_3-adrenergic receptor may be risk factors for obesity.[65]

PHARMACOGENETICS IN CONTEXT: GENE REGULATION AND DEVELOPMENT

Pharmacogenetic defects must be considered as only one part of the complex series of genetic and environmental factors that regulate the level and developmental time course of protein expression. Furthermore, many of these variables have potential relevance for the perinatal pharmacologist. For instance, with regard to drug-metabolizing enzymes, differential activation of transcription of cytochrome P-450 and other oxidative metabolism genes at varying stages of prenatal and postnatal development has been shown to produce age-specific isozyme patterns.[66] Different P-450 genes have been shown to be activated immediately after birth, in the months following birth, at the onset of puberty, or according to neonatal imprinting by sex hormone exposure, most notably in rodent species; certain isoforms also may be specifically suppressed at puberty. Significant differences exist between the forms of P-450 expressed before and after birth.[67-69] Of interest, one isoform of the CYP3A family (CYP3A7), which previously was considered to be fetus-specific, has been demonstrated to display both expressions in all fetal livers tested and polymorphic expression among adult human livers.[70] On the other hand, recent studies suggest that differences in the content and activity of cytochromes P-450 in early life are unlikely to account for the increased clearance of many drugs in children.[71]

In addition, it is known that the expression of certain classes of P-450 isozymes is induced (at the level of transcriptional activation) by specific drug or hormonal exposures.[72] For example, polycyclic aromatic hydrocarbons present in cigarette smoke may cause elevation in the liver content of CYP1A isozymes; this response has been shown to be both genetically and developmentally variable.[73] Expression of CYP1A2, which appears to be responsible for the metabolism of caffeine, theophylline, phenacetin, and a variety of aromatic amine procarcinogens, develops slowly (possibly through exposure to environmental inducing agents) over the first several months of extrauterine life,[74] and in women, its activity is drastically impaired during pregnancy.[69] It also has been suggested that a glucocorticoid-inducible P-450 isozyme may be involved in the process of oxygen contracture and subsequent closure of the ductus arteriosus at birth.[75]

The consequences of allelic variation in phase II enzymes for drug therapy also may be important for the pediatric pharmacologist, especially because both genetic and regulatory factors may govern the expression of metabolizing capacity during development.[76] For instance, longitudinal NAT2 acetylator phenotyping studies in developing infants[77] have demonstrated that all neonates are phenotypically slow acetylators, suggesting that the manifestation of the isoniazid acetylation polymorphism develops relatively slowly over the first 1 to 2 years of life. NAT1 activity has been detected in human fetal liver, adrenal glands, lung, kidney, and intestine, although at a lower level than that observed in adult tissues.[78] NAT1 also is present in the placenta[79] and in human preimplantation embryos[80]; in view of its ability to metabolize a catabolite of folic acid,[81] a role for this enzyme in neural tube development has been suggested.[82] As a consequence of the different substrate specificities and tissue-selective patterns of expression of NAT1 and NAT2, exposures to certain arylamine chemicals may be expected to result in different patterns of elimination or toxicity (or both), depending on the levels of expression of these enzymes during fetal and neonatal development. Obviously, further work is required to delineate these patterns of expression and their consequences for drug therapy and toxicity.

From the previous examples, it is apparent that when genetic factors, which may differ between mother and child, interact with such developmental and gene regulatory processes, a vast potential exists for variation in metabolizing capacities for drugs and xenobiotics. Thus clinical outcomes (adverse drug reactions, fetal toxicity) are dependent not only on the chemical agent but also on the genetic constitution of mother and child and the time point of exposure in relation to the functional integrity of the drug biotransformation systems.[83-85]

INTERACTION BETWEEN ONTOGENY AND PHARMACOGENETICS: CLINICAL PEDIATRIC PHARMACOGENETICS

The examples just cited highlight how rapidly the field of pharmacogenetics has grown in the last several years, aided greatly by advances in analytical techniques, cellular assays and most especially genomic technologies. One of the main challenges has been to provide unified approaches to both genotype and phenotype for the handling of specific drugs and other xenobiotics. Recognition of the specificity of drug metabolic pathways and the ability to determine which pathways are critical for handling a new drug before its widespread use in humans may lead to decreased population variability and risk of untoward side effects. Use of in vitro techniques employing human hepatic microsomes (with specific inhibitors of drug metabolizing enzymes) and recombinantly expressed enzymes can determine whether a new drug is likely to be metabolized polymorphically in the population and what other drugs may potentially interact with the new drug to alter its kinetics. In vivo probe compounds (such as dextromethorphan for CYP2D6 and caffeine for NAT2) can help map regulation and development of expression of metabolic pathways safely in large populations. As outlined in the previous section, one of the main challenges in pediatric developmental pharmacology is to integrate the growing body of data on specificity of metabolic pathways with their ontogeny.[66,76]

The interaction of genetics and ontogeny of drug metabolism has been the topic of several reviews.[83-86] For example, the availability of potentially toxic electrophilic metabolites to mediate toxicity often depends on the interaction of several metabolic pathways, one of which may be genetically polymorphic, whereas others are developmentally regulated. Although the genetic abnormality may potentially be critical to the ultimate toxic outcome, risk may be modulated by developmental expression of other pathways, perhaps explaining why some drug-induced toxicities (such as hepatotoxicity from valproic acid[87]) may be more prevalent among younger patients. Knowledge of specificity of metabolic pathways also can help in the design of clinical pharmacokinetic studies of new drugs. For example, if a new drug is metabolized by CYP1A2, an enzyme that appears to develop over the first postnatal year of life and then undergoes

down-regulation during puberty (at earlier Tanner stages in girls than in boys[88]), studying patients by age-groups of 6 to 12 years and 13 to 18 years (with children in various Tanner stages) may lead to increased and difficult-to-interpret variability. It may make better sense to group patients by Tanner stage, studying smaller numbers of more homogeneous patients to assess the effects of puberty on handling of the compound. A similar approach can be taken for the newborn—determining the pathways of clearance for the drug, elucidating the ontogeny of those pathways, and thereby more rationally choosing doses for clinical studies.

As with any new field during a period of rapid scientific expansion, it is difficult to predict the ultimate impact of pharmacogenetics on "real-life" pharmacotherapy. The advances of the last several years, however, suggest that the field will have a direct impact on patient care.[10,12,89-92] The field is clearly expanding beyond just drug metabolism. Molecular approaches will lead to (1) disease susceptibility markers, early preclinical "diagnosis" of disease diathesis for early therapeutic intervention; (2) syndrome diagnostic markers, to determine more specific causes of complex human symptoms and thus improve specificity of therapeutic choices; (3) pharmacogenetic markers for drug metabolism and efficacy variants; and (4) markers to individualize idiosyncratic adverse drug reaction risk, minimizing both individual and population risk of adverse drug effects. Routine availability of molecular diagnostics in the clinical laboratory will usher in a new era for clinical pharmacogenetics.

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24 Drug Distribution in Fetal Life

Marianne Garland

Drug distribution and clearance determine the concentration of drug that will be attained at the site of drug action. Drug targets include cell surface receptors, intracellular receptors, enzymes, transcriptional mechanisms, ion channels, and molecular transport systems.These targets may be within the circulatory system, in well-perfused tissues, in less well-perfused tissues, or behind specialized endothelial or epithelial barriers.The fetus lies behind one of these circulatory barriers—the placenta. The placenta keeps the maternal and fetal circulations separate but brings them into close apposition for transport of nutritional needs and removal of waste products. In addition, this interface is the major route of drug delivery to and elimination from the fetus.The fetus also has specialized circulatory arrangements designed for intrauterine life that require additional considerations in the understanding of fetal drug distribution. Furthermore, developmental differences in body composition, drug metabolism, renal clearance, and specialized barriers make fetal drug distribution distinct from that in the infant, child, and adult.

Most drug action is predicted on the basis of plasma drug concentrations. It is not easy, even in experimental models, to measure tissue concentrations, particularly when extracellular versus intracellular concentration warrants consideration. Hence major emphasis is placed on the determinants of fetal plasma concentration. An appreciation of this aspect of pharmacokinetics requires an understanding of physicochemical properties of drugs, placental transfer of drugs, and fetal clearance of drugs. Many of these concepts also pertain to tissue distribution. Drug delivery to the central nervous system of the fetus is of particular interest and serves in this chapter to illustrate the concepts relevant to tissue distribution. In considering developmental

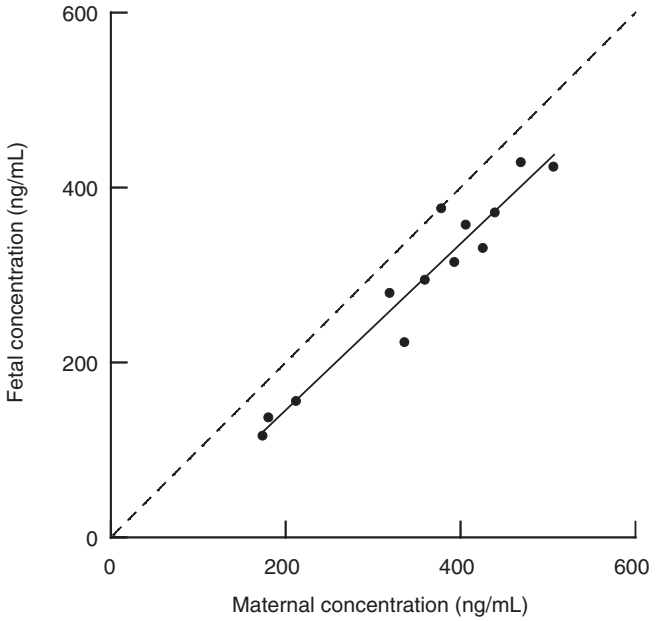


Figure 24-1 Paired fetal and maternal zidovudine concentrations in plasma samples obtained at steady state in the chronically catheterized baboon infused with 150 to 350 μg/minute of zidovudine.

issues relevant to fetal disposition of drugs, an important point is that drug targets also have complex developmental trajectories. Understanding fetal drug distribution may allow prediction of drug concentration at the site of drug action, but prediction of drug action, which is the true goal, requires understanding the interaction between the drug and its target.

DETERMINANTS OF FETAL PLASMA CONCENTRATION

The plasma concentration of the drug in the mother is the main factor determining the fetal plasma concentration. Figure 24-1 shows the concentrations of zidovudine measured in paired samples of maternal and fetal baboon plasma under steady-state conditions.[1] Doubling the maternal concentration essentially doubles the fetal concentration. Of note, the fetal concentration of zidovudine is always slightly less that that in the mother. This observation suggests that other factors also influence the fetal plasma concentration. How placental permeability, fetal drug elimination, drug ionization and protein binding, and, in non–steady-state situations, volumes of distribution affect fetal drug levels is the focus of this chapter.

Once the maternal concentration is known, fetal distribution can be divided into three segments: transfer across the placenta, modification of the fetal plasma concentration, and tissue distribution. In order to describe how differences in these various contributors affect fetal drug levels (be it plasma, extracellular, or intracellular), an integrated pharmacokinetic approach, with graphic representations, is used throughout.

MATERNAL PLASMA CONCENTRATION

The maternal plasma concentration is the driving force for drug delivery to the fetus. For many drugs, physiologic changes of pregnancy lead to altered drug absorption, distribution and clearance in the mother, and thus different plasma concentrations from those seen in the nonpregnant state.[2-9] Overall, plasma drug concentrations tend to be lower in pregnancy. There is an increase in the volume of distribution from an increased plasma volume and increased fat deposition as well as addition of the fetal compartment. Maternal renal clearance is enhanced owing to increased cardiac output and renal blood flow. Hepatic clearance also may be enhanced as a consequence of increased hepatic blood flow or hormonal stimulation of drug-metabolizing enzymes.[10,11] In some cases, however, pregnancy hormones may inhibit drug-metabolizing enzymes.[3] Unfortunately, good comparative data are available for only a small number of drugs. For fetal considerations, these numerous physiologic changes of pregnancy that alter maternal drug distribution can be bypassed by measuring the concentration of the drug in maternal plasma.

DRUG TRANSFER ACROSS THE PLACENTA

The placenta is the specialized interface between mother and fetus across which drug distribution occurs. Most drugs are believed to cross the placenta by passive diffusion; accordingly, the surface area provided by the placenta and the nature of the interface, together with drug characteristics, determine placental permeability. The role of transporters in the placenta is beginning to emerge as a contributor to fetal drug disposition. Transporter activity modifies transfer of several important drugs and is discussed further on.

Structural Development of the Placenta

The following discussion is a synopsis of placental development highlighting the aspects relevant to drug transport,[12] with a focus on the relationship among the maternal and fetal circulations, the surface area of exchange, and the nature of the diffusional barrier.

During implantation, the trophoblastic tissue invades and becomes surrounded by decidua. The placenta develops at the embryonic pole with trophoblast in contact with the rest of the decidua gradually breaking down. Spaces develop within the expanding trophoblastic tissue to form the lacunae that lie between the villous structures. The uterine spiral arteries supplying the decidua and the veins draining the decidua are invaded by trophoblast in such a manner that these maternal vessels open directly into the lacunae and maternal blood bathes the villous structures. Anchoring villi extend the full thickness of the trophoblast layer; most villi have the appearance of treelike structures (Figure 24-2, *A*). As pregnancy advances, the placental surface area increases by increasing the number of villi and the number of branches. As with most epithelial transport surfaces, the luminal plasma membrane of the villus trophoblast has microvilli that further increase surface area (see Figure 24-2, *C*). Later in gestation the diffusional capacity of the placenta increases mostly by thinning of the trophoblast layer where it overlies fetal vessels within the villi. The villus itself consists of a stromal core to support the fetal blood vessels and is surrounded by a single layer of syncytial trophoblast attached to a basement membrane (see Figure 24-2, *B*).[13] Cytotrophoblasts and some Hofbauer cells (placental tissue macrophages) lie between the two. The syncytial trophoblast is a multinucleate cellular structure formed by the fusion of trophoblastic cells to form a syncytium. Underlying cytotrophoblastic cells add to the syncytiotrophoblast by fusion and, near term, few cytotrophoblasts are present within the villus. The fetal arterioles branch into a capillary bed also surrounded by a basement membrane.[14] The capillaries are nonfenestrated and have variably spaced tight junctions between endothelial cells.[15-17] In the mature placenta, the contact zones between the syncytiotrophoblast and endothelial cells are free of nuclei and are thinner than other regions (see Figure 24-2, *B*). The layers between maternal and fetal blood over which diffusion occurs are shown in Figure 24-2, *C*. In addition to the microvillus surface, the luminal membrane of the syncytiotrophoblast (that in contact with maternal blood) contains clefts. The abluminal membrane also has infoldings. The continuous nature of the syncytiotrophoblast precludes

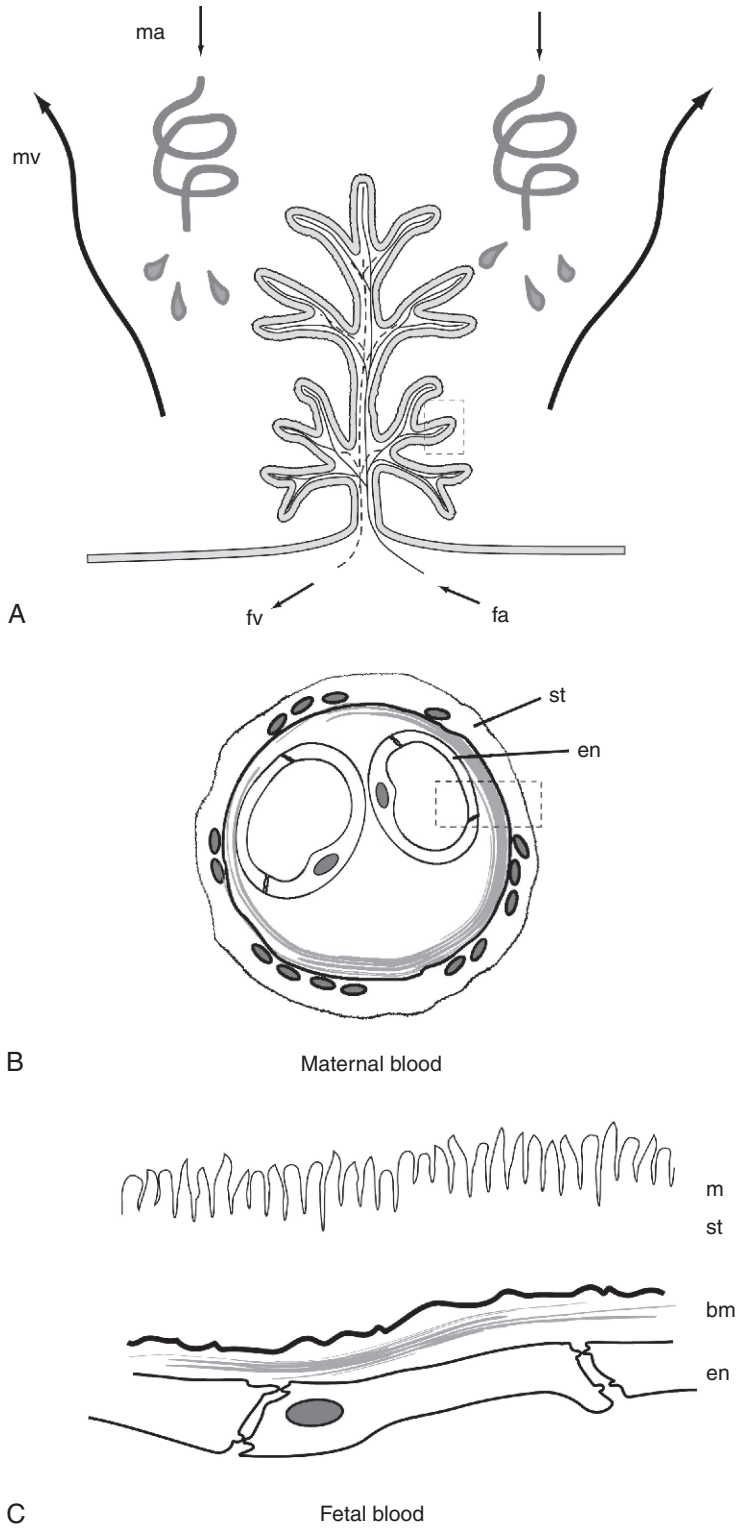


Figure 24-2 Schematic diagram of mature human placental structure. A, The placental villus supports vessels from the fetus and is in direct contact with maternal blood. **B,** The syncytial trophoblast forms a continuous cellular layer over the fetal villi. Areas in close apposition to fetal capillaries are thinner and lack nuclei. **C,** The layers that drugs must cross to transfer from one circulation to the other are depicted. Area surrounded by the *dashed line* is enlarged in the succeeding diagram; *arrows* indicate direction of blood flow. *en,* Endothelium; *fa,* fetal artery; *fv,* fetal vein; *m,* microvilli; *ma,* maternal artery; *mv,* maternal vein; *st,* syncytiotrophoblast.

intercellular spaces through which transport can occur. By contrast, the endothelium does allow some paracellular transport of low-molecular-weight hydrophilic substances. Placental capillaries are less permeable than most other capillaries present in tissues with continuous capillaries; however, they are still more permeable, by two orders of magnitude, than those present in the brain.[17]

Models of Placental Transfer of Drug

From an anatomic perspective, many placental characteristics are important in the transfer of drug to the fetus. The most striking features are the very large exchange surface and the very thin syncytial-endothelial barrier between the circulations, supporting passive transfer of substances (see Figure 24-2). Visualization of the human placental structure suggests a crosscurrent exchange interface; however, experimental data at best support a concurrent model (detailed in Chapter 14, Mechanisms of Transfer Across the Human Placenta).

The simplest and perhaps most illustrative way to view the maternal-fetal dyad with respect to drug distribution is as a two-compartment model[1,18-20] (Figure 24-3). This model differs from the standard peripheral compartment model in that the fetal compartment includes an elimination route independent of the placenta. In addition, at least in experimental models, this compartment can be sampled. Rate equations for the model describe how the amount of drug in each compartment changes with time and are determined by considering how much drug is entering and leaving each compartment. The amount of drug *(D)* that leaves in a given time period (mass/time) equals the clearance (Cl, volume/time) multiplied by the mean concentration (*c*, mass/volume) for that time period as follows (see parameter descriptions in Figure 24-3):

Rate equation for maternal compartment:

$$\frac{dD_m}{dt} = R_m - Cl_m \cdot c_m - Cl_{mf} \cdot c_m + Cl_{fm} \cdot c_f \quad (1)$$

Rate equation for fetal compartment:

$$\frac{dD_f}{dt} = Cl_{mf} \cdot c_m - Cl_{fm} \cdot c_f - Cl_f \cdot c_f \quad (2)$$

Solutions of these rate equations can be used to generate concentration-time plots for each compartment, to illustrate the effects of decreasing placental permeability after administration to the mother of a single oral bolus of drug (Figure 24-4) or when drug is administered as a continuous infusion (Figure 24-5). Both Figures 24-4, *A,* and 24-5, *A,* show the effect of decreasing placental permeability after a single oral bolus of drug (for Figure 24-4) and when drug is administered as a continuous infusion (for Figure 24-5) to the mother. The assumption is that drug crosses the placenta by passive diffusion, so the clearance will be the same in both directions. In Figures 24-4, *A,* and 24-5, *A,* no direct fetal clearance occurs—that is, fetal nonplacental clearance is set at zero. The only parameter that varies is placental clearance. (Figures 24-4, *B-D,* and 24-5, *B-D,* are discussed further on.) Placental permeability will affect peak fetal drug concentrations when administered by bolus, but total fetal drug exposure, represented by the area under the curve, is the same in each case. Under steady-state conditions (obtained with continuous intravenous infusion or repeated bolus doses), for drugs with low placental permeability, fetal levels will be as high as those achieved for highly permeable drugs if continued for an extended time.

A much-debated question is whether fetal drug concentrations can exceed those in the mother with passive placental transfer. During the elimination phase after bolus administration, fetal concentrations are higher than maternal—but the peak fetal concentration will not exceed the peak maternal concentration (see Figure 24-4). Even for rapidly transferred substances, the peak concentration will be blunted. In addition, mean steady-state concentrations will not exceed maternal concentrations; however, during the elimination phase, fetal concentrations may exceed maternal levels (see Figure 24-5). Also, total drug exposure in the fetus will not exceed that in the mother. In Figures 24-4, *A,* and 24-5, *A,* when there is no direct fetal clearance, not only is the area under the curve (measure of total drug exposure) the same in each fetus, but the area under the curve is the same in the mother as in the fetus. In the absence of direct fetal elimination, mean steady-state concentrations (or areas under the concentration time curves) in the fetus are equal to those in the mother. This is an important concept to grasp, because single maternal-fetal drug determinations after bolus drug administration have caused considerable confusion in the understanding of fetal drug distribution. In certain situations, mean active drug concentrations in the fetus exceed maternal concentrations—for example, in the presence of active transport from the maternal to fetal circulation and after prodrug administration, when drug metabolite concentrations can be higher in the fetus than in the mother. These circumstances are explored later on.

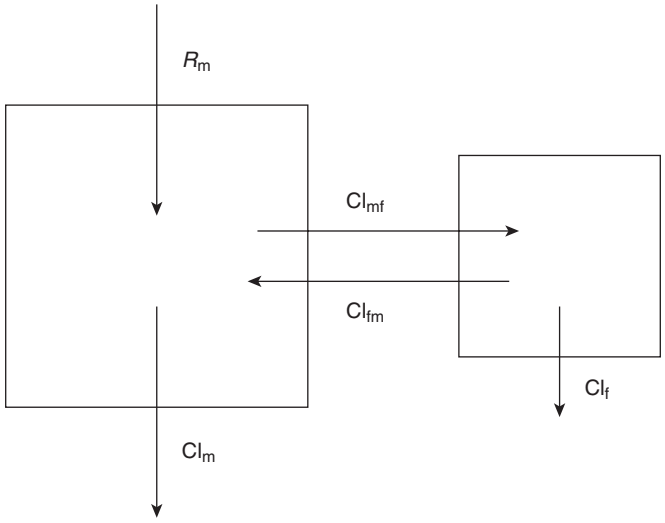


Figure 24-3 The two-compartment model representation of the maternal-fetal dyad. Drug movement into and out of each compartment is indicated by the *arrows.* Clearance parameters are used to characterize the elimination from or transfer between compartments. *Cl,* Clearance; *f,* fetal; *m,* maternal; *R,* rate of infusion of drug to maternal compartment.

Passive Transfer of Drug Across the Placenta

It is thought that most drugs cross the placenta by passive diffusion. *Passive diffusion* is the movement of substances in solution across a *semi-permeable membrane,* in this case, the placenta. This process uses the kinetic energy of the molecules, rather than any energy provided by cellular mechanisms. As molecules bounce around in solution, some will cross to the other side of the membrane. The percentage that crosses is determined by the number of molecules in solution (concentration) and the ease with which molecules cross. Subsequently, as a result of the random movements, a certain percentage of these molecules will in turn cross back to the other side, again dependent on the concentration and ease of transfer. Because the membrane is essentially the same in both directions, more molecules will cross from the side with the higher concentration, and net transfer will be to the side of lower concentration. Once equilibrium is reached, the concentrations on the two sides of the membrane will be the same, with no net transfer. Because net transfer is proportional

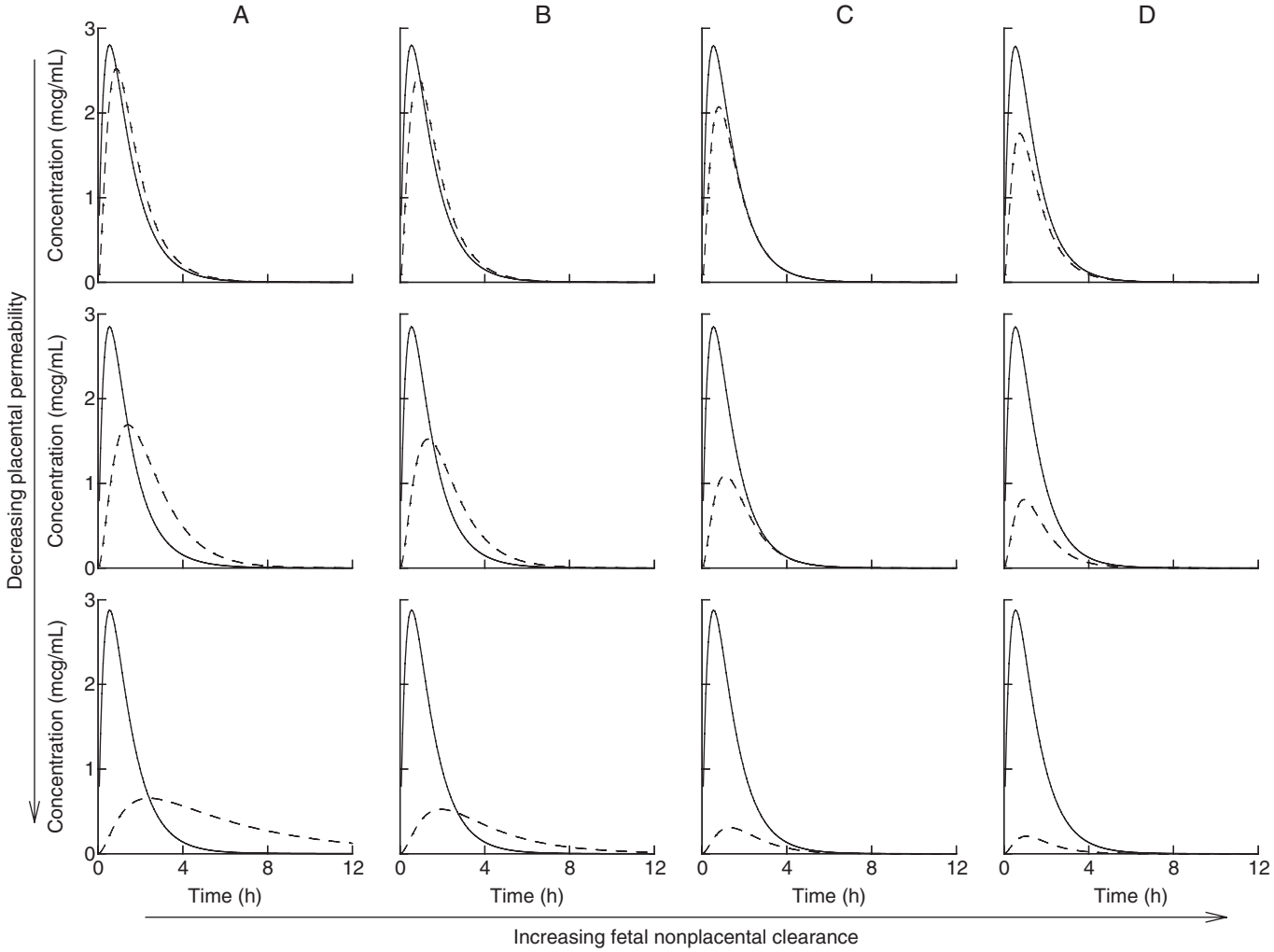


Figure 24-4 Drug concentration–time curves for the mother *(solid line)* and the fetus *(dashed line)* after an oral bolus of drug. These plots represent the solutions for the rate equations of a two-compartment model following an oral bolus of drug where placental clearance (a measure of placental permeability) and fetal nonplacental clearance (direct fetal elimination) were varied while all other system parameters remained constant. The general parameters used to make these plots are those obtained experimentally in the pregnant baboon following zidovudine administration.[1,28] The *top row* represents high placental permeability in that placental clearance is 20% of maternal clearance. In the *middle row,* placental clearance is 5% of maternal clearance (that observed experimentally). The *bottom row* represents limited placental permeability in that only 1% of drug administered to the mother will cross the placenta to the fetus. Levels of direct fetal elimination increase with progression from **A** to **D: A,** no direct fetal clearance; **B,** fetal clearance is 1% of maternal clearance (that observed experimentally); **C,** fetal clearance is 5% of maternal clearance, as would be the case when fetal metabolic activity expressed per tissue mass is similar to that in the adult; **D,** fetal clearance is 10% of maternal clearance, as would occur only when fetal enzyme activity is higher than that in the adult.

to the concentration gradient across the membrane, it is not a measure of the permeability of the placenta.

Permeability is the ease with which a molecule (or drug) crosses a membrane and is a function of the membrane itself and properties of the molecules. The *placental barrier,* as described previously, consists of a closely apposed layer of syncytiotrophoblast and fetal endothelial cells. Cell membranes consist of *lipid bilayers* and allow the passage of small, lipid-soluble molecules relatively easily. Tight junctions between endothelial cells in the placenta minimize paracellular transport. The composition of lipid membranes may alter transport characteristics. Known influences on lipid composition include diet, hormones, and pregnancy.[21,22]

Permeability can be considered per unit of placental tissue or as the placental unit as a whole. Traditional membrane studies express permeability in terms of surface area and thickness of the membrane. In the placenta, the membrane is very thin, and permeability is much more dependent on surface area. Because it is difficult to estimate placental surface area, permeability usually is expressed per unit tissue mass or in terms of the whole placenta. For comparisons in different experimental situations, placental perfusion studies often report relative permeabilities comparing test drugs with known substances, usually antipyrine.

Figures 24-4 and 24-5 show examples of passive diffusion systems. The concentration of drug in maternal plasma and the initial lack of drug in fetal plasma provide the gradient across the placenta. In the case of bolus administration (see Figure 24-4), net transfer of drug occurs from mother to fetus, and the fetal concentration increases until the concentration is equal in the maternal and fetal compartments. At this point, net transfer is zero. From this point on, the maternal concentration is less than the fetal concentration, and net transfer is from the fetus to

the mother. In the case of a constant infusion (see Figure 24-5), the fetal concentration increases owing to net transfer from mother to fetus until steady state is achieved. In Figure 24-5, *A,* with no elimination from the fetus, an equilibrium is established whereby the fetal and maternal concentrations are equal and no net transfer occurs in either direction. Drug molecules are still randomly crossing back and forth across the placenta as determined by the permeability and drug concentration, but at the same rate in both directions. When the drug infusion is stopped, the maternal concentration will fall, setting up a gradient from fetus to mother. This fundamental process provides the foundation for all distributive properties of drugs.

Rates of transfer from mother to fetus can be determined in experimental models. Using the Fick principle, measuring the change in concentration over either the uterine or umbilical circulations (at specific flow rates) will determine the amount of drug removed from maternal plasma or the amount of drug taken up by fetal plasma. These measurements can be achieved by in situ perfusion of whole placentas of small animals, controlling placental and umbilical blood flows, or in chronically instrumented sheep models using flow probes on uterine and umbilical circulations.[23] In addition, the human placenta can be perfused ex vivo.[24-26] The calculated measures of placental clearance obtained from these experimental models provide a measure of placental permeability. Of note, this measure has the same units as those for placental clearance (volume/time). This method also can determine the amount of drug eliminated by the placenta.

Another method uses mean steady-state concentrations in the mother and fetus applied to a two-compartment pharmacokinetic model (see Figure 24-3).[20] This method calculates the placental clearances in both directions across the placenta and is used in sheep and primate models.[27-30] In the absence of active transport and fetal metabolism, these placental clearances are measures of the permeability of the whole placenta. The advantage of this model is that blood flow measurements are not required. It does require drug administration to "steady state" (in both compartments) and drug determinations from both circulations. Neither of these methods can be utilized clinically, so extrapolations from animal and ex vivo placental perfusion studies are required to predict drug concentrations in the human fetus. The two-compartment model under steady-state conditions better reflects long-term drug therapy. In addition, parameters derived under steady-state conditions can be used to model single-dose situations. Most of the equations and graphs in this chapter are generated from this model and provide a framework for understanding the effects that physiologic parameters have on fetal distribution.

Molecular size and *solubility* are the drug characteristics that determine drug permeability. Lipid-soluble drugs with molecular weights of up to 600 are readily transferred across the placenta. Water-soluble drugs up to 100 daltons in size also readily cross; larger hydrophilic molecules cross according to their coefficient of diffusion in water.[24] Placental perfusion studies comparing substances of varying sizes and solubilities demonstrate relative permeability rankings that are a combination of the two factors.

Active Drug

When drugs are present in plasma, they may be bound to plasma proteins, or if they are weak acids or bases, they may exist in an ionized state. Only drug that is *unbound* and *nonionized* is available for transfer across the placenta; in this state, it often is referred to as *active* drug. The percentage of drug bound to protein is determined by the number and affinity of binding sites, whereas the percentage of drug ionized is determined by the pK_a of the drug and the pH of the plasma. In both cases, drug will either bind or dissociate—that is, it will shift between ionized and nonionized forms. On the maternal side, the active drug concentration decreases as it is transferred across the placenta to the fetus. Subsequently, drug bound to proteins will dissociate, and ionized drug will shift from an ionized to a nonionized form; newly formed active drug will then be available for further transfer. On the fetal side, active drug increases in concentration and will bind to proteins and ionize to achieve the appropriate proportions determined by the conditions. If protein-binding attributes and pH were the same in mother and fetus, total drug concentrations in the mother and the fetus would be the same at equilibrium. Because differences exist in protein binding and fetal pH is slightly less than maternal pH, the drug concentrations will be different in the fetal and the maternal compartments.

The two major proteins involved in binding drugs are albumin and α_1-acid glycoprotein, the latter particularly involved in binding basic drugs.[5,31,32] During pregnancy, maternal albumin concentrations fall, although total albumin is increased.[31,33,34] In the fetus, albumin levels increase with gestation and, toward term, exceed those in the mother.[31] α_1-Acid glycoprotein levels tend to be rather variable in both the mother and fetus, but fetal levels are almost always less than maternal levels.[31] Protein binding is expressed as percent of drug protein bound. Equation 3 and Figure 24-6 describe the relationship between protein binding in the mother (M) and fetus (F) and the effect on total drug concentration at steady state (C_{ss}).

$$C_{ssFtotal} = \frac{C_{ssMunbound} / C_{ssMtotal}}{C_{ssFunbound} / C_{ssFtotal}} C_{ssMtotal} \quad (3)$$

Binding differences less than 40% to 60% tend to be insignificant. For drugs with high protein binding, however, a major effect on the fetal-to-maternal difference in total drug may be seen. Although bound drug may seem to be of little relevance, because it is not active, it serves as a depot that may prolong fetal or newborn exposure, particularly when affinity is high or when drug clearance in the newborn is poor.

The pK_a of a drug determines the degree of *ionization* at a specific pH. Usually only 0.1 pH unit difference exists between mother and fetus, so the difference in ionization is minimal. The effect of maternal and fetal pH differences on fetal-to-maternal concentration ratios of drugs that are weak acids and bases can be calculated from the Henderson-Hasselbalch equation:

The Henderson-Hasselbach equation:

$$pH = pK_a + \log \frac{[base]}{[acid]} \quad (4)$$

For weak acids:

$$C_{ssFtotal} = \frac{1 + 10^{(pH_F - pK_a)}}{1 + 10^{(pH_M - pK_a)}} C_{ssMtotal} \quad (5)$$

For weak bases:

$$C_{ssFtotal} = \frac{1 + 10^{(pK_a - pH_F)}}{1 + 10^{(pK_a - pH_M)}} C_{ssMtotal} \quad (6)$$

Weak acids are less ionized and weak bases are more ionized at lower pH. As fetal pH decreases below maternal pH, the amount of total drug in the fetus will change (Figure 24-7); weak acids will decrease in amount, whereas weak bases will increase. While the pH differential is maintained, drug effect will not be altered, because active drug levels are not affected. In the fetus, transition back to a normal pH is likely to be gradual, and placental redistribution will occur without exposure of the fetus to excessive (for basic) or subtherapeutic (for acidic) concentrations of active drug. If at the time of delivery the fetal pH is low, total concentration of basic drugs may be higher than in the mother, and as pH corrects in the newborn and drug quickly returns to an active nonionized state, adverse drug effects may occur. For acidic drugs, drug concentrations in the newborn infant may be less that that required for therapeutic benefit after resolution of fetal acidosis.

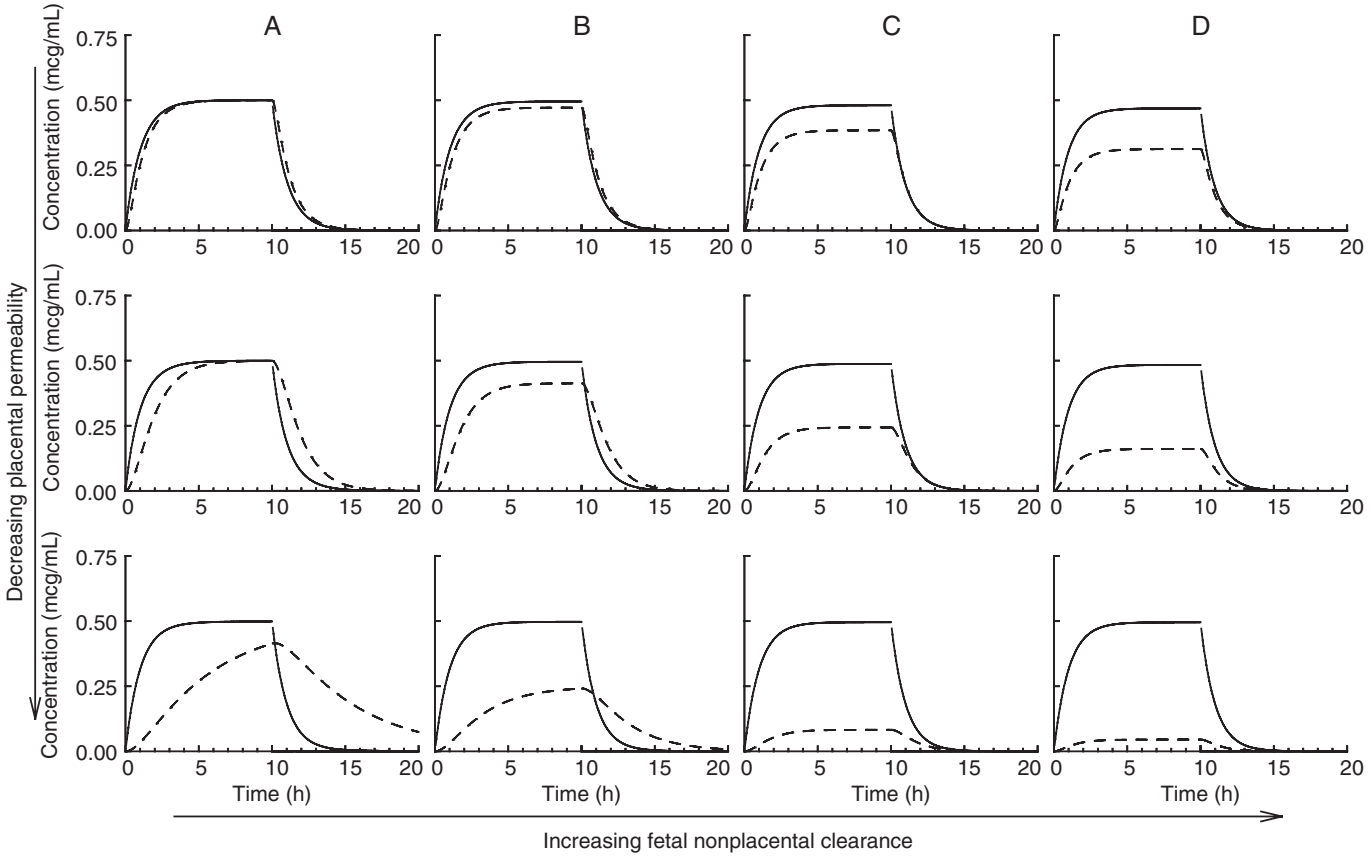


Figure 24-5 Drug concentration–time curves for the mother *(solid line)* and the fetus *(dashed line)* during and after a constant infusion of drug. These plots represent the solutions for the rate equations of a two-compartment model during and after infusion of drug wherein placental clearance (a measure of placental permeability) and fetal nonplacental clearance (direct fetal elimination) were varied while all other system parameters remained constant. Steady-state concentrations provide a good estimate of fetal exposure in relation to maternal concentrations. See Figure 24-4 for details on the specific parameter values.

Placental Blood Flow

The two sides of the placenta are perfused independently. Maternal placental blood flow is about twice that going through the umbilical circulation (Chapter 14, Mechanisms of Transfer Across the Human Placenta). Experimental situations show that decreasing blood flow, particularly in the umbilical circulation, will decrease delivery of freely diffusible drugs across the placenta (flow limited) but will have little effect on drugs that are "permeability limited." Placental circulations are fairly stable at most times in pregnancy, but will show gradual increases over gestation. Fetal compromise and uterine contractions are two clinical entities in which decreased fetal drug transfer has been demonstrated clinically. Changes in blood flow are unlikely to affect steady-state levels achieved unless the increased blood flow enhances nonplacental clearance mechanisms in the mother or the fetus.

Active Transport of Drug Across the Placenta

Cell membranes, including those of the syncytiotrophoblast and villous capilliary endothelium, contain large numbers of transport proteins that require cellular energy to function.[35,36] The specificity, orientation (into or out of the cell), and position (whether luminal or basal membrane) are important factors in determining the effect placental transporters will have on drug disposition.[37] Few drugs appear to be entirely dependent on active transport for transfer across the placenta to the fetus. Most drugs will cross to the fetus by passive diffusion. However, placental transport proteins may modify the final concentrations achieved.

The transporters involved in drug disposition belong primarily to two classes.[36] These are the ATP-dependent binding cassette proteins ("ABC transporters") and the solute-linked carrier proteins ("SLC transporters"). In the placenta, ABC transporters cause efflux of drug from the syncytiotrophoblast or fetal endothelium.[38] Energy in the form of ATP is required for activity. ABCB1 (P-glycoprotein), and ABCG2 (breast cancer–related protein [BCRP]) are located in the apical membrane directing drug toward the maternal circulation. ABCB2 (multidrug resistant protein 2 [MRP2]) and ABCC1 (multidrug resistant protein 1 [MRP1]) are on the basolateral membrane and direct drug toward the fetus.[38,39] ABCG2 also is on the fetal endothelium and can direct drug toward the fetus.[38] The SLC transporters are a more diverse group and include organic anion and cation transporters.[40-42] Those found in the placenta include OAT1 and OAT4, OCT1 to OCT3, and OCTN1 to OCTN3, with OAT4 and OCT3 localized to the basolateral membrane.These transporters use the electrochemical gradient of the cell for energy and can transfer substrates in both directions.While solute exchange is not obligatory, solute exchange can allow transfer against a concentration gradient by using the electrochemical gradient of the co-substrate.

The overall impact of transporters on drug disposition is still being delineated, though, in general, their action tends to protect the fetus from drug exposure. Studies in placental culture systems, the perfused human placenta, and mouse models wherein P-glycoprotein was inhibited show significant increases in drug levels on the fetal side.[43-45] In mice lacking ABCB1, increases in drug toxicity have been demonstrated in

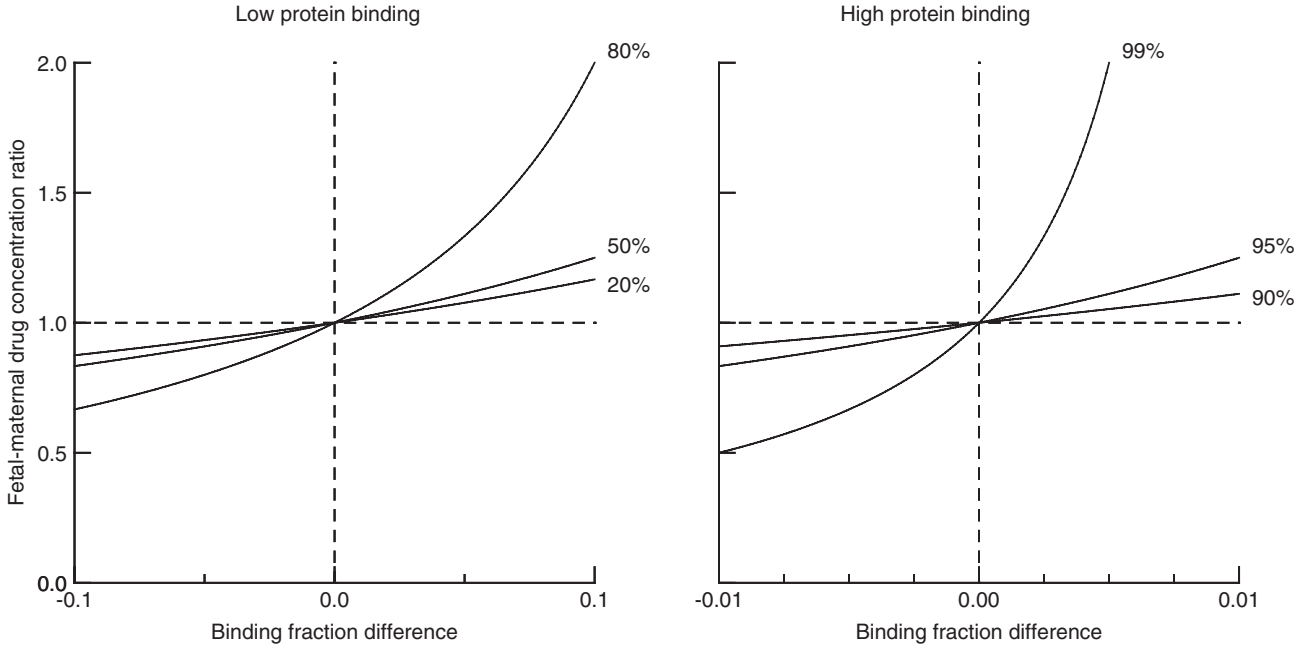


Figure 24-6 Effect of fetal-maternal differences in protein binding on steady-state fetal to maternal total drug concentration ratios. Recall that unbound drug concentrations will be the same in the fetus as in the mother. Drugs that exhibit low protein binding (up to around 50%) exhibit only small differences in the fetal-to-maternal concentration ratio, even with binding differences up to 10%. As binding increases, more marked effects are seen. For drugs that are highly protein bound, even small differences (less than 1%) can have marked effects on fetal-to-maternal total drug ratio. Graphs are plotted from Equation 3 for various levels of maternal protein binding, with fetal protein binding ± 10% of maternal for left-hand graph and ±1% of maternal for right-hand graph.

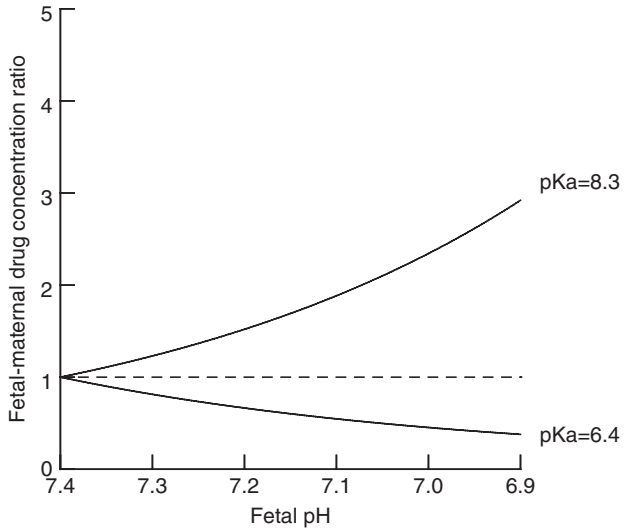


Figure 24-7 Effect of fetal-maternal pH differences on steady-state fetal-to-maternal total drug concentration ratios. Recall that nonionized active drug concentrations will be the same in the fetus as in the mother. Total concentrations of weak acids will be less in the fetus, whereas total concentrations of weak bases will be higher in the fetus. Graph is plotted from Equations 5 and 6 with maternal pH set at 7.4.

the fetus.[46] Placental ABC transporters are differentially regulated by hormone concentrations, inflammatory cytokines, and growth factors.[38] In the two compartment model, active transport will be reflected by differences in the maternal-to-fetal and fetal-to-maternal clearances. Active efflux transport from fetus to mother will effectively reduce fetal drug exposure.

Other Transport Mechanisms

Facilitated diffusion processes are known to occur in the placenta for disposition of nutrients and waste products and also have a role in drug transfer.[47] Specific macromolecules are taken up across the placenta by pinocytosis. An example is IgG, which can be administered therapeutically.[48] Aquaporins present in the placenta may provide bulk flow channels for small hydrophilic drug molecules.[49]

Placental Metabolism

The placenta is a highly metabolic organ producing and modifying many different hormones. However, the extent to which drugs are metabolized is not well delineated. Several members of the cytochrome P-450 subclasses are present and capable of metabolizing drugs at the maternal-fetal interface though expression is often less than other tissues.[50,51] Using sensitive methods, conjugation enzymes have also been detected in the placenta.[52,53] Again the extent to which they diminish fetal exposure is unclear. The placenta will act as a first pass clearance system and decrease drug concentrations reaching the fetus. However the fetus may well be exposed to metabolites of drugs that are not always harmless.[54] In the two compartment model with maternal administration, placental metabolism would have the same effect as fetal metabolism.

MODIFIERS OF FETAL PLASMA CONCENTRATION

Fetal Circulation

Streaming of oxygenated blood from the umbilical vein, through the ductus venosus and foramen ovale in the heart leads to the delivery of better oxygenated blood to the head and upper extremities. Thus the concept of having different distribution in separate regions is not new.[55] The high extraction of oxygen

from fetal blood maintains this differential across the placenta and thus, the difference in carotid arterial and descending aortic oxygen tensions is able to persist. A similar phenomenon is likely to occur with drugs that cross the placenta, although it has never been clearly documented. This difference will be most pronounced when the umbilical arteriovenous difference is greatest—most likely to occur after bolus administration or for drugs highly metabolized by the fetus.

The fetal circulation also affects drug distribution in the liver.[56] The fetal liver is the first organ encountered by fetal blood returning from the placenta. In the healthy fetus, 50% to 90% of blood flow is diverted through the ductus venosus.[57] Pathologic conditions can lead to redistribution of hepatic blood flow with enhanced hepatic metabolism of drug. Similarly, the same constraints pertaining to umbilical arteriovenous differences hold.

Fetal Drug Clearance

Thus far the discussion has been limited to systems in which the fetal nonplacental clearance is zero. As noted previously, that is not the case with zidovudine, for which the fetal steady-state levels can be less than those in the mother. However, differences cannot be attributed to differences in protein binding or pH, or to active transport.[28] Clear evidence demonstrates that the fetus can metabolize zidovudine to its glucuronide metabolite.[28] Figure 24-3 and Equation 2 include an elimination component directly from the fetus. The effect of direct fetal clearance on fetal drug concentrations is shown in Figure 24-3, *B* to *D,* with oral administration and Figure 24-4, *B* to *D,* with intravenous administration. Direct fetal clearance lowers fetal drug concentrations; this decrease is most marked when placental permeability is reduced. Of note, this effect differs from those of protein binding and ionization in that fetal clearance affects active drug concentrations and hence reduces *active* drug concentrations in the fetus.

The Two-Compartment Model at Steady State

In many of the examples given, the effect of changes in physiologic parameters is best appreciated by comparing steady-state concentrations in the mother and the fetus. A relationship between fetal and maternal plasma concentrations can be deduced from the rate equations for the two-compartment model under steady-state conditions. *Steady state* is defined as the condition in which the amount of drug in a compartment does not change with time—that is, the amount of drug being added to the system is the same as that leaving the system.

Rate equation for maternal compartment at steady state:

$$\frac{dD_m}{dt} = R_m - Cl_m \cdot C_m - Cl_{mf} \cdot C_m + Cl_{fm} \cdot C_f = 0 \qquad (7)$$

Rate equation for fetal compartment at steady state:

$$\frac{dD_f}{dt} = Cl_{mf} \cdot C_m - Cl_{fm} \cdot C_f - Cl_f \cdot C_f = 0 \qquad (8)$$

Rearranging the fetal equation gives an expression that relates fetal steady-state plasma concentration to the maternal concentration.

$$\frac{C_{ssF}}{C_{ssM}} = \frac{Cl_{mf}}{Cl_{fm} + Cl_f} \qquad (9)$$

This expression shows the interdependence of fetal clearance mechanisms and placental permeability in determining fetal drug exposure. For drugs that cross the placenta by passive diffusion, Cl_{mf} will be equal to Cl_{fm} (clearance being an attribute of the placenta and a measure of placental permeability). When fetal nonplacental clearance and placental clearance are equal, the fetal concentration is 50% of maternal concentration (see Figure 24-5, *C, middle row,* and Figure 24-5, *B, bottom row*). Furthermore, the same degree of fetal or nonplacental clearance will have a more marked decrease on fetal plasma concentration when placental clearance (or permeability) is low (see Figures 24-4 and 24-5).

Fetal Elimination Mechanisms

Drug-metabolizing enzymes are both present and active in the fetal liver, albeit at reduced levels compared with the adult liver for most enzymes.[28,50,51,58-63] As seen in the model in Figure 24-5, even small amounts of fetal clearance can have marked effects on the fetal-maternal drug ratio, depending on placental permeability. As might be expected, drug-metabolizing capacity will increase as gestation advances; however, placental permeability also increases with advancing gestation. In the sheep, fetal metabolism of acetaminophen increases concordant with growth.[61] In the baboon, fetal morphine metabolism does not appear to change over a similar period of time.[64] Another potential issue is induction of drug-metabolizing enzymes. Maternal smoking is known to induce many placental enzymes, but little is known about corresponding induction of hepatic enzymes prenatally.[54] Phenobarbital has been used successfully near term to reduce hyperbilirubinemia by induction of glucuronosyltransferase UGT1A1.

The *products of metabolism* also must be considered. These often are inactive; however, some metabolites are known mutagens, some are pharmacologically active, and others are true drugs in that a *prodrug* was administered.[65] Rate equations for metabolite formation can be written for the two-compartment model. The solution for these equations is clearly more complex, even at steady state, but fetal metabolite concentration can be expressed as a function of maternal concentration.[66] In this instance, because metabolite is being formed in the fetus and because placental clearance for metabolites often is less than for the drug, the tendency is for fetal metabolite concentrations to exceed those in the mother unless other clearance pathways exist.

In adults, the other major route of drug elimination is urinary excretion. The *fetal urinary excretion system* is unique in that fetal urine, a major component of amniotic fluid, is swallowed by the fetus, thereby providing the potential for any drug excreted in fetal urine to be absorbed back into the fetus through the fetal intestinal tract. The extent to which this occurs is not known. Clearly, not all drugs are reabsorbed in their entirety, because many can be detected in meconium. Under steady-state conditions, drug excreted into urine that is reabsorbed would not contribute to fetal nonplacental clearance; however, drug that becomes sequestered in meconium would contribute to that clearance. Recent research suggests almost complete reabsorption (personal communication).

TISSUE DISTRIBUTION

Volume of distribution is a term used to describe the volume a drug appears to occupy based on the plasma concentration and has little bearing on the physical system. Volumes of distribution vary from drug to drug based on the solubility of the drug in different tissues reflective of the tissues lipid and water content. Individual patients also differ in body composition. The fetus has a very high water content, so lipid-soluble drugs will not have a large volume of distribution. For water-soluble drugs, the volume of distribution would be expected to be higher (per tissue mass) than in the adult, particularly if the amniotic fluid compartment cannot be separated out. Fat deposition occurs during the last trimester, so during this period the capacity for transfer of lipid-soluble drugs would be greater. Drug would concentrate into fat tissues until steady state is achieved. Because the fetal volume of distribution is small compared to that of the mother, the effect of fetal changes is unlikely to be detectable in maternal plasma

concentrations. The total amount of drug delivered to the fetus, however, could be substantially higher.

Fetal Blood-Brain Barriers

The brain has evolved a complex set of protections to shield it from external influences and yet still provide passage for the essential nutrients and communication signals. The capillaries that supply the brain are continuous (nonfenestrated), have extremely tight junctions preventing paracellular transport, and are surrounded by astrocytic foot processes.[67,68] Several transporters are present in the endothelium, transporting drugs among other substances from the endothelium back to the circulation.[69-72] Pial membranes carry blood vessels across the surface of the brain. Where the pial membrane comes in contact with the ependyma of the ventricular system choroidal tissue develops.[68] The capillaries within the choroidal tissue are fenestrated; however, the overlying ependyma is highly specialized with very tight junctions; therefore transfer is essentially all transcellular. Several drug-metabolizing enzymes have been identified in choroidal ependymal tissue and are thought to protect the brain from substrates.[73-77] In addition, many transporters are present on both luminal and abluminal sides.[69-71] With respect to drugs, P-glycoprotein is on the abluminal side, removing drugs or their metabolites from the ependymal cell back to the capillary. Other transporters are present on the luminal and abluminal membranes transporting substances into and out of the ependymal cells. The remainder of the ependyma that is in contact with the ventricular system forms a loose network of cells with minimal barrier function. Thus, once in the cerebrospinal fluid, drugs have access to target neurons that lie near brain surfaces. Within the brain itself, certain cells have been identified as containing drug-metabolizing enzymes, although whether their function is protective or activational is not clear.[76,78] Transporters also are present on neuronal cells. Although not all of the details are known at this time, the distribution of drug metabolizing enzymes and specific transporters at specific sites give some hint of the complexity to unfold.[79,80]

The fetal blood-brain barrier is more robust than was once thought, although developmental changes are still likely. Much ongoing research in neonatology is directed toward neuroprotection of the fetal and neonatal brain. Hence understanding brain distribution of potential agents requires prediction of the concentration at the site of action to optimize effectiveness. Again, plasma concentration is the driving force for drug movement by passive diffusion of drugs down a concentration gradient modulated by metabolism and active transportation.

CLINICAL APPLICATIONS

Most drugs taken during pregnancy are for the mother's benefit. Use of drugs for fetal benefit is increasing, however. Various clinical scenarios are likely to be encountered, with potential ramifications for the patient and the unintentional recipient. Optimally, a drug that achieves therapeutic concentrations in the mother while minimizing fetal concentrations will be selected for maternal treatment, whereas a drug that achieves therapeutic concentrations in the fetus while minimizing maternal adverse effects will be selected for fetal treatment. An appreciation of fetal drug distribution, as summarized in Box 24-1, and drug properties will allow selection of appropriate drugs to attain these goals. It must be kept in mind that drugs are developed primarily for use in nonpregnant adults, and desired characteristics include good oral bioavailability, good tissue distribution, and long half-life—properties that generally lend themselves to placental transfer and hence fetal exposure. Furthermore, what may generally be seen as improvements in drug formulation may alter distributive characteristics unfavorably for the fetus.

BOX 24-1

Key Points in Fetal Drug Distribution

- The maternal plasma concentration is the driving force for drug delivery to the fetus.
- Drug size and solubility determine a drug's placental (and tissue) permeability.
- Protein binding and ionization of drugs can lead to prolonged exposure for the neonate after birth.
- Fetal elimination mechanisms decrease fetal plasma concentration most markedly for drugs with low permeability.
- Products of fetal metabolism (drug metabolites or active moieties of prodrugs) can reach higher levels in the fetus than those in the mother.
- Drug transporters and drug metabolizing enzymes at the tissue level are important regulators of drug concentrations at the site of action.

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25

James F. Padbury and Robert A. Berg

Developmental Pharmacology of Adrenergic Agents

For hemodynamic responses to adrenergic agents, classic pharmacodynamic theory describes differences among agents in receptor specificity, potency, and pharmacologic profile based on parameters such as the median effective dose (ED_{50}) or half-maximal concentration. These pharmacodynamic parameters, however, are derived exclusively from in vitro studies. This chapter describes clinical studies in neonates, infants, and children based on a method of analysis that permits derivation of the *threshold,* or minimum plasma drug concentration, associated with discernible effects. The threshold is derived by graphic analysis of in vivo dose-response data. Examples are provided of both animal and human data, which demonstrate agreement of experimental results with predicted responses. This approach permits more direct comparison of studies with different patient populations and studies from different investigators. Also provided in this chapter is evidence of first-order clearance kinetics of dopamine and dobutamine over the ranges of dosages used normally in neonates, infants, and older children.

In numerous instances in clinical neonatology, pharmacologic principles developed in adult humans and animals have been used to guide pharmacologic therapy in newborns. Because newborns are not just small adults, however, this approach has resulted in many untoward drug-related incidents, including the historic chloramphenicol "gray baby syndrome,"[1-3] errant aminoglycoside drug doses,[4-6] the excessive absorption of topical antiseptics,[7-11] and the benzyl alcohol "gasping syndrome."[12,13] Inotropic drugs are among several classes of drugs whose initial use in neonates and children was based on data generated in adults. Drug manufacturers generally have avoided costly clinical trials of inotropic agents in neonates and children. Industry has instead relied on the experience of clinicians who care for newborns and who base the use of such drugs in their patients on information generated in adults. Thus information on safety and efficacy in newborn and pediatric patients has been the result of practical experience generated in the clinical setting. Adverse effects of drugs that enter the clinical setting in this fashion are reported only after use has become widespread.

To compound this situation further, the plasma concentrations of all of the widely used inotropic agents were well below the level of detection of available assays at the time of the initial pharmacologic investigations. This technical problem inhibited assessment of the pharmacokinetics and pharmacodynamics of these inotropic drugs. The development and application of sensitive assays for commonly used inotropic drugs subsequently permitted evaluation of their actions in the clinical setting. We have used this approach to evaluate the pharmacokinetics and pharmacodynamics of inotropic drugs in neonates, older infants, and children. This chapter reviews the results of such studies, which provide a rational basis for the use of inotropic drugs in critically ill newborns and children.

PHARMACOLOGIC PRINCIPLES

Pharmacokinetics

Pharmacokinetics is the study of in vivo drug disposition. In most circumstances, plasma drug levels are measured, and their concentrations at different time intervals are analyzed by comparison against classic models. A derived parameter that reflects the rate of drug disposition is then calculated. The pharmacokinetic parameter most familiar to clinicians is the half-life. Figure 25-1 shows a typical graph of concentration versus time. Drug is administered as a bolus at time zero. Plasma drug concentration rises rapidly to a short-lived peak concentration. The plasma concentration rapidly decreases as the drug is equilibrating in the plasma compartment. This equilibrium period is referred to as the *distribution phase* or *α phase.*

A second, more prolonged period of decay in plasma drug concentration also is observed. In this latter period, the rate of decay of plasma drug concentration is dependent on mechanisms for elimination or clearance, such as excretion and metabolism. This is called the *elimination phase* or *β phase.* For drugs whose mechanisms of elimination have not been saturated, the β or elimination phase of the decay curve is linear when displayed on a semilogarithmic plot. Such drugs are said to have *first-order* or *linear kinetics.* First-order kinetics simply describes nonsaturability within the range of dosages that were studied. Drugs whose elimination kinetics are saturable have nonlinear exponential decay curves.

The plasma half-life, or the time required for drug concentration to decrease by 50%, is determined graphically or by computer analysis of the decay curves. For drugs with extremely rapid rates of disposition, the short half-lives are difficult to determine accurately. Small errors in the timing of samples during the elimination phase lead to large errors in half-life determination. In addition, drugs with extremely short half-lives, such as inotropic drugs with half-lives of 1 to 2 minutes, usually are administered by continuous infusion. In such circumstances, a more appropriate pharmacokinetic parameter is the *plasma clearance rate.* Clearance is calculated by the following formula:

$$\text{Plasma clearance rate (mL/kg/min)} = \frac{\text{Dose of infusate (g/kg/min)}}{\text{Steady-state concentration (g/mL)} - \text{baseline concentration (g/mL)}}$$

For drugs with first-order kinetics, a linear relationship exists between the steady-state plasma drug concentration and dose. The calculated clearance rate is independent of dose, a finding suggesting nonsaturability of elimination mechanisms over the range of dosages studied. Experimentally, this rate is determined by measurement of steady-state plasma drug concentration at several different stable infusion rates. Clearance usually is normalized to body weight, whereas half-life is independent of body mass. Although clearance rate is a more appropriate derived parameter for drugs with short half-lives, it is a concept less familiar to clinicians.

We examined elimination kinetics for the inotropic agents dopamine and dobutamine in neonates, older infants, and children in the critical care setting.[14-17] In each study, the drug was infused with a calibrated infusion pump. Samples for measurement of plasma drug concentration were collected at several infusion rates. Data were analyzed by computer-based graphic analysis. Figure 25-2 is a representative plot of data for plasma dobutamine concentration versus dose in newborns and older children. For each patient, a linear relationship between dose and plasma drug concentration was found, confirming first-order kinetics. The clearance rate of dopamine and dobutamine

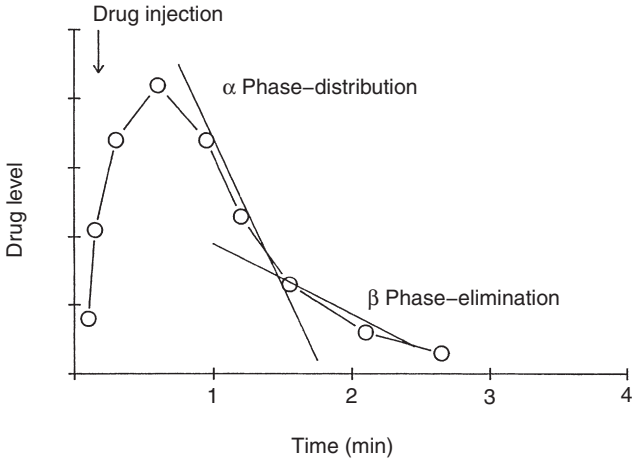


Figure 25-1 Typical concentration versus time result used to generate drug half-life.

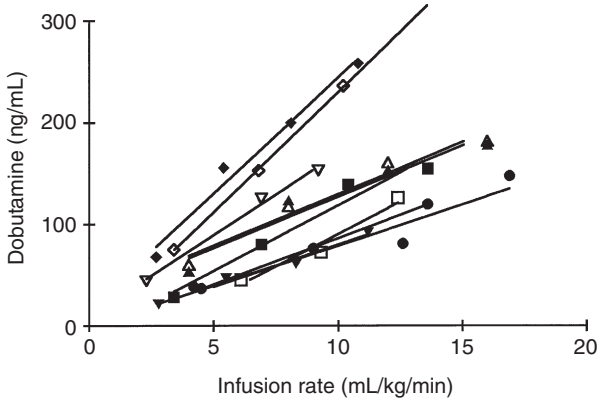


Figure 25-2 Plasma dobutamine level (ng/mL) versus infusion rate. The correlation between dobutamine level and infusion rate was significant ($r = 0.97$; $p < .001$). *(From Habib DM, Padbury JF, Anas NG, et al: Dobutamine pharmacokinetics in pediatric intensive care patients,* Crit Care Med *20:601, 1992.)*

in neonates and older children is independent of dose, a finding that further supports first-order kinetics over the range of dosages studied.[14-17] The more remarkable observation was the wide range of actual clearance rates. Although mean clearance was 75 to 90 mL/kg/minute, the range was 30 to 170 mL/kg/minute. A similar wide range of dopamine clearance levels has been observed. Figure 25-3 illustrates the mean and range of dopamine and dobutamine clearance rates in multiple studies.[14-18] Significant interpatient variation was noted for both dopamine and dobutamine plasma clearance rates in each of the clinical circumstances studied. Initially, it was not clear whether this was the result of specific illnesses in these patients or the result of other factors. Although it is important to generate the pharmacokinetic data under conditions of actual drug use in critically ill patients, physiologic derangements may have contributed to the high interpatient variations. To address this issue, we examined the clearance rate of dobutamine in a set of healthy normal patient volunteers using identical methods.[17] In each of the individual patients, a linear relationship between dose and plasma drug level was noted. The range of clearance rates, 38 to 218 mL/kg/minute, was similar to that seen in critically ill patients. This observation suggests that the high variation in clearance rate among individual patients is not caused by illness itself.

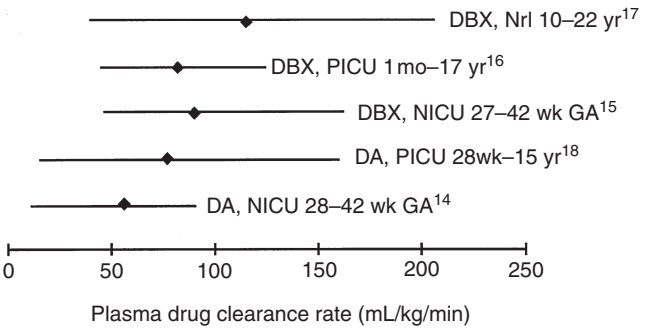


Figure 25-3 Ranges of clearance values in different studies.[14-18] Patients were in either neonatal or pediatric intensive care units (NICU or PICU) at the ages indicated. *DA,* Dopamine; *DBX,* dobutamine; *GA,* gestational age; *Nrl,* normal.

These pharmacokinetic data have significant clinical implications: The first-order clearance kinetics for these agents indicates that plasma drug level is directly and linearly related to dose. In the dosage range studied, clinicians need not be concerned that increasing dose leads to a sudden and exponential increase in plasma level, as seen with drugs with saturable kinetics such as phenytoin or aminoglycosides.

Special Pharmacokinetic Considerations in Neonates: Endotracheal Epinephrine

During cardiopulmonary resuscitation, establishment of an airway and adequate ventilation is generally the first priority. When epinephrine administration is indicated, the endotracheal route often is chosen as a matter of expediency. Animal models and case reports in neonates and children have established that endotracheal epinephrine during cardiopulmonary resuscitation can effectively restore spontaneous circulation.[18-26] In addition, endotracheal epinephrine administration to animals with normal circulatory status, hypovolemia, or asphyxia-induced bradycardia can increase heart rate, mean arterial pressure, and cardiac output.[27-29] The pharmacokinetics of endotracheal epinephrine depends on effective dispersion of the drug over the respiratory mucosa, pulmonary blood flow, and matching of ventilation (i.e., drug dispersal) to perfusion.[21,26]

During cardiopulmonary resuscitation, gross perturbations of ventilation and perfusion occur. Preexisting medical conditions, such as pulmonary edema, pneumonia, and airway disease, also affect the pharmacokinetics of endotracheally administered drugs.[30] Furthermore, epinephrine is a local vasoconstrictor and therefore inhibits its own uptake in the local pulmonary vasculature.[27,31] It should not be surprising that endotracheal administration results in lower peak plasma concentrations and a prolonged depot effect.[21-33] Administration of typical intravenous emergency epinephrine doses (0.01 mg/kg) by the endotracheal route does not significantly affect arterial epinephrine concentration during cardiopulmonary resuscitation in adults, whereas the same dose delivered intravenously increases arterial epinephrine concentration.[30] Animal models and studies in adult humans demonstrate that with use of the endotracheal route, a 5- to 10-fold increase in epinephrine doses generally is necessaryto attain hemodynamic effects comparable with those achieved using the intravenous route.[18,20-34]

The appropriate "endotracheal epinephrine" dose for neonates is not known. In comparison with intravenous doses, higher endotracheal doses of epinephrine are necessary for similar effects, yet prolonged hypertension from depot effects may be particularly dangerous to preterm infants at risk for intracranial hemorrhage. In addition, data are limited regarding metabolism of catecholamines after cardiopulmonary resuscitation in

neonates. Schwab and von Stockhausen[35] demonstrated that in premature neonates, free epinephrine was effectively inactivated by sulfoconjugation after endotracheal administration of 0.25 mg/kg during cardiopulmonary resuscitation. These investigators did not note any adverse effects, yet they documented 10-fold higher plasma epinephrine concentrations 1 and 2 hours after resuscitation.[32]

Pharmacodynamics

Classic pharmacodynamic theory suggests that the laws of mass action can describe the effects of administered agents such as adrenergic agonists. For a single ligand (e.g., exogenous agonist or endogenous catecholamine) interacting with a single class of receptors, the dose-response relationship is characterized by a sigmoidal dose-response relationship.[36] The actual data used to derive these curves usually are obtained after cumulative, stepwise-increasing doses. Boluses of the drug are most commonly infused. Larger doses are administered after return of the preparation to steady-state or baseline conditions. Concentrations ranging over 4 to 6 logarithms are needed to construct the sigmoidal dose-response curves. The data are fitted either graphically or by means of computer-based modeling programs to the expected sigmoidal response. Derived parameters such as the concentration necessary for 50% maximal effect or inhibition (ED_{50} or ID_{50}) and potency ratios are then used to define the pharmacologic profile. These parameters also are used to compare individual drugs, to compare subjects being studied under different physiologic or clinical conditions, or to compare subjects at different developmental ages. The assumptions underlying this methodology are as follows: (1) the administered agent achieves a steady-state concentration and effect at the receptor site after the bolus administration; (2) the maximum effect observed experimentally is indeed maximal, accurately measured, and physiologically meaningful; and (3) the maximum effect is relevant to the proposed clinical application of the drug or agent.

These experimental prerequisites preclude their measurement in human subjects in vivo. Maximal effects are difficult to achieve, and such doses may lead to dangerous side effects. Clinical administration of drugs rarely varies over a 4- or 5-log concentration range. Pharmacologic literature on the clinical application of newer inotropic agents reveals that the major portion of basic information used to define the pharmacologic profile, to determine their potency in comparison with other agents, and to ascertain their potential clinical relevance is derived largely from in vitro data.[37-42] It is clear that the generation of data from cumulative responses in isolated tissues is not relevant to how these drugs are used clinically. Nonetheless, a derived parameter is necessary to permit comparison of drug effects from different investigations. Ample information suggests that age, diagnosis, and intercurrent clinical problems significantly affect response,[40-43] metabolism,[14-17,44-45] or side effects[46] of inotropic agents.

Our goal in optimizing administration of inotropic agents was to develop an alternative approach to study the pharmacodynamics of inotropic drugs based on the relationship of the plasma concentration during clinical administration to the hemodynamic effects. Previous investigations comparing responses to infused catecholamines had suggested that no change occurred in a particular response until the catecholamine concentration reached or exceeded a "physiologic threshold."[47] Beyond the threshold value, a log-linear relationship holds between the plasma catecholamine concentration and the physiologic response. This concept is illustrated in Figure 25-4, in which the classic sigmoidal dose-response curve is shown and the location of the ED_{50} and the threshold on the classic dose-response curve are illustrated. The threshold model at the lower concentrations of the sigmoidal dose-response curve also is shown. A zero-order equation ($y = a$) relates the response to the log of the plasma catecholamine or drug concentration (pg/mL) below the threshold concentration. Above the threshold concentration, a linear increase occurs in response to logarithmic increases in plasma catecholamine concentrations ($y = m \log x + b$). A computerized nonlinear, least-squares regression program was developed to fit experimental data to these assumptions.[48] The *threshold* is defined as the catecholamine concentration at the intersection of these two lines. The program determines the best fit for the baseline value of the response, the threshold drug concentration at the threshold for that response, and the slope and correlation coefficient of the log-linear regression line beyond the threshold value. Goodness of fit to this model can be determined statistically.

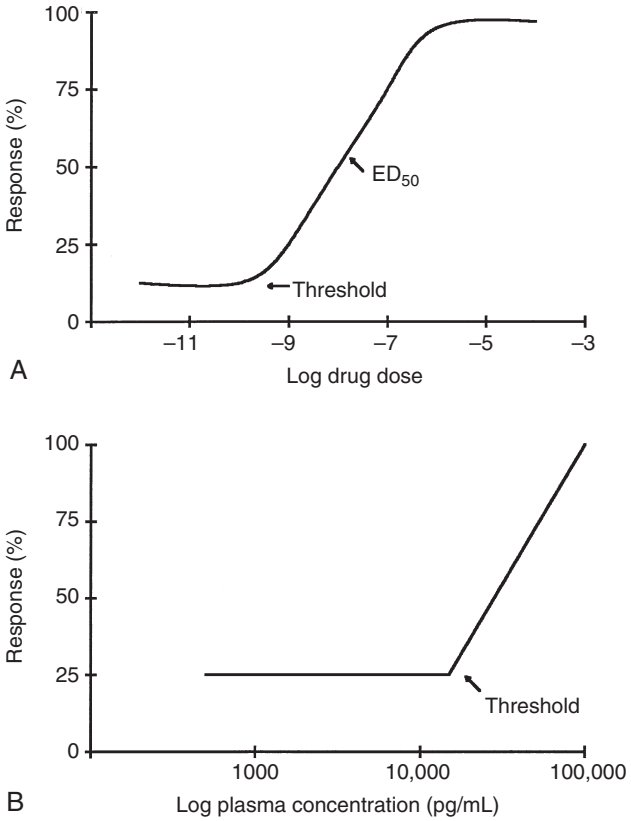


Figure 25-4 **A,** Dose-response curve showing the approximate location of the median effective dose (ED_{50}) and threshold. **B,** Log transformation of dose-response curve. The location of threshold is indicated as the intersection of the zero-order and log-linear responses.

To confirm the validity of this approach for in vivo studies, we first performed experiments in human infants and children. Preliminary studies confirmed that steady-state plasma concentrations and hemodynamic responses were achieved within 15 minutes. Thresholds for individual hemodynamic responses, including increases in blood pressure, cardiac output, and heart rate, were determined for each patient. Excellent agreement was observed between the predicted log-linear dose-response relationship and the measured data in newborns, older infants, and children. Figure 25-5 is a series of representative curves plotted from data for several children depicting the log-linear relationship of plasma dobutamine concentrations and hemodynamic parameters. Thresholds, similar to clearance rates, varied substantially between patients. Nonetheless, the data for most patients demonstrated the predicted log-linear relationship between plasma inotropic drug concentration and responses. Using the apparent threshold and plasma clearance rate for each patient, it is possible to calculate the minimum infusion rate that would

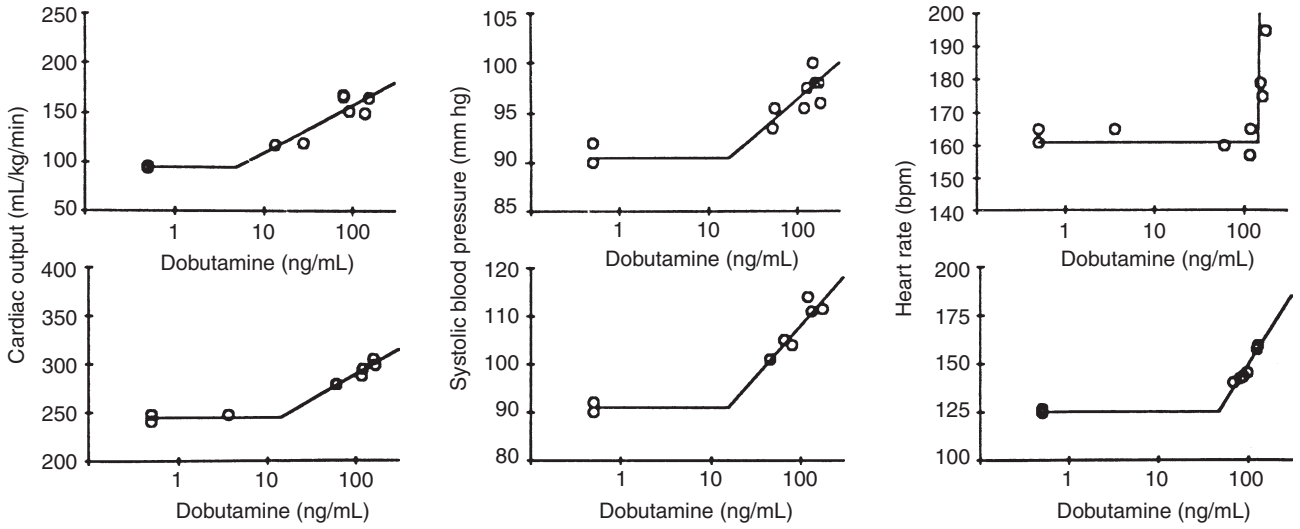


Figure 25-5 Representative dose-response curves. Plasma dobutamine levels are plotted on the logarithmic horizontal axis. Each response is plotted on a vertical linear axis. The *lines* indicate the computer-generated best fit. See Figure 25-3 for abbreviations. *NICU,* Neonatal intensive care unit; *Nrl,* normal; *PICU,* pediatric intensive care unit. *(From Habib DM, Padbury JF, Anas NG, et al: Dobutamine pharmacokinetics in pediatric intensive care patients,* Crit Care Med *20:601, 1992.)*

result in plasma drug concentrations equal to or greater than the threshold. The threshold for hemodynamic responses to dopamine occurred at plasma concentrations of dopamine achieved at an infusion rate of approximately 1.0 mcg/kg/minute and was fairly uniform among patients. For dobutamine, the plasma threshold was achieved at infusion rates of approximately 2.0 mcg/kg/minute.

These results agree with those of other studies demonstrating the effectiveness of dobutamine as an inotropic agent in the newborn,[49-51] despite contradictory reports.[43] In most of the patients we studied, dobutamine demonstrated a lower threshold for increases in cardiac output than for changes in blood pressure. This finding suggests that the hemodynamic effects of dobutamine in this age-group are the result of a direct inotropic response. Clearer evidence of a direct inotropic effect was indicated by the improvement of a load-independent index of myocardial contractility (the relationship of left ventricular end-systolic wall stress to rate-corrected velocity of fiber shortening) after dobutamine was infused. Generation of threshold data permits comparisons of different agents or among patients of different gestational ages. For example, comparison of the ratio of the cardiac output threshold to heart rate threshold (inotropic-chronotropic ratio) indicates a ratio of 2.5:1 for dopamine versus 6:1 for dobutamine in neonates.[14,23] This finding suggests a greater margin of efficacy versus adverse effects for dobutamine.

As noted during the study of the plasma clearance rate of dopamine and dobutamine, a wide interpatient variation was seen in the plasma threshold for the cardiovascular effects of both agents. The threshold for increases in systolic blood pressure in neonates in response to dopamine infusion ranged from 3 to 45 ng/mL of dopamine, which would have been achieved at infusion rates of 0.2 to 2.5 mcg/kg/minute. For dobutamine, the ranges were similarly quite wide. In infants and children, the threshold for dobutamine-mediated increase in cardiac index was observed at infusion rates of 0.2 to 9 mcg/kg/minute. The data are presented graphically in Figure 25-6. The mean and range of thresholds for each study group are shown. The interpatient variation in threshold was nearly 2 logs wide. It is possible that physiologic derangements were the source of the high interpatient variation in sensitivity to the pharmacodynamic effects of inotropic drugs. To address this issue, the clearance rate of dobutaminewas investigated in a set of healthy normal patient volunteers.[17] In these normal volunteers,

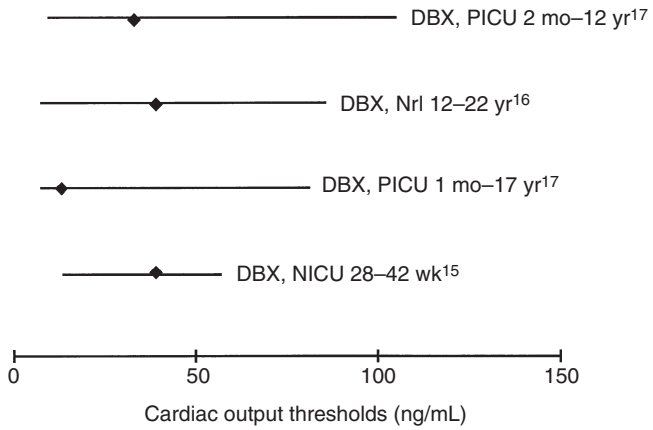


Figure 25-6 Ranges of threshold values in different studies.[15-17] Patients were in either neonatal or pediatric intensive care units (NICU or PICU) at the ages indicated. *DBX,* Dobutamine; *Nrl,* normal.

the range of thresholds for improvements in cardiac index was 4 to 20 ng/mL. The mean threshold for the entire group was achieved at dobutamine infusion rates of approximately 2 mcg/kg/minute.

The threshold approach provides several important advantages. First, it is based on actual data from clinical subjects. Pharmacologic data point to major differences in pharmacokinetics under different clinical situations, such as congestive heart failure, renal disease, or liver dysfunction.[44,45] Extrapolation of pharmacokinetic or pharmacodynamic data derived in experimental animal preparations may not be applicable to these conditions. Second, the traditional pharmacologic approach has used bolus drug injections for generation of dose-response curves. In this setting, drug doses and presumably plasma level or concentration at the receptor vary over 4 to 6 log units. Various investigators (ourselves included),[14,15,45,52] however, have shown that plasma concentrations of these agents during clinical use vary only over 1 to 2 log units. For example, plasma dobutamine concentrations in critically ill neonates averaged 23 ng/mL at the recommended

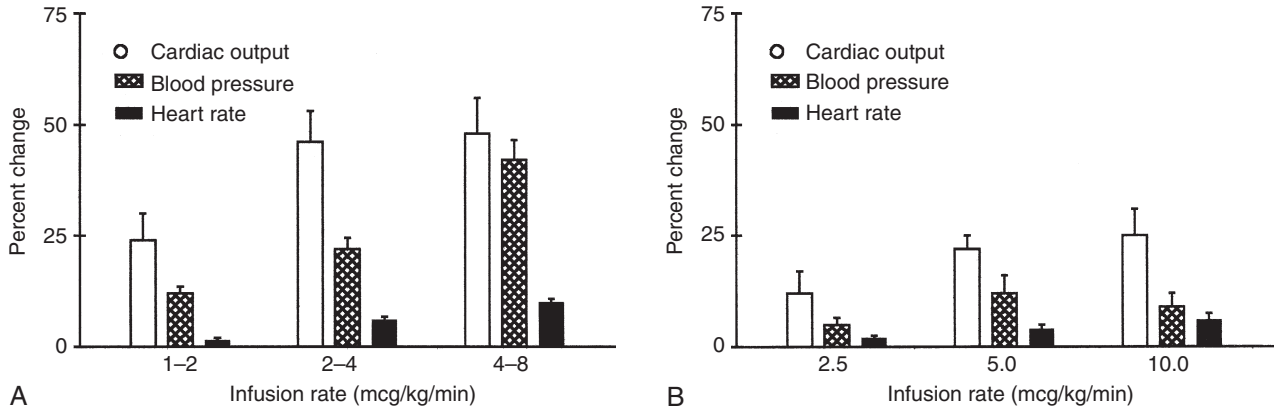


Figure 25-7 Comparison of hemodynamic responses to dopamine and dobutamine in neonates. Results are shown as a percentage of increase in cardiac output *(open bars),* blood pressure *(hatched bars),* or heart rate *(solid bars)* above pretreatment levels. *(Data from Padbury JF, Agata Y, Baylen BG, et al: Pharmacokinetics of dopamine in critically ill newborn infants,* J Pediatr *117:472, 1990; Martinez AM, Padbury JF, Thio S: Dobutamine pharmacokinetics and responses in critically ill neonates,* Pediatrics *89:47, 1992; Habib DM, Padbury JF, Anas NG, et al: Dobutamine pharmacokinetics in pediatric intensive care patients,* Crit Care Med *20:601, 1992; and Berg RA, Padbury JF, Donnerstein RL, et al: Dobutamine pharmacokinetics and pharmacodynamics in normal children and adolescents,* J Pharmacol Exp Ther *265:1232, 1993.)*

starting dose of 2.5 mcg/kg/minute and 92 ng/mL at 7.5 mcg/kg/minute. Because the ED_{50} was developed from a series of drug doses substantially beyond the range in clinical use, it may represent dose-response relationships quite different from those in the clinical arena. The threshold dose is based on steady-state, in vivo conditions and reflects the direct relationship between drug dose and effect during actual clinical conditions.

Finally, this experimental approach demonstrates the most rational use of these drugs in the clinical setting. The stepwise increase in dose with titration to the desired clinical end-point maximizes the beneficial effects of these agents while minimizing the adverse effects such as tachycardia or other arrhythmias (and also desensitization). Because a log-linear relationship exists between dose and response, a "doubling dose"–response curve, as was used in our dopamine studies (2 to 4 to 8 mcg/kg/minute), gives the best results. Likewise, weaning by successive 50% reductions in dose also is more rational than minute decreases with each change.

Special Pharmacodynamic Considerations in Neonates

Developmental changes in myocardial structure and function would be expected to modulate the responses of the newborn to catecholamines. The neonatal myocardium has a greater percentage of noncontractile tissue compared with that in adults and older children and has incomplete sympathetic innervation.[53] In addition, oxygen consumption and myocardial demands are relatively higher in the neonate. Although neonatal myocardial function is excellent at rest, myocardial reserve is limited. Contractility is near maximal at rest. Preload reserve, contractility reserve, and heart rate reserve all are limited.[53,54] It has been presumed that these issues would limit the neonatal myocardial response to catecholamines, although such limited response is not well documented. Similarly, differences in the degree of peripheral maturation and the mechanisms for vascular constriction and relaxation may affect the newborn's responses to inotropic agents.

When catecholamine infusions are used in neonates, the underlying disease states often are associated with pulmonary hypertension. Studies in infants and adults have demonstrated that higher doses of dopamine can increase pulmonary artery pressure, although these results are not consistent.[55-57] Pulmonary α-adrenergic receptors lead to pulmonary vasoconstriction, whereas dopaminergic receptors lead to pulmonary vasodilatation. In most circumstances, increased cardiac output and aortic blood pressure decrease baroreceptor-mediated sympathetic activity, thereby ameliorating pulmonary vasoconstriction. Adverse effects on pulmonary vasculature are not well documented in the neonatal population, and their occurrence has not limited the popularity of these agents in critically ill infants with clinical evidence of pulmonary hypertension.

In the neonate, cardiovascular shock generally is the result of intravascular volume depletion and myocardial dysfunction secondary to hypoxic injury. Therefore intravascular volume repletion is recommended for initial treatment of cardiovascular shock.[55] Myocardial dysfunction or vasoregulatory disturbances, however, frequently accompany shock. In this circumstance, inotropic and vasoactive therapy with catecholamines can improve myocardial function and oxygen delivery.[55-62] We compared the hemodynamic responses of newborns to either dopamine or dobutamine in the studies described earlier to generate the threshold data.[14-17] When responses are expressed as a percentage of improvement in cardiac output, blood pressure, or heart rate above initial level, as shown in Figure 25-7, dopamine has a greater effect on both cardiac output and blood pressure than that seen with "equivalent" (equimolar) doses of dobutamine. Dobutamine has a greater proportional effect on cardiac output than on blood pressure, consistent with its "selective inotropic effect" observed in many studies in adults.[63] This finding implies that in clinical disorders such as congestive cardiomyopathy, which frequently accompany perinatal asphyxia, dobutamine is a preferred inotropic agent. By contrast, cardiovascular dysfunction secondary to neonatal sepsis may be accompanied by substantial derangements in peripheral vascular function;in this setting, therefore, dopamine, with its greater effects on blood pressure and peripheral vascular contractility, is preferred.

Other investigators have suggested that dopamine may be more effective than volume expansion or dobutamine for management of hypotensive preterm infants during the first few days of life.[55,59-62] In this population, hypotension may be more frequently caused by poor peripheral vasoregulation with or without myocardial dysfunction than by volume depletion.[55,59,60] The greater chronotropic effect of dopamine can be a clinical limitation to its usefulness. Concomitant treatment with both drugs is frequent and not necessarily discouraged; however, direct pharmacodynamic evidence of differences, if any, in dose-response relationships when these drugs are given together is limited.

Neonates in congestive heart failure have high levels of endogenous catecholamines[62] and frequently require exogenous catecholamines for hemodynamic support.[64] Epinephrine infusions in isolated intact newborn piglet hearts resulted in left ventricular systolic and diastolic dysfunction, associated with irreversible ultrastructural abnormalities.[65] The clinical relevance of these worrisome in vitro findings is not clear.

SUMMARY AND CLINICAL IMPLICATIONS

The approach to pharmacokinetic and pharmacodynamic studies of inotropic agents presented in this chapter provides insights not afforded by more traditional methods. An experimental approach to the generation of a derived parameter, the threshold, is provided to describe dose-response results for in vivo data. This derived parameter permits comparison of responses among different subjects and results of different investigators. This approach avoids some of the flaws inherent in extrapolation of in vitro data to the clinical setting and allows new insights into the comparison of drug effects between different agents, in different clinical settings, and at different developmental ages. It also demonstrates the most rational approach to clinical use of these agents. Volume expansion is indicated before administration of inotropic agents. With an inadequate response in clinical (perfusion) or physiologic parameters (blood pressure, urine output, acid-base status, or cardiac output or contractility), inotropic support is indicated. The clinician should start at a low dose, near or just above the predicted threshold. The administration rate should be increased by doubling the dose every 15 to 30 minutes until the desired clinical response is observed or until undesired side effects (usually tachycardia) are observed. In a similar fashion, a rational approach to weaning by 50% reductions in dose is based on recognition of the log-linear relationship between dose and response. Therefore incremental adjustments in dose should be 25% to 50% reductions, with continued monitoring of clinical and physiologic responses. These approaches maximize beneficial responses while minimizing both adverse effects and desensitization.

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26

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Maternal Drug Abuse: Effects on the Fetus and Neonate

GENERAL PRINCIPLES

Substance abuse by pregnant women is the leading *preventable* cause of psychologic, physical, and mental problems in infants and children. Women of reproductive age, like many other demographic groups in today's society, have been steadily increasing their nonmedical consumption of drugs to the point of abuse. *Substance abuse* is defined as an excessive self-administration of licit and illicit chemicals to alter perception of the user's cognitive status.[1] The most commonly abused substances are alcohol, cocaine, marijuana, tobacco, and psychotherapeutic agents. Among the opioids, morphine, heroin, and methadone appear to be the ones most frequently used by women of child-bearing age.

Although the maternal effects of substance abuse may be tolerable and desired by the user, the effects on the fetus and neonate often are serious and manifested in neurobehavioral and physical anomalies. With the thalidomide disaster of the late 1950s and early 1960s, it became evident that the placenta is not a protective barrier against all therapeutic and abused substances. Although several compounds are human teratogens, most agents given to pregnant women do not pose significant reproductive hazards when used in recommended doses.[2] Some teratogenic drugs are continued throughout pregnancy, however, because the therapeutic benefit to the woman outweighs the risk to the unborn child.[3]

Numerous attempts have been made to describe the nature and extent of maternal drug abuse during pregnancy and its potential impact on the fetus and neonate. The National Institute on Drug Abuse's National Pregnancy and Health Survey[4] revealed that 5.5% (221,000) of the approximately 4,000,000 women who gave birth to live babies in the United States in 1992 had used some licit or illicit drug during pregnancy. The most frequently used drugs in pregnancy among these 221,000 women were: 1.1% (45,000) used cocaine (all forms), 0.1% (3600) used heroin, 2.9% (119,000) used marijuana, 0.1% (3400) used methadone, 0.1%(4,500) used methamphetamine, 0.3% (12,100) used inhalants, 0.2% (8,700) used hallucinogens, and 1.5% (61,000) reported the nonmedical use of psychotherapeutic agents at some point during their pregnancy. In addition, but not included in the aforementioned 221,000 women, 18.8% (757,000) of women reported the consumption of alcohol and 20.4% (820,000) reported that they smoked cigarettes during their pregnancy.[4] The significant impact of maternal substance abuse also is reflected in the 2002 to 2003 National Survey on Drug Use and Health, which found that of pregnant patients 15 to 44 years of age, 4.3%, 18%, and 9.8% used illicit drugs, tobacco, and alcohol, respectively. The approximate numbers of births in the United States complicated by maternal use of drugs in 2002 were 172,934 for illicit drugs, 723,911 for tobacco, and 394,129 for alcohol.[5] Hence, the impact of substance abuse during pregnancy in the United States extends far beyond only the risks to the health of the mother, affecting a large number of unborn children as well.

Maternal drug abuse during pregnancy is an indication of the seriousness of psychological dependence (addiction) on the nonmedical use of drugs because the evidence suggests a trend among mothers to decrease the rate of drug consumption from 3 months before pregnancy and throughout the gestational period, rather than to achieve complete discontinuation of drug use.[4] In addition, the tabulated figures provided by the National Pregnancy and Health Survey[4] may actually be underestimates, in view of the underreporting of live neonates born to substance-abusing women.

Physicians dealing with high-risk pregnancies attributable to maternal drug abuse must recognize that fetal and neonatal complications are confounded by polydrug use in these patients.

Studies have indicated a strong association between cigarette smoking and alcohol consumption and use of illicit drugs. For example, of those women who reported both alcohol and cigarette use during pregnancy, 20.4% also used marijuana and 9.5% used cocaine.[4] Therefore fetal and neonatal dynamic outcomes represent a composite of all of the substances used by mothers during pregnancy. In addition, maternal drug and polydrug abusers are at higher risk for sexually transmitted diseases and poor prenatal care, and they generally lack the appropriate and necessary resources to care for the neonate with complications associated with in utero drug exposure.

Significant differences in total rates of substance use were found among four sociodemographic variables related to pregnant women[4]: marital status, level of education, employment status, and method of hospital payment. The rates of illicit drug use during pregnancy of marijuana, cocaine, and cigarettes were highest for women who were unmarried, unemployed, had less than 16 years of formal education, or relied on public aid to pay for hospital expenses.[4] The highest rates of alcohol use during pregnancy were by women who were married, employed, had completed college, or had private medical insurance.[4]

It is important for emergency and obstetric medical staff to recognize that the prevalence of substance abuse differs among ethnic minorities and different age-groups, to be able to execute efficient implementation of the most appropriate management algorithms. Overall, 11.3% of African-American women, 4.4% of Caucasian women, and 4.5% of Hispanic women used illicit drugs while pregnant.[4] While African-American women had higher rates of drug use, the estimated population of Caucasian women who used illicit drugs was much larger than that of the African-American or Hispanic populations. Therefore, in terms of actual numbers of users, most women who took drugs while they were pregnant were Caucasian (113,000) followed by African-American and Hispanic women (75,000 and 28,000, respectively).[4] Alcohol and cigarettes were abused by Caucasian women more often (22.7% and 24.4%, respectively); cocaine was most often used by African-American women (4.5%).[4] Rates of marijuana use were highest (3.5%) in the youngest age-group (younger than 25 years of age), whereas cocaine use was highest (1.6%) among older women (25 years of age and older).[4]

This chapter presents an overview of current knowledge about substance abuse during pregnancy and the potential associated fetal and neonatal effects. A general course of treatment also is described for each substance of abuse. In addition, studies on how drugs of abuse eventually reach the fetus and the neonate to elicit their harmful effects are well documented and covered elsewhere in this text and other publications.[6-35]

MATERNAL DRUGS OF ABUSE DURING PREGNANCY

Alcohol

Alcohol is an anxiolytic analgesic with a depressant effect on the central nervous system (CNS). Women who drink heavily (defined as consumption of an average of at least 2 drinks per day, or 1 drink per day and occasional binges of 5 or 6 drinks in one sitting, or 45 drinks or more per month)[36] have a higher predilection for polydrug use and exhibit other confounding risk factors—including older age and parity or, conversely, an increased likelihood of being a younger, single, separated, or divorced woman—that may complicate pregnancy and may jeopardize fetal and neonatal outcome.[37,38] How much drinking during pregnancy is too much? *Risk drinking* during pregnancy, sufficient to potentially damage offspring, has been defined as an average of more than 1 drink (0.5 ounce) per day, or less in the case of binge drinking (i.e., more than 5 drinks per drinking episode).[39] Most reports of adverse prenatal alcohol exposure are related to heavier drinking. More recent research, however, has demonstrated adverse effects for children exposed prenatally to even a small amount of alcohol. Detection of maternal alcohol exposure is particularly challenging because no reliable biologic marker for such exposure has been identified.

The adverse effects of alcohol on the developing fetus make up a spectrum of structural anomalies and behavioral and neurocognitive disabilities, collectively called fetal alcohol spectrum disorders (FASDs).[40] Children with the severe form of such disorders—those with the complete phenotype—are identified as having *fetal alcohol syndrome* (FAS), representing one end of the spectrum. Heavy maternal consumption of alcohol during pregnancy generally is accepted as the factor responsible for FAS.[41] FAS is the most commonly known cause of mental retardation (surpassing Down syndrome) and is characterized by fetal and neonatal growth restriction, severe cognitive impairment resulting from abnormal neural development, microphthalmia, short palpebral fissures, midfacial hypoplasia, abnormal palmar creases, cardiac defects, and joint contractures.[42-45,45a] No single finding is considered pathognomonic, and not all features are present in every neonate who has FAS.

The association of FAS with ethanol dose is not always clear. In many cases, only a few of the typical characteristics are present. Neonates with partial-expression FAS (namely, those with only one or two of the syndrome's characteristics) exhibit *fetal alcohol effects.* This condition is observed in the infants of women who consume fewer than two drinks per day. It remains unexplained, however, why only 10% of fetuses exposed to large amounts of ethanol per day (the ethanol content of six maternal drinks per day) exhibit full-blown FAS. Possible explanations include variations in placental and fetal vasculature, different rates of organogenesis, different rates of ethanol metabolism and elimination by the fetus, and differences in fetal susceptibility to teratogenesis.[46-48] Acetaldehyde has been suggested as a possible causative factor. Some evidence points to substantially higher levels of acetaldehyde in mothers with alcoholism who give birth to children with FAS than in those mothers who have normal babies.[49] Although no definite exposure threshold has been identified, a dose-response relationship is recognized.[50] Furthermore, placental perfusion experiments have shown that the human placenta metabolizes alcohol and contributes to the acetaldehyde transferred into the fetal circulation.[51]

FAS occurs in approximately 1 to 3 infants per 1000 live births in the United States; 2.5 to 10% of women with alcoholism have a child with FAS.[52,53] It generally is accepted that FAS occurs when maternal alcohol consumption reaches six to seven drinks per day (one standard drink is the equivalent of 12 ounces of beer or a 5-ounce glass of wine, or 1.5 ounces of liquor, or approximately 15 g [0.5 ounce] of absolute alcohol). It has been shown that having two drinks per day does not increase the risk for FAS; however, having three drinks per day during pregnancy is associated with a lower intelligence quotient (IQ) at the age of 4 years in children born to women with this drinking history.[1,54]

Pharmacology of Alcohol

The level of blood alcohol (ethanol—C_2H_5OH) depends on the type of beverage (e.g., beer slows absorption) and its ethanol concentration, the gastric contents, and gastrointestinal motility.[55] From 80% to 90% of the absorption of ethanol occurs within 30 to 60 minutes of its consumption. Food may delay absorption for 4 to 6 hours.[55] Absorption occurs primarily in the small intestine, whereas 20% to 30% is rapidly absorbed through the stomach. Ethanol distributes rapidly throughout the fetal circulation because it is both water- and lipid-soluble. The ethanol molecule is mildly polar so it easily crosses cell membranes, the blood-brain barrier, and the placenta. The volume of distribution is estimated to be 0.6 L/kg in adults and 0.7 L/kg in children; this corresponds to total body water. The volume of distribution is slightly smaller in women because of their lower water and higher fat content compared with men. Because of the rapid distribution of alcohol,

early determination of the blood alcohol concentration provides a reliable estimate of ethanol consumption.

Once ethanol is absorbed into the bloodstream, it is the metabolite acetaldehyde that causes the pharmacodynamic effects in the consumer. Approximately 95% of ethanol is converted by hepatic oxidized nicotinamide adenine dinucleotide (NAD^+)-alcohol dehydrogenase to the obligate intermediate acetaldehyde, which is more toxic than alcohol. Acetaldehyde is oxidized in the liver by NAD^+-dependent aldehyde dehydrogenase to form acetate. Acetate also is oxidized once in the bloodstream. The kidneys, muscles, lungs, and intestines also contribute to ethanol metabolism. Up to 5% to 10% of ethanol is excreted unchanged in the breath, urine, and sweat. The average rate of alcohol metabolism is 10 mL/hour. Alcohol is esterified with fatty acids to produce fatty acid ethyl esters, which accumulate in neonatal meconium and may be a promising marker for use in a screening test for heavy maternal drinking during pregnancy.[56,56a]

Effects of Alcohol Consumption on the Fetus and Neonate

Early diagnosis of FAS in a neonate or infant is essential to minimize the development of subsequent secondary disabilities. FAS is diagnosed when characteristic facial dismorphology (short palpebral fissures, flat philtrum, thin upper lip), growth restriction, CNS impairment, and neurodevelopmental abnormalities are present with or without confirmation of prenatal alcohol exposure. Multiple approaches to the diagnosis of FAS have evolved, including the guidelines by the Institute of Medicine (IOM) from 1996 (revised 2005), the 4-Digit Diagnostic Code (created using data from the Washington State Fetal Alcohol Syndrome Diagnostic and Prevention Network of Clinics, revised 2004) (Table 26-1, *A*) and the Canadian Guidelines (created in 2005), which also harmonize the IOM and 4-Digit Diagnostic Code approaches (see Table 26-1, *B*).[57] FAS diagnostic variables are presented in Table 26-2. Diagnosis of FAS in a neonate or infant also can be based on the presence of at least one sign in each of the first two categories listed in Table 26-2; a neonate or infant also must have at least two of the three characteristics in category 3.[58] Although the approaches are different, the underlying fundamental criteria are similar. The 4-Digit Diagnostic Code is now being used throughout the United States and Canada (see Table 26-1A).

Other features associated with FAS may include specific minor anomalies of the face—epicanthal folds, a flat nasal bridge, a thin vermilion border of the upper lip, a hypoplastic maxilla, and a flattened midface. Strabismus, ptosis, poorly formed external ears, congenital heart defects, neural tube defects, renal abnormalities, and unusual palmar creases also have been documented. All infants with FAS have evidence of intrauterine growth restriction (IUGR): Birth weight, body length, and head circumference are reduced for gestational age.[59] Infants with FAS may not experience catch-up growth and may remain small in comparison with opioid-exposed infants born small for gestational age (SGA).[60] Mild to moderate developmental delays are commonplace, and delays in motor and language development, hyperactivity, hypotonia, hyperacusis, and tremulousness are often described by caregivers.[1]

Pregnant women who are heavy drinkers are at increased risk for spontaneous abortion.[53,61,62] In a study of 1248 women,[62] an estimated minimum harmful dose was 1 ounce of absolute alcohol (two drinks) twice per week. The investigators showed a 25% miscarriage rate in women drinking 1 ounce of absolute

TABLE 26-1A

4-Digit Diagnostic Code Criteria for Fetal Alcohol Syndrome Disorders (FASDs)

Rank	Growth Deficiency	FAS Facial Phenotype	CNS Damage or Dysfunction	Gestational Exposure to Alcohol
4	*2 Significant* Height and weight below 3rd percentile	*3 Severe* All 3 features: PFL 2 or more SDs below mean Thin lip: rank 4 or 5 Smooth philtrum: rank 4 or 5	*4 Definite* Structural or neurologic evidence	*4.1 High risk* Confirmed exposure to high levels
3	*5 Moderate* Height and weight below 10th percentile	*6 Moderate* Generally 2 of the 3 features	*7 Probable* Significant dysfunction across 3 or more domains	*8 Some risk* Confirmed exposure Level of exposure unknown or less than rank 4
2	*9 Mild* Height or weight below 10th percentile	*10 Mild* Generally 1 of the 3 features	*11 Possible* Evidence of dysfunction but less than rank 3	*12 Unknown* Exposure not confirmed present or absent
1	*13 None* Height and weight at or above 10th percentile	*14 Absent* None of the 3 features	*15 Unlikely* No structural, neurologic, or functional evidence of impairment	*16 No risk* Confirmed absence of exposure to birth

CNS, Central nervous system; *FAS,* fetal alcohol syndrome; *PFL,* palpebral fissure length.

TABLE 26-1B

Harmonization of the Institute of Medicine (IOM) Nomenclature and 4-Digit Diagnostic Code

IOM Nomenclature	Growth Deficiency	FAS Facial Phenotype	CNS Damage or Dysfunction	Gestational Exposure to Alcohol
FAS (with confirmed exposure)	2, 3, or 4	3 or 4	3 or 4	3 or 4
FAS (without confirmed exposure)	2, 3, or 4	3 or 4	3 or 4	2
Partial FAS (with confirmed exposure)	1, 2, 3, or 4	2, 3, or 4	3 or 4	3 or 4
ARND (with confirmed exposure)	1, 2, 3, or 4	1 or 2	3 or 4 (2 for <6 years)	3 or 4

ARND, Alcohol-related neurodevelopmental disorder; *CNS,* central nervous system; *FAS,* fetal alcohol syndrome.
Data from Chudley AE, Conry J, Cook JL, et al: Fetal alcohol spectrum disorder: Canadian guidelines for diagnosis. CMAJ 172(Suppl 5):S1, 2005.

TABLE 26-2
Diagnostic Variables in Fetal Alcohol Syndrome

Category	Variables
1	Prenatal or postnatal growth restriction
	Weight, length, or head circumference below 10th percentile after correction for gestational age
2	Central nervous system involvement
	Neurologic abnormalities: neonatal irritability and childhood hyperactivity
	Developmental delay
	Cognitive impairment: mild to moderate mental retardation
3	Characteristic facial dysmorphology
	Microcephaly: head circumference below third percentile
	Microphthalmia or short palpable fissures
	Poorly developed philtrum and vermilion border; flattened or absent maxilla

alcohol or more, compared with 14% in those women who drank less. This effect was independent of smoking, gestational age, or a history of spontaneous abortion. In another study[61] comprising 32,000 women, a significantly higher risk for second-trimester spontaneous abortion was noted in those consuming one or two drinks per day than in those who consumed less than one drink per day.[61] Among women reporting the consumption of more than three drinks per day, the risk was even higher. A recent population-based, case-control study suggested that drinking during pregnancy is associated with an increased risk of miscarriage, although the effects appeared to be concentrated among those who drank regularly (at least once a week to daily) and possibly among those who drank 14 units of alcohol (in this study, one alcohol unit was half a pint of beer or a small glass of wine) a week.[63]

An increased risk for abruptio placentae has been reported in heavy drinkers.[64,65] A recent study reported that alcohol consumption may be an independent risk factor for placental abruption.[66] Considerable controversy remains regarding the association among maternal alcohol consumption, duration of pregnancy, and the incidence of stillbirth.[53,67-69] The major problem in research efforts to explain poor fetal and neonatal outcomes is the inability to separate high maternal ethanol intake from other confounding variables such as cigarette smoking, polydrug use, and poor prenatal care. A study that adjusted for such confounders found that low to moderate levels of alcohol consumption during pregnancy were associated with slightly higher rates of stillbirth, but the increased rate was not statistically significant.[70] Many investigators have studied the effects of maternal ethanol use on fetal and neonatal growth, as well as on the development of fetuses exposed to ethanol in utero. An average of two drinks per day before conception correlated with a marginally lower birth weight (mean of 91 g) (in comparison with birth weights of infants of lighter drinkers), independent of smoking.[71] A study of 31,600 pregnancies[1] confirmed this observation. In addition, the risk of IUGR is increased with an alcohol consumption of one to two drinks per day.[46-48,72-75] Alcohol interference with the transplacental transfer of amino acids across the placenta may alter embryonic development by inhibiting protein synthesis and prenatal growth. The literature suggests that the level of alcohol consumed by the father also may have an effect on fetal birth weight.[76] A prospective cohort study[77] of 359 neonates that assessed the critical period of alcohol exposure during pregnancy revealed that craniofacial abnormalities are definitively related to first-trimester alcohol exposure.

A recent study from Finland suggested that prenatal alcohol exposure alters long-term brain metabolism in multiple brain areas. These changes corroborate previous findings that long- standing biochemical alterations may occur in response to prenatal exposure to alcohol.[78] A behavioral study[72] of 124 infants born to women classified according to their alcohol consumption found that tremors, time spent with the neonate's head turned to the left, and low activity levels increased progressively with the level of maternal alcohol intake. Infants of women who consumed two to four drinks per day during pregnancy had a lower score for mental and psychomotor development.[74] A 10-year, long-term follow-up study[75] showed severe growth deficiencies and intellectual limitations in those children diagnosed with FAS. Kalberg and colleagues completed a study of 14 children with FAS, ranging in age from 20 to 68 months, in which they assessed motor function in children exposed prenatally to alcohol and in children who had no prenatal alcohol exposure. A majority of these FAS-affected children showed clinically important delays in their motor development.[79] In a longitudinal study of children born to 500 women who were heavy smokers and drinkers,[80] a 7-point decrease in IQ was associated with maternal consumption of more than two drinks per day during pregnancy. A recent behavioral study involving 537 children showed that those who were exposed prenatally to alcohol during maternal binge drinking were 1.7 times more likely to have IQ scores in the mentally retarded range and 2.5 times more likely to exhibit clinically significant levels of delinquent behavior.[81] Postnatal exposure of infants to alcohol also may be harmful. In that regard, infants exposed to alcohol through breast milk scored lower on motor development tasks than unexposed infants at 1 year of age.[82]

Neonatal alcohol withdrawal and neurobehavioral disturbances have been documented in otherwise clinically normal neonates born to women who drank throughout pregnancy.[1] Ethanol exposure during the third trimester increases the probability that the newborn will experience postpartum ethanol withdrawal. In a study by Coles and colleagues,[83] abnormalities were observed in motor performance, autonomic regulation, and reflexive behavior during the first postpartum month in neonates exposed to alcohol in utero. Hyperactivity, hyperacusis, hypotonia, and tremulousness were commonly described in young infants, in addition to withdrawal symptoms characteristic of narcotic withdrawal in neonates.[72,74]

Clarren and associates[84] described multiple brain malformations attributable to fetal alcohol exposure. The most frequently seen malformations were leptomeningeal and neuroglial heterotopias. These investigators found aberrant neural and glial tissue covering large parts of the surface of the brain, as well as extensive brain disorganization from errors in neuronal and glial migration and rudimentary cerebellum and brain stem disorganization.

Abnormalities on the electroencephalogram (EEG) consisting of prominent δ bands and atypical slow waves in the posterior head region have been observed in neonates with FAS.[85] The intellectual deficit secondary to abnormalities in brain growth is the most important component of FAS. Full expression of FAS generally occurs with long-term daily consumption of at least 2 g/kg of alcohol.

Another investigation[86] studying the effect of maternal alcohol ingestion on the incidence of respiratory distress syndrome suggested that among infants born before 37 weeks of gestation, the incidence of respiratory distress syndrome decreased with increased maternal alcohol consumption. The researchers proposed that maternal alcohol ingestion enhanced fetal lung maturation. No direct or indirect epidemiologic evidence or basic scientific information either supports or refutes an association between sudden infant death syndrome (SIDS) and maternal alcohol consumption during pregnancy.[87]

Optic nerve hypoplasia is a nonprogressive congenital anomaly associated with a decreased number of axons in the involved nerve.[88,89] The mother's use of alcohol during pregnancy has been associated with the development of optic nerve

hypoplasia.[90] Optic nerve hypoplasia has been reported in 48% of children with FAS.[91]

Clinical Management of Heavy Drinkers During Pregnancy

It is well known that self-reports of drinking have a definite tendency to minimize the real incidence of drinking episodes and amounts of alcohol consumed.[92] In view of that limitation, accurate determination of the amount of alcohol consumed by the mother during pregnancy is essential because the extent of fetal damage is dose-dependent. The age of the mother, other disease conditions, dietary deficiencies, use of other drugs, heavy smoking, and emotional stress are additional teratogenic factors that must be evaluated when the risk to fetal development from maternally consumed alcohol is assessed.

No specific prenatal diagnostic measures exist to identify fetuses with FAS. Alcohol exposure screening tools such as Alcohol Use Disorder Identification Test (AUDIT); Cut Down, Annoyed, Guilty, Eye Opener (CAGE); Tolerance, Worried, Eye Opener, Amnesia, (K)Cut Down (TWEAK); Tolerance, Annoyance, Cut Down, Eye Opener (T-ACE); and Michigan Alcoholism Screening Test (MAST)[81,93,94] have been used for years in the primary care setting. Of note, however, not all of these screening tools have been developed and validated for use in pregnant women. Guidelines from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) recommend that any woman who drinks more than 7 drinks per week or more than 3 drinks on any given day should be further assessed for alcohol-related problems.[95] Some evidence suggests that T-ACE and TWEAK are effective screening tools to identify women who would benefit from intervention for alcohol use during pregnancy.[57] It has been suggested, however, that a comprehensive screening tool should assess both mother and fetus to identify in utero alcohol exposure.[96]

Timeline FollowBack (TLFB) calendars, which include the date of conception and the date of pregnancy recognition, may be used to accurately assess the timing, frequency, and amount of alcohol consumed during pregnancy.[96] Combining the screening tools to include both mother and fetus may increase the accuracy and effectiveness of such tools; this will require validation work before use in the clinic. These screening tools are not diagnostic; their purpose is to identify women who may benefit from early intervention or treatment for alcohol consumption during pregnancy. Novel biologic markers may help to overcome dependence on self-reporting and may be a critical step in ensuring an accurate diagnosis of FAS in cases in which effects of in utero alcohol exposure are not yet apparent in the infant. Promising maternal serum biomarkers include mean corpuscular volume (MCV), γ-glutamyltransferase (GGT), and hemoglobin-associated acetaldehyde (HAA).[97]

Meconium fatty acid ethyl esters (FAEEs) also have been proposed as biologic markers for alcohol exposure.[98] Placental studies suggest that FAEEs do not cross the human placenta.[98] Therefore FAEEs detected in neonatal matrices are likely direct from the fetus. Although meconium FAEE studies have demonstrated the promise of using FAEE levels as the first biologic marker of alcohol exposure during pregnancy, these FAEEs are available for collection only in the first 1 or 2 days of life, reflecting a very narrow window of opportunity for collection.[98]

Serial measurements of alpha fetoprotein may suggest the occurrence of FAS because alpha fetoprotein is produced by the fetal liver, which may be damaged when exposed to alcohol. In one study, 13 women gave birth to infants with FAS; each woman had low levels of alpha fetoprotein and pregnancy-specific beta-1-glycoprotein.[99] The authors concluded that low alpha fetoprotein and pregnancy-specific beta-1-glycoprotein may be primary or secondary effects of alcohol abuse during pregnancy and may be useful in predicting FAS.[99]

Currently, the best-established markers for the detection of alcohol abuse in pregnant and nonpregnant women are increased levels of serum γ-glutamyltransferase activity and increased mean blood cell volumes.[100]

Prenatal ultrasonography has been used to visualize and catalogue the fetal malformations associated with numerous drugs and chemicals. Ultrasound examination of fetuses exposed to alcohol may reveal many associated anomalies[101,102] (Box 26-1).

Abstinence is the treatment goal in heavy social drinkers or women who are physically dependent on alcohol. If alcohol consumption totals more than 80 g/day of absolute ethanol, a gradual reduction in alcohol consumption over 4 days is recommended.[52] During detoxification and throughout pregnancy, intense supportive counseling must be provided. A diazepam (Valium)-loading regimen has been used successfully in alcohol withdrawal treatment.[103] β-Blockers can reduce the signs and symptoms of mild to moderate alcohol withdrawal.[104]

BOX 26-1

Fetal Malformations Associated With Maternal Substance Abuse Visualized by Prenatal Ultrasonography

General Anomalies
Microcephaly
Diaphragmatic hernia
Renal defects
Hypospadias
Labial hypoplasia
Fetal growth restriction
Pulmonary atresia

Cardiac Anomalies
Heart malformations
Ventricular septal defect
Atrial septal defect
Double outlet of right ventricle
Dextrocardia
Patent ductus arteriosus
Tetralogy of Fallot

Skeletal Anomalies
Short nose
Hypoplastic philtrum
Hypoplastic maxilla
Micrognathia
Small mandible
Pectus excavatum
Bifid xiphoid
Scoliosis
Poorly formed orbits
Microphthalmia
Cleft lip
Cleft palate
Radioulnar synostosis

Cocaine

Cocaine is the most potent naturally occurring stimulant. Cocaine blocks the reuptake of the biogenic amines serotonin, dopamine, and norepinephrine in the CNS and causes these neurotransmitters to bind with their receptors.[105,106] Enhanced receptor binding increases neuronal excitability, resulting in the typical cocaine "high." In addition to its effects on the CNS, cocaine acts peripherally to inhibit nerve conduction by blocking the reuptake of norepinephrine at the sympathetic nerve terminals.[107] This action increases norepinephrine levels, with consequent vasoconstriction, tachycardia, and hypertension.

Exposure to cocaine decreases blood flow to the fetus by constricting the placental vasculature. In addition, cocaine causes the myometrium to contract, potentially increasing the risk for premature rupture of the membranes with the need for induction of labor.[108,109] Vasoconstriction of the uterine arteries, causing circulatory insufficiency, has been suggested as the mechanism leading to reduced birth weight, fetal growth restriction, and labor-induced fetal stress.[110]

Cocaine abuse traditionally has involved concomitant intravenous injection of heroin. Currently, cocaine is insufflated or smoked, rather than injected. In the 1980s, in the United States, cocaine use had dramatically increased, especially among young adults. In the mid '80s, 40% of cocaine addicts used the drug nasally, 30% by free-base smoking, 20% by injection, and the remaining 10% by combining these routes.[111] According to the 2004 National Household Survey on Drug Use and Health, substance abuse during pregnancy increased over the past three decades in the United States; 4.6% of American women reported use of an illicit drug during pregnancy. Previous studies on illicit drug use during pregnancy and perinatal outcome showed inconsistent results.[5]

Pharmacology of Cocaine

A peak plasma cocaine concentration is attained within 15 to 60 minutes after insufflation.[112] The peak plasma concentration after oral administration of cocaine is reached in 50 to 90 minutes. The bioavailability of cocaine ingested orally is 60%, which is similar to that of cocaine administered intranasally. Free-base smoking and intravenous cocaine administration have comparable kinetics: Both are characterized by rapid and complete absorption of the drug. The elimination half-life of cocaine is between 20 and 90 minutes.[112] After an intranasal dose of 1.5 mg/kg, cocaine can be detected in the urine for 8 hours. Benzoylecgonine, the primary metabolite of cocaine, can be detected in the user's urine for 48 to 72 hours with chromatographic techniques and for 90 to 144 hours with radioimmunoassay.[54a]

Cocaine readily diffuses across the blood-brain and placental barriers and also transfers into breast milk. Cocaine rapidly crosses the placenta because of its low molecular weight (305), high lipid solubility, and low protein binding in human plasma. The rate of transfer of cocaine is approximately 10 times that of benzoylecgonine. Therefore the major portion of the administered dose is available to equilibrate with the fetal circulation.[22] The placenta serves as a reservoir for large amounts of cocaine and consequently offers some protection to the fetus after bolus dosing. Conversely, fetal exposure may be prolonged as cocaine and benzoylecgonine leach out of the placenta.[22] Fetal lamb studies[113] also indicate that cocaine and its metabolites in the amniotic fluid may prolong fetal drug exposure. Measurable levels of cocaine and benzoylecgonine have been detected in breast milk as late as 36 hours after consumption of the drug has ceased.

Cocaine is metabolized primarily by esterases and by cytochrome P-450 enzymes. Cytochrome P-450 accounts for the *N*-demethylation metabolism (20%) of cocaine into the bioactive metabolite norcocaine, which is more vasoactive than cocaine.[114] The remainder of the drug is extensively metabolized by plasma and liver cholinesterases, which hydrolyze the drug into an active metabolite, ecgonine methyl ester. Cocaine is spontaneously converted to benzoylecgonine in the plasma by nonenzymatic hydrolysis. The metabolites are more polar than cocaine and are readily excreted by the kidney. Less than 10% of cocaine is excreted unchanged in the urine.

The mean cocaine elimination half-life for intranasal administration is 75 minutes, for oral administration 48 minutes, and for intravenous administration 54 minutes. The volume of distribution is 1.2 to 1.9 L/kg. Differences in individual cholinesterase levels explain the variation seen in the half-life of cocaine. The elimination half-life of benzoylecgonine is considerably longer than that of cocaine, but the benzoylecgonine molecule is more polar, so the drug distributes less rapidly than cocaine.[115]

In adults, more benzoylecgonine than cocaine is found in the blood, brain, and liver.[116] In neonates, the distribution of cocaine in the tissues differs from that in adults. Klein and associates,[115] in their description of the distribution of cocaine and benzoylecgonine in a fetus born to a woman who used cocaine, detected both drugs in the fetal brain, scalp, liver, kidney, heart, placenta, blood, and hair. The neonate's liver contained substantially more cocaine than the other tissues, whereas the brain contained more benzoylecgonine than any other tissue.

Effects of Cocaine Use on the Fetus and Neonate

The potential deleterious effects on the fetus and neonate of maternal cocaine use during pregnancy are numerous. Cocaine use during pregnancy has been associated with an increased risk for spontaneous abortion, abruptio placentae, premature labor, precipitous delivery, stillbirth, and meconium staining.[117-122] After intravenous cocaine use, spontaneous abortion and abruptio placentae are more prevalent.[123] These effects may be associated with increased maternal blood pressure, increased uterine contractility and placental constriction, and decreased uterine blood flow, all of which cause fetal hypoxia.

Decreased birth weight, shorter body length, smaller head circumference, a higher malformation rate, and a more frequent need for medical support and resuscitation have been documented in newborns exposed to cocaine in utero.[118,119,122,124,125] The effects of cocaine exposure during pregnancy on intrauterine growth and growth deviation during gestation have been described by Bada and colleagues.[126] These investigators found that there is a significant interaction between cocaine exposure and gestational age. Percentile estimates for birth weight, length, and head circumference showed growth deceleration, evident after 32 weeks gestation, in infants exposed to cocaine during pregnancy. After controlling for confounders, at 40 weeks gestation, cocaine exposure was estimated to be associated with a decrease of 151 grams, 0.71 centimeters, and 0.43 centimeters in birth weight, length, and head circumference, respectively.[126]

Infants exposed to cocaine in utero also exhibit depressed interactive behavior and poorly organized responses to external stimuli. Marked tremulousness, irritability, and deficiencies in mood control and interactive behavior also have been reported in the literature.[121,123,124,127,128] In a series of 12 infants,[127] 11 exhibited poor visual attention and abnormal flash-evoked potentials. A large-scale, prospective, blinded study of 172 infants who were exposed to cocaine prenatally did not identify an increased number or consistent pattern of malformation. The study did, however, suggest that infants exposed to cocaine prenatally had a significantly smaller birth weight, length, and head circumference in comparison with unexposed infants.[129]

Doberczak and associates[130] studied the relationship between maternal cocaine abuse during pregnancy and neonatal neurologic abnormalities in 39 infants. These investigators found that 34 of the neonates displayed CNS irritability, with bursts of sharp waves and spikes, which were multifocal in 14 of 17 EEG tracings. EEG abnormalities persisted in 17 of 38 infants during the first week of life. In one neonate, an EEG was not done. In nine of these 17 infants, EEG tracings remained abnormal through the second week of life. Between 3 and 12 months later, however, only one of these infants had an abnormal EEG tracing. These findings did not suggest a correlation between fetal cocaine exposure and clinical seizures or neurologic abnormalities.

A rare but serious CNS condition associated with maternal cocaine use near delivery is perinatal cerebral infarction.[131] In the first reported case, right hemiparesis with right-sided focal seizures developed in the newborn infant of a cocaine abuser on the first day of life. The mother had used cocaine excessively (5 grams, intranasally) 3 days before delivery.[131a] A computed

tomography scan and a lumbar puncture showed evidence of cerebral infarction in the brain of the infant.

Neonates exposed in utero to cocaine showed significant differences in organizational response and interactive behavior compared with control neonates when they were assessed with the Brazelton Neurobehavioral Assessment Scale.[119,132] Oro and Dixon[122] described a prevalence of excessive tremor, hypertonia, hyperreflexia, poor feeding, and sleep disturbances among cocaine-exposed and cocaine-methamphetamine–exposed neonates. It appears that the developing brain is sensitive to cocaine exposure during early development. However, the literature does contain conflicting studies on this issue. Chasnoff and colleagues[132] showed that neonates exposed to cocaine solely in the first trimester exhibited neurobehavioral abnormalities but no growth anomalies. A large cohort of children exposed to cocaine were followed by Lewis and co-workers[133] from birth to 4 years of age. The results of their study suggested that compared with unexposed children, children exposed to cocaine had milder receptive language delay and were less likely to have higher expressive abilities. Children exposed to cocaine who were placed in foster care demonstrated language skills superior to those in children exposed to cocaine who remained in the biologic mother's care.[133] These findings support suggestions that a cocaine-specific effect on language skills in early childhood may be modified by an enriched environment. Conversely, the Motherisk group[134] showed that babies exposed to cocaine only during the first trimester had no greater risk of delayed cognitive development than that in their control groups.

Cocaine-mediated vasoconstriction of the fetal cerebral vasculature decreases cerebral blood flow.[135] Several clinical reports[136-138] described neonatal stroke (cerebrovascular accident) after prenatal cocaine exposure in the setting of abruptio placentae or birth asphyxia, each of which is a known risk factor for neonatal stroke. A vascular cause is suggested by the cases of three neonates with porencephaly who were exposed to cocaine in utero and that of one neonate with bilateral occipital infarcts after an uncomplicated delivery.[139] Ultrasound examination of the head demonstrated an increased incidence of intracranial hemorrhage and cystic lucencies among cocaine-exposed neonates, at rates (30%) similar to those seen in infants with hypoxic-ischemic encephalopathy.[140] In addition, Doppler examination revealed increased cerebral blood flow in neonates with recent cocaine exposure.[141] Coupled with systemic hypertension, such an increase in cerebral blood flow could elevate intracranial pressure, leading indirectly to intracranial hemorrhage.

To date, most studies assessing the reproductive neurotoxicity of cocaine have focused on neonatal and infant cognitive function. It is possible, however, that other neurologic damage may occur, causing attention deficit disorders or learning disabilities.[142] A recent study assessed learning abilities and intellectual functioning at age 7 of 212 full-term children with prenatal cocaine exposure. No differences were found in the estimate of relative risk for impaired intellectual functioning (IQ below 70).[143] Children exposed to cocaine, however, had a 2.8 times greater risk for development of learning disabilities by age 7 than that in unexposed children (95% CI = 1.05-7.67; $p = .038$; IQ cutoff of 70 or higher).[143]

Abnormal sleep patterns in neonates and infants also have been observed after prenatal maternal cocaine use.[122,144] The risk of SIDS attributable to maternal cocaine use during pregnancy was investigated by several groups of workers. In a study comprising 996 women who used cocaine during pregnancy,[145] 1 of the 175 infants (0.6%) in the cocaine-exposed group died of SIDS, whereas 4 of the 821 infants (0.5%) in the nonexposed control groups died. The investigators concluded that cocaine did not increase the risk of SIDS in cocaine-exposed pregnancies.

Conversely, other studies[120,121] postulated an increased risk of SIDS in neonates who were exposed to cocaine in utero. In these studies, 10 (18%) of 56 cocaine-exposed neonates died of SIDS. This figure is substantially different from other reported values and may reflect a sample-size effect or an inclusion or reporting bias. Chasnoff and co-workers[146] conducted a prospective study of cardiorespiratory patterns in 32 cocaine-exposed neonates and in 18 methadone-exposed neonates. Eight of the 32 cocaine-using mothers also abused heroin and were started on methadone maintenance therapy. Although 12 of 32 cocaine-exposed neonates and 1 of the 18 methadone-exposed neonates had abnormal pneumograms, no deaths were attributable to SIDS. Despite the methodologic weaknesses in the study design, the investigators concluded that prenatal exposure to cocaine appeared to be linked to cardiorespiratory abnormalities. It was not, however, specifically concluded that cocaine exposure was linked directly to SIDS. The same link was previously suggested by Ward and colleagues.[147] The relationship between cocaine use during pregnancy and SIDS is still not clear.[87]

A meta-analysis done by Fares and collegues[148] showed that an increased risk for SIDS could not be attributed to intrauterine cocaine exposure alone after institution of controls for the confounding variable of concurrent use of other drugs. The increase in risk for SIDS was found not to be specific to cocaine, but rather was associated with intrauterine exposure to illicit drugs in general.[148] The pathophysiology of SIDS is not completely elucidated. Many studies support the concept of abnormal CNS regulation of breathing as the most plausible mechanism.[149-151] Cocaine-mediated changes in the content of the biogenic amines, which are important respiratory control neurotransmitters, may be responsible for neonatal hypoxia. Alternatively, through the direct effect of cocaine-induced hypertension or, indirectly, through repeated episodes of hypoxia secondary to cocaine-mediated vasoconstriction of the placental vasculature, cocaine may disrupt brain structures essential to respiratory regulation.[152-154]

The effects of maternal cocaine abuse on the neonatal auditory system were studied in 18 newborns.[155] The auditory brain stem responses of neonates exposed to cocaine showed prolonged interpeak and absolute latencies (evoked potentials) compared with those of nonexposed control neonates. These abnormalities suggest a neurologic impairment or dysfunction such as delayed myelination, as well as altered DNA synthesis requiring further audiologic and neurologic follow-up evaluation.

The risk of major structural malformations attributable to in utero cocaine exposure is still undetermined. Results of a meta-analysis failed to show increased teratogenic risk associated with cocaine use alone after controlling for use of other drugs of abuse.[156] Cocaine use during pregnancy also has been associated with increased risk for the development of transient myocardial ischemia in the neonate.[157] Transient ST-segment elevation and a lower ratio of low- to high-frequency power in the spectral analysis for heart rate variability reflected increased vagal activity in cocaine-exposed neonates.[157]

The fetal cardiovascular response to cocaine is postulated to be a combination of direct and indirect effects. An indirect effect of placental hypoperfusion, fetal hypoxia, and subsequent catecholamine release has been reported.[152] The direct effect of cocaine is at the level of the fetal noradrenergic nerve terminal.[152]

The principal focus of published research on cocaine use is on the fetal risks associated with heavy maternal cocaine users, whose practices and general health also add numerous confounding variables such as polydrug use, concomitant use of cigarettes and alcohol, higher risk for vaginal bleeding, hepatitis B carrier state, and poor prenatal care. Because constituents of smoke such as carbon monoxide and nicotine are known fetotoxicants,[158] Forman and associates[125] examined the outcome for neonates exposed to cocaine alone, and to cocaine and maternal smoking, to ascertain the effects attributable to cocaine, as opposed to those attributable to tobacco. The analysis revealed that most of the detrimental effects on the fetus and neonate were the result

of cigarette smoking.[125] These results are in agreement with those of an earlier meta-analysis.[159]

Very little research has focused on the potential risks associated with social maternal cocaine use. Most social cocaine users seem to discontinue their cocaine use once they are pregnant. A comparison[142] of social cocaine users and drug-free control subjects produced no evidence that neonates born to social cocaine users have an increased risk of perinatal complications, dysmorphology, or abnormal neurobehavioral development. Analysis of all available studies pertaining to maternal social cocaine consumption suggests that cocaine is not a major human teratogen and that neonates born to social cocaine users are normal in their physical and neurobehavioral development,[142] as evidenced by Bayley scores at the age of 18 months.[1]

A specific subgroup of fetuses has been recognized to be susceptible to the adverse effects of cocaine, probably because of variability in the maternal metabolism of cocaine,[160] the placental transfer of cocaine,[161] and the placental-vascular response to cocaine, as well as fetal pharmacodynamic variability.[142] Cocaine toxicity tends to reduce the activity of the choline esterase enzyme that metabolizes cocaine. Therefore in women using similar doses of cocaine, placental and fetal exposure will vary significantly.

The reliability of the evaluation of the reproductive risks associated with cocaine use during pregnancy is dependent on the verification of fetal drug exposure, or of nonexposure in the control groups. Fetal drug exposure is based on the mother's self-reports of gestational cocaine use and a positive result on the neonatal urine test, or a negative result on the neonatal urine test in the control groups. Recall that cocaine has a short elimination half-life (see section on "Pharmacology of Cocaine"). The detection of cocaine and its metabolite in blood and urine is limited by the short elimination half-life of these compounds; they are often not detectable in urine beyond a maximum of 7 days after last consumption of the drug.[54a, 161a] However, in chronic, high-dose users taking more than 10 grams/day of cocaine, benzoylecgonine has been detected in urine for up to 22 days after last consumption.[54a,161b] If a woman chooses to deny her cocaine use and the urine test result is negative, her neonate may be erroneously identified as a nonexposed control subject.[161c]

To overcome this limitation, a cocaine hair test can be used. Cocaine and benzoylecgonine incorporate into a growing hair shaft.[161c] As is the case with nutrients, cocaine transfers into the growing hair shaft through the capillaries nourishing it. The cocaine hair test remains positive for the entire life span of the hair shaft in both the mother and the baby, often long after the urine test results have become negative.[161c]

The detection of cocaine in neonatal hair is an indicator of maternal cocaine use during the last months of pregnancy. The initial fetal hair, lanugo, sheds at 5 to 6 months of gestation. Lanugo hair is replaced by neonatal terminal hair that grows throughout the third trimester of pregnancy at an approximate rate of one centimeter/month, incorporating benzoylecgonine into its matrix, and remains intact for several months after birth.[162,162a,162b] Therefore, detecting intrauterine drug exposure becomes a problem after this time. Because maternal hair grows at a rate of 1.0 to 1.5 cm per month, the hair test also may be used to determine the time of fetal drug exposure during gestation from an analysis of the maternal hair in sections. The clinician can use the results of this analysis to predict the extent and type of deleterious effects on the fetus attributable to the drug (based on the duration of drug exposure in utero), to plan a course of treatment for the fetus and neonate.

Neonatal signs such as abnormal sleep-wake cycles, tremulousness, poor feeding, hypotonia, and hyperreflexia have been documented in infants exposed in utero to cocaine.[121,122,127] These neonatal signs may reflect the effects of cocaine's slow elimination in the newborn infant.[131] A study[120] of eight neonates exposed to cocaine in utero reported no withdrawal symptoms. Ascertaining the existence of a neonatal cocaine withdrawal syndrome is further complicated by the maternal cocaine abuser's concomitant use of opioids that are known to cause neonatal withdrawal signs and symptoms.

Clinical Management of Pregnant Cocaine Abusers

A specific clinical management algorithm for the treatment of cocaine abuse is dependent on the extent of the mother's cocaine use and all associated medical and social problems. Irregular use of cocaine does not imply that the dependence on the drug is less severe. Most patients can be managed on an outpatient basis, although some women need hospitalization. Maternal cocaine withdrawal is characterized by periods of depression ranging in duration from hours to days, hypersomnolence, and hyperphagia, followed by a period of high energy and an intense craving for cocaine.[163] Unless the depression is severe, no pharmacologic therapy is recommended. A careful history is always essential, because cocaine abusers frequently use other drugs in combination with heroin, for which treatment may be necessary.

Neonatal hyperexcitability can be treated with the administration of benzodiazepines as necessary for effective control of symptoms. Withdrawal symptoms should be managed supportively.

Cigarette Smoking

Epidemiologic data indicate that cigarette smoking in the Western world has been gradually declining, more so in men than in women. The prevalence of smoking during pregnancy decreased from 40% to 25% between 1967 and 1980.[164] This decrease was seen primarily in highly educated populations. Despite extensive negative publicity, tobacco use continues in approximately 25% of all pregnancies in the United States, overshadowing illicit drugs of abuse, including cocaine.[165] Almost half of all adolescents smoke during pregnancy in the United Kingdom.[166] The societal cost of maternal smoking is seen most readily in underweight newborns; in high rates of perinatal morbidity, infant death, and SIDS; and in persistent deficits in learning and behavior.[167]

Pharmacology of Nicotine

In addition to the well-described long-term health hazards attributable to smoking, exposure to carbon monoxide from cigarette smoke produces a progressive rise in maternal and fetal carboxyhemoglobin concentrations that parallels a fall in tissue oxygen delivery as the smoking day progresses. Carbon monoxide readily crosses the placenta. Several hours are required for the fetal carboxyhemoglobin concentration to equilibrate with maternal concentrations. Final fetal concentrations eventually become higher than maternal concentrations because fetal hemoglobin has a greater affinity for carbon monoxide and because the elimination half-life from the fetal circulation is longer than that from the maternal circulation.[158] One smoking day may result in a maternal carboxyhemoglobin concentration of 12% and an even higher fetal concentration (by 10% to 15%). A 10% carbon monoxide level means that the delivery of oxygen to the fetus may actually be reduced by as much as 20% to 25% because carbon monoxide also is bound to the cytochrome enzymes. In addition, other compounds in tobacco smoke, such as thiocyanate and other cyanides, bind irreversibly to fetal and maternal hemoglobin to reduce oxygen delivery even further.

Intense uterine vasoconstriction[1,168] and a rapid fall in the uterine blood flow have been attributed to nicotine concentrations similar to those measured in arterial blood after smoke inhalation. These effects are produced by nicotine-mediated catecholamine release from the adrenal glands. Fetal hypoxemia often accompanies this vasoconstrictive effect.

Nicotine is readily absorbed by the oral mucosa, respiratory tract, gastrointestinal tract, and skin. From 80% to 90% of nicotine is metabolized in the liver, and smaller amounts are metabolized in the lung and kidney. The volume of distribution of nicotine is 2.0 L/kg in smokers and 3.0 L/kg in nonsmokers.[169] Nicotine is rapidly transferred from the maternal serum to the breast milk. Nicotine concentrations in breast milk are linearly correlated with those measured in maternal serum.[170] Both nicotine and cotinine (the major metabolite of nicotine) transfer into breast milk.[171-173] The mean elimination half-life of nicotine in breast milk is 92 ± 15 minutes, and in plasma it is 81 ± 9 minutes. Heavy maternal smoking may alter the milk supply and may cause nausea and vomiting in the neonate.[1] Nicotine and its metabolites are excreted primarily by the kidneys. Cotinine has an elimination half-life of 10 to 20 hours.[174]

Effects of Cigarette Smoking on the Fetus and Neonate

Studies[72,175] have shown an association between maternal smoking throughout pregnancy and increased risks of spontaneous abortion, placenta previa, and abruptio placentae; early or late gestational bleeding; and IUGR. Placentas of women who smoke are markedly different morphologically from those of nonsmoking women. In smokers, the placenta tends to be heavier, with a greater degree of infarction and calcification, as well as an increased frequency of vascular anomalies. Maternal smoking directly dysregulates cytotrophoblast expression and differentiation, which helps to explain the mechanisms by which smoking negatively affects human pregnancy outcome.[176]

In a review conducted to ascertain whether perinatal fetal mortality rates were increased among mothers who smoked,[177] 5 of 17 studies found significantly increased risks for stillbirth affecting infants born to smoking women. The risk of perinatal death may not be equally distributed and may vary with maternal age, parity, the ethnic group, and socioeconomic status.[178] A study from Sweden[179] found that smoking was associated with a higher risk for early neonatal death.

A reduction in fetal growth has been associated with maternal smoking. In that regard, Scholl and colleagues[180] demonstrated that smokers were 3.1 times more likely than nonsmokers to give birth to an SGA infant. Neonatal weights averaged 272 g less than those in the nonsmoking group. A quantitative dose-and-effect relation between tobacco exposure and decreased gestational age at birth and size of the neonate has been reported.[181] In light smokers (1 to 5 cigarettes per day), the risk of giving birth to an SGA baby was increased by a factor of 1.76 for girls and 1.72 for boys. In heavy smokers (more than 21 cigarettes per day), the risk of SGA was increased 3.15-fold for boys and 3.51-fold for girls.[182] The lower oxygen saturation in infants of heavy smokers may adversely affect fetal growth.

Alternatively, a reduction in epidermal growth factor (EGF)–stimulated autophosphorylation may be associated with IUGR.[183] EGF normally is involved in human embryouterine signaling and implantation,[184] in vitro stimulation of syncytiotrophoblast formation,[185,186] and modulation of placental endocrine functions.[185-188] Receptor autophosphorylation is required for EGF to elicit normal placental cell division and differentiation.[183] Conversely, abnormal development of the placenta may interfere with fetal growth.[189] Gabriel and associates[183] and Wang and coworkers[190] showed that EGF receptor autophosphorylation was significantly reduced in the placental membranes of smokers with SGA newborns compared with that in the placental membranes of smokers who gave birth to infants without growth restriction and those of nonsmokers. Gabriel and associates[183] concluded that IUGR in infants of women who smoked during pregnancy is associated with an alteration in the EGF receptor bioactivity of the placenta and suggested that the regulatory role of EGF in placental growth and differentiation is defective in smokers.

Smoking during pregnancy is a strong predictor for low birth weight, suggesting that focusing on education within smoking cessation programs for pregnant women, particularly those who may also be illicit drug users, could help reduce the adverse health consequences of low birth weight.[191]

A case-control study[192] of childhood cancer described a dose-response relationship between the number of cigarettes smoked per day by the mother during pregnancy and the risk of cancer in her children. A 50% higher incidence of cancer was found in infants exposed prenatally to cigarette smoke than that observed in control infants. The risks for the development of Hodgkin disease, acute lymphoblastic leukemia, and Wilms tumor also were higher. A more recent case-control study[193] (the Northen California Childhood Leukemia Study) included 327 children with acute lymphocytic leukemia (ALL), 46 children with acute myeloid leukemia (AML), and 416 control subjects matched for age, sex, race, and ethnicity. Maternal smoking was not associated with an increased risk for either ALL or AML, but paternal preconception smoking was significantly associated with increased risk for both.[193] Inherent difficulties with retrospective assessments of exposure led the investigators to no definitive conclusion.

The incidence of neonatal respiratory distress syndrome is reduced by maternal smoking.[194] Children exposed to smoke during pregnancy, however, tend to manifest a higher incidence of bronchitis and pneumonia during their first year of life.[195-198] A higher incidence of neonatal respiratory tract infections also has been linked to maternal cigarette smoking.[199] Smoking has been shown to decrease the production of breast milk.[200] Said and co-workers[201] suggested that maternal smoking during pregnancy may contribute to the incidence of neonatal colic.

Epidemiologic evidence clearly supports a link between maternal smoking and SIDS, but these studies fail to make the critical differentiation between fetal nicotine exposure and passive postpartum nicotine exposure, consequently weakening the association. Naeye and associates[202] stipulated that the strength of this correlation increased with maternal smoking of 6 or more cigarettes per day. Passive inhalation of smoke also increases the risk for SIDS in infants.[203] In another study,[204] researchers found that maternal smoking during pregnancy doubled the rate of SIDS from 2.3 to 4.6 per 1000 births. None of these studies, however, attempted to differentiate between intrauterine exposure and passive exposure to smoke after birth.

Malloy and colleagues[205] studied the association of maternal smoking with infant age and the specific cause of infant death among Caucasion women having singleton pregnancies, rather than using infant mortality in general as the outcome measure. This group of investigators concluded that maternal smoking was associated with a nearly two-fold increase in SIDS. Haglund and Cnattinguis[206] reported that maternal smoking of up to 9 cigarettes per day doubled the risk of neonatal SIDS, and smoking 10 or more cigarettes per day tripled the risk of SIDS, over that in infants of nonsmoking control subjects. Both Malloy and colleagues[205] and Haglund and Cnattinguis[206] acknowledged that a study of passive smoking by either partner after birth was not conducted, and passive smoking may in fact contribute to the incidence of neonatal SIDS. A two-fold increase in the rate of SIDS associated with maternal cigarette smoking also was suggested by Anderson and collegues.[207] Infants exposed to smoke were 1.9 times more likely to die of SIDS.[207]

To investigate the effects of maternal smoking on the cognitive development of 3-year-old children, a study of 700 mother-child pairs[208] was conducted. Extensive information on growth and development, including cognitive development test results, was included in the study variables. The investigators concluded that the cognitive performance of infants born to women who continued to smoke throughout their pregnancies was significantly compromised, at least to the third year of life, after correcting for known confounding variables. One study that prospectively

followed children up to the age of 4 years suggested that in utero exposure to cocaine resulted in a deficit in special language skills.[133] Although scientists and the public continue to pay far more attention to fetal cocaine effects than to the effects of nicotine or tobacco use, a change in focus to concentrate on tobacco use during pregnancy and after birth could have a much larger impact on human health.

Clinical Management of Smokers During Pregnancy

Smoking in pregnancy remains a major public health problem. The goal of maternal therapy for smoking during pregnancy is to improve fetal outcome by preventing associated prenatal complications. Education and support are required for pregnant smoking women. Nicotine replacement therapy (NRT) in pregnancy has been tried, but with low effectiveness.[209] This poor success rate may reflect the doubling of the clearance rate of nicotine seen in late pregnancy, thereby rendering replacement levels subtherapeutic.[210,211] NRT is effective for smoking cessation in nonpregnant people, but because women metabolize nicotine and cotinine much faster in pregnancy, it is unclear whether NRT can be effective for smoking cessation in this population.[212] Bupropion is an effective agent for smoking cessation. The manufacturer's registry suggests that it is not teratogenic when used in clinical doses.

Marijuana

After alcohol, the second most commonly used recreational drug in the United States is marijuana. Prevalence of marijuana use during pregnancy in developed nations is estimated to be approximately 10%.[210]

Marijuana, or cannabis, is produced from the flowering tops of hemp plants, from which at least 61 cannabinoids and more than 300 naturally occurring compounds can be extracted. The isomer 1-Δ^9-tetrahydrocannabinol(THC) is the most potent psychoactive compound among the cannabinoids.THC and other cannabinoids are C_{21}-substituted monoterpenoid compounds.[1]

The exact mechanism responsible for the pharmacodynamics of marijuana is unknown. Marijuana smoking produces an approximately 5-fold greater increase in the blood carboxyhemoglobin level than that produced by cigarette smoking.[213,213a] This may be due to an approximately two-thirds larger puff volume, a one-third greater depth of inhalation, and a 4-fold longer breath-hold-time with marijuana than cigarettes ($p < 0.01$).[213a] This results in a greater respiratory burden due to increased carbon monoxide exposure when smoking marijuana. The resulting high carboxyhemoglobin concentration may affect fetal oxygenation and, consequently, fetal growth and development.[1,214]

Pharmacology of Marijuana

The amount of systemically absorbed THC from a single inhaled dose is between 10% and 50% (mean, 18%). The bioavailability of an oral dose is 5 to 10 times lower.[215-218,218a] The peak plasma concentration after oral administration is attained within 45 minutes and remains relatively constant for 4 to 6 hours.[219] The peak plasma concentration after smoking marijuana is attained after 7 to 8 minutes. At 30 to 60 minutes from the initial time the user consumes the drug, tachycardia and euphoria occur.

THC is almost 100% protein-bound. The drug is highly lipid-soluble. Plasma levels decline rapidly after inhalation, and a slow elimination phase ensues.The volume of distribution is estimated to be 10 L/kg.[120] The elimination half-life in long-term users is 28±1 hours, compared with 57±4 hours in nonusers of marihuana (normal volunteers without previous exposure to cannabis preparations), primarily because of the faster metabolism of the long-term user.[219-222]

THC is converted almost entirely to the active metabolite 11-hydroxy-THC on first pass through the liver. The effect of the active metabolite is identical to that of the parent drug. This metabolite is subsequently biotransformed to inactive metabolites, which are excreted by the kidneys. Less than 1% of THC is excreted unchanged in the urine. Metabolites excreted in the bile can be reabsorbed. Fecal excretion is the major route of elimination for unconjugated cannabinoid metabolites.

Both animal and human studies[223-225] have shown that Δ^9-THC crosses the placenta. The maternal blood concentration of the drug is 2.5 to 6 times higher than that in fetal blood. Duration of exposure increases the fetal-maternal concentration ratio. In heavy users, an eight-fold accumulation of Δ^9-THC in breast milk over that in maternal plasma has been reported.[226,227] The nursing neonate can therefore consume Δ^9-THC from the mother's breast milk.

Effects of Marijuana Use on the Fetus and Neonate

As with other drugs of abuse, data collected from mothers who use THC must be interpreted cautiously, because these users may practice polydrug abuse and any self-reported statistics may be grossly inaccurate. In fact, polydrug use may account for the poorer prenatal care received by marijuana users.[228]

The effect of THC on pregnancy outcome has received much attention. Documented effects of cannabinoids on pituitary and ovarian function, prolactin secretion, hormonal activity, and uterine contractility have been amassed from animal and human studies.[229] No relationship between marijuana use during pregnancy and the length of gestation, duration of labor, or birth weight is evident.[230] Heightened tremors, abnormal startle responses, and altered visual responsiveness have been observed in neonates born to women who abused THC.

In a study of pregnancy course and outcome in 35 regular marijuana users and 36 age- and parity-matched nonusers,[231] the neonates of marijuana users exhibited a common tendency toward anemia, poor weight gain, and IUGR.The results were not statistically significant, however, which may be indicative of confounding variable clustering. A higher proportion of THC users (29 mothers) than of nonusers (3 mothers) experienced prolonged, protracted, or arrested labor.[231] These findings have been confirmed in other studies.[232-234] Marijuana abusers also exhibited a higher frequency of fetal distress and more often required manual removal of the placenta.[231] The course of labor appeared to be more hazardous in the THC user group than in the nonuser control group.[231]

Among THC users, meconium passage by the fetus, associated with an increased risk of neonatal death, was found to occur with increased frequency.[1,235] By contrast, Fried and associates[236] did not find any evidence to support increased prevalence of meconium staining among newborn infants of heavy marijuana users.

The effects of marijuana use during pregnancy on infant birth weight and length of gestation are controversial. In a study by Fried and colleagues,[232] neonates born to women who consumed marijuana six or more times per week during pregnancy demonstrated a significant reduction of 0.8 weeks in the length of gestation, after controlling for the effects of nicotine and alcohol, parity, maternal prepregnancy weight, and the gender of the infant. No reduction in the birth weight of these neonates was noted. In another study,[233] however, the investigators reported contradictory results: Reduced birth weight and no change in the length of gestation were associated with marijuana use. The difference between these two study outcomes may be attributed to the younger age and lower socioeconomic status typical of the latter study population. Moreover, it is possible that alcohol effects may have caused these minor differences, because many women abusing THC also consume large amounts of alcohol.[225,236]

Any association between minor physical anomalies and prenatal marijuana exposure is controversial. No difference was observed in the frequency of such anomalies in the children of

25 marijuana-using women and the children of 25 matched nonuser control subjects.[217] Similar observations have been reported in other studies.[237,238]

A link between prenatal marijuana exposure and the associated features of FAS has been demonstrated.[217,233,234,237] Thirty-one (2%) of 1384 neonates born to women who smoked marijuana had typical features of FAS. The likelihood of giving birth to an infant with manifestations of FAS is five times higher in cannabinoid users than in nonusers.

The Brazelton Neonatal Behavioral Assessment scale[239] was used to correlate smoking cannabinoids during pregnancy with a decrease in neonatal response to light directed at the eyes. Forty-six percent of the neonates born to users of cannabinoids did not respond to the light, in contrast with 16% of those born to matched nonusers.[240,241] Neonates born to methadone-using mothers showed a similarly poor visual response. In the infants with both marijuana and methadone exposure, the neonatal response to auditory stimulation was normal.[242,243] Other characteristic features seen in neonates born to heavy cannabinoid users include marked tremor, mainly around day 9 of postnatal life, and a startle reflex. Cortical electrical responses in the exposed neonates appeared to be slightly slower than those in age-matched control infants. Similar neonatal outcomes have been documented in cases of in utero exposure to alcohol and nicotine. Other visual abnormalities, including myopia, strabismus, and unusual appearance of the optic disks, occur at a higher frequency in marijuana-exposed (33%) versus nonexposed (6%) neonates.[244,245]

The findings of neurologic dysfunction could be interpreted as manifestations of neonatal drug withdrawal. In the adult population, however, abstinence after heavy THC use produces mild withdrawal reactions that encompass restlessness, sleeplessness, anorexia, nausea, vomiting, dreaming, and sweating.[1] Neonates exposed in utero to THC are not more irritable than neonates born to women in the non–marijuana-smoking control groups.[1] Few studies have studied the effects of prenatal marijuana exposure on academic achievement; results tend to be conflicting. As children grow, different variables such as socioeconomic status, the child's emotional well-being, social support, and family stressors may affect academic achievement. Therefore these factors have to be considered during evaluation of differences in prenatal drug exposure and academic performance. The Ottawa Prenatal Prospective Study (OPPS) suggested that prenatal cannabinoid exposure was not a significant predictor of performance in 5- to 6-year-old marijuana-exposed children, nor did it predict school achievement at the 6- to 9-year follow-up evaluation.[246] This study represents a lower- to middle-class population, however, in which the environment may ameliorate the effects of drug exposure.[246]

Goldschmidt and collegues[247] evaluated prenatal marijuana and alcohol exposure on school achievement at the age of 10 years. These investigators reported that daily exposure to one or more marijuana joints during the first trimester predicted deficits in Wide Range Achievement Test-Revised (WRAT-R) reading and spelling score and a lower rating on teacher evaluation of performance.[247] Second-trimester marijuana use was significantly associated with reading comprehension and underachievement, whereas third-trimester marijuana exposure was not associated with any change in the measures of academic achievement. This study was conducted in low-income women; the effects of marijuana use persisted even after institution of controls for confounding factors.[247]

Clinical Management of Marijuana Smokers During Pregnancy

Marijuana may be consumed concomitantly with multiple drugs and cigarettes, in which case treatment must be initiated to treat the deleterious effects of the more dangerous drugs and chemicals being used during the pregnancy. Education and support for pregnant marijuana smokers are paramount.

Opioids

Opiates are natural compounds derived from the opium poppy; *opioids* are synthetically derived analgesic compounds that have morphine-like pharmacodynamic activity. More than 500,000 opiate and opioid addicts live in the United States, of whom 70,000 are enrolled in methadone maintenance treatment programs.[248] Neonates exposed in utero to either heroin or methadone account for 3% to 5% of live births in New York City municipal hospitals.[248]

Heroin or diacetylmorphine is derived from morphine and produces pharmacologic effects identical to those of the parent drug. Heroin has a greater solubility and crosses into the CNS more rapidly than morphine, thereby making heroin the drug of choice among opioid addicts. Methadone is a synthetic opioid with a different chemical makeup from that of morphine, but it has nearly identical pharmacodynamic effects. Methadone is used in clinical medicine principally to treat heroin addiction[249] and to manage pain.

The abuse of oxycodone in the form of the controlled-release formulation (OxyContin) is a recognized public health issue and a focal point for law enforcement agencies.[250] Cicero and associates examined the prevalence and magnitude of abuse of prescription drugs in the United States using the Researched Abuse, Diversion, and Addiction-Related Surveillance (RADARS) system; drug abuse experts (e.g., pain and methadone health care specialists) were used as the source of data.[251] The results, showed, in rank order, that the prevalence of abused drugs was as follows: OxyContin > hydrocodone > other oxycodone > methadone > morphine > hydromorphone > fentanyl > buprenorphine; the magnitude of abuse (5 or more cases per 100,000 persons in a three-digit ZIP code area) was most pronounced with OxyContin and hydrocodone.[251] In a retrospective chart review of data for pregnant women conducted at the University of Kentucky Chandler Medical Center, OxyContin was mentioned most frequently in patient interviews, followed by, in order of decreasing frequency, Lortab or Lorcet (hydrocodone with acetaminophen), methadone, Percocet (oxycodone with acetaminophen), hydrocodone, and morphine.[252] The average dose of OxyContin was 154.1 ± 147.8 mg (n = 68).[252] The preferred route of administration was snorting (in 35.3% of patients), but the use of intravenous OxyContin was reported to be more widespread than it was 10 years earlier, probably because of the decreased amount of drug needed to produce euphoric effects and the associated lower cost.[252]

Lower socioeconomic class, a long history of drug abuse, frequent and multiple medical complications, poor diet, heavy smoking, and the concomitant use of alcohol, marijuana, cocaine, barbiturates, or tranquilizers are the characteristic hallmarks of opioid addiction. Adverse effects of heroin and methadone addiction are encountered frequently.

Pharmacology of Opioids

Opioids affect the activity of neurons involved with respiration, pain perception, and affective behavior. Morphine is an opioid analgesic that exerts its primary effect on the CNS and organs containing smooth muscle. The drug functions as an agonist at specific, saturable opioid μ receptors in these regions. Pharmacologic effects include analgesia, decreased gastric motility, suppression of the cough reflex, respiratory depression, CNS depression, nausea and vomiting, euphoria and dysphoria, sedation (fetal), mental clouding, and alterations of the endocrine and autonomic nervous systems.[253]

Morphine is readily absorbed by the gastrointestinal tract and undergoes significant first-pass metabolism in the liver. The oral

bioavailability of morphine is less than 5% in most users. Peak blood concentrations after intramuscular or subcutaneous injection are attained within 30 minutes. Tissue concentrations are quite low within 24 hours after the last dose, because morphine does not persist in the tissue for extended periods.

The elimination half-life of morphine in adults is between 2 and 3 hours (mean, 1.9 ± 0.5 hours). Significant variation is reported in the mean duration of action, with values ranging from 2.5 to 4.2 hours. Morphine is 35% ± 2% protein-bound,[254] and the clearance of the drug is 2.02 L/hour/kg in healthy young adults and 1.66 L/hour/kg in elderly patients.[255] The elimination half-life of morphine in neonates is between 6 and 14 hours.[256-258] The neonatal clearance of morphine is 1.43 L/hour/kg.[258-261] Published information on the oral bioavailability of morphine in neonates is lacking. However, the neonatal bioavailability of morphine probably is incomplete, as it is in older children.[256]

Approximately 10% of an administered dose of morphine is excreted through the bile; the remainder is excreted in the urine as a glucuronide conjugate. Morphine is metabolized primarily by glucuronidation. Glucuronic acid conjugation of the phenolic hydroxyl group by uridine diphosphate–glucuronyltransferase causes the conversion of morphine into morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), and normorphine. Although M3G is quantitatively the major metabolite, M6G is up to 40 times more potent than the parent compound and is thought to be the substance responsible for the analgesic properties of morphine. Morphine is *O*-methylated to produce codeine. Heroin is deacetylated to form morphine and 6-monoacetylmorphine (6-MAM). 6-MAM has been shown to be pharmacologically equipotent to heroin. It is thought that the characteristic pharmacodynamic effects of heroin are mediated by 6-MAM and morphine. Morphine, heroin, and methadone cross the placenta by simple diffusion.

Methadone is well absorbed from the gastrointestinal tract; peak plasma concentrations are attained within 4 hours. No good correlation has been found between plasma levels and analgesia.[262] A plasma level of 211 mcg/L has been associated with the best rehabilitation results in patients receiving methadone maintenance therapy.[263] The oral bioavailability of methadone is 92% ± 21%,[254] and the drug is 89% plasma protein-bound. Methadone has a volume of distribution of 3.8 ± 0.1 L/kg and a clearance of 0.08 ± 0.03 L/hour/kg. The clearance of methadone may increase during pregnancy. A study from the United Kingdom measured plasma methadone concentrations to investigate the rate of clearance of methadone prescribed for heroin dependence in the first, second, and third trimesters of pregnancy. The weight-adjusted clearance rates gradually increased from a mean of 0.17 to 0.21 L/hour/kg as the pregnancy progressed.[264] The drug is metabolized to form pyrrolidines and pyrrolines, which are excreted in the urine and bile; 24% ± 10% is excreted in the urine unchanged.[254] The elimination half-life is longer for methadone than for morphine (14.3 hours for the α phase and 54.8 hours for the β phase,[265] versus 6 to 14 hours for morphine), and methadone also causes less sedation and euphoria. Nausea and vomiting are rare.

Oxycodone is a semisynthetic opioid analgesic that is available in immediate- and controlled-release formulations. The drug has a half-life of 4 to 6 hours, a volume of distribution of 1.8 to 3.7 L/kg, and an oral bioavailability of 0.42 to 0.45.[266] Peak plasma concentrations in nine volunteers who received a large oral dose of 0.28 mg/kg had an average of 0.038 mg/L at 1 hour after administration; the elimination half-life averaged 5.1 hours.[267] Average peak plasma concentrations in adults (N = 28) given a single 20-mg prolonged-release tablet were 0.023 mg/L for oxycodone at 3.2 hours, 0.015 mg/L for noroxycodone at 4.3 hours, and 0.001 mg/L for oxymorphone at 4.5 hours.[268] In naltrexone-blocked volunteers, in whom enhanced absorption would be expected, single doses of 40 and 80 mg in controlled-release tablet form produced a mean area under the curve (AUC) (in nanograms × hour per milliliter) (with % coefficient of variation), C_{max} (in ng/mL), and T_{max} (in hours) of 423.1(33.3) and 1085.5(32.3), 39.3 (34.0) and 98.5 (32.1), and 3.1 (77.4) and 2.1 (52.3), respectively.[269] Oxycodone is metabolized by *N*- and *O*-demethylation to oxymorphone, a potent analgesic, and to the relatively inactive metabolite noroxycodone.[266] Between 33% and 61% of a single dose of oxycodone is excreted in a 24-hour urine collection as free (13% to 19%) and conjugated oxycodone (7% to 29%), conjugated oxymorphone (13% to 14%), and an unknown amount of noroxycodone.[266]

In a study of 22 infants undergoing surgery,[270] oxycodone pharmacokinetics were characterized after administration of an intravenous bolus dose of 0.1 mg/kg oxycodone HCl. Infants were stratified by age: younger than 1 week (n = 10), 1 week to 2 months (n = 6), and 2 months to 6 months (n = 6). The median results (with range) for clearance were 9.9 (2.3 to 17.2), 20.1 (3.7 to 40.4), and 15.4 (14.8 to 80.2) mL/min/kg, respectively.[270] Volumes of distribution at steady state were 3.3 (1.9 to 4.7), 5.6 (1.3 to 8.5), and 3.2 (1.8 to 6.0) L/kg, respectively, and the elimination half-lives were 4.4 (2.4 to 14.1), 3.6 (1.6 to 11.6), and 2.0 (0.8 to 3.9) hours, respectively.[270] Clearance and half-life were correlated with age (p <.05).[270] Variation in the results probably is due to the time required for demethylation mechanisms to mature in the newborn. These data indicate that there is large intraindividual oxycodone pharmacokinetic variability in the newborn; the dose of oxycodone should be titrated on an individual basis.

Effects of Opioid Use on the Fetus and Neonate

A significantly lower birth weight, shorter length, and smaller head circumference have been observed in neonates who have been exposed in utero to heroin or methadone.[271] The effects on infant growth variables are thought to be a direct result of fetal opioid exposure. These infants catch up in weight and length by 1 year of age. The head circumference of the opioid-exposed infant does not exhibit catch-up growth to an appropriate size for age until at least the age of 2 years.[1]

Early stunting of growth may be a direct effect of methadone on the hypothalamic-hypophyseal axis of the neonate.[272] Neonatal recovery is associated with decreased methadone plasma and tissue concentrations.

Growth restriction in infants born to heroin addicts is attributable mainly to a decrease in cell numbers.[180] Neonates born to women addicted to heroin alone and to the combination of heroin and methadone had mean birth weights of 2490 g and 2535 g, respectively.[273] Neonates in the control group born to women who did not use heroin or methadone had a mean birth weight of 3176 g. In heroin- and methadone-addicted mothers, a higher incidence of SGA neonates (35% and 22%, respectively) has been reported.[274] By contrast, a lower incidence of SGA (16.5%) was reported in 830 neonates born to drug-addicted women.[275] A 20% incidence of Apgar scores less than 7 at 1 minute post partum has been documented for neonates of "drug-addicted" or heroin- or methadone-addicted women, representing a two-fold higher incidence over that of infants in the control groups. A similar trend in Apgar scores has been reported at 5 minutes post partum.[273,276] These findings have been recently confirmed by an Australian group of investigators who completed a retrospective audit of obstetric and perinatal outcomes in women who had used opiates during pregnancy. Infants exposed to opiates were more likely to be born before 37 weeks of gestation, have birth weights of less than 2.5 kg, be SGA, and require admission to special care units (i.e., neonatal intensive care units) and resuscitation.[277] In addition, a two-fold increase in premature deliveries over a 10-year period was observed in a series of 384 neonates born to heroin-addicted mothers.[278] The incidence of premature deliveries was only slightly higher (7%) in methadone-addicted

mothers.[215] Premature delivery (29%) was confirmed by Fajemirokun-Odudeyi and collegues[279] in a study of 108 heroin-addicted women. This study also suggested that compared with babies of women who used only methadone, babies of women who used heroin in late pregnancy experienced a more severe neonatal abstinence syndrome (NAS) and required longer hospital stays.[279]

Infants born to heroin addicts exhibit a lower incidence of hyaline membrane disease than those born to nonaddicted women.[280] Opioids such as heroin may induce enzymes to accelerate the production of pulmonary surfactant in the fetus. In neonates exposed to opioids during pregnancy, a shift to the right in the oxyhemoglobin-dissociation curve is noted for umbilical cord samples, and 2,3-diphosphoglycerate concentrations in the neonatal red blood cells are increased.[281] 2,3-Diphosphoglycerate decreases fetal hemoglobin affinity for oxygen. Therefore the enhanced ability of red blood cells to deliver more oxygen to the tissues may contribute to the decreased incidence of hyaline membrane disease. Moreover, neonates exhibiting heroin withdrawal symptoms and signs have respiratory alkalosis in the first few days of life. This may be another protective mechanism, because acidosis is a predisposing factor for development of respiratory distress syndrome. In the first 3 days of neonatal life, a less common severe hyperbilirubinemia occurs in neonates of opioid-addicted women.[274,282]

The incidence of SIDS is increased 5- to 10-fold in neonates born to opioid-addicted women.[283,284] However, an association between SIDS and prenatal opioid exposure has not yet been clearly established, primarily because no comparative drug study has been conducted, other than studies of the opioids used by pregnant women. For neonates exposed to opioids in utero, it may be prudent to initiate cardiorespiratory monitoring. Moreover, neonates with apnea may benefit from administration of 1.5 mg/kg of theophylline (Theo-Dur) every 6 hours.[285] In a study of 1760 cases of SIDS,[286] the risk ratio for SIDS was 3.6 for methadone, 2.3 for heroin, 3.2 for methadone plus heroin, and 1.1 for cocaine plus methadone or heroin consumption (after controlling for associated high-risk confounding variables). Despite the consensus linking maternal opioid use to SIDS, no proof of causation has been developed. The most plausible hypothesis suggests that morphine and related opioids reduce brain stem responsiveness to carbon dioxide[287] and depress the pontine and medullary centers that control respiratory rhythmicity.[288] Most of these data, however, are derived from adult studies. Studies of the prolonged effects of opioids on infants after intrauterine exposure are scarce. In one such study, Olsen and Lees[289] examined the ventilatory response to carbon dioxide in nine methadone-exposed neonates and four control neonates. The investigators showed a minimal response to carbon dioxide in drug-exposed neonates during the first 4 days of life that persisted for an average of 15 days. No relationship was evident between ventilatory response and the maternal dose of methadone, serum methadone half-life, or the need to treat for neonatal abstinence.

A weaker link between maternal opioid use and neonatal SIDS was postulated by Naeye,[290] who suggested that infants dying of SIDS actually were suffering from chronic hypoxia and chronic hypoxemia. The tissue markers noted during autopsies that support this postulate are listed in Box 26-2.[290] None of these markers, however, has been specifically studied in opioid-exposed neonates who died of SIDS. In addition, no evidence exists to indicate that chronic postnatal hypoxia occurs in such infants.[87] Prenatal growth restriction and meconium staining are considered hallmarks of fetal hypoxia and have been observed in neonates exposed to heroin.[291] Hence predisposition of the neonate to SIDS may be the result of opioid-induced chronic intrauterine hypoxia, causing damage to the brain centers regulating respiration—consistent with the brain stem gliosis described by Naeye.[87]

BOX 26-2

Tissue Markers of Chronic Hypoxia and Hypoxemia in Infants Dying of Sudden Infant Death Syndrome

- Carotid body gliomic tissue changes
- Increased tissue in the adrenal medulla
- Abnormal gliosis in brain stem centers controlling respiratory drive
- Thickened pulmonary arterial walls
- Increased weight of the right cardiac ventricle
- Retention of periadrenal brown fat
- Increased hepatic erythropoiesis

Developmental outcomes were unfavorable for infants of drug-dependent mothers (IDDMs) who used heroin or heroin and methadone in combination during pregnancy compared with the developmental outcomes of infants of mothers who were not drug-dependent or infants in the general population.[292] Unfavorable outcomes in IDDMs were: decreased gestational age at birth and lower birth weight; impaired physical growth, neurologic development, and intelligence; abnormal behavior and social competency; and disturbed social state (e.g., foster care). Prenatal care with social support and methadone substitution were critical factors for favorable outcome. IDDMs in foster care exhibited even more unfavorable outcomes than those for children living with their biologic parents.[292]

In a study that used the Brazelton Neonatal Behavioral Assessment scale,[271] opioid-exposed neonates exhibited significant impairment of interactive abilities, motor maturity, and organizational response compared with their nonexposed counterparts. These neonates were more irritable and tremulous compared with control neonates, who were not exposed to prenatal opioid consumption. Opioid-exposed neonates also demonstrated deficits in general cognitive abilities and in visual, auditory, and tactile perception. Mothers of neonates exposed to methadone have noted sleep disturbances in their infants.[293] These disturbances include irritability, excitability, inability to nap, and various other sleep irregularities. A significant reduction in the complications attributable to opioid abuse was demonstrated when addicted, pregnant women were given comprehensive care that included enrollment in a methadone maintenance regimen of 20 mg/day or less.[294]

Rates of malformation in infants of opioid users typically are no different from those of control groups. In addition, no homogeneous pattern of malformation has been established. Nevertheless, in a study of 830 opioid-exposed neonates, 2.4% demonstrated major malformations, compared with 0.5% in a control group.[273] The findings of this study should be viewed with caution, however, because the rates of major malformation are known to range between 1% and 3% in the general population. In another study, Harper and associates[282] found malformations in 5.9% of neonates born to women in a methadone treatment program. These figures may not represent an increased risk, however. Several other studies[274,295] failed to demonstrate an increased risk for malformations in neonates prenatally exposed to opioids.

No increased frequency or discernible pattern of teratogenic effects has been observed in infants born to women abusing heroin.[296,297] In reports implicating heroin in congenital anomalies, the findings have been questioned, because the patient population was poorly defined for confounding variables such as polydrug use by the pregnant women. Low birth weight and an increased incidence of meconium staining were reported as perinatal adverse effects associated with maternal heroin use. After prolonged and continuous heroin use by the mother during

pregnancy, narcotic withdrawal occurs in many neonates. Strabismus has been observed in 24% of drug-exposed neonates (including those exposed to opioids), compared with 2.8% to 5.3% in the general population.[298,298a]

Morphine is believed to be excreted into breast milk in small, clinically insignificant amounts.[299] Despite the widespread licit and illicit use of morphine, information on exposure to the drug through breast milk is very limited. With the advent of highly sensitive radioimmunoassays and specific high-performance liquid chromatography (HPLC) analytical techniques, new data are becoming available to discern morphine kinetics in the nursing neonate. Robieux and colleagues[300] presented a case report of a nursing woman with systemic lupus erythematosus who was receiving morphine therapy to treat severe arthritic back pain. The neonate had a morphine plasma concentration of 4 ng/mL; breast milk concentrations ranged from 10 to 100 ng/mL. The dose needed to achieve the observed morphine plasma concentration in the infant was calculated to range between 0.8% and 12% of the maternal dose. This case demonstrates that neonates experience significant exposure to morphine even when the mother receives morphine at low doses.

For mothers being treated with methadone, the amount of methadone that passes into the breast milk is small (2% to 4%).[301] Methadone reaches its maximum levels 2 to 4 hours after maternal dosing. Even at this low level of exposure, a baby can experience withdrawal. Breast-feeding on a regimen of medium to high doses of methadone probably is safe according to conventional criteria, because the dose is less than 10% of the relative infant dose.[301] Because the absolute dose of methadone received by the infant is dependent on the maternal dose, however, the risk-benefit ratio should be considered in each case. The dose of methadone received in breast milk is unlikely to be sufficient to prevent drug-induced NAS. The benefits of breast-feeding outweigh the risks associated with subsequent withdrawal.[301]

Oxycodone is excreted into the breast milk and has a milk-to-plasma ratio of 3.4:1. Maternal use of this drug is classified as a pregnancy risk factor B (risk factor D if the drug is used for prolonged periods or in high doses at term).[302] In a recent study, Seaton and colleagues[303] investigated the relationship between maternal ingestion of oxycodone after cesarean section and the resulting maternal plasma, breast milk, and neonatal plasma drug concentrations up to 72 hours postpartum. The results showed that oxycodone administered at any dose in a 24-hour period was detectable in the breast milk, with a significant correlation between maternal plasma and milk levels ($R^2 = 0.81$); this correlation decreased (to $R^2 = 0.59$) over the next 48 hours.[303] The milk-to-plasma ratio was 3.2:1. Oxycodone levels up to 168 ng/mL were measured in the breast milk, but only 1 neonate ($n = 41$) had oxycodone detected in the plasma.[303]

Neonatal withdrawal syndrome is evident primarily after maternal heroin and methadone abuse during pregnancy. The syndrome also can manifest after prenatal codeine, pentazocine, and propoxyphene exposure. The signs and symptoms are similar but are more frequent and more severe in methadone-exposed neonates than in newborn infants of heroin-addicted women.[274,304] The amount of methadone consumed by the mother appears to correlate with the severity of the ensuing neonatal withdrawal syndrome. Although the time to onset of signs and symptoms may vary, ranging from minutes to 2 weeks post partum, in most neonates, withdrawal manifests within 48 hours. The duration of acute neonatal withdrawal ranges from a few days to several weeks.[216] CNS excitation, altered gastrointestinal tract function, respiratory distress, and autonomic symptoms characterize the neonatal opioid withdrawal syndrome. Irritability is the most common manifestation, followed by tremors and hypertonicity. Seizure activity may appear between 3 and 34 days post partum in 2% to 11% of addicted neonates.[305] Neonates born to opioid-addicted women occasionally demonstrate seizure activity.[305] It appears that the incidence of seizure activity is higher in methadone-exposed neonates than in those exposed to heroin.

Neonates exposed to low doses of morphine in utero or through breast milk may not always exhibit the morphine withdrawal syndrome. The reason for this lack of the syndrome may be the extended half-life of morphine during early life, which results in a slow decline in the concentration of morphine in neonatal plasma.[258] Alternatively, the neonate may have developed tolerance to the opioid after long-term exposure in utero and throughout the breast-feeding period.[300] Rao and Desai reported a case in which a neonate was diagnosed with withdrawal signs and symptoms after maternal OxyContin use; presence of the oxycodone metabolite oxymorphone was measured by gas chromatography–mass spectometry (GC-MS) opiate analysis.[306] Treatment consisted of administration of paregoric followed by chloral hydrate.

The Collaborative Perinatal Project[306a] found no evidence to suggest a relationship between first-trimester oxycodone exposure and large categories of major or minor malformations or to individual defects. Other data also do not support an association between oxycodone and congenital defects.[302] Additional work is needed to determine the effects, if any, of in utero exposure to oxycodone.

Clinical Management of Opioid-Addicted Women and Neonates

Mild withdrawal from opioids usually does not necessitate treatment. The treatment of neonatal withdrawal syndrome is primarily symptomatic. Symptomatic neonates may require pharmacologic intervention. Pharmacologic preparations used to treat neonatal withdrawal include diluted tincture of opium (DTO), paregoric (camphorated tincture of opium), morphine (Astramorph), methadone (Dolophine), diazepam (Valium), chlorpromazine (Thorazine), phenobarbital (Barbita), and clonidine (Catapres).[307-309]

The American Academy of Pediatrics recommends the use of DTO for NAS that is due to opioid withdrawal.[310] Many neonatal intensive care units use oral or intravenous morphine solutions; methadone also is used in the treatment of neonates withdrawing from opioids.[311] In a small case series of inpatients, methadone at a dose of 0.1 mg/kg three times a day was found to reduce peak Finnegan scores (neonatal abstinence syndrome scoring system) faster than tincture of opium administered at 0.1 mg/kg every 3 hours.[312] Paregoric was prominent in earlier reports of treatment of infants exposed to opioids but is now less frequently used, probably because of the potential hazards of the added preservatives.[313,314] Phenobarbitone frequently is used for management of polydrug NAS and is a second-line agent for treatment of opioid withdrawal.[311] Controlled trial data supporting the efficacy of any one agent are limited; the evidence is weakened by the methodologic quality of published trials. Opioid-related NAS, however, is best treated with opioids.[310,315,316]

A recent survey, showed that opioid medications are the most commonly used drugs for the treatment of opioid and polydrug withdrawal.[311] The results of a recent double-blind, randomized trial of morphine versus phenobarbitone support the use of opioids in the treatment of opioid-related NAS.[317] Infants treated with morphine had a shorter duration of treatment, reduced frequency of admission to the neonatal intensive care unit, and a reduced need for the use of second line treatments.[317] Pacifico and co-workers showed that morphine given as a single agent relieved opioid-related NAS symptoms better than did a combination of phenobarbitone and diazepam or a combination of morphine, phenobarbitone, and diazepam.[318] Morphine mixtures of variable strength also are frequently used to treat opioid-related NAS because of the short half-life of morphine.[314]

Paregoric contains 0.4 mg/mL of anhydrous morphine and is therefore a more physiologic substance because it substitutes

for the drug that is causing the withdrawal symptoms. The recommended dose for full-term neonates is 0.2 to 0.5 mL (0.08 to 0.20 mg of anhydrous morphine) administered orally every 3 to 4 hours until the signs and symptoms are well controlled.[319] The initial paregoric dose is dependent on the severity of the withdrawal symptoms. The severity is assessed based on the Finnegan Scoring System.[320] If within 4 hours no clinical improvement is observed, the paregoric dose is increased by 0.05 mL every 4 hours until it has an effect (Finnegan score ≤4). When the withdrawal score is sustained for 48 hours, the total daily dose of paregoric should be reduced by 10% each day while a constant dosing interval is maintained. The recommended dose for paregoric in premature infants is 0.05 mL/kg every 4 hours initially, with increments of 0.02 mL/kg every 4 hours until symptoms are controlled. Neonates treated with paregoric exhibited more physiologic sucking patterns, higher caloric intake, better weight gain, and improved control of seizure activity and gastrointestinal disturbances in comparison with neonates who were treated with phenobarbital.[309,321]

Overall, the opioid paregoric appears to be more effective than diazepam, phenobarbital, or paraldehyde in ameliorating withdrawal. In a clinical trial in which infants were randomly assigned to receive either paregoric or phenobarbital,[307] however, no significant difference in clinical efficacy was found between these two agents. The advantages of using phenobarbital to treat neonatal withdrawal include the drug's ability to mitigate signs and symptoms referable to the CNS and its safety. Phenobarbital is safer than an opioid[322] when sedation is required. The disadvantages include the inability to titrate the dose because of the long elimination half-life, the presence of other chemicals in the formulation, and the lack of standard dosing guidelines for phenobarbital. A chart review comparing paregoric and methadone for the treatment of neonatal opioid withdrawal showed no difference in outcome measures (time to resolution of withdrawal symptoms, rate of decrease in symptom severity, and length of hospitalization) between the two drugs; rates of adverse events were similar.[323]

Clonidine has been used in pediatrics to treat postoperative withdrawal from long-term opiate administration.[324,325] In a single, small case series reported in the literature, seven methadone-exposed infants were treated with 3 to 4 mcg/kg/day; six of the infants responded.[326] Clonidine may cause profound hypotension; additional work with this agent is required to determine its utility in treating NAS.[314] In a small case series study, diazepam was investigated in the treatment of NAS. Treatment of 23 infants born to opioid-dependent women treated over 7 days resulted in birth weight recovery by postpartum day 10; mean weight loss was 6.5%.[327] Most of the mothers in this study were polydrug users, with drugs of abuse including alcohol, cocaine, and benzodiazepines. The success of diazepam may have been due to its blocking of effects of the nonopioid drugs. Kron and colleagues,[328] however, found that diazepam significantly depressed sucking rate, nutritive consumption, and percentage of sucking time when compared with phenobarbitone. Further study of diazepam for this indication is required.

The duration of therapy is dependent on the severity of the maternal addiction and the duration of fetal exposure. A treatment regimen spanning 10 to 20 days is required in approximately 50% of opioid-exposed neonates. Another 25% require treatment for a longer duration, and the remaining 25% require a shorter course of treatment.[329] A significantly longer period of active treatment is required with use of paregoric than with use of phenobarbital, probably because of the longer half-life of the latter drug.

In the management of addicted women during pregnancy, the clinician must be acutely aware that two patients are actually being treated. Therefore a treatment regimen must be chosen that maximizes the benefits for both mother and fetus. In late pregnancy, abrupt maternal withdrawal may be accompanied by fetal withdrawal, typically characterized by hyperactivity, hypoxia, passage of meconium, and death.[330,331] Opioid antagonists such as naloxone (Narcan) should be administered to the pregnant woman only if she experiences a life-threatening overdose. Naloxone crosses the placenta to exert its antagonist effects on the fetus, thereby potentially exacerbating the withdrawal reaction in the neonate.

Heroin addicts should be encouraged to enroll in a methadone maintenance program.[332] Methadone treatment during pregnancy offers overwhelming advantages over the less acceptable option of medical detoxification or the unacceptable option of leaving heroin-addicted women dependent on street drugs. Methadone suppresses withdrawal symptoms and, in high doses, may block the opioid effect. In addition to decreasing illicit drug use, these programs improve fetal and subsequently neonatal outcome. An obstetrician who is skilled in monitoring high-risk pregnancies should be part of the maternal treatment team because of the potential adverse effects on the fetus attributable to maternal detoxification. With an accumulated experience of over 25 years, methadone maintenance has been shown to be invaluable and essential in improving the health of women during pregnancy, in improving the outcomes of these pregnancies, and in offering opiate-addicted women a chance to improve both their own lives and the lives of their families.[333]

Psychotherapeutic Drugs

The psychotherapeutic drugs include amphetamines, sedatives, tranquilizers, and analgesics. On the basis of the results of the National Pregnancy and Health Survey,[4] it was estimated that 1.5% of women using some illicit substance in the United States during pregnancy used psychotherapeutic drugs. The nonmedical use of these agents was highest among black women (3.1%), followed by women of Hispanic descent (1.8%) and white women of non-Hispanic origin (1.1%).

Benzodiazepines are widely used for therapeutic reasons and frequently are consumed by polydrug users.[334] Abuse of the benzodiazepines in high doses is not as common as it has been, but some women have been maintained on long-term therapeutic doses for many years. Because of the limited availability of these drugs, abuse of barbiturate and nonbarbiturate hypnosedatives also is not as prevalent as was described previously. These drugs include chloral hydrate (Noctec), glutethimide (Doriden), meprobamate (Equanil), and methaqualone. Butalbital (Fiorinal) is the only frequently abused barbiturate.[335] It is available only as a proprietary combination of aspirin and caffeine with or without codeine.

Use of methamphetamine, the most commonly abused type of amphetamine in pregnant women, is increasing in the United States. A recent study, primarily evaluating methamphetamine use in pregnant women, reported that 10.7% of mothers have used illicit drugs during pregnancy.[336] Amphetamine levels are found in approximately 2% of cases amassed from small, random analytical surveys of emergency department toxicology screens.[337] Illicit amphetamine formulations vary dramatically in potency and purity.[338]

Pharmacology of Psychotherapeutic Drugs

Benzodiazepines are weakly basic drugs absorbed most effectively at the high pH found in the duodenum. The oral absorption of diazepam and desmethyldiazepam, the active metabolite of clorazepate, is faster than that of the other benzodiazepines. Oral absorption is extremely rapid for triazolam. After intramuscular injection, the bioavailability of several benzodiazepines, including chlordiazepoxide and diazepam, may be unreliable. The barbiturates are weak acids that are very rapidly absorbed into the blood from the stomach and small intestine.[339]

The benzodiazepines and other sedative-hypnotic drugs cross the human placenta, thereby exposing the fetus. The rate at which the maternal and fetal blood concentrations equilibrate is slower than the rate at which the maternal blood and CNS equilibrate, partially because of the lower blood flow to the placenta.[339] Benzodiazepines are highly plasma albumin–bound; binding ranges from 60% to more than 95%.

The elimination of benzodiazepines occurs through hepatic metabolism. Most benzodiazepines undergo microsomal oxidation reactions such as *N*-demethylation and aliphatic hydroxylation. The metabolites are conjugated by glucuronosyltransferase to form glucuronides, which are excreted in the urine. Many benzodiazepines have pharmacologically active metabolites. A wide range (12 to 150 hours) in elimination half-life has been documented for the various benzodiazepines. Only insignificant amounts of barbiturates (except phenobarbital) are excreted unchanged. The major barbiturate metabolic pathway involves oxidation by hepatic enzymes. Few barbiturate metabolites are pharmacologically active. The elimination half-life of the barbiturates ranges from 18 hours (secobarbital and pentobarbital) to 4 to 5 days (phenobarbital).[339]

After an oral dose of 10 to 25 mg, peak plasma levels of amphetamines occur within 1 to 2 hours. Protein binding ranges between 15% and 34%, depending on the specific drug.[340] Amphetamines are rapidly absorbed from the gastrointestinal tract, and absorption usually is complete within 4 to 6 hours.[340] A consideration in polydrug users is that amphetamines decrease gastric emptying and intestinal motility, thereby extending the time for absorption of other drugs as they pass through the stomach and intestines. The elimination half-life of amphetamines is in the range of 8 to 12 hours after therapeutic dosing, and 18 to 24 hours after abusive consumption. Amphetamines are metabolized by deamination to form phenylacetone, which is subsequently oxidized to benzoic acid and excreted as a glucuronide conjugate.[341] β-Hydroxylation produces the active metabolite *O*-hydroxynorephedrine, which accounts for some of the drug's effects in long-term users.

Effects of Psychotherapeutic Drugs on the Fetus and Neonate

Increased risk of cleft lip and cleft palate after first-trimester exposure to benzodiazepines has been reported,[342,343] although a subsequent case-control study[344] did not confirm the initial observations. Late third-trimester use and exposure during labor may be associated with greater risk for the development of floppy infant syndrome or marked neonatal withdrawal symptoms and signs ranging from mild neonatal sedation, hypotonia, and poor suck to apneic episodes, cyanosis, and impaired metabolic responses to cold stress.[345] No specific neonatal abnormalities have been reported in cases of prenatal exposure to the newer benzodiazepines. A fetal benzodiazepine abstinence syndrome[346] was reported in seven cases; however, because of inadequate controls, a causative role remains speculative.[347] Published reports of human teratogenicity attributable to benzodiazepine use during pregnancy are lacking.[348] A meta-analysis of 23 studies (both cohort and case-control studies) pointed to an increased risk of congenital malformations associated with the use of benzodiazepines during the first trimester of pregnancy. Data from the cohort studies revealed no association between fetal exposure to benzodiazepines and the risk for oral clefting or development of any other malformations.[349] Data from case-control studies revealed a marginally significant two-fold increase in the risk for major malformations or oral cleft alone.[349] The case-control study populations were heterogeneous, however, thereby decreasing the reliability of the results. The absolute risk of clefts, if present, remains small (i.e., the incidence of oral clefting in the general population is approximately 1 per 1000).[349] No reports of long-term adverse effects on the neurodevelopment of children born to mothers who took benzodiazepines during pregnancy are found in the literature.[349]

Manifestations of neonatal benzodiazepine withdrawal, including seizures, are indistinguishable from those of narcotic or barbiturate withdrawal.[350] Neonatal withdrawal signs and symptoms have been reported even when the mother had received therapeutic doses of chlordiazepoxide[351,352] or diazepam,[346,353] with onset soon or several days after birth. Case reports also have described neonatal withdrawal from hydroxyzine (Atarax),[354] ethchlorvynol (Placidyl),[355] and glutethimide.[356] Withdrawal from these agents would not be unexpected. Because of maternal polydrug practices, however, withdrawal attributable to phenobarbital, diazepam, and heroin, respectively, cannot be excluded. An initial study[357] reported a three- to four-fold greater risk for development of cleft palate among neonates exposed in utero to diazepam. This observation was supported by other reports in the literature.[358,359] Neither Czeizel[360] nor Rosenberg and colleagues,[344] however, could confirm this association. Most of the studies from which conclusions were drawn were retrospective case-control studies that relied heavily on maternal recall. In a prospective study,[361] a diazepam-mediated cleft palate malformation was not demonstrated in neonates. Furthermore, prenatal exposure to triazolam and alprazolam in more than 600 cases was not associated with adverse neonatal outcome (data on file, Upjohn Pharmaceuticals, Kalamazoo, Mich).

Available evidence suggests that barbiturates are not teratogenic.[362] Nevertheless, the teratogenicity issue is not completely resolved. Butalbital and secobarbital have not been associated with congenital malformations. Information concerning amobarbital is controversial.

Neonates exposed in utero to barbiturates may exhibit the signs and symptoms associated with neonatal abstinence syndrome.[363-365] Desmond and colleagues[363] reported that all 15 neonates exposed prenatally to barbiturates or phenobarbital given for maternal treatment of anxiety, sedation as part of treatment of hypertension during pregnancy, or seizure control demonstrated hyperactivity, restlessness, disturbed sleep, and excessive crying, and most of the infants also were tremulous, hyperreflexic, and hyperphagic. The clinical manifestations of drug withdrawal appeared later (at approximately 1 week postpartum) in barbiturate-exposed neonates than in the narcotic-exposed group.[363] This delay is not unexpected because of the long β-elimination half-life of barbiturates. Women receiving secobarbital[364] and butalbital[365] also manifest barbiturate withdrawal. Neonates withdrawing from barbiturates can experience generalized seizures, in addition to exhibiting increased neuromuscular excitability.[366] Maternal abuse of phenobarbital during pregnancy has been shown to cause a hemorrhagic syndrome in neonates secondary to hypoprothrombinemia, interference with vitamin K metabolism, and platelet deficiencies.[367] Phenobarbital use during pregnancy increases the incidence of major malformations in neonates born to women with epilepsy.

Newborn infants of women consuming amphetamines during pregnancy have exhibited initial CNS hyperexcitability.[122] After the hyperexcitable state subsides, some infants manifest marked lethargy and require tube feeding. This state of profound lethargy is thought to be indicative of a true withdrawal syndrome, comparable with that observed during cocaine and methamphetamine withdrawal in the adult population.[368] Similar reactions in newborn infants of amphetamine-abusing women have been described.[369,370] Ramer[369] reported a case in which a neonate whose mother had been receiving parenteral amphetamine had episodes of diaphoresis, restlessness, and miotic pupils during the first day of life. By day 3, the infant was listless, with decreased muscle tone and a glassy-eyed stare. Symptoms and signs abated by postpartum day 9.

In a prospective study[362] examining 367 neonates exposed to amphetamine during the first trimester, no increased risk of

any malformation was found. Conversely, a retrospective study[371] found that congenital heart malformations were more prevalent when infants had been exposed prenatally to amphetamine. More recently, a multicenter longitudinal study was completed that assessed the neonatal effects of prenatal methamphetamine exposure. Infants in the methamphetamine-exposed group (84 subjects) were 3.5 times more likely to be SGA and to have lower birth weight than those in the unexposed group.[372] These findings suggest that prenatal methamphetamine use is associated with fetal growth restriction after adjustment for confounding factors.[372]

Clinical Management of Maternal Psychotherapeutic Drug Abusers and Exposed Neonates

A tapering regimen with diazepam obviates the drug concentration fluctuations that occur with short-acting benzodiazepines. In a socially stable woman, tapering may be achieved on 5 mg of diazepam per week, with weekly outpatient supportive counseling.[373] Abstinence usually can be achieved within 6 to 8 weeks. If the outpatient approach fails to result in abstinence, rapid diazepam tapering can be initiated in hospital. In cases of neonatal benzodiazepine withdrawal, 0.1 mg/kg intravenous diazepam every 12 hours is recommended.[350] Adjustments to the dosing interval and dose may be required to control neonatal withdrawal symptoms adequately.

Women consuming more than 400 mg of barbiturates per day are at risk for development of a withdrawal syndrome characterized by tonic-clonic seizures and delirium. These women should be admitted to the hospital for detoxification. Phenobarbital is the drug of choice, administered either on a tapering regimen[374] or by an oral loading technique.[375] Phenobarbital also is the drug used to treat neonates who exhibit signs and symptoms of barbiturate withdrawal.[350] Therapy should be initiated if the neonate manifests seizure activity, moderate to severe hyperreactivity, irritability, poor feeding, or failure to gain weight. An intravenous or intramuscular loading dose of 20 mg/kg, followed by intravenous or oral maintenance therapy at 4 mg/kg/day, should be administered.[350] The maintenance dose may be adjusted as necessary for effective management of symptoms and signs.[364] It is important to monitor serum phenobarbital concentrations to avoid toxicity. Once withdrawal symptoms have been effectively controlled for a period of 1 week, the dose can be reduced by 25% per week.[350]

CNS hyperexcitability associated with prenatal amphetamine exposure should be treated with benzodiazepines as necessary.[376] Withdrawal symptoms should be managed supportively.

SUMMARY AND CLINICAL IMPLICATIONS

Substance abuse is one of the major problems and challenges for society. Establishing the true prevalence of substance abuse among pregnant women is difficult. First, illicit drug use often occurs concurrently with licit drug use (e.g., cocaine plus alcohol). Second, obstetric complications of drug use may be complicated by coexisting factors such as a chaotic family environment or malnutrition. Third, social stigma continues to be attached to identified abusers, making women unwilling to admit alcohol and drug use, particularly during pregnancy.

Substance abuse during pregnancy is associated with significant maternal and fetal morbidity. The optimal management of pregnant, addicted women should be multidisciplinary, with input from obstetricians, pediatricians, midwives, social workers, drug counselors, and other related services professionals and agencies. Medical management of the infant should involve not only pharmacologic treatments but also assessment of parenting abilities, social support, and surveillance for transmission of infections. A multidisciplinary approach with active participation of social workers, physicians, and educators is critical for successful outcome.

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27 Drug Excretion During Breast-Feeding

Michael J. Rieder

BREAST-FEEDING

The optimal feeding of human infants is with breast milk; in addition to providing psychologic benefits, breast-feeding is associated with a decreased risk for gastrointestinal and respiratory disease in infancy.[1-3] Breast-feeding has been demonstrated to be an effective part of programs to reduce neonatal mortality, and some data (albeit controversial) suggest that breast-feeding may influence ultimate neurodevelopmental outcome. Therefore promotion of breast-feeding is a major public health initiative worldwide.

The prevalence of breast-feeding among the mothers of newborn infants in the United States and Canada declined steadily from the beginning of the 20th century to 1970, falling from 70% in 1915 to the nadir of 25% in 1970.[4] Since 1970, breast-feeding among mothers of newborn infants increased steadily until 1984, when more than 60% of mothers breast-fed their newborns. Although a decline in breast-feeding in the United States was noted in the early 1980s (although breast-feeding of newborns continued to increase in Canada), recent data suggest that the number of American women who breast-feed has continued to rise, notably in the first 12 weeks post partum, and that numerous national and local initiatives have been implemented and appear to have increased the rate of breast-feeding.[5,6]

As noted, the optimal choice for feeding infants in the first 6 to 12 months of life is breast-feeding. Of major concern in this context ismore than 90% of women take medications during this time.[7,8] Many patients are aware of the possibility that medication taken by a lactating mother can pass into breast milk, from which it can be ingested by the nursing infant. Thus the potential effects of maternal medication transmitted in breast milk on nursing infants constitute an important clinical consideration.[4]

BREAST MILK

Composition

Breast milk is a complex fluid, the most abundant component of which is water, constituting on average approximately 88% of content.[9] Milk also contains carbohydrate (5.6% to 6.9%), lipids (3.4% to 4.4%), and protein (1.7% to 2.2%).[10-15] The main carbohydrate present in breast milk is the disaccharide lactose synthesized within the alveolar cell, with small amounts of α-glucosides present as well. The lipids in breast milk are almost entirely triglycerides, contained in fat globules surrounded by membranes formed from cellular material produced by the alveolar acini (98%). These membranes consist of a complex blend of phospholipids, glycolipids, triglycerides, cholesterol, and proteins, which act to emulsify lipids in the aqueous environment prevalent in breast milk. The most abundant fatty acids are oleic acid and palmitic acid, with relatively high concentrations of linoleic acid and linolenic acid. The protein in human breast milk is a mixture of casein, albumin, lactalbumin, immunoglobulins, and lysozyme with an aqueous whey-micellar casein ratio of 60-65:35-40. In addition to protein, human breast milk also contains nonprotein nitrogen in the form of free amino acids and small compounds such as urea, which make up approximately 25% of the nitrogen content of breast milk.

Breast milk is acidic with reference to plasma, the average pH of human breast milk being 7.08 (range, 6.35 to 7.65). A biphasic change in breast milk pH occurs over time. The pH of colostrum is 7.45; it falls to 7.0 in transition milk and then slowly increases to reach an average pH of 7.4 by age 10 months.[4,16] The pH of breast milk also changes during the course of feeding, foremilk (expressed during early feeding) having a more acidic pH (as well as a lower fat content) than that of hindmilk.

The composition of human breast milk is not uniform because the constituents of breast milk change over time and to some extent over the course of feeding, most markedly in the first several weeks of life. Colostrum, the first "milk" produced by a lactating mother, is produced in the first 4 or 5 days after birth; transition milk is then produced, followed at 2 or 3 weeks after birth by the production of mature milk.[17]

The protein content of breast milk is subject to changes in both concentration and content. Colostrum has a higher protein content than that of mature milk, primarily owing to increased immunoglobulin content, whereas the albumin content remains relatively constant.[18] Breast milk produced by mothers of preterm infants has much more protein and less lipid in comparison with breast milk produced by mothers of mature infants.[14] The differences between the milk produced by lactating mothers of term and preterm infants are most marked in the immediate perinatal period; changes lessen as time from delivery increases.[4] On average, the lipid content of breast milk tends to increase over time.[4]

A diurnal variation also has been noted in lipid and protein content of breast milk:The fat content and amount of breast milk per feed are highest in the morning and fall in the late evening.[19-21] Protein content reaches its nadir in the early afternoon.[20]

Production

Human breast milk is produced in the alveolar acini of mammary tissue located in the breast, in response to hormonal stimuli.[22] During gestation, estrogen and progesterone (regulated by follicle-stimulating hormone and luteotrophic hormone) stimulate the maturation of mammary tissue in the breast and inhibit milk production. The rapid decrease in estrogen and progesterone secretion immediately before parturition leads to loss of inhibition of milk production.[4] Infant suckling stimulates release of prolactin from the pituitary gland, which provides the principal stimulus for milk production and secretion.[23] Drugs containing estrogen and estrogen derivatives can inhibit breast milk production; commonly used oral contraceptives reduce the production of breast milk by 20% to 30%.

Alveolar cells are the component of mammary tissue that produces milk. Clusters of these cells are arranged around a central lumen. Milk flows down the lumen to the ducts, which join in increasing size to meet under the nipple. Milk flow from the acini to the nipple is promoted by oxytocin-mediated contraction of the myoepithelial cells surrounding the acini and ducts.[4]

A linear relationship exists between blood flow and milk production: As blood flow to the alveolar acini increases, so does milk production.[22] Average breast milk production by mothers of 6-month-old infants is approximately 800 mL/day (ranging from 600 to 1000 mL/day).[24-26]

PRINCIPLES OF DRUG EXCRETION INTO BREAST MILK

Many medications can be excreted into breast milk, thereby exposing the nursing infant. Drug exposure by this route requires three processes: transfer of the drug into breast milk, absorption of the drug from breast milk by the infant, and subsequent drug

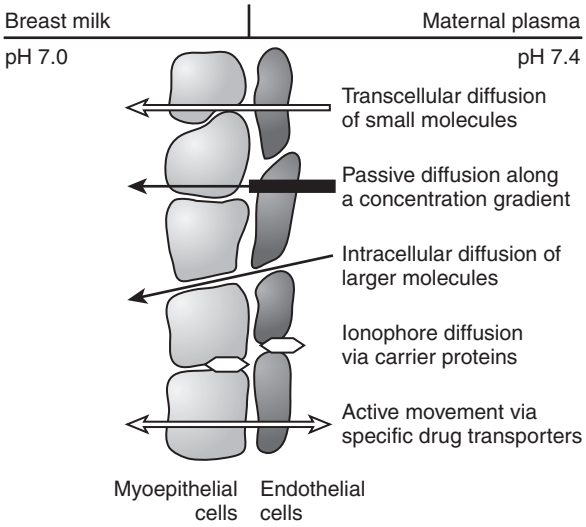


Figure 27-1 Steps involved in transfer of drugs into breast milk.

metabolism in the infant.[4,27] In most cases, drug transfer to breast milk requires the presence of the drug in the maternal circulation.[4,28] The only exception to this rule is with use of a topical agent applied to the nipple or areola, in which case direct transfer of the drug into breast milk may occur.[29] This is an uncommon but nonetheless potentially important mode of drug exposure.

Transfer of Drug From the Maternal Circulation Into Breast Milk

Drugs enter breast milk through the alveolar acini in the mammary gland. Major determinants of the amount of drug transferred to breast milk are maternal serum drug concentration and the physicochemical nature of the drug. Most drugs taken by lactating women are given orally, although less commonly, drugs may be given by the intravenous, topical, or aerosol route. The steady-state drug concentration in the maternal circulation is determined by the balance of drug absorption, distribution, metabolism, and excretion (Figure 27-1).

Drugs can be transferred from and to the maternal circulation to breast milk by several mechanisms (see Figure 27-1)[27]:

- Transcellular diffusion of small molecules, which are nonionized and have high lipid solubility, such as ethanol, for which plasma and breast milk concentrations are similar[27,30]
- Intercellular diffusion of drugs that pass between alveolar cells—possibly accounting for the passage of larger molecules, such as immunoglobulins, into breast milk[27]
- Passive diffusion along a concentration gradient
- Ionophore diffusion of polar substances by binding to carrier proteins[27]
- Active movement of specific compounds by specific drug transporters

Of note, diffusion of drugs into breast milk is a bidirectional process: Not only can drugs diffuse into breast milk from plasma, but they also can transfer from breast milk back into plasma. In addition, the principles of drug transfer into and out of breast milk apply equally to drug metabolites. In most instances, it appears that the passage of drugs from the maternal circulation to breast milk occurs by passive diffusion down a concentration gradient. This concentration gradient is across the capillaries feeding the alveolar acini and into the alveolar cells. To cross from the circulation into the alveolar acini, a drug must be present in the maternal circulation in an unbound, nonionized form. Therefore the concentration driving diffusion of drugs from the maternal circulation into breast milk is that of the free fraction of drug in the circulation.

For example, propranolol is highly protein bound, so a relatively smaller amount is available as free drug.[31] In addition, the plasma protein binding of some drugs changes during pregnancy; a decrease in the plasma protein binding of sulfisoxazole, phenytoin, diazepam, and dexamethasone occurs during gestation, with a return to prepregnancy values at 1 to 2 months after delivery of the infant.[32] Drug passage across lipid membranes by passive diffusion is a function of molecular size and lipid solubility. In order for a drug to diffuse from the maternal circulation to the acinar cells, it must be in the nonionized form.

Because most drugs are weakly acidic or basic, drug partitioning between blood and breast milk is at least partially dependent on pH.[4,33-36] The ratio of the amount of drug present in milk to the amount present in plasma, the M/P ratio, can be estimated by the following equations[4]:

For drugs that are weak acids:

$$\text{M/P ratio} = 1 + 10^{(\text{pH}_m - \text{p}K_a)} / 1 + (\text{pH}_p - \text{p}K_a)$$

For drugs that are weak bases:

$$\text{M/P ratio} = 1 + 10^{(\text{p}K_a - \text{pH}_m)} / 1 + 10\,(\text{p}K_a - \text{pH}_p)$$

where pH_m = the pH of breast milk, pH_p = the pH of plasma, $\text{p}K_a$ = dissociation constant, and $\text{p}K_a$ = the $\text{p}K_a$ of the drug of interest.[4] For acidic drugs, the lower pH of breast milk (versus plasma) leads to presence of a larger portion of the drug in the ionized form in plasma, with a lower M/P ratio; this is known as *ion trapping*. For weak bases, the opposite is the case.[4]

The question of whether breast tissue can metabolize drugs present in breast milk is unknown for most compounds.[27] Conjugation has been demonstrated for some animal species, but human, bovine, and rodent breast tissue does not appear to possess appreciable concentrations of phase I enzymes such as cytochrome P-450.[27] Both ribonuclease and reverse transcriptase have been identified in human milk; the significance of these enzymes is uncertain.[37,38] It is increasingly clear from studies of other organs that drug metabolism is both more widespread and more important that has previously been appreciated, and it is likely that this will also apply to some drugs and the human breast. Of interest, Ito and co-workers have described the differential induction of drug metabolizing enzymes in infants fed with formula as opposed to breast milk.[31] Whereas formula-fed infants demonstrate a marked increase in caffeine clearance, presumably as a result of induction of Cyp1A1, breast-fed infants do not; this appears to occur by way of pathways dependent of the aryl hydrocarbon receptor, despite the fact that the concentrations of ligands for this receptor are higher in breast milk than in formula.[31] The long- and short-term clinical relevance of this observation is currently being studied.

A pharmacokinetic model for drug transfer into breast milk has been described for drugs that have low lipid solubility. This model assigns a deep compartment for breast milk, with transfer occurring from a central compartment (maternal circulation) to the deep compartment.[28] For most drugs, a two-compartment model provides a valid pharmacokinetic model for the study of the transfer of drugs into breast milk.[39,40]

In addition to the well-known mechanisms, as described, for transfer of drugs from the maternal circulation into breast milk, emerging evidence suggests that for certain drugs, active transport systems may function to transfer these substances into or out of breast milk.[4] It appears that such transporters can be a major determinant of drug transfer into breast milk. To illustrate, the concentration of ranitidine in breast milk in vivo is markedly higher than would be predicted on the basis of simple diffusion and ion trapping, suggesting the existence of an active transport system for organic cations in human mammary glands.[34] Many drug transporters expressed in animal models—such as the organic anion transporters 1 to 4—have not been found in human

tissue, but a number of other drug transporters appear to be expressed in the human mammary gland.[34] The putative biologic relevance of the presence of drug transporters in breast milk may relate to a role for these systems in nutritional transport, wherein drug transport is a biologically unintended consequence of the presence of such transport systems. For example, it appears that the ATP-binding cassette transporter BRCP (*b*reast *c*ancer *r*esistance *p*rotein), which is highly expressed in human breast tissue, may be involved in folic acid transport.[34] The activity, specificity, and clinical relevance of drug transporter expression in human mammary gland constitute an area of ongoing research.

Transfer of Drug from Breast Milk to the Infant

For most pharmacologic agents in routine clinical use, as a consequence of the effect of differences in molecular size and polarity on the processes just described, a predictable portion of drug in serum will pass into breast milk. It must be appreciated, however, that drug absorption from breast milk is not analogous to the enteral administration of drug to an infant for therapeutic purposes. Rather, with a drug that has passed into breast milk, it typically is present in dilute form in a relatively large volume of liquid, which includes other elements that can interact with the drug and alter the amount of drug available for absorption.

Protein and lipid components of breast milk can interact with drugs present in the aqueous phase; hence drugs with extensive protein binding will not be as available for absorption.[31] This decreased availability can be corrected for by calculating M/P ratios for these drugs on the basis of the amount of drug in skim milk.[4,40-43]

Drugs in breast milk also can interact with the lipid content of milk. The partition of highly lipid-soluble drugs in breast milk can be corrected by calculating the M/P ratio and using a correction factor for the octanol-water partition coefficient of the drug of interest.[4,42] Drugs with high lipid solubility, such as phenobarbital, can be trapped in the fat globules in breast milk, with a relative elevation in the amount present in milk versus plasma.[27]

Estimation of the Drug Dose Delivered in Breast Milk

A widely used method to estimate the amount of drug that lactating infants receive through breast milk is to calculate the drug dose in milk using the M/P ratio.[4,28,36,43] To use this method, it is necessary to know the M/P ratio and maternal steady-state plasma or serum drug concentration (C_{ss}) of the drug of interest and the volume of milk produced per day. The dose available to the infant can then be calculated using the equation

$$\text{Dose (mg/kg/day)} = C_{ss} \times \text{M/P ratio} \times \text{milk volume (mL/kg/day)}$$

To use this equation, three pieces of data must be known. The first is the C_{ss}, which can be determined by therapeutic drug monitoring of maternal plasma or serum. The second item is the M/P ratio, which is listed in various reference sources.[4] The final piece of data is an estimate of the milk volume; for term infants, the average milk volume is approximately 150 mL/kg/day (in this case, the weight is the weight of the nursing infant[22]).

Use of this equation to calculate the amount of drug received by a nursing infant is illustrated in the following example: A breast-feeding woman with epilepsy takes 300 mg of phenytoin once daily. Therapeutic drug monitoring reveals a steady-state serum concentration of 15 mcg/mL. Two M/P ratios for this drug have been reported in the literature, 0.45 and 0.13, with both values derived from small groups of infants. Using these values, the amount of drug delivered in breast milk can be calculated:

$$\text{Dose} = 15 \text{ mcg/mL} \times 0.45 \times 150 \text{ mL/kg/day} = 1.01 \text{mg/kg/day}$$

$$\text{Dose} = 15 \text{ mcg/mL} \times 0.13 \times 150 \text{ mL/kg/day} = 0.29 \text{mg/kg/day}$$

Given that the usual therapeutic maintenance dose of phenytoin for children is 5 mg/kg/day, these equations suggest that the dose of phenytoin anticipated in breast milk would be in the range of 6% to 20% of the usual therapeutic dose. An important consideration with regard to the implications of these estimates is that a three-fold difference between the M/P ratios was found in the two studies.

This equation can be useful to estimate the dose delivered to a nursing infant, but several caveats are in order with use of any equation involving the M/P ratio for determining the safety of breast-feeding.[40] First, for the equation to be accurate, the maternal serum concentration must be determined at steady state. It is possible that drugs may accumulate in breast milk over time; thus estimates made before the mother's serum concentration reaches steady state may underestimate the amount of drug present in breast milk.[4] In situations in which the C_{ss} is not available, it may be possible to estimate C_{ss} if the dose rate, bioavailability, and clearance are known.[4] Second is the accuracy of this equation is the M/P ratio itself. Although many studies have reported M/P ratios for various drugs, some of these studies have derived the M/P ratio from a single, simultaneously obtained determination of plasma (or serum) and milk from small numbers of infants.[4] Therefore, if with any temporal variation in the M/P ratio (as is suggested in the case of phenytoin by the wide range of values obtained), the estimates produced by this equation may not be accurate. The effect of changes in breast milk composition during feeding and over time (with progression from colostrum to mature milk) on the M/P ratio has not been evaluated for most drugs.[4,40] This changing composition may be particularly germane if transporter systems are involved in drug transfer into breast milk, as the expression and variability of these systems between individual patients and over time is just now being appreciated.[44] In addition, transporters can have efflux or influx capacity, so the summative effect may be difficult to predict. A better method for calculating the M/P ratio is to use multiple time points to calculate an area under the curve (AUC) for drug concentrations in milk and plasma and then use the AUC values to calculate the M/P ratio. Additionally, it has been suggested that combining use of the M/P ratio and estimates of drug clearance may be a more relevant way to evaluate the potential exposure of developing infants to drugs in breast milk.[43] Moreover, the effects of drug metabolites, especially for drugs that undergo biotransformation to active metabolites, are not considered as part of the usual M/P ratio. Given that the same considerations with respect to breast milk transfer apply to drug metabolites, the use of the M/P ratio alone may give a misleading impression of potential biologic effect(s) for compounds extensively transformed to active metabolites.

For many compounds, no M/P ratios have been described. It is possible to estimate an M/P ratio based on the physicochemical nature of the drug, using data such as the acidic strength (pK_a) of the drug, the amount of protein binding, and the octanol-water partition coefficient of the drug.[4]

An alternate approach taking advantage of the availability of sensitive analytical technology provides a method for estimation of drug dosage in breast milk by directly measuring the drug of interest in breast milk. The drug concentration can then be used to calculate the drug dose presented to the nursing infant. Ideally, multiple determinations should be made to discover any temporal variation in drug concentration during and between feeds. In addition, therapeutic drug monitoring in the infant can be used to follow the plasma or serum drug concentrations.

Finally, the value of any estimation of drug concentration in breast milk lies in the determination of potential effects on the infant. A critical and often underevaluated part of this estimation is assessment of the ability of the infant to clear the drug in question and the probable pharmacologic effects on the infant. As an

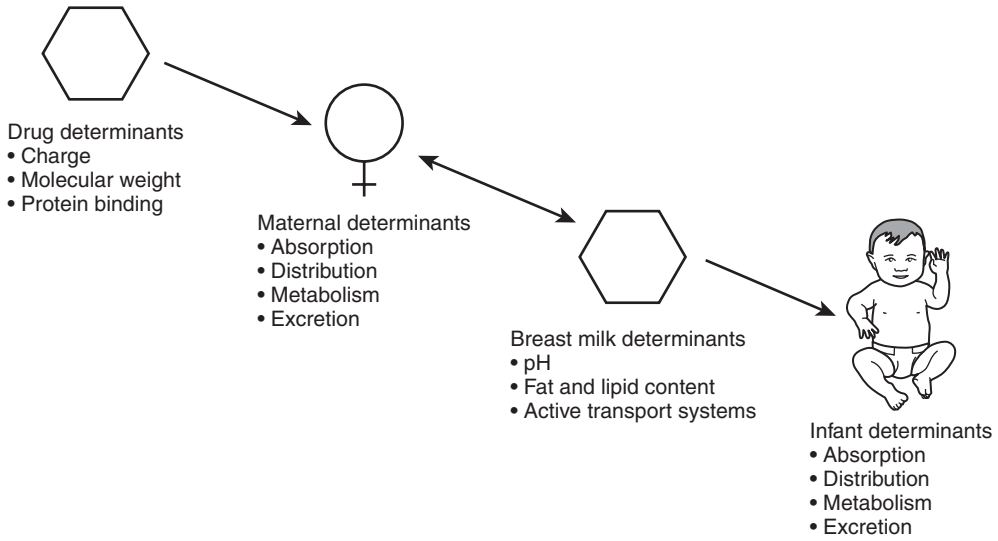


Figure 27-2 Factors affecting the potential impact of drugs in breast milk on infants.

example, ranitidine—which has an M/P ratio of 5—commonly is used in infants and has a wide margin of safety, thus being compatible with breast-feeding, whereas phenobarbital—which has an M/P ratio of 0.5—is inefficiently cleared by infants owing to differences in maturation of drug metabolism mechanisms and thus can accumulate, with the potential for adverse effects in breast-fed infants.

Clinical Implications of Drug Excretion During Lactation

The most common clinical indication for estimating drug dose in breast milk is to assess whether the drug will exert an adverse effect on a nursing infant (Figure 27-2). Fortunately the dose in breast milk (in most cases) is much lower than the usual therapeutic dose, and biologic effects (untoward and otherwise) would not be anticipated. In addition, the final determinant of the effect of any drug in breast milk on the infant is the ability of the infant to clear the drug.

As a rule, caution is warranted in prescribing drugs for lactating mothers. In instances in which therapy is clearly indicated, however, the demonstrated benefits of maternal therapy almost always outweigh theoretical risks of adverse drug effects in the nursing infant.A useful set of guidelines for a general approach to drug excretion during lactation, based on studies of drug excretion into breast milk, have been proposed by Berlin.[27] In general, it is reasonable to assume the following:

- A majority of all drugs (and environmental chemicals of molecular weight less than 200) cross from maternal plasma to breast milk.
- The time-concentration profile of drugs in breast milk parallels the time-concentration profile of drugs in plasma, with M/P ratios usually ranging from 0.5 to 1.0.
- The concentration of drugs in breast milk is almost always too low and too unpredictable to permit treating infants by giving medication to their mothers.
- The half-lives of drugs in plasma and in breast milk are similar after a single maternal dose, implying rapid transfer of the drug from plasma to milk.
- The amount of drug available in breast milk for absorption by the infant usually is less than 1% of the maternal dose.This amount can be minimized by breast-feeding during times when maternal plasma drug concentrations are low, such as just before the next dose.
- Few studies have described drug transfer in the setting of long-term maternal therapy over days or weeks; current understanding of how changes in drug transfer may occur over time is limited.
- In the case of most drugs, even those with potent pharmacologic effects, the risk for adverse drug effects in nursing infants usually is negligible, especially if attention is given to planning the nursing schedule around the time of minimal drug exposure.
- Potent drugs should be administered to breast-feeding mothers only after careful consideration, and parent counseling as to monitoring for potential adverse effects should be part of the prescribing process.This decision also should include consideration of clearance of the drug of interest by infants.

The penultimate point has been reinforced by a prospective study of breast-feeding mothers conducted by the Motherisk Program, a teratogen information service in Toronto. Between 1985 and 1991, 838 mother-infant pairs were assessed for exposure to a number of drugs taken by the mothers; no major adverse events were described among the infants.[4] Minor adverse effects were described in 11% of the infants, none of whom required medical attention.[4]

The last point in the list is highlighted by a recent report with respect to concerns when potent drugs are administered to nursing mothers.[45] Koren and associates[45] described the death of a nursing infant whose mother was receiving an analgesic containing acetaminophen and codeine. The infant died on day 13 of life of opiate overdose; detailed pharmacogenetic and drug analysis revealed that the mother's milk contained high concentrations of morphine, the active metabolite of codeine. In addition, the mother's genotype indicated that she was an ultrarapid metabolizer for CYP2D6 substrates, of which codeine is one.[45] Thus the mother metabolized codeine to morphine in much larger concentrations than would have been anticipated. Newborn infants are known to have a reduced capacity to metabolize morphine, which in this case resulted in a fatal outcome. In contrast with most case reports, in which isolated adverse effects that may be related to drug exposure through breast milk do not establish either causality or reproducibility, this report clearly correlates a fatal outcome associated with drug exposure from breast milk with a common pharmacogenetic variant.[45-48] Case reports must be interpreted with caution, however, in assessing possible risks associated with therapy in lactating mothers.[46] In this context, the ultrarapid metabolizer phenotype of CYP2D6 is seen in 1%

BOX 27-1

Drugs Contraindicated for Nursing Mothers

- Antimetabolites
  - Azathioprine
  - Cyclophosphamide
  - Doxorubicin
  - Methotrexate
- Antiocoagulants
  - Phenindione
- Ergot alkaloids
  - Bromocriptine
  - Ergotamine
- Gold
- Iodine-containing compounds
  - Amiodarone
  - Potassium iodide
  - Povidone-iodine (extensive topical use)
- Oral contraceptives (relative contraindication)*
- Psychoactive drugs
  - Lithium (relative contraindication)*
  - Phenobarbital
- Radiopharmaceuticals*†
- Recreational chemicals
  - Amphetamines
  - Cocaine
  - Ethanol
  - Heroin
  - Marijuana
  - Phencyclidine (PCP)
  - Nicotine (tobacco smoking)

*Relative contraindication; see text.
†See Table 27-1 for specific agents.

or less of Northern Europeans but can be seen in up to 10% of persons of Mediteranean extraction and in as many as 30% of people in populations from the Horn of Africa.[45] Thus the decision to prescribe codeine for the breast-feeding mother should be made only after careful consideration,and part of the prescribing process should include counseling about how to monitor for adverse effects in the newborn infant such as sedation.

With long-term therapy in a breast-feeding woman, her infant may be exposed to the potentially harmful drug for a period of weeks or months. In many instances, the therapy may have been started before delivery, and in such situations it is likely that the infant will be exposed to smaller concentrations through breast milk than the fetus was exposed to in utero. In the case of certain compounds, such as barbiturates, accumulation is possible, and the infant should be monitored for signs of toxicity. For most agents used in long-term therapy, however, thoughtful care and using therapy as indicated to ensure optimal maternal health are of greater importance in maintaining the health of the infant than is a nihilistic policy of drug avoidance regardless of potential effects on maternal health.

DRUGS CONTRAINDICATED FOR NURSING MOTHERS

A number of drugs are contraindicated during lactation, or preclude breast-feeding, as listed in Box 27-1.

Antimetabolites

Therapy with antimetabolites, especially for the therapy of neoplastic disease, is contraindicated during lactation. Although the absolute dose present in breast milk is small, the pharmacologic potency of these agents and the potential for serious long-term toxicity usually is believed to preclude lactation when therapy with these agents is required. The commonly used agents for immunosuppressive therapy include azathioprine, methotrexate, cyclosporine, and tacrolimus.These drugs (and the methotrexate metabolite 6-mercaptopurine) can be detected in breast milk in low concentrations.[49-51] It has been suggested that breast-feeding may be safe for mothers on low-dose therapy with methotrexate; however, this remains controversial.[52] Cyclosporine is transferred into breask milk in low concentrations. Although the older literature discourages breast-feeding during cyclosposorine therapy, in fact no adverse events have been reported among breast-fed infants of cyclosporine-treated mothers. The breast milk concentrations of cyclosporine typically are in the range of 1% to 2% of the maternal dose. On the basis of two reports of detectable cyclosporine concentrations in the blood of breast-fed infants of mothers treated with the drug, however, it would be prudent in such clinical situations to monitor nursing infants by means of serum or plasma drug level measurements. Almost no data are available on the safety of mycophenolic acid and sirolimus with respect to breast-feeding. When the parents have decided to continue with breast-feeding after being counseled about potential effects of these agents on the nursing infant, careful monitoring of the infants with respect to immunologic and hematologic status, with determinations of milk and plasma concentrations of the drug of interest, is recommended.[4]

Anticoagulants

The various warfarin (Coumadin) derivatives appear to be excreted into breast milk in differing amounts. Warfarin has not been detected in breast milk, and the infants of women receiving warfarin therapy have not had problems with coagulation[53,54] Similarly, no bleeding was found among the infants of a large cohort of women treated with dicumarol (bishydroxycoumarin).[55] This evidence suggests that therapy with warfarin and dicumarol is compatible with breast-feeding.[47,56] By contrast, the anticoagulant ethyl biscoumacetate has been detected in breast milk, and in one study, bleeding complications developed in 5 of 42 nursing infants whose mothers were being treated with this agent.[57,58] Phenindione (not available in the United States) has been detected in the milk of lactating mothers and has been associated with significant bleeding complications in the nursing infant.[56,59,60] Heparin is too large a molecule to be excreted into breast milk, and it appears that maternal use of the low-molecular-weight heparins—which again are too large for the transfer of significant concentrations into breast milk—is compatible with breast-feeding.

Ergot Alkaloids

The ergot alkaloid bromocriptine suppresses prolactin and among other indications is used clinically to prevent lactation.[61] Thus it is not surprising that bromocriptine is contraindicated when breast-feeding is planned. Ergotamine is excreted into the breast milk of mothers receiving this agent in doses used for the treatment of migraines, and ergotism, with clinical manifestations including vomiting, diarrhea, and seizures, has been described among breast-fed infants of mothers treated with this drug; moreover, ergotamine can suppress.[56,62] Only small concentrations of sumatriptan pass into human milk, and adverse effects would not be anticipated among breast-fed infants of sumatriptan-treated mothers.[63] No adverse effects have been associated with the short-term use of methylergonovine to promote uterine involution.[4]

Gold

Gold can be detected in the breast milk of women treated with that agent.[64-68] It has been estimated that nursing infants can be exposed to as much as 20% of the maternal dose through

breast milk, the clinical significance of which remains unclear.[67] In various reports, the presence of gold in breast milk was associated with no apparent adverse effects on the nursing infant; conversely, exposure to gold in breast milk has been described as potentially related to a constellation of adverse events; however, a causal link has not been established.[64,66] Some investigators think that gold salts are contraindicated during breast-feeding, although the Committee on Drugs of the American Academy of Pediatrics considers use of these agents compatible with breast-feeding.[56,67] When women breast-feeding their infants are being treated with gold, the infants should be closely observed during follow-up for adverse effects, and gold concentrations should be determined in breast milk and the infant's serum.

Iodine-Containing Compounds

Iodine is excreted into breast milk, which can lead to iodine-induced goiter and hypothyroidism in breast-fed infants.[69] This risk has been identified forboth oral preparations such as potassium iodide and topical povidone-iodine preparations.[56,70,71] Therefore compounds containing iodine should be avoided during lactation. Amiodarone is an antiarrhythmic agent that contains 75 mg of iodine in each 200-mg dose.[72] The drug is excreted into breast milk, and although no reports of adverse effects have been published in the literature, the high iodine content of this compound and the long half-life in adults suggest that amiodarone should be used onlywith great caution, if at all, during breast-feeding.[72,73]

Oral Contraceptives

The use of oral contraceptives is a relative contraindication in certain groups of nursing mothers. Use of such agents during the early phases of breast-feeding is associated with shortened duration of lactation, decreased milk production, and decreased nitrogen and protein content in milk (all of which can decrease infant weight gain).[74-78] These effects are less marked with agents containing only progesterone and have not been observed with newer combined oral contraceptives.[78] The effect of oral contraceptives on weight gain may be especially important for the infants of malnourished mothers. Therefore it has been recommended that oral contraceptives be avoided in nursing mothers who are malnourished.[4,79] It also has been suggested that oral contraceptives be avoided for the first 6 weeks after delivery.[4,76]

Both estrogen and progesterone can enter breast milk, although with currently available low-dose combination products, the estrogen content of breast milk from whose mothers are being treated with these products does not appear to be higher than that of breast milk from women who are not receiving any hormonal agents.[48] A single case report described the development of mild breast hypertrophy in a child exposed to a large dose of estrogen.[48] Use of oral contraceptives is acceptable in women who have successfully established breast-feeding.[56,76]

Psychoactive Drugs

Lithium can be detected in breast milk in variable concentrations which on occasion can result in milk concentrations similar to those of serum.[80-82] Concerns with respect to possible long-term adverse effects of exposure to lithium have led the Committee on Drugs of the American Academy of Pediatrics to consider lithium contraindicated during lactation.[56] An alternate approach is to measure lithium concentrations in breast milk and the serum of the nursing infant, to determine on an individual basis if lithium therapy can be continued during lactation; recent data have suggested that under these conditions therapy is likely to be be (in most cases) safe.[82]

TABLE 27-1

Duration of Excretion of Radioactivity into Milk After Maternal Administration of Radiopharmaceuticals

Drug	Duration of Radioactivity in Milk
Copper-64	50 hours
Gallium-67	2 weeks
Indium-111	20 hours
Iodine-123	36 hours
Iodine-125	12 days
Iodine-131	2-14 days*
Radioactive sodium	96 hours
Technetium-99	1-3 days

Modified from Committee on Drugs, American Academy of Pediatrics: The transfer of drugs and other chemicals into human milk. *Pediatrics* 93:137, 1994. Used with permission of the American Academy of Pediatrics.

*Varies depending on type of study.

Phenobarbital is excreted into breast milk, with M/P ratios between 0.4 and 0.6.[80,83-85] Elimination of phenobarbital is slower in infants than in adults, and serum phenobarbital concentrations in the infant can actually exceed those in the mother, producing sedation in the infant. Therefore, in treating lactating women with phenobarbital, infants should be followed closely for signs of sedation. Additionally, breast milk and serum concentrations of phenobarbital in the infant should be determined at periodic intervals.

Radiopharmaceuticals

Many commonly used radiopharmaceuticals are excreted into breast milk.[56] Therefore it is generally recommended that breast-feeding be avoided over the time during which the radioisotope in question is being excreted into breast milk.[56] The time interval for clearance of the radioisotope varies with the compound used for the investigation in question (Table 27-1). When this time to clearance is in doubt, an expert in nuclear medicine should be consulted. It may be possible to plan for this eventuality in advance by banking frozen milk that can be used to feed the infant while the breast milk containing the radioisotope is pumped and discarded until radioactivity has cleared from the milk.[4]

Recreational Chemicals

Recreational chemicals are a group of compounds that includes drugs used socially and drugs used under circumstances of abuse. As a general rule, all of the active compounds present in these agents can be excreted into breast milk. Thus, in view of the potent pharmacology of many of these drugs, the use of these agents is contraindicated during pregnancy and lactation.[4]

Amphetamines can be concentrated in breast milk, with M/P ratios that can range from 2.8 to 7.5.[86] The Committee on Drugs of the American Academy of Drugs considers amphetamines to be contraindicated during breast-feeding.[56] The same consideration applies with methamphetamine. Cocaine enters breast milk readily because of its low molecular weight and high fat solubility. Cocaine and benzoylecgonine, the principal metabolite of cocaine, have been detected in the urine of nursing infants, and some of these infants have had clinical signs compatible with cocaine exposure.[87,88] Signs and symptoms also have been reported in an infant whose mother used cocaine as a topical analgesic on her nipples.[29] Cocaine should be avoided at all times by lactating mothers.[56]

Ethanol is a small, lipid-soluble molecule that freely passes into breast milk at concentrations equivalent to plasma

concentrations.[30,89] By contrast, acetaldehyde, a toxic metabolite of ethanol, apparently does not enter breast milk in appreciable concentrations.[30] The elimination of ethanol from breast milk appears to be similar to that of ethanol in plasma, which follows zero-order kinetics.[30,89] The consumption of small amounts of ethanol on an infrequent basis does not appear to be associated with untoward effects in nursing infants, because that the amount of ethanol in breast milk is likely to be small.[4] It has been appreciated for some time, however, that ingestion of large amounts of ethanol in a single sitting can result in high concentrations of ethanol in breast milk, with significant signs and symptoms, including sedation, in the nursing infant.[90] It is controversial as to whether chronic ethanol use during lactation is associated with neurodevelopmental effects on the infant, although common sense suggests avoiding regular ethanol ingestion during breast-feeding.[91,92] A prudent recommendation is for lactating mothers to avoid chronic ethanol use while nursing their infants. The Committee on Drugs of the American Academy of Pediatrics considers moderate ethanol use to be compatible with breast-feeding, although caution is recommended.[56] In addition to possible effects on the infant, ethanol in high doses blocks release of oxytocin.[93]

Heroin and other opiates can be detected in breast milk. In the case of maternal heroin use, sufficient heroin is excreted into breast milk to produce dependence among nursing infants.[94] Use of heroin and other opiates, especially under circumstances of abuse, is contraindicated during lactation. As noted prevously, drug effects may be fatal in vulnerable infants.[45]

The active ingredient of marijuana is δ-9-tetrahydrocannabinol (THC); THC has been found in the breast milk of women who have smoked marijuana.[95] It appears that infants absorb THC from breast milk, which is then further metabolized.[95] No evidence has emerged to show adverse clinical consequences after exposure to THC through breast milk.[56] Long-term follow-up studies beyond 1 year of age, however, have not been performed, and many confounding variables that affect the developmental outcome of drug-exposed children have been recognized. The Committee on Drugs of the American Academy of Pediatrics regards marijuana use as being contraindicated in breast-feeding mothers.[56]

Phencyclidine can be detected in the breast milk of women who have used the drug. Furthermore, it can be detected for a considerable time after the last dose.[96,97] With respect to other hallucinogens, little information is available regarding transfer into breast milk. Of note, these drugs are small-molecular-weight compounds with considerable potency, so untoward effect(s) on nursing infants are highly probable. Thus phencyclidine and other hallucinogens, including lysergic acid diethylamide (LSD), are contraindicated during breast-feeding.

Nicotine and cotinine, the major metabolite of nicotine, have been found in the breast milk of mothers who smoke cigarettes.[98-100] Cotinine has been found in the urine of nursing infants whose mothers smoke, suggesting absorption of nicotine metabolites from breast milk or exposure to second-hand smoke.[4,101-103] Adverse effects, including increased rates of respiratory illness and possibly an increased rate of colic, have been reported in the infants of mothers who smoke.[103,104] In addition, cigarette smoking may inhibit production of breast milk.[105] The transfer of nicotine and cotinine falls sharply when a nicotine patch is used by breast-feeding mothers, suggesting that smoking cessation therapies that involve the use of the nicotine patch are safe during breast-feeding and are preferable to continued smoking.[98]

In summary, recreational chemicals pass readily into breast milk and may alter the production, composition, and volume of breast milk, with the potential for adverse effects in the nursing infant. Accordingly, breast-feeding mothers should be strongly advised to restrict their intake of recreational chemicals.[90]

DRUG CLASSES COMMONLY USED BY LACTATING WOMEN

Analgesics

Acetaminophen, aspirin, and nonsteroidal antiinflammatory drugs such as ibuprofen and ketorolac are secreted into breast milk in small concentrations. The use of these agents in usual therapeutic doses has not been associated with adverse effects on nursing infants. Salicylism has been reported in the nursing infant of a mother treated with aspirin; the pharmacokinetic profile, however, suggested that exposure occurred by routes other than breast milk.[106] Opiates transfer into breast milk; in the case of morphine, transfer to breast milk is limited. Although codeine in doses used for the ambulatory patient has been considered to be compatible with breast-feeding, the fatal case just described suggests caution, and monitoring should be considered when codeine is prescribed for breast-feeding mothers, especially if therapy will necessitate more than one or two doses.[45]

Antiasthma Agents

Asthma is among the most common chronic diseases requiring therapy in lactating women. β_2-Adrenergic selective agents are the drugs of choice for the acute therapy of asthma and typically are given by aerosol. Absorption of albuterol (salbutamol) is minimal when this drug is given by inhalation; as well, abundant data are available on the safety of direct administration of this drug in infants as young as a few months of age.[107] Thus significant excretion of these agents into breast milk is unlikely, and effects on nursing infants are highly improbable. The same considerations apply for inhaled adrenocorticosteroids, notably when the total dose is less than 1000 mcg/day. Prednisone is excreted into breast milk in small concentrations.[108] Because these concentrations are unlikely to be associated with adverse effects in the nursing infant, use of both prednisone and prednisolone is compatible with breast-feeding, especially in view of the considerable experience with the use of corticosteroids in infants and children.[56] Theophylline is excreted into breast milk in small concentrations, with an estimated infant dose that is less than 1% of maternal dose.[109] A report of irritability in the nursing infant of a mother taking a rapidly absorbed theophylline preparation suggests that nursing mothers who require theophylline therapy should be treated with slow-release preparations. Nonetheless, theophylline is considered to be compatible with breast-feeding.[56] Limited data are available with respect to the transfer of newer agents such as montelukast into breast milk, but a reasonable assumption is that a small amount is likely to enter breast milk; on the basis of the experience with these agents in children, it also is likely that they are compatible with breast-feeding.[110]

Antimicrobial and Antiinfective Agents

Essentially all of the antimicrobial and antiinfective drugs transfer into breast milk. The β-lactam antibiotics are compatible with breast-feeding. Tetracycline passes into breast milk in low concentrations, where it can associate with calcium in milk. Absorption of tetracycline from milk appears to be negligible, because tetracycline has not been detected in the blood of nursing infants whose mothers were treated with this agent.[111,112] Although use of tetracycline in younger children normally is contraindicated because of effects on developing teeth and bones, the lack of evidence of absorption has led the Committee on Drugs of the American Academy of Pediatrics to consider tetracycline therapy to be compatible with breast-feeding.[56] Erythromycin is present in breast milk during maternal therapy, but such therapyalso is compatible with breast-feeding. Clarithromycin is compatible with breast-feeding. Azithromycin is excreted into breast milk and may accumulate because of the lipid solubility of the drug and ion trapping; in

view of the pharmacokinetics of this drug, the possibility of accumulation is unlikely to be a contraindication to breast-feeding.[113]

Caution must be exercised in the use of nalidixic acid, nitrofurantoin, quinine, and sulfonamides for therapy in lactating women who belong to populations at risk for glucose-6-phosphate dehydrogenase deficiency, because drug transfer through breast milk may be associated with a risk for hemolysis in the infant.[4] The recommendation that sulfonamides be avoided during the first postnatal week to avoid displacement of bilirubin from albumin is a theoretical one.

The quinolones—norfloxacin, ciprofloxacin, ofloxacin, grepafloxacin, levofloxacin, and gemifloxacin—appear to be excreted into breast milk in low concentrations.[114] Less data exist with respect to breast milk transfer for the newer agents in this class than on the first- and second-generation drugs (ciprofloxacin, norfloxaxin, and ofloxacin). Animal studies demonstrating an arthropathy in a beagle pup model have been cited as a rationale for avoiding use of quinolones in pregnancy and lactation.[4] The expanding experience with these drugs in pediatrics, however, has not demonstrated adverse effects in humans and suggests that, when clinically indicated, quinolones are compatible with breast-feeding.[115,116] Chloramphenicol is excreted into breast milk. Caution has been recommended with use of chloramphenicol in lactating women because of the possibility of an idiosyncratic reaction or bone marrow toxicity (or both).[4] Therefore an appreciation of the severity of potential adverse reactions is essential in prescribing chloramphenicol for this group of patients. Metronidazole is excreted into breast milk.[117] Although concern has been expressed with respect to the potential mutagenic effects of metronidazole, such effects have not been demonstrated in humans.[4] In conventional dosages, metronidazole is compatible with breast-feeding. If metronidazole is given in a single large dose (such as the single 2-g dose recommended for therapy of trichomoniasis), however, the breast should be pumped and milk discarded for 12 to 24 hours after the dose, to allow for elimination of metronidazole.[56]

Isoniazid and the isoniazid metabolite acetylisoniazid are excreted into breast milk in large concentrations.[118] Although no reports of toxicity among nursing infants of mothers treated with isoniazid have appeared in the literature, it is recommended that when lactating mothers are being treated with isoniazid, nursing infants should be monitored for signs of peripheral neuritis or hepatitis.[119]

Acyclovir has been detected in breast milk in much higher concentrations than would be anticipated from the physicochemical nature of the drug.[120] This finding suggests that acyclovir may have a unique active or facilitated transport process for entry into breast milk.[120] Based on the poor oral absorption of this agent, it has been estimated that the amount absorbed by nursing infants of mothers treated with acyclovir should be minimal.[120] Therefore the Committee on Drugs of the American Academy of Pediatrics considers acyclovir therapy to be compatible with breast-feeding.[56]

Recent work has demonstrated that zidovudine transfers into breast milk in concentrations roughly equivalent to those seen in serum.[120] Postnatal transmission of humanimmunodeficiency virus (HIV) has emerged as a major public health problem worldwide; a number of regimens to reduce mother-to-infant transmission have been developed, but the optimal regimen to prevent transmission in breast milk remains controversial.[121] Many of the antiretroviral drugs, such as nevirapine, lamivudine, and zidovudine, enter breast milk in reasonably high concentrations; whether such levels are sufficient to treat or provide prophylaxis for HIV infection remains controversial.

Antihypertensives and Diuretics

β-Adrenergic blocking agents are excreted into breast milk; the extent of drug transfer is determined by the degree of protein binding. Agents such as atenolol and sotalol, which are relatively less protein bound, pass into breast milk in greater quantities than an agent such as propanolol, which is more highly protein bound.[122-124] Maternal use of propranolol, nadolol, and metoprolol is compatible with breast-feeding. Acebutolol accumulates in breast milk and should be avoided in breast-feeding mothers. Atenolol accumulates in breast milk in concentrations exceeding maternal plasma concentrations, probably owing to ion trapping; breast-fed infants of atenolol-treated mothers have had very low plasma atenolol concentrations. Data on the transfer of esmolol and pindolol into breast milk are limited. The degree of transfer of sotalol and effects on the nursing infant remain controversial; with maturation of renal function, effects in infants older than 2 months of age would not be anticipated.[122] Nursing infants of mothers being treated with a βadrenergic blocking agent should be monitored for signs of β-adrenergic blockade, notably bradycardia.

Captopril and other angiotensin-converting enzyme inhibitors are excreted into breast milk in low concentrations. The use of these agents and drugs such as hydralazine, methyldopa, and verapamil is considered to be compatible with breast-feeding.[56]

Diuretics such as chlorothiazide and hydrochlorothiazide are excreted into breast milk in low concentrations.[33] Although thiazide diuretics can suppress lactation, in the usual therapeutic doses these agents are compatible with lactation.[56] Furosemide also is excreted into breast milk and is compatible with lactation.[56]

Anticonvulsants and Psychoactive Drugs

Carbamazepine, phenytoin, and valproic acid are excreted into breast milk in small amounts; use of these agents is compatible with breast-feeding.[56] Ethosuximide is found in breast milk in concentrations roughly equivalent to those in maternal serum; however, the Committee on Drugs of the American Academy of Pediatrics considers ethosuximide therapy to be compatible with breast-feeding.[56] As described previously, ongoing phenobarbital therapy is contraindicated during lactation, primarily owing to delayed clearance by the infant. Primidone is metabolized to phenobarbital, and the same considerations with respect to phenobarbital therapy apply to primidone. Lamotrigine is transferred into breast milk in appreciable concentrations, and although this agent is not contraindicated during breast-feeding, infants should be closely monitored (a serious lamotrigine rash has been described in a breast-fed child). Topiramate, gabapentin, and valproic acid are present in low concentrations in milk and are considered compatible with breast-feeding. In the case of valproic acid, however, infants are known to be a high-risk group for valproic acid–associated hepatic failure, and it has been suggested that nursing infants of mothers treated with valproic acid should be closely followed.

As described previously, lithium therapy is believed by many authorities to be contraindicated during lactation but can be used if accompanied by a monitoring program. Most psychoactive drugs used, including the phenothiazines, benzodiazepines, and classic antidepressants, are excreted into breast milk. These agents should be used with caution in lactating women, notably if used in combination therapy.[56] Diazepman specifically has a long half-life in breast milk, and alternate agents are recommended if benzodiazepine therapy is needed. Olanzepine is transferred into breast milk in low concentrations, and maternal therapy with this agent is considered compatible with breast-feeding.

The selective serotonin reuptake inhibitors (SSRIs), a very widely used class of drugs, are excreted into breast milk in variable and usually small amounts.[125-129] In the case of fluoxetine, drug transfer into breast milk is appreciable, and although adverse events in terms of reduced weight have been noted in only a single study, it would nonetheless be prudent to consider use of

an alternate agent for breast-feeding mothers. Although isolated case reports of toxicity have appeared in the literature, series of mother-infant pairs have demonstrated minimal infant exposure to most of the SSRIs tested, notably escitalopram, paroxetine, and venlafaxine, with measured breast milk concentrations less than 10% of the maternal dose.[125-129]

Cardiovascular Drugs

Digoxin is excreted into breast milk in small concentrations and is compatible with lactation, [122] as are flecainide, mexiletine, and quinidine.[4] Procainamide and its active metabolite *N*-acetylprocainamide also are excreted into breast milk.[130] Although adverse effects associated with procainamide use by lactating mothers have not been reported, the effects of long-term exposure are unknown. Therefore infants of lactating mothers treated with procainamide should be followed closely. Disopyramide is present in milk in relatively high concentrations. Although no adverse effects have been reported among infants of lactating mothers treated with disopyramide, clinicians should observe these infants closely and consider monitoring drug concentrations in breast milk and the infant's blood.[4] Quinidine is excreted into breast milk and is compatible with breast-feeding.[56,131] A systematic review has suggested that the use of angiotensin-converting enzyme inhibitors and many of the calcium channel blockers is safe during breast-feeding.[132]

Endocrine and Gastrointestinal Drugs

Thyroid replacement therapy with agents such as levothyroxine is compatible with breast-feeding. The antithyroid agent propylthiouracil is excreted into breast milk in small concentrations; maternal therapy with total daily doses of 50 to 300 mg is considered compatible with breast-feeding, although follow-up evaluation of the infant's thyroid function on a regular basis is recommended.[133]

Tolbutamide and chlorpropamide are excreted into breast milk in low concentrations, which are unlikely to influence the infant's blood glucose concentration.[134] The molecular weight of insulin precludes entry of this agent into breast milk.

Adverse effects have not been described after maternal use of a number of laxatives, including mineral oil, phenolphthalein, and senna derivatives.[4] It has, however, been recommended that lactating mothers who require laxatives be treated with agents that are poorly absorbed, such as psyllium.[4]

Ranitidine and cimetidine are excreted into breast milk in small amounts, and use of these agents is considered to be compatible with breast-feeding.[56] Transfer of omeprazole, pantaprazole, and esomeprazole into breast milk is minimal; data on transfer of rabeprazole and lansoprazole into breast milk are very limited. Antacids that contain sodium and aluminum are considered to be compatible with breast-feeding.[135] Metoclopramide and domperidone both are compatible with breast-feeding and have been used to increase milk production.[4]

Disease-Modifying Agents and Biologic Therapies

Since 2000, a number of disease-modifying agents have become available for clinical practice, often using targeted molecules of biologic origin. Many of these agents are large-molecular-weight compounds that would not be anticipated to transfer into milk to any significant degree, infliximab being an example. Although the indications for these agents often have been among patients unlikely to breast-feed, the use of such agents is expanding into different populations, so study of their transfer into breast milk as well as their impact on breast milk production is needed.

Over-the-Counter Drugs

Numerous over-the-counter drugs used for symptomatic relief of common problems, notably self-limited upper respiratory tract infections and environmental allergies, transfer into breast milk. In usual therapeutic doses, these agents are compatible with breast-feeding; however, because these agents have the potential for causing sedation, care should be taken if they are used for a prolonged period.[22,47,48] A single case report described the development of irritability and a high-pitched cry in an infant receiving breast milk from a woman taking the antihistamine clemastine. Single or intermittent doses of clemastine, however, are compatible with breast-feeding.[48]

Herbal Medicines

Herbal and other traditional medications frequently are used by patients, and recent estimates are that up to one third of all patients take some type of complementary or alternative medicine preparation on a regular basis. Patients often assume that herbal medications are safe because they are "natural." Nevertheless, the active ingredients in herbal medications, like those in most other medications, are likely to enter breast milk. Two concerns have emerged: First, with herbal medication, the possibility of adulteration must be borne in mind if infants exhibit unusual signs or symptoms. Second, it is possible that the active elements of some herbal medications, such as St. John's wort, may be absorbed by breast-feeding infants. Although recent work has suggested that some commonly used herbal agents such as St. John's wort and gingko may be safe when used by breast-feeding women, further research is needed on the safety of herbal medications during breast-feeding.[136,137]

INFORMATION SOURCES ON DRUGS AND BREAST-FEEDING

Drug use is common, and drug utilization studies have demonstrated that a wide range of compounds and drug classes are used by patients. For example, a recent study of drug use in children—thought to be a low drug utilization group—has demonstrated that over 1 year, 4 prescriptions were dispensed per child from a list of 1400 distinct chemical entities.[138] Drug use among lactating women is even more diverse. Today's practitioners, therefore, need access to a valid and readily available information source. In addition to textbooks such as the classic *Drugs in Pregnancy and Lactation* by Briggs, Freeman, and Yaffe, the valuable website LacMed, sponsored by the NIH, provides concise and thoughtful reviews of the current literature on breast milk transfer of a number of commonly used medications (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?LACT). The practitioner who routinely cares for women of reproductive age should ensure that they have ready access to resources to enable them to address questions relative to the potential effects of drugs transferred in breast milk on breast-feeding infants.

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SECTION IV

Intrauterine and Postnatal Growth

28

Frederick C. Battaglia

Circulatory and Metabolic Changes Accompanying Fetal Growth Restriction

Fetal growth restriction (FGR) is an important clinical problem in obstetrics and gynecology. Because of its importance, it has stimulated considerable clinical research that not only has elucidated aspects of the pathophysiology of the growth restriction but also has led to a better understanding of normal human biology. This chapter summarizes some of the work that has been directed at describing how the fetus adapts to placental dysfunction.

A plethora of terms have been used in describing the problem of FGR. In part, this potentially confusing terminology has a historical foundation. The first attempts to recognize growth restriction in newborn infants came from studies using gestational age and birth weight information.[1-3] The infants were classified as being small for gestational age (SGA) if their birth weights fell below the 10th percentile. Later reports used terms such as small for dates (SFD), basing the definition on standard deviations for the birth weight gestational age distribution. Many studies brought out the differences in birth weight–gestational age distribution data among different populations of peoples. All of these attempts to define FGR, however, were based on birth weight information to establish the norms for populations. It was clear from the beginning that preterm birth weights could hardly be considered "normal" data of in utero growth for a population. It also was clear that growth-restricted infants were not a homogeneous group but instead included some babies who were small but normally grown infants. The term *intrauterine growth retardation* (IUGR) was used initially to identify SGA infants who had truly grown more slowly in utero because of one or another disease processes, usually those affecting placental development. Later, because of concerns that "retardation" is a term parents may associate with mental retardation, the term was modified to *fetal growth restriction.* This latter designation has come to be used more and more widely and interchangeably with IUGR.

The approach to defining FGR was changed fundamentally once ultrasound techniques permitted determination of fetal body size in utero, a measurement that could be done repeatedly during the pregnancy. This capability removed the necessity to use only birth weight data at the completion of a pregnancy. It also permitted examining the rate of fetal growth, that is, the change in fetal growth with time, in any particular pregnancy. The wide application of ultrasound-derived fetal growth curves made possible the early detection of FGR. Currently, the common practice is to use equations derived from local population data to calculate an estimated fetal weight. Such equations usually rely on measurements of head circumference (HC), abdominal circumference (AC) and femur length. It is not clear, however, that such a calculation of estimated fetal weight is significantly better than relying on the actual measurements made. Alternatively, FGR may be defined as growth impairment in which the infant's abdominal circumference is two standard deviations below the norm for gestational age.

The diagnosis of FGR can be further sharpened by the use of standards that incorporate maternal and paternal size. Essentially, this approach seeks to distinguish fetuses that are small because the parents are small from those with growth restriction resulting from one or another disease process. Focusing on this approach, Gardosi and colleagues have reviewed this subject extensively.[4] Nomograms for particular countries are available.[5,6] The most compelling evidence, however, that a fetus is truly growth-restricted comes from accumulating functional evidence for those physiologic characteristics that have been shown to be altered in FGR pregnancies.

Distinguishing babies who are small but are fulfilling their growth potential (i.e., those who are normally grown) from those with FGR (i.e., those who are growth-restricted as a result of some pathology) is important for a number of reasons: (1) Intensive obstetric surveillance can be applied more effectively to the fetus; (2) neonatal intensive care can better anticipate problems for affected babies; and (3) FGR has important long-term implications for development of the infant, not only through childhood but also into adult life. Thus it behooves today's clinicians to approach the diagnosis of FGR using all available information.

In recent years, studies have begun to sort out normally grown, SGA babies from those with growth restriction by consideration of functional aspects, as well as simply the body size of the fetus and of the parents. These aspects are evident in a consideration of some of the vascular and metabolic changes clearly associated with FGR, as discussed next.

PLACENTAL DEVELOPMENT

A great deal of work has been done to delineate the changes in placental development that are associated with FGR pregnancies. The histologic changes in the placenta, including the vascular changes, have been reviewed in multiple studies.[7-9] These changes are consistent with increased uneven perfusion and increased diffusion limitations in the small FGR placenta. Surface area is significantly reduced in FGR pregnancies, along with placental mass. These findings have led to the hypothesis that the characteristic may be triggered in part by placental hyperoxia in the maternal placental circulation.[8] This hypothesis has received support from both animal studies[10] and clinical studies[11] of respiratory gas tensions in the umbilical and uterine circulations in FGR pregnancies. For many years, obstetricians believed that uterine artery blood flow was reduced in FGR pregnancies. Although such reduction may or may not

occur in the earliest stages of pregnancy, it has now been well established by Pardi and associates[11] that uterine blood flow is increased in FGR pregnancies relative to the O_2 requirements of the pregnant uterus. This relative increase is an important physiologic characteristic of FGR pregnancies and, as stated earlier, is consistent with findings in an ovine model of FGR.[10] An important point in this context is that the human placenta simulates a venous equilibrator. This subject has been reviewed by Meschia, who has brought out the implications of this similarity for both the uterine and umbilical circulations[12]: Regardless of the complexity of the fetal circulation and of perfusion patterns within the intervillous space, the human placenta is functioning as an exchange system in which the uterine venous Po_2 is the major determinant of the umbilical venous Po_2 in the fetal circulation.

FETAL VELOCIMETRY CHANGES

The fetal circulation has been studied extensively in terms of the velocity waveform changes occurring in different fetal vessels.[13] These changes do not occur in all FGR pregnancies. In the classification proposed by Pardi and associates,[14] pregnancies without velocimetry changes represented approximately one third of all FGR pregnancies. The changes in fetal velocimetry are presumed to reflect two pathophysiologic processes: (1) an increased impedance within the placental vascular bed and (2) a redistribution in fetal cardiac output secondary to fetal hypoxia. The evidence for each of these changes is reviewed next.

In normal pregnancies, the umbilical artery pulsatility index (PI) starts at a high value in early gestation and decreases until term.[15] In FGR pregnancies, however, the PI is increased over normal values even relatively early in gestation. In the ovine FGR model described previously, the increased PI is associated with an increased blood pressure in the fetal circulation and with a reduced umbilical blood flow.[10] This observation provides clear evidence of an increased impedance in the fetal vascular bed. Both an increased PI and a decreased umbilical blood flow[16] have been reported in human FGR pregnancies. Thus it is not unreasonable to assume an increased placental vascular impedance in human FGR pregnancies.

Hypoxia and lacticacidemia occur in a relatively high percentage of FGR pregnancies. Both hypoxia and lacticacidemia were found in approximately two thirds of the FGR pregnancies in which both umbilical artery velocimetry values and fetal heart rate were abnormal.[14] Fetal hypoxia has been well studied in animal models. In the ovine fetus, the changes in the distribution of fetal cardiac output that occur with acute fetal hypoxia were well described many years ago.[17] These changes include vasoconstriction to some fetal vascular beds, such as skeletal muscle, and vasodilatation to other vascular beds, such as the cerebral circulation. A number of more recent studies have addressed the high perinatal mortality and morbidity rates that are associated with these fetal velocimetry changes.[18] The same data, however, can provide additional clinical information if the sequence of velocimetry changes that occur in human FGR fetuses is considered. The physiologic significance of the velocimetry changes in different fetal vessels can be interpreted properly only by viewing these changes as a sequence of circulatory adjustments by the fetus.

The sequence of changes in fetal velocimetry has been studied in a group of FGR pregnancies that would ultimately require delivery for FHR abnormalities as well.[19] This study brought out that some vessels show alterations in velocity waveform relatively early in relation to the time of required delivery of the infant and others occur much later, within a few days of delivery. An important point is that even the "late" changes occurred some days before the FHR became abnormal, the latter triggering delivery of the fetus. Also, within any single vessel, velocimetry waveforms may show progressive changes—for example, in the umbilical artery, progressing from an increased pulsatility index (PI) to absent end-diastolic flow (AEDF) to reversed end-diastolic flow (REDF). These velocimetry changes have been described for a number of fetal vessels. Another study examining the sequential changes in fetal velocimetry pointed out that many of these changes precede any change in the biophysical profile, emphasizing the importance of velocimetry and blood flow studies.[19] It may well be that clinical practice may move toward utilizing fetal velocimetry data for decisions about the optimal delivery time for a growth-restricted infant. This change in practice rests on whether follow-up studies document that leaving the unborn infant in utero after "late" velocimetry changes have occurred, but before FHR is abnormal, contributes to potential CNS injury to a greater extent than severe prematurity. At this point, the data are not available to make this decision.

Blood Flow Measurements

From a physiologic standpoint, blood flow is the critical determinant of tissue perfusion, not the velocity of the blood. As techniques have developed to accurately determine the diameter of a vessel, it has become feasible to measure blood flow in the vessel from the diameter and the velocity of blood in the vessel. This approach has been applied in human pregnancies to measure the blood flow in three important sites:

- Uterine artery
- Umbilical vein
- Ductus venosus

Measurement of blood flow in each of these vessels is very important to an understanding of the pathophysiology in FGR pregnancies.

Uterine Artery Blood Flow

Of note, extensive research has examined the significance, frequency, and severity of uterine arterial velocimetry changes in FGR pregnancies. Measurement of uterine artery velocity waveforms has proved useful in FGR pregnancies, especially because measurement of actual blood flow has proved difficult.[20-25] Unfortunately, the few sequential studies of the changes in umbilical velocimetry did not include information on the uterine arteries so a correlation between the changes in the two circulations cannot yet be made.

Measurements of uterine artery blood flow in human pregnancies were first reported in the 1990s.[26-28] In the relevant studies, estimates of uterine blood flow were reported as approximately 500 mL/min^{-1} at term in normal pregnancies. Meschia[12] has pointed out that these estimates were likely to be too low in the context of published data for uterine venous O_2 saturation, maternal hemoglobin concentration, and estimates of uterine O_2 consumption. On the basis of these oxygen data, he calculated that uterine blood flow in human pregnancy should be approximately 1 L/min^{-1} at term. More recently, Konje and co-workers[29] used color power angiography to measure uterine blood flow in uncomplicated human pregnancies. These workers reported a blood flow at term of 970 ± 193 mL/min^{-1}, not significantly different from that estimated by Meschia from the O_2 data. Thus a blood flow of 1 L/min^{-1} appears to be a reliable estimate for normal human pregnancy. Unfortunately, no measurements of uterine blood flow in FGR pregnancies have yet been reported. Furthermore, blood flow measurements in the uterine artery probably underestimate total flow, because some flow may be contributed by the ovarian or cervical arteries as well. Because of the technical difficulties in the measurement of uterine blood flow, including the necessity to measure flow

of each uterine artery, studies of the velocity waveform in the uterine artery are likely to prove more clinically useful in the immediate future.

Umbilical Vein Blood Flow

A number of studies have reported umbilical blood flow rates at different gestational ages in uncomplicated human pregnancies.[30-35] Umbilical blood flow (mL/min^{-1}) increases exponentially during gestation, in a pattern roughly similar to the increase in fetal weight. Several other studies, however, have shown that blood flow does not increase in a fixed proportion relative to fetal body weight. When umbilical blood flow is expressed per kg of fetal weight, it decreases slightly during gestation from approximately 130 mL/min^{-1} at 20 weeks of gestation to approximately 100 mL/min^{-1} at term. Both the umbilical vein diameter and the mean velocity increase during gestation, although the increase in diameter is the dominant factor leading to the exponential increase in umbilical blood flow during gestation.

For obvious reasons, umbilical blood flow uncorrected for fetal size is low in FGR pregnancies because the fetus is smaller. But if blood flow is expressed per kg of fetal weight or normalized to fetal body mass by some other biometric index such as fetal HC or AC, it is still significantly reduced in most FGR pregnancies.[16] This is one of the more important findings in FGR pregnancies—that umbilical blood flow to the placenta is reduced even when corrected for fetal body size. Furthermore, the reduction is quite large. For example, at 34 weeks of gestation, the umbilical flow (mL/min^{-1}) in FGR pregnancies is reduced by approximately 70%. The data from a study by Ferrazzi and associates are presented in Figure 28-1.[16] In this figure, blood flow has been normalized for estimated fetal weight, which also is reduced in FGR pregnancies. The reduction is even more striking if normalized for head circumference, because head circumference is spared in FGR pregnancies. The study by Ferrazzi's group demonstrated that the decrease in flow is entirely attributable to the decrease in umbilical blood flow velocity, not in the diameter of the vessel. The reduction in umbilical blood flow can occur quite early in gestation—that is, at the time the diagnosis of FGR is first made by serial ultrasound examinations (24 weeks). As noted in Figure 28-1, umbilical blood flows in some FGR pregnancies are within the normal range. It will be important through follow-up studies to determine what differences in outcomes, both long- and short-term, are associated with a reduction in umbilical blood flow of this magnitude. These first studies were cross-sectional in design, but a subsequent longitudinal study established that these flow changes were persistent in a pregnancy.[36] A reduced umbilical vein blood flow makes it more difficult to deliver O_2 and nutrients to the fetal tissues and increases the risk for fetal hypoxia and acidosis.

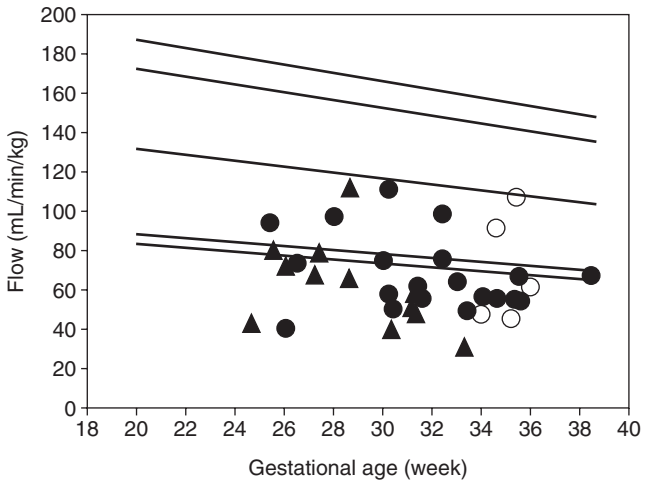


Figure 28-1 Umbilical vein flow per unit head circumference plotted against gestational age in growth-restricted fetuses: group 1, ○; group 2, ●; group 3, ▲. *Continuous lines* represent the 5th, 10th, 50th, 90th, and 95th percentiles from 70 normally grown fetuses. *(From Ferrazzi E, Rigano S, Bozzo M, et al: Umbilical vein blood flow in growth-restricted fetuses,* Ultrasound Obstet Gynecol *16:432-438, 2000.)*

Fetal Hepatic and Ductus Venosus Blood Flow

The third fetal vessel for which blood flow data in human pregnancies are now available is the ductus venosus.[37,38] As with the uterine artery, most studies have focused on the velocity waveform, rather than on measurements of actual blood flow. These studies[39-42] have pointed out the clinical significance of an increased shunt through the ductus venosus and of the presence, in severe FGR, of reversed flow in the left portal vein. In the human fetus, the ductus venosus is a small, trumpet-shaped vessel connecting the umbilical vein and inferior vena cava. Its purpose is to shunt some of the highly oxygenated umbilical vein blood away from the fetal liver and directly into the inferior vena cava. Because of its conical shape (its inlet diameter is smaller than its outlet diameter), the velocity waveform changes along the length of the vessel. More recently, in a series of studies, some of which were directed at computerized modeling of the velocity waveform as a function of conicity, several groups of investigators have measured the blood flow in the ductus venosus.[43-46] This measurement permits determination of the amount of umbilical venous blood shunted through the ductus as well as the amount perfusing the fetal liver. These flows, as well as umbilical flow, have been measured together in normal human pregnancies throughout the latter half of gestation. Figure 28-2 illustrates the fact that the percent ductal shunt decreases as pregnancy progresses. This is brought out in the figure by the fact that the ductal flow does not increase as much as the umbilical blood flow during gestation. The change is rather striking. The ductal shunt, expressed as percentage of umbilical flow, decreases from 40% at 20 weeks to 15% at term. Figure 28-2 abstracts data obtained in three studies on blood flows in human pregnancies. The uterine blood flow data are from a study by Konje and co-workers,[29] those for umbilical flow from a study by Barbera and colleagues[30] and those for the ductus, from a study by Bellotti and associates.[38] Figure 28-2, *A*, presents the flow rates in mL/min^{-1}. Figure 28-2, *B*, presents flows per kg of fetal body weight; it is clear that the increase in umbilical blood flow during gestation most closely approximates the growth of the fetus, because flow per kg decreases only slightly during gestation in normal pregnancies.

Several studies have pointed out that the ductal shunt is increased for gestational age in some pregnancies with clinically severe FGR. Because fetal hepatic blood flow is determined in large part by the umbilical blood flow and inversely by the percent ductal shunt, a growth-restricted fetus with a reduction in umbilical blood flow and an increased ductal shunt may have an enormous reduction in hepatic blood flow. Bellotti and colleagues have shown that this combined effect does indeed occur in some FGR pregnancies.[47,48] It is not surprising, in view of these vascular changes, that two different groups of investigators have reported hepatic injury unrelated to infection in growth-restricted newborns.[49,50] Obviously, this area needs more study, because it may affect the management (particularly nutritional management) of affected babies in the immediate neonatal period. A more recent study has reported an increase in ductal shunt in pregnancies in which maternal undernutrition is present; this finding is the first link between

maternal nutrition and cardiovascular redistribution in the fetal circulation.[51]

METABOLIC CHANGES IN PREGNANCIES WITH FETAL GROWTH RESTRICTION

Carbohydrates

Lacticacidemia, hypoxia, and acidosis have already been discussed as frequent complications of FGR pregnancies, particularly in those with "late" velocimetry changes such as REDF in the umbilical artery. With regard to lactic acidemia and acidosis, it is important to emphasize the unique metabolic characteristics of the fetal liver and placenta with regard to the metabolism and transport of lactate and pyruvate. In animal studies, it has been well established that the fetal liver has a large net output of pyruvate and net uptake of lactate from the fetal circulation. In view of the fact that the fetal liver is perfused with a high percentage of umbilical venous blood, the most-oxygenated blood of the fetus, it is clear that the fetal liver could play an important role in the defense of acid-base balance during fetal hypoxia. Figure 28-3 presents in graphic form some of the changes in acid-base balance and lactate concentration during severe and persistent fetal hypoxia taken from the study by Wilkening and colleagues.[52] The striking finding is that despite persistent, severe fetal hypoxia for 24 hours, the fetal acidosis is largely completely corrected as reflected in fetal arterial pH, although the lactate concentration is still elevated. This correction of metabolic acidosis occurs because of the continued uptake of lactate and release of pyruvate from the fetal liver. This important finding, that the fetal liver continues to take up lactate and release pyruvate even during persistent severe fetal hypoxia,[53] was derived from studies done on normal pregnancies, which were then made hypoxic. In FGR pregnancies, however, in which the ductus venosus shunt may be increased, this defense mechanism may be further compromised. It is

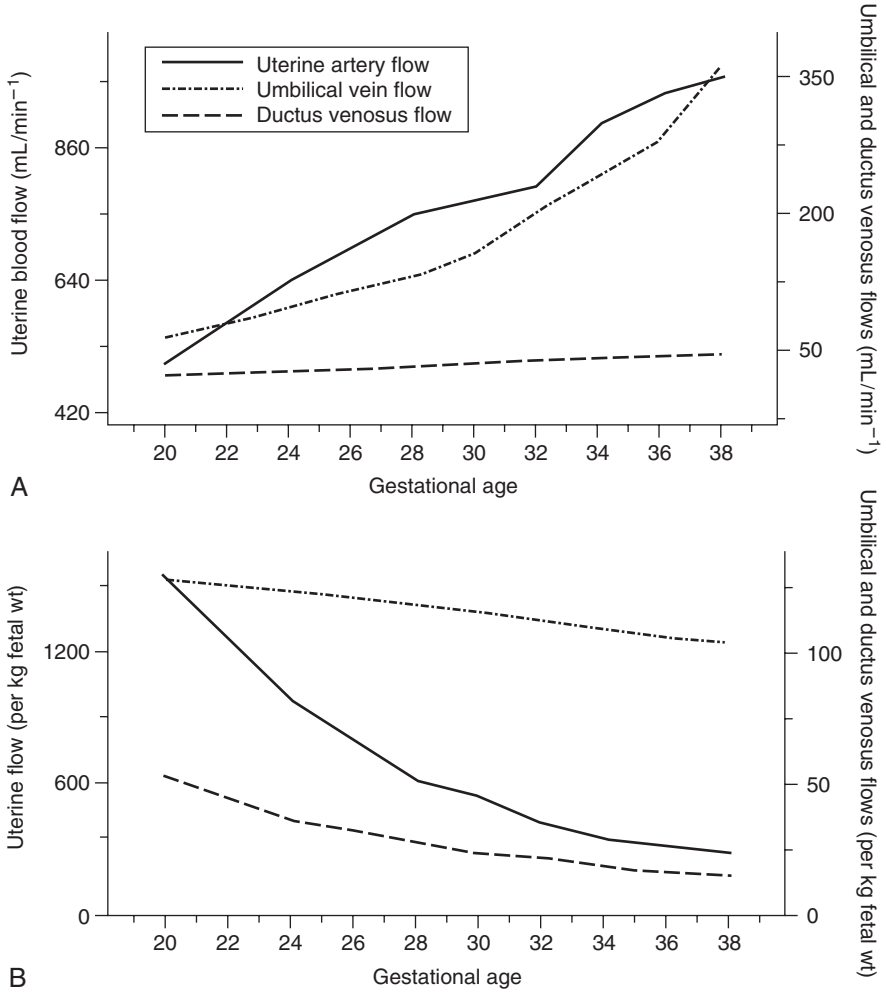


Figure 28-2 The three vessels for which flow measurements are available in human pregnancies are the uterine artery, with flow plotted as a *solid line;* the umbilical vein, with flow plotted as a *dotted-dashed line;* and the ductus venosus, for which flow is plotted as a *dashed line.* **A,** Flows in milliliters per minute = uterine artery flow. **B,** Flows in the same three vessels expressed as milliliters per minute per kilogram of fetal weight. In human pregnancies, the uterine flow is much greater than the umbilical flow. The umbilical flow, in milliliters per minute per kilogram of fetal weight, declines only slightly over the course of gestation; this finding establishes that the rate of increase in umbilical flow is roughly parallel to the rate of increase in fetal weight. *(Data from Konje J, Kaufmann P, Bell S, Taylor D: A longitudinal study of quantitative uterine blood flow with the use of color power angiography in appropriate for gestational age pregnancies,* Am J Obstet Gynecol *185:608-613, 2001; and Barbera A, Galan H, Ferrazzi E, et al: Relationship of umbilical vein blood flow to growth parameters in the human fetus,* Am J Obstet Gynecol *181:174, 1999.)*

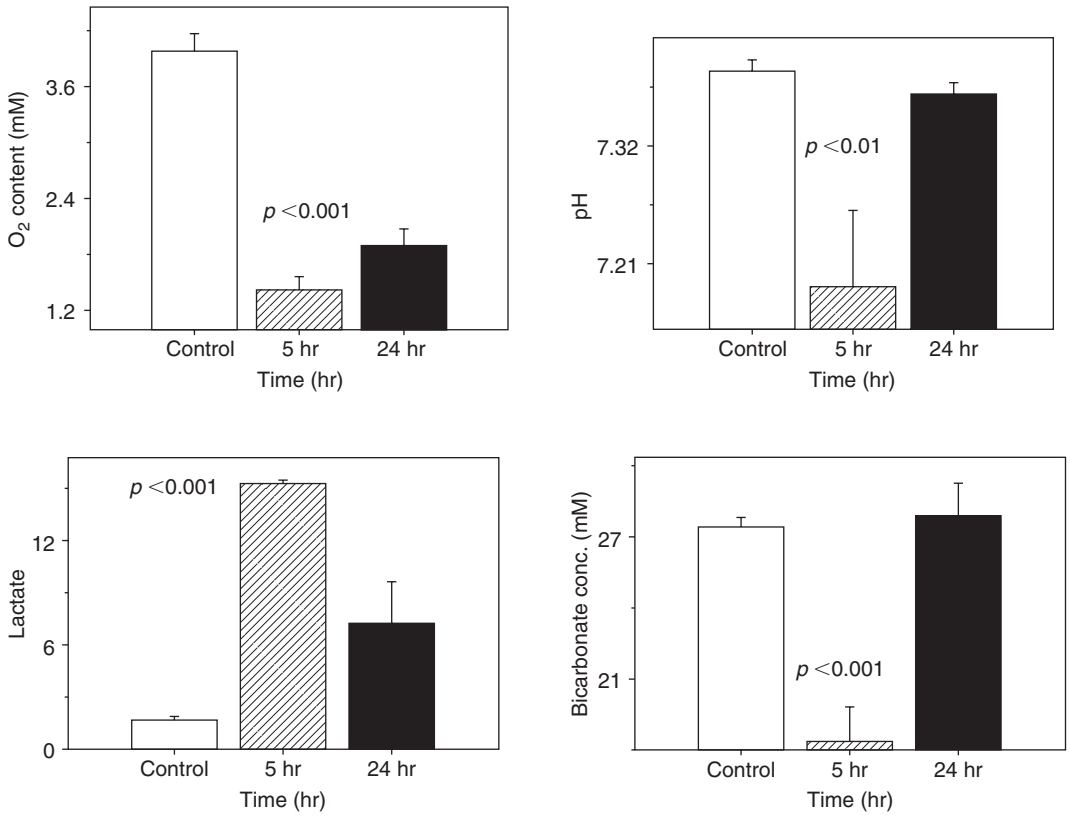


Figure 28-3 Changes in acid-base balance and lactate concentration during severe and persistent fetal hypoxia. *(From Wilkening RB, Boyle D, Meschia G: Fetal pH improvement after 24 h of severe, nonlethal hypoxia,* Biol Neonate *63:129-132, 1993.)*

the reduced hepatic perfusion in some FGR pregnancies that makes the growth-restricted fetus particularly vulnerable to fetal hypoxia.

Glucose metabolism also is altered in FGR pregnancies. In the sheep model, glucose uptake into the fetal circulation from the placenta is maintained within the normal range when expressed per kilogram of fetal weight or expressed per gram of placental weight.[54] The maintenance of a normal glucose uptake despite the small placenta in FGR pregnancies is accomplished by an increase in the transplacental glucose gradient. In women, umbilical glucose uptake cannot be measured directly. The glucose gradient across the placenta in normal human pregnancies, however, has been well described by a number of investigators.[55-59] Figure 28-4, from a study by Marconi and co-workers,[55] presents data obtained at the time of cordocentesis for the maternal "arterialized" and fetal umbilical venous glucose concentration differences throughout gestation for normal human pregnancies. It illustrates the fact that the maternal-fetal concentration difference increases with advancing gestation. As in the ovine model of FGR, however, the maternal-fetal glucose concentration difference is increased significantly over that in normal pregnancies at the same gestational stage. Figure 28-5 illustrates this increase, as well as bringing out that the magnitude of the glucose concentration difference correlates with clinical severity of the growth restriction, defined by umbilical artery velocimetry and FHR data.[55] Thus the changes in glucose gradients are linked to the fetal velocimetry changes as well. Studies of glucose transporter-1 (Glut-1) expression in human FGR pregnancies have shown that Glut-1 expression is not altered in FGR pregnancies, consistent with the foregoing data pointing to the increased transplacental glucose gradient as the primary adaptation to maintain fetal glucose uptake.[60] These findings also are consistent with the hypothesis that Glut-1 expression in the human placenta is altered in type 1 diabetes associated with high maternal glucose concentrations in early pregnancy[61] but not in gestational diabetes, in which maternal glucose concentrations are increased only in late pregnancy.[62]

Scaling of all metabolic data is critically important in studies of FGR pregnancies. Because both the fetus and the placenta in such pregnancies are smaller than normal, all of the values for uptake of nutrients and for blood flow will be reduced unless the data are normalized for body size. The smaller conceptus mass significantly reduces the metabolic demand on the mother. In human pregnancies, this issue has been addressed only for glucose.[59] Marconi and co-workers examined the relationship among three variables: (1) maternal plasma glucose disposal rate at steady state, a measure of maternal glucose utilization; (2) conceptus mass (fetal weight plus placental weight); and (3) maternal plasma glucose concentration.[59] These studies brought out the fact that, at comparable maternal glucose concentrations, the smaller the mass of the conceptus, the smaller the maternal glucose utilization rate.

Another aspect of glucose metabolism in FGR pregnancies is the question of whether significant gluconeogenesis occurs. Figure 28-6 summarizes the normal fluxes into and out of the fetal liver in the ovine fetus in late gestation (control period) taken from the study by Teng and associates.[63] Of note, the fetal liver has a very large uptake of lactate and gluconeogenic amino acids. Despite this large uptake of potential glucose carbon, no significant glucose release from the fetal liver occurs under normal conditions. Instead, carbon leaves the liver primarily as glutamate, serine and pyruvate. It is only when fetal gluconeogenesis is stimulated by a fetal infusion of glucagon that net glucose release from the liver can be found and, in response, glutamate release is virtually shut off. In human FGR pregnancies, stable isotopically labeled glucose has been infused into

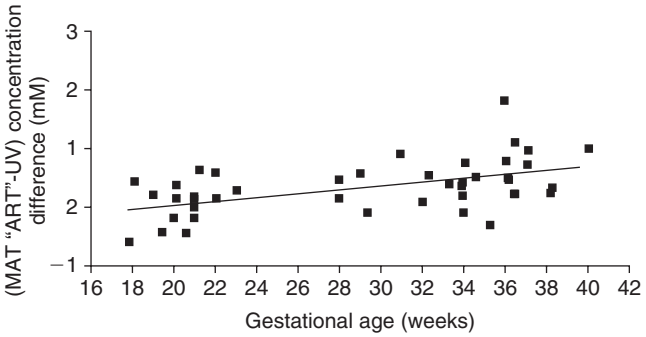


Figure 28-4 Maternal "arterial"-umbilical venous glucose concentration difference versus gestational age in AGA pregnancies. *AGA,* Appropriate for gestational age. *(From Marconi AM, Paolini C, Buscaglia M, et al: The impact of gestational age and fetal growth on the maternal-fetal glucose concentration difference,* Obstet Gynecol *87:937-942, 1996.)*

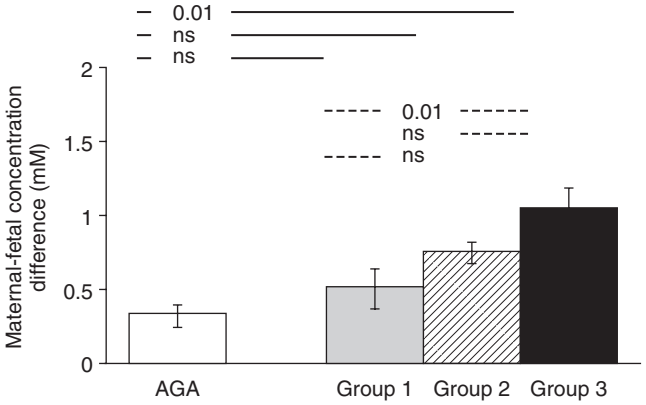


Figure 28-5 Measured mean ± SD values of maternal "arterial" umbilical venous glucose concentration (CONC) differences in appropriate-for-gestational age (AGA) fetuses and fetal growth restriction (FGR) cases of groups 1, 2, and 3. The *p* values refer to the significance of the differences for the intercepts of AGA versus FGR groups *(solid lines)* and among groups of FGR *(dashed lines)* for the regression analysis of the maternal-fetal difference versus gestational age, because there were no significant differences (ns) among the slopes of these regressions. *(From Marconi AM, Paolini C, Buscaglia M, et al: The impact of gestational age and fetal growth on the maternal-fetal glucose concentration difference,* Obstet Gynecol *87:937-942, 1996.)*

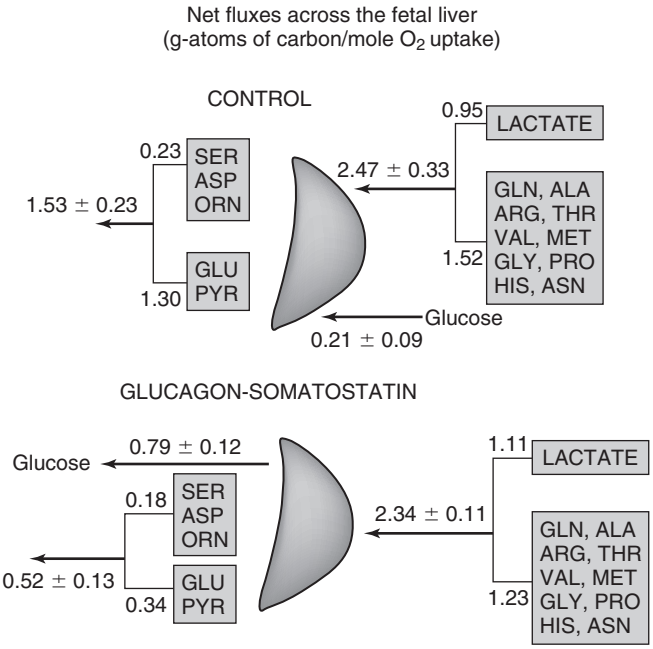


Figure 28-6 Fetal hepatic uptake and output of glucose carbon and glucogenic substrate carbon under normal physiologic conditions (control) and during a glucagon-somatostatin infusion into the fetal circulation. Each number represents a substrate carbon-to-oxygen uptake molar ratio. *ALA,* Alanine; *ARG,* arginine; *ASN,* asparagine; *ASP,* aspartic; *GLN,* glutamine; *GLU,* glutamate; *GLY,* glycine; *HIS,* histidine; *MET,* methionine; *ORN,* ornithine; *PRO,* proline; *PYR,* pyruvate; *SER,* serine; *THR,* threonine; *VAL,* valine. *(From Teng C, Battaglia F, Meschia G, et al: Fetal hepatic and umbilical uptakes of glucogenic substrates during a glucagon-somatostatin infusion,* Am J Physiol *282:E542-E550, 2002.)*

the maternal circulation to investigate whether dilution of the fetal plasma glucose enrichment occurs in FGR pregnancies.[64] Despite the fact that these measurements were carried out after an overnight fast, no evidence of dilution of the fetal glucose enrichment was found. Thus, in this study, an FGR pregnancy did not stimulate detectable fetal gluconeogenesis.

Amino Acid Transport and Metabolism

As an aspect of placental nutrient transport, amino acid transport and metabolism have been the subject of considerable research interest over the past decade. This work was stimulated in large part by advances in molecular biology that have permitted the identification of specific transporter proteins on the two plasma membrane surfaces of the trophoblast. The field of placental amino acid transport has been reviewed by several different groups of investigators.[65-67] As it applies to human FGR pregnancies, the following characteristics have been reasonably well established.

First, in microvesicle preparations from either the basal (fetal) surface or microvillus (maternal) surface of the trophoblast, transport capacity is reduced on both surfaces of the placenta in these pregnancies.[65] These defects are not present for all transport systems, nor are the problems the same on each surface. Changes in amino acid transport in vivo in FGR pregnancies can reflect changes not only in membrane transporter activity but also in placental utilization or production of an amino acid, as well as changes in perfusion of the placenta.

Figure 28-7 outlines the transplacental fluxes of an essential amino acid, leucine, in diagrammatic form. The data were obtained in normal pregnant sheep in late gestation.[68,69] Of note, the nutritionally important variable—namely, the net uptake of leucine into the fetal circulation—is determined principally by three different fluxes of approximately equal magnitude: (1) the transplacental flux from maternal plasma to fetal plasma; (2) the backflux from the fetal plasma into the placenta; and (3) the leucine flux from placental protein breakdown into the fetal plasma. In the ovine model of FGR, it has been shown for two essential amino acids, leucine and threonine, that the transplacental flux is significantly reduced.[69,70] This decrease holds even when the flux is expressed per gram of placenta. In those studies, the fetal adaptation to the reduced transplacental flux is a lower fetal plasma amino acid concentration. This change leads in turn to a lower fetal amino acid oxidation rate and a reduced backflux into the placenta. Both of these changes would tend to divert these amino acids to fetal protein synthesis. A reduced transplacental flux in ovine FGR pregnancies led

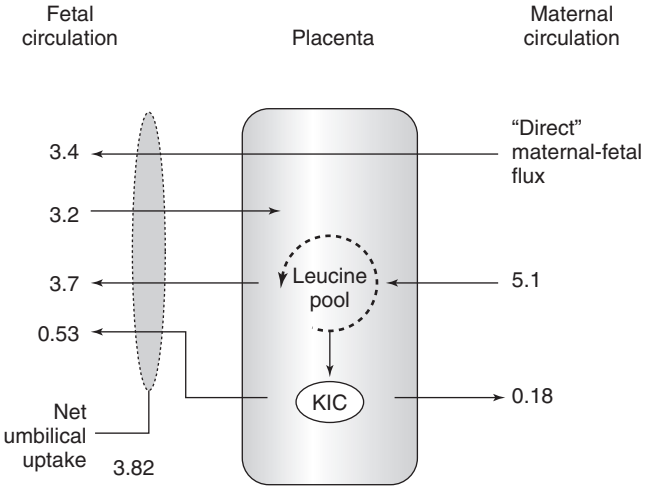


Figure 28-7 The unidirectional fluxes of leucine into and out of the placenta (μmol • min^{-1} • $kg_{fetal\ wt}^{-1}$) measured in vivo under steady-state conditions are presented. (Data are abstracted from studies by Loy [68] and Ross[69] and their colleagues.) The three major fluxes that together determine the umbilical uptake are of approximately equal magnitude. *KIC,* Alpha-ketoisocaproic acid. *(From Battaglia FC, Regnault TR: Placental transport and metabolism of amino acids,* Placenta *22:145-161, 2001.)*

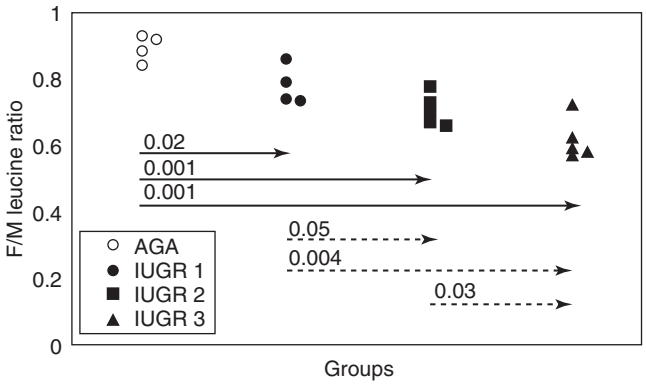


Figure 28-8 Leucine fetal-maternal (F/M) enrichment ratio in appropriate-for-gestational-age (AGA) and intrauterine growth restriction (IUGR) pregnancies at fetal blood sampling. *(From Marconi AM, Paolini C, Stramare L, et al: Steady state maternal-fetal leucine enrichments in normal and intrauterine growth-restricted pregnancies,* Pediatr Res *46:114-119, 1999.)*

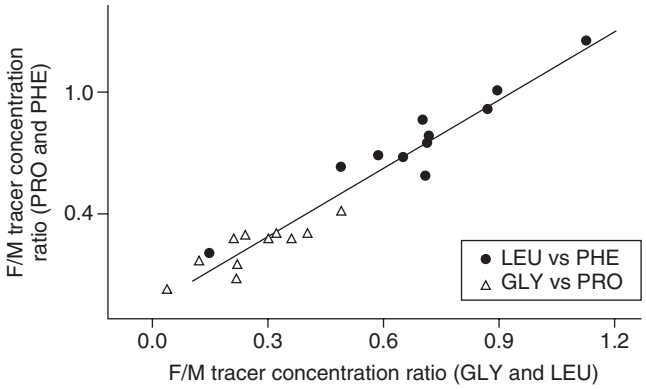


Figure 28-9 The fetal-maternal (F/M) plasma tracer concentration ratios are plotted with the values for glycine (GLY) and leucine (LEU) along the abscissa and those for proline (PRO) and phenylalanine (PHE) along the ordinate. The regression is highly significant: F/M $y = -0.0511 + 1.1274$, F/M x, $R_2 = 0.94$; $p < .001$. *(From Paolini CL, Marconi A, Ronzoni S, et al: Placental transport of leucine, phenylalanine, glycine, and proline in intrauterine growth–restricted pregnancies,* J Clin Endocrinol Metab *86:5427-5432, 2001.)*

to a significantly lower fetal-maternal (F/M) enrichment ratio for both leucine and threonine. These studies led to clinical studies of the F/M ratio in normal and FGR pregnancies. The clinical data for the leucine F/M ratio are presented in Figure 28-8, from a study by Marconi and co-workers.[71]

Two aspects of these data merit further discussion: First, the data clearly demonstrate a significant reduction in the F/M enrichment ratio for leucine in FGR pregnancies, presumably reflecting a significant reduction in the maternal-to-fetal transplacental flux. Second, the magnitude of this effect correlated with the classification of clinical severity in FGR pregnancies proposed by Pardi and colleagues,[14] which was based on fetal umbilical arterial velocimetry and FHR data. Just as with glucose data, leucine metabolic data correlated with clinical severity defined by fetal vascular changes. Studies of amino acid transporter activity and expression in vesicles prepared from the microvillus surface have provided additional information, although the interpretation of the findings is not always clear. For example, the taurine transporter TauT has been localized almost exclusively to the syncytiotrophoblast microvillus membrane.[72] Its expression is not altered in FGR pregnancies. This lack of effect could imply a reduced activity of the transporter or simply reflect the decrease in surface area represented by terminal villi in the FGR placenta. By contrast, leucine transporter activity is reduced in FGR placentas—an effect that would be magnified by the reduction in terminal villi surface area. [73]

A study by Paolini and associates in ovine pregnancy had used similar non–steady-state methodology to compare the transport rates of the nine essential amino acids in normal ovine pregnancy.[74] One of the observations coming from that study was that the three branched-chain amino acids and phenylalanine and methionine appeared to cross at similar rates, and much more rapidly than all other essential amino acids. Figure 28-9 from that study illustrates the fact that differences for the pulse fluxes from the maternal to the fetal circulation for these five amino acids were determined by differences in their normal concentrations in maternal plasma. Similar methodology has been used in human normal and FGR pregnancies to examine the relative placental transport rates for four amino acids: leucine, phenylalanine, glycine, and proline.[75] Figure 28-9 also compares the F/M plasma enrichment ratio of leucine with phenylalanine and that of glycine with proline. No significant differences in transport rate were found between leucine and phenylalanine, or between glycine and proline. However, leucine and phenylalanine crossed much more quickly than glycine and proline. Furthermore, in FGR pregnancies, the F/M ratio of leucine and phenylalanine was significantly reduced compared with that in normal pregnancies. Thus, with amino acids, it is becoming increasingly possible to assess placental function in human FGR pregnancies in vivo.

SUMMARY AND CLINICAL IMPLICATIONS

It is clear that in FGR pregnancies, the fetus experiences problems in transport of O_2 and nutrients. Most of these problems can be attributed directly to the maldevelopment of the placenta, which not only is reduced in size but also has structural abnormalities in its vasculature. These changes in the placenta, with the additional factor of chronic hypoxia, can lead to fetal circulatory

changes in many fetal vascular beds detectable by both velocimetry and blood flow studies. Circulatory and metabolic studies have demonstrated a wide range in clinical severity of FGR based on fetal size for gestation. The importance of delineating more homogeneous subsets of FGR pregnancies in terms of severity of the growth restriction stems from the potential clinical usefulness of such information: It can help direct optimal timing of delivery in pregnancies that feature severe FGR. It can form the basis for adjustments in neonatal care of affected infants. Finally, such information can provide the foundation for more focused follow-up studies in childhood and in adulthood.

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29 Endocrine Factors Affecting Neonatal Growth

Dennis M. Styne

Growth reflects a complex interaction of genetic and epi-genetic, endocrine, and nutritional factors which, in turn, regulate cell division and differentiation affecting function. Various phases of growth appear "programmed" to occur within specific time intervals, which, if missed, may not be recoverable. Ever-increasing evidence indicates that the pattern and rapidity of fetal and neonatal growth affect the endocrine environment in later life. Thus, the role of endocrine factors in modulating growth during this critical "epoch" of development and the resulting long-term effects are reviewed.

PATTERN OF NEONATAL GROWTH

The greatest growth rate occurs during fetal life; in fact, the development of a single fertilized cell into a 3.5-kg neonate entails an increase in length of 5000-fold, an increase in surface area of 61×10^6, and an increase in weight of 6×10^{12}. The greatest postnatal growth rate occurs just after birth,[1] followed by slower growth during midchildhood, only to increase once more in puberty before the final cessation at epiphyseal fusion.

Both longitudinal studies (mostly from outside the United States) and cross-sectional studies (by U.S. investigators) have described the growth process after birth. The Centers for Disease Control and Prevention (CDC) released the latest version of the commonly used growth charts from birth until the age of 18 years in 2001* using cross-sectional data from the United States. The charts demonstrate growth between the 3rd to 97th percentiles of length during the first 3 years after birth. Most iterations of the charts show a different color between the 3rd and 97th percentiles. Even though the color change usually found at the 3rd percentile may cause concern in parents of an infant that falls below that line, it is important to recognize that 3% of normal children fall below that lower limit and that only a minority have a condition requiring evaluation or therapy.

Analysis of the factors affecting various phases of growth led to the development of the infant-childhood-pubertal growth charts[2] in which the amount of growth for each of three phases of growth (infancy, childhood, puberty) are considered and their sums plotted. Each component of this model can be described by specific mathematical functions (Figure 29-1).

Infancy

The slowly decelerating component in infancy occurs as the child has a decrease from the rapid fetal growth phase that starts before birth and falls off by age 3 to 4 years. An infancy-child growth spurt occurs at 6 to 9 months of age, which defines the beginning of the childhood phase, as the infant phase decreases.[3] Average total gain in height for Swedish boys (the population from which the plots were originally developed) is 79.0 cm (44.0% of final height) and for girls is 76.8 cm (46.2%).

Childhood

The childhood phase begins during the first year of life and continues until adult height is attained. It is believed to mainly exhibit the strengthening endocrine effects of thyroid hormone, growth hormone (GH), and other agents. Average total gain in height for boys is 85.2 cm (47.4%) and for girls is 78.4 cm (47.3%).

Puberty

The puberty phase is dependent on sex steroids, mainly estrogen, working in concert with GH; in the absence of either, such as in GH or gonadotropin deficiency, growth decreases markedly, as does height. According to this model, average total

*See http://www.cdc.gov/nchs/about/major/nhanes/growthcharts/clinical_charts.htm#Clin%202.

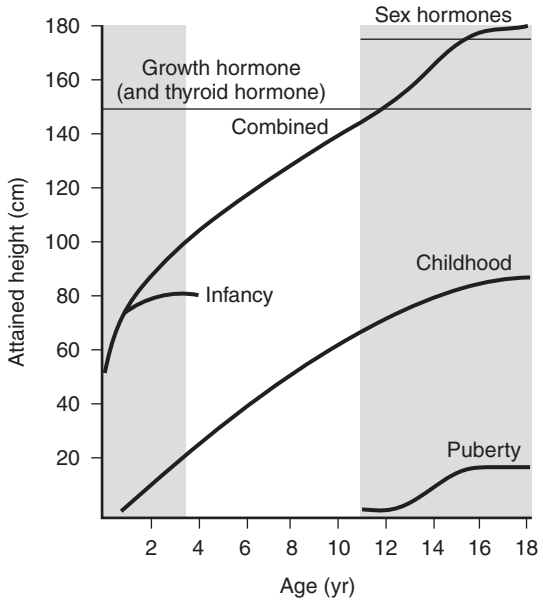


Figure 29-1 Individual components of the infancy-childhood-puberty growth chart and the combined growth curve that results from the sum of them. *(Modified from Karlberg J: On the construction of the infancy-childhood-puberty growth standard,* Acta Paediatr Scand Suppl *356:26, 1989.)*

gain in height for boys is 15.4 cm (8.6%) and for girls is 10.9 cm (6.5%) in this period.

MEASUREMENT OF GROWTH

Weight measurements are the standard method of evaluating neonatal growth. Scales used for this purpose may be accurate to 10 g if they are calibrated regularly. Nonetheless, even this variation when measuring the weight gain of a 1000-g infant during a period of 1 week can lead to an error of as much as 2.9 g per day: If the infant was weighed daily rather than weekly the error might be as much as 20 kg per day.[4]

The accurate measurement of length at birth is just as important as the measurement of weight because the growth velocity can be determined and a pathologic tendency for a baby to cross percentiles in length for age will become apparent.[5] Unfortunately, accurate and reproducible measurement of neonates and infants is difficult to obtain in clinical practice. A study of the measurement of infants as well as inanimate dolls led to inaccuracies of up to 25% in length measurements, and weight measurement errors exceeded 10%.[6] Even if a measurement is assumed to be accurate, the points plotted on the growth chart are also prone to error due to rounding of the measurement by inattentive personnel or to lack of attention to adjustment for gestational age (if the baby is premature).[7,8] Measurement of growth can be performed accurately if two individuals gently straighten the infant and hold the child against a calipers-like device such as a Neo-Infantometer (Graham-Field, Inc., Hauppauge, New York) that expands and contracts to measure the linear distance from a perpendicular plate at the top of the head to a perpendicular plate at the bottom of the feet; staff can be trained to perform this with accuracy.[6,9,10]

Measurement of length is not just a matter of academic interest. Most analyses of infant deaths have been based on birth weight. However, careful analysis of the relationship between birth length and death after premature birth in the Swedish population showed the important effect of length in preterm infants.[11] Although low weight or ponderal index led to a greater risk of early death, those factors at 4 to 5 days of age no longer exerted such a strong effect; however, having a length less than −1 standard deviation (SD) at birth continued to increase the risk of long-term mortality.[11]

Knemometry is a technique that measures minute changes in length of the lower leg[12] and is applicable even to seriously ill children in the neonatal intensive care unit.[13] Although the yearly growth rate is considered linear and consistent, knemometry demonstrates the waxing and waning pattern of growth that occurs over days and weeks; the time between peak growth rate and the nadir of growth rate may be 10 to 20 days.[9,14,15] This fluctuating growth in length may be accompanied by changes in the pattern of weight gain; however, this pattern is innate, perhaps genetic in origin, and is not affected by variations in caloric intake unless frank malnutrition develops.[16] Further, knemometry demonstrated the effect of dexamethasone or chronic lung disease on decreasing growth.[12] Glucocorticoid treatment may increase weight while decreasing length velocity, so measuring weight cannot be a substitute for measuring growth in length. Knemometry is mainly used in research studies, but it adds important information in the clinical setting by some. However, even the knee-to-heel measurement is shown to have problems of accuracy and sensitivity.[17]

Therefore it is apparent that measuring weight and height is important in the evaluation of neonates. Although in the clinical practice of most centers, weight measurements are more accurate than length measurements, with extreme care in measuring length by trained staff, accurate length measurements can be obtained and plotted.

GROWTH CHARTS

The CDC full-term neonatal growth charts were revised and released in 2001 and are based on cross-sectional data for large numbers of infants: thus they can be assumed to be accurate. However, the derivation of premature infant growth charts is another matter. In Chapter 28, Battaglia provided detailed discussion of fetal growth and its measurement. Premature growth charts are based upon data obtained from the birth weight of premature babies at various gestational ages. The gestational age used in these charts are based on the mothers' last menstrual period or postnatal examination, which is considered to be accurate only within 2 to 4 weeks. Furthermore, it has been estimated that up to 40% of infants born at 23 weeks gestation are small for gestational age: Using these data to develop a growth chart to purportedly represent normal premature baby growth is therefore problematic.[18] Thus neonatal premature growth charts may be affected by inaccuracies of average weight for gestational age and indeed inaccuracies of estimated gestational age: Both the *x*- and *y*-axes may be inaccurate.[4] This is important because the American Academy of Pediatrics states that the growth of preterm infants should replicate that of a fetus of the same gestational age. If clinicians do not know what the normal growth of normal fetuses is at a given gestational age, they will have difficulty trying to ensure this happens. Likewise, feeding and postnatal nutritional supplementation practices vary in relation to goals for growth, complicating the situation further. It has been suggested that new longitudinal data collection is needed to allow reexamination of this concept.[19] At present, no consensus has emerged regarding the optimal reference for the growth of premature infants.

An alternative attempt to improve the relevance of neonatal growth charts used a population study in Burgundy, plotting the birth weights in each week of gestation for one cohort with maternal diseases likely to affect fetal growth and another for healthy mothers without these diseases.[20] Regardless of the group in which the mother was placed, small-for-gestational-age (SGA) infants born at term had a increased risk of death,

however, SGA preterm infants born at 32 to 36 weeks gestational age was not associated with increased mortality and morbidity compared with appropriate-for-gestational-age (AGA) term infants. Infants determined to be SGA at 28 to 31 weeks according to the healthy population standards were at higher risk for pulmonary disease and intraventricular hemorrhage, leading to the conclusions that standards based on healthy maternal populations may better indicate those infants at most risk for complications. Customized British birth weight percentile charts based on characteristics of the mother (e.g., height, weight, parity) and the physiologic characteristics of the neonate (e.g., gestational age, sex) are available for that population[21,22] and are likewise believed to be helpful.

Despite these concerns about standards of height, weight, and weight-for-height adjusted for gestational age, such standards are in general use for preterm low-birth-weight infants and are expected to help determine appropriate weight gain, evaluate failure to thrive, and observe patterns of excessive weight gain in low-birth-weight infants.[23,24]

Longitudinal measurement of length in an infant helps predict childhood growth patterns and other factors in the evaluation of neonates. The risk for attaining an adult height 2 SD less than the mean was five-fold higher in children with a low birth weight and seven-fold higher in those with a low birth length.[25] The length of a full-term newborn is closely correlated with the maternal stature and weight[26] and parity status; the babies of short or thin mothers who are born SGA are more likely to have a positive outcome than those who are born SGA to primiparous mothers.[27] Once free from the constraints of the uterus, the full-term infant will often adjust growth to achieve the growth channel of the percentile that will be followed for most of childhood. An increase in percentiles during childhood occurs sooner after birth as the childhood percentile is reached by 11 to 12 months, and a downward adjustment will allow the child to reach the new lower percentile by a later mean age of 13 months of age.[28] These changes are gentle, however, and show no abrupt change in growth velocity, which would indicate pathology. Accurate data on weight and height velocity, as well as body composition changes, are available in neonates.[28a] Determining the percentile or standard deviation score of velocity is more accurate than plotting a single height or weight as the velocity will change first.

Prematurity, Intrauterine Growth Restriction, and Small for Gestational Age

As reviewed by Saenger[28b] in 2007, the definition of intrauterine growth restriction (IUGR) and SGA are often confused; even the medical literature is not always clear. The term IUGR indicates a documented decrease in intrauterine growth noted by two fetal ultrasound examinations; a baby with IUGR may have a temporary problem that will still allow a normal or near-normal birth weight and length. Thus the accuracy of fetal ultrasonography becomes an issue in this diagnosis. Because many infants are born with low birth weights, the description of SGA is in fact a proxy for IUGR in many, but not all, situations.

The description of SGA refers to an infant whose weight (and presumably length) are below a limit, usually weight or crown-heel length at birth is at least 2 SD less than the mean for the infant's gestational age, equivalent to the 2.3 percentile, based on data derived from an appropriate reference population; however, in some publications, SGA refers to babies with birth weights below the 3rd, 5th, or 10th percentile for gestational age, confusing interpretation of clinical studies during various eras. Furthermore, the difference in percentiles used for diagnosis is of practical importance because medical insurance companies may adhere to one diagnosis and eliminate treatment of children fulfilling other criteria. The World Health Organization (WHO) defines "low birth weight" as a weight at birth less than 2500 g in an infant of any gestational age; this inclusion of all gestational ages becomes problematic because long-term outcomes are likely to be different for a baby who was premature but AGA and for a full-term infant who is SGA even if of the same weight.

Recently a consensus conference on treating babies born SGA with GH suggests a diagnostic criterion of a weight or length more than 2 SD below the mean for gestational age[29]; that is the convention used in this chapter. SGA may be further classified as SGA with a low birth weight, SGA with a below-normal birth length, or SGA with a low birth weight and below-normal length, and the difference may change the outcomes of therapy. Two other descriptors of IUGR are used: *symmetric IUGR* denotes normal body proportions (a small head and a small body) and may be considered a more severe and longstanding form of IUGR, dating from the second trimester; *asymmetric IUGR* denotes small abdominal circumferences (due mostly to a small liver), decreased subcutaneous and abdominal fat, reduced skeletal muscle mass, and a head circumference in the normal range, probably resulting from stress effects in the third trimester. These definitions do not consider ethnic background, parity, or maternal size, so further classification is possible but not objectively defined.[29] A low ponderal index (birth weight/length^2) is a marker for asymmetric growth restriction and is associated with an increased risk for cerebral palsy.[30]

Although premature infants are expected to have catch-up growth by 2 years, it is those born after 29 weeks gestation who exhibit catch-up growth; those born before 29 weeks more likely have a decreased rate of length and weight gain, which may be noted in the first week after birth and lasts up to 2 years.[6,31,32] Although it was previously assumed that SGA babies, in contrast to AGA premature babies, did not have catch-up growth in length, evidence from longitudinal (as well as less powerful cross-sectional) studies show that the majority, up to 80%, that started with lengths greater than 2 SD below the mean, do indeed show catch-up in height for age.[25,33,34] It has been demonstrated for decades that catch-up growth can be demonstrated soon after birth if careful measurements are utilized,[35] which seems to be crucial because much of the catch-up growth occurs in this period.[36] Indeed, an acceleration of growth in length is noted in the first 6 months of SGA babies who will catch up.[36,37] In one study, 44% of infants with neonatal length more than 1.28 SD below normal (i.e., −1.28 SD), and therefore considered SGA, caught up to attain a length that was less than −1.28 SD by 3 months, 51% caught up by 3 years, and 73% by 6 years.[38] A large study (n = 3650) of SGA infants found that 88% of healthy singleton SGA infants showed catch-up growth by 2 years, with most showing the catch-up growth by 2 months; those that remained short at 2 years had a greater risk of shorter adult stature.[25] In a prospective study of SGA babies (the criteria for the classifications of the infants in the study appeared to be birth weight and ponderal index, suggesting that SGA rather than IUGR was under investigation), 38% were under the 10th percentile of height at 6 months of postnatal age versus 7% of control babies; proportionate SGA infants had greater catch-up growth than disproportionate SGA babies, but the differences were in the range of 1 cm of increased growth per 6 months.[39] In general, children born SGA will achieve an adult height standard deviation score (SDS) equivalent to their SDS in childhood because skeletal development is normal. Approximately one fifth of extremely short children have a history of IUGR.[40,41]

Controversy exists about the infantile growth rate of very SGA infants (<1500 g); some studies show similar growth in length and head circumference in very SGA infants compared with those of the same birth weight but shorter gestation (preterm but AGA).[42] Others found the opposite—that SGA infants

are shorter and lighter at 3 years and have delayed growth.[43] Of 166 children with birth weight less than 1000 g and SGA, 81% demonstrated catch-up growth in length during childhood, with 78% showing catch-up growth in length by 3 years of age.[44]

Longitudinal evaluation of growth in neonates in intensive care units demonstrates an almost universal growth impairment.[45] Gestational age is the most important predictor of growth impairment, followed by birth weight and length standard deviation scores. The babies born at younger gestational age with higher weights were at greatest risk for both types of growth impairment. Because this early growth impairment appears to be common, it is thought that recreating the intrauterine environment for a premature baby is a virtual impossibility. Not only is growth impaired in the immediate postnatal period, but such impairment continues for months. Enhanced infant formulas have been tried in an attempt to increase growth during these vulnerable periods.

However, catch-up growth is associated with dangers even outside the neonatal intensive care unit.

DANGERS OF RAPID NEONATAL GROWTH

As noted in other chapters, children born with lower birth weight are shown to be at higher risk for impaired glucose tolerance and cardiac disease as adults.[46,47] Rapid growth in such children, however, also leads to adult risk for cardiovascular disease and glucose intolerance,[48-50] as reviewed by Gluckman and colleagues[51] The early weight gain during the first months of life may be more of a risk factor for obesity and the onset of disease related to insulin resistance than later weight gain during childhood, according to the results of longitudinal studies in Avon, Stockholm, and other sites.[52] Infants with the greatest increase in linear growth during this period have decreased flow-mediated endothelium-dependent dilation (FMD) (a measure of vessel dysfunction), which is as significant an event as seen with insulin-dependent diabetes mellitus or smoking in adults.[53] Likewise measures of insulin resistance are higher in those with the greatest weight gain in the postnatal period.[54] It has been suggested that somewhat slow growth in the postnatal period may not be undesirable.[45]

Prematurity itself affects insulin sensitivity. Premature babies who are born AGA and demonstrate slow catch-up weight gain nonetheless have postnatal insulin resistance, just like SGA babies.[55] Rapid, excessive weight gain in full-term neonates also has been correlated with the development of hypertension, obesity, and comorbid illnesses by the age of 30 years.[56,57] However, recent conflicting data from birth cohorts in Brazil, Guatemala, the Philippines, and South Africa indicate that weight gain at any age relates to increased adult blood pressure and that weight gain in infancy does not pose a higher risk than at other ages.[58] It is not clear how to reconcile this finding with those noted previously.

Low birth weight can affect pubertal development in girls. Girls born with low birth weights extending to the classification of SGA defined previously tend to have early onset of puberty.[59] In addition girls with low birth weight have increased prevalence of insulin resistance by 1 year of age[60] and a tendency toward developing polycystic ovarian syndrome characterized by hyperinsulinemic hyperandrogenism. Girls with average birth weight who tend towards early breast development have an extended duration of time between breast development and menarche; therefore the age of menarche is relatively stable even if the age of onset of breast development is more variable. However, girls with low birth weight do not demonstrate such an extension of the time between breast development and menarche; it has been postulated that the hyperinsulinemia characteristic of these girls is related to early menarche.[2] The effect of low birth weight on the reproductive axis in males is not well-known, but aromatase activity due to elevated estradiol levels, 5α-reductase activity reflected by elevated dihydrotestosterone, and elevated inhibin B, reflecting activity of the seminiferous tubules, are inversely proportional to birth weight, but at present the effects of these changes are not known.

The U.S. Food and Drug Administration (FDA) has approved treating SGA infants lacking catch-up growth by 2 years after birth with GH in a dose of 0.48 mg/kg/day. Controversy remains about optimal dose as well as frequency and duration of therapy, but treated children clearly can reach an adult height in the normal range.[61-63] GH is most effective when used at a young age; therefore caregivers of SGA babies should be aware that if catch-up growth does not occur by 2 years of age, they should consider the possibility of using GH. Because the neonatologists who care for the babies at birth do not care for them in the subsequent months and a program of treatment would optimally start at 2 years old, effective communication between the neonatologists and pediatricians who follow the children as well as the endocrinologist who may evaluate the child for GH therapy is important.[64] SGA babies tend towards insulin resistance, and GH therapy can produce insulin resistance. Thus the potential for the development of type 2 diabetes in GH–treated SGA babies is of concern, but so far an increase in the prevalence of type 2 diabetes after the increase in insulin levels that occurs during growth hormone treatment has not been demonstrated.[65,66] However, not enough treatment years have passed to ensure that adult diabetes or abnormalities of lipid metabolism caused by growth from treatment in SGA children does not occur. However, some evidence exists for an increase in proinflammatory tendencies[67] so that GH-treated children must be watched long term.

AGA infants born preterm who grow poorly in the initial postnatal months are considered to be growth restrained; these children grow in a manner similar to SGA babies throughout childhood and reach similar adult size.[2] It has been suggested that these individuals should be approved for the use of GH as SGA babies.[68]

Turner syndrome occurs in one in 2000 girls and is another condition for which the FDA has approved GH to increase height.[69] In addition to low birth weight, Turner syndrome is often diagnosed in the neonatal period because of numerous characteristic, somatic abnormalities, and frequent occurrence of left-sided heart disease, such as coarctation of the aorta and related hypertension. These infants grow poorly starting in early life.

Controversy exists about the long-term effects of SGA on intellectual function. A 26-year follow-up study comprising 1064 infants born SGA (defined as birth weight below the 5th percentile) and 14,189 non-SGA (control) infants found no difference in years of education, employment, marital status, or satisfaction with life between the two groups.[70] However, persons whose birth history included SGA were less likely to be in the top 15% of their high school class and more likely to be recommended for special education; in addition, they demonstrated small but significant defects in academic achievement. They also attained shorter adult heights (−0.55 SDs) Furthermore, persons in the SGA group were less likely to hold managerial or professional employment and had lower incomes compared with those in the non-SGA group. Another 20-year longitudinal follow-up study of persons whose birth history included very low birth weight (less than 1500 g) demonstrated a lower mean intelligence quotient (IQ) (87 versus 92), lower academic achievement scores, lower likelihood of high school graduation, and less post–secondary school study but, on the positive side, less alcohol and drug abuse than those born at normal weight.[71]

Neonatal growth may be affected by metabolic bone disease in preterm infants, probably as a result of vitamin D deficiency

in view of the association with elevated serum alkaline phosphatase. For example, the finding of a peak neonatal alkaline phosphatase of greater than 1200 IU predicted shorter stature to age 12 years. The effect exceeds that of maternal or paternal height, sex, parental educational achievement, and hypertension during pregnancy—other factors shown to affect childhood height.[72,73] Human milk increases bone mass as shown in a 20-year follow-up of preterm infants randomized to various diets in the neonatal period.[74] C-type natriuretic peptide appears to be a useful marker because it plays an essential role in endochondral bone growth and when measured in plasma can be used as a reflection of bone turnover in infants.[75]

MIDCHILDHOOD GROWTH SPURT

Although the striking pubertal growth spurt attracts much attention, two periods after birth are characterized by brief growth spurts before puberty occurs: the infant-childhood growth spurt between 8 months and 3 years and the midchildhood growth spurt between 6 and 7 years. Furthermore, in the context of body weight and fat content, an "adiposity rebound" of accelerating weight gain and rising body mass index occurs in midchildhood after a period of relative stability of weight gain beforehand. An early adiposity rebound is a risk factor for the development of obesity later in childhood and thereafter.

THE HORMONAL CONTROL OF NEONATAL GROWTH

The hormones that mediate postnatal growth do not play the same roles in fetal growth. As noted in chapter 178, GH is present in very high concentrations in the fetus in contrast to the limited presence of GH receptors. Although this discrepancy suggests limited activity of GH in the fetus, GH does play a role in fetal growth that is reflected in the mean birth weight 1 SD below the mean characteristic of GH-deficient infants. Infants with Laron syndrome (OMIM 262500) (i.e., GH resistance due to reduced or absent GH receptors) have elevated GH and low serum insulin-like growth factor (IGF)-1 levels; they have decreased birth length and weight. Insulin excess, as in the infant of a mother with diabetes, causes increased growth. One patient with congenital IGF-1 deficiency (not due to GH receptor defects)[76,77] had a birth length 5.4 SD below the mean and was 6.9 SD below the mean for height at age 15 years old. Because no IGF-1 was found, no feedback to GH (OMIM 608747) secretion was obtained and GH values were high, similar to those typical for Laron dwarfism. In addition, children were reported with IGF-I receptor mutations (OMIM 608747 and 270450) demonstrating intrauterine and postnatal growth retardation, again demonstrating the importance of IGF-I in both periods of growth.[78]

Thyroid hormone deficiency does not directly affect human birth weight, but prolonged gestation is a feature of congenital hypothyroidism, and this factor will itself increase weight. Placental lactogen exerts no apparent effect on birth size in human beings. However, the concentration of placental GH (from the *GHV* gene; see later) is significantly decreased in the serum of a pregnant woman bearing a fetus with IUGR.

In the postnatal phase, these hormones have important but, in many cases, different roles.

GROWTH HORMONE

Human GH is produced as a single chain, 191–amino acid, 22-kDa containing two intramolecular disulfide bonds[79] (Figure 29-2) A 20-kDa variant of pituitary GH accounts for 5% to 10% of circulating GH.[80] The genes for a family of GH-related proteins share a common structural organization with four introns separating five exons. The gene for GH is located on the long arm

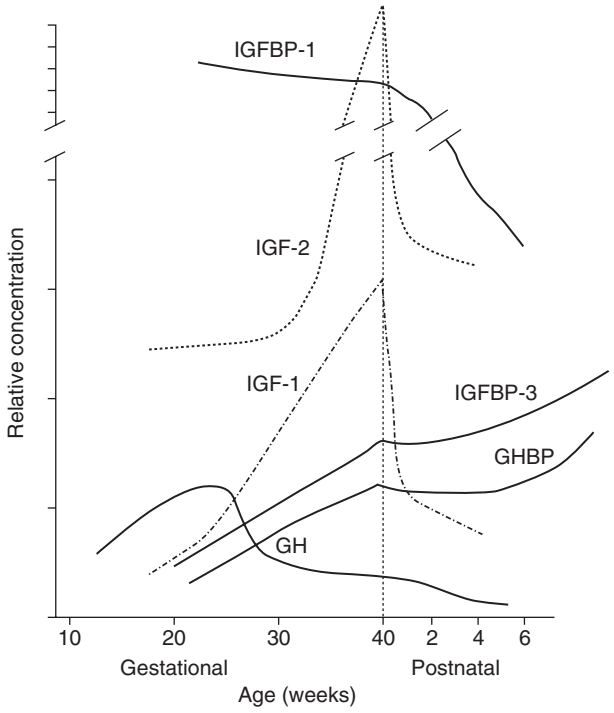


Figure 29-2 Relative concentrations of GH, GHBP, Igfs, and IGFBPs during gestation and perinatally. *(Adapted from Wollman HA,* Horm Res *53[Suppl 1]: 50, 2000.)*

of chromosome 17 in a cluster of five genes with evolutionary relationships: *GHN* codes for human GH (a single 191–amino-acid polypeptide chain with a molecular mass of 22 kDa), *GHV* codes for a variant GH produced in the placenta, *CSH1* and *CSH2* code for prolactin, and *CSHP1* codes for a variant prolactin molecule.[81]

GH is released in a pulsatile pattern in which peaks are rare, which are measurable by most assays and nadirs when only the most ultrasensitive assays can detect GH (e.g., values of 0.04 μg/L). These peaks and valleys of GH are under the control of the two hypothalamic regulatory peptides: GH-releasing hormone (GHRH) a stimulatory peptide also released in a pulsatile manner, and somatostatin (or somatotropin release-inhibiting factor), an inhibitory factor.[82] GHRH contains 44 amino acids, but alternative forms have 40 and 37 amino acids, although the amino terminus of GHRH must be present for biologic activity. GHRH exerts effects by binding to a G protein–related receptor on the pituitary somatotropes, increasing intracellular cyclic adenosine monophosphate (cAMP) production, and subsequently increasing GH synthesis. Somatostatin, however, regulates the timing and amplitude of the pulses of GH but has no effect upon synthesis of GH. In contrast with the action of GHRH on the pituitary, when somatostatin binds to its receptor, it inhibits adenylate cyclase activity and reduces intracellular calcium levels.[83] Pulses of GH secretion occur when a decrease in the release of somatostatin and an increase in the release of GHRH occurs from the hypothalamus; however, when somatotropin release-inhibiting factor is released without GHRH, a nadir of the GH pulse occurs.

A plethora of neurotransmitters regulate the release of these hypothalamic factors including serotonin, histamine, norepinephrine, dopamine, acetylcholine, γ-aminobutyric acid, thyroid-releasing hormone, vasoactive intestinal peptide, gastrin, neurotensin, substance P, calcitonin, neuropeptide Y, vasopressin, galanin, and corticotropin-releasing hormone (reviewed by Reiter and colleagues[84]). The physiologic state also affects

GH secretion including stress, sleep (deep sleep during later childhood), hemorrhage, fasting, hypoglycemia, and, at least in older subjects, exercise. GH-stimulatory tests are employed in the evaluation of GH secretory capacity in neonates if indicated. The secretion of GH is also influenced by a variety of hormones such as estrogen, which in most cases increases basal and stimulated GH secretion (androgens exert their effect through aromatization to estrogens), thyroid hormone, which is essential in allowing GH secretion, and glucocorticoids, which decrease GH secretion when in excess. IGF-1 exerts negative feedback control of GH secretion by interaction with pituitary IGF receptors. Small (6 amino acid) peptides, GH-releasing peptides, regulate GH secretion through pituitary and hypothalamic receptors separate from GHRH and somatotropin release-inhibiting factor. Receptors for ghrelin, a recently discovered GI hormone that stimulates GH secretion, are identified.[85,86] Ghrelin values at term are negatively correlated to postnatal growth as values increase after birth. However, ghrelin values do not correlate with growth in preterm infants.[87]

The cell membrane GH receptor has 620 amino acids and a molecular mass of 70 kDa and contains an extracellular hormone-binding domain, a single membrane-spanning domain, and a cytoplasmic domain. A circulating GH binding protein (GHBP), which has the same amino acid sequence as the extracellular domain of the GH receptor (approximately 55 kDa) is derived from proteolytic cleavage of the extracellular domain of the receptor.[88] The major GHBP in human plasma binds GH with high specificity and affinity but with relatively low capacity because only 45% of circulating GH is bound. Levels of GHBP are low in early life, increase through childhood, and plateau during puberty and adulthood. Patients with abnormalities of the GH receptor (e.g., Laron dwarfism) also have the defect reflected in the serum GHBP concentration in that those with decreased numbers of GH receptors have decreased serum GHBP concentrations.[89] Nutritional state and other factors regulate both GH secretion and GHBP values.

It has long been recognized that cord blood values of GH are higher than in adults[90] and decrease during the 2 weeks to 2 months after birth.[91] Adults exhibit pulsatile GH release, but the difficulty of sampling blood for the analysis of pulsatile GH secretion in neonates has presented technical and ethical difficulties. Recently, safe micro sampling techniques allowing blood withdrawal every 20 to 30 minutes over 12 hours or longer were developed.[92] By using this technique, all infants demonstrate a pulsatile pattern of GH release, but infants, unlike adults, have not yet developed entrainment of the GH secretory rhythm with sleep. At 4 days of age, GH secretion and insulin secretion are correlated, which demonstrates a relationship between feeding and GH secretion.[93] On the first day after birth infants from gestational age 33 to 41 weeks sampled every 20 minutes in 6 hours demonstrated elevated GH (9 to 191 μg/L) released in pulses, with 5 to 6 busts found in 6 hours leading to a pulse periodicity of 73 minutes and a half-life of 18 minutes.[94] This elevation of GH was due to increased secretion rather than decreased clearance. Prolactin was also high but not released in pulses, and prolactin release was not related to the GH peaks. In another study, sampling term infants every 30 minutes for 12 hours demonstrated higher GH pulse frequency and amplitude soon after birth (8 to 40 hours) compared with later (63 to 85 hours), with no difference between boys and girls.[95] Studies of GH secretion at 1 to 13 days after birth revealed higher values of GH in the neonates than in later life, and even higher peaks of GH were found in SGA babies compared to AGA infants.[96-98] Basal mean and peak GH values in AGA and SGA babies were 17 versus 54 and 63 versus 190 μg/L, respectively. The pulse frequency was greater in SGA (5.1 for 12 hours) than in AGA (4.0 for 12 hours), with a pulse periodicity of 180 minutes in AGA and 140 minutes in SGA infants. Premature infants (mean 33 weeks gestation) sampled every 30 minutes for 12 hours at approximately 40 hours after birth had high basal (24 to 26 μg/L), incremental (20 to 24 μg/L), and peak (45 to 51 μg/L) GH values with a frequency of 3 for 12 hours; no difference was seen between males and female infants.[96] Elevated GH is not related to growth rate in premature infants, but persistent elevation of serum GH has been linked to the development of retinopathy of premature infants.[99] Premature infants had higher secretory burst amplitudes, higher production rates, and a higher mass of GH per secretory burst than term infants. The integrated plasma GH concentration exhibited a strong trend toward a higher value in the premature infants.[100] Although serum GH is elevated in SGA infants, values normalized by 1 month of age, and although this elevation was related to the pattern of intrauterine growth, it was not related to postnatal growth.[98] Thus children born SGA have a different profile for GH and IGF-1 from that in infants born at a normal weight; at birth and at 3 days of age, basal GH was high, peak amplitude was low, and frequency of peaks of GH was higher, but serum IGF-1, IGF-binding proteins (IGFBP-3), and leptin were significantly reduced.[25,97,101] When evaluated later in life for GH secretion, however, the children who were SGA at birth showed normal amplitude and normal baseline IGF-I and IGFBP-3.

The bioactivity of GH measured in a Nb2 cell assay changed in a pattern similar to the immunoactive GH with higher values when first measured at 5 days, which decrease by 1 and 4 months in term infants.[102] Urinary GH excretion is higher in SGA and AGA premature and term infants than in older child controls.[103]

Although serum GH is high in the neonate, GH receptors are decreased, as reflected in the lower serum GHBP. An increase in GHBP occurs at 6 months of age, which just predates the phase of increasing IGFF-1 and IGFBP-3 (after approximately 10 months), suggesting a greater GH dependence of GH responsive factors and GH dependent growth in the infant of this age and denotes the end of the infancy phase of growth and the beginning of the childhood phase of growth.[104] Nonetheless, even though infants with congenital GH deficiency are of near normal length at birth, growth rate decreases soon and, even a few months after birth, may descend to more than 3 to 7 SD below the mean.[105] Treatment with GH increases growth rate and catch-up growth proceeds to increase height by 2 years. Recent studies demonstrate that the d3-isoform (genomic deletion of exon 3) of the GH receptor, when present in heterozygous or homozygous patterns, is associated with catch-up growth in SGA infants in the neonate[106] as well as increased responsiveness to GH treatment in non-GH deficient short stature[107] and Turner syndrome. However, adult height is not ultimately increased compared with those who do not have this isoform in patients with severe isolated GH-deficiency treated with GH, suggesting a temporary effect. Remarkably the same isoform is associated with decreased third trimester growth in SGA babies.[108]

Various congenital defects of the pituitary hypothalamic axis lead to deficiency of GH or other pituitary hormones. Defects range from pituitary hypoplasia to other midline defects to holoprosencephaly; any midline defect of the central nervous system area should be suspect as an etiology of hypopituitarism. The pituitary gland resides in the sella turcica near the optic chiasm. A decrease in the size of the sella turcica is an indication of reduced pituitary development. The anterior pituitary and pituitary stalk are often smaller than normal, and a bright spot representing the posterior pituitary is located in the hypothalamus rather than in its normal location. This finding is referred to as an ectopic posterior pituitary. Congenital hypopituitarism is associated with breech birth, forceps delivery, and intrapartum and maternal bleeding. Alternatively a combined defect can be found in the function of the pituitary

gland and visual ability as is found in the syndrome of (septo-) optic dysplasia (OMIM 182230), in which congenital blindness or nystagmus is an indication of potential pituitary defects. This condition is sporadic or due to homozygosity of an inactivating mutation of the *Hesx-1* gene. Although nutrition was always of concern in such cases, 4% of cases of cleft palate or lip are associated with GH deficiency and the prevalence rises to 32% if short stature is associated.

GENETIC FORMS OF HYPOPITUITARISM

Genetic defects account for more than 5% of cases of isolated GH deficiency or combined pituitary hormone deficiencies (reviewed by Parks[109,110]).

Isolated Growth Hormone Deficiency

Autosomal recessive complete deletions of the *GH1* gene have isolated growth hormone deficiency (IGHD) type IA (OMIM 262400). This condition is reported to lead to the development of antibodies to GH after treatment with GH. Birth weight is moderately decreased rather than leading to true SGA. Autosomal recessive IGHD type IB (OMIM 612781) have various sizes and sites of deletions in the GH gene or mutations in the GHRH-receptor gene. Autosomal dominant IGHD type II is caused by mutations in the *GH1* gene. However, the gene responsible for X-linked IGHD type III has not yet been identified; some affected persons have associated immunoglobulin deficiency. In similar terminology, autosomal recessive multiple pituitary hormone deficiency is type I, and the X-linked form is type III (OMIN 262600).

Mutations may be found in the pituitary transcription factors PROP1 (OMIM 601538) (causing luteinizing hormone and follicle-stimulating hormone as well as GH, prolactin, and thyroid-stimulating hormone [TSH] deficiencies and possibly adrenocorticotropic hormone deficiencies in a pattern of recessive inheritance) and POU1F1 (OMIM 173110) (autosomal dominant pattern of GH and prolactin deficiency as well as some degree of TSH deficiency).

Children with Laron syndrome (OMIM 262500) lack GH receptor structure or function[111]; affected children will not grow when treated with GH but must receive IGF-1 treatment. Increased growth was shown in several studies of IGF-1 treatment in such children.[112] Serum GHBP is decreased in those with GH receptor deficiency; others have adequate numbers of receptors, which are inactive or have lesser activity, and these children have normal amount of GHBP.

INSULIN-LIKE GROWTH FACTORS

The effects of GH are mediated mainly by the IGFs (reviewed by Reiter and colleague[84]), originally called sulfation factor, subsequently nonsuppressible insulin-like activity, then somatomedin; see later, but GH also has direct effects such as lipolysis, increased amino acid transport into tissues, and increased protein synthesis in liver. Administration of GH produces a degree of insulin resistance and is a diabetogenic substance, increasing blood sugar. Two IGFs, IGF-1 and IGF-2, share 45 of a 73-amino acids sequence and have structures similar to that of the proinsulin molecule (50% homologous with the A and B chains of insulin with these chains connected by disulfide bonds).[113,114] Both the IGFs and proinsulin have a connecting peptide or a C chain, although the homology is not complete in terms of amino acid structure because the IGF-1 C-peptide region has 12 amino acids and the IGF-2 C-peptide region is 8 amino acids long; neither show homology in amino acid sequence with the C-peptide region of proinsulin. Further, the IGFs have a carboxyl-terminal extension, or D-peptide, unlike proinsulin.

The gene for IGF-1 is located on the long arm of chromosome 12 and contains at least five exons.[115] IGF-1 is produced in most tissues and appears to be exported to neighboring cells to act upon them in a paracrine manner.[116] Thus serum IGF-1 concentrations may not reflect the most significant actions of this growth factor. The liver is a major site of IGF-1 synthesis, and much of the circulating IGF-1 probably originates in the liver; serum IGF-1 concentrations vary in liver disease with the extent of liver destruction. IGF-1 is a progression factor, so that a cell which has been exposed to a competence factor, such as platelet-derived growth factor, in stage G0 of the cell cycle and has progressed to G1 can, with IGF-1 exposure in G1, undergo division in the S phase of the cell cycle.[117] Aside from the stimulatory effects of IGF-1 on cartilage growth, IGF-1 has stimulatory effects upon hematopoiesis, ovarian steroidogenesis, myoblast proliferation and differentiation, and differentiation of the lens.

The IGF-1 cell membrane receptor (the type I receptor) resembles the insulin receptor in its structure of two alpha and two beta chains.[118,119] Binding of IGF-1 to type I receptors stimulates tyrosine kinase activity and autophosphorylation of tyrosine residues in the receptor. This leads to cell differentiation or division or both. The type I IGF receptor messenger RNA is present in almost all tissues except liver. IGF-1 receptors are down-regulated by increased IGF-1 concentrations, whereas decreased IGF-1 concentrations lead to an increase in IGF-1 receptors. The structural similarity of the IGFs and insulin explains why IGFs are able to bind to the insulin receptor and why insulin in excess can bind to the type I IGF receptor (in physiological levels insulin has little binding to the IGF-1 receptor), although the structural differences may explain why insulin cannot bind to the IGFBPs (see later).

IGF molecules may be bound to one of the IGF-binding proteins (IGFBPs), some of which are found in the circulation and some of which are found in tissue.[120] Some IGFBPs are considered to be inhibitory to IGF effects, although there is evidence that some IGFBPs can enhance IGF activity.

IGFBP proteases (for IGFBP 2 to 5) metabolize the IGFBPs by proteolysis, leading to another level of control of the IGF axis. There is evidence that SGA babies have at least two different proteases, which, by digesting IGFBP-3, increase the effectiveness of IGF-1.[121] IGFBP-1 is a 25-kDa protein that inhibits IGF action and is suppressed by insulin but is not affected by GH. IGFBP-1 is present in high concentrations in fetal serum and amnionic fluid. Serum values of IGFBP-1and IGFBP-2 in blood are inversely proportionate to birth weight, suggesting that IGFBP-1 exerts suppressive effect upon fetal growth. This suggests that growth is restricted to some degree by elevated IGFBP-1 but after birth the IGF system becomes more active, increasing the likelihood of catch-up growth.[36] IGF-1 circulates bound to IGFBP-3 and an acid labile subunit in a 150-kDa complex. Serum IGFBP-3 concentrations are directly proportional to GH concentrations as well as to IGF-1 concentrations and are regulated by nutritional status as is IGF-1. Thus, in malnutrition, IGFBP-3 and IGF-1 levels fall while GH rises. IGFBP-3 increases with advancing age throughout childhood, with highest values achieved during puberty.

IGF-2 is a 67-amino-acid acidic peptide coded by a gene on the short arm of chromosome 11, close to the gene for preproinsulin.[115] Information about the function of IGF-2 is limited. The type II IGF receptor preferentially binds IGF-2 and is identical to the mannose 6-phosphate receptor, a single-chain transmembrane protein. Although most of the effects of IGF-2 presently described appear mediated by its interaction with the type I receptor, independent actions of IGF-2 via the type II receptor are described. The ratio of IGF-2 to IGF2-R (a soluble form of the type 2 IGF receptor which exhibits inhibitory effects on IGF-2 action) is directly related to birth weight and is even more closely related to placental weight.[122]

IGF-1 in the fetus is regulated by metabolic factors rather than undergoing significant influence by GH, as is the case in postnatal life (see Chapter 178). One explanation is that there are fewer GH receptors in the fetus than after birth. The GH receptor is first found at about 30 weeks gestation in human fetuses.[123] In the human fetus, serum GH level decreases during later gestation owing to maturation of central nervous system negative control; serum IGF-1 and IGFBP-3 increase during gestation, demonstrating their differing pattern and independence from GH stimulation. Serum GH level in the last half of gestation decreases, but IGF-1 increases, as does IGF-2 and IGFBP-3.[98]

Early studies demonstrated that IGF-1 (then measured as nonsuppressible insulin-like activity) is directly related to gestational age and birth weight.[124] The serum values of IGF-1 are 50% or less of adult values; IGF-2 values are approximately 50% adult values in term neonates. Cord blood IGF-1 values are correlated with birth weight in some but not all studies. IGF-1 concentrations are more highly correlated in monozygotic twins than in same-sex dizygotic twins, indicating a genetic effect upon IGF-1 regulation. Premature infants have lower serum IGF-1 and IGFBP-3, lower GHBP but higher IGFBP-1 and -2 than term newborns; these differences were greater with gestational age.[125] Decreased IGF-2 is also seen in SGA infants. The most severely SGA infants have evidence of increased GH resistance; GH was higher and the GH-dependent growth factors were lower compared with AGA infants in one study of SGA infants.[126] Alternatively, in large-for-gestational-age (LGA) infants, IGF-1, insulin, and IGFBP-3 were increased but IGFBP-1 was decreased.[127] Preeclampsia further lowers cord blood IGF-1 values after correcting for gestational age and for birth weight.[128] IGFBP-1 is elevated in SGA infants and decreased in LGA infants, as is IGF-1. IGF-1 is inversely related to birth weight.[129,130] However, in Spanish infants, IGF-1 and IGFBP-3 had direct relationship to ponderal index, IGF-1, IGFBP-3, and GHBP increased with gestational age and IGFBP-1 decreased.[131]

Some data are available concerning IGF and early postnatal growth. At a mean of 4 days after birth, IGF-1 was higher in AGA than SGA infants, and peak, mean, and incremental GH and IGFB-1 was higher in SGA infants.[93] Measurements during the first week after birth demonstrated the following patterns: IGF-1 decreased in term infants for the first 3 days and then increased at the end of the week, IGF-1 stayed stable in preterm infants, and IGFBP-3 and IGF-2 did not change significantly during this first week.[132]

The pattern of change between birth and 1 to 3 months of age in normal term newborns is one of a decrease in IGFBP-1 and IGFBP-2 and an increase in IGF-2 by 3 months along with a decrease in GH. GHBP values did not change much during this time period in one study[133] but an increase during 30 days is demonstrated in others.[102,125] An increase is demonstrated at 4 and 6 months of age.[102,133] Values of IGF-1 and IGFBP-3 remain relatively low in childhood until a peak is reached during puberty, with values higher than at any other time in life. Because IGF-1 is so low in the neonate, IGFBP-3 values, which are higher in normal neonates, may serve better as an indication of GH deficiency than serum IGF-1. Cord blood IGF-1 correlates with postnatal growth in SGA and AGA infants during the first month.[134] A correlation is found between IGF-1 and IGFBP-3 and catch-up growth for the first year in SGA babies.[135] Some studies show a relationship between catch-up growth and higher IGF-1 and IGFBP-3[98,101,135] and some do not.[121] One study reports a relationship between serum IGF-2 values at 3 months and catch-up growth.[136] IGF-1 and IGFBP-3 are related to length in growing preterm infants on enteral nutrition.[137]

Phosphorylated IGFBP-1 is less active and more inhibitory than nonphosphorylated IGFBP-1. The proportion of nonphosphorylated IGFBP-1 to total IGFBP-1 was higher in infants' cord blood than in mothers' serum, and, although this form did not vary by gestational age, the concentration was lower in SGA infants. However, phosphorylated isoforms were higher in SGA fetuses. Thus the isoforms vary with fetal growth and may be of importance.[138] During an 8-week period after birth, the growth of leg length was related to IGF-1 and rate of increase in weight related to IGFBP-3 values. The ratio of low phosphorylated IGFBP-1 and high phosphorylated IGFBP-1 was correlated with weight growth velocity[139]; this ratio also decreases with advancing gestational age.

Gene manipulation and infusion of growth factors in animals demonstrate the influence these factors exert on fetal growth.[140,141] IGF-1 knockout mice (which produce no IGF-1) have reduced late fetal and postnatal growth as well as diaphragmatic muscular hypoplasia (which ultimately causes their death), organ hypoplasia, and delayed ossification of cartilage.[142,143] IGF-2 knockout mice have an earlier fetal growth deficit than IGF-1 knockout mice even though their growth rate is normal later in gestation.This suggests a role for IGF-2 in growth in early gestation and one for IGF-1 in later gestation. Elimination of type 1 receptors (by gene knockout) leads to a more profound growth failure than is found in IGF-1 knockout mice alone, suggesting that factors other than IGF-1 exert effects upon fetal growth through the type 1 receptor (IGF-2 is one of these factors).

Artificial elevation of maternal IGF-1 can increase growth in mouse and rat fetuses and overcome the restraint imposed by uterine limitations of liter size.[144] Knockout of the maternal gene for the type 2 receptor leads to high birth weight, generalized organomegaly, and other anomalies, usually leading to rapid postnatal death; the type 2 receptor appears necessary for postnatal life.[145,146] Transgenic mice overexpressing IGFBP-1 have smaller birth size, lower birth weight, and poorer postnatal weight gain (as well as more frequent postnatal death) with proportional decrease in the size of all organs except the brain, which is much smaller in proportion to other organs; fasting hyperglycemia and impaired glucose tolerance occurs.[147,148] Early postnatal growth restriction occurs in mice experiencing excess IGFBP-1, suggesting an inhibitory role for IGFBP-1 in growth in late gestation and thereafter. Overexpression of IGFBP-3 in mice led to organomegaly of the spleen, liver, and heart, although birth weight was not different from that in wild-type mice.[147,149] Thus excess IGFBP-1 stunts fetal growth, but excess IGFBP-3 leads to selective organomegaly. IGF-1 exerts prenatal effects in the primate[150]: An increase in spleen, thymus, and kidney weights and an increase in small intestinal length were seen in fetal rhesus monkeys given synthetic IGF-1.

INSULIN

Although insulin is a major regulatory factor for carbohydrate metabolism, many lines of evidence demonstrate its importance in fetal growth as well. Macrosomia is a well-known effect of fetal hyperinsulinism and may be seen in the infant of a mother with diabetes.[151,152] These infants have increased body fat at 1 year[153] and by 10 years of age have a higher prevalence of obesity than that seen in nonmacrosomic infants. At the other end of the spectrum is the SGA infant born to a mother with diabetes with vascular disease, or under extremely tight control, or who has eclampsia or preeclampsia; this demonstrates that limited nutrient delivery compromises the growth of the infant. These SGA infants have long-term metabolic defects and severe hypoglycemia (described in Chapter 18). They have a tendency to develop renal, hepatic, and adrenal tumors and others. Postnatal growth is rapid and tall adult heights are achieved.

Errors in the normal pattern of IGF-2 gene expression from the paternal chromosome and type II IGF receptor for IGF-2 from the maternally derived gene may underlie the pathogenesis of Beckwith-Wiedemann syndrome (OMIM 130650).[154] Affected infants are large and have elevated insulin concentrations.

Just as increased insulin stimulates fetal growth, syndromes of fetal insulin deficiency such as are found in congenital diabetes mellitus, pancreatic dysgenesis, or fetal insulin resistance (e.g., leprechaunism [OMIM 147670]) are characterized by IUGR and decreased muscle and fat mass.[155,156] The ability of insulin to either signal metabolic processes or to stimulate growth seems to relate to the ability of insulin to autophosphorylate insulin receptor substrate-1; gene knockout of the insulin receptor substrate-1 leads to a growth-restricted fetal mouse that is born viable but has fetal and postnatal growth deficiency and hyperglycemia (knockout of insulin receptor substrate-2 leads to diabetes).[157] Insulin values are lower in SGA than in normal subjects, but values are elevated in LGA babies.[132]

Two states of decreased insulin effects are known; congenital absence of insulin in pancreatic aplasia and congenital absence of the insulin receptor as found in Donohue leprechaunism syndrome. Infants lacking insulin receptors have severe postprandial hyperglycemia, as expected from the lack of insulin effect. Remarkably, they have preprandial hypoglycemia at first but fasting hyperglycemia months after birth. They also are able to demonstrate some effects of insulin, such as suppression of ketone formation, and the inverse relationship between insulin and IGFBP-1 is maintained, suggesting some insulin effects upon the liver.[158] GH is low as well in leprechaunism. It is possible that the insulin effects are caused by the extremely elevated insulin concentration acting through the type I IGF receptor. Survival is limited to a matter of months. Other infants with insulin deficiency due to absence of the islets of Langerhans have severe diabetic ketoacidosis in the untreated state[159] and have poor growth, which may be less related to the lack of insulin effects than to the severe illness the children experience.

Recent studies evaluated the effects of insulin infusion in very-low-birth-weight infants.[160] Infants characteristically have decreased serum IGF-1 levels, which has been linked to the development of retinopathy of prematurity: Insulin infusion decreased blood sugar to more normal levels and increased IGF-1, but as yet it is not known whether this will decrease the retinopathy.

LEPTIN

Leptin is produced in adipose tissue and in older individuals is correlated with fat mass, although androgens decrease leptin values so that values are higher in females than in males.[161] Leptin also correlates with body weight and body fat mass in cord blood.[162-164] SGA infants have lower leptin than controls because SGA infants have lower fat mass.[165] This direct relationship to birth weight is independent from IGF-1 control mechanism.[166]

Although SGA and control infants follow a similar pattern of body mass index change during the first year, body mass index is lower in the SGA group. However, the customary relationship between leptin and body mass index is present in the control infants but not in the SGA infants in a period of 2 years; nor was there a sexual dimorphism as is normally found.[167] The higher leptin in SGA suggests a degree of leptin resistance during this period of catch-up growth or an adipocyte defect.

THYROID HORMONE

Thyroid hormone is a major factor in postnatal growth, although, like GH, it has relatively little effect in the growth in length of the fetus. The pattern of thyroid function in the fetus is detailed in Chapter 182. After birth, TSH is acutely released, followed by a rapid decline during the first 24 hours and a slower decline during the next 5 days. The initial peak of TSH causes an increase in serum T_4 and T_3 (owing to increased conversion from T_4) by a few hours after birth. Thus thyroid hormone values change by the hour and day in the neonatal period. During the next weeks after birth, serum T_4 and T_3 decrease.

The effects of thyroxine in the neonate and infant are best reflected in the growth of patients with untreated and treated congenial hypothyroidism. Although endemic goiter due to iodine deficiency is the most common cause of hypothyroidism worldwide, in most of the developed world, anatomic or metabolic defects of the thyroid gland, rather than nutritional causes, prevail. Congenital hypothyroidism is found in approximately 1 of 4000 infants; defects in the development of the thyroid gland (thyroid dysgenesis) account for 90% of infants; approximately one third of those have complete aplasia,[168] although most of the remainder of these neonates have an ectopic location of thyroid tissue. The two interior and hypothalamic deficiency of TSH and thyrotropin-releasing factor (TRF), respectively, were initially thought to be rare—in the range of 140 of 60,000. Recent studies in the Netherlands suggest that the prevalence may be closer to 1 in 20,000. The infusion of TRF is recommended as a diagnostic modality to establish the diagnosis of TRF deficiency.[169,170]

However, TRF has not been available in the United States for several years. Screening programs in the United States utilize TSH levels, looking for elevated levels characteristic of primary hypothyroidism. Children who lack TSH or TRF effects will have decreased TSH and therefore will not be identified by the screening programs; TSH or TRF deficiency is known as secondary or tertiary hypothyroidism, respectively. The genes *TTF-1, TTF-2,* and *PAX-8* are important transcription factors for thyroid development; mutations of the *PAX-8* gene are described in some affected neonates with either hypoplasia or ectopy. Alternatively blocking antibodies may be transferred from the mother that caused the thyroid defect. In addition to the anatomic defects, metabolic defects of thyroid function, usually associated with goiters and usually transmitted in autosomal recessive patterns, cause congenital hypothyroidism. TSH deficiency may be isolated or may occur in cases with *Pit-1* mutations. Infants born with resistance to the actions of thyroid hormone may demonstrate elevated T_4 without euthyroidism and may have growth failure as found in hypothyroidism.

Infants with congenital hypothyroid are of normal length or, because gestation is often prolonged, length might be increased. Careful study of the growth rate demonstrates a decrease in growth in length in the untreated state during the first few weeks of age with profound growth failure lasting as long as the infant is not treated. Analysis of infant growth using the infant-childhood-pubertal chart demonstrated a delay in the onset of the childhood component even if thyroxine therapy is begun early. Thus, an effect of thyroxine is exerted on the onset of the childhood component of growth in the last months of the first year of life.[171] Remarkably, growth is decreased in congenital hypothyroidism compared with normal controls during the first 2 and 4 weeks of thyroid therapy; this demonstrates that the early dependence of neonatal growth on thyroid hormone lasts for weeks after institution of therapy. A greater decrease in growth rate is seen in those infants most deficient in thyroid hormone in the weeks after the onset of therapy.[172]

In addition to a direct effect on epiphyseal cartilage and growth, thyroid hormones have a permissive effect on GH secretion because individuals with hypothyroidism have decreased spontaneous GH secretion and blunted responses to GH provocative tests. A small study of congenital hypothyroid infants demonstrated a decrease in GHBP for the first 6 months of therapy with thyroxine suggesting a degree of GH resistance in congenital hypothyroidism that continues even after replacement therapy.[173] Serum IGF-1 is so low in normal children that patients with congenital hypothyroidism appear to have no decrease in IGF-1 and IGFBP-3 at the time of diagnosis if the diagnosis is made within the first month after birth.[174,175] However, if the

diagnosis is not made early, the values of IGF-1 and IGFBP-3 decrease below control levels. With appropriate therapy with thyroxine, the values of IGF-1 and IGFBP-3 increase to age appropriate levels.[176]

Untreated hypothyroidism starting after the first 2 years of the postnatal period can cause profound growth failure and virtual arrest of skeletal maturation, although intellectual impairment is not permanent. A bone age determination in an infant is performed on radiograph of the foot and leg, which shows calcification of the distal femoral epiphysis at 40 weeks gestation, proximal tibial epiphyses at 38 weeks, and cuboid epiphysis at 36 weeks; an infant with severe intrauterine hypothyroidism will have absence of some or all of them. Treatment with thyroid hormone after a long delay results in rapid catch-up growth, which is typically accompanied by marked skeletal maturation; if doses are excessive epiphyseal fusion may be overly rapid and adult height may be compromised. Epiphyseal dysgenesis is seen when calcification of the epiphyses progresses with thyroxine treatment after initial delay in development.[177] The normal decrease in the upper to lower segment ratio with age is delayed (and therefore this ratio elevated) owing to poor limb growth in hypothyroidism in infancy and childhood.

Congenital hypothyroidism diagnosed at birth and treated appropriately soon thereafter will lead to normal height and weight during infancy. Early treated congenital hypothyroidism is compatible with normal height at 1 year[178] and 3-4 years.[179] If the diagnosis is delayed up to 1 year, the child may still achieve catch-up growth and normal height by 10 months after starting therapy,[179-181] and even if thyroxine therapy is started as late as 2 years of age, catch-up growth in length appears to be possible (although IQ will not be optimal).[182] However, a study from Canada showed an inverse relationship between bone age at birth and length of time until treatment begins and height at 9 years, demonstrating long-term effects of intrauterine hypothyroidism on long-term growth.[183] Adult height is related to genetic potential in early treated congenital hypothyroidism because complete catch-up growth occurs in those treated in the first month of life.[184,185]

The starting dose of thyroxine in neonates is now 10 to 15 μg/kg, higher than previously recommended, although no effect has been reported of this change in recommended dose upon growth velocity.[186] In fact, a review of cohort studies of different doses of thyroxine therapy for congenital hypothyroidism demonstrated no clear trend of the effects of high (10 to 15 μg/kg) versus low dose (4 to 8 μg/kg) thyroxine on growth or cognitive development of behavior.[187] The long-term intellectual development of patients with congenital hypothyroidism treated early is usually normal, although evidence of decreased IQ and abnormal coordination or other neurological manifestations is also found in the literature. In general, the use of the higher replacement doses of thyroxine (10 to 15 μg/kg/day) and maintenance of high normal serum T_4 (upper one half to one third of the normal range) are usually associated with normal IQ and neurological functioning in most studies.[171,186,188-191]

Due to the interruption of the normal development of the hypothalamic thyroid axis, premature infants, especially those with very low birth weights, have low serum thyroxine levels.[192] Some have treated these low levels in the assumption that they are pathologic. Unfortunately no randomized controlled studies prove the efficacy or even the safety of this process. A study followed very-low-birth-weight children treated with thyroxine for 10.5 years for this probable physiologic hypothyroxinemia and demonstrated improvement in school performance and motor function in infants born before 28 weeks; however, worsening of function occurred if treatment was administered to those born after 29 weeks gestation.[193] Clearly, more evaluation is necessary before this therapy can be recommended.

NEONATAL HYPERTHYROIDISM

Neonatal hyperthyroidism is temporary and usually results from the transplacental passage of thyroid stimulating immunoglobulin from the affected mother to the fetus causing a temporary disorder.[194] Infants with congenital hyperthyroidism may manifest IUGR. After birth they have poor weight gain that will tend to limit growth during the temporary hyperthyroid phase.[195,196] In older children with hyperthyroidism, if nutrition is adequate, increased growth may occur but this tendency is overcome by the hypermetabolic state.

SEX STEROIDS

Gonadal sex steroids exert an important influence on the pubertal growth spurt, but absence of these factors is not noticeable in prepubertal growth.[197] But even in early life, gonadal and adrenal sex steroids in excess can cause a sharp increase in growth rate as well as the premature appearance and progression of secondary sexual features. Sex steroids exert a direct effect on long bone growth, mainly through conversion to estrogen, and can increase GH secretion, once GH receptors are present in adequate supply. Untreated virilizing congenital adrenal hyperplasia is compatible with survival, sometimes for years, as long as severe hypoglycemia or shock does not develop; in those children who have achieved such a state of stability, growth acceleration may occur early in infancy; if the adrenopathy also is of the salt-losing type, the failure to thrive will mask any tendency toward increased growth rate, and the child will grow poorly until treatment is supplied or the child dies from hyponatremia, hyperkalemia, and hypoglycemic shock. Familial Leydig and germinal cell maturation may also cause increased growth early in the first year. If unabated, increased sex steroids will cause advancement of skeletal age, premature epiphyseal fusion, and short adult stature. Thus, just as growth deceleration requires evaluation, growth acceleration can be just as abnormal and may be a sign of precocious puberty or virilizing congenital adrenal hyperplasia.

GLUCOCORTICOIDS

Although glucocorticoids are necessary for normal metabolic function, endogenous or exogenous glucocorticoids in excess will quickly decrease or stop growth; this effect occurs more quickly than weight gain. An infant treated with excess glucocorticoids for, say, incorrect diagnosis of congenital adrenal hyperplasia will exhibit a remarkable decrease in growth rate.[198] The absence of glucocorticoids has little effect on growth if the individual is clinically well in other respects (i.e., if hypotension and hypoglycemia are absent).

Cord blood cortisol was inversely related to growth achieved in the first 3 months of life in a preliminary study of normal term infants.[199] When IUGR infants are considered, an inverse relationship between serum cortisol and catch-up growth in the first 6 months of postnatal life is seen.[200]

SOCIOECONOMIC AND NUTRITIONAL FACTORS

Worldwide, the most common cause of poor growth and short stature is poverty and its effects. Thus poor nutrition, poor hygiene, and poor health, including infectious disease, influence growth both before and after birth.

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30

Margit Hamosh

Human Milk Composition and Function in the Infant

Human milk, like the milk of many other mammals, is specifically adapted to the needs of the newborn. Transfer of nutrients and bioactive components from mother to infant occurs through the placenta before birth and in colostrum and milk after birth. The substitution of infant formula for human milk deprives the infant not only of nutrients that are more accessible from human milk than from formula but also of many bioactive and immunoprotective factors directed specifically against pathogens in the infant's environment. In many instances, human milk components compensate for immature function in the newborn, in whom endogenous production of digestive enzymes, immunoglobulin A (IgA), taurine, nucleotides, and long-chain polyunsaturated fatty acids (LC-PUFAs) is insufficient. Many of these components are resistant to pasteurization, indicating that it may be advisable to feed pasteurized human donor milk to infants whose mothers are unable to nurse. The benefits of these bioactive components are the reason for the superiority of human milk to even the best infant formulas.

MILK VOLUME

Milk volume seems to be relatively constant regardless of maternal nutritional status. In general, healthy infants have an average daily intake of 750 to 800 mL milk within the first 4 to 5 months after birth.[1,2] A wide range of intake of volumes, from 450 to 1200 mL/day, has been reported, however.[1,3] Similar findings are reported from developing countries,[1,3] although maternal nutritional status may be subject to seasonal variation and may be less adequate than in industrialized countries. Increasing the intake of fluid does not seem to affect milk volume; therefore lactating women should maintain adequate fluid intake, but they should be aware that "fluids consumed in excess of natural thirst have no effect on milk volume."[1]

MILK COMPOSITION

Table 30-1 presents estimates of the concentration of nutrients in mature human milk (i.e., after the colostrum-producing stage of early lactation). The nutrients in milk have three different sources: some are synthesized de novo within the lactating mammary gland (e.g., medium-chain fatty acids), some are modified within the mammary secretory cells from precursors taken up from the circulation (e.g., long-chain fatty acids incorporated into milk triglycerides), and some are transferred into milk directly from plasma (e.g., vitamins and minerals, which are taken up from the circulation, without modification within the mammary gland). Endocrine changes specific to lactation are known to modulate mammary gland function and milk composition. For example, the marked increase in mammary gland lipoprotein lipase activity and the concomitant sharp decrease in adipose tissue at the onset of lactation[4] (both under the effect of prolactin[5]) result in channeling of dietary fat to the mammary gland for incorporation into milk lipids, rather than deposition in adipose tissue.

Lactose is synthesized within the mammary gland from the precursors galactose and glucose provided from the circulation. Lactose, present at a concentration of 7 g/dL, is the most constant nutrient of human milk and is not affected by maternal nutrition. The protein content of mature human milk is

TABLE 30-1

Estimates of the Concentrations of Nutrients in Mature Human Milk

Nutrient	Amount*
Major Nutrients	***g/L***
Lactose	72.0 ± 2.5
Protein	10.5 ± 2.0
Fat	39.0 ± 4.0
Minerals	
Macronutrient Elements	**mg/L**
Calcium	280 ± 26
Chloride	420 ± 60
Magnesium	35 ± 2
Phosphorus	140 ± 22
Potassium	525 ± 35
Trace Elements	**mg/L**
Chromium	50 ± 5
Copper	250 ± 30
Fluoride	16 ± 5
Iodine	110 ± 40
Iron	300 ± 100
Manganese	6 ± 2
Molybdenum	NR
Selenium	20 ± 5
Zinc	1200 ± 290
Vitamins	
Fat-Soluble	**mg/L**
Vitamin A, in RE	670 ± 200 (2230 IU)
Vitamin D	0.55 ± 0.10
Vitamin E	2300 ± 1000
Vitamin K	2.1 ± 0.1
Water-Soluble	**mg/L**
Vitamin B_6	93,000 ± 8000
Vitamin B_{12}	0.97
Biotin	4 ± 1
Vitamin C	40,000 ± 10,000
Folate	85 ± 37
Niacin	1500 ± 200
Pantothenic acid	1800 ± 200
Riboflavin	350 ± 25
Thiamine	210 ± 35

From Hamosh M, Dewey KG, Garza C, et al: Human Lactation, Vol 2: Maternal and Environmental Factors. In Institute of Medicine: et al: *Nutrition During Lactation.* Washington, DC, National Academy Press, 1991, p 116.

NR, Not reported; *RE,* retinol equivalents.

*Data = means ± SD.

TABLE 30-2

Factors That Affect Milk Fat Content and Composition

Variable	Change
Gestation	LC-PUFA levels higher in preterm* and transitional milk
Lactation	Phospholipid, cholesterol higher in colostrum (preterm > term)
	LC-PUFAs decrease during lactation (3 mo–1 yr) in term milk, but remains constant in preterm milk for 6 months
Parity	P10+: lower endogenous synthesis of fatty acids (C_6-C_{16})
Volume	Low–milk fat concentration associated with high volume
Feed	Fat: foremilk < "midmilk" < hindmilk
Diet	
High carbohydrate intake	Increase in endogenous synthesis of fatty acids (C_6-C_{16})
Low caloric intake	Increase in palmitic acid (C_{16})
High margarine intake	Increase in *trans* fatty acids
Pregnancy weight gain	Positively associated with gain in milk fat content
	Weight loss increase

From Hamosh M: Lipid metabolism in pediatric nutrition, *Pediatr Clin North Am* 42:839, 1995.

LC-PUFA, Long-chain polyunsaturated fatty acid.

**Preterm* or *term* refers to milk or colostrum of women whose infants are born prematurely or at term, respectively.

approximately 0.9 g/dL,[1] and based on studies in industrialized and developing countries, it seems to be unaffected by maternal diet or body composition.[1] Limited information is available, however, indicating that short-term high-protein diets increase the protein and nonprotein nitrogen content of human milk, whereas limited maternal food supply leads to lower milk protein levels.[1]

Most milk proteins participate in the immune and nonimmune protection of the newborn from infection. These proteins, IgA, IgG, IgM, lactoferrin (LF), and lysozyme, have various functions in the newborn.[6] Whether the level of these protective proteins in milk is affected by maternal diet is not clear at present.[7-10]

Although the amount and composition of carbohydrate and protein remain relatively constant in mature human milk, the composition of fat is highly variable and to a large extent is immediately (within several hours) affected by maternal nutrition. Table 30-2 lists the factors that affect milk fat content and composition. Length of gestation and duration of lactation particularly affect the content of lipids that constitute the milk fat globule membrane, phospholipid and cholesterol.[11] Levels of the latter are higher in the early stage of lactation (colostrum and transitional milk) because the milk fat globules are much smaller than in mature milk.[12,13] The total membrane lipid level is therefore higher in colostrum and transitional milk.

LC-PUFAs—C20:4 *n*-6 and C22:6 *n*-3, arachidonic acid (AA) and docosahexaenoic acid (DHA)—are essential for growth, brain development, and retinal function.[14] These fatty acids are stored in the fetus only in the last trimester of pregnancy, and preterm infants depend on human milk for the provision of these essential nutrients. Using measurements of mean melting points of plasma phospholipids as an indicator of fatty acid composition, Holman and colleagues[15] reported lower levels of LC-PUFAs in pregnant and lactating women, a finding suggesting preferential transfer of these essential fatty acids from mother to fetus or to the newborn through milk, even at the cost of possible depletion of maternal reserves. Studies in lactating and suckling ferrets showed that the adipose tissues of the suckling animals contain approximately three-fold higher concentrations of LC-PUFAs than maternal adipose stores.[16] The latter contain, however, only slightly lower levels of LC-PUFA than the adipose depots of virgin females, a finding suggesting that the suckling ferret's higher LC-PUFA adipose tissue reserves result from increased storage of these fatty acids by the suckling newborn.[16]

It has long been known that milk fat content changes drastically during each feeding,[17,18] and milk fat composition is markedly affected by maternal diet.[19] Studies have shown that the mechanism for endogenous synthesis of fatty acids seems to be exhausted in women of high parity,[20] that infants who receive low-fat milk tend to nurse more frequently and for longer periods, thereby causing an increase in milk volume,[21] and that a strong positive relationship exists between weight gain during pregnancy and milk fat content.[22]

As mentioned previously, the vitamin content of human milk depends on the mother's vitamin status, and when maternal

intake of specific vitamins is chronically low, these vitamins are also present at lower levels in the milk. With supplementation of vitamins, levels in milk also increase. Water-soluble vitamins in milk generally are more responsive to maternal dietary intake than are fat-soluble vitamins.[1] The relationship between maternal intake and milk concentration varies among vitamins, however. For example, excess vitamin C does not further increase the level in milk (above that associated with adequate intake), whereas milk vitamin B levels continue to rise with higher intakes; milk folate levels remain normal even at the expense of maternal folate stores and do not decrease until the latter are depleted.[1] Based on the concentrations of fat-soluble vitamins in human milk and infant needs, it is advised that in the United States all newborns should receive 0.5 to 1 mg of vitamin K by injection or 1 to 2 mg orally immediately after birth[2]; infants should receive 5 to 7.5 mg vitamin D per day if exposure to sunlight seems inadequate.

The concentration of trace minerals varies as a function of length of lactation (iron, copper, zinc, selenium). Although the concentrations in milk seem to be independent of maternal nutrition for iron[23, 24] or fluoride,[25] concentrations of manganese,[26] iodine,[27] and selenium[28] are dependent. Iodine is unique among trace elements in that it is avidly accumulated by the mammary gland. It is recommended that breast-fed infants be supplemented with fluoride if the family water supply has a low fluoride content (0.3 ppm). Because of the high bioavailability of iron in human milk, exclusively breast-fed infants do not need iron supplements during the first 6 months of life. When supplementary foods are introduced (as recommended after 4 to 6 months of exclusive breast-feeding), iron should be supplemented in the diet.

It is important to assess not only the concentration of milk components but also the amount delivered to the infant. Thus, although some milk components are present at a higher concentration in colostrum than in milk, the marked differences in volume must be taken into consideration: Colostrum amounts to approximately 100 mL/day, whereas average milk volumes are 750 to 850 mL/day.

The recommendation by the American Academy of Pediatrics is that breast-feeding should be of longer duration, 12 months, as compared with the previous recommendation by the Institute of Medicine of the American Academy of Sciences (4 to 6 months).[1] Therefore the need for proper supplementation after 4 to 6 months of exclusive breast-feeding should be strongly emphasized to patients, especially in view of the resurgence of rickets[29-31] and zinc deficiencies[32] in breast-fed infants. It is known that in some populations, human milk does not provide sufficient vitamin D during the first 6 months of lactation[33] (e.g., persons with dark skin or limited exposure to sunlight)[34-36]; this deficiency can be overcome by provision of vitamin D supplements. Furthermore, the global increase in atmospheric pollution may put mothers and breast-fed infants everywhere at risk of vitamin D deficiency.[37] The report of hypovitaminosis D, in 42% in young black women and 4.2% in the same age-group of white women,[38] emphasizes the importance of supplementation during pregnancy and lactation.

Zinc supplements are indicated for breast-fed infants during the second half of the first year of life if the complementary foods are low in zinc. Furthermore, some infants may require supplementation even during early lactation because of a low milk zinc content secondary to a defect in zinc transfer from maternal serum to breast milk.[39] Although zinc homeostasis and normal milk levels during the first 2 months of lactation in a population with low zinc intake seem to be achieved by high fractional absorption and intestinal conservation,[40] clinical zinc deficiency during breast-feeding is reported to occur because of a combination of low fetal zinc accumulation and insufficient zinc in breast milk.[41,42]

Although iodine is unique among trace elements in that it is widely accumulated by the mammary gland,[1] the iodine content of human milk varies greatly according to geographic region and maternal intake, from a low of 15 mcg/L in areas in which iodine deficiency disorders are prevalent to 150 mcg/L in areas with sufficient iodine.[43] Despite the importance of iodine for normal growth and cognitive development, only a few studies have measured its concentration in human milk. In light of the newest recommendations for iodine (minimum 8 mcg/100 kcal and maximum 35 mcg/100 kcal),[1,24] further studies worldwide are necessary to estimate accurately the daily intakes of iodine by breast-fed infants throughout lactation.

GROWTH OF BREAST-FED INFANTS

The growth of breast-fed infants in affluent populations differs from that of formula-fed ones.[44,45] When evaluated using the 1977 and 2000 National Center for Health Statistics growth charts, breast-fed infants gain weight rapidly during the first 2 to 3 months of life, followed by a downward trend in percentile ranking thereafter.[44,45] The earlier growth charts were obtained by evaluating mainly formula-fed infants, and when breast-fed infants were included, no information on exclusivity or duration of breast-feeding was available. The acceptance of a clear definition of breast-feeding[46] and the availability of newer studies that measured infant growth during breast-feeding for up to 12 to 18 months (breast-feeding exclusively for the first 4 to 6 months, followed by quantified complementary feeding)[44,45] resulted in a reevaluation, led by the World Health Organization (WHO), of the specific growth pattern of breast-fed infants, with the aim of creating specific reference growth curves for these infants.[47]

The major differences in growth patterns between breast-fed and formula-fed infants can be summarized as follows: For weight, breast-fed infants gain less than formula-fed infants during the first year of life (at 12 months, this difference is 600 to 650 g); for length, linear growth seems to be unaffected by type of feeding, with no difference or only slightly lower gain in the breast-fed group; for head circumference, feeding mode makes no difference. Breast-fed infants generally are leaner than formula-fed ones (after approximately 4 months of age), a finding consistent with the observed association between duration of breast-feeding and greater decline in weight-for-length Z-scores during the first 12 months of life.[44,45,48] The physiologic reason for slower weight gain is that breast-fed infants self-regulate their energy intake[1,44,45,49,50] at a lower level than that observed in formula-fed infants. This lower activity may be related to the lower body temperature and metabolic rate of breast-fed infants.[51] It also may be associated with the different endocrine environment of breast-fed as compared with formula-fed infants,[49,51] which could be affected by the marked differences in protein content of human milk and infant formula.[51] The question of whether formula feeding (which is regulated by the mother) leads to a higher incidence of obesity than that associated with breast-feeding (which is infant-regulated) is still open, although a difference in adiposity by mode of feeding persists at least until the age of 18 to 24 months, with overweight infants amounting to 13% versus 35% of those fed human milk versus formula-fed groups, respectively.[44]

The slower weight gain documented with breast-feeding does not seem to be associated with short- or long-term deleterious effects. A recent metaanalysis on the topic indicates that mortality rates from infectious diseases are lower in breast-fed infants, and cognitive development seems to be enhanced by this mode of feeding. Furthermore, only a modest protective effect of breast-feeding on childhood obesity has been documented.[52] Indeed, reduced morbidity and long-term health benefits of breast-feeding may become apparent only in childhood

and adolescence.[53] The 12th International Conference of the International Society for Research in Human Milk and Lactation (in 2004, in Cambridge, United Kingdom) was devoted to the topic "Breast Feeding: Early Influences on Later Health."[54]

BIOACTIVE MILK COMPONENTS

This section focuses on milk nutrients that protect the infant against infection. Support for the relatively immature state at birth of the human infant derives in part from the mother's milk, engineered to provide many components that facilitate the postnatal development of specific organs such as the brain, the immune system, and the digestive system. During this critical period when the newborn is exposed to infectious agents, protective factors in the mother's milk are essential to the infant's health. Provision of digestive enzymes facilitates the digestion and absorption of nutrients, and the provision of specific nutrients ensures normal development of the brain and other organs. Thus what is unique to human milk, and so far has not been duplicated, is the bioactive function of many human milk nutrients.

Many nutrients in human milk have to be considered multifunctional agents. First, they provide an essential bioactive function; subsequently, they are digested and used as nutrients.

Protection Against Microorganisms

The antiinfective properties of human milk were recognized millennia ago (as in the ancient Indian medical treatise *Sushruta Samhita*), but only recently has the rationale for this protection from infection (i.e., the immaturity of the immune system in the newborn and the reciprocal relationship between protection by human milk and the postnatal development of the newborn's immune system) been explored extensively. Furthermore, in addition to providing protective agents, human milk components also modulate the development of immune function in the newborn. Indeed, the thymus of breast-fed infants is twice as large as that of formula-fed infants, and a dose-dependent response of thymus size to human milk intake has been reported.[55]

Earlier studies carried out mainly in developing countries that showed protection against diarrhea, infectious diseases, and otitis media from breast milk were found to be methodologically flawed.[56,57] In the 1990s, however, carefully designed studies confirmed without any doubt that breast-feeding protects the newborn from diarrhea, respiratory infections, and otitis media, not only in developing countries (where inocula may be higher)[58] but also in industrialized nations.[59-61] Furthermore, early observations that if infection occurs, it is less severe in breast-fed than in formula-fed infants,[1] have been complemented by studies showing that breast-feeding may protect against nosocomial infections.[62]

Ongoing research since the 1980s has started to elucidate the nature of the protective factors in milk, as well as the mechanisms by which this protection is provided. These studies have shown that protection from infection is provided by two main mechanisms: classic immune protection provided by IgA, IgG, and IgM in human milk, as well as by many other milk components that act as ligands for bacteria and viruses; and factors that refine the interaction among these agents and that also may enhance the maturation of the infant's own immune potential. The protective agents in milk share several characteristics that enable them to be active in the infant[63]: They act at mucosal sites and are well adapted to resist the environment of the gastrointestinal tract (hydrolytic enzymes, changes in gastric and intestinal pH, presence of bile salts). Furthermore, action on microorganisms often is accomplished synergistically, and protection is achieved without triggering inflammatory reactions. As is the case for many milk components, secretion of many protective factors during lactation is inversely related to the infant's capacity for their release at mucosal sites.

Immunoglobulins

The protective role of immunoglobulins is well reviewed in the literature.[64,65] Briefly, whereas IgG, IgM, and IgD are present in human milk and may protect both the lactating mammary gland and the newborn from infection, polymeric IgA is the main component of human milk (consumption by the infant is 4 g on day 1 and approximately 1 g/day after day 4 postpartum). In addition to the resistance of IgA to proteolysis, the functional advantages of IgA are the presence of four to eight antigen-binding sites, carbohydrate moiety–mediated antiadherence proteins, and inhibition of complement activation. Although the protective effect of IgA is exercised mainly at mucosal surfaces, internalization by cells expressing various IgA receptors suggests additional protective functions.[66]

Nonimmune Protection

Nonimmune protection is provided by many components in human milk that act in a nonspecific way and thus provide a broad spectrum of antiinfective activity (Tables 30-3 and 30-4). These components are reviewed only briefly here, because many excellent reviews on this topic are available in the literature.[53,64-72]

Proteins

Lactoferrin (LF), a 79-kDa, 692–amino acid, single-chain glycosylated protein, consists of two globular lobes, each containing one iron-binding site (Table 30-5). Although this protein was once considered an iron transporter and its antiinfective

TABLE 30-3

Multiple Functions of the Major Nutrients of Human Milk in the Infant

Nutrients	Amount	Function
Protein	***mg/dL***	
SigA	50-100	Immune protection
IgM	2	Immune protection
IgG	1	Immune protection
Lactoferrin	100-300	Antiinfective, growth-promoting, iron carrier (?)
Lysozyme	5-25	Antiinfective
α-Lactalbumin	200-300	Induces tumor apoptosis, part of lactose synthase, ion carrier (calcium)
Casein	200-300	Ion carrier, inhibits microbial adhesion to mucosal membranes, growth factor for probiotics
Carbohydrate	***g/L***	
Lactose	6.5-7.3	Energy source
Oligosaccharides	1.0-1.5	Microbial ligands
Glycoconjugates		Microbial and viral ligands
Fat	***g/L***	
Triglyceride	3.0-4.5	Energy source
Long-chain polyunsaturated fatty acids		Essential for brain and retinal development and for infant growth
Free fatty acids, monoglycerides*		Antiinfective

*Free fatty acids and monoglycerides produced from triglycerides during fat digestion in the stomach and intestine.

function was ascribed to its high affinity for iron (which could deprive microorganisms of needed iron), more recent studies cast doubt on both functions[73,74] and showed that LF-associated protection is provided by several other mechanisms. LF is bactericidal, antiviral, and antiinflammatory, and it modulates cytokine function. The bactericidal activity is provided by an 18–amino acid residue loop formed by a disulfide bond between cysteine residues 20 to 37 of human LF and 19 to 36 of bovine LF. This domain, called lactoferricin and located in the N-terminus of LF in a region distinct from its iron-binding site, has broad antimicrobial properties.[75] Partial gastric hydrolysis of LF actually enhances antiviral activity against herpes simplex virus (HSV), cytomegalovirus, and human immunodeficiency virus (HIV), and it occurs during the early stage of infection, probably at the stage of virus adsorption or penetration. Immunomodulating activity leads to reduced release of interleukins 1, 2, and 6 and tumor necrosis factor–α from monocytes and of prostaglandin E_2 from macrophages. This activity is the result of LF binding to LF receptors on these cells.[76] Other LF activities include activation of natural killer cells, modulation of complement activation, effects on coagulation, and inhibition of adhesion of enterotoxigenic *Escherichia coli*[77] and of adhesion and invasiveness of *Shigella flexneri.* The last is caused by LF-induced disruption of outer membrane virulence proteins required for invasion (IpaB) and for intracellular and extracellular spread (IcsA).[78] Antiinflammatory activity involves scavenging of iron, which inhibits iron-catalyzed formation of hydroxyl radicals from superoxide and hydrogen peroxide,[77] inhibition of lipopolysaccharide-mediated activation of neutrophils,[79] and endotoxin-induced cytokine release.[80] Large fragments of LF are present in the stool[81] and urine[82] of breast-fed infants, suggesting that protection is provided within the gastrointestinal tract and systemically. Enhancement of neonatal intestinal growth,[83,84] hepatic protein synthesis, intestinal recovery from injury[83,84] (associated with avid LF interaction with nucleic acids),[85] and stimulation of growth of probiotic intestinal bacteria are additional LF-mediated mechanisms to reduce intestinal infections and the development of protein allergy.[86]

Lysozyme, a 14.4-kDa, 130–amino acid containing glycoprotein, hydrolyzes the β1-4 linkage between *N*-acetyl glucosamine and *N*-acetylmuramic acid in bacterial walls. Lysozyme, like LF, is present in other exocrine secretions and lyses gram-positive and a few gram-negative bacteria. In constrast with the other protective proteins in human milk (IgA and LF), concentrations of which decrease during lactation, those of lysozyme increase with prolonged lactation. Concentrations in milk are higher than in serum. In addition, lysozyme concentrations are severalfold higher in human than in bovine milk.[69,87]

κ-*Casein,* a 30- to 40-kDa, 162–amino acid, highly glycosylated human milk protein, has been shown to inhibit adherence

TABLE 30-4

Protective Functions of Minor Nutrients in Human Milk

Immune Protection	Function
IgA, IgG, IgM, IgD	Specific antigen-targeted antiinfective activity
Nonspecific protection	Antibacterial, antiviral, and antimicrobial toxins; enhance newborn's immune system maturation
Major nutrients	See Tables 30-1, 30-3, and 30-5
Minor nutrients	
Nucleotides	Enhancement of T cell maturation, NK cell activity, antibody response to certain vaccines, intestinal maturation and repair after diarrhea, growth factors for probiotics
Vitamins	
• A (β-carotene)	Antiinflamatory (scavenging of oxygen radicals)
• C (ascorbic acid)	Antiinflamatory (scavenging of oxygen radicals)
• E (tocopherol)	Antiinflamatory (scavenging of oxygen radicals)
Enzymes	
Bile salt–dependent lipase	Production of free fatty acids with antiprotozoan and antibacterial activity
Catalase	Antiinflamatory (degrades hydrogen peroxide)
Glutathione peroxidase	Antiinflamatory (prevents lipid peroxidation)
PAF acetylhydrolase	Protects against necrotizing enterocolitis (hydrolysis of PAF)
Hormones	
Prolactin	Enhancement of the development of B and T lymphocytes, affects differentiation of intestinal lymphoid tissue
Cortisol, thyroxine, insulin, and growth factors	Promotion of maturation of the newborn's intestine and development of intestinal host defense mechanism
Cells	
Macrophages, PMNs, and protective agents	Microbial phagocytosis, production of lymphokines and cytokines, interaction with and enhancement of other lymphocytes
Cytokines	Modulation of function and maturation of the immune system

Modified from Hamosh M: *Pediatr Clin North Am* 45:53, 2001.

IgA, IgG, IgM, IgD, Immunoglobulins A, G, M, D; *PAF,* platelet-activating factor; *PMNs,* polymorphonuclear leukocytes.

TABLE 30-5

Protective Function of Human Milk Proteins

Protein/Normal Levels	Protective Function
Lactoferrin 500-600 mg/dL in colostrum, 150 mg/dL in mature milk	Iron chelation: Bacteriostatic for siderophilic bacteria and fungi Lactoferricin, 18–amino acid residue loop with broad-spectrum antimicrobial action Antiviral activity (HIV, CMV, HSV), probably by interfering with virus adsorption or penetration Immunomodulating activity: reduced release of IL-1, IL-2, and IL-6 and TNF-α from monocytes and of prostaglandin E_2 from macrophages; activation of NK cells, effect on complement activation and coagulation Antiadhesive for *Escherichia coli* and antiinvasive for *Shigella flexneri* Affects neonatal intestinal growth and recovery from injury, thereby reducing intestinal infections
Lysozyme 5-25mg/dL; increases with prolonged lactation	Bacterial lysis: hydrolysis of β1-4 link between *N*-acetyl-glucosamine and *N*-acetylmuramic acid in bacterial walls Immunomodulating activity: muramyl dipeptide enhances IgA production, macrophage activation Binding to bacterial lipopolysaccharides: reduces endotoxin effect
α-Lactalbumin 200-300mg/dL	Causes apoptosis in tumor cells after structural change induced by association with oleic acid
κ-Casein <100 mg/dL	Antiadhesive: Inhibits binding of *Helicobacter pylori* to human gastric mucosa and *Streptococcus pneumoniae* and *Haemophilus influenzae* to human respiratory tract epithelial cells
Casein macropeptide	A strong growth-promoting factor for the probiotic *Bifidobacterium bifidum*

CMV, Cytomegalovirus; *HIV,* human immunodeficiency virus; *HSV,* herpes simplex virus; *IL,* interleukin; *NK,* natural killer; *TNF–α,* tumor necrosis factor–α.

of *Helicobacter pylori* to human gastric mucosa and of *Streptococcus pneumoniae* and *Haemophilus influenzae* to human respiratory tract epithelial cells. The C-terminal proteolysis product of κ-casein is a strong growth-promoting factor for *Bifidobacterium bifidum,* an acid-producing anaerobe that reduces the growth of intestinal pathogenic microorganisms in breast-fed infants.[69] The genes for the foregoing human milk proteins have been cloned.

α-Lactalbumin and Apoptosis of Malignant Cells

α-Lactalbumin is present in the mammary gland and in human milk and is part of the lactose synthase complex. Studies showed that it also may prevent growth of human adenocarcinoma cells in culture.[86] Detailed structural studies showed that a partial unfolding of the protein and association with oleic acid result in a variant able to induce apoptosis in tumor cells and immature cells, whereas healthy cells are resistant to this effect.[89] Conditions in the newborn's stomach favor formation of this complex, which may explain the lower incidence of cancer in persons who were breast-fed as infants.

Other proteins with potentially protective functions in human milk are fibronectin, protectin, and components of the complement system.[90] The extent to which milk proteins provide protection to the breast-fed newborn has yet to be quantitatively evaluated, although good data are currently available for immunoglobulins and LF. Whether supplementation of infant formula with recombinant human milk proteins will have protective effects remains to be evaluated in the future.

Most proteins in human milk are heavily glycosylated[87] and are therefore resistant to proteolysis after ingestion by the infant,[69,72] as well as after short-term storage (for 4 to 24 hours) at elevated ambient temperatures (15° to 38° C).[91]

Glycoconjugates and Oligosaccharides

These milk components act as ligands for microorganisms, viruses, and their toxins and thereby inhibit binding of pathogens to epithelial surfaces (Table 30-6). These components are present in the milk fat globule membrane and in skim milk and amount to 1.5 g/dL. Thus they are the third most abundant components in human milk. Among the best-known components in this group are mucin-1 (400 kDa)[92] and lactadherin (46 kDa)[72]; mucin decreases adhesion of *S*-fimbriated *E. coli* to buccal epithelial cells,[82] and lactadherin inhibits replication of rotavirus in vitro[93] and protects infants against symptomatic rotavirus infection.[94] Other glycoconjugates in milk inhibit binding of the HIV envelope glycoprotein gp 120 to its host cell receptor CD_4 (chondroitin-rich glycosaminoglycan in the milk fat globule membrane),[95] and they act as receptor analogues for *Vibrio cholerae* and *E. coli* heat-labile toxins, as well as for *V. cholerae* El Tor and enterotoxigenic *E. coli.* Oligosaccharides in human milk prevent the attachment of *H. influenzae* and *S. pneumoniae* to the respiratory epithelium.[69]

No significant digestion of milk oligosaccharides occurs during intestinal transit, with resulting excretion of 40% to 50% of intact milk oligosaccharides in the breast-fed infant's stools.[96,97] The presence of these oligosaccharides in urine[97,98] suggests that they provide protection not only in the infant's intestinal tract but also systemically.[97-99] The extent of fucosylation of oligosaccharides, which affects the binding and inhibition of bacteria, is controlled by fucosyl transferase and fucosidases present in human milk at different levels of activity in the course of lactation.

Milk glycolipids such as the ganglioside Gb_3, the receptor of the β subunit of Shiga toxins, are present in the cream fraction (the milk fat globules) of human milk. Shiga toxin–associated diarrhea and the more serious complications of hemolytic uremic syndrome are caused by *Shigella dysenteriae* and some strains of *E. coli* (O157:H7 and O11:NM). Protection against this disease may be accomplished by the combined action of Gb_3 and specific IgA antibodies.[100]

TABLE 30-6

Protective Functions of Oligoconjugates in Human Milk

Structure	Distribution in Milk	Function*
Oligosaccharides	Skim milk	Protect against heat-stable *Escherichia coli* enterotoxin, attachment of *Haemophilus influenzae* and *Streptococcus pneumoniae* to respiratory epithelium, *Vibrio cholerae* hemagglutinin activity
Glycoproteins	MFGM/skim milk	Prevent binding of *V. cholerae* El Tor
Mucin	MFGM	Prevents binding of *S*-fimbriated *E. coli*
Lactadherin	MFGM/skim milk	Prevents binding of rotavirus
Gangliosides	MFGM	Receptor analogues for heat-labile toxins of *V. cholerae* and *E. coli*
Glycosaminoglycan	MFGM	Inhibits binding of HIV glycoprotein g 120 to CD4 receptor

Modified from Hamosh M: *Pediatr Clin North Am* 45:53, 2001.
HIV, Human immunodeficiency virus; *MFGM,* milk fat globule membrane.
*Most of the listed activities are based on in vitro studies; however, lactadherin prevents rotavirus-caused diarrhea in mice and human infants.

Lipids

Milk fat globules, the second most abundant component of human milk, protect the infant from infection by two separate mechanisms. As noted earlier, the membrane glycoconjugates act as specific bacterial and viral ligands, whereas the digestive product of the core triglycerides, free fatty acids, and monoglycerides have a detergent-like lytic action on enveloped viruses, bacteria, and protozoa[101,102] (Table 29-7). Although products of lipolysis are qualitatively similar in formula-fed and breast-fed infants, quantitatively the breast-fed infant benefits from higher rates of gastric lipolysis[102] and duodenal lipolysis associated with the structure of milk fat globules and the presence of milk bile salt–dependent lipase, respectively.[102] Hydrolysis of milk triglyceride during storage at 4° to 38° C probably contributes to resistance to bacterial and viral growth in milk.[91] Indeed, addition of lipases or free fatty acids and monoglycerides to human milk or formula increases antiviral (respiratory syncytial virus, HSV-1) and anti–gram-positive microbial (*H. influenzae,* group B streptococci) activity.[69,90,103] Antiprotozoal activity against *Giardia lamblia* was shown to be directly related to the release of free fatty acids from milk triglycerides by bile salt–dependent lipase and lipoprotein lipase.[69]

The structural determinants associated with the highest level of microbial and protozoal lysis are fatty acid chain length (C12:0), degree of unsaturation (C18:2), and nature of the active group (highest with carboxyl-containing lipids, i.e., fatty acids). Lauric acid (C12:0) and linoleic acid (C18:2) amount to 5% and 15% of total fatty acids in human milk, respectively, and are the most readily released during gastric lipolysis.[69]

Antiinflammatory Components

Antiinflammatory components in human milk that consist of antioxidants (e.g., vitamins A, C, and E and enzymes such as catalase and glutathione peroxidase) are reviewed in detail in the literature.[67,104] Therefore they are not discussed here.

TABLE 30-7

Protective Functions of Lipids in Human Milk

Feature	Example(s)
Structural determinants of antiinfective activity	Free fatty acids and monoglycerides, most active: lauric acid (C12:0) and linoleic acid (C18:2)
Role of digestion	Milk triglycerides (98% of milk fat) have to be hydrolyzed in the stomach* and intestine to free fatty acids and monoglycerides†
Antiinfective function	Antiprotozoal: *Giardia lamblia* Antimicrobial: *Haemophilus influenzae,* group B streptococci, *Staphylococcus epidermidis* Antiviral: respiratory syncytial virus, herpes simplex virus 1

Modified from Hamosh M: *Pediatr Clin North Am* 45:53, 2001.

*Gastric lipolysis of milk triglycerides is higher in human milk–fed infants (25%) than in those who are formula-fed (14%).

†Human milk bile salt–dependent lipase enhances intestinal fat digestion in breast-fed infants.

Cells in Human Milk

The topic of cells in human milk[64,65] has been studied extensively and reviewed in the literature, so it is not discussed here.

Nucleotides in Human Milk

Nucleotides are considered conditionally essential nutrients because of their insufficient synthesis by the newborn.[105] Nucleotides present in human milk differ qualitatively and quantitatively from those in bovine milk and are another agent shown to enhance intestinal repair after injury[106] and to potentiate the immune response to certain vaccines.[107] Certain nucleotides also may promote the growth of *Lactobacillus bifidus,* which is known to suppress the growth of enteropathogens in the newborn's intestine.

Enzymes in Human Milk

Human milk, like the milk of other species, contains many enzymes[87,101]; however, the specific enzymes and their activity levels vary among species. Only those milk enzymes that may benefit the human infant are considered here. Among these enzymes are those involved in protective function (see earlier) such as lysozyme (see Table 30-3), peroxidase, the antiproteases, catalase, glutathione peroxidase, and platelet-activating factor acetylhydrolase (see Table 30-4). Milk enzymes have been shown to aid in the digestion of carbohydrates and fats during the period of pancreatic exocrine immaturity, which results in low pancreatic lipase levels and the absence of amylase for up to 2 months after delivery at term.[87,108,109] Milk bile salt–dependent lipase and amylase are secreted into human milk and have been shown to be active in the infant,[87,108,109] thereby providing the breast-fed premature and full-term babies with better digestive potential (Table 30-8) than that of formula-fed infants.

Probiotics and Prebiotics

Prebiotic and probiotic substances have received much attention as potential protective factors. *Probiotics* consist of viable organisms that have a beneficial effect on intestinal microbial balance. Human milk feeding stimulates the growth of lactobacilli and bifidobacteria, lactic acid–producing microorganisms that generate an intestinal milieu inhibiting the growth of pathogens such as *E. coli* and *Clostridium difficile.*[86] *Prebiotics* are nondigestible food ingredients that enhance the growth or metabolic activity of certain bacteria in the colon. For example, *L. reuteri* produces reuterine, a bacteriocin with broad antimicrobial activity.[86]

TABLE 30-8

Enzymes With Digestive Function in the Infant

Characteristic	Bile Salt–Dependent Lipase	Amylase
Maternal factors		
Parity (=10)	?	Low activity
Diurnal and within feed activity	Constant	Constant
Secretion		
Prepartum*	Present	?
Postpartum	Constant	Decreases before first month
Preterm versus term delivery†	Equal activity	Equal activity
Milk characteristics		
Distribution	Skim milk	Skim milk
Storage (−20° C, −70° C)	Stable years	Stable years
Pasteurization (62° C, 30 min)	Inactivated	85% of activity maintained
Storage (15-38° C)	Stable (at least 24 hr)	Stable (at least 24 hr)
Stable at low pH (stomach)	>3.0	>3.0
pH optimum	7.4-8.5	6.5-7.5
Enzyme characteristics	Identical to pancreatic cholesteryl ester lipase	Isoenzyme of salivary amylase
Evidence of activity in infants	Yes	Yes
Present in milk of other species: primates, carnivores, rodents		?

Modified from Hamosh M: *Pediatr Clin North Am* 45:53, 2001.

†Infants delivered preterm (26 to 36 weeks gestation) and term (37 to 40 weeks gestation.)

*Prepartum: 2 to 3 months secretion.

BREAST-FEEDING AND ATOPIC DISEASE

In contrast with the protective effect of breast-feeding against infectious diseases in infants,[53-65] the relationship between breast-feeding and infant allergy is poorly understood.[111] It seems that allergy-preventing effects, if present, may be limited to infants with a genetically determined increased risk of atopic disease.[111] Deleterious effects of breast-feeding also have been reported; thus an abnormal ratio between LC-PUFAs of the *n*-6 and *n*-3 series in mother's milk is associated with the development of allergic disease in infants during the first year of life.[112] Furthermore, although breast-feeding is associated with lower rates of wheezing illnesses in infancy, this effect varies according to maternal asthma status. In children of asthmatic mothers, the development of childhood asthma increases significantly with duration of exclusive breast-feeding.[113] Exclusive breast-feeding and development of cow's milk allergy also have generated controversy. Studies showed that although early feeding of cow's milk increases the risk of allergy development, exclusive breast-feeding does not seem to reduce the risk. [114]

LATE EFFECTS OF BREAST-FEEDING

Among the late-onset diseases that may be affected by the mode of infant feeding are diabetes mellitus, cancer, inflammatory bowel disease, and celiac disease.[53,115] Controversy is ongoing regardingt the specific role of breast-feeding in the origin of these diseases, which are known to have multiple causes, including environmental factors (e.g., early feeding pattern) and genetic susceptibility.[53,115-117] The apoptosis-inducing effect of modified α-lactalbumin[89] lends support to the epidemiologic data indicating lower incidence of certain forms of cancer in children who were exclusively breast-fed during infancy.[115]

The controversy about the role of breast-feeding in protection against celiac disease[53] seems to have been resolved by studies showing that the gradual introduction of gluten-containing foods into the diet of infants while they are still being breast-fed reduces the risk of celiac disease in early childhood and probably also subsequently.[118]

The controversy about whether breast-feeding protects against obesity in later life remains unresolved.[49,119,120] This issue was one aspect of the previously mentioned conference dealing with the effect of infant feeding modality (breast milk or infant formula) on later health (such as obesity, sensory perception, type 2 diabetes, cardiovascular disease, hypertension, and some cancers arising later in life).[54]

Human milk contains a number of growth factors or hormones that affect food intake and energy regulation, such as leptin,[130-132] ghrelin,[133,134] adiponectin,[135,136] and motilin.[137] These factors are present in the milk secreted by women whose infants were born at term as well as prematurely and may affect appetite regulation, development of gastrointestinal function, and growth. Whether these early effects are expressed in later life and contribute to differences in metabolism of adults who were exclusively breast-fed as infants, as compared with those who were formula-fed, is currently unknown.

Breast-Feeding, Long-Chain Polyunsaturated Fatty Acids, and Cognitive Function

LC-PUFAs are essential for normal growth and development.[121-123] The two families of LC-PUFAs, the *n*-3 derived from α-linolenic acid and the *n*-6 originating from linoleic acid, have specific functions. DHA (22:6n3) has an important role in retina and brain development, and AA (20:4n6) is the precursor of prostaglandins, leukotrienes, and other eicosanoids. These highly unsaturated fatty acids affect many aspects of membrane function including membrane order, permeability, and lipid-lipid as well as protein-lipid interactions. Specific interactions of membrane proteins with particular lipids may in turn affect receptor function, enzyme activities, signal transduction, and membrane excitability. For the developing infant, DHA is of major importance because it is the major PUFA in cerebral cortex and retinal membranes. The photoreceptor outer segments of the retina contain very high concentrations of DHA, and most molecular species contain at least one DHA moiety. Retinal DHA is important for normal photochemical activity of the visual pigment rhodopsin.

LC-PUFA requirements in the newborn are a major focus of research.[121-129] The reason for this intense interest is based on several observations. Accretion of LC-PUFAs occurs primarily during the last trimester of pregnancy, thereby making the preterm infant particularly vulnerable to LC-PUFA deficiency. Plasma levels of LC-PUFAs decrease markedly after birth in formula-fed preterm infants, as compared with nearly constant levels in breast-fed infants of comparable gestational age, and brain levels of DHA are lower in formula-fed than in breast-fed full-term infants.

Studies indicate that the newborn can synthesize LC-PUFAs from essential fatty acid precursors; however, the extent of de novo synthesis seems to be insufficient to meet demand at this age.[122-124] Postnatally, human milk provides LC-PUFAs to the newborn. Maternal LC-PUFA reserves depend on diet and can be improved by supplementation of DHA and AA during pregnancy and lactation. This, in turn, affects fetal LC-PUFA accretion and postnatal provision through mother's milk. Supplementation of formula-fed preterm or full-term infants with DHA and AA leads to plasma and red blood cell LC-PUFA levels similar to those in breast-fed infants.

Studies conducted in larger numbers of formula-fed infants clearly showed that full-term infants do not benefit from supplementation with DHA and AA,[123-125] whereas definite visual and cognitive benefits are found in preterm infants.[126] Indeed, the Life Sciences Research Office's assessment of nutrient requirements for term infant formulas prepared for the U.S. Food and Drug Administration (FDA) did not recommend supplementation of formulas for term infants with DHA or AA.[126] DHA and AA are now supplemented in preterm human formulas.

In this context, the effect of breast-feeding, to provide the infant with mother's milk containing the LC-PUFAs DHA and AA, is supported by numerous studies in infants as well as studies showing that 80% of the FAs in retinal photoreceptors is DHA. Furthermore, recent studies have shown that DHA may affect photoreceptor signal transduction.[138] The beneficial effect of breast-feeding on visual function has led to recommendations for supplementation of infant formulas with both DHA and AA. The role of DHA in brain function is much less understood. It is known that DHA is present in high concentration in neural gray matter, and that it regulates genes involved in synaptic plasticity and the functions derived from the latter.[127] Low brain DHA is associated with lower cytochrome *c* oxidase activity and glucose uptake[138]; the latter could have important functional consequences because of the high metabolic activity of the developing brain.[127] Low DHA activity in the developing brain also has been shown to affect dopamine and serotonin metabolism,[140,141] which in turn can affect cognitive function during early development.

The effect of mode of infant feeding on subsequent cognitive function has been investigated for the past 80-some years[142-154] (Table 30-9). IQ and motor development measured during infancy, in early or later childhood, or even in adulthood showed that feeding breast milk rather than formula has beneficial effects on the infant. The effects were small, however, especially with correction for confounding factors such as maternal age and education, socioeconomic status, and birth order. More recent studies indicate a relationship between duration of breast feeding and IQ in adolescence and adulthood. It is interesting that breast feeding for more than 9 month is associated with a decrease in IQ levels. Whether this is due to lack of proper nutritional complementation during prolonged breast feeding is not known.

Although cognitive benefits may be associated with DHA in human milk, the latter may affect the former through additional mediators such as growth factors, especially nerve growth factor (NGF) in human milk and with other components. Furthermore, breast-feeding also may be an indicator of material involvement in the infant's well-being and development not only during infancy but well beyond it. Well-designed, prospective studies are needed to ascertain the exact role of human milk and its components on cognitive development and function.

RECENT FINDINGS OF INTEREST

Although much of the research conducted since publication of the previous edition of this book has been rather repetitive of the seminal work carried out in 1970s to the 1990s, several more recent reports on various aspects of breast milk function and composition warrant mention.

Atopic disease: Studies have continued on the link between higher LC-PUFA *n*-6:*n*-3 ratio and eczema or asthma in the offspring, often with attention to parental, especially maternal allergies and asthma,[155-159] as well as the effect of growth factors and cytokines in maternal milk on allergy in infants.[160]

Trans *fatty acids:* Because of the detrimental effect and their known presence in human milk,[161,162] research has focused on the effect of lower consumption of these fatty acids by lactating women on their level in breast milk. Recent studies show that *trans* fatty acids in human milk have indeed decreased in Canada, where their amount in food has to be listed.[163] These fatty acids are still found at much higher levels in human milk in the United States[164] and Canada[163] than in Europe[161] or especially Africa,[165] however.

TABLE 30-9

Effects of Breast-feeding or DHA Supplementation on Cognitive Function

Study	Duration of Breast-Feeding*	Effect (IQ Points)	Age at Assessment
Hoefer and Hardy, 1929[141†]	4-9	Higher IQ	7-13 years
Menkes, 1977[142]		Less breast-feeding in children with learning disability Higher IQ (small difference)	
Fergusson et al., 1982[143]		Higher IQ (small difference)	5-7 years
Rodgers, 1978[144]		Higher IQ (small difference)	8-15 years
Morrow-Tlucak and Hande, 1988[145]	4 months	Higher IQ (small difference)	1-2 years
Florey et al., 1995[146]		Higher IQ (small difference)	18 months
Rogan and Gladen, 1993[147]		Higher IQ (small difference)	
Lucas et al., 1992[148‡]		Higher IQ (7-8 point difference)	7.5-8 years
Lanting et al., 1994[149]		Higher IQ	9 years
Horwood and Fergusson, 1998[150§]		Higher IQ	18 years
Gale and Martyn, 1996[151§]		Higher IQ	
Mortensen et al., 2002[152†]	1-9 months	Higher IQ (7 points)	adults
Helland et al., 2003[153‖]		Higher IQ (4 points)	4 years
Gustafsson et al., 2004[154¶]		Higher IQ	6.5 years

DHA, Docosahexaenoic acid; *IQ,* intelligence quotient; *LPC,* lysophosphatidylcholine.

*If duration is not indicated, breast-feeding lasted for several months.

†Breast-feeding duration longer than 9 months was associated with the poorest cognitive development.

‡Preterm infants were gavage-fed expressed human milk.

§Increase in IQ related to length of breast-feeding.

‖Maternal supplementation with *n*-3 LPC during pregnancy and lactation; higher IQ attributed to exposure to higher *n*-3 LCP level during pregnancy.

¶Controlled study; milk long-chain polyunsaturated fatty acid (LC-PUFA) assay was performed at 1 and 3 months of lactation, and correlation between total IQ and verbal and performance IQ was measured. Length of breast-feeding contributed significantly to total IQ.

Free amino acid concentration: Amino acid levels have been shown to be high in human milk,[166] especially that of glutamic acid,[167,168] so breast milk is a good source of nitrogen for the nursing infant.

Iodine: Ongoing controversy debates whether iodine is currently higher than that previously reported in human milk[169] or lower because of the effect of perchlorate.[170]

Vitamin K: This nutrient is still found to be present in insufficient amounts in human milk.[171,172]

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31 Physiology of Lactation

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Unlike most other organs in the body the mammary gland achieves its mature function, the secretion of milk, only after a developmental program that takes place in the adult, guided by reproductive hormones. Formation of the glandular structure of the breast, however, is initiated during embryonic development and reaches maturity during puberty. We first describe development of the functional anatomy of the mammary gland through puberty and pregnancy to its completion several days after parturition when milk assumes mature composition. We next describe the functional changes that take place during the first week post partum and that transform a prepared, but nonsecretory, gland into a fully functioning organ. Most lactation problems arise during this period. This section is followed by a description of the synthetic and secretory processes that are integrated during lactation to produce milk and the physiologic processes that regulate milk production and its ejection through the ducts and out the nipple. We conclude with a discussion of the effects of lactation on the mother.

OVERVIEW OF THE FUNCTIONAL ANATOMY OF THE BREAST

The human female breast is composed of a tubuloalveolar parenchyma embedded in a connective and adipose tissue stroma. The glandular component of the mature breast is composed of radially organized lobes, each of which is connected to the nipple by a single milk duct. Each lobe is composed of multiple lobules containing the alveolar structures responsible for milk production and an associated network of small ducts that join to form larger ducts that drain into the milk duct. Most textbooks describe the mature breast as containing 15 to 20 lobes on the basis of cadaver dissections; however, more recent ultrasound measurements put this number at approximately 9, with some individuals having as few as 4 and others as many as 14.[1] Figure 31-1 depicts the developmental origin of the lobuloalveolar units (referred to as *terminal duct lobular units* by pathologists) starting with embryogenesis, which begins in the 18- to 19-week fetus, at which time a bulb-shaped mammary bud can be discerned extending from the epidermis into the dense subepidermal mesenchyme (see Figure 31-1, *A*).[2] A nipple forms in the epidermal portion of the structure, and ducts invade the fat-pad precursor, branching and canalizing to form the rudimentary system that is present at birth.[2,3] Limited milk secretion occurs in the infant after birth because of changes in maternal hormones. The immature gland remains as nipple with a set of small branching ducts that grow in parallel with the child.

At puberty, estrogen[4] and a pituitary factor that is probably a growth hormone[5] stimulate the further elongation and branching of the mammary ducts. In early puberty, bare ducts course through the fat pad (see Figure 31-1, *B*). With the onset of menses and ovulatory cycles, the progesterone secreted by the ovary during the luteal phase brings about some lobular development as shown in Figure 31-1, *C*. The lobular clusters are dynamic structures that increase in size and complexity during each luteal phase but tend to regress with the onset of the menses and the loss of hormonal support.[4]

The hormones of pregnancy bring about expansion and functional differentiation of alveoli. In addition to increasing levels of progesterone, a lactogenic hormone, either prolactin or human placental lactogen, possibly aided by growth hormone, is thought to be essential for the final stages of mammary growth and differentiation.[6] By midpregnancy, the gland has developed extensive lobular clusters (see Figure 31-1, *D* and *E*), and, indeed, small amounts of secretion product are formed. However, differentiation continues until parturition, with full secretion being inhibited by the high circulating concentrations of progesterone. Secretory activation represents the onset of *copious* milk secretion and takes place during the first 4 days post partum. A major volume increase that occurs around 40 hours post partum is often referred as the *coming in* of the milk. Both the fall in progesterone, resulting from loss of the placenta, and maintenance of prolactin secretion from the pituitary gland are necessary for milk secretion.

Lactation is the process of milk secretion; it will continue as long as milk is removed from the gland on a regular basis. In women prolactin is required to maintain milk secretion, and oxytocin is required to produce the let-down that allows the infant to extract milk from the gland. An example of the swollen ducts and alveoli that occur when milk is not removed from the gland is shown in Figure 31-1, *E*. The actual volume of milk secreted is adjusted to the requirements of the infant by as yet unidentified local factors that may be activated by stretching and flattening of the mammary epithelial cells.

The later parts of this developmental program are repeated during each pregnancy because the glandular epithelium responsible for the secretion of milk regresses after weaning to a state resembling that of the prepregnancy condition. Involution takes place when regular extraction of milk from the gland ceases or, in many but not all species, when prolactin is withdrawn. Like lactogenesis this stage involves an orderly sequence of events that include cessation of milk secretion, apoptosis of the mammary epithelium,[7] and remodeling of the gland, which return it approximately to a prepregnant state.

SECRETORY ACTIVATION

The most critical time in the establishment of lactation is its onset during the transition from pregnancy to lactation, a period we now call *secretory activation* (previously termed *lactogenesis stage II*). Secretory activation takes place after birth in women, in contrast to many animal species where it occurs concomitantly with parturition.[6] Most lactation problems arise during this period as the result of stress of childbirth or problems with the mechanics of suckling. If unresolved these problems can lead to lactation failure.

Milk Volume and Composition During the First Week Post Partum

Figure 31-2, *A,* shows milk volumes in 11 American women who weighed their infants before and after every feeding for the first week post partum. Although there is wide variation among individuals, the trend is revealed by the averaged data in Figure 31-2,

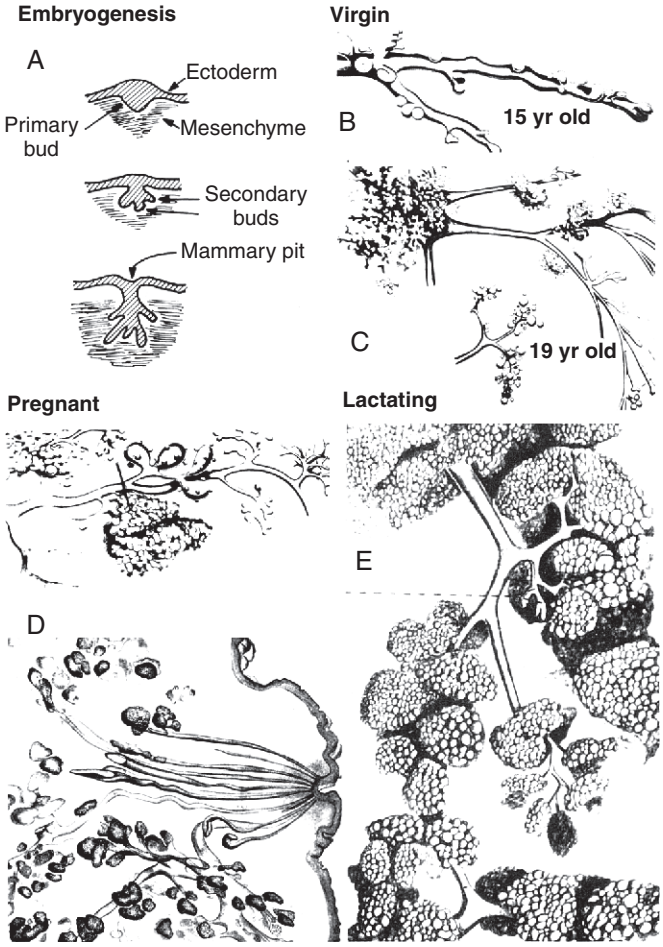


Figure 31-1 Human mammary development. A, A thickening of the ectoderm called the placode pushes branches into the underlying stroma to produce the rudimentary mammary gland with the nipple present at birth in both sexes. **B,** At the onset of puberty, ductal structures branch and elongate into the fat pad. **C,** With the onset of the menstrual cycles, progesterone secretion during the luteal phase brings about budding of alveolar complexes called terminal duct lobular units from the sides and ends of the ducts. **D,** The terminal duct lobular units undergo extensive development during pregnancy. **E,** This picture of the lactating gland was drawn from a section of the breast of a woman who died 2 days after last suckling her infant. Note that each alveolus is engorged with milk. *(The camera lucida drawings of sections of the human breast are from Dabelow A: Die postnatale Entwicklung der menschlichen Milchdruse und ibre Korrelationen,* Morphol J *85:361-416, 1941. With permission for publication from Springer-Verlag.)*

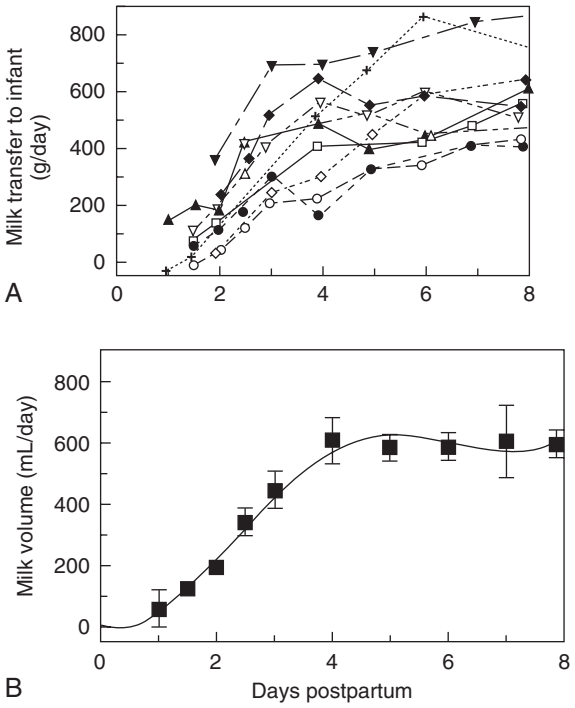


Figure 31-2 Milk volumes during the onset of lactation in American women. A, Eleven multiparous women weighed their infants before and after every feeding for 7 to 8 days post partum. Milk output was averaged by 0.5-day intervals for the first 3 days and then daily for the remainder of the experiment. All women had successfully breast-fed at least one previous infant. Note the extensive variation in the volumes of milk produced. All these women breast-fed successfully for at least 6 months. **B,** Mean output in the women shown in **A.** *(Plotted from data in Neville MC, Keller RP, Seacat J, Lutes V, Neifert M, Casey CE, et al.: Studies in human lactation: milk volumes in lactating women during the onset of lactation and full lactation,* Am J Clin Nutr *48:1375-1386, 1988. Graphs from Neville MC: Lactogenesis in women: a cascade of events revealed by milk composition. In Jensen RD [ed]:* The Composition of Milks. *San Diego, Academic Press, 1995, pp 87-98. Used by permission.)*

B: volumes of breast secretion of less than 100 mL per day characterize the first 2 days post partum, after which a rapid increase in milk volume leads to production of an average of 600 mL/day by the fourth day post partum. A detailed study of milk composition over this period reveals a carefully programmed cascade of events that leads to a mammary secretion product with a composition close to that of mature milk. Although the secretion product in the first few days is usually called *colostrum*, we avoid that term here because it implies a secretion product with a fixed composition. In fact, as we shall see, milk composition changes dramatically during the first 3 to 4 days post partum.

The first compositional change to occur after delivery is a fall in the sodium and chloride concentrations in the milk and an increase in lactose levels (Figure 31-3, *A*). These modifications commence immediately after delivery and are largely complete by 72 hours post partum.[6] They precede the rapid increase in milk volume by at least 24 hours and can be explained by closure of the tight junctions that block the paracellular pathway during lactation. With the paracellular pathway closed, lactose, produced by the epithelial cells, can no longer pass into the plasma, and sodium and chloride can no longer pass directly from the interstitial space into the milk space and must be secreted by the cellular route.

The next changes to occur are increases in the concentrations of serum immunoglobulin (Ig) A and lactoferrin (see Figure 31-3, *B*).[8] The concentrations of these two important protective proteins remain high for the first 48 hours post partum, the two together comprising as much as 10% by weight of the milk before they decrease rapidly after day 2 post partum, as a consequence of both dilution as milk volume increases and an actual decrease in the rate of secretion, particularly in the case of Igs. By day 8 post partum, these protective proteins together comprise less than 1% of the total weight of the milk; however, their secretion rate is still substantial, amounting to 2 to 3 g/day for each protein. Concentrations of oligosaccharides in milk are also high in

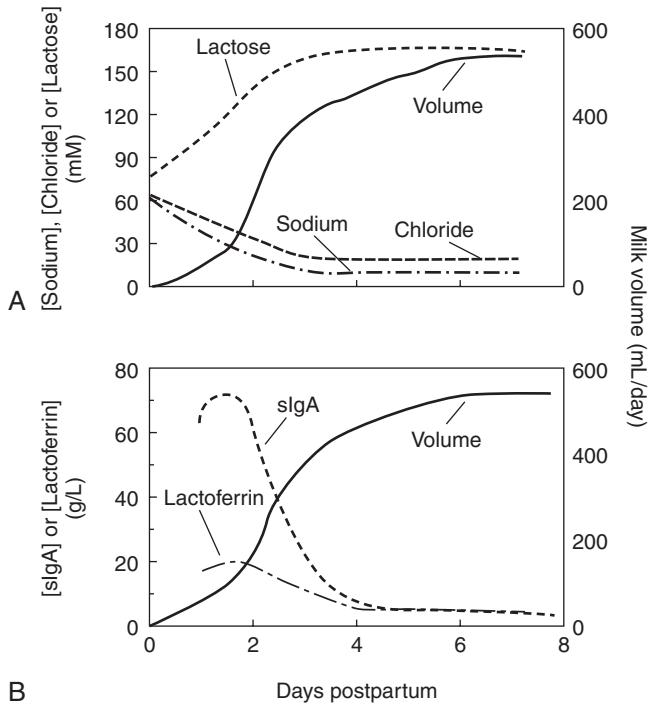


Figure 31-3 Changes in the concentration of certain milk components during the first week post partum. A, Time course of changes in the lactose, chloride, and sodium concentrations contrasted with the mean milk volume transfer to the infant. Changes in these milk components begin immediately post partum and are complete at least 24 hours before achievement of a steady state in milk volume. As described in the text, the decrease in sodium and chloride and increase in lactose concentration reflect closure of the tight junctions between the mammary epithelial cells. *(Data from Neville MC, Allen JC, Archer PC, Casey CE, Seacat J, Keller RP, et al: Studies in human lactation: milk volume and nutrient composition during weaning and lactogenesis.* Am J Clin Nutr *54:81-92, 1991.)* **B,** Changes in the concentration of secretory IgA and lactoferrin during the onset of lactation in women. *(Data replotted from Dils R, Clark S, Knudsen J: Comparative aspects of milk fat synthesis. In Peaker M [ed]:* Comparative Aspects of Lactation*. London, Academic Press, 1977, pp 43-55. Figure modified from Neville MC, Keller RP, Seacat J, Lutes V, Neifert M, Casey CE, et al:* Pediatr Clin North Am *48:35, 2001. Used by permission.)*

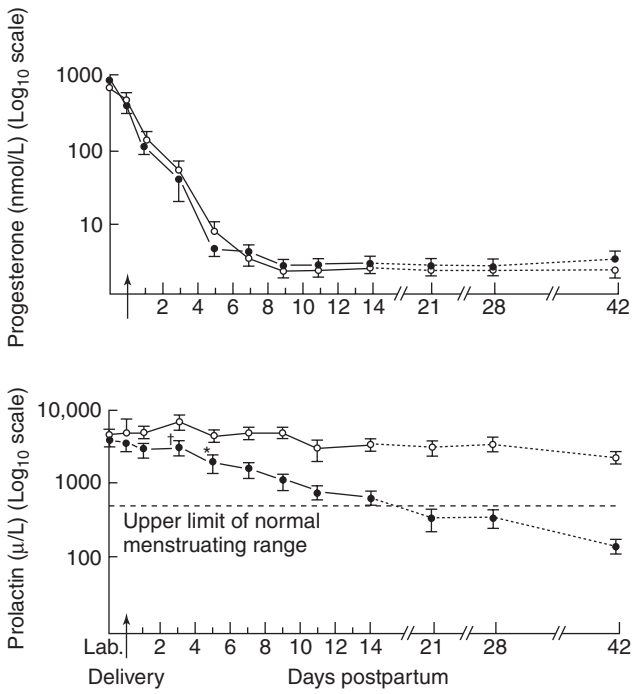


Figure 31-4 Maternal hormone levels after parturition in breast-feeding and non–breast-feeding women. Breast-feeding subjects (n = 10; *open circles*); non–breast-feeding subjects (n = 9; *filled circles*); $p = .01$. *(From Martin RH, Glass MR, Chapman C, Wilson GD, Woods KL, et al: Human alpha-lactalbumin and hormonal factors in pregnancy and lactation,* Clin Endocrinol *13:223, 1980. Reprinted with permission.)*

the early secretion product of the mammary gland, amounting to as much as 2% of milk weight on day 4 post partum. These complex sugars are also considered to have substantial protective effect against a variety of infections including human immunodeficiency virus.[9-11]

Finally, 36 to 48 hours post partum, milk secretion begins in earnest (see Figure 31-3). This volume increase is perceived by the parturient woman as the coming in of the milk and reflects a massive increase in the rates of synthesis and secretion of almost all the components of mature milk including but not limited to lactose, protein, lipid, calcium, sodium, magnesium, and potassium.

Hormonal Requirements for Secretory Activation

It has been clear for nearly three decades that the major inhibitor of milk production during pregnancy is progesterone.[6] Once birth occurs, a developed mammary epithelium, the continuing presence of high levels of prolactin, and a fall in progesterone are necessary for the onset of copious milk secretion. The levels of these hormones are shown in Figure 31-4. Evidence is clear for the inhibitory effect of progesterone during pregnancy in women. Thus removal of the placenta, the source of progesterone during pregnancy in this species, has long been known to be necessary for the initiation of milk secretion.[12] Conversely, retained placental fragments with the potential to secrete progesterone have been reported to delay secretory activation.[13] The conundrum that progesterone does not inhibit established lactation was solved when Haslam and Shyamala[14] showed that progesterone receptors are lost in lactating mammary tissues.

In most species high levels of plasma prolactin also appear to be essential for secretory activation; although the level declines with continued lactation some prolactin is thought to be necessary for active milk secretion. In women bromocriptine and other analogues of dopamine, drugs that effectively prevent prolactin secretion, inhibit the onset of lactation when given in appropriate doses.[15] Conversely, an older hypothesis that a prolactin surge is the trigger for secretory activation is probably incorrect. Although a biphasic rise in prolactin is associated with the stress of parturition in women,[16] it precedes the onset of copious milk secretion by 2 days.

Delays in Secretory Activation

A delay in the onset of milk secretion is a significant problem for the initiation of breast-feeding in a large number of women. For normal women the timing of secretory activation has been determined carefully from milk volume or composition in a few studies of middle-class white women.[6] Most of these data are in reasonable accord, but some do suggest that either parity

or previous lactation experience may influence the timing of secretory activation. Various pathologic conditions have been reported to delay secretory activation in women including cesarean section, diabetes, obesity,[17] and stress during parturition.[18] The role of cesarean section is controversial, no effect having been found in a large study from the laboratory of Hartmann[19]; a small effect, statistically significant, was found in a more recent study where breast fullness and the appearance of casein in the secretion were measured.[18]

Women with poorly controlled diabetes, studied in both the United States[20] and Australia,[21] often had a delay in secretory activation. In a recent, well-controlled study, stress during parturition, accompanied by an increase in cord blood glucose level, had the same result, and milk production on day 5 post partum was significantly decreased. Another cause of delayed milk secretion, obesity, has come to our attention only recently. In studies reviewed by Rasmussen and associates,[17] women with a high prepartum body mass index had more difficulty initiating lactation. Is there a common thread in all these conditions? Poorly controlled type I diabetes is associated with higher blood glucose level. Both type II diabetes and obesity can be associated with increased plasma insulin level. It is not clear how exposure to increased glucose or insulin during pregnancy could alter the onset or course of lactation, but these factors require serious investigation in the future.

CELLULAR MECHANISMS FOR MILK SYNTHESIS AND SECRETION

Milk components are secreted into the alveolar lumina by five distinct pathways as illustrated diagrammatically in Figure 31-5.[22] Four secretory processes are synchronized in the alveolar cell: (1) exocytosis, (2) fat synthesis and secretion, (3) secretion of ions and water, and (4) transcytosis of immunoglobulins and other substances from the interstitial space. These processes are summarized briefly here, followed by an introduction to the role of a fifth pathway, the paracellular pathway, in determining the composition of the mammary secretion.

Exocytosis

Most components of the aqueous phase of milk are secreted by the exocytotic pathway (see pathway I, Figure 31-5). The basic mechanisms of exocrine secretion, worked out in the pancreas more than 40 years ago by Palade,[23] are thought to apply essentially to all exocrine cells including milk-secreting cells. Like the exocytotic pathway in all cells, exocytosis in the mammary gland ultimately begins in the nucleus with the synthesis of messenger RNA molecules specific for milk proteins. The mature messenger RNA serves as a template for protein synthesis. The protein molecules are transported into the endoplasmic reticulum where they are folded and transported through the Golgi system to take part in secretory vesicle formation. In the Golgi, molecules destined for release are packaged in vesicles as a highly condensed granule matrix visible in the electron microscope as electron-dense structures. Distinct mechanisms appear to exist for concentrating proteins in secretory granules.[24] Pancreatic exocrine granules, for example, exhibit pH-dependent aggregation of granular proteins and require Ca^{2+} for stability, whereas caseins, the major type of protein found in milk, are packaged into electron-dense granules as micellar aggregates that are cross-linked by colloidal calcium phosphate.[25] Once formed, secretory vesicles move to the apical membrane where they discharge their contents into the alveolar lumen by exocytosis. Several specialized reactions within this pathway give the composition of milk its final form. For example, in addition to secretion of milk proteins such as casein, lactose and the oligosaccharides, which together comprise almost 8% of the weight

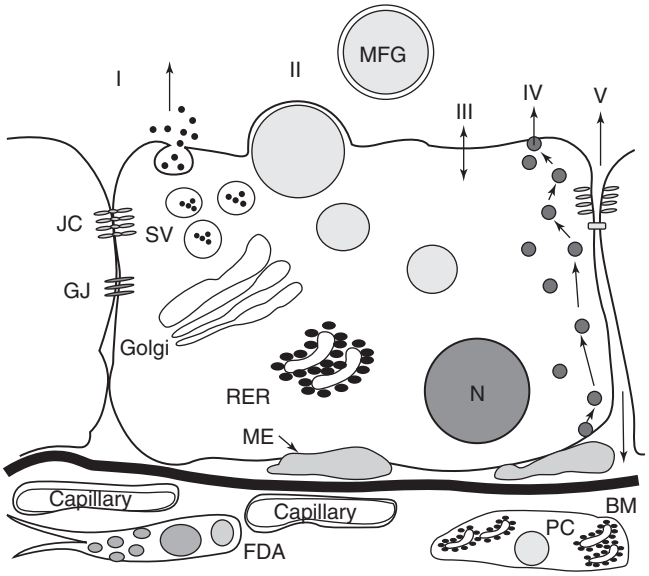


Figure 31-5 Cartoon of a mammary epithelial cell showing pathways for milk secretion. *Pathway I,* Exocytotic pathway for secretion of milk proteins, lactose, calcium, and other components of the aqueous phase of milk. *Pathway II,* Milk fat secretion with formation of cytoplasmic lipid droplets that move to the apical membrane to be secreted as a membrane-bound milk fat globule (MFG). *Pathway III,* Transporters for the direct movement of monovalent ions, water, and glucose across the apical membrane of the cell. *Pathway IV,* Vesicular transcytosis of proteins such as immunoglobulins from the interstitial space. *Pathway V,* The paracellular pathway for plasma components and leukocytes. Pathway V is open only during pregnancy and involution and in inflammatory states such as mastitis. *SV,* Secretory vesicle; *RER,* rough endoplasmic reticulum; *BM,* basement membrane; *N,* nucleus; *PC,* plasma cell; *FDA,* fat-depleted adipocyte; *JC,* junctional complex containing the tight and adherens junctions; *GJ,* gap junction; *ME,* myoepithelial cell. *(Redrawn from Neville MC, Allen JC, Watters C: The mechanisms of milk secretion. In Neville MC, Neifert MR:* Lactation: Physiology, Nutrition and Breast-Feeding. *New York, Plenum Press, 1983, p 50. Used by permission.)*

of human milk, are synthesized and secreted by this pathway. In mammary glands of most species, lactose is an important regulator of the osmotic movement of water into milk and maintenance of isoosmolarity. Lactose is synthesized in the Golgi from uridine diphosphate–galactose and glucose, which enter from the cytoplasm, by the enzyme β-galactosidase with α-lactalbumin acting as a cofactor. The high concentration of lactose present in the Golgi during lactation osmotically stimulates the influx of water that contributes to the fluidity of milk.[24] The importance of this mechanism in the maintenance of milk fluidity was established by the observation that α-lactalbumin–deficient mice produce highly viscous milk.[26]

Lipid Synthesis and Secretion

The majority of lipid in milk exists as triacylglycerols (98%).[27] Triacylglycerols are synthesized from precursor fatty acids and glycerol by enzymes of the smooth endoplasmic reticulum membrane of the mammary alveolar cell. They then undergo assembly into protein and phospholipid–coated structures referred to as cytoplasmic lipid droplets.[28] After transport to the apex of the cell (see pathway II, Figure 31-2), these droplets become enveloped in apical plasma membrane and are

secreted into milk as plasma membrane bilayer–coated structures (milk fat globules) by a process that is distinct from the classical secretory pathway used for lipid secretion by hepatocytes and enterocytes.[24] The occasional inclusion of a crescent of cytoplasm within the membrane-bound globule[29] enables any substance contained in the cytoplasm to enter milk. The membrane surrounding the milk fat globule is the primary dietary source of phospholipids for the breast-fed infant and serves to prevent the triglyceride globules from coalescing into large fat droplets that might interfere with milk removal. In addition this membrane may enhance the uptake of lipid and lipid-soluble vitamins by the neonatal intestine.[30]

Transport of Small Molecules Across the Apical Membrane

Unlike other pathways for milk secretion, the pathways for the direct transport of substances across the apical membrane of the mammary alveolar cell are poorly understood (see pathway III, Figure 31-5). Linzell and Peaker[31] devised a clever technique to determine which molecules could use this pathway, infusing isotopes of small molecules up the teat of a goat and calculating how much of the substance left the milk and entered the blood. They found that sodium, potassium, chloride, and certain monosaccharides, as well as water, directly permeated this membrane, but calcium, phosphate, and citrate did not. Although the mechanisms are not understood, it is clear that apical membrane transport pathways are limited to a modest number of small molecules that also include glucose and possibly amino acids. Recently it has become clear that calcium is also transported across the apical membrane by a well-studied transporter called PMCA 2, a plasma membrane calcium adenosine triphosphatase.[32]

Transcytosis of Interstitial Molecules

Intact proteins in the interstitial space surrounding the basal and lateral surface of the mammary alveolar cell can cross the mammary epithelium in two possible ways: by transcytosis and through the paracellular pathway. Because the paracellular pathway is closed during lactation, plasma proteins must enter milk through transcytosis (see pathway IV, Figure 31-5). The best-studied molecule in this regard is IgA.[33] IgA is synthesized by plasma cells that reside in the interstitial spaces of the mammary gland or elsewhere in the body. Dimeric IgA binds to receptors on the basal surface of the mammary alveolar cell, after which the entire IgA-receptor complex is endocytosed and transferred to the apical membrane, where the extracellular portion of the receptor is cleaved and secreted together with the IgA. The many proteins including serum albumin, insulin, prolactin, IgF1, and possibly other growth factors that find their way into milk from the plasma are also thought to be secreted by a similar, but much less well-studied, pathway.

The Role of the Paracellular Pathway in Milk Secretion

Pathway V (see Figure 31-5) involves passage of substances between epithelial cells, rather than through them, and for this reason is designated the *paracellular pathway.* During lactation the passage of even low-molecular-weight substances between alveolar cells is impeded by a gasketlike structure called the *tight junction* that joins the epithelial cells tightly, one to another.[34] Although immune cells apparently can elicit diapedesis between epithelial cells to reach the milk,[35] the junctions seal tightly behind them and leave no permanent gap. During pregnancy, and probably mastitis, these tight junctions are leaky and allow components of the interstitial space to pass unimpeded into the milk. At the same time, milk components can enter the plasma. This leakiness is useful during these periods, inasmuch as secretion products are allowed to leave the gland and inflammatory cells can enter. It is not clear when junctions open during involution, but when milk volume drops below approximately 400 mL/day, the concentration of sodium and chloride in the mammary secretion increases suggesting a change in the permeability barrier.[36] When the junctions are open, mammary secretions have a high concentration of sodium and chloride, a fact that is sometimes useful in diagnosing breast-feeding problems.[37]

REGULATION OF MILK SYNTHESIS, SECRETION, AND EJECTION

Milk is synthesized continuously and secreted into the alveolar lumen where it is stored until it is ejected from the breast by a let-down reflex. This means that two levels of regulation must exist: regulation of the rate of synthesis and secretion and regulation of milk ejection. Although both processes ultimately depend on sucking by the infant or other stimulation of the nipple, the mechanisms involved, both central and local, are very different. Prolactin mediates anterior pituitary regulation of milk secretion, but its influence is greatly modified by local factors that depend on milk removal from the breast. Oxytocin, on the other hand, participates in a neuroendocrine reflex that results in stimulation of the myoepithelial cells that surround the alveoli and ducts. When these cells contract, milk is forced out of the alveoli to the nipple. Only then does it become available to the suckling infant. If the let-down reflex is inhibited, milk cannot be removed from the breast and local mechanisms bring about an inhibition of milk secretion. With consistent partial removal of milk, these local factors adjust milk secretion to a new steady-state level.[38] If milk removal ceases altogether, involution sets in and the gland loses its competency to secrete milk within days to weeks depending on the species.

MILK VOLUME PRODUCTION IN LACTATING WOMEN

A metaanalysis of the volume of milk secreted by exclusively breast-feeding women showed that milk volume at 6 months post partum is remarkably constant at about 800 mL/day in populations throughout the world.[38] Milk volume increases 5% to 15% in women with very low body fat because of secretion of milk with reduced lipid content, which has a caloric density of up to 15% lower than milk with normal fat content. This observation illustrates the important principle that *milk volume secretion in lactating women is regulated by infant demand.* Thus when the milk has a lower caloric density, increased sucking by the infant is thought to result in increased emptying of the breast, in turn bringing about an increase in milk secretion. Mothers of twins, and occasionally even triplets, are able to produce volumes of milk sufficient for complete nutrition of their multiple infants. Studies of wet nurses, completed in the 1930s, show that at least some women are capable of producing up to 3.5 L of milk per day. Conversely, if infants are supplemented with foods other than breast milk, milk secretion is proportionately reduced. For example, in countries such as Peru and the Gambia where infants' diets are customarily supplemented with small amounts of food at mealtimes, but the infants are given several breast-feedings a day, the daily milk production remains at approximately 600 mL/day for 12 months or longer.

The mechanisms by which the volume of milk is regulated are still under study. Although prolactin is necessary for milk production, and prolactin levels are consistently above baseline for the duration of lactation,[39] they are not proportional to milk

volume secretion. Local mechanisms, including autocrine and stretch, have been implicated in the regulation of milk volume production.[40-42] Definitive evidence in support of either mechanism remains elusive. However, cellular transduction of stretch responses remains an attractive mechanism for regulating milk production, because stretch is a known regulator of signal transduction and gene expression in physiologic systems affected by mechanical tension[43,44] and stretch-dependent effects on gene expression have been reported for mammary epithelial cells.[45]

OXYTOCIN, MILK EJECTION, AND SUCKLING

Milk removal from the breast is accomplished by the contraction of myoepithelial cells, the processes of which form a basketlike network around the alveoli where milk is stored. When the infant is suckled, afferent impulses from sensory stimulation of nerve terminals in the areolas travel to the central nervous system where they promote release of oxytocin from the posterior pituitary. In the woman, oxytocin release is often associated with such stimuli as the sight or sound, or even the thought, of the infant, indicating a significant psychologic component in this so-called neuroendocrine reflex. The oxytocin is carried through the bloodstream to the mammary gland where it interacts with specific receptors on myoepithelial cells, initiating their contraction and expelling milk from the alveoli into the ducts and subareolar sinuses. The process by which milk is forcibly moved out of the alveoli is called *milk ejection* or *let-down* and is essential to milk removal from the lactating breast.

During correct suckling, the nipple and much of the areola are drawn well into the mouth so that a long teat reaching nearly to the infant's soft palate is formed.[46] The mammary sinuses extend into this teat. Milk is removed not so much by suction as by the stripping motion of the tongue against the hard palate. This motion carries milk through the teat into the baby's mouth. The sinuses refill as the continued action of oxytocin forces milk from the alveoli into the ducts.

It has been known for some time that psychologic stress or pain decreases milk output. Recently the basis for this finding was shown by Ueda and colleagues[47] to be inhibition of oxytocin release. In relaxed, undisturbed women suckling their infants, oxytocin release begins with the onset of suckling or even before suckling when the infant cries or becomes restless. When the suckling women were asked to carry out difficult mental calculations or were fed traffic noise through earphones while nursing the infant, the number of oxytocin pulses was significantly reduced. Interestingly, the prolactin response to suckling was not impaired by psychologic stress, implicating different neural pathways in the release of the two hormones.

Alcohol and drugs of abuse may have effects on let-down. Building on earlier studies showing that ethanol inhibits milk ejection in a dose-dependent manner, Coiro and colleagues[48] measured plasma oxytocin concentrations in response to breast stimulation in nonlactating women and found that 50 mL of ethyl alcohol completely abolished the rise in oxytocin levels. Minor effects of long-term maternal ethanol consumption on motor development of breast-fed infants in a well-controlled study in humans[49] were attributed to alcohol transfer to the infant rather than suppression of milk secretion. A potent effect of morphine on oxytocin release has been described in rats,[50] but the effects of opioids and other drugs of abuse on lactation have not been studied in women.

EFFECTS OF LACTATION ON THE MOTHER

Nutritional Effects

In many species such as dairy cattle and laboratory mice, a large portion of the metabolic output is directed to milk synthesis so that careful management of nutrient intake is critical for lactation success. In humans the energetic costs of milk production are estimated to be about 700 kcal/day,[51,52] which can represent as much as a third of a woman's metabolic output. Typically the increased energy requirements of lactation are met by relatively small increases in food intake or decreased metabolic rate in healthy individuals.[53] Nonetheless, it is important to remember that, as already stated, the amount of milk produced is almost entirely regulated by infant demand. If a woman is breast-feeding twins, she will produce double the usual volume of about 800 mL/day.[38] Under these circumstances the nutritional demands may be substantial and attention should be paid to nutritional intake, particularly in malnourished women, so that maternal nutrient depletion does not result. One maternal effect that seems clear is that lactating women whose menses have not yet returned have a significant loss of calcium from their bones.[54] Both secretion of parathyroid hormone (PTH)–related peptide from the mammary epithelium and the lack of estrogen caused by postpartum amenorrhea may be involved. That PTH-related peptide plays an active role in mobilizing bone calcium during human lactation is suggested by findings that plasma calcium and alkaline phosphatase are elevated in lactating women and that the calcium regulatory hormone PTH is decreased.[54] After weaning, bone calcium levels return to normal.

Effects of Lactation on Reproduction

The most marked maternal effect of lactation is on fertility. In the early postpartum period, secretions of the ovarian follicle remain suppressed for a period of time the duration of which depends to a substantial extent on the pattern of suckling.[55] In non–breast-feeding women, fertility returns about 6 weeks post partum as pulsatile secretion of gonadotropin-releasing hormone returns to normal. In breast-feeding women who suckle their infants at regular intervals, follicle-stimulating hormone secretion returns to normal follicular phase levels during the course of lactation, but pulsatile secretion of luteinizing hormone tends to remain in a suppressed state so that the preovulatory surge of luteinizing hormone does not occur. The contraceptive effects of lactation may also reflect effects of breast-feeding intensity on maternal metabolism and associated reproductive hormone suppression.[56]

Postpartum suppression of fertility is thought to play a significant role in birth spacing on a population basis in developing countries where prolonged breast-feeding may be the rule and the use of supplementary feedings delayed. In developed countries, women who wish to ensure against pregnancy during lactation are usually advised to use other contraceptive means, often progesterone-containing compounds that do not interfere with milk secretion.[15]

SUMMARY

Milk secretion is a robust process that proceeds according to plan in at least 85% of women post partum. With assistance in the techniques of breast-feeding, anecdotal evidence suggests that at least 97% of women can successfully breast-feed their infants. The reasons for lack of success in breast-feeding are not well understood because it is easy to substitute formula when infants fail to thrive on the breast, at least in Western societies. Although this is not the place to discuss possible pathologic mechanisms, it should be noted that breast-feeding failure usually takes place in the first week or so post partum, and a much better understanding of the mechanisms by which milk secretion is initiated during this period may help us to understand why some women have serious problems with lactation. Other areas that require attention are the behavioral correlates of breast-feeding and the transfer of drug and toxins into milk. The latter may have a long-term impact on infant health and should receive additional attention.

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32 Nutritional Assessment

Gilberto R. Pereira

Newborn infants, especially those born prematurely or who are ill, are at great risk for the development of nutritional deficiencies that can adversely affect their postnatal growth.The susceptibility of the neonate to the development of such deficiencies results from the rapid growth velocity of the newborn period, the metabolic immaturity of several organ systems, and the difficulty in meeting the additional nutritional needs imposed by illness, surgical stress, or excessive nutrient losses.

The purpose of this chapter is to outline a practical approach to the nutritional assessment of neonates through the combined evaluation of the following: (1) medical history; (2) physical examination; (3) nutritional intake; (4) body composition; (5) anthropometry; and (6) biochemical markers.

MEDICAL HISTORY

The nutritional evaluation of the newborn infant begins with the analysis of both the maternal medical history during the pregnancy and the neonatal medical history.

The maternal medical history can be obtained by interviewing the mother and by reviewing her medical record. Both are often necessary to retrieve pertinent data, such as (1) maternal weight gain during the pregnancy; (2) maternal nutritional intake before and during the pregnancy; (3) history of chronic illness; (4) family history of chromosomal, metabolic, and endocrinologic disorders; (5) results of amniocentesis and fetal ultrasonographic examinations; (6) use of medications during pregnancy; (7) complications of pregnancy known to cause either retardation or acceleration of fetal growth (Table 32-1); and (8) specific maternal nutrient deficiencies (e.g., iron, folate, and vitamin B_6).

The medical history of the neonate from birth should be reviewed, including the identification of factors known to alter energy requirements such as medications (theophylline, corticosteroids), medical illnesses (respiratory distress, bronchopulmonary dysplasia, congestive heart failure, sepsis, surgery, cold stress), and nutrient losses (chronic diarrhea, excessive drainage from chest tubes, ostomies, and fistulas). Special attention should also be paid to the use of medications known to decrease the metabolic rate by limiting physical activity such as sedatives and muscle-paralytic agents.

PHYSICAL EXAMINATION

The general physical examination of the newborn infant offers a unique opportunity for the evaluation of nutritional status. By simple inspection, one can assess the lack or presence of physical activity, muscle wasting, and subcutaneous fat. In addition, the examination of the head configuration, skin, hair, and mucous membranes can be instrumental in identifying the deficiency of specific nutrients. By palpation, one can assess the presence of craniotabes, raised skin rashes, and bone prominences such as rachitic rosary and healing fractures (Table 32-2). The use of anthropometry for protein-energy assessment is described in a separate section of this chapter.

NUTRITIONAL INTAKE

Optimal nutritional intake by neonates is considered as the diet that supports appropriate growth according to standards without imposing stress on the infants' immature metabolic and excretory functions.[1] Other models for the estimation of nutritional requirements include net uptake of nutrients from the umbilical circulation,[2] nutrient balance studies,[3,4] infusion of stable isotopes,[5,6] intake of human milk,[7] and the determination of the optimal intake that prevents nutritional deficiencies and allows for favorable growth and developmental outcome.[8] The estimation of dietary intake in neonates is much simpler than that in older children and adults because of the lack of diversity in the neonatal diet, the practice of routinely recording the infant's dietary intake, and the well-documented composition of milk, infant formulas, parenteral nutrition solutions, and nutritional supplements. Accurate estimation of dietary intake in breast-fed infants, however, is more complicated because of the known variability in the composition of human milk among individual mothers and the difficulty of estimating milk intake during feedings. In practice, the composition of human milk is assumed to approximate the average values described for preterm and term human milk, and the volume of breast milk ingested is estimated by weighing the infant immediately before and after feeding, using electronic scales.[9] Computerized programs for assessment of dietary intake are now commonly used by nutrition support services to facilitate the calculation of nutritional intake for patients receiving parenteral and enteral nutrition.

TABLE 32-1

Common Conditions Leading to Acceleration and Retardation of Fetal Growth

Growth Acceleration	
Maternal	Diabetes classes A and B
	Obesity
	Hyperglycemia resulting from steroid treatment
	Use of lithium during pregnancy
Fetal	Transposition of the aorta
	Beckwith-Wiedemann syndrome
	Soto syndrome (cerebral gigantism)
Growth Retardation	
Maternal	Malnutrition
	Pregnancy-induced hypertension
	Chronic hypertension
	Drug addiction (heroin, alcohol)
	Placental insufficiency
	Diabetes classes C, D, and E
	Smoking
	High altitude
Fetal	Multiple pregnancy
	Congenital anomaly
	Congenital infection
	Radiation

TABLE 32-2

Clinical Findings in Nutritional Deficiency in Neonates

Clinical Finding	Deficiency
Lethargy	Protein, calorie
Pallor	Iron, copper, folate, vitamin B_{12}
Muscle wasting	Protein, calorie
Edema	Protein, zinc
Craniotabes, frontal bossing	Vitamin D
Hair depigmentation	Protein, zinc
Keratomalacia (eyes)	Vitamin A
Angular stomatitis	Vitamin B_2
Glossitis	Niacin
Goiter	Iodine
Follicular hyperkeratosis	Vitamin A
Dry skin, scaly dermatitis	Essential fatty acids
Acrodermatitis	Zinc
Petechia, ecchymosis	Vitamin C
Rachitic rosary, bone thickening	Vitamin D
Osteopenia	Calcium, phosphorus

TABLE 32-3

Nutritional Needs of the Full-Term and Premature Infant

	PREMATURE		
Requirement per kg/day	**<1 kg**	**1-2.5 kg**	**Full-Term**
Protein (g)	4	3.5	2
Na (mEq)	3.5	3	3
Cl (mEq)	3.1	2.5	2.3
K (mEq)	2.5	2.5	2.4
Ca (mg)	210	185	130
P (mg)	140	123	70
Mg (mg)	10	8.5	5
Fe (mg)	2-4	1-2	2
Biotin (μg)	1-1.4	1-2	1-2
Pantothenic acid (mg)	5-9	1-1.4	1-1.4
Choline (mg)	5-9	5-9	5-9
Requirement per day			
Fluoride (mg)	0.1(?)	0.1(?)	0.1(?)
Cu (mg)	0.17	0.1-0.5	0.5-1
Zn (mg)	1.5	1.5-3	3-5
Mn (mg)	0.01-0.02	0.02-0.04	0.5-1
Cr (μg)	2-4	2-6	10-40
I (μg)	5	5-10	10-15
Se (μg)	1.5-2.5	1.5-7.5	10-60
Mo (μg)	2-3	2-7.5	30-80
Vitamin A (IU)	1000	1000	1000
Vitamin D (IU)	400	400	400
Vitamin E (IU)	5-25	5-25	4
Vitamin K (μg)	5	5	5
Vitamin C (mg)	60	60	35
Vitamin B_1 (mg)	0.2	0.2	0.2
Vitamin B_2 (mg)	0.4	5	5
Vitamin B_6 (mg)	0.2	0.2	0.2
Vitamin B_{12} (μg)	0.15	0.15	0.15
Niacin (mg)	5	5	5
Folic acid (μg)	50	50	50

Resting energy expenditure (REE) in premature and in full-term infants can be measured by open circuit indirect calorimetry or by the elimination of stable isotope doubly labeled water ($^2H_2{}^{18}O$); both methods show comparable results.[10] The estimation of total energy requirement is done by adding the REE to the energy cost of the following: (1) physical activity; (2) thermoregulation; (3) growth; (4) thermic effect of food; and (5) fecal and urinary losses. Energy requirements in healthy full-term neonates receiving enteral feedings vary from 100 to 120 kcal/kg/day.[11] Energy intake as high as 148 kcal/kg/day has been recommended for premature infants to promote weight gain at in utero rates.[12] Nevertheless, this level of intake is currently considered excessive because it leads to fat retention at rates significantly greater than those observed in the fetus of similar gestational age.[13] Higher energy requirements than those presented earlier may be present in patients with fever, increased work of breathing,[14] and hypothermia, and in those with ostomy losses or exhibiting catch-up growth. On the contrary, lower energy requirements should be prescribed for patients receiving parenteral nutrition (85 to 100 kcal/kg/day) (because energy is neither used for intestinal digestion and absorption of feedings nor lost in the stools) and for infants with limited physical activity due to administration of skeletal muscle relaxants or heavy sedatives. Failure to recognize these changes in energy requirements may have a detrimental effect on the infant's postnatal growth velocity.[15]

The evaluation of nutritional intake in neonates should encompass the period from birth to the time of assessment. The adequacy of nutritional intake can be evaluated by comparing actual intake of a nutrient to a recommended optimal level. Daily nutritional requirements for premature and full-term infants are given in Table 32-3.

BODY COMPOSITION

At birth, the body composition of the neonate varies both with the length of pregnancy and with the occurrence of intrauterine complications that affect fetal growth. Ziegler and co-workers[16] and Widdowson[17] have been foremost in describing the changes in body composition that fetuses undergo with increasing gestational age. These changes include a progressive decrease in total body water, extracellular water, sodium, and chloride, and a progressive increase in intracellular water, potassium, calcium, and magnesium. In addition, the body stores of protein, fat, glycogen, and mineralized bone also increase with gestational age (Table 32-4). Complications of pregnancy known to either retard or accelerate fetal growth can influence the body composition of the neonate. For example, infants who are small for gestational age (SGA) have reduced total body fat, whereas infants who are large for gestational age (LGA) have increased total body fat when compared with infants who are appropriate for gestational age (AGA). Enzi and associates[18] demonstrated that the body fat content approximated 12% in SGA infants, 14% in AGA infants, and 18% in LGA infants. Similarly, Fee and Weil[19] reported a total body fat content of 21% in the LGA infant of a diabetic mother.

Significant changes in body composition are observed with growth during the neonatal period and throughout the first year of life (Figure 32-1).These changes include (1) a progressive increase in the amount of adipose tissue (which peaks between 4 and 6 months of postnatal life[20] and declines slightly by 12 months of age) and (2) a progressive decrease in total body water, with a relative increase in the amount of intracellular water.[21] The proportion of body weight represented by minerals and protein remains relatively constant from birth to 1 year of age (see Figure 32-1).

Widdowson and Dickerson[22] described the contributions of various organ systems to the total body mass of premature and full-term infants compared with that of adults (Table 32-5). From their data, it can be seen that certain organ systems such as the heart, bony skeleton, kidneys, and liver maintain the same percentage of total body weight at 24 weeks and 40 weeks gestational age, as well as during childhood. The skin and the brain make up a larger proportion of neonatal body weight than of adult body weight, whereas skeletal muscle represents a relatively lower percentage of neonatal body weight. These studies, performed by chemical analysis of infant cadavers, are still considered the gold standard for measurements of body composition.

TABLE 32-4

Water, Protein, Fat, and Mineral Content of the Fetus

Body Weight (g)	Gestational Age (wk)	Water (g)	Fat (g)	Protein (g)	Ca (g)	P (g)	Mg (g)	Na (g)	K (g)	Cl (g)	Fe (mg)	Ca (mg)	Zn (mg)
500	22	433	6	43	2.4	1.5	0.09	1.1	0.8	1.2	30	1.7	8.9
1000	26	850	23	86	5.7	3.4	0.21	2.1	1.7	2.3	64	3.8	17.6
1500	29	1240	60	135	9.8	5.6	0.33	2.9	2.5	3.3	101	6.0	25.9
2000	32	1598	120	188	14.7	8.3	0.45	3.7	3.4	4.2	141	8.1	33.8
2500	35	1925	208	244	19.9	11.0	0.57	4.5	4.2	4.7	183	10.1	41.2
3000	38	2217	330	334	25.1	13.9	0.69	5.5	5.0	5.3	227	12.0	48.1
3500	42	2380	525	446	30.3	17.3	0.80	6.5	5.8	5.7	283	14.3	53.6

Modified from Widdowson EM: Growth and composition of the fetus and newborn. In Assali NS (ed): *Biology of Gestation,* vol II. *The Fetus and Newborn.* New York, Academic Press, 1972, pp 1-44.

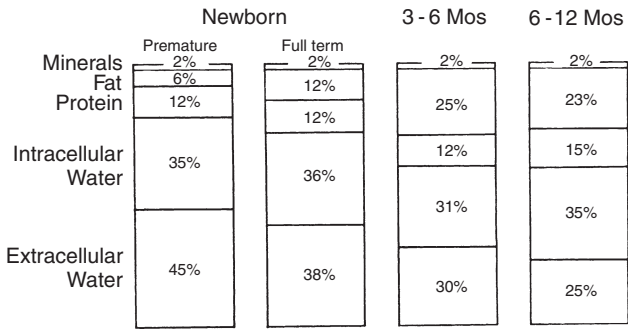


Figure 32-1 Body composition from birth to 12 months of age. *(Data from Fee B, Weil WM: Body composition of a diabetic offspring by direct analysis,* Am J Dis Child *100:718, 1960; and Westerterp KR, Lafeber HN, Sulkers EJ & Sauer PJJ: Comparison of short-term indirect calorimetry and doubly labeled water method for the assessment of energy expenditure in preterm infants,* Biol Neonate *60:75, 1991.)*

TABLE 32-5

Contribution of Organs and Major Tissues to Body Weight (%)

Organ or Tissue	Premature (20-24 wk)	Full-Term Newborn	Adult
Skeletal muscle	25.0	25.0	40.0
Skeleton	22.0	18.0	14.0
Heart	0.6	0.5	0.4
Lungs	3.3	1.5	1.4
Liver	4.0	5.0	2.0
Kidneys	0.7	1.0	0.5
Brain	13.0	12.0	2.0

Modified from Widdowson EM, Dickerson JWT: Chemical composition of the body. In Cormac CL, Broonnea F (eds): *Mineral Metabolism,* vol 2. *The Elements.* New York, Academy Press, 1964, pp 1-247.

Over the past several decades, various methods have been developed for the indirect assessment of body composition in living infants. Although some of these methods have technical limitations, they have the advantages of being noninvasive and applicable for longitudinal measurements in growing infants.

The total body potassium (K40) counter has been used to assess lean body mass (LBM) because potassium resides only in the LBM and not in adipose tissue. This method is based on the supposition that naturally occurring potassium (K39) contains a fixed proportion of the radioactive K40, which can be measured by a counter. Fat mass is determined by subtracting the calculated LBM from total body weight. Limitations to the use of this method include the insufficient sensitivity of the K40 counter for small infants and the variable potassium content of viscera and skeletal muscle in children of different ages. Spady and colleagues[23] used this method to assess the effect of maternal smoking on infant body composition.

Neutral fat does not bind water and, therefore, methods to estimate total body water have been developed to determine the nonfat compartment of the body. The oral administration of doubly labeled water, with a known amount of the stable isotopes deuterium (^2H) and oxygen 18 (^{18}O), has been used for the estimation of total body water in adults.[24] This method is based on the dilution of the ^2H and ^{18}O isotopes in body fluid such as saliva, urine, or serum, after a period of equilibration.[24] The changes in the water content of the LBM, which occur at various ages and among different individuals, represent a major limitation of this method, which is further explained in the stable isotope section of this chapter.

Total body electrical conductivity (TOBEC) has been used to estimate fat-free mass (FFM) by detecting a change in the electromagnetic field. This change is caused by shifts in the amount and volume distribution of electrolytes that are present in the FFM.[25] Fat mass can be estimated by subtracting the FFM from the total body weight. This method is safe, noninvasive, and rapid, but it has some limitations. First, the model used to calibrate this method was the infant miniature pig and not the human neonate. Second, the TOBEC scan is costly, not portable, and not uniformly available. Standard values for FFM and for total body water in infants using TOBEC have been reported by Fiorotto and associates.[25]

A dual-energy x-ray absorptiometry (DEXA) method has been proposed for the determination of lean and fat body masses.[26] This technique employs low-radiation x-ray sources that provide alternating pulses of 70 and 140 kV. After passing through the infant's body, the rays are filtered for analysis. Estimates of body composition derive from the x-ray absorption capacity of different body tissues. This method is safe, rapid, and noninvasive and allows for measurements of bone mineral content as well as lean and fat body masses.

Magnetic resonance imaging (MRI) is a noninvasive method that allows for fast and reproducible measurements of adipose tissue content in neonates with low intracoefficients and intercoefficients of variability.[27] The MRI measurements of total body fat have been validated against stable isotope dilution methods in term infants with reasonable correlation.[28] A comparison of the various methods of body composition employing different methods is presented in Table 32-6.

ANTHROPOMETRY

Anthropometric assessment provides insight into the quality and quantity of growth in newborn infants. The assessments done at the time of birth reflect the pattern of fetal growth, whereas longitudinal assessments reflect postnatal growth.

The three single measurements most often used for the nutritional assessment of neonates at birth are weight, length, and

TABLE 32-6

Body Composition of Neonates as Assessed by Various Methods

Methods	TBW (%)	ECW (%)	FFM (%)	TBF (%)
Total body potassium counter				
Term infant[23]	—	—	89.1	10.9
TOBEC				
Term infant[25]	69	—	86.7	13.2
DEXA				
Preterm infants[137]	—	—	—	9-11
Term infant[26, 135, 136, 137]			82	13.4-18
MRI				
Term infant[28]	53-69	—	—	17-22

ECW, Extracellular water; *DEXA,* dual-energy x-ray absorptiometry; *FFM,* fat-free mass; *MRI,* magnetic resonance imaging; *TBF,* total body fat; *TBW,* total body water; *TOBEC,* total body electrical conductivity.

head circumference (HC). When plotted on standard growth charts against gestational age, these measurements indicate whether the infant is AGA, SGA, or LGA. Pathophysiologic conditions that cause aberrant fetal growth increase the risks for perinatal morbidity and mortality.[29] Numerous standard curves for the assessment of birth weight, length, and HC have been published, and major differences among them can be observed.[30-37] These differences appear to be related to the various ethnic, socioeconomic, and environmental characteristics of the reference population. Measurements of weight, length, and HC are also used longitudinally for the assessment of postnatal growth. In the hospitalized neonate, these measurements are performed frequently to determine whether adequate growth is occurring on a particular nutritional regimen. Nevertheless, it should be acknowledged that changes in nutritional intake or in infant needs might take several days to be reflected in anthropometric parameters.

There are several growth charts available for postnatal assessment of premature infants, some of which are based on intrauterine standards.[29-35] Among them, the Fenton growth chart[35] was recently developed to update the commonly used Babson and Benda's growth chart[31] published in 1976. Advantages of this newer chart include: (1) the assessment of premature infants with gestational ages as early as 22 weeks, instead of 26 weeks; (2) the display of the growth curves in percentiles, instead of standard deviation and; (3) the use of 100-g increments, instead of 500-g increments, for plotting weight measurements. There are also premature growth charts that are based on postnatal growth rates.[36-38] The most contemporary of these charts[38] was based on a large multicenter sample of 1660 premature infants with birth weights between 500 and 1500 g. These charts display growth curves for weight, head circumference, length, and midarm circumference (MAC) for the first 17 weeks of life. All these charts encompass the early neonatal and postnatal periods and are therefore suitable for in-hospital use.

Growth charts for long-term growth assessment of premature infants are also available.[39,40] These charts were developed by the Infant Health and Development Program with basis on the premise that premature infants grow differently than full-term infants during infancy and early childhood. The growth percentiles were based on a large sample of premature infants born with less than 37 weeks of gestation with birth weights of less than 1500 g or between 1501 and 2500 g. These charts are suitable for follow-up of premature infants up to 38 months of gestation-adjusted age and are available for premature boys and girls.

Growth standards on long-term assessment of full-term infants were published by the Centers for Disease Control and Prevention (CDC) in 2000.[41] More recently, growth charts for the assessment of full-term infants who were partially or exclusively breast-fed were made available by the World Health Organization (WHO).[42] These standards were based on a large sample of infants from a multicountry population derived from Brazil, Ghana, India, Norway, Oman, and the United States. A study comparing the CDC and the WHO charts demonstrated significant differences in growth between the two samples. Infants in the WHO chart had faster growth rates during early infancy and lower rates of weight gain and greater linear growth throughout infancy.[43] These differences were mostly attributed to differences in dietary intake during infancy of the two study samples: breast-feeding for the WHO chart and formula feeding for the CDC chart.

Weight

Weight for age is often considered the gold standard for the assessment of postnatal growth. Hospitalized infants are routinely weighed on a daily basis. However, care must be taken to weigh the infant unclothed and to correct for the weight of attached equipment, such as endotracheal tube, chest tube, intravenous board, gauze, and tape. The assessment of weight gain velocity over time is more useful than one single measurement of weight for age. Premature infants are expected to gain weight at rates comparable to in utero standards.[44] Although this appears to be the best approach available, a number of investigators have questioned the validity of this assumption.[36,37] Dancis and associates[36] published growth curves that take into account the birth weight of the preterm infant and the expected percentage of weight loss during the first 50 days of life. These curves have been modified, demonstrating that advances in medical care and nutritional support have increased the rates of postnatal growth in premature infants.[37] In a recent observational study from our institution of AGA infants with birth weights less than 2000 g, 48% of these infants had weight values that fell below the 5th percentile on the growth chart, based on intrauterine standards at some time during their hospital course despite adequate nutritional intake. In addition, one must be cautious when interpreting weight loss in the first postnatal week because it is caused predominantly by loss of extracellular water.[45] Weight measurements alone cannot be considered an accurate indicator of lean body mass, nor can they distinguish between gains in lean body mass and fluid gains. The expected rate of fetal weight gain during the last trimester of pregnancy is 10 to 15 g/kg/day.

Length

Length for age is an extremely useful measurement of nutritional status in older children.[46] Neonates, however, are difficult to measure accurately. An accurate length measurement in neonates can be obtained when two trained observers use a rigid surface containing a stationary headboard, a movable footboard, and a built-in centimeter scale. When interobserver and intraobserver reliability can be assured, serial measurements of length are excellent for following longitudinal growth. Unlike changes in weight, changes in length are not influenced by fluid status. The expected rate of fetal growth in length during the last trimester of pregnancy is 0.75 cm/week.

Head Circumference

The measurement of HC provides an indirect measurement of brain growth and is, therefore, an important part of the nutritional assessment, both at birth and in longitudinal studies. At birth, the detection of microcephaly indicates retarded brain growth during fetal life, which may result from various causes, such as chromosomal abnormalities, maternal drug addiction, infection, or lack of nutrient availability secondary to placental insufficiency. The lack of brain growth associated with a lower number of cells indicates an insult that occurred during the first trimester of pregnancy.

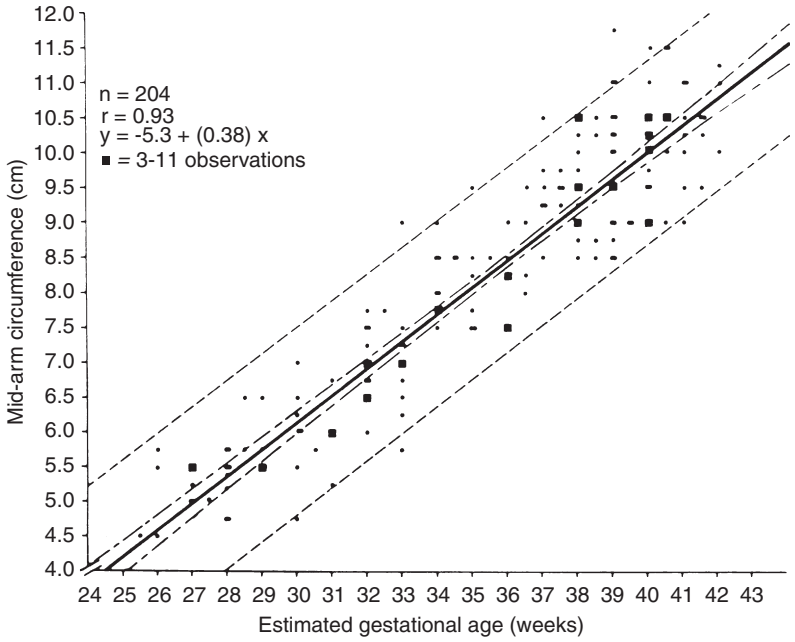


Figure 32-2 Correlation between midarm circumference and gestational age. — represents mean midarm circumference; -—- represents 95% confidence limits on predicted mean; --- represents 95% confidence limits for one observation.

Lack of brain growth caused by small cell size generally indicates a chronic insult throughout pregnancy. Neurologic outcome can be poor in microcephalic infants, particularly if no catch-up head growth is observed during the neonatal period.[47-52]

When measured longitudinally, HC is a useful tool for nutritional assessment, except in the presence of hydrocephalus. Decreases in HC are not unusual during the first few days of life, while head molding and scalp edema are still resolving. For that reason, the HC should be remeasured 3 days postnatally. In the newborn period, the brain is spared during mild to moderate malnutrition. In premature infants, a decrease in nutritional intake that results in slowing of weight and length velocities might not necessarily affect head growth velocity. In addition, in premature infants on a regimen of advancing caloric intake, head growth precedes the onset of growth in weight and length. The maintenance of normal head growth in the neonatal period is important because a rapid period of brain growth, evidenced by DNA synthesis, formation of dendritic synapsis, and myelination, occurs between 28 and 44 weeks after conception.[34-36] Infants who are born with normal head size but who become microcephalic during the postnatal period are at significantly increased risk for poor developmental outcome.[48] The expected rate of fetal head circumference growth during the last trimester of pregnancy is 0.75 cm/week.

Midarm Circumference

The MAC reflects the combination of muscle mass and fat stores in the upper arm and rapidly diminishes when protein and fat stores are depleted.[46] Standard values for MAC obtained from neonates born between 24 and 42 weeks gestation have been reported by Sasanow and colleagues.[53] These data document a predictable linear rise in MAC with increasing birth weight and gestational age (Figure 32-2). MAC is measured at the midpoint of the upper arm, which is found by measuring the distance between the acromion and the olecranon with the arm held in a horizontal position. In one study, serial measurements of MAC were more sensitive than those of weight or length for assessing nutritional status in growing premature infants.[53]

Skinfold Thickness

The simplest way to estimate body fat is to measure the thickness of the subcutaneous tissue.[54] Standard measurements of skinfold thickness for premature and term infants have been reported.[55] In addition, serial measurements of skinfold thickness at the triceps or at the scapular site can provide information on the rates of fat accretion in premature and term infants.[56] The correct technique for measurement of skinfold thickness requires firm placement of the calipers without slippage for a minimum of 15 seconds. Several investigators have shown that holding the calipers for 60 seconds while measuring the skinfold thickness of the triceps is useful in eliminating false values resulting from edema.[57,58] Dauncey and colleagues[59] have estimated total body fat percentages from measurements of skinfold thickness from multiple sites. However, the rapidly changing distribution of fat accretion in premature infants makes it difficult to generate a consistent equation for predicting total body fat.[60] Ultrasonography has been used to measure skinfold thickness; however, these measurements appear to be less reproducible than those obtained with skinfold calipers.[61]

Arm Muscle and Arm Fat Areas

Once the measurements of MAC and triceps skinfold thickness at 15 seconds (TSF_{15}) and at 60 seconds (TSF_{60}) are obtained, one can then calculate the arm area (AA), the arm muscle area (AMA), the arm fat area (AFA), and the arm water area (AWA), using the following formulas.[62,63]

$$AA = MAC^2 / 4\pi$$

$$AMA = (MAC - \pi TSF_{15})^2 / 4\pi$$

$$AFA = AA - (MAC - \pi TSF_{60})^2 / 4\pi$$

$$AWA = AA - AMA - AFA$$

The measurement of TSF_{15} is thought to reflect subcutaneous fat and water; however, the TSF_{60} measurement is thought to reflect fat only, assuming the water has been squeezed out

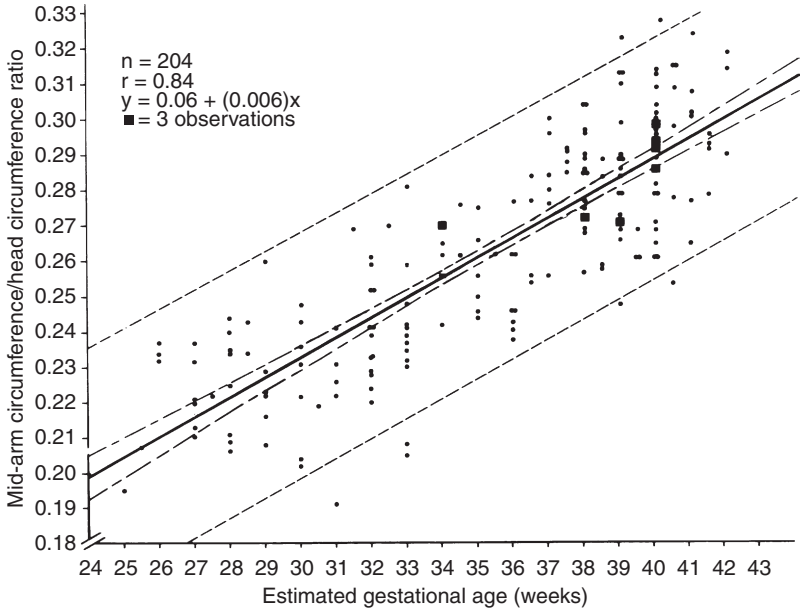


Figure 32-3 Correlation between midarm circumference/head-circumference ratio and gestational age. -—- represents 95% confidence limits on predicted mean; --- represents 95% confidence limits for one observation.

of the subcutaneous tissue.[58] Standard measurements of arm muscle and fat areas for infants at term have been published.[62]

Measurements of arm muscle and fat areas can be useful in the assessment of newborn infants who have experienced either fetal growth restriction or acceleration. For example, these measurements can identify reduced muscle or fat stores in growth-restricted infants who might be classified as AGA by weight criteria. Similarly, the measurements of TSF and AFA are proportionately greater in infants of diabetic mothers.[64,65] These infants are known to have a greater percentage of their body mass as fat, compared with infants born to nondiabetic mothers.[19]

Ponderal Index and Midarm-Head Circumference Ratio

The ponderal index and the MAC/HC ratio provide information on the proportionality of body growth. As such, they potentially allow the estimation of stunting and wasting that has occurred either in utero or postnatally. When measured at birth, both indices have been shown to be more specific than weight for age alone in determining the risk for perinatal morbidity associated with intrauterine growth disorders.[55] When used longitudinally, these indices can assess whether length is spared at the expense of weight, or whether head growth is spared at the expense of somatic growth.

The ponderal index is calculated by using the following formula[66]:

$$\text{Weight (g)} \times 100 / \text{length (cm}^3\text{)}$$

The ponderal index has been shown to vary with the gestational age of the infant[67] and is useful for the identification of growth-restricted infants who are at risk for neonatal hypoglycemia.[66] The ponderal index can be greatly misleading if measurements of length are performed inaccurately because this potential error is cubed during the calculation. The ponderal index has proved not useful for screening macrosomic infants at risk for hypoglycemia;[68] its use for longitudinal assessment of nutritional status in neonates has yet to be studied.

The MAC/HC ratio has proved useful as an index of body proportionality in neonates because it compares an anthropometric measurement likely to be affected by lack of nutritional intake (MAC) with another measurement that is less likely to be affected by nutritional intake (HC). The rationale for its use is based on the observation that head growth is spared relative to somatic growth in malnourished infants. Standard values for the MAC/HC ratio obtained from infants born between 24 and 42 weeks gestation have been reported by Sasanow and colleagues.[53] The MAC/HC ratio values increase progressively with the gestational age of the neonate (Figure 32-3).

When used at birth, the MAC/HC ratio is more sensitive than either the ponderal index or weight for age in identifying neonates at risk for morbidity in association with intrauterine growth restriction or acceleration.[68] We have compared the serial measurements of the MAC/HC ratio and weight and length for age measurements in a large group of premature infants during the first few weeks of life. Although the two indices had comparable sensitivity, the MAC/HC ratio had greater specificity in identifying protein-energy malnutrition in these infants.[69] We have also serially measured the MAC/HC ratio in premature infants to determine the proportionality of body growth throughout the first year of life. Our results indicate that premature infants, despite their small size at birth, maintain proportional growth similar to that of term infants (MAC/HC ratios <0.3) during the first year, even though they demonstrate no catch-up weight gain. Thus, it appears that premature infants have a regulatory mechanism that keeps their growth proportional as long as they receive adequate nutritional intake.[69]

Thigh Circumference and Thigh-Head Circumference Ratio

Ultrasonographic measurements of thigh, head, and abdominal circumferences have been used for the estimation of fetal weight during the last trimester of the pregnancy.[70] The chronology of adipose tissue deposition, being last in the lower extremity after occurring in other parts of the body, explains the benefits of the thigh circumference (TC) and the thigh-head ratio (THR) over other fetal parameters. Measurements of the TC are known to be affected by the position of the lower leg at the time that the measurement is being obtained. Ideally, the leg should be maintained at 90 degrees of flexion in relation to the thigh. Because of the inability to control leg position in utero, Warda and colleagues[71] identified a specific plane in the

thigh (transitional plane), at the level of the nutrient foramen of the femur, that allows for ultrasonographic measurements to be performed in an accurate and reproducible manner.

Standard values for TC and THR have been reported for neonates born at gestational ages varying from 27 to 41 weeks and have been plotted against birth weight and gestational age.[72] The same study also reported a good correlation between the THR and the ponderal index. The usefulness of the TC and THR for the evaluation of infants who are either large or small for gestational age needs further study.

BIOCHEMICAL ASSESSMENT

Measurements of various biochemical markers in serum and in whole blood have been used to assess protein, mineral, and vitamin status in newborn infants. Although some of the data on the use of these indices are extrapolated from older children and adults, most of the data presented in this section are derived from studies performed in neonates. A major limitation to the interpretation of the serum concentration of any nutrient as a marker of nutritional status is that the intravascular space generally represents a small portion of the total body pool and, therefore, might not reflect total body stores. This principle can be applied to most minerals, vitamins, and trace elements. The only exceptions are the serum proteins and some electrolytes such as sodium and chloride, which are predominantly concentrated in the extracellular fluid.

Serum Albumin

Serum albumin concentrations have been the mainstay of the biochemical assessment of nutritional status in neonates, children, and adults for several decades.[73] Serum albumin levels are lower in premature infants than in full-term infants because of a more rapid turnover of the albumin pool and a presumed decrease in the rate of synthesis by the immature liver. Albumin levels increase progressively with gestational age, varying from 2.5 to 3.5 g/dL in premature infants and 3.5 to 4.5 g/dL in term infants.[74] The serum half-life of albumin ranges from 14 to 21 days in the full-term neonate and from 5 to 7 days in the premature neonate, which makes it ideal for long-term assessment of nutritional status, but less than optimal for assessing short-term responses to changes in nutritional intake.[75]

Serum Transferrin

The serum concentration of transferrin has been used to assess protein status after nutritional rehabilitation in surgical adult patients and in pediatric cancer patients.[75-77] Transferrin has a serum half-life of 8 to 12 days, which potentially makes it a more responsive marker than albumin to recent changes in protein intake. However, serum transferrin represents 80% of the total iron-binding capacity, and for that reason the serum level of this protein is elevated during iron deficiency, regardless of the protein status.[78] Serum levels of transferrin range from 90 mg/dL in neonates born at 25 weeks gestation to 300 mg/dL in those born at term.[79] Like other serum proteins, transferrin concentrations increase with gestational age and body weight. LGA babies have higher concentrations than do AGA babies. Growth-restricted infants also have elevated serum levels of transferrin, most likely on the basis of iron deficiency.[79,80] Despite its usefulness for nutritional evaluation in the pediatric population, we were unable to demonstrate a role for serum transferrin in the longitudinal assessment of protein status in premature infants.[80,81] In our study, changes in serum transferrin concentration, unlike serum transthyretin changes, neither reflected changes in protein intake nor correlated with weight gain velocity.[82-84]

Serum Transthyretin (Prealbumin)

Transthyretin is a minor thyroid-binding globulin and a co-carrier of vitamin A with retinol-binding protein (RBP).[85] Its half-life of about 2 days makes its serum concentrations responsive to recent changes in protein-energy intake. In contrast, serum transthyretin levels are not particularly useful for long-term nutritional assessment. The serum concentrations of transthyretin at birth range from 5 to 20 mg/dL and are lower in preterm than in term infants.[86,87] The serum concentrations of this protein in adults are almost twice the values observed in newborn infants.[88] In newborns, increases in the serum concentrations of transthyretin occur rapidly with increases in protein intake, and these increases can predict weight gain velocity more accurately than the actual protein intake per se.[82] Nevertheless, several nonnutritional factors can affect the serum levels of transthyretin, thus limiting its usefulness for nutritional assessment. Transthyretin can act as an acute-phase reactant during neonatal sepsis.[88] In addition, either liver or renal disease can affect the production and degradation of transthyretin, thereby affecting its serum concentrations. Corticosteroids are known to increase the liver synthesis of transthyretin. For this reason, infants who receive dexamethasone for the treatment of bronchopulmonary dysplasia and infants whose mothers received betamethasone prenatally have elevated serum concentrations of transthyretin for about 2 weeks after these medications are discontinued.[89,90]

Retinol-Binding Protein

RBP is synthesized in the liver and has a serum half-life of about 12 hours. RBP is the main carrier of retinol (vitamin A), and its synthesis and release from hepatocytes are modulated by the availability of vitamin A. The serum concentrations of RBP at birth range from 1.2 to 3.5 mg/dL, and the values in premature infants are lower than those in term infants.[87-91] Because premature infants can become relatively vitamin A deficient after birth, a low serum level of RBP might represent vitamin A deficiency rather than protein deficiency. Like transthyretin, the RBP serum concentrations can be elevated significantly by the administration of corticosteroids.[89-90] To date, no studies have been undertaken to evaluate the usefulness of RBP in determining protein status in newborn infants.

Plasma Aminogram

The plasma concentration of amino acids is a dynamic function of the following variables: (1) recent protein intake (usually from the previous 24 hours), (2) gastrointestinal assimilation of dietary protein, and (3) uptake of amino acids from the plasma for protein synthesis in the liver. The plasma aminogram pattern has been used for the evaluation of both the quantity and quality of protein ingested by the neonate. The standard pattern of the plasma aminogram has been defined as the one observed postprandially in growing newborn infants fed human milk. It remains to be determined whether this pattern should also be used for nutritional evaluation in sick or malnourished infants. Rassin and colleagues[92,93] and Gaull and co-workers[94] have studied the plasma aminogram in low-birth-weight infants fed formula containing either high (3 g/100 kcal) or low (1.5 g/100 kcal) protein content; infant feedings containing two types of protein with a predominance of either whey or casein were also evaluated in these studies. These investigators concluded that low-birth-weight infants fed formulas containing high protein with a predominance of casein had a higher concentration of plasma amino acids than did infants fed human milk. They also demonstrated that low-birth-weight infants had a limited capacity to convert methionine to cysteine and taurine, or phenylalanine to tyrosine, thus suggesting that

the amino acids cystine, taurine, and tyrosine are essential for the premature infant, but not for the full-term infant. Heird and associates[95] reported a study in which the plasma aminogram was used for the evaluation of a pediatric amino acid solution. The study documented that infants receiving that particular amino acid mixture had plasma amino acid levels within the 95% confidence limits of the plasma concentration observed in breast-fed infants, except for one amino acid, phenylalanine. The study also showed that the ability of low-birth-weight infants to tolerate parenterally administered amino acids is not as limited as was previously thought.

3-Methylhistidine

This amino acid, a constituent of muscle protein, has unique properties in that it is not available for protein synthesis in the liver, and on its release from the muscle it is excreted unchanged in urine. Therefore, the excretion of this amino acid in the urine has been regarded as a reflection of skeletal muscle catabolism. Although the renal excretion of 3-methylhistidine has been shown to correlate with nitrogen balance in adults, this may not be clinically applicable to the neonate for several reasons: (1) the relative contribution of skeletal to total body mass is smaller in neonates than in adults (25% vs. 40%); (2) the muscle content of 3-methylhistidine in neonates is lower than that in adults (1.7 vs. 4.2 mL/g protein); and (3) the gastrointestinal tract of the neonate might provide a greater portion of 3-methylhistidine than does that of the adult.[96-99]

NUTRIENT BALANCE STUDIES

Nutrient balance studies have long been used to estimate nutrient retention in infants.[100,101] One can measure the total body balance of virtually any substance, including nitrogen, minerals, vitamins, and trace elements, by estimating intake and excretion.[100-103] Balance studies are labor intensive because of the need for meticulous methodology. A common goal of balance studies is to define parenteral and enteral nutrient requirements based on accretion and retention of administered compounds. Such studies have been reported for nitrogen,[98] fat,[102] calcium,[102-104] magnesium,[105] zinc, and copper,[106] among others. The techniques employed in these studies are not practical for routine clinical evaluations. Simplified balance studies have been performed with the goal of modifying a patient's nutritional regimen. Most often, these types of studies are used to give an estimate of energy and nitrogen balance, or to document excessive losses of specific nutrients in stools, urine, or other body fluids.

The single greatest limitation to any nutrient balance study is the accuracy with which specimens are collected.[107] This limitation is greater when applying balance techniques to young infants because the loss of collected specimens, especially urine, is a frequent occurrence. Fomon and Owen[108] have emphasized that a significant overestimation in nitrogen retention can result from a small error either in the measurement of dietary intake or in the volume of urine collected. Another limitation of this method is that, although information can be obtained about net nutrient absorption and retention, no conclusions can be drawn about how the retained nutrient is used.

Balance studies imply measuring the differences between the enteral-parenteral intake and the excretion of nutrients. The nutrients can be excreted whole or in the form of metabolites in urine, feces, sweat, or exhaled gases. Skin losses, particularly in extremely premature infants, can be large. Urine collections can be performed with relatively simple equipment such as urine bags and diapers, but this approach is fraught with problems because of specimen losses. Bag urine collections from female infants are particularly problematic, especially if the balance study lasts longer than 24 hours.[109-110] The use of metabolic beds ensures accuracy in the quantitation of urine and stool collected during balance studies.[107]

Several factors must be considered when balance studies are being interpreted for clinical or experimental purposes. Assuming adequate collection technique, the next issue is whether the data are representative of the infant's nutritional status. Typically, the shorter the collection period, the greater is the chance that the data are not representative. Six-hour urine collections for nitrogen balance have been criticized on this basis. Furthermore, it is important to know the expected length of time that the body will retain a nutrient. If the excretion of a particular nutrient that was administered enterally is to be measured in the stool, one should mark the feedings with a nonabsorbable dye, such as carmine red, to increase the accuracy of the collection period.[107] Balance studies of enterally fed nutrients generally require 72- to 96-hour collections because of the slow intestinal transit time of the neonate. On the other hand, the balance of exclusively parenterally administered nutrients, particularly in patients who are not stooling, can be done over shorter periods of time and extrapolated to a "daily" nutritional requirement. This is frequently done when assessing sodium, potassium, and nitrogen balances in critically ill neonates.

Nitrogen balance can be used to determine protein requirements in neonates by determining the difference between nitrogen intake and excretion (urinary and fecal nitrogenous compounds). It is important to measure the total urinary nitrogen, as opposed to the more commonly used, but incomplete, urea urinary nitrogen, when recording urinary nitrogen losses. The estimation of protein requirements from balance studies is predicated on the assumption that the dietary requirement is satisfied once maximal nitrogen retention is attained. Protein requirements in neonates based on nitrogen balance studies range from 1.6 to 4.2 g/kg/day, depending on the type of protein received by the infant, the infant's clinical status, and the infant's gestational age.[94,111]

Daily energy requirements are not typically assessed by classic balance studies; rather, they are estimated by energy utilization techniques. These include direct and indirect calorimetry. Indirect calorimetry using an open circuit metabolic gas analyzer has been extensively validated in the neonatal literature. These studies can be done in patients who require mechanical ventilation if there is no air leak around the endotracheal tube, or in patients who are in an oxygen tent. Measurements are made of oxygen that is entrained and then removed from the system, with a subsequent calculation of the rate of oxygen consumed by the patient per unit of time. This oxygen consumption rate can then be converted to resting energy expenditure (REE) by using the Weir equation.[112] The REE value approaches, but is not equal to, the basal metabolic rate. Measurements of REE can be used to identify infants with increased energy needs (such as those with bronchopulmonary dysplasia or congestive heart failure) and to then prescribe adequate amounts of energy to meet these infants' basal requirements. It is important to perform the study at various points in a 24-hour period, especially if infants are being enterally fed. This ensures that the thermic effect of feeding and other energy-consuming activities is included in the overall REE value. Infants must be studied in the resting state because the variance of data generated when they are active renders the values uninterpretable. Typically, less than 5% to 10% variation during the study is the expected norm. Finally, it must be realized that the total energy intake must exceed the REE value by approximately 50 kcal/kg to meet basal energy requirements and to maintain a weight gain of approximately 10 to 15 g/kg/day.

Direct calorimetry can also be performed in neonates. The infant is placed in a chamber and the amount of heat generated is measured. This lost energy is directly related to oxygen consumption. The routine use of this technique in the nursery

is precluded by the need for specialized equipment required for the measurement. Longer-term assessment of energy needs can be estimated by using doubly labeled water, a technique that is discussed in the section on stable isotopes.

SPECIAL STUDIES

Stable Isotopes

Stable isotopes are naturally occurring atoms that contain one or more neutrons in their nuclei, which makes them heavier than their parent species (i.e., ^{13}C for ^{12}C [carbon]; ^{15}N for ^{14}N [nitrogen]; and ^{2}H for ^{1}H [hydrogen]). Stable isotopes are suitable for clinical studies because they naturally occur in a predictable ratio to one another, which allows for their quantification in body fluids by measurement of their isotopic ratios using mass spectrometry. The use of stable isotopes in humans is considered safe because they do not decay and, therefore, do not emit ionizing radiation. In general, the stable isotope methodology for clinical studies includes the administration of a known amount of an isotope-labeled substrate, followed by the quantification of the isotope present in body fluids, such as blood or urine, to determine the metabolic fate of the labeled substrate.

Several aspects of glucose metabolism in neonates have been studied by using stable isotopes. For instance, continuous administration of a known amount of ^{13}C glucose and the subsequent measurement of the isotopic ratio of ^{13}C glucose in blood, at steady state, allow for the estimation of the rates of endogenous glucose production. Furthermore, the concomitant measurement of the ^{13}C isotopic ratio in expired carbon dioxide (CO_2) allows for the determination of the rates of glucose oxidation. Studies by Bier[113] and Kalhan[114] and their co-workers demonstrated that the rates of glucose production in neonates vary from 4 to 6 mg/kg/minute. Studies by Denne and Kalhan[115] estimated that approximately 53% of the glucose produced by the neonate is oxidized. Other studies[116,117] have shown that the endogenous production of glucose can be suppressed by the exogenous infusion of dextrose at rates exceeding 8 mg/kg/minute.

The use of stable isotopes in the form of ^{15}N amino acids provides a method to measure the absolute rates of protein synthesis and degradation. Such studies are based on the assumption that there is a single metabolic pool of nitrogen into which nitrogen enters from either diet or protein catabolism and exits by either protein synthesis or excretion in the form of ammonia or urea. Once the ^{15}N amino acid is administered and a steady state is attained, the flux of nitrogen entering and leaving the pool is equal, thus allowing estimation of the rates of protein synthesis and degradation.^{15}N-glycine has been safely administered intravenously and intragastrically to newborn infants with a variety of problems.[118] Stable isotope technology has permitted determination of fractional catabolic and synthetic rates for a large number of proteins[119] and can be used to determine which amino acids are essential for the premature neonate.[120]

Doubly labeled water has been used for the determination of rates of CO_2 production and oxygen (O_2) consumption, and values were found to be comparable to those obtained using indirect calorimetry.[121-123] The doubly labeled water technique has been validated for nutritional studies in neonates.[119] This method involves the administration of both $^{2}H_2O$ and $H_2{}^{18}O$ and the subsequent measurement of the enrichment of these isotopes in blood, urine, or saliva. This method is based on the principle that the disappearance of $H_2{}^{18}O$ from body fluids reflects the elimination of CO_2 and H_2O, whereas the disappearance of $^{2}H_2O$ reflects the elimination of H_2O only. The rate of CO_2 production and, therefore, its elimination are represented by the difference between the turnover of H_2O and that of CO_2. A major concern about the use of doubly labeled water in neonates is that the body composition changes rapidly after birth, and this methodology requires body water volume to remain constant throughout the study period.

Bone Mineralization Measurements

Decreased bone mineralization (osteopenia) has often been reported in sick premature infants.[124-127] Most premature babies with osteopenia are initially asymptomatic and may remain so for several weeks until the occurrence of pathologic fractures or overt rickets. Therefore, the early recognition of osteopenia is of clinical importance for the nutritional assessment of sick premature infants.

Vitamin deficiency has been clearly identified as a potential cause of osteopenia and rickets in premature infants.[128] However, current practices of routinely supplementing vitamin D to parenteral nutrition solutions and to enteral feedings have eliminated vitamin D deficiency as a common etiologic factor. Studies indicate that mineral deficiency is the major cause of osteopenia in premature infants being treated in contemporary neonatal intensive care units. Factors contributing to this mineral deficiency include (1) inability to provide adequate amounts of calcium and phosphorus in parenteral nutrition solutions,[129] and (2) increased renal losses of calcium due to the chronic use of diuretics, such as furosemide.[130]

Assessment of bone mineralization can be performed noninvasively by densitometry with the use of single- or dual-photon absorptiometry techniques.[131-137] The standard values for bone mineral content in premature and in full-term infants have been developed by several investigators. Their results are summarized in Table 32-7. There are several factors that are known to affect the rates of bone mineralization in neonates. At birth bone mineralization can be adversely affected by prematurity and postnatally by a variety of conditions, including the type of feeding; the type of dietary fat; the intakes of vitamin D, calcium, and phosphorus; the infant's body size; and the degree of physical activity.

TABLE 32-7

Bone Mineral Content in Premature and Full-Term Infants Measured by Single- and Dual-Photon Absorptiometry

		BONE MINERAL CONTENT (mg/cm)	
	Site	**Full-Term**	**Premature**
Single-Photon Absorptiometry			
Steichen et al., 1976[131]	Distal radius/ulna	103	—
Minton et al., 1979[132]	Distal radius/ulna	91	41 (<32 wk); 58 (>34 wk)
Greer et al., 1983[133]	Distal radius/ulna	94	26 (<24 wk); 71 (37 wk)
Congdon et al., 1990[140]	Mid-forearm	194	109 (25 to 34 wk)
Lapillone et al., 1997[134]	Total body scan	65 (41 wk)	20 (32 wk)
	Site	**Bone Mineral Content (g)**	**Bone Density (g/cm)**
Dual-Photon Absorptiometry			
Braillon et al., 1992[132]	Lumbar spine (L1 to L5)	1.7 (34 wk) to 2.9 (40 wk)	0.22 (34 wk) to 0.28 (40 wk)
Salle et al., 1992[136]	Lumbar spine (L1 to L5)	1.5 (32 wk) to 2.5 (40 wk)	0.2 (32 wk) to 0.27 (40 wk)
Truscott et al., 1996[139]	Total body scan (portable)		0.04 (23wk) to 0.1 (41 wk)
Rigo et al., 1998[137]	Total body scan	10 (1 kg) to 75 (4 kg)	
Hubner et al., 1999[138]	Total body scan	58.3 (39 wk)	0.3 (39wk)

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SECTION V

Perinatal Iron, Mineral, and Vitamin Metabolism

33

Jorge A. Prada

Calcium-Regulating Hormones

CALCIUM

Calcium accounts for 1% to 2% of adult human body weight.[1] More than 99% of total-body calcium is found in teeth and bones in hydroxyapatite form;and the remaining fraction is in intracellular fluids and soft tissues. Close to 1% of the calcium present in the skeleton serves as a reservoir and is freely transferable with the extracellular fluid. Although this is a small percentage, it is approximately equal to the total content of calcium in the extracellular fluid and soft tissues.

The extracellular concentration of calcium ions (Ca^{2+}) is approximately 10^{-3} M, whereas the concentration of Ca^{2+} in the cytosol is approximately 10^{-6} M. In the blood, Ca^{2+} exists in three forms: 40% is present as a nondiffusible complex with protein; 5% is a diffusible but undissociated complex with citrate, bicarbonate, and phosphate; and 55% is present as free ionized calcium. The total normal concentration is between 2.25 and 2.75 mM. The nondiffusible complex is bound largely to the carboxyl groups in albumin, and this binding is highly pH-dependent. Acute acidosis decreases binding and increases free ionized calcium, and acute alkalosis increases binding with a decrease in free ionized calcium.

Alteration in extracellular calcium concentration must be sensed to allow rectification by the homeostatic system. The recent cloning of a Ca^{2+}-sensing, G protein–coupled receptor from bovine and human parathyroid, and from rat kidney and brain, has confirmed that Ca^{2+} can function as a conventional first messenger. The calcium-sensing receptor (CaR) is a membrane-bound G protein–coupled receptor present in parathyroid and kidney cells that monitors the level of extracellular calcium and transduces signals involved in serum calcium regulation. Like other family members, it contains seven hydrophobic helices that anchor it in the plasma membrane. The large extracellular domain (spanning approximately 600 amino acids) is known to be critical to interactions with extracellular calcium. The receptor also has a rather large cytosolic tail (approximately 200 amino acids long) (Figure 33-1) Activation of the calcium sensor has two major signal-transducing effects: (1) activation of phospholipase C, which leads to generation of the second messengers diacylglycerol and inositol trisphosphate, and (2) inhibition of adenylate cyclase, which suppresses intracellular concentration of cyclic adenine monophosphate (cAMP). The sensor also can activate the mitogen-activated protein kinase pathway, suggesting an ability to influence nuclear function. The calcium sensor is expressed in a broad range of cells, including parathyroid cells and C cells (parafollicular cells) in the thyroid gland. Functional studies and investigation of animals with mutations in the calcium sensor gene have confirmed that the calcium sensor directly affects secretion of parathyroid hormone (PTH) and calcitonin.[2]

The calcium sensor also is expressed in several cell types in the kidney, osteoblasts, a variety of hematopoietic cells in bone marrow, and the gastrointestinal mucosa. A surprising finding is that it also is present in the squamous epithelial cells of the esophagus. Such a broad distribution of expression supports the concept that calcium, acting as a hormone, has direct effects on the function of many cell types. Elucidation of the role of the calcium sensor in calcium homeostasis has benefited greatly from the study of mutations in the human gene encoding this receptor:

Inactivating mutations: A large number of different mutations in the calcium sensor gene have been identified in patients affected by a type of calcium resistance known as familial hypocalciuric hypercalcemia. The different mutations result in a spectrum of calcium sensor dysfunctions, ranging from total inactivation to a moderate decrease in affinity of the receptor for calcium. A prominent clinical consequence of such mutations is an abnormal set point or sensitivity of the parathyroid gland to blood calcium concentration, resulting in hypercalcemia. Abnormalities in renal excretion of calcium also are observed, resulting in hypocalciuria.

Activating mutations: Certain types of mutations lead to a calcium sensor with an elevated sensitivity to calcium. The clinical consequence of such mutations is familial hypercalciuric hypocalcemia—basically the opposite of what is seen with inactivating mutations in the sensor gene. High blood calcium affects the normal calcium sensor to suppress PTH secretion. If the calcium sensor is constitutively more active, then a type of hypoparathyroidism results.

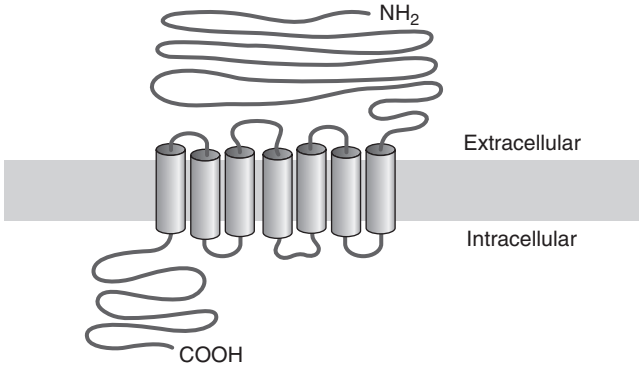


Figure 33-1 Calcium-sensing receptor (CaR). The CaR is a G protein–coupled receptor that contains seven plasma membrane anchoring hydrophobic helices. The large extracellular domain is known to be critical to interactions with extracellular calcium. *NH_2,* Amino terminus; *COOH,* carboxyl terminus. *(Reprinted with permission from Brown EM: Physiology and pathophysiology of the extracellular calcium-sensing receptor,* Am J Med *106:238, 1999.)*

Identification of the calcium-sensing receptor (CaR), expressed in the plasma membrane of parathyroid and kidney cells in particular, along with some aspects of CaR function, has been a major advance in elucidating mechanisms in control of extracellular calcium homeostasis.[3,4]

Cellular Calcium Metabolism

The control of cellular calcium homeostasis is multifaceted (Figure 33-2). The following discussion highlights some of the most important roles of the calcium ion in cellular physiology.

Extracellular fluids contain approximately 10^{-3} M of Ca^{2+}, whereas in the cytoplasm, the concentration of ionized calcium is approximately 10^{-6} M, or $\frac{1}{1000}$ that present in the extracellular fluids. The mitochondria and microsomes contain 90% to 99% of the intracellular calcium. The low calcium concentration in the cytosol is maintained by three pump transport systems: an external system, located in the plasma membrane, and two internal systems, located in the microsomal membrane and the inner mitochondrial membrane, respectively. Each pump acts like a Ca^{2+} outlet from the cytosol and requires energy to operate. Calcium ion is the most important factor linking excitation and contraction in all forms of skeletal and cardiac muscle.[5] Depolarization of the plasma membrane is accompanied by the entry of a small amount of extracellular calcium into the cell. The rise in cytosolic calcium engenders interaction with troponin, a specific calcium-binding protein, leading to a conformational change and the actin-myosin interaction that constitutes muscle contraction. In a number of cells, calcium serves as a second messenger, mediating the effects of membrane signals on the release of secretory products (e.g., neurotransmitters, amylase, insulin, aldosterone).[5] The calcium messenger system involves two pathways: (1) a calmodulin pathway and (2) a C-kinase pathway. It is now recognized that the calcium messenger system and the cAMP messenger system are related and integrated in such a way that the net cellular response to a given stimulus is determined by a complex interplay between these two systems.[5]

Many important aspects of life are regulated by the free cytosolic Ca^{2+} concentration. The regulation of cytosolic Ca^{2+} homeostasis is essential for all cells, particularly vascular smooth muscle cells. The extracellular calcium concentration, which itself is the first informative hormone-like messenger in this system, plays an important role. Expression of the calcium-sensing receptor protein, which belongs to the C subfamily of seven transmembrane-spanning G protein–coupled receptors, in tissues with functions not linked to systemic calcium homeostasis, indicates that extracellular calcium operates as a first messenger to regulate diverse cellular functions.[2,6] The biology of the low-affinity, G protein–coupled CaR and the effects of its activation in various tissues play a significant role in the regulation of PTH secretion and

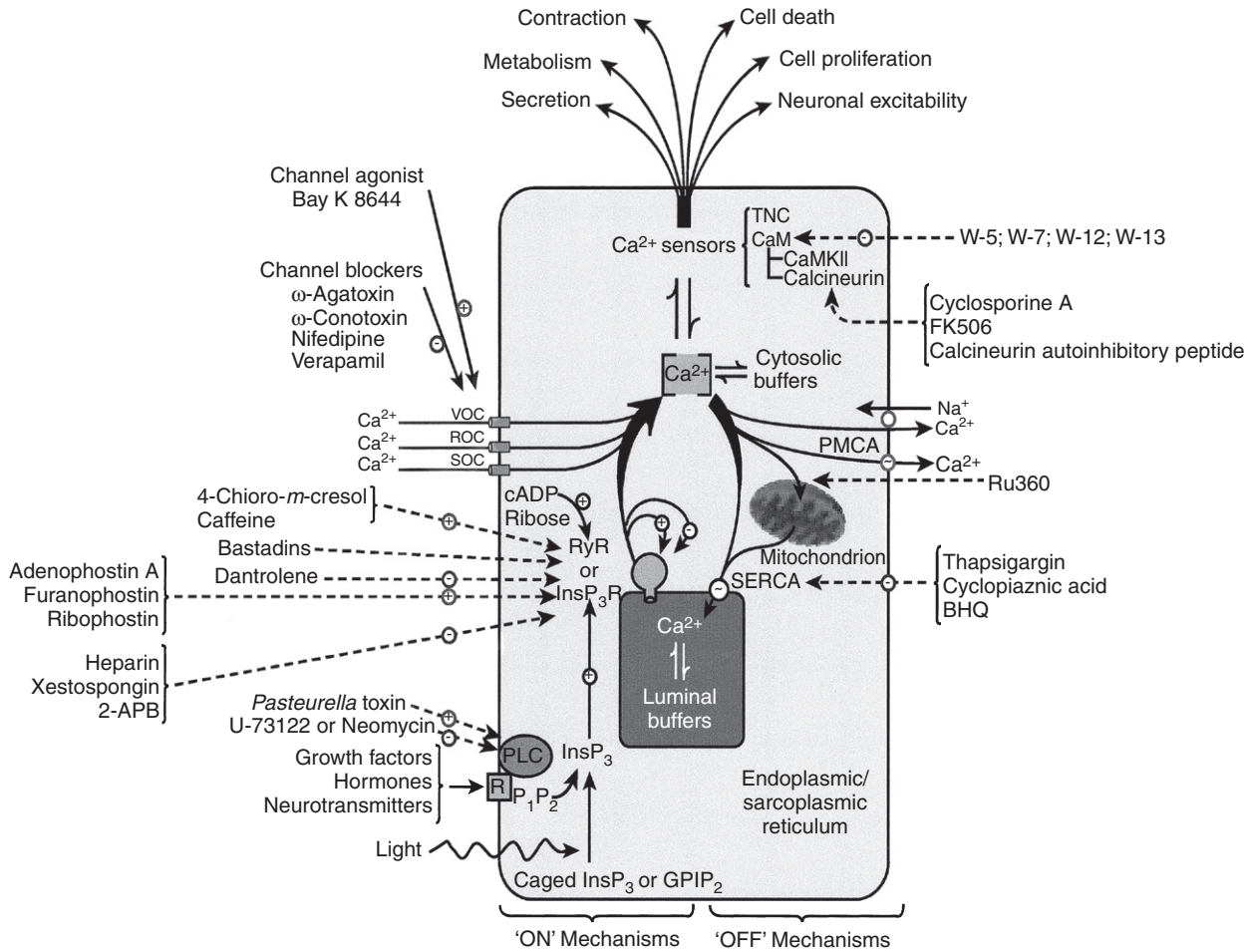


Figure 33-2 ON and OFF mechanisms for modulation of intracellular calcium. Many cellular processes are regulated by the second messenger Ca^{2+}, which is derived from two separate sources. The ON mechanisms depend on Ca^{2+} entry through channels in the plasma membrane or Ca^{2+} release through ryanodine receptors (RYRs) or inositol triphosphate receptors ($InsP_3Rs$). The OFF mechanisms remove Ca^{2+} from the cytoplasm using pumps. *BHQ,* 1,4-Benzhydroquinone; *cADP,* cyclic adenosine diphosphate. *(Courtesy Calbiochem, 2001.)*

urinary calcium excretion by small changes in extracellular Ca^{2+}. CaR also affects the renal handling of sodium, magnesium, and water.[7-9]

Calcium Ion Balance and Mechanism for Systemic Mineral Homeostasis

The total extracellular amount of calcium in an adult is approximately 900 mg. This amount is in dynamic equilibrium with calcium entering and exiting by way of the intestine, bone, and renal tubules. In zero balance, bone resorption and bone formation are equivalent, and the net quantity of calcium absorbed by the intestine (approximately 175 mg/day in an adult) is excreted into the urine. Under normal conditions, net calcium absorption provides a surplus of calcium that exceeds systemic requirements. Normal adults lose 1% of their skeleton yearly after the age of 20 years; this is the equivalent of a calcium loss of approximately 25 mg/day.

The ionized fraction of calcium is the physiologically important fraction of calcium. It is regulated by fluxes occurring at the levels of bone, kidney, and intestine; these fluxes are controlled by calciotropic hormones, which in turn are controlled by the calcium-sensing receptor protein.[6] The human body is equipped with an efficient protection system against hypocalcemia. Any modification of the extracellular calcium concentration triggers a series of integrated hormonal homeostatic reactions that incorporate PTH, 1,25-dihydroxycholecalciferol ($1,25\text{-}[OH]_2D_3$), and calcitonin.[10]

PARATHYROID HORMONE

PTH is synthesized and secreted by the chief cells of each of the four parathyroid glands. Its primary role is the maintenance of the normal concentrations of circulating calcium and inorganic phosphate, but many other tissues that are not involved in Ca^{2+} regulation also express PTH receptors.[11] PTH is a single-chain 84–amino acid polypeptide with a molecular weight of 9500. The structures of human, bovine, and porcine PTH are shown in Figure 33-3.

The PTH molecule initially is synthesized as a 115–amino acid precursor polypeptide, pre-proparathormone.[12] In the endoplasmic reticulum,[13] the amino-terminal (N-terminal) 25–amino acid sequence is proteolytically cleaved to form a 90–amino acid polypeptide proparathormone, and in the Golgi complex, proparathormone is successfully cleaved to yield the final hormonal product, the 84–amino acid PTH. The most N-terminal portion is required for the classic actions of the hormone on mineral ion homeostasis, mediated by type 1 PTH receptor (P1R) activation.[12,14-17] Proteolysis of the 84–amino acid PTH results in two or more fragments: PTH(37-84) and a large N-terminal fragment PTH(1-36).[18,19] PTH has a half-life of approximately 4 minutes and is metabolized mainly in the liver by Kupffer cells into a biologically inactive carboxyl-terminal (C-terminal) fragment and a biologically active N-terminal fragment.[20] PTH is synthesized and secreted continuously at normal plasma calcium concentrations in a pulsatile fashion,[20] with peak plasma levels occurring in the early morning hours.[21,22]

Human parathyroid glands are functionally active as early as 12 weeks of gestation.[23] PTH does not appear to cross the placenta in either direction. Maternal parathyroid dysfunction, however, can adversely affect fetal parathyroid function through variations in calcium concentration. Maternal hyperparathyroidism results in maternal hypercalcemia, which leads to fetal hypercalcemia and suppression of fetal and neonatal parathyroids. Conversely, untreated maternal hypoparathyroidism leads to maternal hypocalcemia, fetal hypocalcemia, and secondary fetal and neonatal hyperparathyroidism. Under normal circumstances, serum PTH levels measured by radioimmunoassay are lower in the fetus than in the mother, owing to the relative hypercalcemia that exists during fetal life.[24] After birth, with the abrupt termination of maternal calcium supply, the serum calcium concentration decreases, and serum PTH increases correspondingly. Both term and preterm infants show a PTH response to a falling serum calcium concentration.[25] Infants of diabetic mothers (IDMs), however, demonstrate impaired PTH production during the first days of life.[26] Infants with birth asphyxia also may exhibit a decreased PTH response to hypocalcemia.[27]

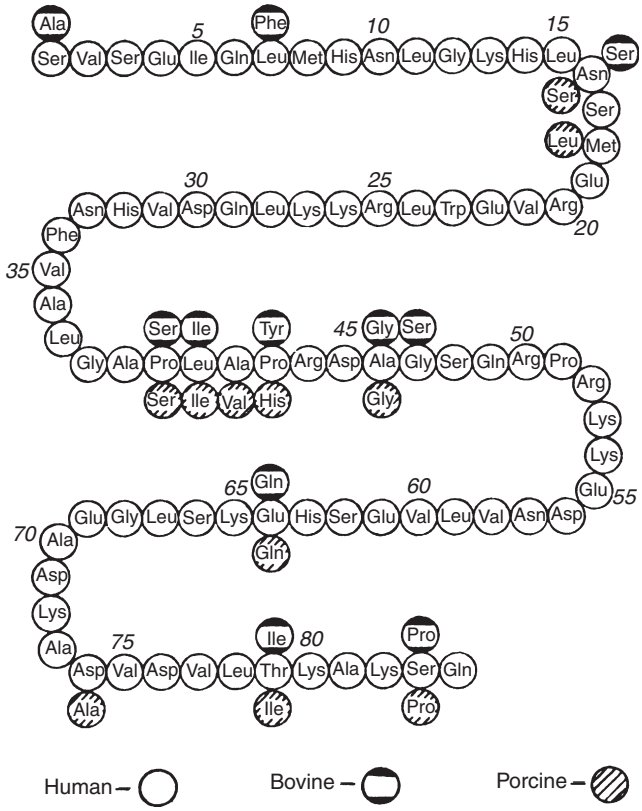


Figure 33-3 The molecular structure of parathyroid hormone. The human, bovine, and porcine sequences are illustrated. The fully active portion comprises amino acids 1 to 34. Regions 28 to 34 and 34 to 48 are produced by enzymatic cleavage. Positions 48 to 84 are the biologically inactive C-terminal region. *(Reprinted with permission from Keutman HT, Sauer MM, Hendy GN, et al: The complete amino acid sequence of human parathyroid hormone,* Biochemistry *17:5723, 1978. Copyright 1978, American Chemical Society.)*

Secretion

The extracellular fluid calcium concentration sensed by the CaR is the main determinant of PTH secretion.[28] PTH is secreted in response to small declines in ionized calcium concentration in the extracellular fluid, according to a tight feedback control mechanism. Decreases in ionized calcium of as little as 0.4 mmol/L are transduced by the CaR and detected in the plasma membrane of the parathyroid gland to stimulate PTH secretion.[29-31] Extracellular calcium has been shown to regulate the vitamin D receptor expression by parathyroid cells independently of calcitriol ($1,25\text{-}[OH]_2D_3$); by this mechanism, hypocalcemia may prevent the feedback effect of calcitriol on the parathyroids.[32]

Catecholamines,[33] aluminum,[34] histamine,[35,36] and hormones, including active vitamin D metabolites,[37] glucagon,[38] cortisol,[39] and calcitonin,[40] also have been shown to influence PTH

secretions. Acute decreases in magnesium stimulate PTH secretion[41,42]; however, chronic magnesium deficiency paradoxically decreases PTH secretion, probably by altering the calcium-sensitive magnesium-dependent adenylate cyclase involved in PTH secretion.[43] PTH secretion dramatically increases when Ca^{2+} levels are decreased to approximately 1 mM; however, further lowering of the calcium concentration does not elicit an additional increase in PTH secretion.

Metabolism

Intact PTH is cleared rapidly from the circulation by hepatic and renal tissues, with a half-life of less than 4 minutes.[19,44-46] The more efficient hepatic clearance involves three cell types: macrophages, Kupffer cells, and hepatocytes.[47,48] Kupffer cell clearance is high and does not discriminate between active and inactive PTH, whereas uptake by hepatocytes specifically removes active hormone. Renal clearance mechanisms are complex. A small amount of biologically active hormone is removed by the basal-lateral surface of tubule cells. This mechanism may be receptor-mediated. Both intact and C-terminal fragments are removed by glomerular filtration, however.[49] C-terminal fragments are more abundant than intact PTH and are released into the circulation where they have a half-life several times longer than intact PTH.[50] C-terminal fragments may account for up to 70% to 95% of the circulating immunoreactive PTH.[51] Radioimmunoassays specifically measuring the intact PTH molecule are believed to reflect PTH secretion more accurately.

Mechanism of Action

PTH regulates calcium homeostasis and phosphate metabolism by a complex interaction affecting both bone turnover and renal excretion of calcium and phosphate. In addition, PTH increases the synthesis of 1,25-$(OH)_2D_3$, which promotes intestinal calcium absorption. PTH administration leads to a release of calcium from the surface of bone.[52] Chronic administration of PTH leads to an increase in the number and activity of osteoclasts[53]; intermittent administration, however, results in increased deposition of trabecular bone.[54] PTH increases the serum calcium concentration directly, by increasing bone resorption and by increasing renal calcium reabsorption, and indirectly, by increasing renal synthesis of 1,25-$(OH)_2D_3$ through activation of 25-(OH) D_3-1-hydroxylase in the proximal tubular cell. Administration of PTH also causes a release of phosphate from bone and increased phosphaturia by inhibiting reabsorption of phosphate in the proximal (and in part distal) tubules. Although serum phosphorus may increase as a consequence of PTH-induced resorption of bone, PTH lowers the serum phosphorus concentration through its phosphaturic action, thereby minimizing the possible adverse effect of hyperphosphatemia on calcium homeostasis.[55] The direct result of all of these actions is an increase in the concentration of blood calcium and a decrease in the concentration of blood phosphate.

Cellular Actions

Two PTH receptor subtypes, type 1 (PTH1R) and type 2 (PTH2R), have been identified.[56] Type 1 PTH receptor binds both PTH and N-terminal peptides of parathyroid hormone–related protein (PTHrP). This molecule is a G protein–coupled receptor with seven transmembrane segments. The extracellular domain has six cysteine residues. Binding of ligand to this receptor activates both adenyl cyclase and phospholipase C systems, generating protein kinase A and protein kinase C signals, respectively. The cAMP–protein kinase A pathway is predominant. As might be expected from the actions of PTH, the messenger RNA (mRNA) encoding the type 1 receptor is most abundant in bone (especially in chondrocytes at growth plates) and kidney. The mRNA also is expressed at lower levels in many other tissues, probably reflecting its use as a receptor for PTHrP. The type 2 PTH receptor binds PTH but shows very low affinity for PTHrP. This molecule is expressed in only a few tissues, and its structure and physiologic significance are poorly characterized. Like the type 1 receptor, it is coupled to adenyl cyclase, and ligand binding induces a rise in intracellular concentration of cAMP. Their complementary DNA (cDNA) sequences share 70% similarity with those of members of a subfamily of G protein–coupled receptors to which the receptors for calcitonin, secretin, and glucagon also belong.[57,58] Expressions of recombinant receptors have established that type 1 and type 2 PTH receptors each alone are capable of stimulating increases in Ca^{2+} and intracellular cAMP.[59-62] Although PTH acts on bone to increase osteoclast number and activity, osteoclasts surprisingly contain no PTH receptors, and PTH has no effect on isolated osteoclasts.[63] PTH receptors are found on bone-forming osteoblasts, which secrete factors that stimulate osteoclast activity. In the kidney, PTH blocks the sodium-dependent phosphate cotransport[64] in proximal and distal tubule cells. In the distal tubule cell, however, PTH also stimulates calcium absorption against an electrochemical gradient.

PTH does not act directly in the cytoplasm of target cells; instead, PTH binds to specific receptors on the surfaces of those cells. This binding stimulates the release of cytoplasmic "second messengers" that mediate the distal effects of PTH. Binding of PTH to its receptors requires the first 34 amino acids of PTH, and the function of the carboxyl portion is unclear. PTHrP, produced by certain tumors, resembles PTH in its first 13 amino acids.[65] Peptide fragments of this protein bind to PTH receptors in bone and kidney[66]; this proclivity explains many activities of the protein and the similarities between hyperparathyroidism and the humoral hypercalcemia of malignancy.

The major mediator of PTH action is cAMP. Exposure of bone or kidney cells to PTH increases intracellular cAMP, and administration of cAMP analogues mimics many of the effects of PTH. Binding of PTH to its receptor on the cell stimulates the release of guanosine diphosphate (GDP) from the G_s protein, and enables the binding of intracellular guanosine triphosphate (GTP) to G_s. GTP G_s then stimulates membrane-bound adenylate cyclase. The cAMP produced by adenylate cyclase binds to the regulatory subunit of cAMP-dependent protein kinase A. This binding causes the regulatory subunit to dissociate from the catalytic subunit. The free catalytic subunit then phosphorylates specific serines and threonines in target proteins.[67] PTH also stimulates phosphatidylinositol turnover in culture cells,[68] causes a rapid rise of free cytosolic calcium,[69] and promotes a shift of protein kinase C to the cell membrane.[70] These effects are different from the actions of cAMP, suggesting that multiple second messengers may mediate some actions of PTH. A review of the molecular structure of PTH and its interaction with various receptors, as well as recent technical advances in PTH assays, is available.[71]

PARATHYROID HORMONE–RELATED PROTEIN

PTHrP comprises a family of protein hormones produced by most if not all tissues in the body. PTH and PTHrP bind to the same receptor, but PTHrP also binds to several other receptors. PTHrP was discovered as a protein secreted by certain tumors that caused hypercalcemia in affected patients. It was soon shown that the uncontrolled secretion of PTHrP by many tumor cells induces hypercalcemia by stimulating resorption of calcium from bone and suppressing calcium loss in urine, similar to what is seen with hyperparathyroidism. It quickly became apparent, however, that PTHrP had many activities not seen with PTH. PTHrP is an important paracrine and autocrine regulator of proliferation, apoptosis, and differentiation of several normal cell types.[72,73] Many tissues produce PTHrP, which plays a number

of physiologic roles, particularly in the mother during pregnancy and in the developing fetus.[73,74] PTHrP has been isolated from malignant tumors[75,76] and has been identified as being primarily responsible for "humoral" hypercalcemia of malignancy.[77] PTHrP expression in normal and tumor cells may be regulated by glucocorticoids,[78] epidermal growth factor,[79] tumor necrosis factor-α,[80] and vitamin D.[81]

Hormone Structure

PTHrP contains 141 amino acids; 8 of the first 13 are identical to those in PTH.[82,83] A single gene that is highly conserved among species encodes PTHrP. It should probably be described as a polyhormone, because a family of peptide hormones is generated by alternative splicing of the primary transcript and through use of alternative posttranslational cleavage sites. To make matters even more complex, some cells appear to use alternative translational initiation codons to produce forms of the protein that are targeted either for secretion or nuclear localization.

The diverse activities of PTHrP result not only from processing of the precursor into multiple hormones but also from use of multiple receptors. It is clear that N-terminal peptides of PTHrP share a receptor with PTH, but they also bind to a type of receptor in some tissues that does not bind PTH. In addition to the secreted forms of this hormone, considerable evidence indicates that a form of PTHrP is generated in some cells that is not secreted and, by means of nuclear targeting sequences, is translocated to the nucleus, where it affects nuclear function. The consequences of this "intracrine" mode of action are not yet well characterized, but this hormone may modulate such important activities as programmed cell death.

PTHrP is secreted from a large and diverse set of cells, during both fetal and postnatal life. Among tissues known to secrete this hormone are several types of epithelium, mesenchyme, vascular smooth muscle, and central nervous system tissue. Although PTHrP is found in serum, a major portion of its activity appears to reflect paracrine signaling. PTHrP can interact with PTH receptors in classic PTH target tissues such as bone and kidney.[84,85] Synthetic peptides that include the N-terminal region PTHrP(1-34) mimic all of the classic activities of PTH on bone and kidney, as well as nonclassic activities such as smooth muscle relaxation and vasodilation.[86]

Physiologic Effects

One thing to recognize about PTHrP is that its name is inadequate to describe its activities. As with PTH, some of the effects of PTHrP result from its effects on transepithelial fluxes of calcium, but many of its actions have nothing to do with calcium homeostasis. Most prominently, PTHrP peptides exert significant control over the proliferation, differentiation, and death of many cell types. They also play a major role in development of several tissues and organs.[87]

Much of the current understanding of the biologic effects of PTHrP comes from experiments with genetically altered mice. Mice with targeted deletions in the PTHrP gene (knockout mice), mice that overexpress PTHrP in specific tissues (transgenic mice), and crosses between knockout and transgenic mice have been critical in delineating many effects of this hormone. Humans with mutations in the PTHrP gene or the parathyroid receptor have also played a role in confirming the activity of PTHrP. Some of the physiologic effects of PTHrP collected from these studies are described next.

Cartilage and Bone Development

Mice with homozygous inactivation of the PTHrP gene die at birth, if not earlier. They manifest severe chondrodysplasia and premature epiphyseal closure, reflecting a developmental defect in proliferation and differentiation of cartilage. These and other types of studies indicate that PTHrP stimulates the proliferation of chondrocytes and suppresses their terminal differentiation. These effects of PTHrP appear to be due to interaction of the PTH-like peptide with the PTH receptor.[74]

Mammary Development and Lactation

The mammary glands of female mice with homozygous inactivation of the PTHrP gene fail to develop, except for the earliest stages. Development of the mammary gland depends upon a complex interaction between epithelial and mesenchymal cells that apparently requires PTHrP. In normal animals, mammary epithelial cells secrete large amounts of PTHrP, which suggests a role of this hormone in adapting maternal metabolism to the calcium demands of lactation. PTHrP concentration in milk is 10,000 times the normal circulating levels and 1000 times higher than average plasma concentrations in hypercalcemic cancer patients.[88] Because PTHrP production can be induced by lactation,[89] it has been suggested that this "milk protein" serves to condition the gastrointestinal absorption of calcium during breast-feeding.[88]

Placental Transfer of Calcium

The "midregion" peptide of PTHrP has been shown to control the normal maternal-to-fetal pumping of calcium across the placenta. In the absence of fetal PTHrP, this gradient is not established. PTHrP and the common PTH and PTHrP receptors have been identified in gestational tissues, including myometrium, fetal membranes, and placenta.[90-97]

Smooth Muscle Functioning

PTHrP is secreted from smooth muscle in many organs, usually in response to stretching. It acts to relax smooth muscle, serving as a vasodilating hormone. Transgenic mice that express PTHrP in vascular smooth muscle manifest hypotension. PTHrP may also have effects on contraction of muscle in the bladder, uterus, and heart. Studies also indicate that endogenous PTHrP is essential for optimal differentiation of keratinocytes.[98,99] PTHrP and its mRNA have been identified in syncytiotrophoblast and cytotrophoblast cells.[100,101] Potential autocrine, paracrine, and endocrine roles for PTHrP during pregnancy include regulation of cellular growth and differentiation,[102,103] vasodilation of the uteroplacental vasculature,[91,104] relaxation of uterine muscle,[105-107] and stimulation of placental calcium transport.[108] Because of its wide distribution, PTHrP may utilize novel (non-PTH) signal transduction systems in autocrine and paracrine fashion in controlling normal physiologic processes.[109]

Other Effects

PTHrP is highly expressed in skin. Transgenic mice that overexpress PTHrP in skin show alopecia, and treatment of mice with a PTHrP antagonist leads to increased numbers of hair follicles and a shaggy appearance. Another interesting defect in PTHrP-knockout mice is that their teeth develop normally but fail to erupt. Finally, both PTHrP and its receptors are widely expressed in the central nervous system and appear to influence neuronal survival by several mechanisms. A recent study has suggested that activation of the calcium-sensing receptor is responsible for increased synthesis and secretion of PTHrP.[110]

VITAMIN D

Vitamin D is synthesized in the skin on exposure to ultraviolet (UV) light. The initial step in the production of vitamin D_3, the conversion of 7-dehydrocholesterol (7-DHC) to previtamin D_3, requires a UV spectrum that peaks at 295 nm, with conversion dropping to zero at 315 nm.[111] This waveband, known as UVB, is at the short wavelength limit of solar radiation reaching the

earth's surface. When the sun is low in the southern sky, the incoming radiation must travel further and is subject to more scattering and absorption than when the sun is directly overhead. Because Rayleigh scattering (scattering of light waves by particles with dimensions much smaller than their wavelengths) and UV absorption at more northern latitudes are strongly wavelength-dependent, skin synthesis of previtamin D_3 is affected by latitude, season of the year, and weather conditions.

Vitamin D also is absorbed from dietary sources in the duodenum and jejunum, as either vitamin D_3 (from animal sources) or vitamin D_2 (ergocalciferol, from vegetable sources). The metabolism of vitamin D_3 does not differ appreciably from that of vitamin D_2. Regardless of its origin, vitamin D is transported to the liver, where it is converted into 25-hydroxyvitamin D, and subsequently to the kidney, where it is transformed to the final, active metabolite 1,25-$(OH)_2$ vitamin D. In the liver, 25-hydroxylation is a fairly unregulated process; 25-hydroxyvitamin D is the major circulating vitamin D metabolite and its concentration is an indicator of vitamin D status, reflecting both intake and sunshine exposure. By contrast, renal 1-hydroxylation, leading to the active metabolite, appears to be tightly regulated. The main factors increasing the synthesis of 1,25-$(OH)_2D_3$ are PTH, hypocalcemia, and hypophosphatemia. 1,25-$(OH)_2D_3$ is a steroid and binds to a receptor in target tissues, leading to synthesis of calcium-binding (calbindin-D) proteins.[112]

Three major sites of action for 1,25-$(OH)_2D_3$ are known: (1) intestine, where it increases the absorption of calcium and phosphorus; (2) bone, where it mobilizes calcium and phosphorus; and (3) kidneys, where it increases calcium reabsorption. Thus vitamin D is necessary to maintain normal calcium and phosphorus homeostasis. The most completely studied target tissue for 1,25-$(OH)_2D_3$ is the intestine, which depends on the hormone for adequate absorption of dietary calcium in adults.[113,114] In the intestinal mucosa, the steroid receptor complex induces synthesis of a specific calcium-binding protein (molecular weight, 28,000). In addition to the endocrine actions of 1,25-$(OH)_2D_3$, interaction of 1,25-$(OH)_2D_3$ with hematopoietic cells[115] and with the immune system[116] has been documented. The actions of 1,25-$(OH)_2D_3$ appear to involve changes in gene expression in target cells.[117-119]

Regulation and Metabolism

1,25-$(OH)_2D_3$ is synthesized by the kidney. In the kidney mitochondrion, 25-hydroxyvitamin D_3 (25-[OH]D_3), the major circulating form of vitamin D, is hydroxylated to either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ by either 1α- or 24-cytochrome P-450 hydroxylating enzymes, respectively[120] (Figure 33-4). The predominant product formed depends on vitamin D and PTH status. In the vitamin D–deficient state, 1-hydroxylase activity is high and 24-hydroxylase is low; 1,25-$(OH)_2D_3$ (the product of 1-hydroxylase) represses 1-hydroxylase and induces 24-hydroxylase activity. Directly or by depleting renal cortical inorganic phosphate concentration, PTH increases 1-hydroxylase activity and decreases 24-hydroxylase activity. cAMP mediates the effect of PTH, but protein kinase C also may be involved in 25-$(OH)D_3$ metabolism.

In a vitamin D sufficiency state, 1-hydroxylase activity is low and 24-hydroxylase activity is high. Once synthesized by the kidney, 1,25-$(OH)_2D_3$ can be converted by enzymatic oxidation into different metabolites. These include 1,24,25-$(OH)_3D_3$; 1,23,25-$(OH)_3D_3$; 24-keto-1,23,25-$(OH)_3D_3$; 1,25-$(OH)_2D_3$-(23-26) lactone; and 1α-(OH)-24,25,26-tetranor-23(COOH) vitamin D_3.[121]

Molecular Mechanisms of Action

1,25-$(OH)_2D_3$ can generate biologic responses by activation of voltage-dependent Ca^{2+} channels coupled to appropriate signal transduction pathways.[120,122,123] A target cell must have three key components for its genes to be regulated by a steroid hormone: (1) a protein receptor for the hormone that contains a unique binding domain for the steroid *and* a DNA-binding domain for the receptor to locate the genes in the nucleus of the cell

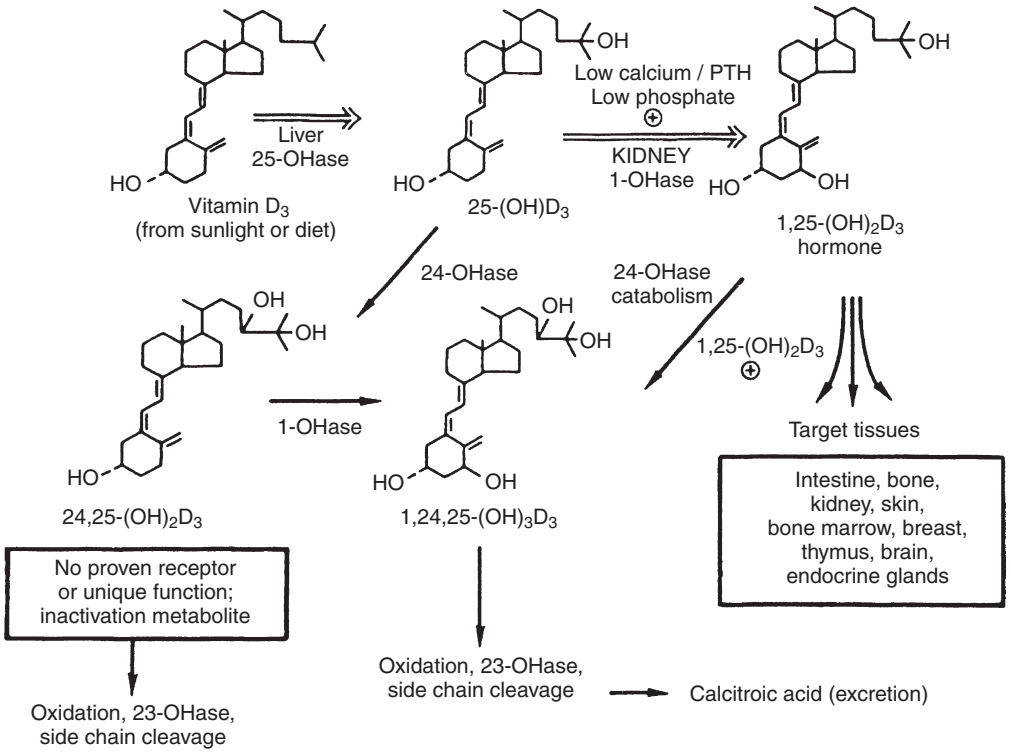


Figure 33-4 Schematic depiction of the metabolic pathways of the vitamin D endocrine system. *(From MacDonald PN, Dowd DR, Haussler MR: New insight into the structure and functions of the vitamin D receptor,* Semin Nephrol *14:101, 1994.)*

it will regulate; (2) responsive elements that consist of specific sequences of DNA nucleotides that promote an interaction between the occupied receptor and the genes to be regulated; and (3) access to the steroid hormone.

The vitamin D receptor is a member of the steroid hormone receptor superfamily.[124] The receptor is primarily a nuclear protein (51,000 daltons) that functions as a transactivating factor.[117] Although this protein is universally termed *vitamin D receptor,* in reality it binds to 1,25-$(OH)_2D_3$, the metabolite with hormonal activity, with much higher affinity than for any other vitamin D metabolite.[125] Vitamin D receptor is a DNA-binding protein, with a typical highly conserved DNA-binding domain located near the N terminus of the molecule and a hormone-binding domain at the carboxyl end (C terminus).[117,124] Transcriptional regulation occurs by way of the variable N-terminal domain, which also may be involved in regulating DNA binding. Although the precise mechanisms of protein-DNA interaction are not totally known, it is thought that after binding to 1,25-$(OH)_2D_3$, the vitamin D receptor undergoes conformational changes that increase its affinity for DNA.[118] The vitamin D receptor is widely distributed in classic and nonclassic target tissues. Tissues in which binding sites have been described include the hematopoietic, lymphatic, and reproductive systems; endocrine glands; the skin, liver, and lungs; and skeletal and smooth muscle.[120] Initial studies for the expression of the cloned vitamin D receptor seem to confirm the hypothesis that a single receptor mediates the protein actions of vitamin D metabolites in the different target cells.[120]

Biologic Actions

The classic target organs for vitamin D are bone, intestine, and kidney. The actions of 1,25-$(OH)_2D_3$ on bone cells are essential for bone formation: Hypovitaminosis D leads to rickets or osteomalacia. In hematopoietic cells, 1,25-$(OH)_2D_3$ promotes the differentiation of promonocytes into monocytes and macrophages, and finally into osteoclasts, which promote bone resorption but are devoid of receptors for 1,25-$(OH)_2D_3$.[126] If administered at high doses, however, 1,25-$(OH)_2D_3$ induces bone resorption by a receptor present on osteoblasts. The biologic effects of 1,25-$(OH)_2D_3$ on bone are indirect and transduced from the osteoblast to the osteoclast.[127] The transduction may occur through the secretion of a soluble factor by the osteoblast. 1,25-$(OH)_2D_3$ stimulates the synthesis of noncollagenic matrix proteins, such as osteocalcin,[128,129] matrix G1a protein,[130] osteopontin,[131,132] and fibronectin,[133] and increases alkaline phosphatase activity.[134] It is thought that stimulation of matrix proteins is transcriptional. The regulatory actions of 1,25-$(OH)_2D_3$ on all of these important components of bone matrix imply an important role in bone matrix formation.

1,25-$(OH)_2D_3$ stimulates the transcription of the gene encoding calbindin-D,[114,135] which is a calcium-binding protein that mediates Ca^{2+} transport through the intestinal epithelium.[136] 1,25-$(OH)_2D_3$ also induces nongenomic changes in Ca^{2+} transport in intestinal cells. These include stimulation of Ca^{2+} uptake in the intestinal epithelium,[137] increased net Ca^{2+} transport in duodenal loops,[138] and release of lysosomal enzymes.[139] 1,25-$(OH)_2D_3$ also stimulates phosphate transport[140] and induction of enterocyte growth and differentiation.[141,142] The actions of 1,25-$(OH)_2D_3$ on the kidney have not been studied as well as they have been in other target organs. 1,25-$(OH)_2D_3$ is involved in calcium and phosphate transport.[120,143] Stimulation of phosphate transport in proximal tubule brush border membranes occurs by altering plasma membrane fluidity.[144] An important action of 1,25-$(OH)_2D_3$ on the kidney is its feedback inhibition on 1α-hydroxylase activity and the associated stimulation of 24-hydroxylase, the first step in the inactivation pathway of vitamin D metabolites.[145,146]

Knowledge of the spectrum of biologic actions of 1,25-$(OH)_2D_3$ and broad distribution of vitamin D receptors has expanded current understanding of the role of the hormone as an important regulator of cell development and proliferation in many tissues, as well as in neoplasia. Further discovery of effects of 1,25-$(OH)_2D_3$ on tissues other than those regarded as classic targets is anticipated. Reviews on this subject are available in the literature.[120,147]

Biologic Concentration of Vitamin D and Its Metabolites

The normal adult range for blood 25-$(OH)D_3$ concentrations for healthy subjects is 10 to 80 ng/mL, and the range for 24,25-$(OH)_2D_3$ is 1 to 4 ng/mL. The normal circulating concentration of 1,25-$(OH)_2D_3$ is 15 to 70 pg/mL.[148] 25-$(OH)D_3$ appears to cross the placenta, whereas little transfer of 1,25-$(OH)_2D_3$ seems to take place.[149,150] Fetal vitamin D status seems to depend on maternal transfer of 25-$(OH)D_3$.[151] In term infants, serum 1,25-$(OH)_2D_3$ concentrations are low at birth but increase to a normal range by 24 hours of life, possibly reflecting the need for increased intestinal absorption of calcium and phosphorus.[149] Studies from the United Kingdom have suggested that maternal vitamin D deficiency may predispose infants to "late" neonatal (at 1 week of age) hypocalcemia. In extremely premature infants, end-organ resistance to 1,25-$(OH)_2D_3$ may exist, theoretically predisposing them to the development of hypocalcemia.[152] The vitamin D concentration is 20 to 60 IU/L in human milk, approximately 400 IU/L in regular milk (fortified) formulas, and 500 to 1200 IU/L in "preterm" formulas. A daily intake of 200 to 400 IU/day is recommended for both preterm and term infants. Breast-fed term infants with adequate sunshine exposure (30 to 120 minutes per week, with only face and hands exposed) do not appear to require supplemental vitamin D.[153]

CALCITONIN

Calcitonin is a 32–amino acid peptide with a molecular weight of 3600. It is secreted primarily by the parafollicular or C cells of the thyroid gland (Figure 33-5). Its main biologic effect is to inhibit osteoclastic bone resorption. Secretion of calcitonin varies with acute changes in serum calcium concentration: It increases when the serum calcium concentration increases and decreases when the serum calcium concentration decreases. The calcitonin receptor has been cloned and shown to be a member of the seven-transmembrane, G protein–coupled receptor family. The U.S. Food and Drug Administration (FDA) currently approves calcitonin for the treatment of Paget disease, osteoporosis, and the hypercalcemia associated with malignancies. The kidney and the liver metabolize calcitonin.

Physiologic Actions

A diverse set of effects has been attributed to calcitonin, but in many cases, these were seen in response to pharmacologic doses of the hormone. The physiologic effects of calcitonin are opposite those of PTH; however, calcitonin and PTH share similar target tissues. Calcitonin plays a role in calcium and phosphorus metabolism. Calcitonin can decrease blood calcium concentrations at least in part by effects on two well-studied target organs, bone and kidney.

Calcitonin suppresses resorption of bone by inhibiting the activity of osteoclasts, a cell type that "digests" bone matrix, through production of cAMP, and increments in cytosolic calcium in the osteoclast,[154-156] releasing calcium and phosphorus into blood. Calcitonin inhibits tubular reabsorption of these two ions, leading to increased rates of their loss in urine.

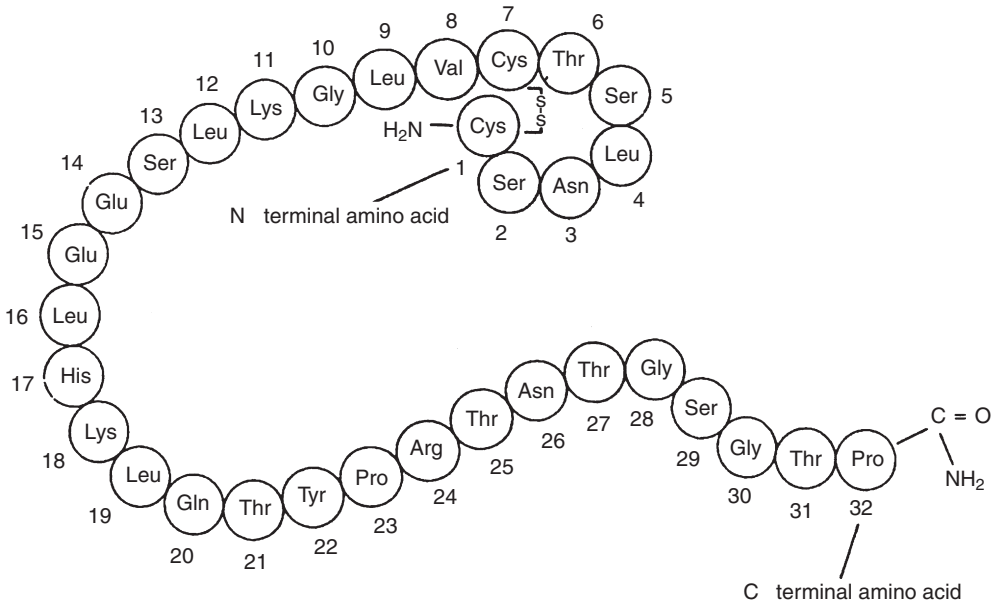


Figure 33-5 The molecular structure of calcitonin. Calcitonin, a hormone produced by the thyroid gland, is a single-chain, 32–amino acid polypeptide with a molecular weight of 3600. *(From Miacalcic Calcitonin-Sandoz:* A Profile of Sandoz's Salmon Calcitonin, *London, 1989, Meditext Ltd, p 13.)*

In the kidney, calcitonin is involved in transport of ions such as Na^+, K^+, Cl^-, Mg^{2+}, and Ca^{2+}. Calcitonin also enhances calcium excretion in tubule cells.[157] Despite the effects on calcium described earlier, it seems that calcitonin has at best a minor role in regulating blood concentrations of calcium. As evidence to support this statement, humans with chronically increased (medullary thyroid cancer) or decreased (surgical removal of the thyroid gland) levels of calcitonin in blood do not show alterations from normal in serum calcium.

In the central nervous system, calcitonin may be involved in the modulation of prolactin secretion.[158] Evidence for a physiologic role of calcitonin in the gastrointestinal tract is circumstantial. It is possible that some of the reported actions of calcitonin in the gastrointestinal tract may be secondary to cross-reaction with receptors of structurally related peptides.[159,160] From more recent studies it appears that calcitonin also may play an important role in a variety of processes ranging widely from embryonic or fetal development to sperm function and physiology.[161]

A large number of diseases are associated with abnormally increased or decreased levels of calcitonin, but pathologic effects of abnormal calcitonin secretion per se generally are not recognized. Calcitonin has several therapeutic uses. It is used to treat hypercalcemia resulting from a number of causes and has been a valuable therapy for Paget disease. Calcitonin also appears to be a valuable aid in the management of certain types of osteoporosis. A review of the literature on this subject is available.[161]

Biochemistry

Twelve species of calcitonin have been identified (including human), and common features include a 1- to 7-amino acid disulfide bridge that causes the N terminus to assume the shape of a ring, a C-terminal proline amide residue, and a glycine at residue 28. Five of the nine N-terminal residues are identical among many species.[162] The calcitonin gene encodes two distinct hormones in the initial gene transcript: a 141–amino acid precursor for calcitonin and a 128–amino acid precursor for calcitonin gene–related peptide (Figure 33-6). Calcitonin is the major processed peptide in C cells, whereas calcitonin gene–related peptide is the processed peptide in neurons.

Biosynthesis and Metabolism

Calcitonin is secreted by mitochondria of thyroid parafollicular C cells. Other tissue sources of calcitonin are the pituitary, neuroendocrine cells, and carcinomatous lesions such as medullary thyroid carcinoma and small cell lung cancer.[163,164] Calcitonin is therefore used as a tumor marker for those cancers. The serum calcium concentration is the most important regulator of calcitonin secretion.[162]

The effects of chronic hypercalcemia and chronic hypocalcemia are controversial.[165] C cells respond to hypercalcemia by increasing calcitonin secretion. If the hypercalcemia is severe and prolonged, however, the C cells exhaust their secretory reserve.[162] Calcitonin concentrations are lower in women than in men.[166,167] The mechanism responsible for this gender difference is unclear, but the effect of gonadal steroids on calcitonin secretion may play an important role. Newborn infants have high serum concentrations of this hormone, and a significant rise in blood immunoassayable circulating calcitonin (iCT) occurs between the ages of 6 and 12 years, concurrent with a fall in PTH.[168] Calcitonin may condition skeletal remodeling in the growing child by modulating the rate of bone resorption. The metabolism of calcitonin is complex and involves many organ systems. Degradation of the hormone occurs in kidney, liver, bone, and thyroid gland.[169] Inactivation is more important than excretion, and relatively small amounts of calcitonin can be detected in urine. The physiologic significance of calcitonin in calcium homeostasis is not well established.

CALCITONIN GENE–RELATED PEPTIDE

Two calcitonin gene–related peptides (α- and β-CGRP), in addition to calcitonin, form the calcitonin family of regulatory peptides.[170] β-CGRP is the product of a separate gene, but it is structurally similar to α-CGRP, differing only by three amino acids; both peptides have similar biologic properties.[171-173] α-CGRP has been identified in brain, thyroid, heart, smooth muscle, and nerve fibers.[170,174,175] Neurons containing CGRP have been identified in the heart, where it is considered an essential regulator of

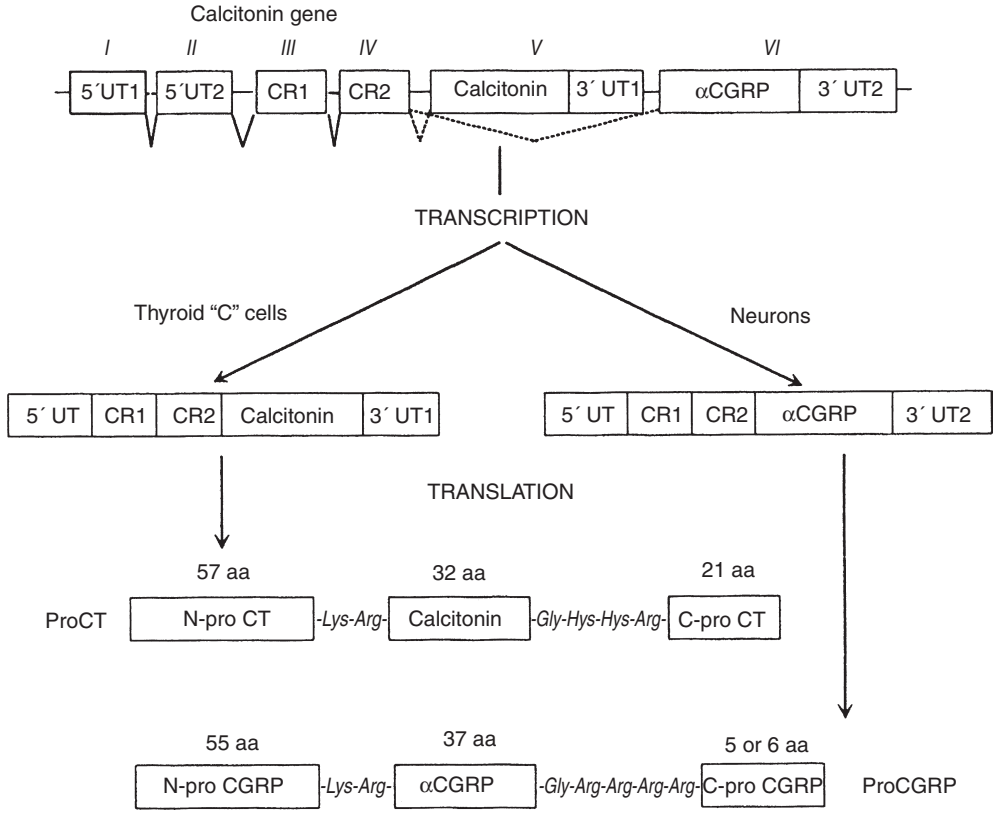


Figure 33-6 The structure of the calcitonin gene. The calcitonin (CT) gene is composed of six exons that encode the calcitonin (exon V) and the calcitonin gene–related peptide-α (α-CGPR, exon VI). *(From Civitelli R, Avioli LV: The biochemistry and function of calciotropic hormones. In Crass MF, Avioli LV [eds]:* Calcium-Regulating Hormones and Cardiovascular Function, *Boca Raton, FL, 1995, CRC Press.)*

cardiovascular function and vascular tone.[156,174,176] The calcitonin and CGRP molecules are approximately the same size, and both contain a disulfide bridge at the N terminus. These similarities may account for the ability of these hormones to bind to each other's receptor, and to produce similar in vivo biologic responses.[43,159,177]

Because very little CGRP is released into the circulation, CGRP is thought to exert its effects in a paracrine-autocrine fashion.[178] CGRP's potent vasodilatory activity is induced by its binding to two CGRP receptors: CGRP-1 and CGRP-2.[178] CGRP-1 and CGRP-2 receptors have been identified in the human fetoplacental unit[91]; however, only CGRP-1 receptors are demonstrable in the human uterine artery.[179] The mechanism of action of CGRP is unclear. It appears that CGRP interacts with receptors on smooth muscle cells that are coupled to cAMP production.[180] In rat aorta, CGRP exerts endothelium-dependent relaxant effects by releasing nitric oxide.[178] CGRP activity in human placenta, however, is independent of nitric oxide.[91]

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34 Calcium, Phosphorus, and Magnesium Transport Across the Placenta

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PLACENTAL TRANSPORT OF CALCIUM

During mammalian pregnancy, the placenta transports large amounts of calcium to allow rapid fetal skeletal mineralization. In all species that have been studied so far, the rate of maternofetal calcium transfer increases dramatically during the last third of pregnancy. In the human, two thirds of the 28 g of calcium accumulated in a healthy term fetus is transported during the third trimester.[1] Similarly, in the rat, fetal calcium content increases from 0.45 mg on day 17 of gestation to 12.3 mg at term (22 days).[2]

Active Calcium Transport

In late pregnancy, the concentration of total, ultrafilterable, and ionized calcium in fetal plasma is higher than in maternal plasma (Table 34-1); in the human, this maternofetal gradient is present as early as 20 to 26 weeks of gestation.[3,4] It is likely, therefore, that maternofetal transfer of calcium uses active *transcellular* mechanisms that overcome the concentration gradient across the placenta. As indicated by physiologic evidence, however, placental *paracellular* water-filled channels could be used by any solute, although the morphologic correlate of these channels is difficult to identify. The relative contribution of transcellular and paracellular calcium transport is unclear, but the latter mechanism may be of greater significance in the human.[5] The remainder of this discussion deals only with active transport of calcium across the placenta.

Considerable evidence has accumulated to support the concept of active maternofetal calcium transport in the placenta. Using a method in which the fetal circulation of the in situ rat placenta is artificially perfused, Štulc and Štulcová[6] noted that the maternofetal clearance of ^{45}Ca (37.4 ± 0.8 μL/minute) is considerably greater than that of inert, electrically neutral, hydrophilic molecules, such as sucrose (2.6 ± 0.3 μL/minute), which are believed to move across the placenta by passive diffusion. In earlier studies, perfusion of the fetal circulation of the in situ guinea pig placenta with plasma from an adult guinea pig[7] and the in situ ovine placenta with autologous blood[8] resulted in an increase in the ionized[8] and total[7,8] Ca^{2+} concentrations of the perfusate effluent compared with the concentration in the inflowing fluid.

Further evidence of active maternofetal placental calcium transport has been provided by numerous observations. Shaw and co-workers[9] reported a significant decrease in the unidirectional maternofetal clearance of ^{45}Ca across in situ perfused rat placenta after the addition of potassium cyanide (metabolic inhibitor) to perfusion media (Figure 34-1), or after lowering the temperature of the perfusate (Figure 34-2), findings confirming the earlier report by Štulc and Štulcová.[6] Using in vitro perfusion of a human placental lobule, Abramovich and colleagues[10] reported an increase in both ionized and total Ca^{2+} concentrations in the fetal perfusate reservoir during recycling perfusion. Finally, Štulc[5] provided evidence that the transcellular component of unidirectional maternofetal calcium flux ($^{Ca}J_{MF}$) across the isolated human placental cotyledon is saturable and sensitive to cyanide.

Thus, maternofetal transport of calcium across the placenta takes place against a concentration gradient, is temperature dependent, is inhibited by metabolic inhibitors such as cyanide, and is considerably faster than that of molecules that cross the placenta by simple diffusion. All of these findings support the conclusion that placental calcium transport involves active transfer processes.

Bidirectional Placental Calcium Transport

Studies employing radioactive isotopes of calcium as tracers have revealed that transport of calcium across the mammalian placenta is bidirectional, with marked species variation in the relative magnitudes of unidirectional maternofetal, unidirectional fetomaternal, and net calcium fluxes. In the sheep near term, placental calcium transport is highly asymmetric, with a calculated $^{Ca}J_{MF}$ of 215 mg/day/kg of fetal weight and a $^{Ca}J_{FM}$ of 12 mg/day/kg.[11] A similar situation probably prevails in the rat because the value for net calcium flux estimated from the increase in fetal calcium content between days 20 and 21 of gestation (100 ± 4 nmol/minute) is close to the $^{Ca}J_{MF}$ (100 ± 7 nmol/minute) estimated in nonanesthetized animals at 21 days.[6] By contrast, in the rhesus monkey, the magnitudes of bidirectional placental calcium fluxes are comparatively similar to one another ($^{Ca}J_{MF}$, 391 mg/kg/day; $^{Ca}J_{FM}$, 326 mg/kg/day).[11] In the human, bidirectional clearances of calcium also are similar (unidirectional maternofetal clearance, 40.9 ± 14.7 μL/minute/g of placental weight; unidirectional fetomaternal clearance, 39.1 ± 21.8 μL/minute/g).[5] These fluxes and clearances are considerably higher than the rate of daily fetal calcium accumulation or the net flux, which in the rhesus monkey at term has been estimated to be 40 mg of calcium per day/kg of fetal body weight,[12] and in the human, 200 mg of calcium per day during the last trimester.[1] A similar situation also exists for placental transfer of sodium across the human placenta.[13]

Mechanisms of Placental Calcium Transport

Although the placental content of total calcium is high,[14] the free cytosolic Ca^{2+} concentration must be maintained in the submicromolar range, by analogy with other transporting epithelia,[15] if viability is to be preserved. In isolated placental cells, the cytosolic concentration of free calcium is in the nanomolar range.[16] Therefore the large traffic of calcium ions across the trophoblast poses a threat to normal cellular function, and efficient mechanisms for intracellular calcium buffering and translocation from the maternal-facing cytosol to the fetal-facing cytosol must exist. Whereas bidirectional movements may occur by diffusion through water-filled paracellular channels, asymmetric active

TABLE 34-1

Maternal and Fetal Plasma Calcium Concentrations (mmol/L)

	MOTHER				FETUS			
Species	**Total**	**(n)**	**Ultrafilterable/Ionized**	**(n)**	**Total**	**(n)**	**Ultrafilterable/Ionized**	**(n)**
Human	2.13 ± 0.15	(115)	1.12 ± 0.06[i]	(115)	2.65 ± 0.19	(115)	1.41 ± 0.09[i]	(115)
Monkey	2.05 ± 0.008	(7)	1.05 ± 0.006[i]	(5)	2.10 ± 0.008	(7)	1.33 ± 0.005[i]	(5)
Rat	2.23 ± 0.06	(9)	1.10 ± 0.03[i]	(17)	2.75 ± 0.002	(49)	1.42 ± 0.03[i]	(18)
Guinea pig	1.70 ± 0.06	(13)	1.23 ± 0.05[u]	(13)	2.30 ± 0.03	(13)	1.53 ± 0.04[u]	(13)
Sheep	2.12 ± 0.05	(40)	0.99 ± 0.09[i]	(5)	3.30 ± 0.05	(42)	1.59 ± 0.16[i]	(6)
Pig	2.53 ± 0.02	(19)	0.79 ± 0.03[i]	(5)	3.10 ± 0.04	(22)	1.12 ± 0.14	(5)

From Sibley CP, Boyd RDH: Control of transfer across the mature placenta. In Clarke JR (ed): *Oxford Reviews of Reproductive Biology,* vol 10. By permission of Oxford University Press, *1988*.

Values are ± SEM.

i, Ionized; *u,* ultrafilterable.

transport necessitates a transcellular route. Transcellular placental calcium transport is likely to involve three steps:

1. Influx or entry of Ca^{2+} from maternal plasma across maternal-facing placental membrane
2. Movement of calcium through trophoblastic cytosol
3. Efflux or exit of Ca^{2+} from cytosol across the basolateral or fetal-facing placental membrane

Little is known of the mechanisms involved in Ca^{2+} influx from maternal plasma into trophoblastic cytosol, but it is possible that the entry mechanism involves a variety of apical calcium channels.[17] The unidirectional influx of calcium is higher on the fetal than on the maternal side of the placenta in the guinea pig,[18] and the fluxes are equal on both sides of the human placenta.[19] The net maternofetal flux, however, is higher than the fetomaternal flux in both placental types. In studies involving uptake of ^{45}Ca from artificial media used to perfuse both fetal and maternal circulations of guinea pig placenta, van Kreel and van Dijk[20] demonstrated that calcium enters the trophoblast as a charged ion. Thus placental calcium transport is likely to be similar to that in the intestine, in which entry of calcium ions across the brush-border membrane occurs by passive diffusion down an electrochemical gradient. As in the gut,[15] this process in the placenta is saturable.[5,18] Evidence of a carrier has been obtained by examining calcium uptake into human placental microvillous membrane vesicles in which a two-site model was characterized with kinetic estimates of the Michaelis constant (K_m) of 18 μM and 1.5 mM, and maximum velocity (V_{max}) of 3.2 and 47 nmol/mg/minute, respectively. The K_m of calcium uptake in intestine is approximately 1 mM,[21] much higher than the K_m for efflux, which is in micromolar concentrations. Therefore the rate of calcium influx into the cells is probably the rate-limiting step for transcellular Ca^{2+} flux. In the intestine, 1,25-dihydroxyvitamin D_3 (1,25-$[OH]_2D_3$), the biologically active metabolite of vitamin D, appears to increase the V_{max} of calcium influx, without altering the K_m.[22] In other words, 1,25-$(OH)_2D_3$ probably increases the synthesis of Ca^{2+} carrier or channel units in this organ.

Little is known about passage of calcium through the trophoblastic cytosol. Clearly, in all calcium-transporting epithelial cells, an efficient mechanism for efflux of Ca^{2+} as well as intracellular compartmentalization or buffering of calcium ions in transit must exist if the cytosolic Ca^{2+} concentration, which is approximately 1000 times lower than the extracellular Ca^{2+} concentration, is to remain unaffected. This maintenance function may be the role of calcium-binding proteins (CaBPs), a family of proteins involved in the transport and binding of calcium, which may serve as an intracellular calcium buffer or an intracellular shuttle and may thus facilitate transcellular calcium transport.

CaBPs have been identified in numerous species. Placental CaBP in the rat is immunologically identical to the vitamin D–dependent CaBP in the intestine,[23] but it seems to lack the classic dependency of the latter on 1,25-$(OH)_2D_3$ in intact animals.[24-26] However, the administration of 1,25-$(OH)_2D_3$ to thyroparathyroidectomized pregnant rats does result in an increase in the concentration of placental CaBP.[27] Thus the role of maternal vitamin D in control of placental CaBP is not entirely clear.

In the rat, placental concentration of CaBP increases rapidly during the period of increased rate of fetal growth and calcium accumulation in late gestation,[23,28] suggesting a role for this protein in placental Ca^{2+} transport. This concept is supported by the finding of a temporal relationship between CaBP messenger RNA (mRNA) expression and $^{Ca}J_{MF}$ of calcium in the placenta of the rat[29] and by the demonstration that uptake of calcium by human placental microsomal membrane vesicles is inhibited by preincubation with antihuman CaBP antibodies.[30] Investigators have shown that CaBP enhances diffusion of Ca^{2+} through an aqueous compartment,[31] and in the presence of CaBP, the rate of cytosolic Ca^{2+} diffusion in enterocytes is 60 to 70 times higher than across cytosol of CaBP-depleted cells.[32] Thus CaBP may act as an intracellular shuttle to facilitate the diffusion of Ca^{2+} through trophoblastic cytosol.

Intracellular organelles may serve as cytosolic calcium buffers and reservoirs in the placenta. In the guinea pig placenta, the subcellular distribution of ^{45}Ca is mainly in the nuclear fraction (48%), 28% is bound to cytoplasmic proteins, and approximately 10% is in the mitochondrial fraction.[20] Electron microscopy of human placenta has revealed granular calcium deposits, localized to endoplasmic reticulum and mitochondria, and in vesicles within the trophoblast.[33] Intracellular calcium sequestration is supported by the observation that a human placental microsomal membrane fraction can be reconstituted into vesicles that exhibit a magnesium (Mg^{2+})– and adenosine triphosphate (ATP)–dependent Ca^{2+} uptake mechanism.[34]

Mechanisms involved in the efflux of Ca^{2+} from cytosol across the fetal-facing trophoblastic membranes to fetal plasma against a steep concentration gradient are not clearly delineated. In other absorptive epithelia (e.g., the intestine and the kidney), at least two mechanisms are involved in efflux of Ca^{2+} out of the cytosol across the basolateral cell membrane:

- Calcium- and magnesium-dependent adenosine triphosphatase (Ca^{2+}-ATPase)
- The Ca^{2+}-Na^+ exchanger (NCX)

Growing evidence suggests that, in the placenta, a Ca^{2+}-ATPase may be responsible for the translocation of calcium from trophoblast cytosol to fetal extracellular fluid across the basal plasma membrane. Hydrolysis of ATP could provide the required energy to overcome the Ca^{2+} concentration gradient. Ca^{2+}-ATPase has been identified in placentas of guinea pigs,[35] mice,[36]

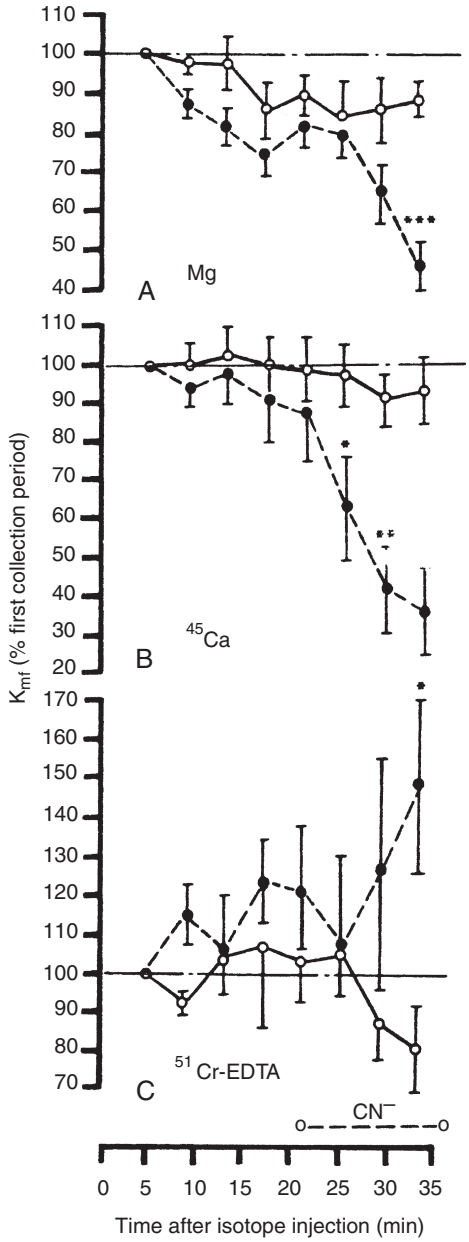


Figure 34-1 Effects of potassium cyanide (CN) (1 mmol/L final concentration) to perfusate fluid on maternofetal clearance (K_{mf}) of magnesium (Mg) **(A),** calcium (^{45}Ca) **(B),** and chromium 51 ethylenediaminetetraacetic acid (^{51}Cr-EDTA) **(C)** across in situ, fetally perfused rat placentas (n = 6/group). Mean K_{mf} values ± SEM are normalized to that for the first 4-minute perfusate collection period. Statistical significance of differences between treated *(solid circles)* and control *(open circles)* groups: *P <.05; **P <.01; ***P <.001 (Student's t-test, two tailed). *(From Shaw AJ, Mughal MZ, Mohammed T, et al: Evidence for active maternofetal transfer of magnesium across the in situ perfused rat placenta,* Pediatr Res *27:622, 1990.)*

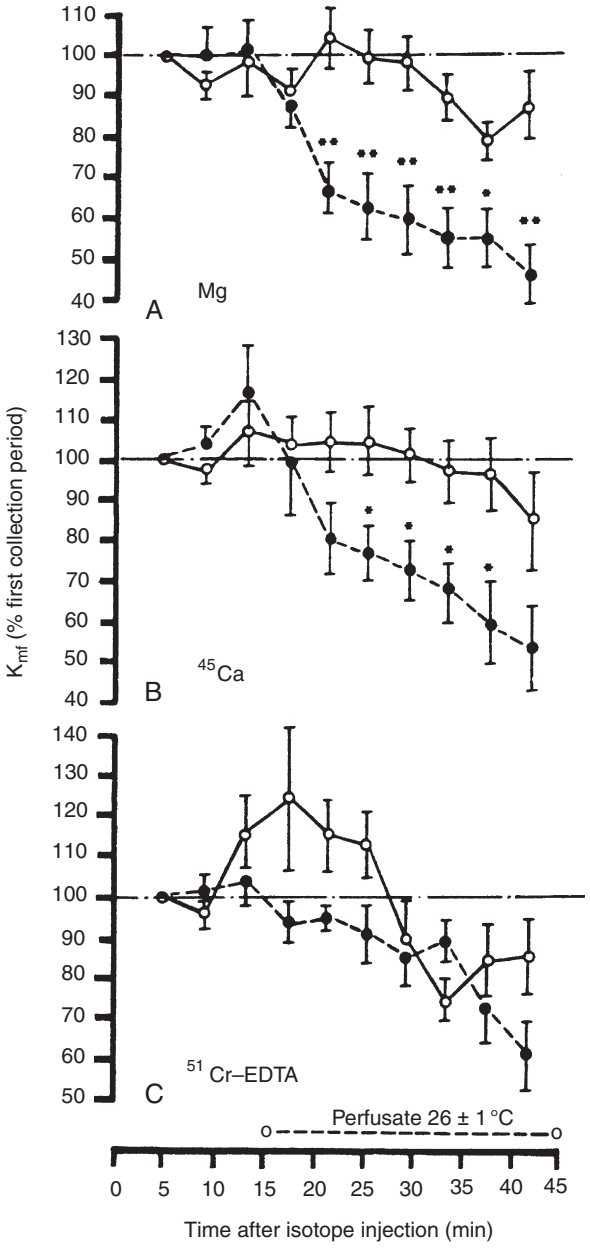


Figure 34-2 Effects of lowering of tempereature of perfusate fluid from 37° C on maternofetal clearance (K_{mf}) of magnesium (Mg) **(A),** calcium (^{45}Ca) **(B),** and chromium 51 ethylenediaminetetraacetic acid (^{51}Cr-EDTA) **(C)** across in situ fetally perfused rat placentas (n = 6/group). Mean K_{mf} values ± SEM are normalized to that for the first 4-minute perfusate collection period. Statistical significance of differences between treated *(solid circles)* and control *(open circles)* groups: *P <.05; **P <.01; ***P <.001 (Student's t-test, two tailed). *(From Shaw AJ, Mughal MZ, Mohammed T, et al: Evidence for active maternofetal transfer of magnesium across the in situ perfused rat placenta,* Pediatr Res *27:622, 1990.)*

cats,[37] and humans.[34,38,40] It is clear that the Ca^{2+}-ATPases are a heterogeneous group of enzymes,[41] not all of which are involved in calcium transport[42,43]; this heterogeneity may explain the reported dissociation between Ca^{2+}-ATPase transport activity and Ca^{2+}-ATPase hydrolytic activity.[34,39] Calcium uptake into highly purified human placental basal plasma membrane vesicles has been described.[44,45] This uptake may be mediated by one of the Ca^{2+}-ATPases. Monoclonal antibodies against the human erythrocyte plasma membrane calcium pump show greater specific staining in the human placental basal membrane and in the fetal-facing membrane of the rat placenta, and they inhibit by 50% transport of calcium into human placental basal plasma membrane vesicles.[46] Expression of Ca^{2+}-ATPase mRNA and protein[47] in both human[47, 48] and rat[29] placenta shows little

change as gestation proceeds; it appears, therefore, that this protein is not rate-limiting in placental calcium transport.

Current evidence indicates that the NCX is unimportant in regulating transplacental Ca movement. Van Kreel and van Dijk,[20] who perfused both the maternal and fetal circulations of the term guinea pig placenta with calcium-free media containing ^{22}Na and unlabeled sodium, found that the addition of 2 mM Ca^{2+} to the media after 30 minutes of equilibration does not affect the release of preloaded ^{22}Na from placental cells into the media. The presence of an NCX has been sought in the in situ rat placenta by perfusing the fetal circulation and measuring the maternofetal flux of calcium in the presence or absence of sodium in the perfusate. In the absence of sodium, maternofetal flux of calcium is either unaltered[49] or decreased.[50] In the latter study, however, the effect on $^{Ca}J_{MF}$ was delayed. In addition, $^{Ca}J_{MF}$ is unaffected during perfusion with sodium-containing perfusate to which ouabain, a potent Na^+,K^+-ATPase inhibitor, had been added; by dissipation of the sodium gradient, an effect on calcium flux would be expected in the presence of an NCX. In vitro placental membrane vesicle studies showed a lack of effect of sodium or ouabain on calcium uptake.[34,45] By contrast, in a later study using human placental basal membrane vesicles, these workers suggested the presence of an NCX in this membrane[51]; however, the number of experiments was small, and the orientation of the vesicles (cytoplasmic side inside versus outside) was mixed, features that call into question the significance of this finding in relation to calcium transport across the basal membrane. Using a cDNA probe to the cloned human cardiac NCX, Kofuji and co-workers[52] demonstrated that the expression of the gene encoding this NCX in human placenta is some 250-fold less than in human heart. The presence of a high-affinity, low-capacity facilitated diffusion transporter in human placental basal membrane vesicles has been reported,[53] but the functional significance of this transporter in relation to maternofetal transfer of calcium is unknown.

Control of Placental Calcium Transport

Factors that may influence the supply of calcium to the fetus include maternal and fetal plasma Ca^{2+} concentrations, maternal and fetal blood flow, and the capacity and efficiency of placental transport mechanisms. In the rat, the maternofetal clearance of calcium per gram of placental tissue increases 70-fold in the last 6 days of gestation,[29] far more than would be expected with an increase in placental surface area or blood flow; uterine horn blood flow increases five-fold per placenta by day 21 of gestation compared with the virgin state.[54] In the human, over the whole of pregnancy, 170 L of maternal plasma would have to be cleared of its calcium content to account for the total amount of calcium accumulated in a term human fetus.[55] Because the estimated uterine blood flow rate in the human at term gestation is 500 mL/minute,[56] placental calcium transfer appears limited by the area and the efficiency of placental transfer mechanisms, rather than by uteroplacental blood flow.[55] Mughal and colleagues,[57] however, observed a marked decrease in the unidirectional maternofetal clearance of ^{45}Ca, but not chromium-51 ethylenediaminetetraacetic acid (^{51}Cr-EDTA, the reference diffusional marker), in growth-restricted rat fetuses induced by uterine artery ligation compared with control fetuses. These investigators speculated that placental ischemia from chronic reduction in uteroplacental blood flow could result in depletion of the necessary energy supply for active placental calcium transport. Thus flow limitation is unlikely to be important for placental calcium transport.

Influence of Maternal Calcium Concentration

As suggested by indirect evidence from human studies, maternal plasma calcium concentration appears to influence placental calcium transport. Maternal dietary deficiency leading to hypocalcemia can be associated with congenital rickets and neonatal hypocalcemia.[58] Transient congenital hyperparathyroidism has been reported in infants born to mothers with hypocalcemia secondary to poorly treated hypoparathyroidism.[59] It is postulated that under these conditions, reduced net placental calcium transport leads to fetal hypocalcemia, which in turn results in fetal hyperparathyroidism. Conversely, transient neonatal hypoparathyroidism caused by untreated hyperparathyroidism has been reported in infants born to hypercalcemic mothers.[60] Thus, in the pregnant human, placental calcium transport appears to be dependent on the maternal plasma calcium concentration. Similar observations have been made in the guinea pig,[61] pig,[62] and rabbit,[63] but not in the sheep,[64] cow,[65] and rat.[66] In the rat, a doubling of maternal plasma Ca^{2+} concentration causes only a 30% increase in the $^{Ca}J_{MF}$ across the in situ perfused rat placenta. It appears, therefore, that in this species an active placental pump must be working at almost maximal capacity under normal conditions.[6] By contrast, studies using the in situ perfused guinea pig placenta have demonstrated that maternal hypercalcemia results in a significant increase in $^{Ca}J_{MF}$,[67] which may result from submaximal saturation of the active Ca^{2+} pump or from the existence of a quantitatively important and possibly unphysiologic paracellular leak pathway in this preparation.[68]

In sheep, maternal hypocalcemia does not lead to any change in fetal plasma calcium concentrations.[69] By contrast, acute maternal hypocalcemia in the rat[70] and monkey[71] results in a fall in fetal calcium concentration, but chronic maternal hypocalcemia induced by maternal vitamin D deficiency and thyroparathyroidectomy does not alter the $^{Ca}J_{MF}$ across in situ perfused placentas.[72] The evidence clearly points to marked species variation in the fetal response to maternal hypocalcemia or hypercalcemia, which may be physiologic or methodologic.

Hormonal Regulation of Placental Calcium Transport

The placenta has no nerve supply. Accordingly, factors that increase placental calcium transport capacity as gestation proceeds must be either genetically preprogrammed or hormones produced by the mother, fetus, or placenta itself. Hormonal signals controlling placental calcium transfer may act on either the maternal or fetal side of the placenta. Possible regulatory hormonal candidates include 1,25-$(OH)_2D_3$, parathyroid hormone (PTH), calcitonin, and other peptides secreted by the parathyroid glands.

Infants born in summer are reported to have a lower bone mineral content at birth when compared with infants born in winter.[73,74] The reason for this difference is not entirely clear but may be related to seasonal variation in maternal and fetal calciotropic hormone levels[74,75] and their speculative effect on placental transport of calcium and magnesium.

1,25-Dihydroxyvitamin D_3, 1α-Hydroxycholecalciferol, and the Vitamin D Receptor

Anecdotal clinical reports have described maternal vitamin D deficiency causing neonatal rickets,[76] consistent with the thesis that maternal vitamin D may be important for placental calcium transfer. This concept is supported by the presence of cytosolic receptors for 1,25-$(OH)_2D_3$ in the human and the rat.[77] Potential sources of 1,25-$(OH)_2D_3$ available to the fetus include that transferred from maternal plasma across the placenta,[78] that synthesized by the fetal kidneys,[79] and the placenta itself.[80] Because fetal plasma concentration of 1,25-$(OH)_2D_3$ falls after fetal nephrectomy in sheep,[81] it appears that in sheep, the fetal kidney is a major source of this metabolite of vitamin D.

The data on the role of maternal vitamin D in placental Ca transfer are conflicting. Administration of 1,25-$(OH)_2D_3$ to pregnant guinea pigs increases fetal calcium content and increases

maternoplacental, but not maternofetal, transfer of ^{45}Ca.[82] In sheep, maternal treatment with 1α-hydroxycholecalciferol (1α-OHCC), the hydroxylated derivative of vitamin D, for 12 days leads to a significant increase in fetal calcium content, as well as maternofetal calcium transfer.[83] In the same species, maternal prolactin injections can stimulate placental calcium transfer[84]; this hormone theoretically could exert an effect on the placenta by stimulating production of 1,25-$(OH)_2D_3$ in the mother. By contrast, in the rat, maternal vitamin D deficiency[72,85] and parathyroidectomy[72] do not affect the maternofetal calcium gradient,[85] the total fetal calcium content,[72,85] or the maternofetal clearance of ^{45}Ca.[72] The concentrations of CaBP[24,25] and its mRNA[26] are significantly reduced in rat maternal intestine, but not placenta, under experimental conditions of maternal vitamin D deficiency. The inconsistent data reported thus far may simply reflect interspecies variation.

The effect of fetal 1,25-$(OH)_2D_3$ on placenta calcium transport also is unclear. Fetal nephrectomy in the rat does not alter the maternofetal total calcium gradient,[86] although injection of a pharmacologic dose of 1,25-$(OH)_2D_3$ into 19-day rat fetuses increases the fetal Ca^{2+} concentration on day 21 of gestation, compared with control fetuses.[87] Fetal nephrectomy in sheep leads to a marked decrease in the fetal plasma concentration of 1,25-$(OH)_2D_3$ associated with reversal of the usual maternofetal calcium gradient, which is restored by infusion of 1,25-$(OH)_2D_3$ into the fetal circulation.[81,88] It is not clear whether these findings reflect control of placental calcium transport by 1,25-$(OH)_2D_3$ synthesized by fetal kidneys or present evidence of short-term dependence of fetal plasma calcium concentrations on mobilization from fetal bone.

In support of a direct permissive effect of fetal 1,25-$(OH)_2D_3$ on placental calcium transfer is the observation of Robinson and colleagues[89] that 1,25-$(OH)_2D_3$ stimulates $^{Ca}J_{MF}$ across in situ perfused placentas of rat fetuses parathyroidectomized by gross decapitation,[90] but not across placentas of intact fetuses. Because PTH is an important trophic stimulator of 1,25-$(OH)_2D_3$ synthesis, it is likely that plasma 1,25-$(OH)_2D_3$ concentration is lower in parathyroidectomized rat fetuses than in intact fetuses. Therefore 1,25-$(OH)_2D_3$ probably stimulates placental calcium transfer only when fetal plasma concentrations are initially low.

Using a vitamin D receptor (VDR) gene–knockout mouse model, Kovacs and colleagues investigated fetal mineralization and maternofetal transfer of calcium in the fetuses of heterozygous males and females. They suggested that the VDR is not required for fetal mineralization or placental maternofetal transfer of calcium.[91] Placental transport of calcium was measured indirectly, however, and the values in the three fetal genotypes were normalized to heterozygous littermates. Further studies directly measuring placental calcium transfer are required to define the role of the VDR.

Parathyroid Hormone and Fetal Parathyroid Hormone–Related Protein

In the rat, fetal parathyroidectomy by decapitation[89,92] or by injection of antibovine PTH serum[93] leads to a fall in fetal plasma total calcium concentration, which is restored by administration of parathyroid extract.[93,94] In the sheep, fetal thyroparathyroidectomy with thyroxine replacement results in a rapid fall in fetal Ca^{2+} concentration within 24 hours.[95-97] Similarly, in the *Hoxa3*-knockout mouse, complete absence of fetal parathyroids results in lower fetal total and ionized calcium concentrations, whereas indirect measurement of placental calcium transfer is unaffected.[98] As previously mentioned, a significant fall in maternofetal clearance of ^{45}Ca occurs across the in situ perfused placentas of decapitated rat fetuses,[89] and clearance is increased (but not returned to normal) by subcutaneous administration of bovine PTH(1-84) to the fetuses. This stimulatory effect of PTH(1–84) on maternofetal ^{45}Ca clearance may result from its direct action on the placental Ca^{2+} pump; alternatively, the hormone may increase fetal 1,25-$(OH)_2D_3$ production. Perfusion of the fetal circulation in intact and decapitated rat fetuses with a medium containing forskolin (an activator of adenylate cyclase) stimulates maternofetal clearance of ^{45}Ca. Synthetic rat PTH amide (r[Nle,Tyr])PTH[1-34]), however, has no effect under these conditions,[99] and because this amide also can increase placental cAMP release,[100] the role of placental adenylate cyclase in calcium transfer is not clear.

Another candidate hormone that may stimulate placental calcium transport is PTH-related protein (PTHrP).[101] The addition of fetal parathyroid gland extract, partially purified PTHrP from a human lung cancer cell line, or recombinant PTHrP, but not bovine PTH(1-84) or rat PTH(1-34),[102] to autologous fetal blood used to perfuse placentas of thyroparathyroidectomized sheep fetuses in situ leads to an increase in the rate of calcium accumulation in the fetal blood reservoir.[95,102-104] In sheep, it appears that mid-molecule fragments of PTHrP may be responsible for the stimulation of placental calcium transport.[95,105,106] When human PTHrP (hPTHrP)(1-34) or hPTHrP(75-86) is infused into the fetal circulation of the in situ rat placenta, these peptides have no effect on calcium transport.[99] Indirect evidence, however, suggests that fetal PTHrP fragments increase maternofetal transport of calcium in murine[107] and human[108] placentas. Surprisingly, net fetal calcium accretion is increased in the absence of fetal PTHrP.[109] Taken together, these findings seem to indicate that, in the absence of fetal PTHrP, maternofetal flux of calcium is decreased, but fetomaternal flux is decreased to a greater extent; the change in the former may be related to PTHrP absence or secondary 1,25-$(OH)_2D_3$ deficiency in fetal plasma, or both.

Calcitonin

In sheep, Barlet[110] demonstrated that chronic maternal calcitonin deficiency (achieved by maternal thyroparathyroidectomy and thyroxine replacement) leads to a significant increase in the total fetal calcium content and fetal accumulation of ^{45}Ca on day 140, but not on day 77, of gestation (term gestation, approximately 150 days). Calcitonin replacement in these ewes normalizes both the fetal calcium content and the maternofetal transfer of ^{45}Ca. Thus, in this species, maternal calcitonin deficiency appears to increase $^{Ca}J_{MF}$. Studies using the calcitonin–calcitonin gene–related peptide-α–knockout mouse model (Ct/Cgrp) suggest that neither maternal nor fetal calcitonin–calcitonin gene–related peptide-α modulate placental calcium transfer.[111]

In situ perfusion of the rat placenta has been used widely in the study of placental transfer mechanisms and their control. The recent validation of this technique in the mouse[112] and its application in transgenic mice is expected to elucidate the control of placental transport at the molecular level.

PLACENTAL TRANSPORT OF PHOSPHORUS

Provision of an adequate supply of phosphorus to the developing fetus is important because of its role in intermediary metabolism and skeletal mineralization. In mammals during the latter part of gestation, the plasma concentration of phosphorus in the fetus is higher than that in the mother (Table 34-2). Thus placental transfer of phosphorus in the maternofetal direction occurs against a concentration gradient and is likely to involve an active transport process. Most fetal phosphorus content is transported to the fetus during the latter stages of pregnancy.[113,114]

Mechanisms of Placental Phosphorus Transport

The flux of phosphorus across the placenta is bidirectional, with flux in the maternofetal direction exceeding that in the fetomaternal direction.[49,115] As shown in Figure 34-3, for a 60-g fetus,

TABLE 34-2

Concentrations of Inorganic Phosphorus in Maternal and Fetal Plasma (mmol/L)

Species	Mother	(*n*)	Fetus	(*n*)
Human*	1.43 ± 0.5	(115)	1.92 ± 0.4	(115)
Sheep†	1.29 ± 0.16	(6)	1.97 ± 0.4	(6)
Guinea pig‡	1.25 ± 0.1	(6)	1.96 ± 0.13	(6)
Rat§	1.75 ± 0.25	(12)	3.29 ± 0.15	(21)

*Data from Schauberger CW, Pitkin RM: Maternal-perinatal calcium relationships, *Obstet Gynecol* 53:74, 1979.

†Data from Mellor DJ, Matheson IC: Variations in the distribution of calcium, magnesium, and inorganic phosphorus within chronically catheterized sheep conceptuses during the last eight weeks of pregnancy, *Q J Exp Physiol* 62:55, 1977.

‡Data from Durand D, Dalle M, Barlet JP: Plasma calcium homeostasis in the guinea pig during the perinatal period, Biol Neonate 42:120, 1982.

§Data from Garel JM, Dumont C, Barlet JP, Care AD: Fetal-maternal calcium relationships in rat and sheep, *J Physiol (Paris)* 64:387, 1972.

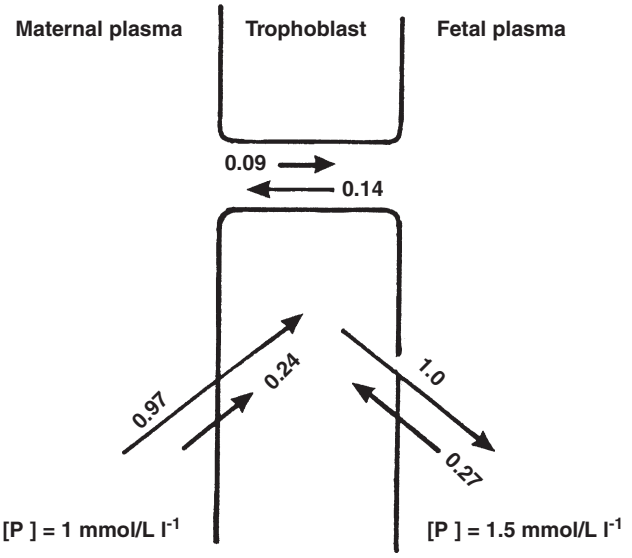


Figure 34-3 The components of phosphorus fluxes expressed in micromoles across fetal[49] and maternal[115] faces of a guinea pig placenta. The net phosphorus flux, 0.7 μmol/minute, is similar to the retention of phosphorus by the guinea pig fetus of comparable weight.[113] The paracellular fluxes constitute only a small fraction of the total bidirectional trophoblastic phosphorus fluxes. *P,* Plasma phosphorus concentration. *(Modified from Štulc J, Štulcová: Transport of inorganic phosphate by the fetal border of the guinea-pig placenta perfused in situ,* Placenta *5:9, 1984. Additional data from Štulc J, Stulcová B, Svihovec J: Uptake of inorganic phosphate by the maternal border of the guinea-pig placenta,* Pflugers Arch *395:326, 1982.)*

the calculated net flux of phosphorus is 0.7 μmol/minute, a value similar to that obtained with retention of phosphorus by guinea pig fetuses of comparable weight.[113] Placental uptake of phosphorus at both the maternal and fetal faces of guinea pig placenta is reduced by anoxic conditions, by the addition of cyanide, or by the reduction or elimination of sodium from the perfusion media.[113] From these results, it appears that the transcellular, bidirectional phosphorus transport is highly asymmetric and depends on a sodiumdependent active transport mechanism. Maternofetal flux prevails over fetomaternal flux and is reduced by anoxic conditions or addition of cyanide.

In vitro phosphorus uptake studies using human placental brush-border membrane vesicles support the presence of a high-affinity (K_m of 87 μM; pH 7; 35° C) sodiumependent transport system[116,117] that is likely to be saturated in vivo. At pH 8, the K_m increases (to 500 μM), whereas an increase in the Na^+ concentration of the incubation medium increases phosphorus uptake by an increase in V_{max} but not K_m. Temperature influences phosphorus uptake by changing both K_m and V_{max}. The uptake of phosphorus by these vesicles is noncompetitively inhibited by glycine, alanine, and proline. Thus, in the human, pH, temperature, and sodium and amino acid concentrations appear to modulate the transport of phosphorus from maternal plasma into the trophoblastic cytosol.

Hormonal Regulation of Placental Phosphorus Transport

Factors that modulate placental phosphorus transport have not been extensively studied. Based on the presence of receptors for 1,25-$(OH)_2D_3$[77] and for PTH[118] in the placenta, it has been suggested that these hormones may regulate placental phosphorus transport. In the rat, maternal vitamin D deficiency does not affect the maternofetal plasma phosphorus concentration gradient or fetal phosphate content at term gestation.[84] By contrast, intravenous administration of 1α-OHCC to pregnant sheep results in significant increase in both maternal and fetal phosphorus concentrations.[119] Similarly, Barlet and co-workers[65] observed that feeding of *Solanum glucophyllum* leaves, which contain 1,25-$(OH)_2D_3$, to pregnant cows results in hyperphosphatemia in both mother and fetus. From these studies, it is not clear whether the maternal 1,25-$(OH)_2D_3$ or its analogues given to the mother increase fetal serum phosphorus concentrations by stimulating net placental phosphorus transport or simply by increasing the concentration of phosphorus in maternal plasma.

There is some evidence of modulation of placental phosphorus transport by vitamin D or its analogues in sheep. Injection of 1α-OHCC to pregnant ewes for 12 days results in increased maternofetal transport of ^{32}P at day 140 of gestation.[83] Lowering of fetal plasma 1,25-$(OH)_2D_3$ by fetal nephrectomy leads to increased fetal plasma concentration of phosphorus, which can be prevented by daily intravenous injections of 1,25-$(OH)_2D_3$ into the fetuses.[88] The investigators speculated that unidirectional fetomaternal phosphorus transport in this species could be regulated by 1,25-$(OH)_2D_3$ produced by fetal kidneys.

The uptake of phosphorus by human placental brush border membrane is inhibited by PTH, with its action possibly mediated by cAMP.[120] Because adenylate cyclase is located in the basal plasma membranes, fetal PTH may reduce placental phosphorus transfer in the fetomaternal direction. Thus 1,25-$(OH)_2D_3$, acting on the maternal as well as the fetal side of the placenta, and fetal PTH may regulate placental phosphorus transport. By contrast, MacIsaac and associates showed that extracts of fetal parathyroid glands and purified PTHrP, as well as recombinant PTHrP(1-84, 1-108, and 1-141), do not stimulate phosphorus transport across the placenta of thyroparathyroidectomized sheep fetuses that are perfused with autologous blood.[97]

PLACENTAL TRANSPORT OF MAGNESIUM

Toward the end of pregnancy, the plasma concentrations of total and ultrafiltrable magnesium[121] in the fetus exceed those in the mother (Table 34-3). Therefore maternofetal magnesium transfer occurs against a concentration gradient and is likely to involve active transport mechanisms. Support for this concept comes from studies by Shaw and colleagues,[9] who observed that the unidirectional maternofetal clearance of magnesium across perfused rat placentas in situ is considerably higher (26.7 μL/g/minute) than

TABLE 34-3

Concentrations of Magnesium in Maternal and Fetal Plasma (mmol/L)

Species	Mother	(*n*)	Fetus	(*n*)
Human*	0.66 ± 0.16	(115)	0.71 ± 0.16	(115)
Sheep†	0.71 ± 0.7	(6)	0.99 ± 0.7	(6)
Guinea pig‡	1.11 ± 0.05	(6)	1.20 ± 0.03	(6)
Rat§	0.66 ± 0.02	(15)	0.98 ± 0.03	(23)

*Data from Schauberger CW, Pitkin RM: Maternal-perinatal calcium relationships, *Obstet Gynecol* 53:74, 1979.

†Data from Mellor DJ, Matheson IC: Variations in the distribution of calcium, magnesium, and inorganic phosphorus within chronically catheterized sheep conceptuses during the last eight weeks of pregnancy, *Q J Exp Physiol* 62:55, 1977.

‡Data from Woods LL, Thornburg KL, Faber JJet al: Transplacental gradients in the guinea-pig, *Am J Physiol* 235:H200, 1978.

§Data from Dancis J, Springer D, Cohlan SQ: Fetal homeostasis in maternal malnutrition, II: Magnesium deprivation, *Pediatr Res* 5:131, 1971.

that of ^{51}Cr-EDTA, the reference diffusional marker (3.2 αμL/g/minute).They also showed that the maternofetal clearance of magnesium is significantly reduced by the addition of cyanide to fetal perfusate (see Figure 34-1) or by lowering of perfusate temperature (see Figure 34-2).

In human pregnancy, fetal magnesium content increases rapidly after the fifth month of gestation, with net fluxes of approximately 4.5 mg of magnesium per day.[122] However, only 0.06% of magnesium injected into the maternal arterial circulation (to achieve twice the physiologic concentration) of in vitro perfused human placental lobule was transferred into the fetal perfusate.[123] Bidirectional magnesium flux has been demonstrated in sheep, with maternofetal flux (0.042 mg/kg of fetal weight/hour) exceeding fetomaternal flux (0.012 mg/kg/hour).[124]

Mechanisms of Placental Magnesium Transport

Little is known of the mechanisms of placental magnesium transport. Influx of Mg^{2+} across the maternal-facing placental membrane may be a passive process depending on the intracellular free Mg^{2+} concentration and transmembrane potential difference. Membrane proteins mediating this influx have not been identified, but they could be Mg^{2+} channels or facilitated-type carriers. The mechanism by which magnesium is translocated across the placental cytoplasm is unknown, but it may be by simple diffusion in either the free or bound state. Mg^{2+} efflux across the fetal-facing placental membrane is likely to be an energy-dependent process. It is clear that the mechanisms involved in placental magnesium transport are separate from those of calcium transport.[9,125,126]

In the rat, administration of amiloride or furosemide reduces maternofetal magnesium flux, consistent with the presence of a Na^+-Mg^{2+} exchanger and an Mg^{2+}-HCO_3^- co-transporter.[127] The presence of a Na^+-Mg^{2+} exchanger is supported by the studies of Shaw and collaborators,[50] who found that the maternofetal clearance of magnesium across the in situ perfused rat placenta is reduced when the perfusate is either sodium-free or contains ouabain, both of which would reduce the electrochemical gradient for sodium. The presence of acetazolamide (a carbonic anhydrase inhibitor) in the perfusate, however, has no effect on the clearance of magnesium, so a role for an Mg^{2+}-HCO_3^- cotransporter remains speculative.[54] Using choriocarcinoma cells as a model for human trophoblast cells, Standley and Standley demonstrated the presence of a functional Na^+-Mg^{2+} exchanger that serves to maintain low Mg^{2+} concentrations within cytotrophoblast cells.[128] Lowering of extracellular Na^+ causes progressively larger, transient increases in Mg^{2+} concentrations within cytotrophoblast cells; these transient changes also are dependent on extracellular Mg^{2+} concentrations.

Control of Placental Magnesium Transport

No direct information on the regulation of placental magnesium transport is available. Insight into placental transfer mechanisms, however, is provided by observing the effect of therapeutic and experimental manipulations of maternal plasma Mg^{2+} concentrations on fetal plasma and carcass Mg^{2+} concentrations and the fetomaternal plasma magnesium gradient. Treatment of preeclampsia with magnesium leads to maternal and fetal hypermagnesemia[129]: either the magnesium-transporting pump apparently is not saturated at physiologic plasma Mg^{2+} concentrations, or a high diffusional permeability to magnesium is characteristic of this species. By contrast, in the rat, maternal intraperitoneal administration of magnesium leads to a three-fold increase in the maternal plasma Mg^{2+} concentration but only a slight increase in fetal plasma Mg^{2+} concentration.[130] Maternal hypomagnesemia in the rat produced by dietary deficiency, however, leads to reduced fetal plasma and carcass Mg^{2+} concentrations and a loss of the fetomaternal magnesium gradient.[121,131] In sheep, neither maternal hypermagnesemia nor maternal hypomagnesemia affects the fetal plasma Mg^{2+} concentration.[132] Thus, in sheep, placental magnesium transport appears to be saturated even when the mother is hypermagnesemic. In addition, any diffusional component of transfer must be small in relation to the carrier-mediated component. The interspecies variation in fetal response to maternal hypomagnesemia and hypermagnesemia may be related to differences in placental structure. Using the Ct/Cgrp-knockout mouse model, McDonald and co-workers[111] showed that serum and total-body magnesium content is significantly lower in fetuses born to Ct/Cgrp-null mothers compared with Ct/Cgrp$^{+/-}$ and wild-type mothers. These workers concluded that Ct/Cgrp may be involved in regulation of placental magnesium transport.

Hormonal Regulation of Placental Magnesium Transport

Some evidence indicates that in sheep, magnesium transport is increased by fetal parathyroid gland extract and mid-molecule fragments of hPTHrP(67-86) and hPTHrP(75-86), whereas the amino-terminal end (hPTHrP[1-34]) has no effect.[95] The same is not true for mid-molecule PTHrP fragments in the rat.[99] These contradictory findings may reflect methodology or species differences.

MINERAL TRANSFER IN DIABETIC PREGNANCIES AND IN INTRAUTERINE GROWTH RESTRICTION

Disturbances in fetal growth commonly are encountered in diabetic pregnancy in both animals and humans. In the rat, fetal growth restriction is a consistent finding.[133] By contrast, infants born to diabetic pregnant women tend to be overgrown at term, although in the first trimester of pregnancies with maternal diabetes the fetus may be growth-restriction and may more closely resemble that in rat pregnancies.[134] Many different qualitative and quantitative changes have been reported in placentas of diabetic pregnancies, but data on measurement of placental nutrient flux in such pregnancies are limited.

An early report using single-photon absorptiometry suggested that the radial bone mineral content in infants of diabetic mothers is reduced by approximately 10% when compared with infants of nondiabetic mothers.[135] By contrast, a recent study in which whole-body bone mass was measured using dual x-ray absorptiometry suggests that bone mineralization is increased in

infants of diabetic mothers compared with reference curves.[136] The effect of maternal diabetes mellitus on fetal mineralization is more consistent in animal studies. Fetal histomorphometric analysis and ashing studies demonstrated reduced mineralization in the offspring of diabetic rats.[133] Whereas the increased incidence of hypocalcemia and hypomagnesemia reported in infants of diabetic mothers may result from disturbances in calciotropic hormone responsiveness,[134-139] the reduced fetal bone mineral accretion in animal studies is likely to be associated with lower net placental transport of bone minerals.

We investigated this situation in more detail by measuring the maternofetal flux of calcium and magnesium across the in situ perfused placenta of the streptozocin-induced diabetic rat.[140] Our results indicate that, in the presence of untreated maternal diabetes, the maternofetal flux of both calcium and magnesium is reduced by between 20% and 40%, accompanied by lower net fetal accretion of calcium and magnesium. These disturbances can be prevented by control of the diabetes with insulin. Fetuses in the untreated diabetic group weighed significantly less than those in the other groups, but even when an allowance was made for this difference, the calcium content, but not the magnesium content, remains significantly lower in the untreated diabetic group of animals. Thus, the net flux of calcium across the diabetic rat placenta is reduced to a greater degree than that expected from growth restriction alone. This situation is different from that in which growth restriction is induced in the rat by reducing uterine blood flow. In that model, net calcium flux is appropriate for fetal size.[57]

The pathophysiology of these disturbances in placental transport of calcium and magnesium during diabetic rat pregnancy is not fully understood, and it is likely that a combination of factors leads to altered placental transport and fetal accretion of calcium and magnesium. Hypomagnesemia[141] and hypermagnesuria[142] are common in diabetes mellitus, and pregnancy has an additive effect on renal Mg^{2+} excretion.[143] However, our observations in the untreated diabetic rat pregnancy do not support reduced cation availability as a cause of the reduced maternofetal flux of these cations,[140] although they do support the finding that CaBP is reduced in the placentas of diabetic rats.[144] Our data, confirmed by other investigators,[145] demonstrate that the expression of CaBP mRNA in placentas of diabetic rats is reduced by a factor of 12 to 15.[140]

Infants who are small for gestational age have a lower bone mineral content compared with those whose size is appropriate for gestational age,[74] even after allowing for seasonal variation.[74] The reasons for this discrepancy are unclear but may be related to differences in calciotropic hormones between infants who are small for gestational age and those of a size appropriate for gestational age.[74] Using an experimental model of fetal growth restriction in the rat (uterine artery ligation), we showed that maternofetal transfer of calcium across the placenta is reduced in proportion to the reduction in body size.[57] This finding may simply reflect the smaller requirement for calcium in a small fetus, but it also may indicate a depletion of the necessary energy supply for active placental calcium transport secondary to a chronic reduction in uteroplacental blood flow.

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35 Fetal and Neonatal Iron Metabolism

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OVERVIEW

Iron is the fourth most abundant element and constitutes approximately 0.0075% of human body composition[1] It is a cofactor in some of the most basic biologic reactions, yet its beneficial effects are tempered by the fact that it is highly toxic in its unbound state and participates in the generation of reactive oxygen species (ROS). In a human term newborn, the whole-body amount of elemental iron has been determined to be 275 to 300 mg, most of which is complexed in hemoglobin.[1,2] In addition to its predominant role in erythropoiesis, iron is important for normal growth and function of all rapidly developing organ systems. Rapidly dividing cells need more iron. Indeed, much of what has been learned about cellular iron homeostasis comes from investigation of cancer cells and bacteria.

The clinical signs of iron deficiency are highly variable because iron-containing proteins are important in many facets of human biology.[3] These include cellular energetics, DNA replication, tissue oxygenation, neurotransmitter synthesis, thyroid status, white cell function, and fatty acid synthesis.[4-8] The consequences of deficiency have been studied most extensively in the hematopoietic system, heart, skeletal muscle, gastrointestinal tract, and brain. The corresponding clinical manifestations include anemia, cardiomyopathy, skeletal muscle weakness, fatigue, intestinal dysmotility, and abnormal motor and mental function.[4-8]

Negative iron balance occurs more commonly during three time periods in child development: the late fetal–early neonatal period (up to 6 months postnatal age), 6 to 24 months of age, and the teenage years. The acute clinical effects and long-term hematologic and nonhematologic sequelae of iron deficiency in the fetal and neonatal period remain largely unknown. The lack of investigation until recently in this field was a consequence of the misperception that neonatal iron deficiency occurs only secondary to severe maternal iron deficiency. The incidence of fetal iron deficiency as a consequence of maternal anemia is low in developing countries because most mothers are supplemented with iron during pregnancy. From 30% to 50% of mothers worldwide, however, are iron-deficient during pregnancy. Moreover, a relatively high incidence of reduced iron stores and frank iron deficiency has been described in certain groups of infants born to iron-sufficient mothers.[9-12] Iron deficiency in these groups is due to reduced placental iron transport secondary to placental vascular disease or to increased fetal iron demand that exceeds the capacity of placental iron transport.

More attention has been focused recently on the negative consequences of iron overload in the fetus and neonate. This condition is rarely due to nutritional iron overload but far more often results from excessive red cell transfusions, congenital hemochromatosis, or reperfusion injuries. Iron overload of any cause is dangerous because iron reacts with oxygen (in the Fenton reaction, as described further on) to create ROS, which damage cell membranes and promote apoptosis and cell death. Studies have tried to delineate the role of iron overload in neonatal diseases characterized by oxidative injury such as bronchopulmonary dysplasia, necrotizing enterocolitis, and retinopathy of prematurity.[13-15]

This chapter begins with a review of the biochemistry of iron-containing proteins, their biologic functions and the regulation of cellular iron accretion. The subsequent discussion of fetal iron balance emphasizes the mechanisms of maternal-fetal iron transport with particular attention to the relative contributions of maternal and fetal iron status to regulating placental iron transport proteins. The section on postnatal iron balance addresses the various sources of iron and the overall iron requirements of term and preterm infants. Finally, the potential physiologic consequences of fetal and neonatal iron deficiency and iron excess are discussed.

GENERAL PRINCIPLES OF IRON METABOLISM

The Biology of Iron

Iron is a ubiquitous mineral that exists in trace conditions in humans. It is vital for the proper function of multiple proteins and is incorporated into their structures, typically as heme moieties or iron-sulfur clusters. The greatest amount of iron in the human is found within red cells in the hemoglobin molecule. Hemoglobin is prototypical of hemoproteins whose function is to carry oxygen or transfer electrons. Closely related compounds (because of their iron-containing porphyrin ring) include the cytochromes that are necessary for electron transport during oxidative phosphorylation and ATP generation. Iron therefore participates in the most basic aspects of cellular respiration during aerobic metabolism. In addition, iron-containing proteins reside in crucial pathways for detoxification (cytochrome P-450), neurotransmitter and hormone synthesis (tyrosine hydroxylase), and fatty acid production. Consequently, iron deficiency manifests as dysfunction of multiple organ systems including red blood cells, white blood cells, brain, heart, and skeletal muscle. Although iron deficiency anemia can produce classic signs and symptoms of fatigue, lethargy, and growth failure, most clinical manifestations are due to primary failure of nonhematologic organ systems dependent on iron-containing proteins and enzymes.

Iron not only is a necessary element whose deficiency produces symptomatology, but also is a highly toxic metal whose excess can cause severe organ damage through its interaction with oxygen in the Fenton reaction to generate ROS, which cause lipid membrane peroxidation. The Fenton reaction to generate ROS is as follows:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$$

Iron generally is found in the ferric (Fe^{3+}) or ferrous (Fe^{2+}) state but in either case is always meant to be protein-bound, whereby the structure of the protein physically shields the iron, effectively keeping it from reacting with oxygen. Multiple degenerative disorders and reperfusion injuries are postulated to be caused in part by free iron–mediated ROS, including Alzheimer disease, Parkinson disease, and brain injury after stroke and myocardial injury after infarction. Neonatal investigators have explored a potential deleterious role for free iron in the pathogenesis of birth asphyxia, necrotizing enterocolitis, bronchopulmonary dysplasia, and retinopathy of prematurity.[13-16]

Regulation of Cellular Iron Homeostasis and Iron Transport

It is clear that iron is a highly necessary but potentially toxic mineral that requires tight regulation to maintain adequate but safe concentrations in blood and tissue. An elegant posttranscriptional system regulates the expression of proteins involved in cellular iron uptake, storage, and export. All cells need iron to survive. Most cells take up iron specifically for utilization within themselves. These cells require a mechanism for iron uptake but typically do not express proteins that excrete iron. Others, such as duodenal epithelial cells, mammary epithelial cells, endovascular endothelial cells in the blood-brain barrier, and the syncytiotrophoblast, additionally take up iron in order to transport it from one compartment to another (Table 35-1). These cells require proteins not only for iron uptake but also for its excretion.

Cellular iron uptake is mediated primarily by transferrin receptor (TfR-1), a transmembrane dimeric glycoprotein that actively internalizes diferric transferrin from the serum as well as from cerebrospinal fluid (CSF) and the brain parenchyma into the cytosol by way of clathrin-coated endosomes.[17-19] Because each transferrin receptor dimer binds two molecules of transferrin, each of which contains two molecules of ferric iron, a total of four iron molecules per TfR-1 unit are internalized. Although most cells use TfR-1 to internalize iron, iron uptake from intestinal lumen into the duodenal epithelial cell does not involve TfR-1 but instead requires the divalent metal transporter-1 (DMT-1) or lactoferrin (in the preweaning stage). The dimeric TfR-1 has a molecular weight of approximately 180 and contains *O*-linked and three *N*-linked carbohydrate chains with a disulfide bridge in its extracellular domain. The extracellular, carboxy terminal component is large, approximately 140 kD in size.[20] For optimal binding to occur, the receptor must be fully glycosylated, with an intact disulfide bridge that binds the two monomers close to the cell membrane surface.[20] TfR-1 isolated from cytosolic membranes demonstrates a lower binding affinity for transferrin[21] because of a presumably immature or incomplete glycosylation pattern. Similarly, truncation of the oligosaccharide side chains, particularly at Asn137, reduces the binding affinity for diferric transferrin.[22] Within the cytosol, the endosome is acidified, releasing the iron molecules from transferrin (see Figure 35-1). The subsequent ferrous (2+) iron is exported from the endosome into the cytosol by DMT-1. Once in the cytosol, the iron is available for incorporation into heme complex proteins or into proteins that contain iron-sulfur clusters.

Within the cell, excess cytoplasmic iron not immediately used for cellular functions is stored in the form of cytoplasmic ferritin. Ferritin is a nonheme iron protein complex consisting of a protein shell (apoferritin) made up of 24 protein subunits.[20] In human tissue, two important subunits have been identified, designated as H (heavy) and L (light). These subunit chains form a shell into which iron can bind reversibly and be stored. Under various conditions, apoferritin has the capability of storing as many as 270 atoms of elemental ferric iron to form ferritin.[23] The greatest concentrations of fetal ferritin are located in the cells of the reticuloendothelial system in the liver, placenta and spleen. Iron in these cells serves as a reservoir for iron used for other fetal organ systems. Late in fetal life, a major shift of iron (and erythropoiesis) from the liver to the bone marrow occurs. Ferritin also is present in all other cells to serve as a short-term storage depot for unused cytoplasmic iron, but cellular concentrations in most tissues outside the reticuloendothelial system are small.[23]

An additional step is necessary for cells that act as conduits for iron from one compartment to another (see Table 35-1). These interfaces include the duodenal epithelium, the syncytiotrophoblast, the mammary epithelial cell, and the blood-brain barrier. Iron export from the basal or basolateral membrane to the receiving compartment is mediated through ferroportin-1, a transmembrane protein that transports ferrous iron.[24] Ferroportin-1 has been localized to the duodenal epithelium, the basal (fetal-facing) surface of the syncytiotrophoblast, and the blood-brain barrier.[24,25] Mutations of ferroportin-1 resulting in iron overload of these transporting cells have been identified.[26] The mechanism by which ferroportin transports ferrous iron across the basal or basolateral membrane remains unclear. However, a positive charge donated from the copper moiety of one of two multicopper ferroxidases, hephaestin[27] or ceruloplasmin,[28] is necessary for converting the transported ferrous iron to ferric iron in anticipation of postextrusion binding by transferrin (Figure 35-1). Ferroportin expression, and thus iron transport activity, are negatively regulated by the liver protein hepcidin.[29] When cells are iron-sufficient or iron-overloaded, hepcidin concentrations increase, repressing ferroportin-mediated iron transport. Of interest, inflammation induces a similar increase in hepcidin activity, which may explain the observation that during inflammatory states, iron is sequestered in the reticuloendothelial system (as ferritin) and therefore is not available in the circulation for cellular accretion and erythropoiesis, leading to the anemia of inflammation.[30,31] Thus it may be worth considering that one aspect of the chronic anemia seen in premature infants despite normal or elevated iron stores may be due to chronic inflammatory states common in this group of infants.

The regulation of cellular iron accretion and storage has been elucidated in erythrocyte, hepatocyte, placental trophoblast, and mammary epithelial cell lines. A tightly controlled regulatory mechanism is necessary to ensure adequate availability of cytoplasmic iron while guaranteeing efficient removal of potentially toxic excess cellular iron. This dual process is accomplished by coordinated regulation of the synthesis of the TfR-1 and DMT-1, responsible for uptake of iron into cells, and of ferritin, responsible for conversion of excess cytoplasmic iron into its storage form.[31,32] The simultaneous regulation of the iron uptake proteins and the iron storage protein occurs through post-transcriptional modification of TfR-1 and ferritin messenger RNAs (mRNAs).[31,33] TfR-1, DMT-1, and ferritin mRNAs have specific binding sites known as iron-responsive elements (IREs). These elements represent conserved palindromic mRNA stem loops with a specific six-base sequence 5′-CAGUGN-3′. One IRE is found on the 5′ untranslated sequence of ferritin mRNA whereas five are found in the 3′ region of TfR-1 and DMT-1 mRNA.[33,34] These structurally and functionally identical sets of IREs are able to bind competitively iron-sensitive–specific cytoplasmic mRNA binding proteins known as iron regulatory proteins (IRPs). Two IRPs are recognized. IRP-1 is constitutively expressed but changes its conformation and binding affinity for the IREs based on whether it is

TABLE 35-1

Vectoral Iron Transport Across Single-Cell Membrane Barriers

Tissue	Source	Single-Cell Barrier	Destination
Intestine	Lumen	Intestinal epithelium	Blood
Placenta	Maternal blood	Syncytiotrophoblast	Fetal blood
Mammary gland	Maternal blood	Mammary epithelium	Milk
Brain			
Vascular	Blood	Vascular epithelium	Parenchyma/ ?astrocyte
Choroid plexus	Blood	Choroid epithelium	CSF
Ventricular ependyma	CSF	Ependymal cell layer	Parenchyma

CSF, Cerebrospinal fluid.

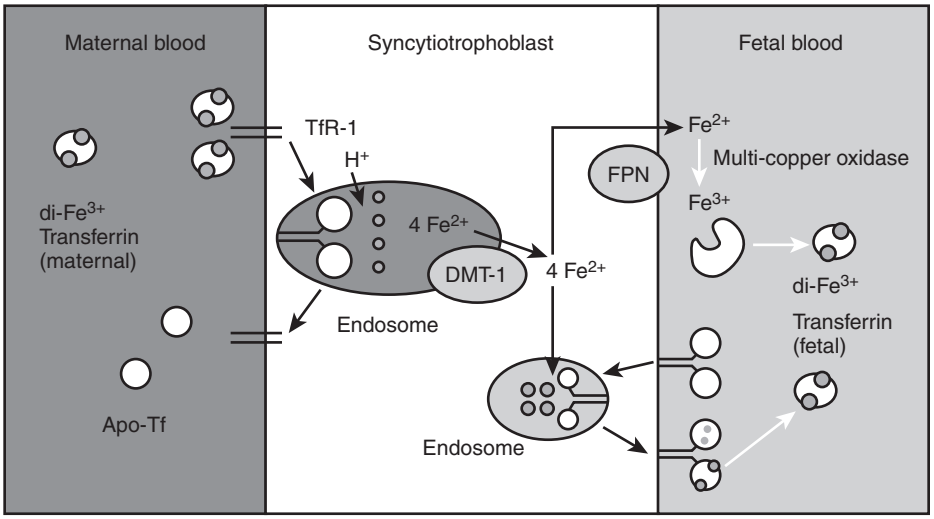


Figure 35-1 Iron transport across the syncytiotrophoblast. *Left panel,* Diferric transferrin is bound to transferrin receptor-1 (TfR-1) at the apical (maternal-facing) membrane. *Middle panel,* Ferrous iron is transported out of the endosome into the cytoplasm by the divalent metal transporter-1 (DMT-1). *Right panel,* Iron export at the basal (fetal-facing) membrane is proposed to occur either through ferrous iron transport by ferroportin-1 (FPN) or through ferric iron transport by TfR-1. See text for details.

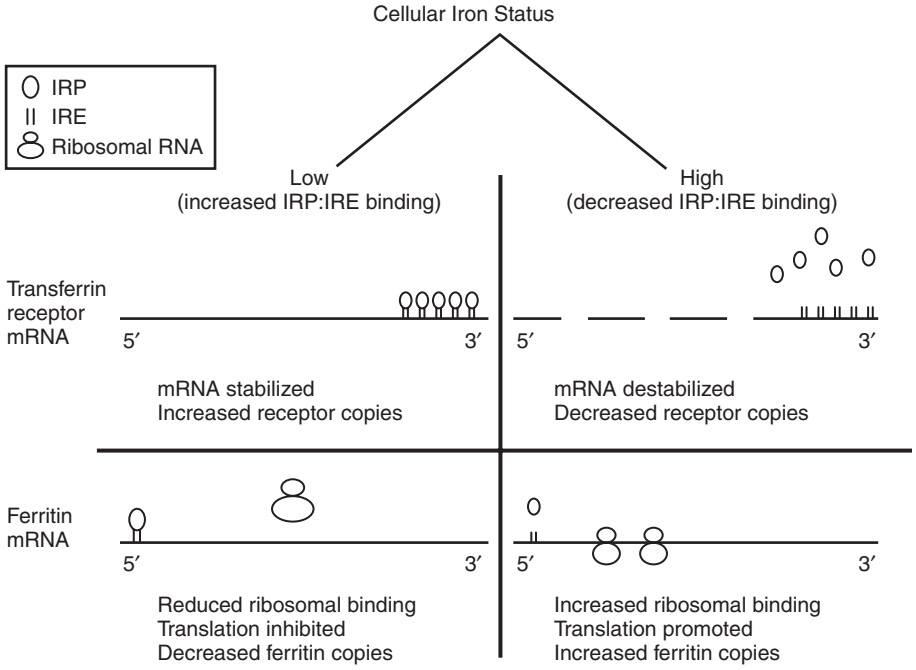


Figure 35-2 Coordinated regulation of transferrin receptor and ferritin recombinant messenger RNAs (mRNAs) by iron-regulatory proteins (IRPs) and iron-regulatory elements (IREs) during low and high cellular iron status. See text for full description of stabilization and translation of mRNA. *(Data from Harford JP, Klausner RD: Coordinate post-transcriptional regulation of ferritin and transferrin receptor expression: The role of regulated RNA-protein interaction.* Enzyme *44:28, 1990; Eisenstein RS: Iron regulatory proteins and the molecular control of mammalian iron metabolism,* Annu Rev Nutr *20:627-662, 2000; and Bierings MB, Baert MR, van Eijk HG, van Dijk JP: Transferrin receptor expression and regulation of placental iron uptake,* Molec Cell Biochem *100:31, 1991.)*

in its iron-sulfur cluster is in its iron-deficient Fe3S4 or its iron-sufficient Fe4S4 form. The Fe3S4 form has higher affinity for the IRE.[33,35] IRP-2 is more classically regulated, with increased mRNA expression in response to iron deficiency. When cellular iron concentrations are low, the IRPs bind the IREs found in the 3′ untranslated region of TfR-1 and DMT-1 mRNA, stabilizing it and producing more copies (Figure 35-2). At the same time, IRP binding of the IRE in the 5′ untranslated region of ferritin mRNA prevents ribosomal binding. The combination results in increased synthesis of the iron importers and decreased synthesis of ferritin. High cytoplasmic iron concentrations lead to reduced IRP binding to the IREs on both mRNAs. This decreased binding leads to destabilization of the mRNAs of the importers while improving the binding of ferritin mRNA to ribosomes, increasing apoferritin synthesis, and promoting of storage of cytoplasmic iron[33,36] (see Figure 35-2). Perinatal models of iron deficiency in humans and rodents demonstrate increased IRP activity in placenta[37,38] and in brain.[39]

Regulation of Placental Iron Transport

The entry point for iron into the fetus is at the trophoblast or syncytiotrophoblast (see Figure 35-1). Human placental iron transport is mediated by TfR-1 localized primarily but not exclusively to the apical syncytiotrophoblast membrane.[18] DMT-1 serves as the intracellular endosomal ferrous iron transporter. The mechanism of basal transport remains elusive, although the presence of ferroportin on the basal membrane suggests that ferrous iron is transported to the fetus at that site.[24,25,38] Nevertheless, the presence of TfR-1 at the basal membrane leaves open the possibility that it is involved in transferrin-mediated iron transport at that site[18] (see Figure 35-1). This receptor-mediated system pumps iron against an ever-increasing gradient from mother to fetus. Mammals with hemochorial placentas use this system, in contrast with other mammalian species that accrete iron by placental erythrophagocytosis.[40]

When the maternal iron status and fetal iron demand are normal, TfR-1 expression is inversely related to placental nonheme iron

content.[41] Placental nonheme iron is found largely in the form of ferritin in fetally derived cells, including Hofbauer cells. These macrophage-derived cells may constitute a form of placental iron storage, much as the Kupffer cells are major iron storage cells in the liver. Placental nonheme iron concentrations are closely correlated with cord blood serum ferritin concentrations and calculated total body storage iron at birth.[37] In clinical studies, the placental nonheme iron status (as well as the fetal storage iron content) correlates inversely with syncytiotrophoblastic TfR-1 expression in both iron-sufficient[41] and iron-deficient fetuses.[42] This finding suggests that increased fetal iron demand positively influences TfR-1 expression. In cell culture, the intracellular iron content of the syncytiotrophoblast determines TfR-1 expression on the apical membrane[19,43,44] by a mechanism that involves stabilization of TfR-1 mRNA.[17,22] Although the intracellular iron status of the syncytiotrophoblast has not been measured in clinical studies, it probably parallels the placental nonheme iron and fetal storage iron concentrations.

Fetal Iron Accretion and the Relationship of Maternal and Fetal Iron Status

The normal human fetus contains 70 to 75 mg of elemental iron per kilogram of body weight during the third trimester of gestation. This represents an accretion rate of 1.35 mg/kg of body weight between 24 and 40 weeks gestation. The iron is distributed in erythrocyte hemoglobin (50 to 55 mg/kg), liver, spleen, bone marrow, hemosiderin, and kidney storage ferritin (totaling 10 mg/kg) and nonstorage tissue iron such as hemoproteins and iron-sulfur proteins, (totaling 7 mg/kg) that are found in virtually all tissues.[1,2,45] The pregnant woman is the source of iron for the fetus, and the placenta is the delivery system. Pregnant women will require increased iron over amounts adequate for the nonpregnant state, especially during the second and third trimesters, characterized by significant fetal growth and maternal blood volume expansion.[46]

Conditions that reduce maternal-fetal iron transport, such as maternal iron deficiency anemia and placental vascular disease, and those characterized by increased fetal iron demand, such as chronic fetal hypoxemia, threaten the normal accretion and distribution of fetal iron. Neonatal iron status is a function of the duration of gestation, fetal sex, maternal iron status, efficiency of placental iron transport, and fetal iron demand. Fetal iron stores as indexed by cord serum ferritin concentrations increase with advancing gestational age.[47,48] A meta-analysis has determined that the mean ferritin concentration for term infants is 134 mcg/L, with 5th and 95th percentiles of 40 and 309 mcg/L, respectively. Preterm infants have lower values, with a mean of 115 mcg/L and 5th and 95th percentiles of 35 and 267 mcg/L, respectively. In term infants, ferritin concentrations are higher in females than in males.[49]

Fetal iron status correlates poorly with maternal iron status when the mother is iron-sufficient.[47] The fetus accretes iron against a concentration gradient through active transport by the syncytiotrophoblast, resulting in higher ferritin concentrations in the fetus than in the mother.[50] With progressive degrees of maternal deficiency, however, fetal iron stores initially are spared,[51] with compromise of those stores occurring later. Infants born to mothers with ferritin concentrations less than 10 mcg/L, but without iron deficiency anemia, have lower but nevertheless normal ferritin concentrations and normal hemoglobin concentrations.[52] Infants born to mothers with frank iron deficiency anemia have low cord blood ferritin and serum iron concentrations. Typically, the risk to the fetus increases when the maternal hemoglobin is less than 100 g/L.[53] These findings suggest that the fetus can accumulate iron normally in the face of maternal iron deficiency,[54] most likely through compensatory up-regulation of placental iron transport,[51] but

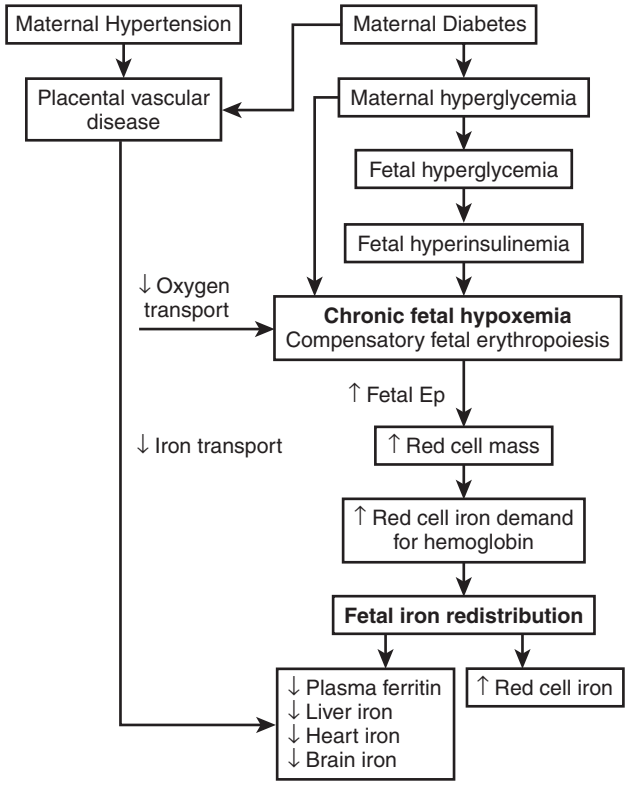


Figure 35-3 Pathophysiology of fetal iron deficiency in pregnancies complicated by maternal hypertension and diabetes mellitus. *(Modified from Petry CD, Eaton MA, Wobken JD, et al: Iron deficiency of liver, heart and brain in newborn infants of diabetic mothers,* J Pediatr *121:109-114, 1992.)*

with a threshold effect, so that during severe maternal iron deficiency, fetal iron status ultimately becomes compromised. The degree of nonheme (e.g., brain, heart) tissue iron depletion in the fetus resulting from severe maternal iron deficiency is unknown.

The fetus also can become iron-deficient despite maternal iron sufficiency in pregnancies characterized by placental vascular disease or in those in which increased iron demand exceeds placental transport capacity[9,35,55] (Figure 35-3). In both circumstances, the reduction in measured or calculated fetal iron stores reflects a true decrease in total body iron, not just an intrafetal redistribution of iron from liver stores into an expanded red cell mass.[9,52,53]

Several studies have shown an increased prevalence of reduced cord serum ferritin concentrations in pregnancies complicated by placental vascular disease,[10-12] most often associated with intrauterine growth restriction. Moreover, up to 50% of infants of appropriate size for gestational age born to mothers with preeclampsia also demonstrate ferritin concentrations below the 5th percentile for gestational age.[10] In an autopsy study of infants who died at birth after pregnancies complicated by uteroplacental vascular insufficiency, the hepatic iron concentrations were reduced by 62% compared with control subjects.[56] The reduction in hepatic iron is likely to reflect the lack of iron transport adequate to fill the iron storage pool. In a similar autopsy study of infants affected by intrauterine growth restriction secondary to Potter's syndrome, a reduction of brain iron by 33% was found.[57]

Increased fetal iron demand also can lead to abnormal iron distribution and iron deficiency at the tissue level. Fetal iron

demand increases most markedly when erythropoiesis is augmented by chronic fetal hypoxemia, especially when it is accompanied by rapid fetal growth. Clinically, this sequence commonly is seen during pregnancies complicated by diabetes mellitus.[58,59] An autopsy study of large-for-gestational-age infants born to diabetic mothers demonstrated that hepatic iron concentrations were 7% of those in control (normal) infants, implying complete depletion of the iron storage pool.[55] Brain and heart iron concentrations were reduced to 61% and 46% of control levels, respectively. The reduction in nonstorage tissue iron is consistent with conventional iron deficiency theory, in that storage iron must be reduced to less than 25% of normal before nonstorage tissue iron concentrations are compromised.[3]

The causes of tissue iron deficiency in pregnancies complicated by diabetes mellitus and chronic fetal hypoxia have been studied in animal models[60-62] (see Figure 35-3). Chronic fetal hyperinsulinemia and hyperglycemia, which are characteristic of poorly controlled diabetic pregnancies, result in increased fetal cellular oxygen consumption in a relatively oxygen-limited environment.[63,64] The resultant chronic fetal hypoxemia stimulates erythropoietin release with consequent expansion of the red cell mass by up to 40%.[9,60,65,66] The red cell mass may be further expanded by the rapid growth of the fetus with the attendant increase in the vascular volume.[59]

The hypoxic fetus has two potential sources for iron to supply this rapidly expanded red cell mass: increased maternofetal iron transport and mobilization of endogenous fetal hepatic iron stores. The latter has been demonstrated in both human[9,10,56,58,67] and animal studies.[60-62] When hepatic iron is depleted to less than 10% of control levels by chronic erythropoietic stimulation in fetal sheep, heart, brain, and skeletal muscle iron concentrations fall in direct proportion to the reduction in organ iron concentration[62] (Figure 35-4). The status of TfR-1 during diabetic pregnancies characterized by fetal iron deficiency also has been studied.[37,67] TfR-1 mRNA and IRP-1 activity both are increased by 40% in iron-deficient diabetic placentas, supporting the hypothesis that up-regulation is possible and probably is controlled by fetal iron demand.[67] However, the diabetic placenta cannot completely compensate the fetus through increased transport, in part because of the decreased binding affinity of TfR-1 for maternal transferrin secondary to hyperglycosylation.[68] The lack of compensation forces a redistribution of fetal iron toward the red cell mass and away from vital organs such as the heart and brain (see Figure 35-3).

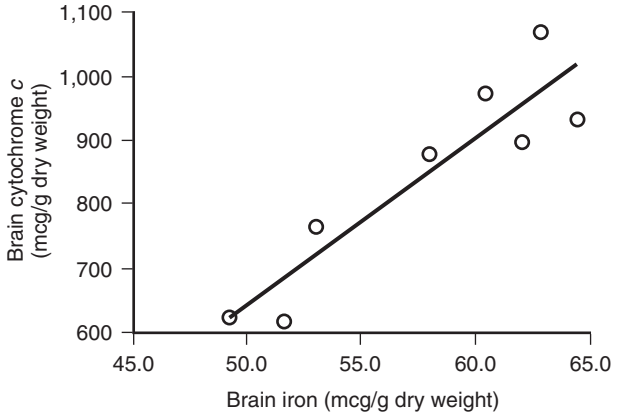


Figure 35-4 Brain cytochrome *c* concentration graphed as a function of brain iron concentration in chronically hypoxic, iron-deficient fetal sheep. $R = 0.91$; $y = -642.79 + 25.70x$; $P = .01$.

POSTNATAL IRON BALANCE

In general, iron deficiency in full-term infants during the first 2 postnatal months has been of minimal concern. An abundance of hemoglobin during fetal life generally ensures an adequate source of iron for the newborn as the red cell mass breaks down in postnatal life. This supply is likely to be particularly abundant if the infant has received a transfusion of red cells from the placenta because of delayed cord clamping. The higher Pa_{O_2} of postnatal life improves the oxygen-carrying capacity of the blood, eliminating the need for high hemoglobin concentrations; thus the first 6 to 8 postnatal weeks are characterized by declining hemoglobin concentrations with subsequent liberation of heme iron. Because this iron is not lost from the body, the healthy term newborn infant is well supplied with iron for the first 2 postnatal months. Thereafter, as the infant grows and the blood volume expands, the iron necessary for hemoglobin synthesis continues to be derived from the reticuloendothelial stores and from absorption of dietary iron. The estimated daily dietary iron requirement for the term infant is 1 mg/kg body weight/day,[69] but the percent absorption from the intestine varies inversely with the iron status of the individual infant.[70,71]

The serum ferritin concentration is a reasonable reflection of iron stores, with each 1 mcg/L of serum ferritin thought to represent 8 to 10 mg of stored iron.[70,71] Serum ferritin concentrations (and iron stores) are higher in newborn infants than in older infants, children, and adults. Full-term newborn infants have slightly higher ferritin concentrations (mean, 134 mcg/L; 5th to 95th percentile range, 40-309 mcg/L) than those measured in preterm infants born at less than 37 weeks of gestational age (mean, 115 mcg/L; 5th to 95th percentile range, 35 to 267 mcg/L) (48). Ferritin concentrations increase during the first postnatal month and then drop precipitously by the age of 6 months,[12,72,73] presumably as storage iron is used for the rapidly expanding blood volume of the growing infant.

Dietary iron is absorbed by the duodenum and proximal jejunum.[74] Absorption is modulated not only by iron need but also by the dietary source of the iron. Heme iron, in the form of hemoglobin and myoglobin, is enhanced by the ingestion of meat and inhibited by calcium. Nonheme iron, which represents the major portion of dietary iron, is absorbed more avidly in the presence of ascorbic acid. Its absorption is inhibited by calcium, phytates (found in soy protein–based formulas), manganese, and polyphenols.[74]

The iron content of breast milk is low compared with that of iron-fortified cow's milk formula, but its bioavailability is high, mostly because of the presence of lactoferrin, which enhances absorption through a species-specific brush border membrane receptor.[75] Iron absorption from human milk may approach or exceed 50%.[75] Lactoferrin concentrations are particularly high in colostrum and decline throughout each feed[76] and with increasing postnatal age.[77] Lactoferrin resists proteolytic digestion in the gut and thus remains largely intact.[78] Iron also is found in xanthine oxidase, a membrane-bound enzyme that accounts for up to 30% of total iron in human milk. Its biologic function is not completely understood, but it may be involved in energy generation.[79] A form of ferritin also is found in human milk; this form of ferritin, however, is unlikely to contribute much to the iron nutrition of the newborn because it is poorly saturated with iron.[80] Although studies assessing iron status of breast-fed infants are difficult to perform because of multiple confounding variables, the bulk of the data supports the concept that infants fed human milk exclusively for 6 months have normal iron status at 6 and 9 months[81] and have higher hemoglobin concentrations than those in infants fed iron-supplemented formula.[82] However, some breast-fed infants may be at higher risk for iron deficiency and iron deficiency anemia, including infants with low iron stores at birth[83] and infants exclusively breast-fed for more than

6 months,[84] particularly in the setting of maternal iron deficiency. This increased risk for iron deficiency may specifically reflect the rapidly declining iron content of breast milk after the age of 6 months. A double-blind, placebo-controlled trial of iron supplementation in breast-fed infants demonstrated higher hemoglobin concentrations, higher mean corpuscular volumes (MCVs), better visual acuity, and better motor development in the iron-supplemented groups,[85] suggesting that some breast-fed infants may become iron-deficient.

The bioavailability of iron from infant formulas is significantly lower than from human milk, accounting for the need for higher iron concentrations in infant formulas. Infant formulas fortified with at least 4 mg/L of iron are most likely to prevent depletion of iron stores and iron deficiency, although infants fed formulas with this amount of iron demonstrate lower ferritin and higher transferrin receptor concentrations than those measured in infants fed formulas with higher iron contents.[86] This observation suggests that 4 mg/L may be close to the minimum concentration of iron content that can support iron sufficiency. Most iron-fortified formulas in the United States contain 12 mg/L. Low-iron formulas are defined by the U.S. Food and Drug Administration (FDA) as containing less than 6.7 mg/L of elemental iron. Current iterations of low-iron formulas contain 4 mg/L of iron or more and appear to present sufficient dietary iron to the young infant who does not have additional risk factors for early iron deficiency. However, because no scientific evidence implicates dietary iron as a cause of common infant symptoms such as colic, constipation, or diarrhea, no compelling reason to feed infants low-iron formulas has been emerged.[87,88] One formula manufacturer has never marketed a low-iron formula, whereas another formula manufacturer in the United States has eliminated low-iron formula from production. Most term infants fed human milk or iron-fortified infant formulas maintain normal iron balance during the first postnatal year.

The iron status of the preterm infant is more labile than that of the term infant. Placental iron transport begins during the first trimester, but two thirds of maternal-fetal iron transfer occurs during the third trimester,[1,2,46] resulting in low iron stores in preterm infants. Preterm infants with intrauterine growth restriction are at risk for further reductions in iron stores owing to placental insufficiency and poor placental iron transfer. In an autopsy study, preterm growth-restricted infants were found to have 40% of normal liver iron concentrations for age.[56]

For all preterm infants, not only are iron stores at birth lower, but postnatal estimated daily iron needs per kg of weight are greater than those in full-term infants, based on estimates of third-trimester fetal iron accretion.[1,2,69] The iron balance of preterm infants can be markedly affected by the management of their hematologic status (i.e., whether they receive red cell transfusions or recombinant human erythropoietin). Iron intake can be compromised because of delayed onset of enteral feedings and a reluctance to give intramuscular or intravenous iron.

Preterm infants undergo a phase of relative anemia (anemia of prematurity), characterized by decreasing hemoglobin concentrations, low reticulocyte counts, and reduced rates of growth. Whether this anemia is due to low rates of erythropoietin synthesis, reduced bone marrow sensitivity to erythropoietin, or lack of iron availability to the bone marrow is a subject of investigation. Evaluation of iron deficiency in preterm infants is difficult, but a growing body of evidence suggests that iron deficiency may contribute to the magnitude and duration of anemia of prematurity. As early as 1977, Lundstrom and associates[89] demonstrated that iron supplementation of preterm infants at 2 weeks of age resulted in a lower incidence of anemia at 3 months of age. Hall and colleagues[90] demonstrated that preterm infants (birth weight less than 1800 g) fed a high-iron (15 mg/L) preterm infant formula had a lower incidence of anemia (hemoglobin less than 9 g/dL) and better iron status at discharge than did infants fed a low-iron formula (3 mg/L). Most recently, Miller and colleagues[91] noted that provision of iron to hospitalized preterm infants (3 to 12 mg/kg/day) resulted in a decrease in the zinc protoporphyrin–heme ratio (ZnPP/H) in treated versus untreated control infants. Because zinc substitutes into protoporphyrin when iron supplies are limited, these data suggest that preterm infants' needs for supplemental iron may be greater than was previously recognized. In previous eras, supplemental iron was administered indirectly through repeated blood transfusions of preterm infants. Many neonatologists now use restricted red blood cell transfusion protocols, and the number of transfusions per infant has declined in recent years.[92] The unintentional effect of this change in management is to decrease the provision of iron to preterm infants, so a fresh look at the iron requirements of preterm infants is needed.

The use of erythropoietin in preterm infants also results in increased iron requirements. Trials of recombinant human erythropoietin to prevent or modify the anemia of prematurity have shown that the young preterm infant is capable of mounting a modest erythropoietic response.[93-95] The stress of augmented erythropoiesis on the iron axis of the preterm infant is considerable[94] and may be rate-limiting in the erythropoietic response.[96] Because these infants receive fewer red cell transfusions, their total body iron is likely to be reduced, and their reliance on exogenous iron is increased.[93] Infants treated with recombinant human erythropoietin require at least 6 mg/kg/day to maintain adequate serum ferritin concentrations.[97] Low-birth-weight infants absorb approximately 30% to 40% of enterally delivered iron regardless of iron status,[98] although the percent of absorbed iron incorporated into hemoglobin varies with the degree of erythropoietic pressure. To this end, Widness and colleagues[99] measured ^{58}Fe incorporation into red blood cells after administration of recombinant erythropoietin to preterm infants and found much higher rates of incorporation in the treated group than in the untreated control group during the first 4 weeks of therapy.

The current recommendation for iron intake for preterm infants is 2 to 4 mg/kg/day.[100] Commercially available iron-fortified preterm formulas contain 1.8 mg Fe/100 calories. An iron-containing human milk fortifier is available that contains 1.44 mg in 4 packets (used to fortify 100 mL of human milk). Use of either preterm formula or fortified human milk at 120 kcal/kg/day will provide preterm infants with the low end of recommended iron intake when goal feedings have been achieved.

After discharge, iron intakes of 2 mg/kg/day are recommended through the first year of life.[100] Breast-fed infants may meet this requirement through the use of multivitamin supplements with iron, which provide 10 mg/mL of iron. However, most infants will outgrow this dose before supplemental foods (such as cereal) are added. Formula-fed infants meet this requirement when taking in 120 calories/kg of body weight. Formula intakes drop over the first 6 months, however, and iron intakes may be low during that time. Some evidence indicates that younger preterm infants may develop iron deficiency anemia[101] before the age of 6 months, and evaluation of hemoglobin is recommended at 4 months postnatal age.

CONSEQUENCES OF FETAL AND NEONATAL IRON DEFICIENCY

The nonhematologic consequences of fetal and neonatal iron deficiency have only recently been considered. When fetal iron is in short supply (as with maternal iron deficiency anemia or uteroplacental insufficiency) or when fetal iron demand is increased (as with chronic fetal hypoxemia), prioritization of available fetal iron will occur. This prioritization takes place among organs and among iron-containing compounds within organs.

The prioritization of iron among organs in postnatal iron deficiency has been well established.[3,4] Typically, hepatic storage iron is depleted before nonstorage tissue iron. Among nonstorage

organs, the erythron, brain, and heart have priority over skeletal muscle and the gastrointestinal tract. A similar pattern appears to exist in utero. In severely iron-deficient newborn infants of diabetic mothers, liver iron is depleted first. Heart and brain iron concentrations decrease only after hepatic iron is less than 25% of normal.[55] A similar relationship exists between liver and brain iron in the iron-deficient perinatal rat.[102] In fetal sheep, chronic erythropoietic stimulation causes reductions in heart, skeletal muscle, and brain iron concentrations to preserve and expand the red cell mass.[62]

Prioritization of iron-containing proteins within an individual organ such as the heart also occurs when placental iron transport is inadequate to meet fetal demand. As with interorgan iron redistribution, preservation of oxygenation has the highest priority. Fetal guinea pigs subjected to both chronic hypoxemia and a decreased maternal iron supply exhibit reduced fetal myocardial iron concentrations.[103] All iron-containing myocardial proteins are not, however, uniformly or proportionately affected. Under conditions of chronic fetal hypoxia and iron deficiency, myocardial myoglobin has high priority in the utilization of iron and is preserved or even elevated despite cellular iron depletion. This preservation of myoglobin compensates for decreased oxygen delivery and improves intracellular oxygen transport in the fetal heart. By contrast, cytochrome *c* decreases in direct proportion to organ iron content, suggesting an intermediate priority.[103] Other nonheme iron proteins such as cellular ferritin are likely to occupy a low priority for myocardial iron utilization in an iron-depleted fetus and are reduced out of proportion to the degree of tissue iron deficiency. Whether this interorgan redistribution of iron occurs in other fetal organs is not known.

The physiologic consequences of fetal and neonatal iron deficiency are based on principles elucidated from postnatal iron deficiency models.[3-6] Potential short-term and long-term effects must be considered for each of the organ systems involved during fetal and postnatal life.

No direct physiologic effects of reduced hepatic storage iron are known. Nevertheless, infants born with low iron stores (infants of diabetic mothers, infants with intrauterine growth restriction) are likely to be at higher risk for postnatal iron deficiency, particularly if the low stores are not due to sequestration of iron in an expanded red cell mass. Thus infants with low serum ferritin concentrations and normal or low hemoglobin concentrations at birth may be at highest risk for later iron deficiency. A group of newborn infants with low umbilical cord blood ferritin concentrations were found to have persistently lower serum ferritin concentrations at 9 months of age.[83]

The physiologic consequences of a postnatal deficiency in cardiac and skeletal muscle iron are well described.[4-6] Myopathies of both organ systems are characterized by decreased contractile force and easy fatigability, contributing to the clinical picture of weakness and lethargy. The biochemical basis for these findings is unknown, although it has been speculated that reduced cytochrome *c* concentrations result in decreased ATP generation, and that reduced myoglobin levels result in reduced oxygen delivery and greater dependence on anaerobic glycolysis. Blayney and coworkers[5] demonstrated decreased mitochondrial *c* content and respiratory function in iron-deficient heart muscle. Similarly, Mackler and associates[6] showed reduced rates of oxidative metabolism in iron-deficient rat skeletal muscle. These findings are clearly independent of the presence and degree of anemia. Furthermore, although newborn infants of diabetic mothers frequently are lethargic and cardiomyopathic, the role of iron deficiency in these conditions has not been defined. The possibility exists that the inability of iron-deficient tissue to withstand hypoxic and hypoglycemic stress contributes to the higher rates of fetal distress and intrauterine death seen in diabetic pregnancies.

The biochemical, anatomic, and neurochemical effects of perinatal brain iron deficiency have been characterized in rat and sheep models. Chronic intrauterine hypoxia in fetal sheep results in reduced brain iron concentrations.[62] Brain cytochrome *c* concentrations parallel the reduction in brain iron (see Figure 35-4). Perinatal iron deficiency in the rat causes reduced oxidative metabolism in the hippocampus and frontal cortex, elevated intracellular glutamate concentrations, reduced dopamine concentrations in the striatum, altered fatty acid profiles, and central nervous system hypomyelination.[102,104-106] Development of the brain is impaired, with truncation of dendritic branching, particularly in the hippocampus.[107] Rodents show behavioral deficits consistent with targeted areas of the brain including trace recognition memory, procedural memory, and spatial navigation. These effects last well beyond the period of iron deficiency, suggesting the possibility of long-term iron deficiency–induced alterations in brain structure and function.[108-112] The effects on energy metabolism, dendritic structure, monamine metabolism, and myelination all appear to work together to alter brain processing, particularly in memory domains.

Growth-restricted infants, infants of poorly controlled diabetic mothers, and preterm infants are clearly at increased risk for poorer long-term developmental outcomes.[113,114] Although the etiology of cognitive impairments in these groups of children is likely to be multifactorial, the relationship of developmental outcomes to perinatal iron status may be of importance, because an iron-deficient brain may be less likely to tolerate hypoxic or hypoglycemic events.[115] Only a few studies have evaluated perinatal iron status and development, but their findings suggest that areas of the brain affected in animal models may map onto behavioral and neurophysiologic abnormalities seen in human infants. For example, in animal models of maternal diabetes, brain iron stores are depleted from portions of the developing explicit memory system as well as from areas that are important for emotion,[116] including the hippocampus and other medial temporal lobe structures. The hippocampus and the hippocampus-dependent memory system develop very early in humans,[117] and recognition memory has been demonstrated in the newborn infant[118-120] using behavioral and neurophysiologic methods of evaluation. In one study, a neurophysiologic technique known as event-related potentials was used to study infants of diabetic mothers with and without perinatal iron deficiency (defined as an umbilical cord blood ferritin level less than 35 mcg/L).[121] Neurophysiologic manifestations of maternal voice recognition were intact in infants of diabetic mothers with normal iron status, but not in infants with perinatal iron deficiency, who demonstrated markedly attenuated responses. At the age of 1 year, infants with perinatal iron deficiency also had lower motor quotients than those in infants with normal iron status.

Wachs and colleagues[122] investigated the relationship between neonatal iron status and temperament development. Iron deficiency results in alterations in areas of the brain such as the amygdala and in neurotransmitters such as dopamine and serotonin[102] that are important in emotional processing. Neonatal anemia, low serum iron levels, and low ferritin levels were associated with higher levels of negative emotionality and lower levels of alertness and soothability, even after adjustment of data for confounding factors.

These two studies evaluated specific aspects of neurodevelopmental function that have been linked to iron deficiency in animal models. Global neurodevelopmental function also has been studied. Armony-Sivan and colleagues[123] evaluated the early development of neurologic reflexes in preterm infants (born before 34 weeks gestation) who were medically stable but anemic with or without low ferritin. Their study found a higher incidence of abnormal reflexes among infants who were anemic with low ferritin concentrations than among nonanemic infants. The findings were thought to be consistent with poor myelination of the brain. Tamura and colleagues[49] reported findings from a large study correlating umbilical cord serum ferritin

concentrations and results of cognitive testing at 5 years of age. Infants in the lowest quartile of umbilical cord ferritin concentration (less than 76 mcg/L) had poorer scores of language comprehension, fine motor skills, attention, and tractability than those in children with umbilical cord ferritin concentrations in the two median quartiles. Taken together, these studies seem to indicate that animal models can be used to reveal areas of the brain affected by perinatal iron deficiency that are consistent with findings in human infants. The long-term development of the brain after perinatal iron deficiency and the potential for behavioral recovery with nutritional mediation have not been adequately evaluated, however.

POTENTIAL IRON TOXICITY IN THE FETUS AND NEONATE

Iron deficiency is not the only iron-related problem that may affect the developing human. The newborn infant also is at risk for iron toxicity due to free radical formation. Newborn infants are considered to be at particular risk for oxidant injury as a consequence of immature antioxidant systems (e.g., vitamin E, selenium, superoxide dismutase) and increased sensitivity of rapidly growing tissues to free radicals.[16] The vitamin C system appears to mature after the age of 2 weeks, so antioxidant abilities improve thereafter.[124] Free radical formation is less likely to occur when iron is tightly bound and hidden by the three-dimensional protein structure of hemoglobin, myoglobin, transferrin, or ferritin. Non–protein-bound or loosely bound iron, however, can generate free radicals through the Fenton reaction, potentially resulting in serious tissue damage from the effects of oxidant stress on cell membranes.[125] Because newborn infants have low levels of iron-binding proteins, rapid infusions of iron may result in oversaturation of the iron-binding capacity, potentially resulting in liberation of free serum iron. Finally, newborn infants are prone to hypoxic-ischemic events, which may result in tissue reperfusion. Tissue reperfusion after hypoxia-ischemia potentiates the likelihood of the Fenton reaction and, consequently, the possibility of iron-related oxidant activity. Indeed, iron deposition has been noted in perivascular areas of the brain associated with neuronal loss in animal models of hypoxia-ischemia.[126] For these reasons, newborn infants are thought to be at particular risk for iron toxicity.

Although these considerations have raised considerable concern about the oxidant properties of iron, only indirect evidence is suggestive of clinical effects of possible iron toxicity in newborn infants. In theory, the highest-risk situation would arise with rapid intravenous boluses of iron, either via parenteral iron infusions or by transfusions of senescent blood cells that hemolyze and release iron. Blood transfusions have been associated with increased free iron levels and an increased risk of chronic lung disease in preterm infants.[13] These findings were not associated with lipid peroxidation; accordingly, the study investigators were not able to provide evidence that blood transfusions result in oxidative injury to the lung.

Regarding the use of oral iron supplements, it is possible that high doses of enteral iron may be sufficient to result in non–protein-bound serum iron and oxidative stress. Investigation on this topic has been minimal, but the available research suggests that current doses of iron given to preterm infants do not result in oxidant stress. Early supplemental iron given during treatment with erythropoietin was not associated with increased malondialdehyde in plasma or F-2 isoprostanes in urine.[127] Miller and colleagues[91] gave 3 to 12 mg/kg of enteral iron sulfate daily to hospitalized preterm infants and found no change in blood and urine isoprostanes, nor did they find any relationship between the iron sulfate dose and the isoprostane levels.

Overall, the relationship among iron toxicity, markers of oxidant stress, and organ damage in preterm infants has been inadequately studied to date. The available evidence, however, suggests that current practices for blood transfusion and oral iron supplementation appear to be safe.

A related consideration is iron overload in the newborn infant. Primary iron overload occurs in the hereditary hemochromatosis syndromes, which are not seen in the newborn infant and are rare even during childhood. Neonatal hemochromatosis previously was thought to be a syndrome of primary iron overload. However, the biochemical abnormalities found in neonatal hemochromatosis also have been shown to occur nonspecifically in severe liver disease, and the patterns of organ injury in neonatal hemochromatosis are not consistent with juvenile or adult forms of hemochromatosis.[128] Although the full-blown disease generally is fatal, a few survivors have been described who have resolution of their liver disease and who do not have ongoing manifestations of hemochromatosis. It is now postulated that iron deposition is a consequence rather than a cause of the liver disease. According to some evidence, the primary inciting factor in many patients may be an alloimmune process targeting an antigen present on fetal hepatocytes. Intravenous immune globulin has been given to fetal siblings conceived after the birth of an affected infant. Nearly all treated infants have shown biochemical evidence of neonatal hemochromatosis but with an apparent reduction in clinical symptoms and no deaths.[129]

Secondary iron overload syndromes have been described in several case reports in which infants with Rh disease had undergone multiple fetal transfusions.[130,131] The affected infants showed severe liver disease, marked elevation of serum ferritin concentrations, and iron overload on liver biopsy. Resolution of symptoms was reported after treatment with desferroxamine.

Despite the number of transfusions received by many premature infants, no cases of secondary iron overload have been reported in the literature. An autopsy study of infants receiving multiple blood transfusions revealed significant correlations between hepatic iron concentrations, volumes of blood transfused and antemortem serum ferritin concentrations, but clinical manifestations of iron overload were not observed in any infants.[132]

CONCLUSION

Iron sufficiency is important for normal growth, development, and function of fetal and neonatal organ systems. The regulation of maternofetal iron transport and intracellular iron accretion and storage continues to be investigated. Certain pregnancy subgroups are associated with an increased risk for fetal and neonatal iron deficiency, either because of inadequate maternal-fetal iron transport, in the case of maternal anemia or intrauterine growth retardation, or because of excessive iron demand, as seen in diabetic pregnancies. In the neonatal period, preterm infants treated with recombinant human erythropoietin and receiving fewer red cell transfusions appear to be at greatest risk for early depletion of iron stores. Iron excess also may play a role in certain neonatal disease processes through peroxidation of lipid membranes. The physiologic consequences of iron imbalances in all of these settings are important to document.

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36 Neonatal Calcium, Phosphorus, and Magnesium Homeostasis

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BODY DISTRIBUTION

Calcium

Calcium is the most abundant mineral in the body and the major inorganic constituent of bone. An adult human body contains approximately 1 to 2 kg of elemental calcium.[1] In term newborn infants, the total-body calcium content is approximately 30 g,[1] most of which (approximately 80%) accrues during the last trimester of pregnancy at a rate of up to 150 mg/kg of fetal weight/day.[2]

At all ages, 99% of total-body calcium is either in bone in the form of hydroxyapatite, integrally associated with collagen fibrils and other components of bone matrix, or in a noncrystalline, amorphous calcium phosphate form (a predominant form in early life).[3] One percent of total-body calcium is contained in the extracellular fluid (ECF) and soft tissues. The calcium of the mineral phase (at the surface of the crystals) is in equilibrium with that in the ECF, but only a minor portion of the skeletal content (approximately 1%) is freely exchangeable with the ECF calcium.[4] Although this exchangeable pool represents a small percentage of skeletal content, it is approximately equal to the total content of calcium in the ECF and soft tissues and serves as a reservoir of calcium. In children 3 to 16 years of age, the size of this total exchangeable pool (TEP) of calcium, measured using stable isotope technique, correlates with age independent of variations in body weight. Bone calcium accretion rate (V_0^+) and the V_0^+/TEP ratio are greater in children than in adults, indicating higher rates of Ca^{2+} flux associated with relatively higher exchangeable calcium pools in children than in adults.[5]

The calcium in plasma is in three forms: as the free ion, Ca^{2+} (50% of total plasma content); bound to plasma protein (40% of total calcium, of which 80% is bound to albumin and the remainder to globulins); and, to a small extent (approximately 10%), as diffusible complexes (complexed with bicarbonate, phosphate, or citrate).[6] Complexed and free (ionized) forms of calcium also are termed *ultrafiltrable* calcium (or non–protein-bound calcium), so that approximately 60% of total calcium in plasma crosses semipermeable membranes. Calcium binding to proteins is reduced at acid pH. Alterations in the concentration of serum albumin can exert a major influence on the measured total serum calcium and ionized calcium concentrations. The serum ionized calcium concentration decreases significantly with addition of serum albumin. Thus a fast infusion of albumin in the human neonate has the potential for acutely lowering the serum ionized calcium concentration.[7] Free calcium—that is, ionized calcium—is the only physiologically active fraction in blood.

Ionized calcium influences many cellular functions and is subjected to tight hormonal control, especially through parathyroid hormone (PTH). Although changes in ionized calcium frequently are reflected in changes in total calcium, and a general correlation exists between serum total calcium and serum ionized calcium, total calcium can be a poor predictor of a specific ionized calcium value, especially in neonates. The ionized calcium can be measured directly with the use of ion-selective electrodes,[8] using capillary blood in newborn infants.[9,10] Several factors, however, may affect ionized calcium determination: Higher ionized calcium values are recorded with the ICA-1 Radiometer than with the Orion SS20 electrode; ionized calcium may be decreased proportional to the concentration of heparin used as anticoagulant; and ionized calcium concentrations vary inversely with blood pH. The serum sample should be analyzed immediately or, alternatively, placed in 5% carbon dioxide–containing tubes and frozen, to minimize pH variations.[11]

Ca^{2+} inside the cell mediates a variety of cellular functions. Most of the cellular calcium is in the form of insoluble complexes (99%) at a concentration of approximately 1×10^{-6} M of cell water. Free calcium (1%) within the cell, which is critical for functional regulation, is present in lower concentrations, approximately 2.5×10^{-7} M.[12] The gradient between plasma and intracellular free calcium is approximately 10,000:1[13] and is tightly regulated.

The Ca^{2+} concentration in the ECF is kept constant by processes that constantly feed calcium into and withdraw calcium from the ECF. Calcium enters the plasma by absorption from the intestinal tract and by resorption of ions from bone. In conditions of calcium balance, rates of calcium release from and uptake into bone are equal. In normal adults, the total serum calcium concentration ranges from 2.2 to 2.6 mmol/L (8.8 to 10.4 mg/dL) and is remarkably constant. The ionized calcium concentration, although subject to changes directed by PTH, calcitonin, vitamin D, and blood pH, also is stable within an individual patient over prolonged periods and ranges from 1.2 to 1.3 mmol/L (4.8 to 5.2 mg/dL).[8]

At birth, an abrupt termination of the maternal-to-fetal calcium supply occurs. To maintain serum calcium homeostasis at this time, it is estimated that an increase of 16% to 20% in calcium flux from bone to extracellular space is required, unless sufficient exogenous intake is achieved.[14] In term newborn infants, cord blood total calcium concentrations are 2.6 mmol/L (10.2 mg/dL), and ionized calcium concentrations are 1.5 mmol/L (5.8 mg/dL).[15] By 2 hours of age, serum total calcium declines by 5%, and by 24 to 36 hours, serum calcium reaches its nadir of 2.3 mmol/L (9.0 mg/dL) for total calcium and 1.2 mmol/L (4.9 mg/dL) for ionized calcium[15] (Table 36-1). After a period of stabilization, serum calcium concentration slowly rises, reaching levels by 1 week of age similar to those found in childhood[16] (Table 36-2). In preterm infants, the mean cord serum ionized calcium concentration is 1.45 mmol/L (95% confidence interval [CI], 1.29 to 1.61 mmol/L), or 5.8 mg/dL (CI, 5.74 to 5.86 mg/dL]), which decreases during the first 24 to 36 hours of life and subsequently rises at approximately 6 days of age to values that exceed original cord blood concentration.[16,17] The early fall in ionized calcium levels may be associated with parathyroid glandular unresponsiveness secondary to prematurity[18] or hypomagnesemia,[19] and severe hypocalcemia may result. Very-low-birth-weight infants are likely to exhibit the lowest nadirs of ionized calcium; in most cases, however, low levels are unassociated with tetany or decreased cardiac contractility.[20,21]

Cord serum calcium concentrations differ by season of birth (being lower in summer-born than in winter-born infants)[22] and may vary in accordance with mode of delivery (lower for elective

cesarean section without labor than for elective cesarean section with labor, spontaneous vaginal delivery, or emergency cesarean section).[23] Cord serum calcium concentrations are unaffected by gender, race, or weight appropriateness for gestation.[24]

Phosphorus

Phosphorus is a major component of bone and of all other tissues and, in some form, is involved in almost all metabolic processes. The total amount of elemental phosphorus in normal adults is approximately 1 kg.[1] In term newborn infants, total-body phosphorus content is approximately 16 g.[1] As with calcium, approximately 80% of the phosphorus contained in term newborn infants is accumulated during the last trimester of pregnancy at a rate of approximately 75 mg/kg of fetal weight/day and is closely linked to the accretion of calcium, with a calcium-phosphorus ratio of 1.7:1.[1,2]

Approximately 85% of total-body phosphorus is in bone, primarily as hydroxyapatite but also found as more loosely complexed amorphous forms of bone crystal,[3] so phosphorus plays a key structural role in bone. In contrast to Ca, phosphorus is widely distributed in nonosseous tissues both in inorganic form and as a component of various structural macromolecules. Approximately 15% of total-body phosphorus is present in the ECF, largely in the form of inorganic phosphate ions, and in soft tissues, almost totally in the form of phosphate esters. Intracellular phosphate esters and phosphorylated intermediates are involved in a number of important biochemical processes, including the generation and transfer of cellular energy.

The phosphorus in body fluids is divided between an organic fraction, composed of phospholipids and phosphoesters, and inorganic phosphate (P_i). The latter comprises two ionic forms, monovalent $H_2PO_4^-$ and divalent HPO_4^{2-}. Divalent HPO_4^{2-} is the major ionic species of phosphorus in serum at pH 7.4. Serum P_i also exists as three fractions: ionized (approximately 55%); protein-bound (11%); and complexed to sodium, calcium, and magnesium (approximately 34%).[6] Approximately 90% of the P_i in plasma is ultrafiltrable. In contrast with calcium, only a small portion (11%) is protein-bound.[6] The intracellular and extracellular concentrations of ionized phosphorus (HPO_4^{2-}) are 1×10^{-4} M and 2×10^{-4} M, respectively.[12] Because so many phosphorus species are present, depending on pH and other factors, it is the convention to express concentrations in terms of mass of elemental phosphorus (i.e., mmol/L or mg/dL). In contrast with the calcium concentration, the serum phosphorus concentration varies quite widely, exhibits daily variations of as much as 50%, and is influenced by age, gender, diet, pH, and a variety of hormones. An adequate serum phosphorus concentration is important in maintaining a sufficient ion product (with calcium) for normal mineralization.[25]

Serum total phosphorus concentrations are higher in children than in adults. Adult concentrations may vary, with a normal range of 1.24 to 1.86 mmol/L (3.0 to 4.5 mg/dL). At birth, the infant's serum phosphorus concentrations are relatively low in comparison with older newborn or childhood values. In normal term infants born at a weight appropriate for gestational age, cord serum phosphorus concentrations do not differ by gender, race, season of birth,[22] weight appropriateness for gestation,[24] or mode of delivery.[23] The relatively low initial values, 2.6 mmol/L (range, 1.5 to 3.4 mmol/L), equivalent to 6.2 mg/dL (3.7 to 8.1 mg/dL), begin to rise shortly after birth. The reason for this acute rise is not well understood. The rise may be due to increased gluconeogenesis and endogenous phosphorus release or secondary to a low glomerular filtration rate and reduced phosphorus excretion. Mean serum phosphorus concentrations increase to 3.4 mmol/L (8.1 mg/dL) by 1 week of age and decrease to 1.7 mmol/L (4.1 mg/dL) in childhood.[26]

TABLE 36-1

Serum Concentrations of Total and Ionized Calcium in Term Infants During the First 24 Hours of Life

	SERUM CONCENTRATION: mg/dL*	
Age	Total Calcium	Ionized Calcium
Birth (cord blood)	10.2 (9.0-11.4)	5.82 (5.22-6.42)
2 hr	9.7 (8.5-10.9)	5.34 (4.84-5.84)
24 hr	9.0 (7.8-10.2)	4.92 (4.40-5.44)

*Values are means, with 95% confidence intervals in parentheses.

Modified from Loughead JL, Mimouni F, Tsang RC: Serum ionized calcium concentrations in normal neonates, *Am J Dis Child* 142:516, 1988. Copyright 1988, American Medical Association.

Magnesium

Magnesium is the second most abundant intracellular divalent cation in the body. Adult humans contain approximately 27 g of elemental magnesium.[1] In term newborn infants, the total-body magnesium content is approximately 0.8 g,[1] and approximately 80% of this is accrued during the last trimester of pregnancy at a rate of approximately 3 mg/kg of fetal weight/day.[2]

Magnesium is distributed in three major compartments in the body: Approximately 65% of the total-body magnesium is contained in bone in crystalline form, 34% in the intracellular space complexed with nucleic acid and protein, and only 1% in the

TABLE 36-2

Serum Calcium, Inorganic Phosphorus (P_i), and Magnesium Concentrations in Term Infants Younger Than 6 Months of Age

		SERUM CONCENTRATION: mg/dL*			
		CALCIUM			
Age	Type of Feeding	Free	Ionized	P_i	Magnesium
2 wk	Human milk	10.1 (9.1-11.1)	3.8 (3.6-4.0)	4.2 (3.4-5.0)	
	Human milk + vitamin D	9.9 (9.1-10.7)	3.8 (3.6-4.0)	5.1 (4.5-5.7)	
	Cow's milk–based formula	10.4 (10.0-10.8)	3.7 (3.5-3.9)	4.9 (4.1-5.7)	
16 wk	Human milk	10.4 (10.0-10.8)	3.9 (3.7-4.1)	4.6 (3.8-5.4)	
	Human milk + vitamin D	9.8 (9.2-10.4)	3.9 (3.7-4.1)	5.0 (4.4-5.6)	
	Cow's milk–based formula	10.2 (9.8-10.6)	4.0 (3.8-4.2)	4.7 (4.1-5.3)	
<6 mo	Human milk	9.75 (9.57-9.93)	5.38 (5.32-5.44)	6.44 (6.24-6.64)	2.04 (1.98-2.10)
	Cow's milk–based formula	9.73 (9.61-9.85)	5.27 (5.23-5.31)	6.98 (6.82-7.14)	2.10 (2.04-2.16)

*All data are expressed as means, with values in parentheses representing the 95% confidence interval.

Modified from Roberts CC, Chan GM, Folland D, et al: Adequate bone mineralization in breast-fed infants, *J Pediatr* 99:192, 1981; and Specker BL, Lichtenstein P, Mimouni F, et al: Calcium-regulating hormones and minerals from birth to 18 months of age: A cross-sectional study. II. Effects of sex, race, age, season, and diet on serum minerals, parathyroid hormone, and calcitonin, *Pediatrics* 77:891, 1986.

ECF.[27] Magnesium in bone is not an integral part of the hydroxyapatite lattice structure but appears to be located on the crystal surface. Only a minor fraction of magnesium in bone is freely exchangeable with extracellular magnesium. In infants aged 4 to 11 months, the calculated apparent magnesium exchangeable pool size, measured using the stable isotope ^{25}Mg, ranges from 5.5 to 7.6 mmol/kg of body weight and diminishes with age.[28] Because only 1% of the total-body magnesium is located in the extracellular space,[29] the plasma concentration does not consistently provide a reliable index of either total-body or soft tissue magnesium content.

The magnesium in plasma exists in three forms: as free ion (55%), bound to plasma protein (30%), and complexed to various anions such as phosphate and oxalate (15%).[29] About 70% of the total magnesium is ultrafiltrable and freely filtered at the renal glomerulus.[30] The protein-bound fraction interacts with carboxyl groups of albumin and is influenced by pH in a fashion analogous to that for calcium. The ionized fraction of magnesium is physiologically important (e.g., to plasma membrane excitability).

In contrast with the low concentrations of intracellular calcium, the concentration of free magnesium ions (Mg^{2+}) is 5×10^{-4} M in the cytosol, and this concentration is rigidly maintained and stable.[12] This stability is a reflection of the many critical roles that magnesium plays in cellular metabolism. Cellular magnesium is important as a cofactor for a number of enzymatic reactions and in regulation of neuromuscular excitability.

The serum concentration of magnesium is maintained within relatively tight limits and is essentially the same for neonates, infants, children, and adults, with a normal range of 0.62 to 1.16 mmol/L (1.5 to 2.8 mg/dL).[31] In the cord blood of normal term infants, cord serum magnesium concentrations are 0.68 (0.6 to 0.76) mmol/L (1.6 [1.45 to 1.83] mg/dL) and do not vary by gender, race, season of birth,[22] appropriateness of weight for gestation,[24] or mode of delivery.[23] Serum ionized magnesium concentrations in cord blood appear to be higher in large for gestational age infants and higher in the umbilical vein than in the umbilical artery (the latter values being similar to maternal venous levels).[32] Preterm infants born at the eighth month of gestation have cord serum magnesium concentrations similar to those of the term infant, with a mean of 0.81 (0.59 to 1.0) mmol/L (1.95 [1.43 to 2.45] mg/dL).[33] After birth, both term and preterm infants initially exhibit a decrease in serum magnesium concentrations. At 48 hours of life, serum magnesium concentrations reach mean concentrations of 0.77 mmol/L (1.87 mg/dL).[33] Subsequently, serum magnesium increases over the first week of life, followed by a decline toward childhood values by the end of the first month[34] (see Table 36-2).

Ionized magnesium concentration varies in several neonatal conditions: It is increased in neonatal hyperbilirubinemia,[35] acidosis,[36] and preterm respiratory distress syndrome (RDS).[37] Plasma Mg^{2+} concentration was significantly higher in term infants with severe hyperbilirubinemia (total bilirubin concentration of 304 ± 83.3 μM) than in infants with moderate hyperbilirubinemia (bilirubin level of 94.1 ± 54.7 μM): 0.6 ± 0.12 versus 0.49 ± 0.1 mmol/L. Increase in plasma ionized magnesium also may be due to extracellular movement of Mg^{2+}, a principal intracellular ion, resulting from generalized cellular injury.[35] Cord Mg^{2+} was significantly increased in infants with acidosis (umbilical arterial cord pH 7.0) over that in both preterm and term control infants (umbilical arterial cord pH 7.3), with values of 0.58 versus 0.51 and 0.49 mmol/L, and declined significantly 2 hours after delivery to 0.49 mM, whereas control infants showed constant Mg^{2+} concentrations during the observation period.[36] In preterm infants with RDS, plasma Mg^{2+} concentration is significantly higher than in infants without RDS (0.8 versus 0.57 mmol/L); and within the study group, significant negative correlations were found between plasma Mg^{2+} levels and values for umbilical cord arterial pH ($r = -0.621$) and base excess ($r = -0.746$), whereas no correlations were observed in the control group. The newborns who died later had significantly higher plasma Mg^{2+} levels than those in infants who survived (0.89 versus 0.63 mmol/L). From these data, it is suggested that increase of plasma Mg^{2+} may be due to extracellular movement of Mg^{2+} as a result of acidosis, hypoxia, and probable cellular injury during the early course of RDS.[37]

REGULATION OF SERUM CONCENTRATION

Calcium

The homeostasis of calcium in blood is maintained primarily through the interaction of three hormones—PTH, calcitonin, and vitamin D—and their actions on the gastrointestinal tract, kidney, and bone.

Parathyroid Hormone

PTH is a peptide hormone produced in the parathyroid glands. Under normal circumstances, a decrease in the serum ionized calcium concentration stimulates production and secretion of this hormone.[38] PTH, in turn, acts directly on bone, stimulating resorption, thereby releasing calcium and phosphorus into the ECF and the circulation. PTH also acts directly on the kidney to increase urinary excretion of phosphorus (which removes the phosphorus released into the circulation by its bone action) and decreases the urinary excretion of calcium. PTH indirectly enhances the gastrointestinal absorption of calcium through its effects on synthesis of 1,25-dihydroxyvitamin D (1,25-$[OH]_2$D).[39]

The integrated actions of PTH on bone resorption, distal renal tubular calcium reabsorption, and 1,25-$(OH)_2$D–mediated intestinal calcium absorption are responsible for the fine regulation of the serum ionized calcium concentration. The precision of this integrated control is such that in a normal person, the serum ionized calcium level probably fluctuates by no more than 0.025 mmol/L (0.1 mg/dL) in either direction from its normal set point value throughout the day. Osteoclastic bone resorption and distal tubular calcium reabsorption are the major control points in serum calcium homeostasis; of these two processes, the effect of PTH on the distal tubule is quantitatively the most important. Together, these effects of PTH on bone and kidney constitute a classic short-loop feedback system on the parathyroid chief cell. Adjustments in the rate of intestinal calcium absorption by way of the PTH–1,25-$(OH)_2$D axis require approximately 24 to 48 hours to become maximal. This axis represents a classic long-loop feedback system. PTH production is closely regulated by the concentration of serum ionized calcium. This feedback system is the critical homeostatic mechanism for maintenance of ECF calcium.[25]

With birth of the infant comes abrupt interruption of the transplacental calcium supply. Regardless of whether the newborn infant is fed enterally or given intravenous fluids, calcium delivery over the first hours to days post partum does not match that enjoyed by the fetus near the end of gestation. This decrement sets the stage for a rapid, dramatic decline in serum total and ionized calcium concentrations. In normal term infants, when serum calcium concentrations fall slightly after birth, PTH concentrations increase appropriately (two- to five-fold increase during the first 48 hours of life) and remain elevated for several days postnatally. The PTH secretion in term newborn infants appears substantial, and blood concentrations negatively correlate with calcium levels. Over the course of the first week of life, the role of PTH appears to be enhanced, and neonates show an appropriate calcemic response when challenged with PTH.[26]

In the cord blood of normal term infants, the mean serum PTH concentration is 2.96 ng/L (range, 0.5 to 18 ng/L) (ng/L units are

equivalent to pg/mL). Cord serum PTH concentrations do not differ by gender, race, season of birth, or weight appropriateness for gestation[22,24] but may vary with mode of delivery (being higher in infants delivered by elective cesarean section without labor versus elective cesarean section with labor, emergency cesarean section, or spontaneous vaginal delivery).[23]

In infants of diabetic mothers, the PTH response is blunted and may relate to the magnesium deficiency state characteristic of the diabetic pregnancy.[14] In very-low-birth-weight infants, the serum PTH concentration rises significantly in association with a decline in serum calcium concentration and is suppressed by intravenous calcium infusion, indicating that the parathyroid glands of very low birth weight infants are responsive to changes in serum calcium levels.[20] In infants and young children undergoing cardiac surgery, the PTH response to both hypocalcemia and rising ionized calcium concentrations appears at least as great as that of adults. Thus, in general, the calcium–parathyroid–vitamin D axis functions in infants and young children in a fashion similar to that in adults.[40]

Calcitonin

Calcitonin is a hypocalcemic peptide hormone produced by the parafollicular cells of the thyroid,[41] which in many ways acts as the physiologic antagonist to PTH. The hypocalcemic activity of calcitonin is accounted for primarily by direct inhibition of osteoclast-mediated bone resorption (decreasing the amount of calcium and phosphorus released from bone)[42] and secondarily by increasing renal calcium and phosphorus excretion (at high doses).[43] These effects are mediated by receptors on osteoclasts and renal tubular cells and depend on the rate of preexisting bone resorption; the greatest effects of this hormone apparently are seen in circumstances in which bone resorption is increased.[44] The net consequence of the actions of calcitonin is to decrease serum concentrations of calcium and phosphorus.

The secretion of calcitonin is under the direct control of blood calcium: An increase in calcium causes an increase in calcitonin, and a decrease in calcium causes a decrease in calcitonin concentrations.[45] An increase in the production of calcitonin may also be observed with an increase in circulating magnesium levels or after ingestion of food (which increases circulating levels of gastrointestinal hormones [gastrin, glucagon, and pancreozymin]). Thus it has been suggested that this gastrointestinal-thyroid-cell system serves to prevent marked increases in serum calcium during food ingestion.[46] Once secreted, calcitonin disappears quite rapidly from the circulation, with a half-life of 2 to 15 minutes.[44]

In the cord blood of normal term infants, cord serum calcitonin concentrations are 44.1 (24.3 to 63.9) ng/L or pg/mL.[22] They do not differ by gender, race, or season of birth[22] but decrease with increasing gestational age. Infants born before 32 weeks gestation have nearly three times the cord serum calcitonin concentrations of term infants.[47] In the newborn infant, cord serum calcitonin concentrations are higher than those of the pregnant mother.[48] In both preterm and term infants, serum calcitonin concentrations further increase after birth, with a peak at 24 to 48 hours of age followed by a decline to childhood values by 1 month of age.[47-49] The physiologic importance of this increase in serum calcitonin is unclear but may relate to its effect on counteracting the bone-resorptive action of PTH.

Vitamin D

Vitamin D is either produced in the skin under the influence of ultraviolet light[50] or ingested in the diet and absorbed from the gastrointestinal tract. Before it reaches its final active form, 1,25-$(OH)_2$D, vitamin D must first undergo two hydroxylation steps—the first in the liver and a second in the proximal tubule of the kidney.[51] 25-Hydroxylation in the liver is a relatively unregulated process, and serum 25-hydroxyvitamin D (25-OHD) concentrations generally reflect the patient's vitamin D status (as determined by intake and sunlight exposure). The production of 1,25-$(OH)_2$D by the renal proximal tubule is enhanced by hypocalcemia, hypophosphatemia, and PTH[52] and appears to be tightly regulated. 1,25-$(OH)_2$D is transported through the blood to its target tissues (small intestine and bone), where it regulates calcium homeostasis. 1,25-$(OH)_2$D acts on the small intestine to stimulate the active absorption of calcium and phosphorus,[53] on bone (in conjunction with PTH) to stimulate bone resorption of calcium and phosphorus,[54] and on kidney to conserve calcium and phosphorus. Thus vitamin D is necessary for maintenance of normal calcium and phosphorus homeostasis. The net action of 1,25-$(OH)_2$D is to increase the serum concentration of calcium.

In the cord blood of normal term infants, the mean serum 1,25-$(OH)_2$D concentration is 76 pmol/L and ranges from 31 to 151 pmol/L (32 pg/mL; range, 13 to 63 pg/mL). It varies by season of birth, being high in summer-born versus winter-born infants[22] (Figure 36-1), and by weight in relation to gestation—concentrations are lower in infants who are small for gestational age (SGA) versus those born at a weight appropriate for gestational age (AGA),[24] but not by gender or race.[22] These variable concentrations of serum 1,25-$(OH)_2$D have been suggested as an adaptive response to low bone mineral content in summer-born newborn infants (in the United States) or may reflect decreased production of 1,25-$(OH)_2$D in SGA infants, possibly as a consequence of reduced uteroplacental blood flow. In normal term infants, serum 1,25-$(OH)_2$D concentrations are low at birth but increase to adult normal ranges by 24 hours of life, possibly reflecting the need for optimum intestinal calcium and phosphorus absorption.[55]

In preterm infants, serum 1,25-$(OH)_2$D concentrations at birth are comparable with reference values for healthy children and adults, increase significantly during the first few days of life,[56-58] and are far above reference values between 3 and 12 weeks of life.[58] In preterm infants, serum concentrations of vitamin D–binding protein are lower, serum total 1,25-$(OH)_2$D concentrations are higher, and the calculated free 1,25-$(OH)_2$D index (total 1,25-$[OH]_2$D/D binding protein ratio, which may represent the physiologically active component of 1,25-$[OH]_2$D) is four to six times higher than in previously studied term infants; the free 1,25-$(OH)_2$D index increases significantly with age and does not correlate with serum phosphorus or PTH.[59]

Cord serum 25-OHD concentrations are influenced by season of birth and maternal vitamin D status. In Cincinnati, human milk–fed infants in the first year of life have low serum 25-OHD concentrations in winter (33% to 50% of summer values),[60] paralleling measures of actual sunshine exposure.[61] Cord serum 25-OHD concentrations in Cincinnati, however, do not differ by season of birth, possibly because a large percentage of women take prenatal vitamins, which would obscure any sunshine-related effects on vitamin D metabolism.[22] In contrast with those reported from Cincinnati, cord serum 25-OHD concentrations of Korean neonates are significantly lower in winter (approximately one third of summer values), and 97% of winter-born infants have subnormal 25-OHD concentrations (less than 28 nmol/L [11 ng/dL]), consistent with suboptimal maternal vitamin D nutrition status and reduced seasonal sunlight exposure.[62] The lower vitamin D status in winter may influence fetal bone mineralization during late fetal life; in one study, the total body bone mineral content in winter-born infants was lower (by 6%) than in summer-born infants in Korea.[62]

Phosphorus

The serum concentration of phosphorus appears to be regulated primarily through the kidney by means of tubular reabsorption of P_i; serum phosphorus concentrations are maintained at a value

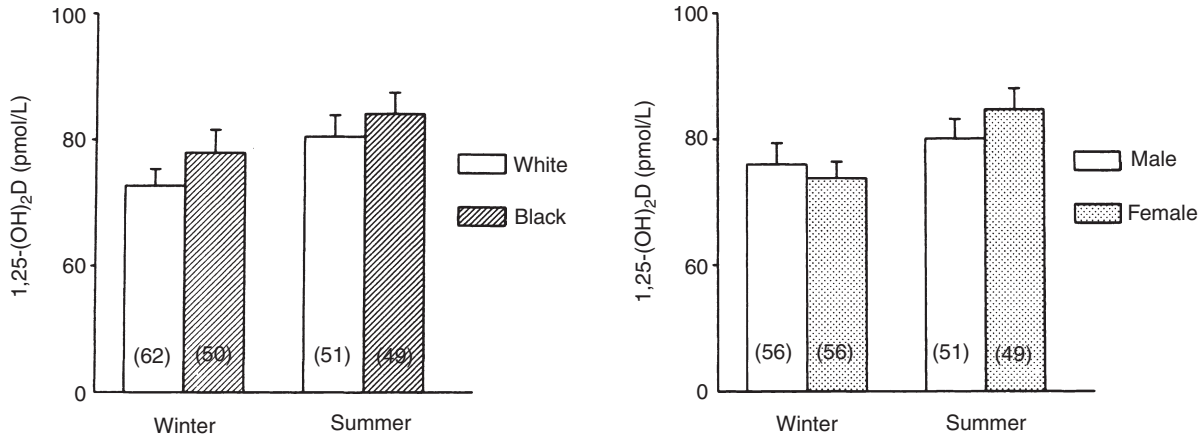


Figure 36-1 Serum 1,25-dihydroxyvitamin D (1,25-$[OH]_2D$) concentrations were higher in summer-born than in winter-born infants (p = .02) and did not differ by race or gender. Values are means ± SE, with numbers of newborns *in parentheses. (From Namgung R, Tsang RC, Specker BL, et al: Low bone mineral content and high serum osteocalcin and 1,25-dihydroxyvitamin D in summer- versus winter-born newborn infants: An early fetal effect?* J Pediatr Gastroenterol Nutr *19:220, 1994.)*

close to the tubular phosphorus threshold, or the tubular maximum for phosphorus/glomerular filtration rate (TmP/GFR).[25] Because of the normal high efficiency and lack of fine regulation of phosphorus absorption in the intestine, only in unusual circumstances is the intestinal absorption a limiting factor in phosphorus homeostasis.

During phosphorus depletion, phosphaturia decreases before the serum phosphorus concentration declines. This adaptive response (i.e., increasing tubular transport when luminal concentrations are decreasing) is an intrinsic property of these cells. The following sequence of events is initiated in the face of a hypophosphatemic challenge: (1) stimulation of 1,25-$(OH)_2D$ synthesis in the kidney, (2) enhanced mobilization of phosphorus and calcium from bone, and (3) hypophosphatemia-induced increase in tubular maximum for phosphorus, resulting in reduced phosphorus excretion. The increased circulating concentration of 1,25-$(OH)_2D$ leads to increases in phosphorus and calcium absorption in the intestine and provides an additional stimulus to phosphorus and calcium mobilization from bone. The increased flow of Ca^{2+} from bone and intestine inhibits the secretion of PTH, resulting in lowered phosphate excretion. The net result of this series of adjustments is a return of serum phosphorus concentration to normal without change in the serum calcium concentration.[25]

The defense against hyperphosphatemia consists largely of a reversal of the just events described. The principal humoral factor that combats hyperphosphatemia is PTH. An acute rise in the serum phosphorus concentration produces a transient fall in the concentration of serum ionized calcium and a stimulation of PTH secretion. This in turn reduces TmP/GFR (increasing urinary phosphorus excretion), leading to a readjustment in serum phosphorus and calcium concentrations. A prolonged rise in the serum phosphorus concentration results in (1) an intrinsic downward adjustment in TmP/GFR that is independent of PTH and (2) a persistent increase in PTH secretion that ultimately can lead to parathyroid chief cell hyperplasia.[25]

The wide variation in serum phosphorus concentrations may be secondary to a few direct regulatory mechanisms. PTH, which has the greatest impact on serum phosphorus concentrations, primarily responds to changes in ionized calcium concentrations, not phosphorus. Phosphorus is freely filtered at the glomerulus and presents to the renal tubule in high concentrations. The renal tubule reabsorbs phosphorus in both the proximal and the distal nephron. In states of low PTH concentrations, the renal tubular cells reabsorb up to 95% to 97% of filtered phosphorus. In states of high serum PTH concentration, reabsorption of phosphorus in proximal and distal tubules is inhibited, resulting in excretion of high concentrations of urinary phosphorus. Although markedly affected by PTH in the usual state, renal tubular cells appear to have a decreased responsiveness to PTH in conditions of severe phosphorus deficiency or overload. Hence, phosphorus is reabsorbed in the face of high circulating PTH in severe phosphorus deficiency, and phosphorus is excreted despite low PTH concentrations when serum phosphorus is high.[63] Other hormones also may have an effect on the excretion of phosphorus by the kidney. Growth hormone, 1,25-$(OH)_2D$, insulin, and possibly somatomedins (insulin-like growth factors) all have been shown to increase renal tubular reabsorption of phosphorus.[63] The extent to which these hormones contribute to the normal regulation of serum phosphorus is unclear.

In preterm infants, a phosphorus depletion syndrome has been observed in infants fed human milk,[64] in infants with restricted enteral mineral intakes (owing to inadequate formula content), and in infants with chronic illnesses who receive prolonged parenteral nutrition without mineral supplements.[65] In this latter group of patients, biochemical signs of phosphorus deficiency are prominent and may be associated with rachitic bone changes; serum 1,25-$(OH)_2D$ concentrations are increased with evidence of increased bone resorption and turnover (increased serum bone resorption marker, cross-linked carboxyl-terminal [C-terminal] telopeptide of type I collagen) and osteocalcin levels in infants with rickets versus controls).[66] These changes appear to be an extreme example of phosphorus depletion because of low enteral mineral intakes during the first month of life in very-low-birth-weight infants.

Magnesium

In contrast with calcium and phosphorus, little is known about the hormonal regulation of systemic magnesium homeostasis. Maintenance of the serum magnesium concentration seems to result from the combined fluxes of magnesium at the levels of intestine, kidney, intracellular fluids, and perhaps the skeleton. Regulation of the total-body magnesium balance resides principally within the kidney and tightly matches the intestinal absorption of Mg^{2+}. The kidney provides the most sensitive control for magnesium balance[67]: Approximately 80% of the total plasma Mg^{2+} is filtered through the glomerular membrane,[68] the major portion of which is subsequently reabsorbed along the nephron[69]; 85%

of the filtered Mg^{2+} in the proximal tubule and the thick ascending limb of Henle, and 5% to 10% in the distal convoluted tubule (DCT). The final urinary excretion of Mg^{2+} is determined mainly by the active reasorption of Mg^{2+} in the DCT.[69] Mg^{2+} transport in the DCT is transcellular in nature and influenced by dietary magnesium restriction[70] and a number of hormones.[71] The molecular details and regulation of this pathway, however, remain largely unknown. With a rise in serum magnesium concentration, the fraction of filtered magnesium that is excreted increases sharply, whereas a decrease in serum magnesium concentration (which occurs when a low-magnesium diet is ingested for a few days) leads to the near-disappearance of magnesium from the urine.[72] The excretion of magnesium is directly related to the renal magnesium threshold and a tubular maximum–limited process and is primarily responsible for maintaining serum magnesium concentration within rather narrow limits.[25]

The body's stores of magnesium appear to be important in the maintenance of calcium homeostasis. Magnesium deficiency may decrease bone calcium release in vitro[73] and increase the percentage of bone calcium, compared with that of magnesium-replete rats in vivo.[74] A heteroionic bone exchange of magnesium for calcium has been demonstrated in vitro.[73] The release of both calcium and magnesium from bone also is stimulated by PTH and decreased by calcitonin.[75]

Congenital rickets may occur in infants whose mothers have received prolonged magnesium infusion for tocolysis.[76] Fetal magnesium toxicity impairs bone mineralization and can lead to serious bone demineralization that may cause fractures in the newborn period that complicate recovery from respiratory disease.[77]

Many of the same hormones that regulate serum calcium concentrations also appear to affect serum magnesium, although the relationships are not as well defined as for calcium. Acute changes in serum magnesium concentration have effects on PTH secretion similar to those for acute changes in serum calcium concentrations, but magnesium is in general a less potent stimulus than clcium.[78] An acute increase in serum magnesium concentration leads to a decline in serum PTH.[79] Reduced activity of PTH may increase the tubular clearance of magnesium, resulting in renal magnesium losses. Conversely, an acute decrease in serum magnesium results in an increase in PTH secretion. The increase in PTH secretion results in an increase in serum magnesium concentrations, probably through increased bone resorption and, more important, decreased losses in the urine. PTH affects tubular reabsorption of magnesium, through a mechanism similar to that used for the transport of calcium and phosphorus involving cyclic adenosine monophosphate (cAMP).[72] It has been difficult, however, to identify a direct effect of PTH on tubular magnesium transport because concomitant changes in serum or urinary calcium or other factors also can affect tubular magnesium transport.[72]

Paradoxically, chronic magnesium deficiency results in decreased PTH secretion and a blunting of the action of PTH on target organs,[80,81] partly because of alterations in the calcium-sensitive, magnesium-dependent adenylate cyclase system that is involved in the secretion of the hormone.[82] Therefore magnesium deficiency may lead to or exacerbate hypocalcemia by inhibiting the parathyroid response to declining serum calcium concentrations.

The role of calcitonin in the regulation of serum magnesium concentrations appears to be minimal. Elevated serum magnesium concentrations increase calcitonin release. In vitro studies have shown that a 100% increase in the magnesium concentration of the extracellular bath leads to a discharge of calcitonin from the thyroid parafollicular cells.[83] This compares with a similar calcitonin response with a 20% increase in the calcium concentration. Because calcitonin inhibits bone resorption, the release of magnesium from bone is apparently decreased by calcitonin.[84] Calcitonin decreases renal tubular reabsorption of magnesium in humans.[85] The physiologic role of calcitonin in the regulation of magnesium homeostasis, however, is not well defined, and there are no major difficulties with magnesium (or calcium) or skeletal homeostasis in humans with congenital or acquired calcitonin deficiency.[86]

Under physiologic conditions, the role of vitamin D in the regulation of serum magnesium concentration probably is of minor importance and appears to have at best an indirect influence on metabolism. Little effect on intestinal magnesium transport or absorption is seen at physiologic concentrations of 1,25-$(OH)_2$D, but increased intestinal magnesium absorption occurs at pharmacologic doses of vitamin D.[87,88] Furthermore, no significant correlation exists between magnesium absorption and plasma 1,25-$(OH)_2$D concentrations[89]; however, magnesium is an essential cofactor for the 25-hydroxylation reaction in the hepatic formation of 25-OHD,[90] and magnesium deficiency is associated with decreased serum 1,25-$(OH)_2$D concentrations in adults, presumably because of its role as a cofactor for 25-$(OH)_2$D–1α-hydroxylase in renal tubular cells.[91]

Other hormones known to have a direct or indirect effect on serum magnesium concentrations are estrogens, thyroxine, and aldosterone, which lower serum magnesium, and epinephrine, glucocorticoids, and progesterone, which increase serum magnesium. The overall effect of these hormones in healthy persons appears to be minimal.[72]

GASTROINTESTINAL TRANSPORT

Calcium

The gastrointestinal tract generally is considered the primary site involved in the long-term regulation of calcium balance. In adults, the average dietary calcium intake is approximately 600 to 800 mg/day, and less than half of this amount is absorbed.[92] In preterm low-birth-weight infants, calcium absorption has been reported to range from 50% to 80% at intakes of 80 to 140 mg/kg/day.[93,94] Calcium absorption increases during periods of rapid growth in children and decreases with advancing age. The percentage of true calcium absorption in preterm infants, however, does not change with birth weight or gestational age.[95]

Most calcium has been considered to be absorbed in the proximal small intestine, and the efficiency of absorption decreases in the more distal small intestinal segments. Several in vivo studies,[96,97] however, have shown that the bulk of Ca^{2+} absorption can be accomplished in the distal small intestine, in particular the ileum, a segment known to have a limited capacity for *active* calcium absorption.[98]

Calcium absorption involves two major processes: *transcellular (active)* movement, predominant in the duodenum and upper jejunum; and *paracellular (passive)* movement through the tight junctions between intestinal cells, throughout the length of intestine.[98,99] When dietary calcium is low, the *vitamin D–dependent transcellular pathway* is thought to play a major role,[98-100] and is down-regulated as total calcium intake rises. At high calcium intakes, the *passive paracellular pathway* is dominant, being linear increase in calcium absorption over a wide range of calcium concentrations (50 to 200 mmol/L) and unchanged as a result of increasing calcium intake.[98,100] The coexistence of mediated and nonmediated transport processes assures the organism of an adequate calcium supply, yet prevents excessive calcium absorption. The passive absorption of calcium does not require metabolic energy and is not subject to acute regulation. According to some studies, however, bile salts and lactose may increase ileal calcium absorption by increasing the permeability of the paracellular pathway.[97,101]

Transcellular transport of calcium is a three-step process consisting of (1) entry across the apical membrane of the enterocyte, by way of Ca^{2+}-selective channels, termed calcium transport

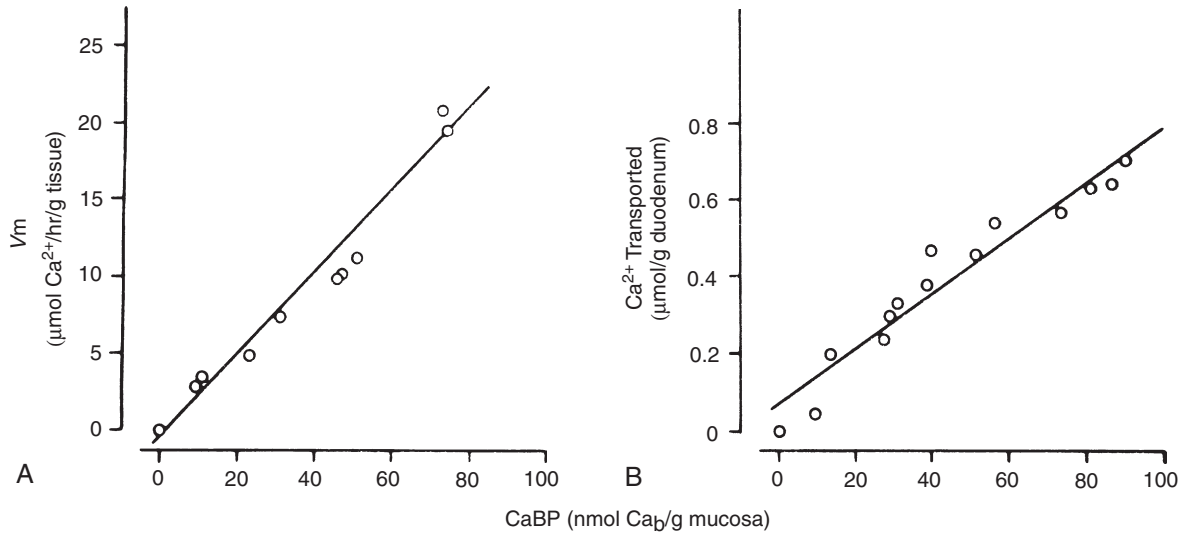


Figure 36-2 The relationship between intestinal Ca^{2+} transport and calbindin-D9k (calcium-binding protein [CaBP]) content. **A,** V_m, calculated from in situ duodenal, jejunal, and ileal loop experiments, shown as a function of CaBP content. (Data from *Pansu D, Bellaton C, Roche C, et al: Duodenal and ileal calcium absorption in the rat and effects of vitamin D*, Am J Physiol *244:G695, 1983.*) **B,** Calcium transport, as evaluated from everted duodenal sac experiments, shown as a function of CaBP content. (*Data from Roche C, Bellaton C, Pansu D, et al: Localization of vitamin D–dependent active Ca^{2+} transport in rat duodenum and relation to CaBP,* Am J Physiol *251:G314, 1986.*) (**A** *and* **B,** *From Bronner F, Pansu D, Stein WD, et al: An analysis of intestinal calcium transport across the rat intestine,* Am J Physiol *250:G561, 1986.)*

protein (CaT1)[102] and epithelial calcium channel (ECaC)[103,104]; (2) intracellular calcium transfer, facilitated by vitamin D–dependent cytosolic calcium-binding protein, calbindin-D9k[105]; and (3) active extrusion across the opposing basolateral membrane against an electrochemical gradient, mediated by a high-affinity ATP-dependent Ca^{2+}-ATPase (PMCA1b) or Na^+-Ca^{2+} exchanger (NCX1)[106,107] (Figure 36-2).

The understanding of calcium absorption has been increased by the identification of the apical calcium channel in Ca^{2+}-transporting epithelia, CaT1 (also known as ECaC2 and recently renamed *transient receptor potential vanilloid* [TRPV6][108]), from the rat intestine,[102,103] which acts as facilitative transporter, mediating cellular uptake of Ca^{2+} down its electrochemical gradient with saturation kinetics.[109] TRPV family members are involved in the absorption of Ca^{2+} in intestine; TRPV5 and TRPV6 have been cloned, initially from vitamin D–responsive Ca^{2+}-transporting epithelia, termed the epithelial calcium channels ECaC1 (CaT2) and ECaC2 (CaT1), and play a major role in the intestinal uptake of Ca^{2+}.[102,103] Functional expression reveals the characteristic features of a constitutively activated Ca^{2+} permeability, a large $Ca^{2+} > Na^{2+}$ selectivity, and a current-voltage relationship showing inward rectification.[110]

The active transport of calcium is under the control of hormones, and 1,25-$(OH)_2$D is the primary regulator of Ca^{2+} transport or absorption. The hormone 1,25-$(OH)_2$D acts through nuclear vitamin D receptors (VDRs),[111] which are present within the enterocytes of the intestine.[112] Possible targets involved in vitamin D–dependent active duodenal calcium absorption are calcium influx through CaT1 and ECaC,[104,113,114] and intracellular calcium transfer by calbindin[105,115] and plasma membrane Ca^{2+}-ATPase (PMCA1b).[116,117] Thus, in wild-type mice and human duodenum, 1,25-$(OH)_2$D up-regulates CaT1 (TRPV6), ECaC, calbindin-D9k, and PMCA mRNA.[116-122] Studies in both humans[123] and animals[105] have demonstrated a direct correlation between mucosal concentration of calcium-binding protein (calbindin-D) and the rate of intestinal calcium absorption, thus supporting a link between the physiologic action of vitamin D and calbindin-D[99] (Figure 36-3).

CaT1 in the intestine is highly responsive to 1,25-$(OH)_2$D and shows both fast and slow calcium-dependent feedback inhibition to prevent calcium overload.[109] Maximum transcellular transport is directly proportional to the cellular content of calcium-binding protein (CaBP), to which greater than 90% of the transported calcium is bound.[113] When Ca^{2+} enters the cytosol, its self-diffusion through the cell interior is enhanced up to 70-fold by calbindin-D9k.[124] Vitamin D deficiency leads to no synthesis of calbindin-D9k and virtually no transcellular calcium transport.[105] Moreover, interference with calcium binding by CaBP interferes with active Ca transport. Theophylline, when added to the incubation medium of everted duodenal sacs prepared from rats on a low-calcium diet, inhibits transcellular calcium transport in a concentration-dependent manner: Calcium binding by CaBP also is depressed by theophylline in a concentration-dependent manner and parallels the inhibition of transcellular calcium transport, supporting a CaBP function as a "calcium ferry" in transcellular transport[125] (Figure 36-4). Thus active calcium transport is regulated by vitamin D or processes that modify the action or metabolism of the sterol. However, there is an opposing view regarding the role of calbindin-D9k in vitamin D–mediated calcium absorption. Recently Akhter and associates[126] produced a calbindin-D9k–null mutant 129/OlaHsd mouse and examined directly whether the mutant mouse can absorb calcium from the intestine in response to the active form of vitamin D. The calbindin-D9k–null mutant mouse is fully able to absorb calcium from the intestine in response to 1,25-$(OH)_2D_3$. From this study, it is suggested that calbindin-D9k is not required for vitamin D-induced intestinal Ca^{2+} absorption.[126]

Calcium extrusion is affected by Ca^{2+}-ATPase, occurs against an electrochemical gradient, and requires a supply of energy.[127] It is not the rate-limiting step, however, because extrusion capacity is more than sufficient to handle the maximum transcellular flux of calcium. It is the flow of Ca^{2+} inside the cell, from the brush-border pole to the calcium pump at the basolateral side, that is rate-limiting.

Intestinal calcium transport declines with age in parallel with the expression of calcium channel,[120] calbindin-D9k,[128] and

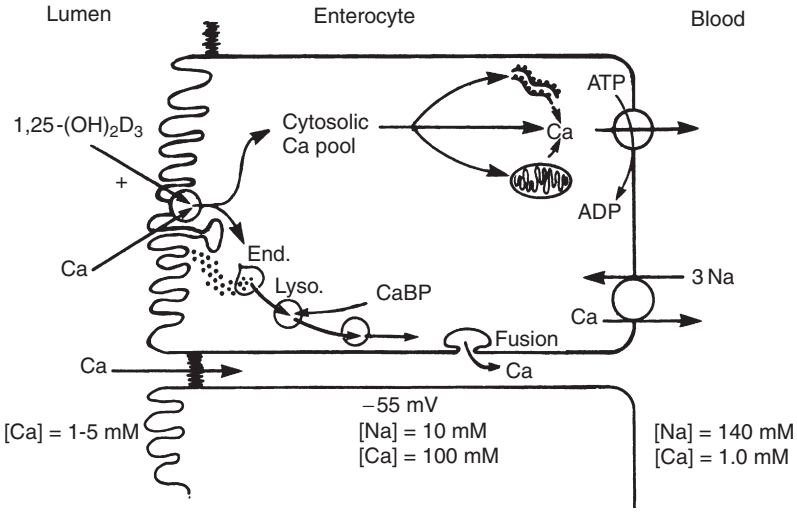


Figure 36-3 The potential pathways, gradients, and mechanisms for calcium transport across intestinal epithelium. *ADP,* Adenosine diphosphate; *ATP,* adenosine triphosphate; *CaBP,* calcium-binding protein; *End,* endoplasmic reticulum; *Lyso,* lysosome. *(From Favus MJ, Tembe V: The use of pharmacologic agents to study mechanisms of intestinal calcium transport,* J Nutr *122[3 suppl]:683, 1992.* © J Nutr, *American Institute of Nutrition.)*

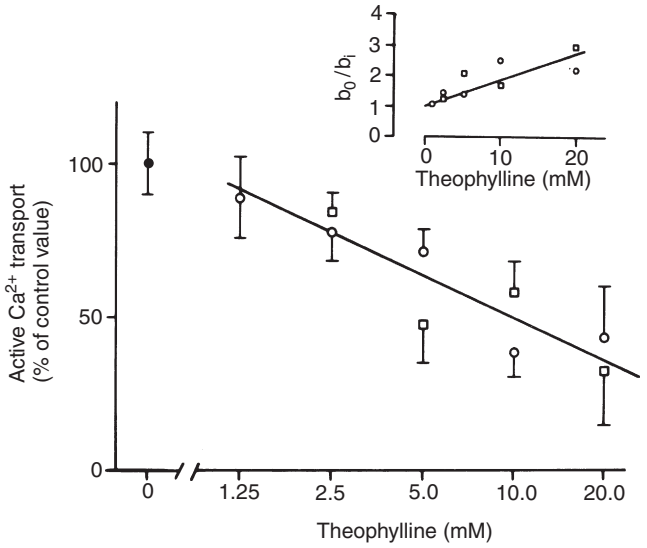


Figure 36-4 Effect of theophylline on transcellular Ca^{2+} transport as evaluated in everted duodenal sacs. Calcium concentration in medium: 0.1 mM (○); 0.25 mM (□). Transport (μmol • h^{-1} • g^{-1} of tissue) in absence of theophylline (●): 0.18 ± 0.02 at 0.1 mM calcium (n = 17), 0.27 ± 0.04 at 0.25 mM calcium (n = 6). *Inset*, K_i • b_0 is mean transport rate without theophylline, and b_i is mean transport inhibited by theophylline. K_i = 10.8 mM, based on least-squares relationship. *(From Pansu D, Bellaton C, Roche C, et al: Theophylline inhibits transcellular Ca transport in intestine and Ca binding by CaBP,* Am J Physiol *257:G935, 1989.)*

Ca^{2+}-ATPase.[129] These age-related changes in the regulation of calcium absorption are reflected in intestinal CaT1,[120] calbindin,[128] and calcium pump levels.[129] This decline in expression may reflect the marked drop in serum 1,25-$(OH)_2$D concentrations from young to adult rats.[128] The capacity of a low-calcium diet and 1,25-$(OH)_2$D itself to increase calcium absorption also declines with age.[120]

The relative importance of active versus passive intestinal absorption of calcium in the human neonate is uncertain. In rats, it has been observed that during the suckling period, before the time of natural weaning, the absorption of calcium is linearly related to the intraluminal Ca^{2+} concentration.[130,131] With increasing age, a decrease in the intestinal permeability of calcium is noted, and the relationship between intraluminal Ca^{2+} concentration and calcium absorption becomes increasingly curvilinear. It is during this time that measurable active intestinal absorption of calcium increases. Just before this increase in active absorption, receptors for 1,25-$(OH)_2$D become identifiable,[132] and a marked increase in mucosal vitamin D–dependent calcium-binding protein is observed.[133] Although an increase in active intestinal absorption of Ca is observed around the time of weaning even in vitamin D–deficient rat pups, the marked increase in absorption that normally is observed occurs only in the presence of 1,25-$(OH)_2$D.[134] Furthermore, the increase in active calcium absorption induced by 1,25-$(OH)_2$D is not observed until the appearance of its cytoplasmic receptor.[134]

Neonates of several species are almost insensitive to 1,25-$(OH)_2$D[134,135] and lack detectable concentrations of calcitriol receptor and calbindin in their mucosa.[132,133] The potential importance of calcitriol-independent neonatal calcium absorption is supported by in vivo studies of calcium absorption in newborn and weaned piglets suffering from pseudovitamin D–deficiency rickets type 1 (PVDRI). In this disorder, renal production of calcitriol is absent.[136] Despite the defect, however, affected animals of this strain are normocalcemic and clinically normal at birth and absorb calcium from the gut in amounts similar to those in control animals during the first 4 weeks of life (although the plasma concentrations of calcitriol are already reduced at birth to an unphysiologically low level[137,138]). By the fourth week post partum, significant calcium malabsorption, hypocalcemia, and typical clinical signs of rickets have developed in PVDRI piglets.[139] In weaned PVDRI piglets, concentrations of Ca^{2+} are significantly lower than in control animals and are associated with significantly lower 1,25-$(OH)_2$D concentrations.[138]

A study by Schroder and colleagues[138] demonstrated that the normal absorption of calcium by newborn PVDRI piglets in the early postpartal period occurs by an *active* mechanism. By measuring unidirectional calcium flux rates using stripped duodenal mucosae (in the absence of an electrochemical gradient), significant net calcium fluxes were found in both newborn (less than 1 week old) PVDRI and control piglets, whereas active net Ca^{2+} absorption was completely absent in weaned (older than 6 weeks of age) PVDRI piglets. Because the impairment of calcium absorption does not develop during the first 4 weeks post partum in newborn PVDRI piglets, it is concluded that *active* Ca^{2+} absorption in neonatal piglets is vitamin D–*independent*. In human neonatal intestine, the possible

presence of vitamin D–independent active Ca^{2+} absorption has not been studied. In newborn PVDRI piglets, active calcium absorption is present despite low 1,25-$(OH)_2$D concentrations and decreased numbers of vitamin D receptors, indicating the presence of a vitamin D–independent active absorption mechanism. In weaned PVDRI piglets, however, active net calcium absorption is absent, with low serum 1,25-$(OH)_2$D concentrations but similar vitamin D receptor number relative to those in control animals.[138]

The timing of similar events in the human is unknown. Because vitamin D–responsive, active calcium absorption occurs in a relatively restricted portion of the small intestine and passive absorption occurs throughout the entire small intestine at similar rates, it should not be surprising that the latter process contributes significantly to the net intestinal absorption of calcium.

In a study of preterm infants 2 to 4 weeks of age (mean gestational age of 32 weeks at birth) using metabolic balance techniques, Senterre and Salle[140] demonstrated that human milk–fed and formula-fed infants supplemented with vitamin D exhibited significantly greater absorption of calcium (expressed as a percentage of intake) than in their unsupplemented counterparts: With human milk feeding, rates in supplemented and unsupplemented infants were 70% and 49%, respectively; with formula feeding, rates in supplemented and unsupplemented infants, 32% and 20%. On the assumption that the absorption of calcium in the unsupplemented groups gives a rough estimate of the passive component, the passive route accounts for a significant portion of absorption in preterm infants. Likewise, the data from the vitamin D–supplemented groups suggest that vitamin D–responsive, active intestinal absorption of calcium was present at the time of study, and that this component of calcium absorption was present before the normal term date. Alternatively, intestinal maturation with regard to vitamin D responsiveness may have been accelerated by preterm delivery.

Although these observations do not establish the relative importance of passive and active mechanisms or the timing of intestinal vitamin D responsiveness with regard to calcium absorption, they do indicate that absorption of calcium is not a limiting issue for the preterm infant. With proper attention to dietary manipulation, adequate calcium balance should be attainable.

Generally, fractional calcium absorption can adapt to high calcium intake. Hardwick and colleagues[141] examined Ca^{2+} transluminal uptake across intact duodenal epithelium at three different levels of the absorptive processes: net absorption in the intact animal, transepithelial transport in everted duodenal sacs, and transapical uptake across intact duodenal epithelium in vitro. In the face of a fivefold difference in calcium intake, net calcium absorption remained relatively constant because of a decrease in fractional absorption from 70% with normal calcium intake to 20% under conditions of high calcium intake. This decrease in fractional absorption could be due to a combination of depressed true absorption and increased intestinal secretion of calcium in response to increased dietary calcium. Previous studies, however, noted that intestinal secretion of calcium is small compared with intake and is not altered by changes in dietary intake.[142] The drop in fractional absorption of calcium probably is due to down-regulation of calcium absorption. This intestinal adaptation to the effect of dietary calcium is well known and is believed to be mediated through changes in 1,25-$(OH)_2$D synthesis.[143] High intake of dietary calcium reduces the production of this metabolite, thereby reducing the rate of intestinal calcium absorption.

In contrast with the findings in experimental animals described previously (the apparent adaptation of the calcium-absorptive rate to high dietary calcium intake), studies in low-birth-weight infants have yielded extremely variable results regarding the fractional and total calcium absorbed and retained.[144-150] Early investigations in preterm infants demonstrated a linear relationship between calcium intake and absorption: As calcium intake increases, so does the intestinal absorption of calcium.[151-153] As noted previously, however, more recent balance studies that measure only net absorption (i.e., true absorption – endogenous fecal excretion) have shown great variability in calcium retention (80 to 170 mg/kg/day) even when the same preterm formula was used,[144,145,148-150] possibly as a result of significant intersubject variability in calcium bioavailability. These variations may represent true differences in the ability of low-birth-weight infants to absorb calcium, the cause of which may relate to as-yet unidentified physiologic or hormonal factors. As indicated by the findings of calcium-absorptive consistency in perimenopausal women,[154] interindividual variability may be an expression of specific absorptive setpoints for different people. Calcium absorption evidently is consistent for each person across differing calcium loads (ranging from 15 to 500 mg). Thus a woman who absorbs calcium poorly with one calcium-containing food is likely to absorb calcium poorly with other foods.

Studies using a dual-tracer, stable isotope technique also have shown variable results. Abrams and colleagues[155] reported that net calcium retention in low-birth-weight infants fed a high-calcium formula is determined primarily by the total dietary calcium absorbed: net calcium retention correlated closely with true calcium absorption ($r = .98$; $p < .001$). The net calcium retention rate for low birth weight infants weighing 1693 ± 174 g (mean ± SD) at 20 ± 10 days of age was 103 ± 38 mg/kg/day at calcium intakes of 216 ± 13 mg/kg/day, which meets the lower estimates of the in utero calcium retention rate (100 to 130 mg/kg/day) during the third trimester.[156] This value was consistent with previous studies of calcium retention determined using mass balance technique, lower than that in other reports describing calcium retention rates of 130 to 170 mg/kg/day,[144,145,149,150] and slightly higher than that in a report of an approximate 80 mg/kg/day retention with this formula.[148] Thus the use of high-calcium formulas may possibly achieve in utero rates of calcium retention in low-birth-weight infants, although some infants retain well below in utero rates and may remain at risk for inadequate bone mineralization.[155]

In preterm infants, feeding strategies can influence the retention of minerals in the first 4 weeks early postnatal period. Schanler and associates[157] have shown that early initiation of feeding (gastrointestinal priming) in preterm infants, using 20 mL/kg/day of milk from days 4 to 14, was associated with better calcium retention (104 versus 78 mg/kg/day) and phosphorus retetention (79 versus 68 mg/kg/day) at 6 weeks of postnatal age. The net retention rates in the early-fed infants approximated the intrauterine accretion rates for calcium and phosphorus. The investigators suggest that early programming is necessary for optimal mineral absorption.

Hillman and colleagues[95] measured the percentage of true absorption and calcium retention rates in low-birth-weight infants (1400 ± 210 g) fed specially formulated preterm formula with differing calcium contents. The percentage of true calcium absorption from the preterm formula studied was around 50%, the same as or slightly higher than that from standard formula (460 mg/L, 47.1%). Increasing the calcium content of formula from the level of 460 mg/L to the levels studied—780 mg/L, 940 mg/L, and 1340 mg/L—resulted in nearly identical levels of true fractional calcium absorption (range, 47.1% to 56.1%) but higher net calcium retention rates with a wide variability. Because multiple factors were different between the formulas studied (e.g., fat composition [content of medium-chain triglycerides], whey-predominant protein, calcium-phosphorus ratio), however, the effect of increased calcium content alone cannot be assessed from the findings in this study.

Significant but small amounts of endogenous calcium are lost daily through intestinal secretion. Substantial quantities of

calcium enter the gastrointestinal tract as constituents of bile and other digestive juices.[158,159] From studies in rats, it also appears that calcium can be secreted into the gastrointestinal tract by way of paracellular channels when the serum ionized Ca^{2+} concentration exceeds the intraluminal Ca^{2+} concentration.[130,159] Although the secreted calcium can participate in normal intestinal absorptive mechanisms, it appears that this portion of the intraluminal calcium is reabsorbed at a level of efficiency less than that of dietary calcium.[160]

Simultaneous administration of both intravenous and oral stable isotopic calcium tracers with subsequent monitoring of fecal and urinary tracers has permitted direct calculation of endogenous calcium secretion. In adults, secretion of calcium into the intestinal lumen is small in amount, constant, and independent of absorption.[160] In children (aged 3 to 14 years), endogenous fecal calcium excretion, measured using a stable isotope technique (^{42}Ca), averages 1.4 ± 0.4 mg/kg/day and is lower than urinary calcium excretion,[161] consistent with values in healthy adults.[4,160] In preterm infants, endogenous fecal calcium excretion is high and quite variable (4 to 150 mg/day) and may exceed even urinary excretion of calcium.[149,162,163] Thus significant amounts of calcium can be lost by way of this route, which might explain some of the variability in reported calcium balance studies, because the mass balance technique does not differentiate between unabsorbed and endogenously secreted fecal calcium. In a study of low-birth-weight infants fed a high-calcium formula, endogenous fecal calcium excretion averaged 15 ± 9 mg/kg/day (7.2% ± 4.1% of intake) and was much greater than concurrent urinary calcium excretion.[155] The reasons for these high rates of endogenous fecal calcium excretion in preterm infants are unknown but may be related to gastrointestinal immaturity.[149]

In a study by Hillman and colleagues,[95] endogenous fecal calcium excretion in low-birth-weight infants fed preterm formula with varying calcium contents ranged from 6.9 to 19.0 mg/kg/day; infants fed human milk excreted 5.1 mg/kg/day. Endogenous fecal excretion is independent of calcium intake (and the true absorption rate), and not related to gestational age or postnatal age at study. A number of carbohydrates (especially lactose) and glucose polymers enhance the intestinal absorption of calcium,[164,165] probably independent of the actions of vitamin D.[164,166] In vitro studies have established that mucosal lactose directly stimulates ileal[167,168] but not duodenal or jejunal calcium absorption.[169] Lactose, in contrast with other monosaccharides and disaccharides, is not hydrolyzed in the proximal small intestine, and large quantities of this sugar are delivered to the ileum.The observation that lactose feeding can reduce duodenal calcium-binding protein content and calcium absorption[170] may be attributed to increased calcium retention from augmented ileal Ca^{2+} absorption. Lactose, by itself, does not solubilize precipitated calcium; it does not maintain calcium in a soluble form in the presence of precipitating agents such as phosphate.[101] The lactose effect on Ca^{2+} absorption may be mediated through its action on the permeability of ileal epithelium, through a paracellular mechanism.[171] The observation that lactose also stimulates intestinal absorption of other divalent cations (e.g., Mg^{2+}, Ba^{2+}, Sr^{2+}, Ra^{2+})[101] further supports the possibility that this sugar may cause a general increase in permeability to divalent ions across the junctional assembly. In rats, osmotic forces that enhance the net intestinal absorption of water also enhance the passive absorption of calcium.[166] It appears from such data that "solvent drag" plays a significant role in the augmentation of calcium absorption, leading to the speculation that this may be the mechanism through which carbohydrates act.

Bile salts stimulate calcium absorption in the chick.[172] The mechanism for bile salt–mediated calcium absorption is not clear, but the stimulatory action is not vitamin D–dependent. Bile salts solubilize lipids for absorption through the formation of micelles.[173] The surfaces of micelles bear negative charges, which strongly bind Ca^{2+} and Na^{+}.The process of micellar solubilization may explain the observation that although the physicochemical environment of ileum favors calcium precipitation,[142] the bulk of calcium absorption occurs in this segment in vivo.[174] In addition to increasing the amount of calcium available for absorption, bile salts also may influence calcium absorption by acting directly on the intestinal epithelium. In a variety of in vivo and in vitro perfusion studies[175,176] using isolated, short-circuited rat and rabbit colons, increased permeability of gastrointestinal barriers has been observed with changes in tight junction complexes. This is reflected by increases in transepithelial conductance[177,178] and permeation of paracellular pathway markers.[178] In addition to the paracellular action, bile salts can also influence enterocytes directly: Bile salts have potent calcium ionophoric effects (mimicking the effect of 1,25-$(OH)_2$D) across a variety of artificial and plasma membranes, including the jejunal brush border membrane.[179]

As mentioned, the bulk of Ca^{2+} absorption occurs in the distal small intestine, in particular the ileum,[96] a segment that is relatively insensitive to 1,25-$(OH)_2$D, devoid of calcium-binding protein, and poorly equipped for active Ca^{2+} absorption.[98] Under in vivo postprandial conditions, the simultaneous arrival in ileum of bile salts and calcium could foster a highly efficient Ca^{2+}-absorptive state.The calcium load, solubilized by bile salts,[175] would create a calcium concentration gradient in the absorptive direction (i.e., $[Ca^{2+}]_m > [Ca^{2+}]_s$).This, in concert with the bile salt–induced increase in the permeability of the intercellular conduit, could promote rapid Ca^{2+} absorption, both economically and in substantial quantities.[97]

Fat malabsorption has the potential to impair intestinal calcium absorption. Preterm infants are not likely to have an intraluminal concentration of bile salts sufficient for the establishment of a micelle phase, which has the potential to reduce the absorption of fat.[180] The unabsorbed free fatty acids would then be available to interact with ionic calcium to form insoluble calcium soaps, which in turn would not be available for absorption. Although steatorrhea can be significantly reduced in preterm infants by administering a significant proportion of dietary fat as medium chain triglycerides (which do not require micelle formation for absorption), it is not clear that this practice significantly improves intestinal calcium absorption.[181] Furthermore, despite these general theoretical considerations, the absorption of calcium during this period is high, particularly in comparison with that in other stages of life.

Fractional intestinal absorption of calcium is enhanced by dietary restriction of calcium or P_i. Under these conditions, rats[182,183] and humans[184] demonstrate increased production of 1,25-$(OH)_2$D, with a resultant increase in active intestinal absorption of calcium.The effect of short-term moderate dietary phosphorus restriction on intestinal net calcium fluxes and external calcium balance was studied in growing rabbits that were fed a phosphorus-deficient diet (containing approximately two thirds of the estimated minimum requirement for phosphorus) for 10 consecutive days.[185] During phosphorus restriction, intestinal calcium absorption rose significantly to reach maximal values within 96 hours; sustained high rates of calcium absorption persisted for the 10 days of phosphorus restriction. When the rabbits were consuming the phosphorus-replenished control diet, calcium absorption fell to control values during the first 9 days of recovery, implying the persistence of mechanisms for calcium hyperabsorption for at least 1 week after this degree of phosphorus restriction. Despite increased intestinal calcium absorption, calcium balance during phosphorus restriction was less positive because the increase in urinary calcium excretion was even greater. The increased efficiency of calcium absorption in response to moderate dietary phosphorus restriction is

consistent with the well-established effect of calcitriol on intestinal calcium transport.[142]

Phosphorus

In comparison with calcium, much less is known about the intestinal absorption of P_i. Fewer direct studies have been performed in humans, so that much of the available information has been derived from animal studies. Phosphorus absorption is remarkably efficient, much more so than calcium absorption. At low levels of intake (less than 2 mg/kg of body weight per day), 80% to 90% of ingested phosphorus in adults is absorbed. Even with levels of intake greater than 10 mg/kg of body weight per day in the form of dairy products, cereals, eggs, and meat, absorption is approximately 70%.[89] In term and preterm infants, the fractional absorption of phosphorus is high, ranging from 86% to 97% regardless of phosphorus or calcium intake.[186] Thus the dietary P_i intake is an important determinant of the amount of phosphorus absorbed.

The absorption of P_i occurs throughout the entire small intestine. On the basis of transepithelial P_i flux measurements and uptake studies with isolated brush border membrane (e.g., of rabbits and rats), small intestinal absorption of P_i has been reported to occur mostly in the duodenum and at the beginning of the jejunum.[187-189] In in vivo segmental P_i absorption analysis in intact rats fed ^{32}P-labeled P_i liquid meal containing a nonabsorbable marker ([^{14}C]-polyethylene glycol), the duodenum, which had the highest transit and absorption rates, accounted for a third of total absorption; the terminal ileum, with a lower absorption rate but a longer transit time, also accounted for one third of the total absorption (equal to P_i absorbed in the duodenum); the remaining one third was absorbed by the intervening small intestinal segments[190] (Figure 36-5).

The movement of phosphorus across the intestinal epithelium can be resolved into two unidirectional fluxes: an absorptive, mucosa-to-serosa flux and a secretory, serosa-to-mucosa flux. Each unidirectional flux, in turn, consists of two components, a *transcellular (active, energy-dependent transport) flux* and a *paracellular (passive, diffusional) flux*. The paracellular flux is driven by transepithelial chemical and physical (electrical and hydrostatic) gradients, whereas the transcellular flux is unaffected by the transepithelial electrochemical gradients, with the movement across the brush border membrane appearing to be the rate-limiting step.[191]

An active, sodium-dependent transcellular P_i-absorptive mechanism has been demonstrated in rat jejunum by measuring net P_i absorptive flux under short-circuited conditions.[192] Sodium stimulates both unidirectional mucosa-to-serosa flux and serosa-to-mucosa flux for P_i (measured in the absence of transepithelial potential differences). Thus absorption at the brush border membrane involves the obligate transport of sodium and P_i and occurs in an electroneutral fashion.[193] The absorbed P_i then traverses the cytoplasm by means of unknown processes; at the basolateral membrane, it moves down its electrochemical gradient by means of simple diffusion[193] or through carrier-mediated facilitated transport.[194] Transcellular transport of P_i in the small intestinal segment is initiated by a sodium-dependent transport step through the apical membrane of the enterocytes. Three classes of sodium-dependent phosphate (Na^+-P_i) cotransporters have been identified: type 1 (NPT1), type 2 (NPT2), and type 3 (NPT3). Among them, type 2 Na^+-P_i cotransporter (NPT2), the major molecule in the renal proximal tubule, is responsible for most resorption of P_i in the kidney and can be strictly controlled by P_i-regulating hormones such as PTH and vitamin D.[195]

Three isoforms of NPT2—NPT2a, NPT2b, and NPT2c—have been identified. NPT2a (type IIa) and NPT2c (type IIc) are expressed mainly in the kidney. NPT2b (type IIb) are expressed specifically in the small intestine and can be regulated by vitamin D.[196,197] Those transporters are found solely in the apical brush border membrane of the renal proximal tubular cells or intestinal epithelial cells.[198] To date, the Na^+-P_i cotransporter NaPi-IIb (SLC34A2)[199] represents the only known protein that mediates Na^+-dependent transport of P_i through the apical membrane of the enterocytes.[200] Intestinal phosphorus absorption is closely linked to expression of the Na^+-P_i cotransporter NaPi-IIb.[200-204]

In a recent study by Radanovic and colleagues,[204] expression of NaPi-IIb and its correlation with Na^+-P_i cotransport activity was assessed in the small intestine of mouse. The highest amount of NaPi-IIb protein was detected in the ileum, whereas in the jejunum, the content of the NaPi-IIb protein was minimal. In agreement, the highest rate of NaPi-IIb cotransport was observed in brush border membrane vesicle (BBMV) isolated from the ileum, whereas in BBMV isolated from the jejunum, sodium-dependent transport of P_i was not measurable.

The regulation of intestinal P_i absorption appears to be centered on the cotransport of sodium and P_i at the brush border. It is at this site that 1,25-$(OH)_2$D acts to enhance the intestinal absorption of P_i.[205] 1,25-$(OH)_2$D increases mucosal border P_i entry, and this stimulating action is mediated through the sodium-dependent component of the total measured influx.[192]

Alterations in dietary P_i intake significantly affect intestinal absorption of Pi. With restriction of dietary P_i, fractional intestinal

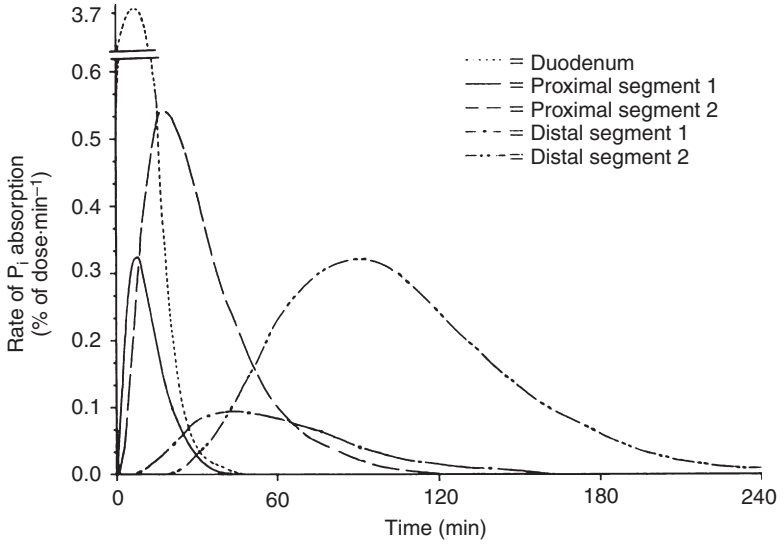


Figure 36-5 Segmental absorption rate profiles for inorganic phosphorus (P_i). Rates for each segment are shown over time. The rates for absorption of P_i from duodenum through distal segment 2 as a function of time were constructed from the compartmental model: The gastrointestinal tract was divided into duodenum and four equal-length segments of small intestine (proximal segments 1 and 2 and distal segments 1 and 2). *(From Kayne LH, D'Argenio DZ, Meyer JH, et al: Analysis of segmental phosphate absorption in intact rats: A compartmental analysis approach,* J Clin Invest *91:915, 1993.)*

absorption of P_i increases.[182-184,206] In growing rabbits,[185] moderate short-term dietary phosphorus restriction induces both intestinal and renal adaptations to conserve calcium and P: Net intestinal phosphorus secretion is observed on the first day of phosphorus restriction, but this changes to increased absorption beginning on the second day, indicating intestinal adaptation. With dietary phosphorus replenishment, adaptations persist to restore the positive mineral balances that were lost because of dietary phosphorus restriction during growth.[185] The mechanisms underlying the increased efficiency (greater fractional absorption) and capacity (hyperabsorption during recovery) of intestinal phosphorus absorption in rabbits in response to moderate dietary phosphorus restriction are not defined, but 1,25-$(OH)_2$D may play a role by increasing the absorptive capacity of the Na^+-P_i cotransporter,[207,208] or by increasing the affinity of this transporter for P_i as well.[208] Selective restriction of dietary phosphorus decreases plasma phosphorus concentration,[209] minimizes cumulative phosphorus balance, and increases plasma 1,25-$(OH)_2$D concentrations in rabbits,[209] humans,[210] and rats.[211] Low-phosphorus diet–induced increases in 1,25-$(OH)_2$D production, however, cannot account for the entire increase in phosphorus absorption in experimental animals,[212] supporting the contention that the regulation of phosphorus absorption is not always predictably a function of 1,25-$(OH)_2$D activities.

Low-P_i diet provokes an increase of the NaPi-IIb mRNA abundance along the entire small intestine. Adaptation to a low-P_i diet results in upregulation of NaPi-IIb and NaPi-IIb cotransport both in the jejunum and the ileum of mouse. From these data, it is suggested that in mouse small intestine, phosphate is absorbed transcellularly by an Na^+-dependent pathway in the ileum, whereas in the duodenum and jejunum, this pathway is of minimal importance. Along the entire mouse small intestine, low-P_i diet affects transcription or the stability of NaPi-IIb mRNA.[204] These data are in agreement with P_i flux measurements, shown in rat small intestine; even when the different transit times are taken into consideration, P_i is absorbed to an equal extent (approximately one third of ingested P_i) in the ileum and in the duodenum.[189,190] However, rat small intestinal localization of the NaPi-IIb cotransporter remains to be determined.

Vitamin D–deficient or thyroparathyroidectomized rats placed on a P_i-restricted diet demonstrated a 50% increase in intestinal P_i absorption as measured using isolated in situ intestinal loops. The presence of active phosphorus absorption in rats severely depleted of vitamin D raises the possibility of non–vitamin D–dependent factors in the regulation of active phosphorus absorption.[213] Bile salts, lactose, and prolactin are examples of agents that can stimulate phosphorus absorption through vitamin D–*independent* mechanisms.[135] An interesting example of vitamin D–independent regulation of phosphorus absorption is the ability of local (perienterocyte) factors to modify or reverse the effect of 1,25-$(OH)_2$D. Because there is often marked fluctuation in luminal concentrations of solutes such as phosphorus, some local regulation of phosphorus absorption may be needed to avoid phosphorus overloading. In support of a locally mediated vitamin D–independent mechanism, Lee[213] has demonstrated that active jejunal transport of phosphorus in vitamin D–deficient rats was not different between rats eating a phosphorus-replete diet and rats eating a phosphorus-deficient diet.

Hydrogen ion concentration also has been observed to influence the activity of the Na^+-P_i cotransport system. Under acidic conditions (media pH ranging from 5.8 to 6.9), rat small intestine BBMV demonstrate high rates of P_i uptake.[214] In rat jejunum, reduction in extracellular pH from 7.4 to 6.0 is associated with a 150% increase in P_i influx, an observation consistent with the reported reciprocal relation between intestinal BBMV P_i uptake and extravesical pH. Because the vesicles are oriented with the intraluminal surface of the BBM facing the bathing media, these results indicate that intestinal absorption is enhanced when the intraluminal environment is somewhat acidic. Paradoxically, when extracellular pH was increased from 7.4 to 8.5, there is an increase (50%) in mucosal P_i influx in intact epithelium studies.[192] In metabolic acidosis (decreased intracellular pH), markedly decreased P_i uptake has been observed in rats as measured by in situ perfused jejunal loops and jejunal BBMV.[214] The observed decrease in P_i transport may represent a decrease in the activity of the Na^+-P_i cotransport system. Treatment of acidotic rats with 1,25-$(OH)_2$D fails to improve the defect in intestinal P_i absorption. It is unclear how changes in pH specifically alter P_i transport. Alterations in pH change the chemical equilibrium between the monovalent and divalent forms of P_i. It is possible that if one of these forms of P_i is transported in preference to the other, changes in pH will shift the chemical equilibrium to either favor or hinder transport. The effective pH of 5.4 at the transporting surface dictates that all P_i in the immediate ambience be in the monobasic form. Furthermore, the properties of the Na^+-P_i cotransport system may be pH-dependent; hence changes in pH may alter the efficiency of this system to transport P_i. The acidic microenvironment, however, does not explain the observation[192] that P_i influx also is increased, though to a much lesser extent, when pH is raised from 7.4 to 8.5. One possibility is that alkaline pH may affect phosphorus permeation through the tight junction. This is a reasonable postulate because electrical conductance is increased at high pH. The mechanisms that mediate changes in P_i influx with changes in either extracellular Na^{2+} concentration or pH remain to be elucidated.

Stauber and colleagues[215] recently demonstrated that metabolic acidosis leads to an increase in intestinal sodium-dependent P_i uptake as a result of to an increased abundance of NaPi-IIb protein in the BBM of the small intestine. The stimulation of intestinal P_i absorption during metabolic acidosis may contribute to the buffering of acid equivalents by providing phosphorus and also may help to prevent excessive liberation of phosphorus from bone.[215]

In preterm and full-term neonates, numerous studies have demonstrated that P_i is well absorbed from the gastrointestinal tract regardless of the type of feeding given, and it is generally independent of the intake of vitamin D.[216,217] Expressed as a percentage of total P_i intake, intestinal absorption ranges between near 70% to greater than 90%. In general, the percentage of P_i absorbed increases as P_i intake decreases, but the absolute absorption of P_i (measured in mg/kg/day) increases proportionally with increasing intake. In infants fed soy-based formulas, the intestinal absorption of P_i is lower than that of a comparable group of infants fed a similar P_i intake from cow milk based formula.[218] Measurements of bone mineral content have also been found to be lower in soy-fed infants compared with cow milk milk-based formula-fed infants, consistent with reduced mineral utilization with soy formulas.[219,220] It is presumed that phytic acid, which is present in soy formulas, complexes with dietary P_i, thereby limiting its bioavailability for absorption. By increasing the calcium and P_i content of soy formulas, however, this problem evidently may be overcome.[219]

Magnesium

Intestinal magnesium absorption is the normal source through which the body repletes its magnesium store or meets the additional demand for magnesium imposed by anabolic states such as growth and pregnancy.

Magnesium absorption, as with calcium absorption, varies with age. In adults, intestinal absorption of magnesium ranges between 34% and 62% of total intake.[89,221] Term and preterm infants have greater intestinal magnesium absorption, in the range of 60% to 86%.[28,222] Magnesium absorption does not appear to vary with gestational age in low-birth-weight infants between 29 and 34 weeks of gestation. Magnesium absorption

and retention increase with increasing postnatal age in very-low-birth-weight infants with low calcium and phosphorus intake or high magnesium intake.[223]

Absorption of magnesium occurs in all segments of the intestine. The predominant site of magnesium absorption, based on transit time and studies using isolated segments of the intestine, is the distal small intestine, in particular the ileum.[224] Because of its relatively greater length and mass, the distal small intestine is likely to be the principal location in which the bulk of magnesium is absorbed. Indeed, the removal of 50% of the distal small intestine results in a 40% drop in magnesium absorption in rats.[225] The colon also is a dominant site for magnesium absorption, as indicated by studies in selected segments of the intestine,[226,227] but not when absorption is measured in the entire undisturbed intestinal tract.[228]

Where and when magnesium absorption normally occurs can be ascertained only in an intestine in which the natural physiologic conditions and regulatory mechanisms are not altered and the transit of the meal is not impeded. Kayne and Lee[229] have demonstrated that significant magnesium absorption occurs in the distal portions of the intestine, using the technique of compartmental modeling to analyze and integrate segmental absorptive events of mineral from a spontaneously propelled liquid meal along the intact intestinal tract: the estimated rate constants for intersegmental transit and segmental absorption reflect in vivo events. Based on the intestinal transit data of a liquid meal labeled by a nonabsorbable marker, ^{14}C-polyethylene glycol, it is apparent that the proximal small intestine has a short exposure time to the rapidly advancing isotope bolus and is therefore responsible only for the modest magnesium absorption observed during the first 2 hours. Thereafter, the food bolus moves more slowly through the ileum and colon, where the bulk of magnesium is absorbed at a steady, higher, and more sustained rate. Thus both transit time through the gastrointestinal tract and availability of magnesium for absorption in various segments may profoundly influence the amount of magnesium absorbed along the intestinal tract. This approach allows the analysis of the mechanism and the regulation of mineral absorption under more authentic in vivo conditions[229] (Figure 34-6).

Intestinal magnesium absorption occurs by two different pathways: a saturable active transcellular transport system and a nonsaturable passive paracellular transport sytem.[230] Saturation kinetics of the transcellular transport system are explained by the limited capacity of active transport. Intestinal luminal Mg^{2+} concentration drives passive diffusional absorption along the paracellular pathway. At low intraluminal concentrations, magnesium is absorbed primarily through the active transcellular route and, with rising concentrations, through the paracellular pathway, yielding a curvilinear function for total absorption. Saturable magnesium absorption is small compared with the total magnesium-absorptive flux, indicating a modest cell-regulated Mg^{2+} flux.[25]

Based on studies in both experimental animals and humans, it appears that passive diffusion through the paracellular pathway accounts for the major portion of magnesium absorbed. Passive diffusion is driven by the electrochemical gradient across the epithelium. Absorption by this process is expected to vary with the luminal Mg^{2+} concentration and not exhibit saturation kinetics. In young, growing rats, luminal Mg^{2+} uptake in duodenum is a linear, concentration-dependent process, not a saturable magnesium-absorptive process, and exhibits no age-related changes.[141] However, it is possible that depletion of luminal Mg^{2+} may loosen the

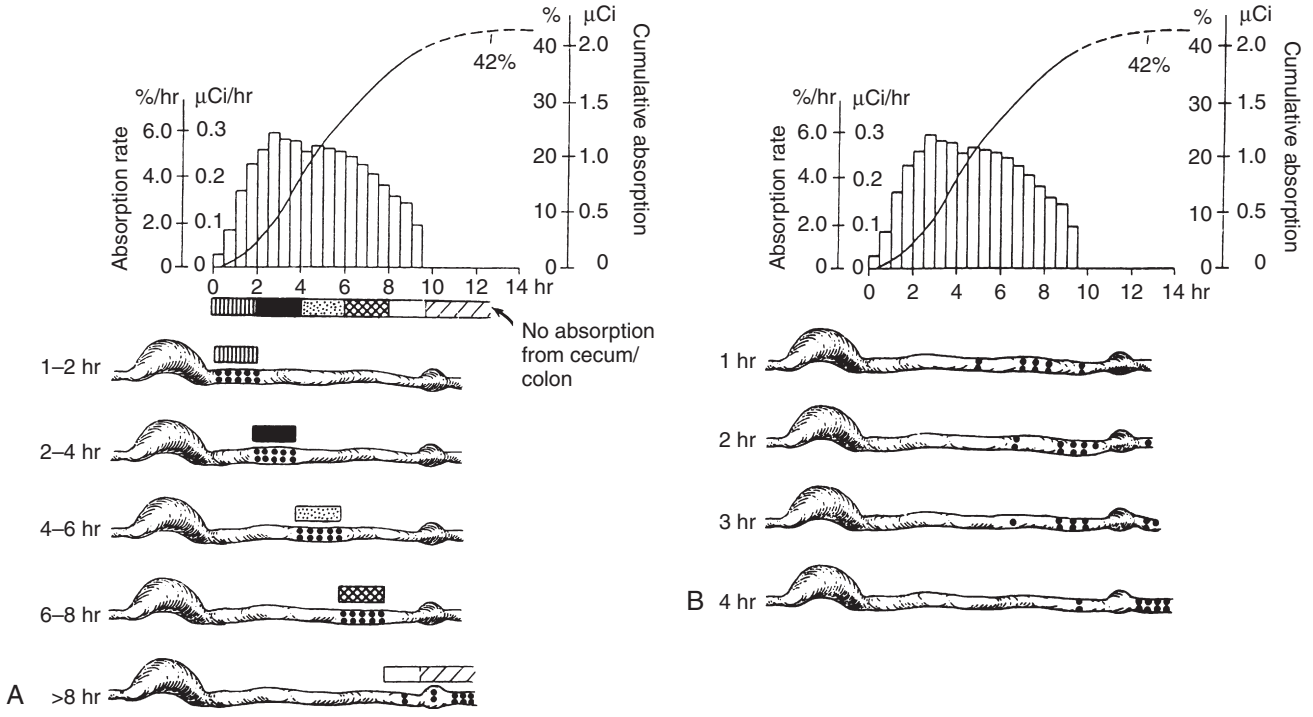


Figure 36-6 **A,** *Top*: *Solid curve* in the graph represents the cumulative absorption of magnesium after administration of 5 µCi of ^{28}Mg. *Columns* represent rates of absorption calculated from the plasma radioactivity. *Bottom*: Schematic depiction of the location and distribution of the bolus *(dark dots)* in the intestinal tract over time, based on conclusions of the study investigators. Marked boxes represent the sites of absorption at different time intervals: 1 to 2 hours *(vertically striped box),* 2 to 4 hours *(black box),* 4 to 6 hours *(dotted box),* 6 to 8 hours *(cross-hatched box),* longer than 8 hours *(open/diagonally striped box).* **B,** The *lower diagram* depicts the movement of ^{14}C-polyethylene glycol over time observed in another study. *(***A,** *From Graham L, Caesar JJ, Burgen AS: Gastrointestinal absorption and excretion of ^{28}Mg in man,* Metabolism *9:646, 1960;* **B,** *from Kayne LH, Lee DB: Intestinal magnesium absorption,* Miner Electrolyte Metab *19:210, 1993.)*

tight junctions, resulting in transepithelial diffusion. Furthermore, a high luminal Mg^{2+} concentration may reduce transepithelial permeability, the latter accounting for the reduction in fractional magnesium absorption observed with a high-magnesium diet.[141]

Net magnesium absorption in adults is linearly related to dietary magnesium over a normal range of intakes (130 to 490 mg/day),[231] consistent with a diet-dependent passive process for magnesium absorption. Fine and colleagues[230] measured magnesium absorption in adults over a wide range of intakes (with a standard meal supplemented with 0 to 80 mEq of magnesium acetate). These investigators observed that net magnesium absorption rose with each increment in intake but that fractional magnesium absorption fell progressively (from 65% at the lowest to 11% at the highest intake), so that the relationship between net magnesium absorption and intake was curvilinear. This observation is consistent with a magnesium absorption process that simultaneously uses (1) a mechanism that reaches an absorptive maximum and (2) a mechanism that endlessly absorbs a defined fraction (7%) of ingested magnesium, indicating that magnesium absorption is mediated by both a saturable carrier and passive diffusion[230] (Figure 36-7).

A saturable component of intestinal magnesium absorption has been consistently reported in animal studies.[224,227] Ross[224] reported curvilinear uptake of Mg^{2+} in the small intestine of rats when the Mg^{2+} concentration was varied from 0.1 to 12 mmol/L. Meneely and associates[227] found that Mg^{2+} absorption was saturable in the colon of adolescent rats but not in the jejunum or ileum, possibly owing to down-regulation of active transport of Mg^{2+} in the colon.[224] Active transport has seemed likely because Mg^{2+} can be absorbed against an electrochemical gradient in the intestine. Studies using the Ussing chamber have documented that Mg^{2+} is actively transported in the descending colon. Karbach and Feldmeier,[232] measuring unidirectional fluxes in the absence of transepithelial electrochemical gradients and under voltage-clamp conditions, have reported active mucosa-to-serosa Mg^{2+} transport in the ileum and in both the proximal and distal colon of the rat. Because no definite evidence supports either an uphill or an energy-dependent Mg^{2+} transport process, however, other investigators have proposed that the observed saturation characteristics are mediated by facilitated diffusion. Active transport of Mg^{2+} has been conclusively demonstrated in the colon, and indirect evidence also points to the existence of an active cellular mediated component of Mg^{2+} transport in the small intestine. This component is probably saturated under conditions of usual dietary magnesium intake and therefore may be critical only to total Mg^{2+} absorption in the intestine under conditions of rapid growth or extremely low dietary magnesium intake.

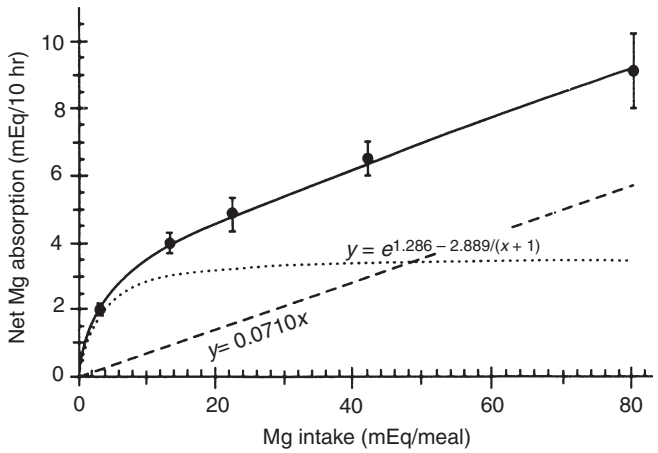


Figure 36-7 Net magnesium (Mg) absorption versus intake as derived by nonlinear regression analysis. The equation for net absorption, $y = e^{1.286 - 2.889/(x+1)} + 0.0710x$, represents a hyperbolic function plus a linear function. These separate components and their respective equations are shown. *(From Fine KD, Santa Ana CA, Porter JL, et al: Intestinal absorption of magnesium from food and supplements,* J Clin Invest *88:396, 1991.)*

As with active Ca^{2+} absorption in the small intestine through epithelial Ca^{2+} channels TRPV5 and TRPV6,[105,106,233,234] transcellular Mg^{2+} transport is a three-step process consisting of (1) entry across the apical membrane of the enterocyte, driven by a favorable transmembrane potential, through the apical epithelial Mg^{2+} channel TRPM6 (*t*ransient *r*eceptor *p*otential cation channel, subfamily *m*elastatin, member *6*), (2) cytosolic diffusion of Mg^{2+}, and (3) active extrusion across the basolateral membrane,[235] with most physiologic studies favoring a sodium-dependent exchange mechanism at this membrane.[236] Mg^{2+} entry seems to be the rate-limiting step and the point of regulation.

The transcellular transport of Mg^{2+}, comprising luminal uptake and basolateral extrusion, requires membrane proteins that increase the low permeability of the membranes and facilitate the movement of Mg^{2+} through these lipid bilayers. The molecular mechanisms mediating transcellular transport are still not completely understood. In recent years, significant progress has been made toward the isolation of genes (and their products) involved directly in transporting Mg^{2+} across biologic membranes in mammalian cells. Identification of the gene defect in patients with hypomagnesemia with secondary hypocalcemia, a disorder characterized by a combined defect of intestinal and renal Mg^{2+} transport, resulted in the discovery of TRPM6 as a crucial component of epithelial Mg^{2+} transport.[237,238] TRPM6, the gatekeeper in transepithelial Mg^{2+} transport, is a member of the TRP gene family of cation channels, and represents *the first molecularly identified protein involved directly in active Mg^{2+} absorption*.[238,239] From the recent electrophysiologic characterization of TRPM6, it is suggested that TRPM6 is an Mg^{2+}-permeable channel localized to the brush border membrane of the Mg^{2+}-absorptive cells in the duodenum, the site of transepithelial Mg^{2+} transport, and is in turn regulated by changes in intracellular Mg^{2+} or Mg^{2+}-ATP level.[235,240] TRPM6 is predominantly expressed in intestinal epithelium and kidney tubule, suggesting a key role of TRPM6 in Mg^{2+} absorption and whole-body magnesium homeostasis. Recently, Groenestege and associates[241] have demonstrated that TRPM6 is expressed predominantly in kidney, lung, cecum, and colon. As indicated by the predominant expression of TRPM6 in cecum and colon, together with the low expression level in duodenum and jejunum, active Mg^{2+} absorption may take place exclusively in the distal part of the intestine. The highly abundant expression of TRPM6 in colon is further supported by the fact that colectomy in rat results in a syndrome of magnesium deficiency, with decreased urinary magnesium excretion, normal serum magnesium concentrations, and decreased bone magnesium content.[242]

Several hormones, including PTH, calcitonin, vitamin D, and sex steroids have been reported to influence the Mg^{2+} balance.[67,243,244] It is suggested that these hormones are only indirect regulators of magnesium homeostasis, because Mg^{2+} lacks specific endocrine control comparable with that for Ca^{2+}.[243] Recently, Groenestege and associates[241] demonstrated that 17β-estradiol (17β-E_2), but not 1,25-$(OH)_2$D or PTH, specifically up-regulates TRPM6 mRNA expression in kidney, pointing to the *first magnesiotropic hormone* in the maintenance of the Mg^{2+} balance. In ovariectomized rats, the renal TRPM6 mRNA level was significantly reduced and subsequently normalized by 17β-E_2 supplementation. It seems that 17β-E_2 increases magnesium reabsorption through an enhanced renal TRPM6 expression. Thus estrogen is involved in magnesium homeostasis through the regulation of the magnesium channel TRPM6 in kidney.

TRPM6 is regulated by dietary magnesium content. Magnesium depletion increased TRPM6 mRNA expression in kidney (but not the colon), whereas a magnesium-enriched diet increased TRPM6 mRNA levels in colon, supporting the gatekeeper function of TRPM6 in transepithelial Mg^{2+} transport. Dietary magnesium restriction in mice resulted in hypomagnesemia and renal Mg^{2+} and Ca^{2+} conservation, whereas a magnesium-enriched diet led to increased urinary Mg^{2+} and Ca^{2+} excretion. Thus it appears that mice can increase their transcellular Mg^{2+} absorption capacity in intestine when fed a magnesium-enriched diet. Physiologically, an excess of Mg^{2+} absorption as a result of high dietary magnesium intake together with TRPM6 up-regulation in colon can be totally corrected by the kidney, by increasing urinary Mg^{2+} and Ca^{2+} excretion. From the unaltered expression levels of TRPM6 mRNA in colon during magnesium restriction, it seems that the Mg^{2+}-absorptive capacity is sufficient to obtain maximal transcellular Mg^{2+} transport. [241]

Mg^{2+} efflux occurs along Na^+-dependent and Na^+-independent pathways.[236,245] Although current data suggest no significant chemical gradient for magnesium across the cell membrane, the significant uphill electrical gradient for the exit of a cation such as Mg^{2+} suggests the participation of an energy-dependent mechanism.[230]

Absolute intestinal absorption of magnesium appears to depend on the magnesium content of the diet and on body magnesium status.[230] Relatively higher Mg^{2+} absorption occurs in patients who have previously ingested low-magnesium diets or in clinical situations characterized by overall negative Mg^{2+} balance, for instance, in pregnancy, lactation, or excessive renal excretion. The factors controlling intestinal magnesium absorption are not well delineated.[232,246] A significant amount of magnesium absorption, however, is vitamin D–independent: It persists under conditions of vitamin D deficiency. Absorption also may be negatively affected by intraluminal complexing agents, such as phytates found in cereal grain.

Repletion of vitamin D in vitamin D–deficient animals and humans is associated with increments in magnesium absorption.[87] In vitamin D–replete animals and humans, pharmacologic doses of vitamin D[87,88] appear to increase magnesium absorption, whereas spontaneous fluctuations in circulating levels of 1,25-$(OH)_2$D have little effect on Mg^{2+} transport. The importance of vitamin D–stimulated magnesium absorption on overall magnesium homeostasis remains uncertain,[87,88] particularly in light of the dramatic increases in urinary excretion of Mg^{2+} that have been associated with vitamin D administration.[246]

For many years, it was assumed that calcium and magnesium were handled similarly at different steps in the intestinal absorptive process. Data from Hardwick and colleagues,[141] however, cast doubt on that assumption. In their studies using young, growing (postweaning) rats, duodenal calcium absorption was mediated both actively and passively (with the former predominating), whereas magnesium absorption was predominantly a concentration-dependent mechanism. It appears, therefore, that calcium and magnesium are handled differently at various steps of the absorptive process.

In rats, several studies have demonstrated that net absorption of magnesium in vivo is depressed by a high calcium intake.[247,248] Karbach and Ewe,[88] however, reported that increasing the calcium concentration in intestinal perfusate from 1.25 to 10 mM/L in vivo had no effect on magnesium absorption in the colon of rats. This study does not rule out that calcium at lower concentrations may significantly alter magnesium absorption.

In humans, several studies have shown that increasing calcium in the diet significantly depresses magnesium absorption.[249,250] In very-low-birth-weight infants,[223] high dietary calcium intakes that allow retentions equivalent to the in utero accretion rates do limit magnesium retention, and a significant magnesium deficiency can occur in comparison with intrauterine accretion.[156] Calcium and phosphorus supplementation reduce magnesium absorption and retention; however, with magnesium intakes approaching 20 mg/kg/day, appropriate retention is achieved in very-low-birth-weight infants. [223]

The effect of increased magnesium intake on calcium absorption and retention is more controversial. Karbach and Ewe[88] demonstrated absence of any limitation in calcium absorption when increasing concentrations of magnesium (1.25 to 10 mmol/L) were used to perfuse the large intestine of the rat. When calcium and magnesium interactions were examined in the descending colon of the rat using the Ussing system, however, magnesium exerted a significant negative effect on net calcium absorption. Specifically, increasing the magnesium concentration to 1.25 mmol/L decreased the mucosa-to-serosa flux of Ca^{2+} by 50% and abolished net calcium absorption. The effect was due to a depression of the voltage-dependent component—that is, the paracellular pathway.

Increasing magnesium in the diet has been reported to increase fecal calcium significantly in humans.[251] Fine and colleagues,[230] however, demonstrated that an increased magnesium intake in adults had no effect on calcium absorption, indicating that even when present in great excess, magnesium does not inhibit active or passive calcium absorption. Urine calcium excretion increases with increased magnesium intake and absorption (Figure 36-8).

Much of the evidence for intestinal interactions between calcium and magnesium has come from studies in which transport of one nutrient was studied in the absence of dietary intake of the other nutrient.[247,252] Moreover, the results of previous studies do not exclude an adaptive effect of calcium on magnesium transport in the intestine. The mechanisms by which calcium and magnesium interact, however, have not been well defined. Several possibilities have been proposed, including competition for a common carrier system, a calcium-induced change in membrane permeability to magnesium, and modulation of a specific magnesium carrier by calcium.[253] Intestinal membrane permeability is sensitive to alterations in luminal Ca^{2+} and Mg^{2+} concentrations.[254] The physiologic changes in these luminal

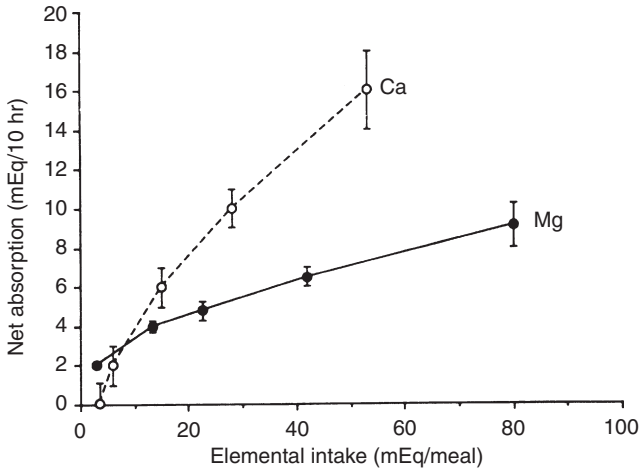


Figure 36-8 Comparison of net magnesium (Mg) absorption with net calcium (Ca) absorption measured from similar meals supplemented with various amounts of calcium carbonate or magnesium acetate. (Mean ± SE values are shown.) At low levels of intake, more magnesium than calcium is absorbed. At intakes greater than 8 mEq, increasingly more calcium than magnesium is absorbed. *(From Fine KD, Santa Ana CA, Porter JL: Intestinal absorption of magnesium from food and supplements,* J Clin Invest *88:396, 1991.)*

concentrations also may influence diffusion through the paracellular pathway. Further investigation is required to test this hypothesis and to determine its importance in short- and long-term regulation of calcium and magnesium transport.

Several other factors can affect intestinal magnesium absorption. Glucose polymers have been observed to enhance jejunal absorption of magnesium.[165] The absorption of magnesium has been found to be directly related to the net absorption of water, consistent with carbohydrate-enhanced magnesium absorption by means of solvent drag. Dietary lactose, a poorly digestible disaccharide, significantly enhances the apparent absorption of magnesium in rats.[261] Theoretically, this effect of lactose may involve a decrease in pH in the intestinal lumen, caused by bacterial fermentation,[255] thereby altering the solubility of magnesium in the digesta. The observed negative relationship between pH in the ileal lumen and intestinal magnesium absorption in the rat supports the notion that the decrease in ileal pH (from 7.5 to 7.2) after lactose feeding is the major mechanism responsible for the improved magnesium absorption.[255] In a study investigating the effect of lactose on magnesium absorption in term infants,[256] magnesium retention was similar between infants fed a formula containing lactose compared with one that was lactose free.

Starch, which is poorly digestible, also influences magnesium absorption by a mechanism similar to that of lactose.[257] In rats fed native starch, magnesium absorption is increased. Furthermore, the ileal pH is significantly lowered, and magnesium concentrations in liquid cecal contents are raised.[257] Whether changing the carbohydrate composition of formula (especially the relatively poorly digestible carbohydrates, e.g., starch) can affect magnesium absorption has not been studied in human infants.

Magnesium absorption may be improved with use of easily absorbed fat in the form of medium chain triglyceride.[258] An increase in dietary protein concentration increases apparent magnesium absorption in rats[259,260] and in humans.[261]

Increased phosphorus intake may decrease magnesium absorption owing to the formation of less soluble phosphorus-magnesium complexes.[262] In full-term breast-fed infants, however, the addition of phosphorus supplements to human milk improved magnesium absorption and retention.[263] In very-low-birth-weight infants, increased dietary phosphorus resulted in a higher fecal loss of magnesium and lower net absorption and retention of magnesium.[264]

Finally, significant amounts of magnesium are secreted into the intestinal tract. Bile, pancreatic juice, and gastric juice all contain large amounts of magnesium. Almost all of the secreted Mg^{2+} is reabsorbed, so that under normal conditions, this fraction accounts for only a small proportion of the total fecal content.[29] In rats, a significant portion of magnesium in feces is of endogenous origin and is changed by dietary protein intake—high levels of soybean protein lead to greater excretion of endogenous magnesium.[28,265,266] By contrast, endogenous Mg^{2+} excretion in feces is significantly depressed in rats fed a threefold higher casein diet, whereas urinary Mg^{2+} excretion is increased.[267] It is uncertain how protein intake affects the excretion of endogenous magnesium in the feces.

Endogenous magnesium losses in adult humans[268] range from 6 to 36 mg/day. In term newborn infants, endogenous magnesium secretion, measured using radioisotopes, ranged from 6.8 to 16 mg/day, similar to the values noted in adults but much greater if expressed per kg of body weight or as percent of total magnesium intake.[28]

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37 Zinc in the Fetus and Neonate

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In a remarkably short time, the general perception of zinc has progressed from that of a rather obscure essential trace mineral of doubtful significance for human health to that of a micronutrient of exceptional biologic and public health importance. This is most evident in relation to early development, both prenatal and postnatal. Space allotted to an overview of trace minerals other than iron and zinc in previous editions of this book is therefore devoted entirely to zinc in this edition, and the reader is referred to other texts for information on other trace minerals.[1, 2] The principal focus of this chapter is the complex biology of zinc that underlies its clinical importance.

BIOCHEMISTRY AND BIOLOGY OF ZINC

The abundance of zinc in the human body (approximately 2 g in the adult)[3] is second only to that of iron among those trace elements for which a nutritional requirement has been established in humans. In contrast with iron and iodine, however, zinc is distributed relatively evenly throughout the body, being found as a component of thousands of zinc metalloproteins or zinc-binding proteins, and also of nucleic acids, so it is less readily detectable. The identification and application of zinc fluorophore sensors are required to assist in the selective and efficient detection of trace Zn^{2+}.[4]

With an atomic weight of 65.39, zinc is adjacent to several first-transition elements of biologic importance, yet its biochemical properties are very different from those of other elements of similar atomic weight. One of the properties of the zinc atom that has proved to be of outstanding value in biology is its ability to participate in strong but readily exchangeable ligand binding.[5] Coupled with this feature is the notable flexibility of the coordination geometry of this metal. These two properties are responsible for the unique ability of zinc to interact with a wide range of organic ligands and thus to be incorporated in myriad biologic systems. The principal amino acids supplying ligands that bind with zinc are histidine, glutamic acid, aspartic acid, and cysteine.[6] In catalytic sites, zinc forms complexes with water and with any three nitrogen-oxygen-sulfur donors, especially histidine. Structural zinc sites have four protein ligands, with cysteine the preferred ligand. Zinc affects tertiary and quaternary protein structure, and the resulting scaffolding of these zinc coordination spheres is important to the function and reactivity of the metal atom.

Zinc can participate in oxidation-reduction (redox) reactions in special circumstances,[7] specifically through the combined biochemistry of zinc, metallothionein, and glutathione. In contrast with iron and copper, however, the zinc atom has no oxidant properties, and it exists virtually entirely in the divalent state. This chemical attribute has simplified the safe transport of Zn^{2+}, both extracellularly and intracellularly, and its incorporation into biologic systems.

As understanding of its biology evolves, the exceptional importance of this micromineral becomes increasingly impressive.

The biologic role of zinc is now recognized in protein structure and function, including those for enzymes, transcription factors, hormonal receptor sites, and biologic membranes. Zinc has numerous central roles in DNA and RNA metabolism,[8] and it is involved in signal transduction, gene expression, and apoptosis.

First to be appreciated were the catalytic properties of zinc, a function of its biochemistry outlined earlier. The number of zinc metalloenzymes with known three-dimensional structures exceeds 200.[9] Although zinc also has a structural role in numerous enzymes, its primary importance is as an active component of the catalytic site. Zinc metalloenzymes have been identified in each of the six major enzyme classifications. Several of the key enzymes involved in nucleic acid metabolism, cellular proliferation differentiation, and growth are either zinc metalloenzymes or zinc-dependent enzymes.[10]

A major advance in the understanding of the biology of zinc was the identification of proteins that contain a *zinc finger motif*.[11] Structurally, the zinc finger motif is a recurring pattern of amino acids with conserved residues of cysteine and histidine at the base to which zinc binds in a tetrahedral arrangement. Subsequently, hundreds of zinc finger motifs were identified. More than 1000 genes in the human genome encode members of three protein families with zinc finger domains alone: C2H2 zinc fingers, RING fingers, and LIM domains.[9] The number of genes containing zinc finger domains exceeds 3% of all identified human genes. Although all zinc fingers are quite similar, they vary in their precise conformation. Steroid hormone receptors, for example, have several such domains that are involved in the structure itself, and one each for binding to DNA and RNA polymerases. Among the identified zinc transcription factors are several involved in early intrauterine development.[12]

Many transcription factor proteins have the zinc finger motif, thus giving this metal a broad role in gene expression. This role perhaps has been best characterized by the self-regulation of this metal of its own metabolism,[13] and that of metallothionein, with which zinc is so closely associated. Gene expression in this context is considered later, in the discussion of zinc metabolism.

Zinc is an important regulator of apoptosis.[14] This micronutrient has cytoprotective functions that suppress major pathways leading to apoptosis and also directly influences apoptotic regulators, especially the caspase family of enzymes. In airway epithelial cells, zinc is colocalized with the precursor forms of caspase 3, mitochondria, and microtubules. A decline in intracellular zinc may trigger pathways leading to caspase activation. An early and direct effect of zinc deficiency not only on proliferation and differentiation but also in inducing apoptosis, has been demonstrated in growth plate chondrocytes of the chick.[15] The development of the human epiphysis also is sensitive to zinc deprivation.[8]

There is evidence of a direct signaling function of zinc on all levels of signal transduction.[16] Zinc can modulate cellular signal recognition, second-messenger metabolism, and protein kinase and protein phosphatase activities. In the brain, zinc is sequestered in the presynaptic vesicles of *zinc-containing neurons,* from which zinc is released into the cleft and is then recycled into the presynaptic terminal.[17] Synaptically released zinc functions as a conventional synaptic neurotransmitter or neuromodulator, but also, analogous to calcium, it functions as a transmembrane neural signal. The best-established physiologic role of this vesicular zinc is the tonic modulation of brain excitability. Vesicular-rich regions such as the hippocampus are responsive to dietary zinc deprivation, which causes brain dysfunction, including learning impairment and susceptibility to epileptic seizures. Normal neuronal function is dependent on normal zinc homeostasis in the brain.[18]

The biology of zinc is closely linked with that of metallothionein, a unique small intracellular protein of less than 7 kDa that is strongly conserved across species. Of the 61 to 68 amino acids, more than one third are cysteine, and it has a unique metal-binding capacity (copper > cadmium > zinc). It consists of two subunits: an α domain, which under typical physiologic circumstances will bind four zinc atoms, and a more reactive β domain, binding three zinc atoms in tetrahedral formation. Of the four known metallothionein isoforms, MT-1 and MT-2 are most abundant, and MT-3 occurs in the brain. Metallothionein occurs in all tissues, including those of the conceptus, and is especially abundant in liver and also in pancreas, intestine, and kidney. Zinc is the major physiologic inducer of metallothionein. Zinc binds directly and reversibly to the zinc finger domains of the metal-response element–binding transcription factor-1 (MTF-1), which functions as a cellular zinc sensor. As a result of this binding, MTF-1 assumes a DNA-binding conformation and translocates to the nucleus, where it binds to metal-response elements of the metallothionein gene thereby initiating transcription.[13] Cytokines, especially the interleukins IL-1 and IL-6 and tumor necrosis factor-α, and stress hormones (glucocorticoids and catecholamines) also are powerful inducers of metallothionein.[19] This finding suggests a key role for zinc in the inflammatory response, as well as in zinc metabolism, and as a metal detoxicant, and several functions for zinc in this context have been hypothesized.[20]

One of these functions is in the role of metallothionein as an antioxidant. The intracellular redox state is intimately related to metallothionein and intracellular zinc metabolism.[7] Increasing intracellular oxidized glutathione can result in the release of all seven zinc atoms, whereas this is inhibited by reduced glutathione. Oxidants cause greater damage to the liver of $MT^{-/-}$ mice than to that of $MT^{+/+}$ mice.[21] Despite its central role in zinc metabolism (see later), metallothionein is not essential for survival. Late-gestation $MT^{-/-}$ mouse fetuses have decreased hepatic zinc and are prone to obesity in later life.[22] More important, they are disadvantaged when the normal environment is compromised, especially when zinc intake is suboptimal. Factors that increase the induction of maternal hepatic metallothionein during early pregnancy may divert zinc from the conceptus to the maternal liver, potentially resulting in a conceptus that is deficient in zinc. Curtailment of fetal zinc uptake can occur with maternal ingestion of alcohol, and this mechanism may be important in the origin of the fetal alcohol syndrome.[23] Close similarities have been recognized between the teratogenicity of maternal zinc deficiency and alcohol administration to mice in early gestation, and this effect can be diminished by parenteral administration of zinc.[23] Cytokines cause a similar disturbance of maternal zinc metabolism and also are teratogenic in rodents.

PHYSIOLOGY OF ZINC

Intracellular Zinc Metabolism

Zinc metabolism is tissue- and organ-specific. Although this specificity is important to an understanding of zinc metabolism, the following discussion is limited to selected aspects of more universal intracellular zinc metabolism. The intracellular concentration of unbound "free" Zn^{2+} is extremely low. An elaborate homeostatic system of proteins regulates cellular Zn^{2+} distribution and perhaps controls a hierarchy of zinc-dependent functions.[7] Eukaryotic zinc transporters have a major, but still only partly defined, regulatory role.[24] These transporters are encoded by ancient gene families that span all phylogenetic levels. The ZIP family is involved in cellular Zn^{2+} uptake. One member of this family, Zrt3, is known to transport stored Zn^{2+} out of a vacuolar intracellular compartment during adaptation to zinc deficiency. The CDF (cation diffusion facilitator) family of transporters mediates efflux of Zn^{2+} out of cells or facilitates intracellular storage. These transporters are regulated by transcriptional and posttranscriptional mechanisms to maintain zinc homeostasis at a cellular and organ level: ZnT-1 and ZnT-4 are expressed ubiquitously, whereas ZnT-2 expression is limited to the small intestine, kidney, placenta, and liver. When zinc intake is restricted, intestinal and kidney ZnT-2

expression is extremely low, and both ZnT-1 and ZnT-2 messenger RNAs (mRNAs) are increased in intestine, kidney, and liver with high zinc intake.[25] Although zinc transporter expression is responsive to variations in dietary zinc intake, many details of the molecular regulation of zinc metabolism and homeostasis await clarification.

The expression of ZnT-1 and ZnT-2 is comparable to zinc-induced changes in metallothionein mRNA levels, suggesting a similar mode of regulation of these genes. Regulation of metallothionein at the protein level by ligand binding[26] and at the gene level by multiple inducers, including zinc, provides a means of controlling the intracellular availability of zinc by regulating the quantity of metallothionein and the ratio of metallothionein to its redox partner thionein (T) (i.e., MT/T). This role of metallothionein is closely linked to both cellular redox and energy status.

Maternal, Placental, Fetal, and Mammary Gland Zinc Metabolism

Maternal Metabolism

A physiologic increase in maternal hepatic metallothionein in pregnancy has been documented[23]; this increase may enhance a maternal store of zinc that is readily available to the conceptus. It is only when zinc is abnormally diverted to the maternal liver, as with alcohol ingestion, that the inappropriately large shift of Zn^{2+} from the plasma compartment is potentially deleterious to the embryo or fetus. A progressive physiologic decline in maternal plasma zinc also occurs as pregnancy advances,[27] and it is only when this decline is excessive because of maternal zinc deprivation or disturbed metabolism that the embryo or fetus is at risk.

Placental and Fetal Metabolism

Uptake of Zn^{2+} into placental villous syncytiotrophoblast is the first step in the transfer of this metal from the mother to the fetus, and it is the rate-limiting process.[28] This zinc is thought to be derived from the low-molecular-weight maternal plasma zinc pool, probably by a carrier-mediated process, and also from zinc bound to serum protein, possibly by an endocytic mechanism.[29] A potassium-zinc transporter is present in the vesicular membranes.[30] α_2-Macroglobulin binds to human trophoblasts in a specific saturable manner, a finding suggesting the possibility of receptor-mediated uptake of zinc.[31] Affinity for zinc (K_m values) by human syncytiotrophoblast microvillous membrane vesicles does not vary with gestational age or with low maternal plasma zinc concentrations. Zinc uptake capacity (V_{max}) is higher in preterm than in term placentas and with low maternal plasma zinc.[32] Fetal plasma zinc is higher than maternal concentrations. Short-term changes in fetal zinc status do not influence placental zinc transfer, suggesting that no ready mechanism is available for adjusting to fetal zinc deprivation once (or if) it occurs.[33] As gestation progresses, marked increases occur in fetal hepatic zinc concentration. These concentrations decline as the third trimester progresses. The increase in hepatic zinc concentration early in the third trimester appears to be secondary to hepatic metallothionein induction, rather than its cause.[34]

Metallothionein has been found in fetal amniotic cells, syncytial trophoblasts, and villous interstitial cells.[35] The mammalian embryo is surrounded by cells that actively express MT-1 and MT-2 genes, suggesting that metallothionein has a functional role in the establishment, as well as the maintenance, of normal pregnancy.[36] MT-3 and MT-4 mRNAs are abundant in the maternal deciduum, in contrast with fetal tissues, and these genes are refractory to induction by either zinc or inflammation in the deciduum.[37] Metallothionein gene expression may have a physiologic role in placental transport of Zn^{2+}.[38]

As with the intestine, further research is essential to determine how the roles of these and other factors, such as zinc transporters, are integrated to achieve regulation of placental Zn^{2+} transport. Only then will it be possible to understand how regulation, and hence the zinc status of the embryo or fetus, may be compromised, especially early in gestation.

Mammary Gland Metabolism

ZnT-1 (present in serosal membrane), ZnT-2, and ZnT-4 (present intracellularly) have been identified in the mammary gland. Milk zinc concentration is regulated through co-coordinated regulation of these mammary gland zinc transporters.[39] Vitamin A, which has substantial interactions with zinc,[40] has a role in the maintenance of zinc homeostasis in the mammary gland.[39] The regulatory changes responsible for the notable decline in human milk zinc concentrations as lactation progresses,[41,42] have not been elucidated, but they may be responsive to changes in the hormonal milieu.

Rarely, the mammary gland has a defect in the ZnT-4 gene resulting in impaired secretion of zinc.[43] Breast milk zinc concentrations are less than the normal range at all stages of lactation. Infants breast-fed by these mothers typically display the classic phenotype of acrodermatitis enteropathica by approximately 2 months of postnatal age. Infants with the clinical syndrome respond rapidly and completely to an oral zinc supplement of 2 mg/kg of body weight/day and then a smaller maintenance dose while breast-feeding continues. This syndrome has been described in term infants but is more likely to affect those infants born preterm, suggesting an increased vulnerability of the preterm infant to clinically significant zinc deficiency syndromes.[44]

Whole-Body Zinc Homeostasis

Postnatally, the gastrointestinal tract, specifically the small intestine and the pancreas, has the major role in maintaining whole-body zinc homeostasis.[45,46] Zinc transporter expression is a major component of homeostatic regulation, including regulation of zinc absorption by the small intestine and excreting zinc through both pancreas and small intestine. A total of 16 zinc transporters have been identified in these organs.[25] When dietary zinc intake is reduced, Zip4 is up-regulated at the apical surface of the small intestine. This transporter is defective in classic acrodermatitis enteropathica.[47] Of note, it is the quantity of zinc ingested rather than zinc status that is the principal factor triggering regulation of expression of the transporters involved with zinc absorption into and through the enterocyte.[45]

Zn^{2+} absorption into and through the enterocyte is a saturable transport process.[48,49] Fractional absorption, therefore, declines with increasing zinc intake, and vice versa. Passive paracellular diffusion also occurs in animal models but has not been detectable in modeling human zinc absorption.[50] When data are adjusted for differences in length of the small intestine, the term infant can absorb ingested zinc as efficiently and with the same capacity as in the adult.[51] This also appears to be true for the preterm infant at 33 weeks after conception,[51] suggesting that synthesis of zinc transporters and other zinc-containing molecules in the enterocyte is mature by this stage of development. However, the length of the small intestine is, of course, shorter. The shorter small intestinal length is commensurate with the lower requirements for absorbed zinc in the term infant. Nevertheless, absorption of adequate zinc is a challenge in the premature infant because of the especially short small intestinal length and the high zinc requirements of this population.

Regulation of the quantity of endogenous zinc excreted has a major role in maintaining whole-body zinc homeostasis in most circumstances.[45,46,52] The zinc exporters ZnT-1 and ZnT-2 exhibit progressive down-regulation, most notably in the pancreas, when dietary zinc is reduced.[25] The ability to regulate endogenous zinc losses through the intestine appears to be well developed in early postnatal life in the term infant.[53,54] In the premature infant, the rate of endogenous zinc excretion is positively correlated with

the quantity of zinc absorbed, as in term infants. In other words, when absorption decreases, endogenous zinc is conserved. Although the premature infant may be able to regulate intestinal excretion of endogenous zinc as efficiently as the term infant, more, unfortunately, is required to help meet the greater needs. Endogenous zinc losses through the kidneys, although small in comparison with the intestinal losses, also are relatively high in the very-low-birth-weight infant on a body weight basis.

Model-based compartmental analysis of zinc metabolism in the adult[55] has identified several pools of zinc that intermix with zinc in plasma within 3 days. The combined size of these pools (exchangeable zinc pool [EZP]) can be estimated from what appears as a single exponential between 3 and 9 days after administration of a zinc tracer and from urine enrichment with this zinc-stable isotope tracer.[56] On a body weight basis, the size of the EZP is approximately seven-fold higher in premature infants at 33 weeks after conception, according to unpublished data of S. Jalla and co-workers.[57] than in adults. This finding may be attributable to more than one possible difference in zinc metabolism or nutrition, but it is explained in part by higher concentrations of metallthionein in the fetal liver earlier in the third trimester.[58]

ZINC DEFICIENCY IN THE CONCEPTUS AND EARLY INFANCY

Embryogenesis

An adequate supply of maternal zinc is critically important for the oocyte and embryo.[12] The teratology in rodents resulting from maternal zinc deficiency is quite exceptional.[59-61] Consequences include a high mortality rate and congenital malformations affecting nearly all organ systems. Relevant data in the human are very limited, and prospective trials of zinc supplementation in carefully selected population groups that encompass the perinatal period will be required. Suggestive evidence points to a causal association between severe maternal zinc deficiency resulting from acrodermatitis enteropathica and congenital malformations including neural tube defects.[62] Although one study did not find any evidence of hypozincemia in the first trimester in pregnancies that resulted in neural tube defects,[63] this lack of correspondence may not apply in maternal populations in which the dietary supply of available zinc is especially limited.[64] Epidemiologic evidence from a presumably well-nourished population points to a link between maternal zinc status and neural tube defects.[65] Preliminary data from West Timor, where intake of available zinc is low and the incidence of cleft lip or palate is high, indicate that maternal zinc supplementation commencing by 8 to 10 weeks gestation is associated with a significant reduction in the incidence of these malformations.[66] The association among maternal alcohol consumption, diversion of maternal zinc from the conceptus, and the fetal alcohol syndrome is discussed earlier. This also is a putative paradigm for any maternal stress or infection that increases cytokine production and consequently increases induction of maternal hepatic metallothionein.

Fetal Development

Maternal zinc restriction during fetal development is associated with intrauterine growth retardation in rodent models.[67] Another reported effect is a decrease in nestin, a marker of proliferation of neural stem cells.[68]

In reported data for humans, findings are conflicting regarding the effects of maternal zinc supplementation on gestational age at delivery and birth weight.[66,69-71] Multicenter, carefully designed, prospective intervention studies will be required to resolve the question of whether maternal zinc deficiency contributes to low birth weight by reducing the duration of gestation or the rate of growth in utero. Perhaps unexpectedly, more consistent evidence suggests that maternal zinc supplementation starting in the second trimester and discontinued at delivery is associated with decreased infectious disease morbidity during the first 6 months of infancy.[72] This effect, which presumably is mediated through an effect of fetal zinc status on the developing immune system, is itself is an important reason for ensuring optimal maternal zinc intake.

Infancy

The effects of zinc deficiency in infancy require consideration both from a global public health standpoint and from an individual perspective. With the latter perspective, cases can usefully be subdivided into acute or severe[73] and mild,[74] but with the recognition that acute or severe zinc deficiency can enter a chronic phase; in addition, apparently mild cases with nonspecific clinical features carry the risk of death[75] as well as severe morbidity.[76,77] Moreover, intermediate clinical presentations, as exemplified by the original descriptions of human zinc deficiency, have been documented.[78]

The prototype of the acute severe presentation is the disorder *acrodermatitis enteropathica,* of autosomal recessive inheritance.[73] Identification of the underlying genetic defect or one of the underlying genetic defects is likely to be imminent. The clinical features of this syndrome manifest between the ages of 2 and 6 months, although presentation may be delayed with the universal zinc fortification of infant formulas in North America and elsewhere. Before this fortification policy, the onset of the disorder in breast-fed infants typically was later than in formula-fed infants, and breast milk was found to be of some therapeutic benefit. The single most important diagnostic feature, although not pathognomonic, is the skin rash. This erythematous rash may be widespread but characteristically is most evident around the body orifices and the extremities. Diarrhea and hair loss, although not inevitable, complete the classic triad of features. Untreated cases are marked by anorexia, marked apathy, progressively severe failure to thrive, and recurrent infections resulting in death in later infancy. Similar syndromes have been reported as a result of exclusive breast-feeding by mothers with a genetic defect in ability to secrete normal quantities of zinc by the mammary gland[42] or secondary to severely restricted zinc intake. The latter was seen quite frequently in parenterally fed patients in the early days of intravenous feeding. Premature infants appeared to be especially vulnerable to a lack of zinc in these infusates.

Many different abnormalities of the immune system have been observed in acrodermatitis enteropathica. Although it has been difficult to separate the effects of zinc deficiency from those of the resulting protein-energy malnutrition, extensive experimental data substantiate the far-reaching effects of zinc deficiency on every aspect of the immune system,[79] defects in which undoubtedly underlie the global public health concerns about zinc deficiency (see later). As one of the systems with the most notable rapid turnover of cells, the immune system is especially vulnerable to the fundamental defects in cellular proliferation, growth, and metabolism that result from an inadequate supply of this mineral.

On a global basis, zinc deficiency is now recognized as a major public health issue. Solid evidence indicates that as many as 20% to 25% of cases of diarrhea and 40% of cases of pneumonia in developing countries can be prevented by preventing zinc deficiency.[80] The risk of malaria also may be reduced. Acceleration of delayed linear growth and weight gain,[81] and probable improvement in brain development and function,[82] have been documented with correction of zinc deficiency in the same or similar populations of young children and also in North America.[74,83] Taken together, these features encompass all of those, with the exception of human immunodeficiency virus infection, that are of greatest public health concern for young children worldwide.

Low-birth-weight infants are at greater risk for zinc deficiency and its sequelae. In India, zinc supplementation from very early postnatal life has resulted in a significant decrease in mortality

rates for infants with intrauterine growth retardation.[75] Improvements in brain function[84] and physical growth velocity and diminution of infectious disease morbidity also have been documented.

EARLY POSTNATAL ZINC REQUIREMENTS OF THE PREMATURE INFANT

For the young, exclusively breast-fed term infant, zinc intake provides an excellent guide to requirements, that is, approximately 2 mg of zinc/day.[41] A rapid rate of physiologic decline in breast milk zinc concentration has been documented, with a resultant decrease in intake to approximately 1 mg of zinc/day for the exclusively breast-fed infant by approximately 4 months, with subsequent further decline.[41] Whether this intake is adequate after 4 to 6 months has not been determined,[85] but complementary sources of zinc clearly are required around this age. For the formula-fed infant, less favorable bioavailability dictates the apparent need for higher intakes, but these have not been well defined.

Estimation of zinc requirements depends primarily on a factorial approach. Such determinations are complex in any population but are especially so in low-birth-weight infants. One reason is that it remains unclear to what extent intrauterine accumulation rates in the premature infant can or should mimic thoseat corresponding gestational ages.[86] As determined in two different studies, postnatal weight gain requires zinc intakes of 17 g/kg body weight/day[87] and 20 mcg/g of weight gain[88]; the very-low-birth-weight infant needs an average daily zinc intake of 340 μg/kg of body weight. How much needs to be retained to meet this intake requirement depends on the extent to which, and the rate at which, hepatic stores of zinc at birth are or can be used by the neonate and young infant. Although the weight of the premature liver is quite small, zinc concentrations are exceptionally high.[58,86] On the basis of several assumptions and complex calculations, the Life Sciences Research Office (LSRO) report determined that 130 μg of zinc/day (or approximately 90 μg of zinc/kg/day) is available from hepatic stores (after 28 weeks gestation) for postnatal growth.[87] Thus retention of exogenous zinc required for growth is 250 μg of zinc/kg/day. With losses of endogenous zinc in feces and urine averaging 215 μg of Zn/kg/day in very-low-birth-weight infants fed either breast milk with fortifier or a premature infant formula (as determined by S. Jalla and co-workers[57]) the estimated average physiologic zinc requirement (amount of absorbed zinc required) is 465 μg of zinc/kg/day. In this study, average fractional absorption from formula and breast milk plus fortifier was 0.25, indicating a dietary requirement approaching 2 mg of zinc/kg/day. The quite strong positive correlation between zinc intake and weight gain in this study suggests, but does not prove, that zinc intakes any lower than this level can limit growth. Relatively high zinc intakes may need to be maintained for many months if lack of adequate zinc is not to limit growth.[89] The results of zinc supplementation studies in early infancy[75,90] suggest that the early postnatal zinc requirements of the small-for-gestational-age infant are at least as high as those of the premature infant.

At a molecular level, more is known about the regulation of excretion of endogenous zinc in response to high zinc concentrations[25] than about the regulation of uptake. The transporter genes that are regulated are ancient, indicating that all eukaryotic cells have developed mechanisms for minimizing the accumulation of zinc in circumstances of environmental excess. This observation suggests that excess zinc is toxic even though little is known about its deleterious effects. In fact, the margin between optimal and toxic levels of zinc may be quite narrow for individual tissues. Moreover, the maintenance of zinc homeostasis by the gastrointestinal tract, although good, is imperfect.[52] Moreover, excess zinc may not need to reach beyond the enterocyte to be deleterious, at least in terms of metal-metal interactions.[91] Therefore appropriate caution is required to ensure that the quantity of zinc supplied to the very-low-birth-weight infant is not excessive. As noted, the margin between optimal and excessive intake may eventually prove to be quite narrow.[92] No evidence has emerged, however, to suggest that the levels of intake from premature infant formulas and human milk fortifiers in current use are excessive. Rather, the foregoing calculations indicate that these are very reasonable, in light of present knowledge.

CONCLUSION

The emergence of the impressive biology of zinc and recognition of the public health implications of zinc deficiency in infants and young children[93] are now alerting the medical community to the need for special attention to this trace element. This need is more apparent in the very-low-birth weight-infant than in any other population group. From a practical standpoint, the most pressing requirement is to delineate the zinc requirements of the infant born very or extremely prematurely, including, as a special subgroup, those with intrauterine growth retardation.

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38 Vitamin A Metabolism in the Fetus and Neonate

Jayant P. Shenai

Vitamin A is a fat-soluble micronutrient recognized since 1912 as a dietary constituent that is essential for growth.[1,2] The vitamin A compounds (retinoids) are present in three natural forms: retinol, retinaldehyde, and retinoic acid (Figure 38-1). Retinol (vitamin A alcohol) is a dietary component present in the form of retinyl esters in food sources of animal origin and also is formed in vivo from its precursor, beta carotene, which is present in food sources of plant origin. Retinyl esters are derived from esterification of retinol and constitute the principal storage forms of vitamin A; the major retinyl esters found in animal and human tissues include retinyl palmitate and stearate and, to a lesser extent, oleate and linoleate. Retinaldehyde, also called *retinal,* is derived from reversible oxidation of retinol and, in combination with various lipoproteins, forms the visual pigment of the retina.[3] The photoisomerization of retinaldehyde is necessary for vision.[4] Retinoic acid is derived from irreversible oxidation of retinaldehyde in the tissues[5] and is the active metabolite of vitamin A in functions related to growth and differentiation.[6]

VITAMIN A METABOLISM

Transport in Plasma

Vitamin A is transported in plasma as retinol bound to a specific carrier protein, retinol-binding protein (RBP) (Figure 38-2). The human RBP molecule consists of a polypeptide chain of approximately 180 amino acid residues; it has a molecular mass of approximately 21 kDa and a single binding site for one molecule of retinol.[7,8] The RBP is synthesized in liver and secreted into plasma as retinol-RBP complex.[9] In plasma, the retinol-RBP complex interacts with another protein, transthyretin, and circulates as an RBP-transthyretin complex.[9] The transthyretin molecule is a stable, symmetric tetramer composed of four identical subunits with a molecular mass of approximately 55 kDa.[10] Vitamin A is distributed to the target tissues in the form of retinol-RBP complex bound to transthyretin. The cellular uptake of vitamin A depends on a specific plasma membrane receptor that recognizes RBP.[11,12] After delivery of vitamin A to the plasma membrane, RBP is returned to the circulation and is partly reused for vitamin A delivery and partly eliminated by the kidney.[13] The mechanisms involved in the processing of transthyretin are not known.

Metabolism in the Cell

The movement of vitamin A within the tissue cells involves two intracellular vitamin A–binding proteins, cellular retinol-binding protein (CRBP) and cellular retinoic acid–binding protein (CRABP)[14] (Figure 38-3). The CRBP molecule consists of a polypeptide chain with a molecular mass of approximately 14.6 kD and a single binding site for one molecule of a specific isomer of retinol, all-*trans*-retinol. The CRBP participates in the enzymatic esterification of retinol into retinyl esters for storage in the cell. The CRABP molecule also consists of a 14.6-kDa polypeptide chain with a single binding site for one molecule of a specific isomer of retinoic acid, all-*trans*-retinoic acid. Given the apparent absence of a plasma delivery system specific for retinoic acid, it seems likely that the retinoic acid within the cell is derived mostly from oxidation of retinol occurring within the cell. The CRABP participates in the interconversion of two specific isomers of retinoic acid, all-*trans*-retinoic acid and 9-*cis*-retinoic acid. Both CRBP and CRABP are suggested as transport proteins for retinoids between the cellular and nuclear membranes.

Processing in the Nucleus

The nuclear uptake of all-*trans*-retinoic acid is mediated exclusively by a family of receptors called *retinoic acid receptors* (RARs) (see Figure 38-3).[15] Three different isomers of RARs—RAR-α, RAR-β, RAR-γ—exist and are expressed in distinct patterns throughout development. The RARs modulate gene expression by binding as homodimers or heterodimers to specific DNA sequences known as *retinoid acid receptor elements* (RAREs). In contrast with that of all-*trans*-retinoic acid, the nuclear uptake of 9-*cis*-retinoic acid is mediated partly by RARs but mostly by another family of receptors called *retinoid X receptors* (RXRs).[15] As with RARs, three different isomers of RXRs—RXR-α, RXR-β, RXR-γ—exist and are expressed in distinct patterns throughout development. RXRs modulate gene expression by binding to specific DNA sequences known as *retinoid X receptor elements* (RXREs). The mechanisms by which retinoic acid influences nuclear metabolism, genomic expression, and growth and differentiation of the target tissue remain under investigation.

Vitamin A Excretion

The excretory end products of vitamin A metabolism are derived largely from retinoic acid.[16] Retinoic acid is excreted partly in the ester form of β-glucuronide through the biliary system and is subject to reabsorption in the gut.[17] The enterohepatic circulation of retinoic acid serves to conserve this biologically active form of vitamin A. The rest of the retinoic acid is excreted largely in the urine in the form of oxidation products.[18]

FETAL ACQUISITION OF VITAMIN A

Vitamin A is transferred from the mother to the fetus during pregnancy, particularly in late gestation, in most animal species.[19-25] The transplacental transport of maternal retinol-RBP complex appears to be the predominant route for providing vitamin A to the fetus in early gestation. In late gestation, RBP synthesized by the fetal liver appears to be involved in extracting vitamin A from the placental circulation. Swallowing of amniotic fluid containing vitamin A[26,27] and transfer of maternal lipoproteins containing retinyl esters[23] are other possible means of vitamin A acquisition for the fetus.

The transplacental transfer of vitamin A in humans has been studied by examining paired samples of maternal blood and fetal or cord blood obtained at various gestational ages.[28-32] The ratio of maternal to fetal concentrations of plasma vitamin A in healthy

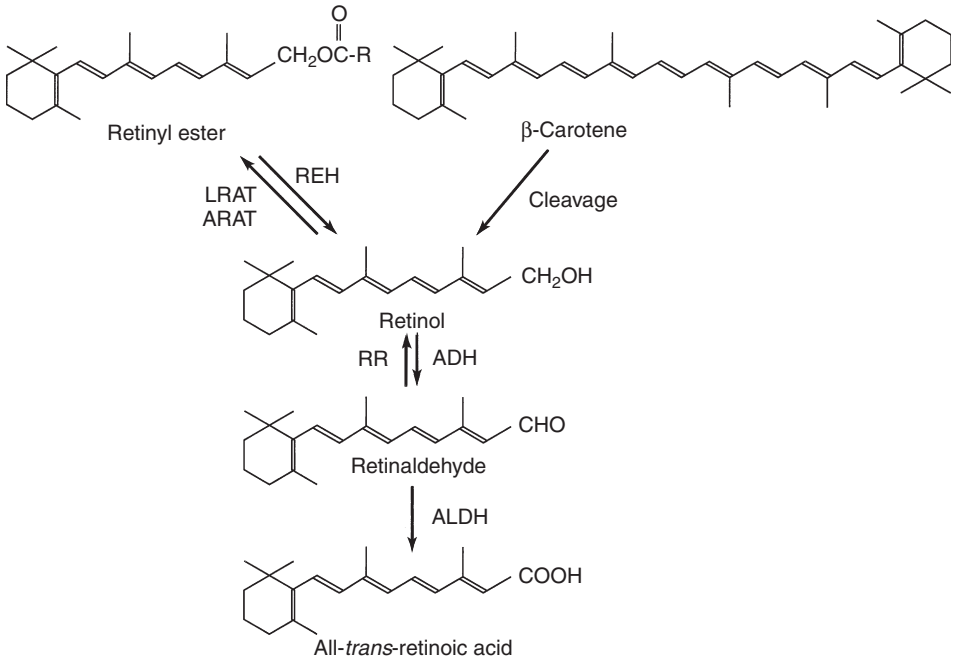


Figure 38-1 Vitamin A compounds. *ADH,* Alcohol dehydrogenase; *ALDH,* aldehyde dehydrogenase; *ARAT,* acyl-CoA-retinol acyltransferase; *LRAT,* lecithin-retinol acyltransferase; *REH,* retinyl ester hydrolase; *RR,* retinaldehyde reductase.

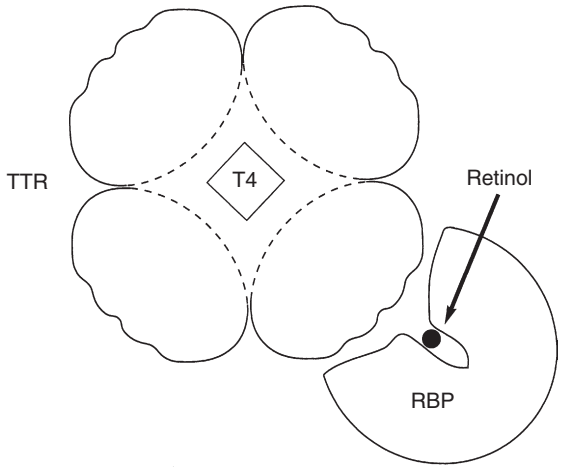


Figure 38-2 Vitamin A (retinol) complexed with its specific carrier proteins in plasma. *RBP,* Retinol-binding protein; *TTR,* transthyretin; T_4, thyroid hormone.

human pregnancies is approximately 2:1. In conditions in which the maternal vitamin A status is marginal or deficient, the fetal plasma vitamin A concentration often is maintained within normal limits and may exceed the maternal plasma vitamin A concentration.[28,33,34] Conversely, in studies involving maternal vitamin A supplementation, the cord blood vitamin A concentration in the supplemented cohort remains similar to that in unsupplemented control infants.[28,35,36] Thus the plasma vitamin A concentration in the fetus appears to be maintained within a normal range despite variations in maternal vitamin A status and intake. The regulatory mechanisms by which this homeostasis is achieved remain unclear, nor is it known whether such mechanisms can compensate successfully for extremes of maternal vitamin A deprivation or excess.

VITAMIN A STATUS AT BIRTH

Plasma Vitamin A Indices

Plasma concentration of vitamin A is the most commonly used biochemical marker of vitamin A status. In healthy human adults, the plasma vitamin A concentration ranges from 20 to 80 mcg/dL.[37] In children, including infants, a plasma vitamin A concentration below 20 mcg/dL is indicative of vitamin A deficiency.[38] Most preterm infants, in contrast with those of term gestation, are born with plasma vitamin A concentrations that are in the deficient range.[39,40]

Plasma concentration of RBP is another biochemical marker of vitamin A status. In healthy human adults, the plasma RBP concentration averages 4.6 mg/dL (± SD of 1.0 mg/dL).[41] The plasma RBP concentrations in infants and children are approximately 60% of the adult values,[30] and those below 2.5 mg/dL are indicative of vitamin A deficiency.[42] Most preterm infants, unlike those of term gestation, are born with plasma RBP concentrations that are in the deficient range.[40,43]

Another useful biochemical marker of vitamin A status involves plasma RBP response to vitamin A administration. This functional measure of vitamin A status is based on the varied rates of secretion of RBP from the liver to the plasma, depending on the availability of vitamin A.[44,45] In vitamin A–deficient animals, RBP secretion is blocked, leading to its accumulation in the liver and resulting in high liver and low plasma concentrations of RBP. When these animals are given vitamin A, mobilization of RBP from the liver occurs, which leads to a rapid decrease in the liver RBP concentration and a concomitant increase in the plasma RBP concentration.[44] In vitamin A–sufficient animals, on the other hand, RBP does not accumulate in the liver, and minimal or no change in plasma RBP concentration is observed after vitamin A administration.[44] The percent increase in plasma RBP concentration (ΔRBP) from its baseline value in response to vitamin A administration is therefore useful for determining the vitamin A status. A high ΔRBP value (above 8%) is indicative of vitamin A deficiency.[46] In preterm neonates, the plasma RBP response to vitamin A administration, characterized by high ΔRBP values, provides further evidence that these infants are vitamin A–deficient

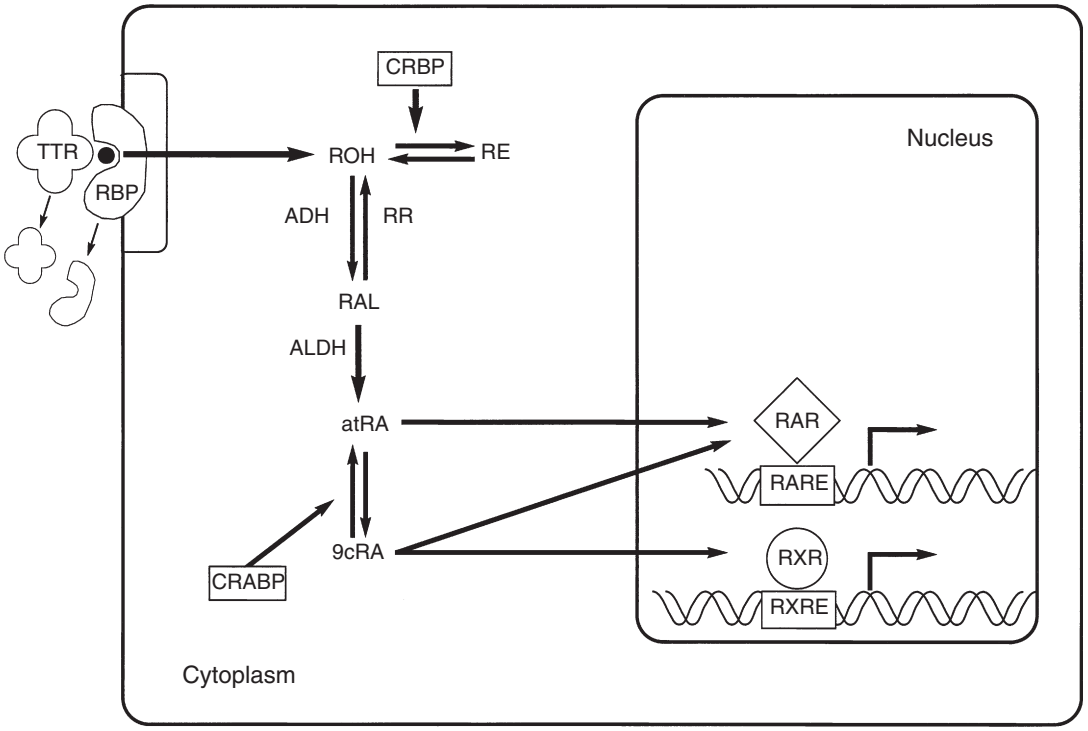


Figure 38-3 Vitamin A metabolism in the cell and nucleus. *ADH,* Alcohol dehydrogenase; *ALDH,* aldehyde dehydrogenase; *atRA,* all-*trans*-retinoic acid; *CRABP,* cellular retinoic acid–binding protein; *CRBP,* cellular retinol-binding protein; *9cRA,* 9-*cis*-retinoic acid; *RAL,* retinaldehyde; *RAR,* retinoic acid receptor; *RARE,* retinoic acid receptor element; *RBP,* retinol-binding protein; *RE,* retinyl ester; *ROH,* retinol; *RR,* retinaldehyde reductase; *RXR,* retinoid X receptor; *RXRE,* retinoid X receptor element; *TTR,* transthyretin. *(Modified from Mangelsdorf DJ, Umesono K, Evans RM: The retinoid receptors. In Sporn MB, Roberts AB, Goodman DS [eds]:* The Retinoids: Biology, Chemistry, and Medicine, *2nd ed. New York, Raven Press, 1994, p 319.)*

at birth.[46] It is likely that such deficiency is due to deprivation of the transplacental vitamin A supply that normally allows accrual of adequate vitamin A by full gestation.

Tissue Vitamin A Storage

As much as 90% of the total body reserve of vitamin A is stored normally in the liver in most animal species.[47] The liver vitamin A concentration ranges from 100 to 300 μg/g in healthy human adults,[48] and it varies with age in children, being low during infancy relative to later childhood.[49,50] A liver vitamin A concentration below 40 μg/g is indicative of low vitamin A reserve, and that below 20 μg/g is considered deficient.[49-51] Most preterm infants, unlike those of term gestation, are born with low liver vitamin A stores.[50,52-55] In the absence of an adequate intake of vitamin A in the postnatal period, therefore, these infants are at an added risk for vitamin A deficiency.

Although the liver is the principal storage site for vitamin A, other organs, including the developing lung, are capable of storing vitamin A.[56-58] In the perinatal rat, significant vitamin A storage occurs in the fetal lung during the last third of prenatal life.[57,58] Depletion of these stores that begins before birth and continues into early postnatal period suggests that the developing lung may depend on these local stores of vitamin A during the period of active growth and differentiation associated with rapid alveolarization of the lung. Prematurely born animals, deprived of adequate stores of vitamin A in the lungs, may be susceptible to the adverse pulmonary effects of vitamin A deficiency. The fetal lung stores of vitamin A can be augmented by prenatal maternal administration of supplemental vitamin A.[59] Conversely, prenatal interventions, such as maternal glucocorticosteroid treatment, can deplete the fetal lung stores of vitamin A.[60] Whether the developing fetal lung of humans is capable of vitamin A uptake, esterification, and storage remains undetermined. Moreover, the possibility that administration of vitamin A to human mothers with imminent preterm delivery may augment the lung vitamin A stores of their newborn infants remains unexplored.

VITAMIN A AND ENTERAL NUTRITION

Vitamin A Content

The vitamin A value of a diet is expressed in international units (IU) or retinol equivalents (RE).[37] One IU of vitamin A is equal to 0.3 μg of retinol; one RE of vitamin A is equal to 1.0 μg of retinol.

The vitamin A content of human milk is variable and influenced by several factors, such as age, parity, and socioeconomic status of the mother; postpartum age; and the volume and fat content of the milk.[61] The vitamin A concentration of human milk in colostrum is high (above 333 IU/dL); levels decrease gradually in the first few months of lactation.[61-64] The vitamin A concentration of mature human milk ranges from 110 to 257 IU/dL.[61-63, 65-67] The milk of mothers giving birth prematurely differs in composition from that of mothers delivering at term gestation. The vitamin A concentration of preterm milk is lower than that of term milk during the first week of lactation, but is higher thereafter.[67-69] At approximately 35 days postpartum, the vitamin A concentration of preterm milk ranges from 277 to 333 IU/dL.[68,69] More than 90% of the vitamin A in human milk is in the form of retinyl esters contained in the core of the milk fat globules; less than 10% of the vitamin A is present as free retinol.[66,70-73]

Various infant formulas designed to meet the nutritional needs of a preterm infant are commercially available. The vitamin A content of these formulas varies, ranging fromapproximately 296 to 1000 IU/dL.[74] Preterm infants receiving full enteral

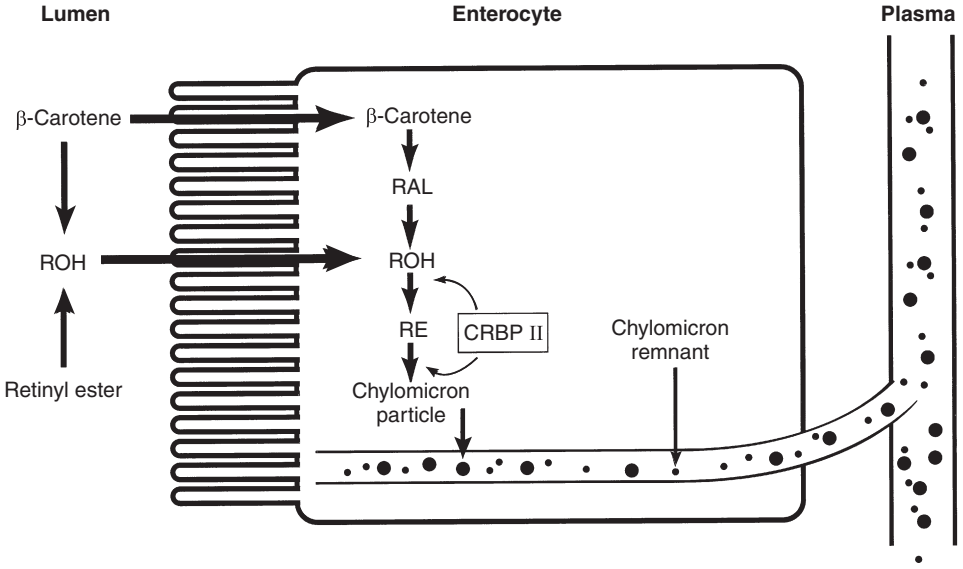


Figure 38-4 Vitamin A absorption in the enterocyte. *CRBP II,* Cellular retinol-binding protein type II; *RAL,* retinaldehyde; *RE,* retinyl ester; *ROH,* retinol.

feedings often are given multivitamin supplements. The vitamin A content of a typical multivitamin supplement is 1500 IU/dL.[74] As in human milk, most vitamin A in the infant formulas and multivitamin supplements is in the form of retinyl esters. Thus the efficiency of use of vitamin A in a neonate fed human milk or an infant formula with or without a multivitamin supplement depends largely on the ability of the infant's gastrointestinal tract to process retinyl esters.

Vitamin A Digestion and Absorption

Dietary retinyl esters are processed by a coordinated series of physical and chemical events in the bowel lumen.[47] These events include dispersion and emulsification of retinyl esters in the stomach, their hydrolysis in the intestinal lumen by pancreatic and other enzymes, and solubilization of retinol (derived from retinyl ester hydrolysis) with bile salts in mixed micelles. These intraluminal events facilitate uptake of vitamin A by the enteral mucosal cells (Figure 38-4). Retinol within the mucosal cells, also partly derived from beta carotene, is largely reesterified with long-chain fatty acids. The retinyl esters are then incorporated, together with other lipids and apolipoproteins, into chylomicron particles. The chylomicrons are secreted by the enteral mucosal cells into the intestinal lacteals and enter the plasma compartment through the intestinal lymphatics, largely through the thoracic duct. Retinyl esters found in the lymph chylomicron are retained almost entirely in the particle during its processing to a chylomicron remnant. The chylomicron remnants and their retinyl esters are almost completely removed from the circulation by the liver.

The reesterification of retinol in the enteral mucosal cells and its subsequent absorption into the lymph are influenced by a specific carrier protein called *cellular retinol-binding protein type II* (CRBP II) (see Figure 38-4).[75] The CRBP II molecule consists of a polypeptide chain with a molecular mass of approximately 16 kDa. In the adult rat, CRBP II is present almost exclusively in the absorptive cells of the small intestine.[76] Its concentration in the small intestine appears to be age-related: Concentrations are is lower in immature fetuses relative to those in mature newborns and adults.[75] A similar gut-specific CRBP II is present in the human small intestine.[77] Its localization in the mature, villus-associated enterocytes of the jejunum suggests that CRBP II may facilitate vitamin A absorption in the gut. The ontogeny of CRBP II during fetal and neonatal development in humans has not been studied. It is possible that the lower concentrations of CRBP II in the small intestine of preterm neonates relative to those in mature term infants may result in differences in vitamin A absorption in these infants.

VITAMIN A AND PARENTERAL NUTRITION

Preterm infants who experience difficulties with enteral feeding often are sustained exclusively with intravenous nutrition. A protein-dextrose solution and a lipid emulsion are commonly used for intravenous nutrition. Various nutrients, including vitamins, generally are administered in the protein-dextrose solution. The vitamin A concentration of the protein-dextrose solution is estimated at approximately 930 IU/dL.[78] In a newborn infant on total parenteral nutrition receiving the protein-dextrose solution at a conventional rate of 120 to 135 mL/kg/day, therefore, expected vitamin A intake ranges from 1116 to 1256 IU/kg/day. Of note, however, vitamin A in protein-dextrose solution is subject to photodegradation, and it binds to intravenous tubing.[79-82] Thus both photodegradative and adsorptive losses of vitamin A render its intravenous administration inefficient. Until such losses can be reduced substantially or eliminated, either through development of new materials for intravenous tubing or with improved administration of fat-soluble vitamins in the lipid emulsion,[83] alternative methods of vitamin A administration, such as by intramuscular route, may be necessary to optimize the vitamin A status of neonates on long-term parenteral nutrition.

VITAMIN A DEFICIENCY

Vitamin A promotes orderly growth and differentiation of epithelial cells, and its deficiency affects various organ systems, including the lung.[84] The histopathologic changes in the respiratory system generally precede other consequences of vitamin A deficiency involving the genitourinary system, eye, and skin.[84] Vitamin A deficiency results in a progressive sequence of changes in the epithelial lining of pulmonary conducting airways.[84-86] These changes consist of necrotizing tracheobronchitis in early stages and squamous metaplasia in advanced stages of the deficiency. The pathophysiologic consequences of these changes include (1) loss

of normal secretions of goblet cells and of other secretory cells; (2) loss of normal water homeostasis across tracheobronchial epithelium; (3) loss of mucociliary transport with resultant predisposition to airway infection; and (4) narrowing of lumina and loss of distensibility of airways with resultant increase in airway resistance and work of breathing. The histopathologic changes of vitamin A deficiency and associated pathophysiologic consequences are reversible with restoration of normal vitamin A status.[87,88]

Preterm infants are susceptible to lung injury from such insults as hyaline membrane disease, barotrauma or volutrauma from mechanical ventilation, oxygen toxicity, and airway infection.[89] These infants are at risk for the development of a form of chronic lung disease called *bronchopulmonary dysplasia* (BPD).[89] The histopathologic changes of BPD—now described as "classic" BPD—consist of necrotizing tracheobronchitis in early stages and squamous metaplasia in advanced stages of the disease, which are remarkably similar to those seen in vitamin A deficiency.[90] Preterm infants who have BPD, unlike those with no lung disease, often manifest clinical, biochemical, and histopathologic evidence of vitamin A deficiency.[91-93] Vitamin A supplementation from early postnatal life in these infants can improve their vitamin A status and also ameliorate the lung disease, as evidenced by a decreased incidence of BPD and of the associated morbidity.[94,95]

Recent advances in neonatal care, including the use of surfactant, continuous positive airway pressure, efficient strategies of mechanical ventilation, and enhanced nutrition, have altered the epidemiology of BPD.[96] In contrast with the classic form of BPD, seen in preterm infants with significant initial lung disease or those requiring high-level oxygen and ventilator support, the "new" BPD is emerging as a disease of the extremely preterm infant, often without much preceding lung illness or need for intensive support. Whereas airway injury—characterized by epithelial metaplasia, smooth muscle hypertrophy, and parenchymal fibrosis—has been the hallmark of classic BPD, the new BPD features a striking decrease in alveolar septation and microvascular development.[97] Normally, the gas exchange region of the immature lung is composed of large saccules.[98] These saccules undergo subdivision by an outgrowth of septa from their walls into smaller, more numerous structures termed alveoli. This septation is critical for increasing the alveolar number and surface area for gas exchange in early lung development. In humans, the alveolar septation occurs in late gestation and during the first few years after birth.[99,100] This septation may be inhibited by several postnatal factors, such as oxygen exposure, stretch injury from assisted ventilation, activation of inflammatory cascades, bacterial infection, and use of drugs such as dexamethasone.[96] These adverse effects on alveolar septation may be reversible with vitamin A. In newborn rats, daily treatment with retinoic acid has been shown to increase the number of alveoli[101] and also to prevent the inhibitory effect of dexamethasone on alveolar septation.[102] Whether such treatment is effective in prevention or amelioration of new BPD in humans remains unexplored.

VITAMIN A TOXICITY

Hypervitaminosis A may result from acute intoxication or from chronic ingestion of vitamin A. Administration of a single large dose of vitamin A in excess of 100,000 IU may cause acute intoxication in infants.[37] The major clinical manifestation of acute hypervitaminosis A is increased intracranial pressure. Prolonged ingestion of vitamin A in excess of 25,000 IU per day may cause chronic intoxication in infants.[37,103,104] The clinical manifestations of chronic hypervitaminosis A include symptoms and signs of increased intracranial pressure, bone and joint pains, and mucocutaneous lesions; hepatomegaly and hepatic injury, hypercalcemia, and hematologic abnormalities are sometimes seen.[105] Radiographic findings in chronic hypervitaminosis A in infants younger than 6 months of age include widened metaphyses, especially of the distal ulna, and radiolucent zones in the radius proximal to the metaphysis.[106] Cortical hyperostosis of multiple long bones and soft tissue changes are seen typically in infants older than 6 months.[106] In hypervitaminosis A, the plasma vitamin A concentration generally is in excess of 100 μg/dL, whereas the plasma RBP concentration usually remains normal.[107]

Hypervitaminosis A is reversible with restriction of vitamin A intake. Infusion of 2-hydroxypropyl-β-cyclodextrin as a means of solubilizing retinoids and enhancing their urinary excretion has been used occasionally as adjunct therapy.[108]

GUIDELINES FOR VITAMIN A NUTRITION

The recommended dietary intake of vitamin A for term newborn infants is 333 IU/kg/day.[109] In preterm infants, a vitamin A intake below 700 IU/kg/day is associated with a marked decline in plasma vitamin A concentrations.[92] Normalization of plasma concentrations of vitamin A and RBP is possible in preterm infants with a vitamin A intake above 1500 IU/kg/day.[46,92-94] Lack of clinical and biochemical evidence of toxicity in studies of vitamin A–supplemented infants and maintenance of their plasma vitamin A concentrations below 80 μg/dL suggest that a total vitamin A intake in the range of 1500 to 2800 IU/kg/day is safe for preterm infants.[46,93,94,110]

The potential role of vitamin A in facilitation of healing from lung injury has led to the development of guidelines for optimizing vitamin A nutrition in preterm infants at risk for BPD.[111] According to these guidelines, preterm infants meeting the following criteria are eligible for vitamin A supplementation: birth weight below 1250 g, estimated gestational age at birth less than 31 weeks, appropriate growth for gestational age, need for mechanical ventilation at 24 hours postnatal age, and absence of major congenital anomalies. In addition to standard vitamin A intake from parenteral and enteral sources, these infants are given supplemental vitamin A (retinyl palmitate) in two phases. Phase I entails an intramuscular injection of 2000 IU/kg/dose on postnatal day 1 and on alternate days thereafter until establishment of full enteral feeding. Full enteral feeding is considered to be established when energy intake by enteral route exceeds 75% of total energy intake. Phase II entails orogastric administration of 4000 IU/kg/dose on the first day of full feeding and daily thereafter until discharge from the neonatal intensive care unit. Each infant is monitored for evidence of vitamin A toxicity by serial measurement of plasma concentrations of vitamin A and RBP, careful physical examination, and periodic determination of blood chemistry profiles, as well as hematologic indices. Ultrasonographic scanning of the brain to detect intracranial hemorrhage and assessment of intracranial pressure by noninvasive methods such as fontanelle tonometry also are useful.

Dexamethasone, a glucocorticosteroid hormone, is being used occasionally in the postnatal treatment of preterm infants with BPD.[112] Postnatal dexamethasone treatment induces a significant, although short-term, increase in plasma concentrations of both vitamin A and RBP in these infants.[113,114] Thus, in the event dexamethasone treatment is initiated, supplemental vitamin A is withheld throughout the course of the dexamethasone treatment. This precautionary measure is intended to avoid the vitamin A toxicity associated with use of this agent. With the guidelines for vitamin A supplementation already discussed, monitoring of plasma vitamin A indices, clinical evaluation for potential toxicity, and careful consideration of concomitant therapies, the goal is to optimize vitamin A nutrition in vulnerable preterm infants at risk for lung disease.

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Lois H. Johnson and Vinod K. Bhutani

Vitamin E Metabolism in the Fetus and Newborn Infant

Although vitamin E has been shown to be essential to maintenance of pregnancy in female rats, such a role has not been adequately demonstrated in humans. This is perhaps because the severe deficiency state that can be induced in experimental animals does not occur spontaneously in healthy women of child-bearing age. Frank deficiency, however, is not the only concern.[1-3] A growing body of evidence documents the need for protection from oxidant free radical damage throughout life.[2-9] In the case of the fetus, this protection involves provision of an antioxidant reserve for combating the increased production of reactive oxygen species associated with complications of pregnancy such as placental insufficiency, preeclampsia, and gestational diabetes.[9] Antioxidant reserves also are needed for protection against potential episodes of vascular compromise, hypoxemia, and ischemia-reperfusion during gestation and the birth process and in early postnatal life; such episodes are recognized to be associated with serious risk for oxidant-mediated injury in the fetus, despite the low-oxygen environment of the fetoplacental unit, and in the neonate.[4,10]

This chapter focuses on studies related to the dependence of the human on not only an adequate supply of dietary vitamin E but also the ability to discriminate among the four naturally occurring tocopherols—α, β, γ, and δ—to maintain at least minimal serum and tissue levels of the α form, by far the most biologically active (Table 39-1). The implications of the physiologic hyperlipidemia of pregnancy for tissue levels of vitamin E in mother and fetus and the associated requirement for vitamin E in the maternal diet are discussed. Also addressed is the importance of defining tissue levels of the vitamin in relation to membrane lipid content, composition, and metabolic function, because the need

TABLE 39-1

Nomenclature and Food Sources of Naturally Occurring Vitamin E Compounds: RRR Isomers*

Common Name Mean ($\bar{X}$) Content† (μg/mL)	Chemical Name	Activity Relative to α-Tocopherol (%)	Food Source(s)	Contribution to Total Vitamin E‡ (%)
α-Tocopherol Plasma $\bar{X}$ = 9.6 RBC $\bar{X}$ = 1.4	5,7,8-Trimethyltocol	100	Nuts, seeds, wheat, rye, rice, vegetable oils, olive, safflower, sunflower Animal fat: butter	20-25
β-Tocopherol Plasma trace RBC n.d.	5,8-Dimethyltocol	25-40	Wheat germ, barley, and oats, lettuce	≤10
γ-Tocopherol Plasma $\bar{X}$ = 1.6 RBC $\bar{X}$ = 0.24	7,8-Dimethyltocol	10-20	Many vegetable oils, especially soybean, corn, and coconut	40-50
δ-Tocopherol Plasma trace RBC n.d.	8-Methyltocol	<10	Soybean, coconut, and peanut oil	~20
α-Tocotrienol Plasma trace RBC n.d.	5,7,8-Trimethyltocotrienol	40-50	Oats, barley, rye (whole cereals)	Minimal
β-Tocotrienol Unknown	5,8-Dimethyltocotrienol	~20	Palm oil	Insignificant
γ-Tocotrienol Plasma trace RBC n.d.	7,8-Dimethyltocotrienol	~10	Coconut oil	Minimal
δ-Tocotrienol Plasma trace RBC n.d.	8-Dimethyltocotrienol	<1	Rubber tree oil	None

*Among the naturally occurring tocopherol and tocotrienol RRR isomers, the most important determinants of activity are the number and position of methyl groups in the benzene ring. The fully methylated compound has the greatest biologic activity.

†On thin-layer chromatography, β-tocotrienol peak could not be separated from α-tocopherol peak. As indicated by its minimal presence in foods, plasma β-tocotrienol level is judged to be negligible. (Data from Chow CK: Distribution of tocopherols in human plasma and red blood cells, *Am J Clin Nutr* 28:756, 1975.)

‡In U.S. diets in 1975.

n.d., Not detectable.

Data compiled from references 14, 17, 80-84.

for antioxidant protection varies from tissue to tissue and during different stages of development and environmental conditions. This variability is especially pronounced during reproduction and periods of rapid growth. The dynamic balance of oxidizing to reducing equivalents (i.e., oxidation-reduction [redox] state) essential for normal differentiation and development during early, mid-, and late gestation has yet to be defined and characterized as a basis for management of pregnancy in general and as a guide for design of treatment protocols to prevent abnormalities in pregnancies characterized by increased oxidant stress.

BRIEF HISTORY OF VITAMIN E

The semipurified diet of Osborne and Mendel—composed of casein, starch, lard, butter fat, salts, and brewer's yeast—was known to contain vitamins A, B, C, and D and to support superior growth and vigor in the laboratory rat. In the pregnant female, however, it often was associated with fetal death and resorption of the conceptus and with testicular degeneration in the young male rat. (The Osborne-Mendel diet probably was not totally deficient in vitamin E because both butter and lard are sources of α-tocopherol.) In 1922, Evans and Bishop demonstrated that the missing dietary factor, which they termed "factor X," was present in wheat germ and fresh lettuce. In 1925, they suggested that this "oil of fertility" be given the name *vitamin E.* The classic 1927 monograph of Evans and Burr summarized these early years, when vitamin E was recognized solely as a dietary factor necessary for fertility in the rat. The related fertility bioassay quantifies the ability of animal tissues, foods, or preparations of vitamin E to prevent fetal resorption when fed to the pregnant, vitamin E–deficient rat during the first 10 days after impregnation. It remains the standard, but no longer the only, assay for assessing in vivo potency of the vitamin.[11,12]

Mechanisms underlying the dependence of mammalian species and nonreproductive as well as reproductive tissues on a dietary supply of vitamin E were poorly understood for many years. In 1928, Evans and Burr described a paralysis in suckling young of mother rats fed a diet low in vitamin E, from which spontaneous recovery occasionally occurred. In 1931, Pappenheimer and Goetsch described encephalomalacia in chickens fed the same semipurified diet used by Evans and Burr. In ducklings, this diet resulted in widespread lesions in the skeletal muscles but no sign of encephalomalacia. These nonreproductive tissue lesions, in all three species, were signs of vitamin E deficiency, but this deficiency was not recognized for another 10 years.

Progress in understanding the role of vitamin E in intermediary metabolism was greatly advanced by the 1936 report of Evans and colleagues on the isolation of an alcohol from wheat germ oil with marked vitamin E biologic activity. They assigned the chemical formula $C_{29}H_{50}O_2$ to this "isolate" of fertility and proposed the name α-tocopherol, from the Greek *tokos,* meaning "childbirth," and *phero,* "to bear or bring forth," with the suffix *ol* to indicate that the substance was an alcohol (Figure 39-1). In 1937, the same workers isolated β-tocopherol and γ-tocopherol from other vegetable oils and made note of their lesser biologic activity. Almost simultaneously, Olcott and Emerson presented evidence that the tocopherols were effective antioxidants, as suggested by the earlier report of Olcott and Mattill on the association of "inhibitols" and vitamin E in lettuce oil.

In 1940, Pappenheimer demonstrated that α-tocopherol prevented the myopathy observed in ducklings fed the Evans and Burr diet, and, in the same year, Mackenzie and colleagues presented evidence that the myopathic changes found in vitamin E–deficient rabbits could be prevented by feeding α-tocopherol. They also noted that simultaneous feedings of cod liver oil and

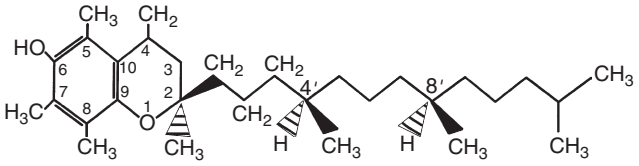


Figure 39-1 Structure of RRR-α-tocopherol, the most active and abundant form of vitamin E. This schema uses the RS nomenclature for specifying configuration at the three chiral centers at carbons 2, 4′, and 8′. *R,* Clockwise (right-handed) orientation; *S,* counterclockwise (left-handed) orientation; *lined triangles,* behind the plane of the paper; *solid triangles,* in front of the plane of the paper.

other lipids high in unsaturated fatty acids inactivated dietary vitamin E, especially in the presence of pro-oxidant ferric salts. These observations presaged the many studies, still ongoing, addressing the importance and interrelationships of prooxidants, oxidant vulnerability, and antioxidant defenses during intermediary metabolism.

ESSENTIALITY OF VITAMIN E AS A CHAIN-BREAKING LIPID ANTIOXIDANT OF CELLULAR MEMBRANES

A large body of work now argues convincingly that the mechanism of action of vitamin E in curing the diverse vitamin E deficiency syndromes depends on its singular effectiveness as a biologic antioxidant, strategically located in every cellular membrane and essential for their structural integrity and function.[13-16] Subtle, radical-mediated perturbations of membrane structure can alter receptor sites, membrane functions, and membrane-bound enzyme activities. Such changes can have serious consequences in some tissues (and some species) and can leave those less vulnerable seemingly unaffected.[16-18] This spectrum of susceptibility would explain much if not all of the variability in vitamin E deficiency syndromes among species, organs, developmental periods, dietary intake, and environmental conditions.[13-15]

For example, vitamin E–deficient rabbits typically exhibit muscular dystrophy, but, in contrast with the vitamin E–deficient rat, they have red blood cells (RBCs) that are resistant to peroxidative hemolysis. In rabbits maintained on standard laboratory chow, RBCs contain two to three times as much linoleic as arachidonic acid, the more highly unsaturated and readily oxidized of the two. By contrast, rabbit muscle contains up to four times more arachidonate than linoleate. The opposite is true of the rat. Therefore rabbit muscle is the target tissue in tocopherol deficiency, whereas in the rat it is the RBC. Supplementing the diet of vitamin E–deficient rabbits with arachidonic acid, however, makes the rabbit RBC susceptible to oxidant damage. Specific membrane phospholipid architecture also influences the different patterns of susceptibility displayed by the rabbit and rat RBC.[19] Disruption of function in membranes deficient in vitamin E also is influenced by the physical location of α-tocopherol in the membrane,[16] the degree of local oxidative stress, and the juxtaposition and adequacy of other antioxidant defense systems.[13,15,16,18,19]

NON-ANTIOXIDANT FUNCTIONS OF VITAMIN E

Although some investigators believe that all functions of vitamin E will eventually be explainable on the basis of antioxidant properties, certain actions, especially in invertebrates and lower mammals, appear to be based on other mechanisms. Such actions include the prevention of fetal resorption in the rat and the influence of vitamin E on the growth, phenotype, and reproduction of *Rotifera,* a freshwater metazoan.[15] Of more pertinence to higher primates and humans is the observation that α-tocopherol functions as an ionophore for potassium and rubidium ions in mitochondria but not for sodium and lithium.[15] Furthermore, α-tocopherol and its oxidation product, α-tocopherol quinone, are specific inhibitors of sodium-potassium adenosine triphosphatase (Na^+,K^+-ATPase) in brain microsomes.[15] Boscoboinik and colleagues[20,21] demonstrated a regulation by the tocopherols of vascular smooth muscle cell proliferation and protein kinase C activity, which is distinct from their activity as antioxidants. Other investigators[22,23] observed an erythropoietic effect of vitamin E in human patients infected with human immunodeficiency virus, especially when they are treated with zidovudine. These observations lend support to the earlier suggestion of Drake and Fitch[24] that humans may have a latent requirement for vitamin E in erythropoiesis. Studies of such possible non-antioxidant functions may lead to elucidation of the role of vitamin E nutrition during gestation.

FUNCTIONAL REQUIREMENTS FOR VITAMIN E

The earliest marker of vitamin E deficiency in the human, as in the rat, is increased hemolysis of RBCs on exposure to peroxide radicals. This effect can be seen before onset of, or in the absence of, clinical anemia.[25-27] Membrane susceptibility to oxidant stress is related to the content of readily oxidized unsaturated fatty acids.[28-31] Of greater clinical importance, however, are the slowly developing signs of oxidant damage to the central and peripheral nervous systems that are seen when vitamin E nutrition is inadequate for an extended period, especially if such dietary inadequacy begins soon after birth. Susceptibility of the RBC and brain to oxidant damage relates to similarities in the lipid composition of their cellular membranes. Furthermore, in the case of the RBC,[32] it also relates to their oxygen-rich, iron-rich environment and to the lability of their vitamin E content, which is readily exchanged with the vitamin E present in the high-density lipoprotein (HDL) and other lipoprotein fractions of the blood.[33,34] Of considerable clinical importance, the amount of vitamin E in the plasma, the RBC membrane, and other membranes of the body is determined largely by the overall concentration of these lipid-rich components of the blood.[35-38] The rate of equilibration between the plasma vitamin E and that of cellular membranes in different tissues varies greatly, however. For example, the content of α-tocopherol is highest in gray matter from the frontal cortex and thalamus, whereas that in gray matter from the cerebellum and spinal cord is significantly lower.[39] Depletion, when it occurs, is seen first in the cerebellum and spinal cord and last in the cerebral hemispheres and medulla and pons.[39-43] Once depleted, the content of vitamin E in the central nervous system is restored only slowly and in the reverse order. Accordingly, tocopherol therapy for prevention or reversal of nervous system lesions must be continued indefinitely, and signs of improvement cannot be expected until long after correction of the RBC defect.

From a practical standpoint, vitamin E deficiency in humans usually is defined as a plasma ratio of vitamin E to total lipids (in milligrams per gram) of less than 0.8, with RBC hemolysis in the standard hydrogen peroxide hemolysis test of greater than 10%. The recommendation for vitamin content of the diet relative to polyunsaturated fatty acid content of dietary lipids is for a ratio of vitamin E to polyunsaturated fatty acids of at least 0.7 (in milligrams per gram).[35,36,44,45] Neither of these lipid ratios is entirely satisfactory, owing to the highly variable fatty acid composition and content of dietary lipids and the difficulty in assessing the effect of dietary polyunsaturated fatty acid on membrane antioxidant requirements.[15,16,19,44,45] As discussed later, an assessment of membrane peroxidizability is the best measure of adequacy of vitamin E nutrition for the rapidly growing and differentiating tissues of the fetus and newborn. The RBC has been widely

used for this purpose because of its accessibility and because the peroxide hemolysis test (despite its limitations) provides useful information and is simple to perform.

HUMAN VITAMIN E DEFICIENCY SYNDROMES

In humans, none of the manifestations of vitamin E deficiency—spinocerebellar degeneration, myopathy, retinopathy, hemolytic anemia—occur in the absence of predisposing conditions such as (1) prematurity coupled with feeding of formulas rich in polyunsaturated fatty acid relative to the tocopherol content,[46-49] especially if the formulas are enriched with iron; (2) chronic pathologic states that interfere with the absorption of fats and fat-soluble vitamins, such as primary biliary atresia, hepatobiliary cholestasis, and fibrocystic disease[37,38,50-52]; and (3) inborn errors of metabolism that interfere with absorption, distribution, and retention of the vitamin, such as the several abetalipoproteinemia syndromes,[53-59] and the mutations in the liver α-tocopherol transfer protein, which cause familial isolated vitamin E deficiency (FIVE) and certain of the Friedreich ataxia syndromes.[60-65] Study of the disposition and metabolism of the tocopherols in these disorders has greatly advanced understanding of a complex and highly regulated system that maintains critical levels of RRR-α-tocopherol (i.e., 2R,4′R,8′-α-tocopherol) in plasma and tissues, even under conditions of dietary restriction.

PRESERVATION OF MEMBRANE: α-TOCOPHEROL

This paucity of vitamin E deficiency syndromes in humans is related not only to the widespread availability of tocopherols in diets of the world and the efficient uptake and retention of α-tocopherol in plasma and tissues but also to the avidity with which membrane content of vitamin E is protected during oxidant stress by other biologic antioxidant systems. Except in the presence of free iron, which is a potent prooxidant, the tocopherol peroxy radical formed in the lipid membrane during encounters with oxidant free radicals results in neither propagation of a free radical chain reaction nor formation of the stable oxidized product, tocopherol quinone. This is the case because the tocopherol peroxy radical is returned to its original state by transfer of the radical to vitamin C and the closely linked selenium-dependent glutathione peroxidase–reduced glutathione–glutathione reductase antioxidant network.[6,13,14,39,66-73] Coenzyme Q_{10} is a lipid antioxidant that interacts directly with the aqueous phase of the cell and also functions to spare vitamin E.[74] Like vitamin C, it is consumed before the membrane content of vitamin E is depleted. For example, in vitamin E–deficient humans and rats, none of these plasma-based systems, the availability of urate or protein sulfhydryl groups, or up-regulation of glutathione peroxidase or superoxide dismutase activity or coenzyme Q_{10} can substitute for α-tocopherol in its role as antioxidant and structural stabilizer for the complex, lipid-rich cellular membrane.[43,66-69]

These considerations underline the importance of general nutritional support for maintaining the body's antioxidant defenses. Such support is especially critical during periods of rapid growth or illness and in the premature infant, with meager nutrient reserves. With the availability of parenteral amino acids, lipid emulsions, and pediatric multivitamin infusate, which includes generous amounts of vitamin E, the management of sick premature infants from the nutritional standpoint has greatly improved.

INTESTINAL ABSORPTION OF VITAMIN E

Vitamin E is absorbed in the upper intestine, together with digested fats, in particular, the medium chain triglycerides.[75,76] An absolute requirement for micellar solubilization of unesterified vitamin E by intraluminal bile acids before it can be absorbed by the enterocyte has been documented.[37,51] If this requirement is met, α-tocopherol and γ-tocopherol, the major forms of vitamin E in the diet, are equally well absorbed by a process of nonsaturable, non–carrier-mediated passive diffusion.[37,45] Within the intestinal cell, reesterified free fatty acids (FFAs), apolipoproteins, phospholipids, cholesterol, α-tocopherol, and γ-tocopherol are incorporated into chylomicrons for secretion by way of the lymphatics and thoracic duct into the systemic circulation.[77-79] Liver uptake of postabsorptive chylomicrons is discussed later, under "Plasma Transport of Vitamin E."

EXCRETION OF VITAMIN E

Although the liver preferentially secretes α-tocopherol in very-low-density lipoproteins (VLDLs) and γ-tocopherol in bile, both forms are consistently found in the bile after oral intake, with the proportion of γ to α decreasing as increasingly generous amounts of both forms are fed. Between 10% and 75% of an oral dose of vitamin E is excreted in the feces, depending on size of the dose and functional health and maturity of the intestinal tract.[77] These phenomena probably explain the relative lack of toxicity of vitamin E compared with that of other fat-soluble vitamins.

SOURCES AND ACTIVITY OF VITAMIN E COMPOUNDS

Vitamin E activity is found in eight naturally occurring compounds in the tocopheryl class, all of which are derivatives of 6-chromanol. Four compounds consist of a tocol structure attached to a saturated phytyl chain, the tocol structure designated αβγδ, depending on the number and position of methyl groups on the benzene ring. Four corresponding tocols with three double bonds in the phytyl side chain are designated tocotrienols (see Table 39-1). The tocopherols have greater biologic activity than the tocotrienols on the basis of standard vitamin E activity assays. Both are widespread in plants such as nuts, seeds, oils, fruits, vegetables, grains, and grasses (mainly in an unesterified form), and they appear to play a developmental role in plant life. For example, the germinating capacity of grain is related to its tocopherol content.[80] Biogenesis of tocopherols in plants probably is through the intermediate of the trienol structure by hydrogenation.[80] Little is known about the importance of tocotrienols for human nutrition, but studies in this regard suggest a role for the tocotrienols in suppression of epidermal growth factor and reduction of protein kinase C (α) activation.[81]

The antioxidant activity of the tocopherols and tocotrienols requires the presence of the free alcohol group on the benzene ring.[82-85] Ester forms are totally inactive as antioxidants until hydrolysis has occurred. Because the free alcohol form is highly susceptible to oxidation, commercial preparations are in the form of acetate, succinate, or nicotinate esters (Table 39-2). Ester hydrolysis occurs readily in the intestine but is only slowly accomplished after parenteral injection. In both dogs and humans, measurable levels of the ester (at least 10% of the peak 4- and 8-hour levels) are still present in the bloodstream after intramuscular administration.[86] One stable preparation of free α-tocopherol has been developed but is not commercially available.[86]

Each of the four tocopherols and the four tocotrienols can occur in eight stereoisomeric forms related to the chirality (handedness) of carbon atom 2. Only the RRR isomers occur in nature and have been shown by various assays to have significantly greater biologic activity than that of synthetic preparations of the other isomers. The chemical composition and configuration[39,87] of free α-tocopherol (molecular weight, 430.66) are shown in Figure 39-1. Biologic activity of the free tocopherol alcohols is modified by the number and position of methyl groups on the benzene ring; full methylation, as in the α form, results in the greatest activity (see Table 39-1). Activity also depends on

TABLE 39-2

Biologic Activity and Stereochemical Configuration of Vitamin E Compounds

1 mg	All-racemic-α-tocopheryl acetate, 2RS,4′RS,8′RS configuration (the synthetic mixture of four mirror-image pairs) Formerly (DL)-α-tocopheryl acetate	1.0 IU*
1 mg	RRR-α-tocopheryl acetate, 2R,4′R,8′R configuration (the natural stereoisomer) Formerly (D)-α-tocopheryl	1.36 IU*
1 mg	All-racemic-α-tocopherol, 2RS,4′RS,8′RS configuration	1.1 IU†
1 mg	RRR-α-tocopherol, 2R,4′R,8′R configuration	1.49 IU†

*Activity expressed as international units (the USP standard) and based on standard biologic assays as confirmed by several investigators (rat fetal resorption, erythrocyte hemolysis, muscular dystrophy, exudative diathesis, encephalomalacia). Most of the difference in activity of the stereoisomers of α-tocopherol results from differences in the chirality at the 2 carbon position, where the phytyl tail meets the chromanol ring. For example, by comparison, 2RS,4′ R,8′R-α-tocopherol (2-ambo-α-tocopherol) and 2S,4′R,8′′R α-tocopherol (2-epi-α-tocopherol, the International Standard before 1956) have approximately 60% and 40% activity, respectively, on the basis of the fetal resorption bioassay. Because of their greater stability, currently available commercial preparations of vitamin E are in the ester form, primarily the acetate, but also the succinate and nicotinate forms.

†Activity calculated on basis of molecular weight compared with the acetate form. The free alcohol forms are available only on an investigative basis.

USP, United States Pharmacopeia.

Data from references 14, 17, 39, 60, 61, 82, 85.

the following: the length of the phytyl side chain (shortening or absence markedly decreases activity); the chirality (stereospecificity) of carbon atom 2, in which the phytyl tail meets the chromanol ring; and, to a lesser extent, the chirality of carbon atoms 4′ and 8′ in the phytyl side chain.

Discrimination in Favor of α-Tocopherol During Intermediary Metabolism

γ-Tocopherol, 7,8-dimethyl tocopherol (molecular weight, 416.66), is the major tocopherol found in soy and corn oil. With the increasing substitution of vegetable for animal fats, γ-tocopherol now accounts for 40% to 50% of vitamin E content of the average U.S. diet. Traditional diets of countries such as Brazil[87] contain predominantly the γ form (see Table 39-1). Because γ-tocopherol has only 10% (at most 20%) of the biologic activity of α-tocopherol, however, it makes only a modest contribution (in the range of 10 to 20%) to overall vitamin E nutrition.[83]

The greater biologic activity of RRR-α compared with that of γ-tocopherol (and the non–RRR-α isomers) results from discrimination, during normal intermediary metabolism, in favor of the α form. This discrimination does not occur in the intestine unless esterified forms are fed, in which case hydrolysis of the RRR esters is faster and more complete.[39] The unesterified α-tocopherol–to–γ-tocopherol ratio in postabsorptive chylomicrons is similar to that in the diet. Once in the bloodstream, postabsorptive chylomicrons undergo triglyceride hydrolysis by action of endothelial lipoprotein lipase, with the formation of chylomicron remnants, excess surface components, and, to a small extent, FFAs and free γ-tocopherol and α-tocopherol. An interchange occurs between apoprotein E (apo E) of HDL and α-tocopherol and γ-tocopherol in excess surface components and chylomicron remnants. Subsequently, the modified remnants and downsized chylomicrons (now containing apo E) are taken up by the liver.[60,61]

During liver uptake of chylomicrons and chylomicron remnants, a major discrimination in favor of α-tocopherol over γ-tocopherol occurs, through the action of a liver binding and transfer protein. This protein, first demonstrated in the rat,[88,89] has 10 times the affinity for α-tocopherol versus γ-tocopherol.[61,77,90] It appears to be critical in the uptake and transfer of α-tocopherol to liver organelles for incorporation into nascent VLDL for secretion into the blood. Catabolism of nascent VLDL, enriched in α-tocopherol, results in a sustained increase in the level of α-tocopherol in the plasma and subsequent tocopherol enrichment of low-density lipoprotein (LDL) and HDL through interchanges between the various lipoproteins. By contrast, the increase in plasma γ-tocopherol is transitory because of its preferential excretion in the bile after liver uptake. As a result, γ-tocopherol accounts for only approximately 15% of vitamin E content of the plasma.[60,61,83,84,91]

Patients with the genetic vitamin E deficiency syndrome FIVE have a missense mutation in the gene that codes for this liver uptake and transfer protein.[60-63] Persons homozygous for this defect have extremely low to undetectable levels of total vitamin E in the plasma, with the level of α-tocopherol consistently less than that required to meet critical tissue needs. As a result, signs of nervous system dysfunction (spinocerebellar degeneration) develop in the absence of any abnormality in fat absorption, lipid metabolism, or dietary deficiency; the age at onset depends on the completeness of the genetic defect. Studies in these patients have shown that the liver transfer protein discriminates not only in favor of α-tocopherol over γ-tocopherol but also in favor of the natural RRR-α isomer over other synthetically prepared isomeric forms, for example, the 2S epimer (2S,4′R,8′R-α-tocopherol).[60,61]

Fortunately, in patients with FIVE syndrome, moderately high doses of α-tocopherol (800 to 1200 IU/day), continued over time, can compensate for the defect in tocopherol enrichment of the VLDL.[60,61] This compensation is accomplished through direct transfer of α-tocopherol in postabsorptive chylomicrons to the HDL and LDL, independent of the liver transfer protein and VLDL.

Discrimination at the Tissue Level

A further discrimination in favor of α-tocopherol occurs at the level of the RBC, brain, and spinal cord, which take up and retain RRR-α-tocopherol in preference to the γ form. As a result, the ratio of α to γ in these (but not other) tissues is greater than that in the plasma.[39,91] As in the case of the liver tocopherol transfer protein, the RBC and central nervous system also retain RRR-α-tocopherol in preference to its other isomeric forms. This tissue discrimination relates to the spatial relationship of the tocopherols and their lipoprotein carriers. A moderate chiral selectivity relative to plasma is exhibited by the RBC in favor of the RRR form, presumably because of accentuation of the chiral properties of phospholipids, cholesterol, and proteins by the more structured lipid environment of the RBC, brain, and spinal cord compared with the more relaxed environment of the plasma lipoproteins.[39] As a result, α-tocopherol comprises approximately 87% of RBC vitamin E, compared with 83% in the plasma.[84]

ROLE OF γ-TOCOPHEROL IN HUMAN NUTRITION

The importance of these relationships for the central nervous system has been demonstrated in feeding studies with α-tocopherol and γ-tocopherol and with natural RRR-α-tocopherol and its other stereoisomers. After 3 months on a low–vitamin E diet, rats fed α-tocopherol and γ-tocopherol for only 24 hours demonstrated 15 times as much α-tocopherol (versus γ-tocopherol) in the brain and 8 times as much α-tocopherol in the adrenal gland.[91] When rats were fed only γ-tocopherol, the brain still contained 3 times as much α-tocopherol as γ-tocopherol, but the adrenal contained 10 times more γ-tocopherol than α-tocopherol.[90] Similarly, Bieri and Evarts,[83] in long-term feeding studies in rats on diets containing up to 4 times as much γ-tocopherol as α-tocopherol, reported that, after 3 months, γ-tocopherol accounted for 60%

of vitamin E in adipose tissue, 50% in muscle, and 40% in kidney and spleen. It remained low in the testes (25%), liver (19%), and plasma (27%). Unfortunately, the relative content of α-tocopherol versus γ-tocopherol was not measured in the RBCs or the central nervous system, which are the tissues most able to discriminate in favor of the α form.[39,40] One would predict (had they been measured) that the brain and RBC would have shown the least accumulation of γ and the greatest retention of α. This prediction is supported by studies by Ingold and colleagues,[39] in which vitamin E was provided as equal amounts of deuterated RRR- and SRR-α-tocopherol. All tissues discriminated in favor of the natural isomer. The greatest discrimination, however, occurred in the brain, which accumulated more than five times more deuterated RRR- than SRR-α-tocopherol, with no decrease in rate of RRR accumulation after 5 months on the diet.[39]

γ-Tocopherol and Vitamin E Nutrition in the Fetus and Young Infant

The importance of γ-tocopherol to overall vitamin E nutrition may relate to its role in sparing α-tocopherol for the brain and RBC when dietary intake of the α form is limited. This effect could have significance for the rapidly growing fetus and newborn, especially under conditions of marginal maternal nutrition. Bieri and Evarts[83] estimated that γ-tocopherol, when present in a diet sufficient in α-tocopherol but at a γ/α ratio of 2 to 3:1, may account for as much as 20% of the total vitamin E activity of the diet. Plasma levels of γ-tocopherol in infants fed a formula (Nutramigen) with three times as much γ-tocopherol as α-tocopherol (total tocopherol content, 29.7 μmol/L) were found to have plasma levels in which the γ form contributed 15% of total plasma tocopherol.[92] Cord blood γ-tocopherol levels of infants in Japan, where the intake of soy products is high, have been reported as accounting for 13% of total tocopherols in cord blood.[93]

Not yet determined, however, is how much γ-tocopherol as opposed to α-tocopherol is advisable in the feedings of infants whose natural food is colostrum and breast milk, both of which are rich in α-tocopherol. At least in young infants, a small study from Japan suggests that the concentration of non-α forms should be limited, even if the amount of α-tocopherol is adequate.[93] This study compared the effects of breast and formula feedings on plasma and RBC concentrations of tocopherol. The formula used contained 2712 μg/dL of total vitamin E, of which large amounts were contributed by β-tocopherol, γ-tocopherol, and δ-tocopherol—2.4%, 45%, and 29%, compared with 24% from α-tocopherol. Breast milk collected 5 to 7 days post partum had a total vitamin E content of 749 μg/dL, of which 10% was γ-tocopherol and 90% was α-tocopherol. The actual concentration of α-tocopherol in the two milks was nearly the same, 671 μg/dL for breast and 645 μg/dL for formula milk, but the total vitamin E content was more than three times higher in the latter. Surprisingly, during the first few months after birth, the mean concentration of α-tocopherol in plasma and RBCs of breast-fed infants was greater ($p < .05$ for both) than in those fed formula: 786 μg/dL ± 47 SEM in plasma and 274 μg/dL packed cells ± 22 SEM in RBCs in the breast-fed infants, versus 596 g/dL ± 60 SEM in plasma and 154 μg/dL ± 9.4 SEM in RBCs in the formula-fed infants. (Non–α-tocopherol forms either were not detectable in the RBC or, possibly, were not measured, because no values were given for these parameters.) Plasma total vitamin E concentrations were comparable in breast-fed and formula-fed infants at 873 and 891 μg/dL, respectively, with non-α forms accounting for 10% (γ) of total plasma tocopherol in breast-fed and 33% (26% γ and 7% δ) in formula-fed infants.[92] These data suggest that, at least in the rapidly growing newborn, a competition exists among the different vitamers[90,92] when the amount of α-tocopherol is relatively low compared with other tocopherol forms. This possibility, of course, amounts to yet another argument in favor of breast milk.

PLASMA LIPOPROTEINS

The principal classes of lipoproteins in the blood are the chylomicrons (density gradients below 0.95), VLDLs (gradients of 0.95 to 1.006), LDLs (gradients of 1.006 to 1.063), HDLs (gradients of 1.063 to 1.21), and very-high-density lipoproteins (VHDLs) (gradients above 1.25). Chylomicrons, on a dry weight basis, are made up of 80% to 95% triglyceride and have a protein content of only 1% to 2%; VLDLs have 40% to 80% triglycerides and 5% to 10% protein; LDLs consist of 10% triglycerides and 25% protein; and HDLs contain 1% to 5% triglycerides and 45% to 50% protein. The primary function of the lipoproteins is transport of lipids, chiefly triglycerides (chylomicrons and VLDLs) and cholesterol (LDLs and HDLs). In addition, the protein component of lipoproteins, the apolipoproteins, has important metabolic functions. The association of apolipoproteins and lipids involves the same sort of noncovalent interactions seen in membrane formation, and the components are quite free to move around, both within the lipid aggregates and between different lipoproteins in the circulation. The apolipoproteins fix the general character of the aggregates but do not create exactly reproducible assemblies. Accordingly, the lipoproteins are heterogeneous mixtures that become even more heterogeneous during transit in the bloodstream.[94]

Plasma Transport of Vitamin E

Vitamin E is transported in plasma in association with lipoproteins and chylomicrons. No specific lipoprotein or lipid is solely responsible for this function. Plasma tocopherol concentration depends largely on plasma lipid concentrations, and a direct and high correlation has been documented between elevated (or depressed) plasma lipid levels and elevated (or depressed) plasma tocopherol levels.[33-38] In patients with primary hyperlipidemia, the lipid primarily responsible for the hyperlipidemia also carries the bulk of the tocopherol. Plasma tocopherol levels may be markedly elevated. To a lesser degree, plasma vitamin E also is elevated in obesity, diabetes mellitus, and hypothyroidism.[51,93] A study in healthy Japanese schoolchildren found a higher plasma tocopherol level and a significantly lower RBC tocopherol level in obese children than in children of normal weight.[93]

If hyperlipidemia is complicated by impaired intestinal absorption of vitamin E, as in cholestatic liver disease, the elevated plasma lipids may compete so successfully with tissues for the limited supply of vitamin E that signs of RBC and central nervous system vitamin E deficiency appear despite plasma tocopherol levels within or above the normal range. When, however, the plasma vitamin E content is corrected for plasma lipid content, the relative deficiency in the plasma becomes evident. A tocopherol–to–total lipid ratio greater than 0.8 (mg/g) has been recommended as an indication of vitamin E sufficiency that takes into account the interdependence of lipid and tocopherol concentrations.[35-38]

Influence of Apolipoproteins on the Distribution of Tocopherol in Plasma

Despite the aforementioned considerations, the distribution of tocopherol between tissue and lipid constituents of the plasma is not entirely passive and nonspecific. The tocopherol content of lipoproteins is influenced by the content of apolipoproteins, as is clearly shown in the work of Behrens and colleagues[95] (Figure 39-2).

The apolipoproteins are grouped into five families designated A to E, all of which have important but incompletely understood

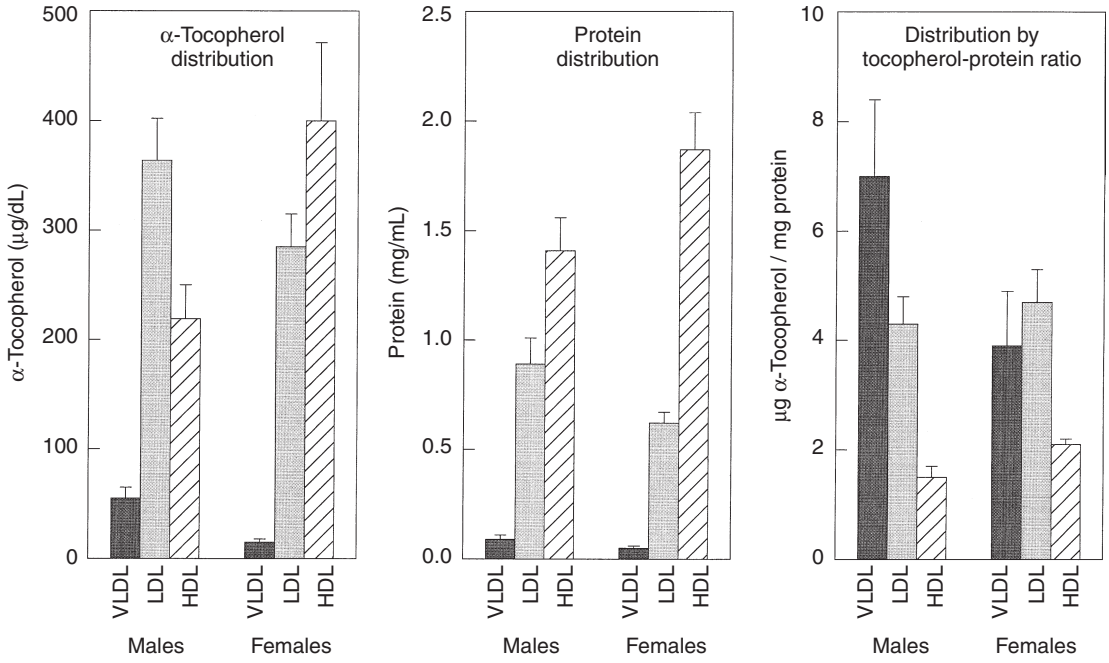


Figure 39-2 α-Tocopherol and protein content of plasma lipoproteins in healthy adults with normal lipid and cholesterol levels (mean ± SD for total tocopherol, μg/dL for plasma; males, 835 ± 82 μg/dL; females, 1035 ± 86 μg/dL.) As has been consistently reported by various investigators, more high-density lipoprotein (HDL) than low-density lipoprotein (LDL) was found in the plasma of women than in that of men in this study. The difference in distribution of tocopherol between the sexes was a reflection of the difference in protein content of the three different lipoprotein classes. Quantitatively, very-low-density lipoproteins (VLDLs) account for only a small proportion of total lipoproteins in either gender. They carry the greatest amount of tocopherol, however, relative to protein. This finding may have relevance to the adequacy of vitamin E nutrition during the hyperlipidemia of pregnancy, which is characterized by a disproportionately large increase in triglycerides and VLDL. The study population was from the Ottawa area of Canada. *(Data from Behrens WA, Thompson JN, Madère R: Distribution of alpha-tocopherol in human plasma lipoproteins,* Am J Clin Nutr *35:691, 1982.)*

metabolic functions. Apo B in VLDL, for example, has a primary role in triglyceride transport. Apo C-II and apo A-I are activators of enzymes involved in lipolysis, lipoprotein lipase, and lecithin cholesterol acyl transferase. Apo E binds to specific liver and endothelial receptors. In adults, the apo B lipoproteins in LDLs are primarily responsible for cholesterol transport to tissues.[78,94] Specific LDL receptor sites are present in the plasma membranes of most cells, and cholesterol, together with vitamin E, is delivered to the cells by this mechanism.[96] HDL apolipoproteins are primarily responsible for cholesterol transport to the liver (catabolic).[94]

The different distribution of the apolipoproteins and tocopherol in the HDL and LDL of men and of women, as seen in Figure 39-2, has been reported by many investigators. When data from men and women are considered together, the correlation of tocopherol with lipoprotein protein is highest for HDL, lowest for LDL, and intermediate for VLDL (HDL: correlation [r] = .93; VLDL: r = .72; LDL: r = .51). When data for men and women are considered separately, however, a significant correlation of tocopherol with protein (within the LDL class) is found in men but not in women.[95] Moreover, in a small but probably significant segment of the population, intake of vitamin E seems to influence the proportion of cholesterol carried by HDL relative to that carried by LDL.[97,98] This response to increased intake of vitamin E is seen most often in young, nonobese men who have high LDL and low HDL cholesterol levels and in patients with spinal cord injury. In such circumstances, an increased intake of vitamin E has been associated with a redistribution of cholesterol in favor of the HDL to an extent of 10% to 20%.[97,98] The significance and mechanism of these phenomena are poorly understood, but in rabbits fed a cholesterolemic diet, supplements of vitamin E result in significantly lower concentrations of serum cholesterol, triglycerides, and VLDL than those found in rabbits not supplemented with vitamin E.[99]

Transfer of Tocopherol to Tissues

Several mechanisms have been identified by which α-tocopherol is transferred from human lipoproteins to tissue. A specific high-affinity LDL lipoprotein receptor mechanism has been shown to mediate the delivery of vitamin E from human LDL to fibroblasts.[96] LDL (and tocopherol) can be taken up and degraded by receptor-independent processes. LDL-specific receptor pathways are therefore not the only mechanism for tocopherol delivery to the tissues. For example, human chylomicrons have been shown to transfer tocopherol to human RBCs and fibroblasts during the hydrolysis of triglycerides by lipoprotein lipase. This process involves binding of the lipase to the cell membrane.[38,90,100] Transfer of tocopherol to tissues by the HDL is less well characterized, but it is known that HDL readily transfers tocopherol to the RBC,[33,34] and high-affinity receptors have been described for tocopherol in the RBC and adrenals.[18,90,101] In adults and newborns, it is probably the HDLs that are the primary means of delivery and exchange of tocopherol with RBCs, which lack LDL receptors.[101]

PATTERN OF PLASMA LIPIDS AND LIPOPROTEINS IN THE NEWBORN

Newborns have low plasma levels of total lipids, apolipoproteins, cholesterol, and tocopherol. Newborn plasma also is characterized by a relatively high content of HDLs, a low content of LDLs,

TABLE 39-3

Concentration and Distribution of α-Tocopherol in Red Blood Cells and the Plasma Lipoproteins of Newborns and Pregnant and Nonpregnant Women*

	PLASMA CONCENTRATION (μg/dL)		
Fraction	Nonpregnant Women	Pregnant (at Term)	Newborn (Cord)
Plasma tocopherol	772 ± 144	1417 ± 388	237 ± 62
RBC tocopherol (packed cells)	193 ± 22	201 ± 34	179 ± 33
VLDL tocopherol	60 ± 31 (9%)	332 ± 184 (25%)	14 ± 8 (6%)
LDL tocopherol	271 ± 58 (41%)	558 ± 197 (41%)	68 ± 20 (30%)
HDL tocopherol	331 ± 53 (50%)	454 ± 131 (34%)	143 ± 43 (64%)

*Values are means ± SD: nonpregnant (n = 8); pregnant (n = 20); newborn (n = 20). In the newborn, 64% of plasma tocopherol is carried by the HDL, which constitutes the major lipoprotein class (greater than 60%) in their plasma. By contrast, only 34% of plasma α-tocopherol in the mother is carried in the HDL, which accounts for a lower proportion of her total plasma lipids. The greatest increase in lipid class during pregnancy occurs in the VLDL, which also shows the greatest increase in tocopherol level. Compared with newborns and their mothers, nonpregnant women have intermediate levels of percent distribution for all parameters. Total plasma lipid levels (mg/dL) are much lower in newborns (193 mg/dL ± 52) than in adults (pregnant women: 800 ± 131 mg/dL; nonpregnant women: 389 ± 29 mg/dL; adult men: 501 ± 106 mg/dL). The percent distribution of VLDL, LDL, and HDL within the total lipids is similar to the percent distribution of total vitamin E among the same lipoprotein fractions. In this small sample, no significant difference in RBC tocopherol levels was noted between either adult group and the newborn cohort, probably because of the predominance in cord blood of HDL, the primary carrier for α-tocopherol to the RBC. The mean RBC tocopherol level in the newborns, however, was somewhat lower. The study population was from the Osaka area of Japan.

HDL, High-density lipoprotein; *LDL,* low-density lipoprotein; *RBC,* red blood cell; *VLDL,* very-low-density lipoprotein.

From Ogihara T, Miki M, Kitagawa M, Mino M: Distribution of tocopherol among human plasma lipoproteins, *Clin Chem Acta* 174:299, 1988.

and a very low content of VLDLs, which are poor in triglycerides but rich in cholesterol esters. Newborn HDLs include the subclasses HDL_2 (density gradients 1.063 to 1.092) and HDL_3 and VHDL (gradients greater than1.125).[102-106] These HDL lipoproteins[107] carry the bulk of plasma tocopherol in the newborn (Table 39-3). After birth, VLDL and LDL concentrations increase dramatically,[108] together with the triglyceride content of VLDL (Figure 39-3). The proportions of plasma cholesterol, apo A-I, and apo A-II carried by the HDLs in cord blood are high compared with adult values.[105,106] Nevertheless, despite low levels of LDL and cholesterol in the plasma, cholesterol transport to the tissues is high.[103-106]

Apolipoprotein E in High-Density Lipoprotein of the Fetus and Newborn Infant

The enhanced transport of cholesterol to the tissues by the HDL of cord blood results from the presence of apo E in HDL (not present in the HDL of most children and adults).[109,110] In the neonate, however, and in patients with abetalipoproteinemia (who lack apo B and LDL and have plasma cholesterol levels less than 50 mg/dL), the HDL contains a subfraction rich in apo E and apo E–apo A-II, which migrates with the HDL. This same HDL subfraction, rich in apo E, also is present in many lower species. Apo E–containing HDLs bind to apo B, apo E receptors in fibroblasts, and many other tissues and are responsible for delivering cholesterol to cells and regulating cholesterol metabolism in a manner comparable to that for the LDLs. These distinctive plasma lipoprotein profiles in the fetus and newborn infant,[102-110] as well as the rapid changes that occur in the first weeks after birth, are poorly understood. They almost certainly have importance for the transport and delivery of nutrients to the tissues and may also influence the prenatal and early postnatal transport of vitamin E.

Hyperlipidemia of Pregnancy

Maternal plasma lipids are elevated during pregnancy,* and levels of total lipids, triglycerides, cholesterol, and phospholipids are significantly higher than in the neonate (Figure 39-4). Maternofetal nutrient transfer through the placenta can be accomplished by different mechanisms, including facilitated diffusion, active transport, and simple diffusion. Simple diffusion is the most important mechanism for lipid-derived moieties. By this process, nutrient molecules are carried from a high-concentration to a low-concentration region, and the rate of movement is directly proportional to the concentration gradient. The rate of transfer is a direct function of the concentration gradient and decreases with molecular size and hydrosolubility.[78,79,111]

The tendency to accumulate fat ceases during late gestation because maternal lipid metabolism changes to a catabolic state. Also characteristic of this gestational stage are hepatic overproduction of triglycerides and enhanced absorption of dietary lipids, which result in a marked progressive increase in maternal circulating triglycerides. Furthermore, during late gestation (when tissue accretion in the fetus is at a maximum), an associated increase in FFAs, glycerol, and triglyceride-rich VLDLs and chylomicrons occurs.[78,79,111] The fetus requires not only essential fatty acids from the mother to support growth and brain development but also nonessential lipids as substrates during early postnatal life. Investigations in guinea pigs, primates, and rats indicate that the amount of fatty acids crossing the placenta exceeds that needed to fulfill lipid storage requirements. Maternal FFAs (esterified fatty acids that have been hydrolyzed at the placental level) and FFAs in unmodified lipoproteins are the potential sources of fatty acids that cross the placenta to the fetus.[78,79,111]

Differences in Fatty Acid Composition of Maternal and Fetal Plasma

Essential and nonessential fatty acids share a common transfer mechanism, regulated by the transplacental nonesterified fatty acid gradient, which consistently favors transport to the fetus. The estimated FFA transfer across the placenta is substantial, although less than for compounds such as glucose and alanine. Placental esterification of FFAs appears to be involved in one type of placental FFA transport. Although maternal circulating triglycerides contribute FFAs directly to the fetal circulation, this probably represents a minor contribution to FFA transport except in conditions of exaggerated maternal hypertriglyceridemia (e.g., in diabetic pregnancies).[111]

The fatty acid mixture entering the fetal circulation from the placenta reflects the maternal FFA concentrations of different fatty acids, with the common exception of arachidonic acid. Concentrations of arachidonic acid and docosahexaenoic acid are higher in cord blood than in maternal plasma. The opposite is the case for linoleic acid, which is present in higher concentrations in maternal plasma.[79,111,113] The fatty acid composition of adipose tissue triglycerides in the newborn also differs from that in the mother in a pattern similar to that for plasma. Placental arachidonic acid synthesis by an active desaturation and elongation system that synthesizes arachidonic from linoleic acid is believed to be important to the supply of arachidonic acid to the fetus. Tissue enrichment in arachidonic acid plays an important role during fetal life by maintaining normal function of biologic membranes and perhaps by providing a substrate for prostaglandin biosynthesis. Although postnatal brain development is

*References 78, 79, 107, 108, 111, 112.

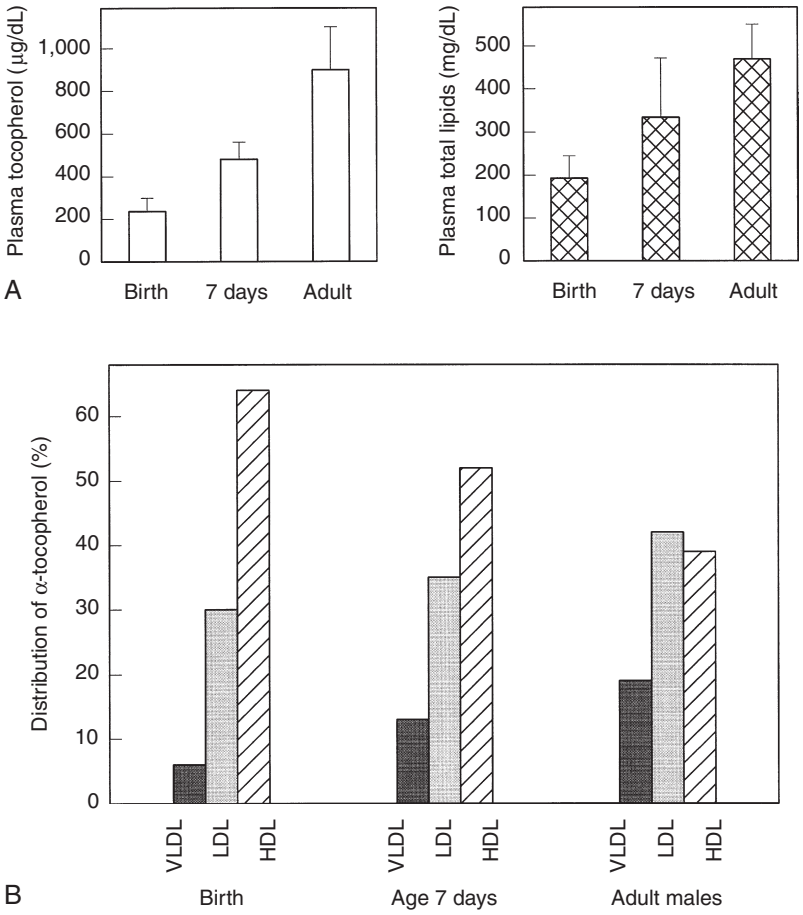


Figure 39-3 **A,** Age-related changes in plasma α-tocopherol and lipid concentrations. **B,** Age-related changes in distribution of α-tocopherol in plasma lipoproteins. In comparison with adults (n = 20), plasma tocopherol levels in the newborns (n = 11) were even lower than plasma lipid levels. More than 60% of plasma α-tocopherol in the cord blood was carried in the high-density lipoprotein (HDL) class. In men, less than 40% of plasma α-tocopherol was carried in the HDL. Newborn very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions increased and the HDL fraction decreased over the first 7 days after birth. Lipoprotein fractions were estimated by ultracentrifugation, and total lipids by the sum of cholesterol, phospholipid, and triglycerides. The study population was from the Osaka area of Japan. *(Data from Ogihara T, Mujake M, Kawamura N, et al: Tocopherol concentrations of leukocytes in neonates,* Ann N Y Acad Sci *570:487, 1989.)*

characterized by an increase in linoleic acid content, chain elongation desaturation products do not increase for several weeks, suggesting that placental transfer of polyenoic fatty acids is of primary importance in fetal accretion of these fatty acids.[79] Such transfer may be compromised under conditions of intrauterine growth restriction.

FETAL ACCUMULATION OF VITAMIN E AND FUNCTIONAL REQUIREMENTS DURING GESTATION

In a series of studies conducted during the 1940s and early 1950s, Mason[114] and Dju and colleagues[115] demonstrated that the concentration of vitamin E, as total tocopherols, in fetal tissues was low and that concentration and body content of vitamin E gradually increased during pregnancy, with a close, direct correlation for content of tocopherol in the body, body weight, and body fat. These early studies were confirmed by Mino and colleagues[116] using more sophisticated biochemical techniques (Figure 39-5). Despite the long-known increased susceptibility of the newborn RBC to peroxide hemolysis, a state of vitamin E deficiency in neonates has been questioned.[44,117,118] In that regard, the ratios of *milligrams of total tocopherol to grams of total fat* present in the body of the newborn (2.7) and the adult (2.5) are comparable. This calculation of this ratio does not take into account the greater proportion of unsaturated to saturated fatty acids in the plasma and RBC membranes of newborns than that in adults, as described previously and shown in Table 39-4. In addition to being highly unsaturated, the lipid content of membranes in newborns is higher than in adults, in whom lipids reside to a greater extent in accumulations of white fat.[31,32,112,113,119]

PATTERNS OF MEMBRANE TOCOPHEROL CONTENT IN THE NEWBORN AND YOUNG INFANT

Because samples are readily obtainable, a large body of data has been accumulated on the uptake, retention, and functional needs of the RBC. Studies of the tocopherol content of platelets, white blood cells, and buccal mucosal cells also have provided important data, but in lesser amounts because of greater assay complexity and difficulty in obtaining an adequate sample size. All of these studies show a decreased membrane α-tocopherol content in newborns compared with that in 1-year-olds, children, and adults[120-124] (Figure 39-6).

Figure 39-6 shows the content of vitamin E for several accessible tissues in the newborn infant, including tissues with and without LDL lipoprotein receptors. All tissues show a low level of α-tocopherol compared with that in older children and adults. Of note is the low concentration of tocopherol in platelets. In human adults and rats, levels of tocopherol in platelets have been shown to reflect changes in the dietary intake of vitamin E more accurately than levels in RBCs, which in turn are more sensitive than levels in plasma lipids and lymphocytes.[125] The low levels of vitamin E in platelets of the newborn probably reflect a marginally adequate supply of the vitamin.[120-125] This limited supply is suggested by the high concentrations of α-tocopherol in colostrum and breast milk, the first foods of the newborn on entry to the oxygen-rich extrauterine environment. Breast milk feedings result in a rapid increase in blood and tissue content of vitamin E, with a peak in platelet tocopherol concentration at age 6 months, which is about the time when other foods, less rich in α-tocopherol, are added to the diet.[124]

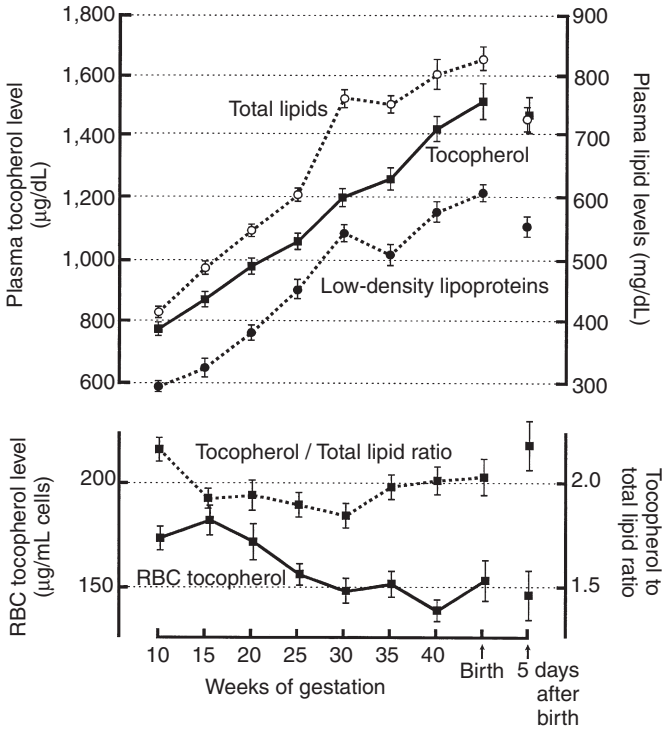


Figure 39-4 Maternal vitamin E nutrition during pregnancy. This graphic summary shows maternal red blood cell (RBC) α-tocopherol in relation to increasing maternal plasma levels of α-tocopherol, total lipid, and low-density lipoproteins during gestation. The greatest relative increase in lipids occurred in the triglyceride class. Despite the rise in plasma E level and the relatively stable α-tocopherol/total lipid ratio, maternal RBC α-tocopherol levels decrease as pregnancy progresses. The study population was from the Osaka area of Japan. *(Data from Mino M, Nagamatu M: An evaluation of nutritional status of vitamin E in pregnant women with respect to red blood cell tocopherol level,* Int J Vit Nutr Res *56:149, 1986.)*

Healthy breast-fed infants not uncommonly have plasma total tocopherol levels of 2.5 to 3.5 mg/dL while they are taking only milk for nourishment. Preliminary studies estimating the delivery of vitamin E to the fetus by measurement of umbilical vein–to–umbilical artery gradients indicate that over a 24-hour period, a surprisingly large per-kilogram transfer occurs to meet the demands of rapid fetal growth. The magnitude of transfer is, of course, greater during the last trimester. This finding is consistent with the much greater difficulty in maintaining plasma vitamin E levels in the sufficient range in small premature infants than in term infants, who have significantly greater stores of the vitamin.[126-129]

IMPLICATIONS OF MATERNAL HYPERLIPIDEMIA FOR FETAL VITAMIN E NUTRITION

Need for Generous Supplies of Vitamin E in the Maternal Diet

The hyperlipidemia of pregnancy involves an increase in triglycerides in the VLDL and LDL fractions.[79,111,112] This increase in plasma lipids usually is associated with an increase in plasma vitamin E level such that the ratio of tocopherol to total lipids is maintained above 0.8. Accordingly, several laboratories have demonstrated a small decrease in the mean maternal RBC tocopherol content in the last trimester of pregnancy,[112] and in some women (as many as 30% in one study), levels of RBC tocopherol are in the deficient range[17] (Figure 39-7). These findings suggest that in a significant number of women at term, the plasma lipids have increased their tocopherol content at the expense of the vitamin E content in RBCs. Data from a Japanese study demonstrating this relationship are shown in Figure 39-4. This finding is relevant to the observation that the concentration of α-tocopherol in maternal RBCs highly correlates with that in the fetus and newborn, after as well as before controlling for differences in lipid levels.[112,119-121]

Effect of Fatty Acid Composition on Functional Requirements for Vitamin E

The decreased resistance to hydrogen peroxide–induced hemolysis in newborns was first described in the classic studies of Rose and Gyorgy[25,-26] and of Gordon and colleagues[27]; this finding

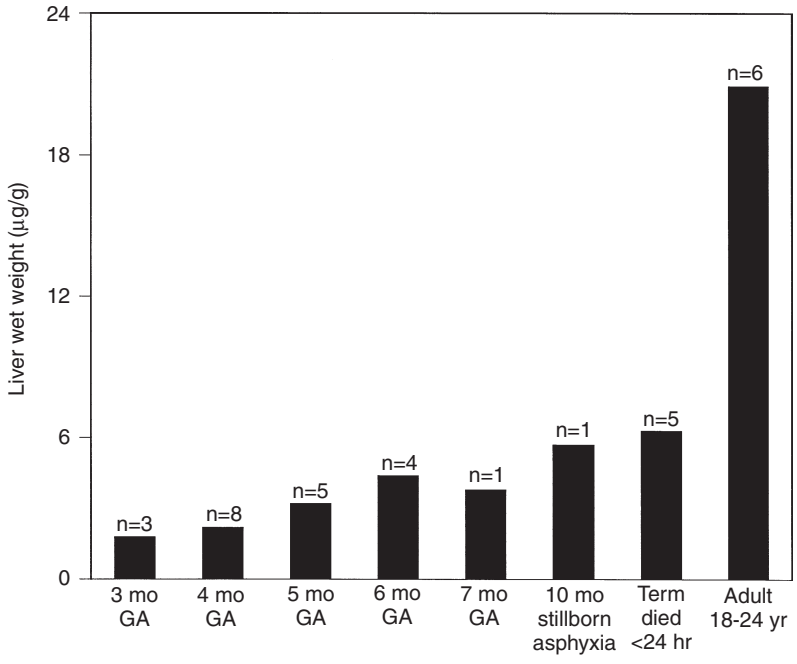


Figure 39-5 Tocopherol concentration in fetal, newborn, and adult human liver. Liver samples were obtained at autopsy after artificial interruption of normal pregnancy, after death from anencephaly or erythroblastosis fetalis on the day of delivery, or after adult accidental death. Liver tocopherol concentrations reported in two studies were in good agreement. *GA,* Gestational age. *(Data from Mino M, Nishino H, Yamaguchi T, Hayashi Met al: Tocopherol level in human fetal and infant liver,* J Nutr Sci Vitaminol (Tokyo) *23:63, 1977; and Dju MY, Mason KE, Filer LJ: Vitamin E (tocopherol) in human fetuses and placentae,* Etudes Neonatales *1:49, 1952.)*

TABLE 39-4

Membrane Fatty Acid Constituents in Cord Maternal and Adult Red Blood Cell Ghosts

Constituent	Cord (n = 10)	Maternal (n = 7)	Adults (n = 11)
Total fatty acids (RBC) μmol/2 mg protein	3.74 ± 0.68*	3.17 ± 1.49	2.24 ± 0.41
Distribution of fatty acids			
% Saturated	54.6*	49.5	53.5
% Monounsaturated	10.9*	13.7	4.0
% Polyunsaturated	31.9*	38.4	30.7
% three or more double bonds	27.2*	20.7	20.1
Principal unsaturated fatty acids			
% Linoleic acid (18:2n-6)	4.7 ± 0.8*	12.7 ± 5.0	10.6 ± 0.9
% Dihomo γ linoleic (20:3n-6)	2.4 ± 0.3*	0.8 ± 0.4	0.8 ± 0.3
% Arachidonic (20:4n-6)	16.4 ± 2.1*	11.1 ± 1.5	11.9 ± 1.7
% Docosahexaenoic (22:6n-3)	6.9 ± 1.3*	7.7 ± 1.3	5.9 ± 1.5
Bis-allylic hydrogen atoms†			
mean per fatty acid molecule	1.94 ± 0.23*	1.79 ± 0.26	1.53 ± 0.25
mean per 2 mg protein	7.25 ± 1.53*	5.60 ± 2.75	3.64 ± 0.79

*p < .05 for cord values as compared with adult values. Maternal values for *bis*-allylic hydrogen atoms are intermediate between those for newborns and nonpregnant adults. The study population was from the Osaka area of Japan.

†Hydrogen abstraction is the initiating step in the chain reaction of lipid peroxidation. Hydrogen in the bis-allylic position is highly susceptible to abstraction.

Modified from Miyake M, Miki M, Yasuda H, et al: Vitamin E and the peroxidizability of erythrocyte membranes in neonates, *Free Radic Res Commun* 15:41, 1991.

has since been extended and confirmed by other investigators[28,31,32,118-122] (Tables 39-5 and 39-6 and Figures 39-8 to 39-12; see also Figure 39-7). The mechanism behind this increased risk of oxidant damage (despite tocopherol RBC concentrations that are at the lower range of normal for adults) was clarified by analysis of the composition of maternal and newborn RBC membranes.[118-122] These studies made it clear that the greater requirement for antioxidant protection of newborn and maternal RBC membranes results from their increased content of lipid, their greater complement of highly unsaturated fatty acids, and their greater number of active (bis-allylic) hydrogen atoms compared with the RBCs of nonpregnant women, older children, and men.[119-121]

These important data and interrelationships are shown in Tables 39-4 to 39-6 and in Figures 39-7 through 39-12, which summarize findings from three different countries on the increased sensitivity of RBC membranes in the fetus and newborn to peroxide injury. The report by Jain and associates,[121] which is summarized in Table 39-6 and in Figures 39-9 to 39-12, examined data for 26 healthy term newborn infants and their mothers from a population with low plasma levels of α-tocopherol. This study noted strong correlations between maternal and newborn plasma and RBC tocopherol concentrations (even without controlling for plasma lipids). Plasma α-tocopherol levels, in paired samples, also correlated significantly, but only after lipid normalization. In a much earlier study involving more than 500 infants, a significant correlation of maternal and newborn plasma vitamin E levels was found without consideration of plasma lipid content.[130]

In a study population of pregnant women with higher plasma tocopherol levels who required investigation at 20 to 40 weeks by percutaneous umbilical blood sampling, analysis of the relation between fetal and maternal plasma tocopherol concentrations showed no such correlation.[120] As in the study by Jain and colleagues,[121] however, strong, consistent correlations were found for concentrations of α-tocopherol in maternal, fetal, and

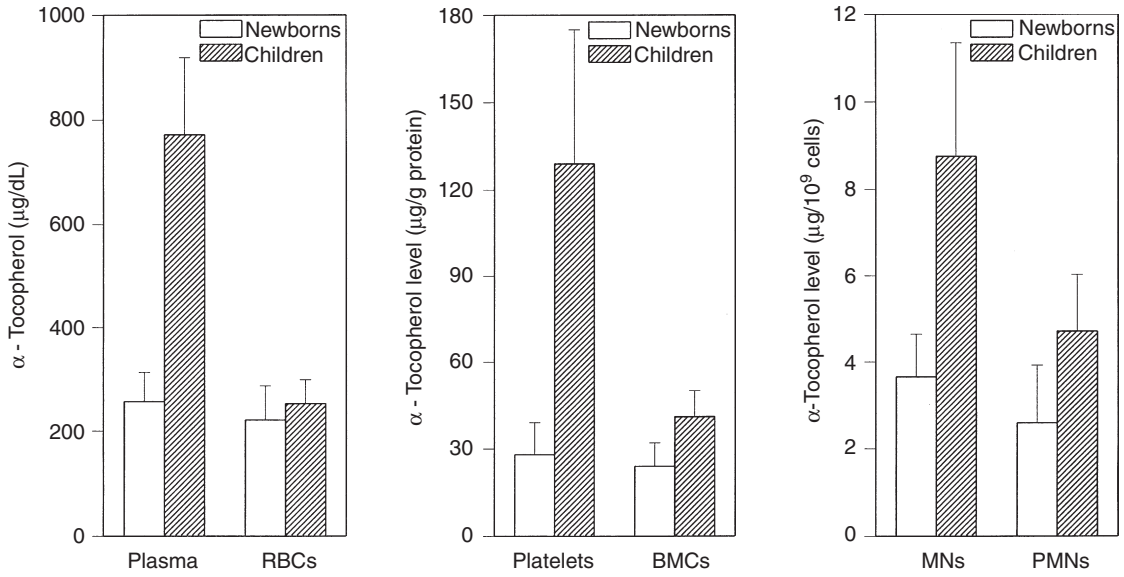


Figure 39-6 Age-related changes in α-tocopherol concentration in plasma, packed red blood cells (RBCs), platelets, buccal mucosal cells (BMCs), and mononuclear (MNs) and polymorphonuclear (PMNs) leukocytes in term newborns and children (3 to 16 years of age). The α-tocopherol level in tissues as well as in plasma was significantly lower in newborns than in children, except for levels in the RBCs, which were comparable. The study population was from the Osaka area of Japan. *(Data from Kaempf DE, Miki M, Ogihara T, et al: Assessment of vitamin E nutritional status in neonates, infants and children—on the basis of alpha-tocopherol levels in blood components and buccal mucosal cells,* Int J Vit Nutr Res *64:185, 1994.)*

newborn RBCs. Similar data also have been reported by investigators in Japan. This consistent finding (i.e., a correlation between concentrations of tocopherol in maternal and fetal RBC membranes) is important because it means that vitamin E supplements to the mother, continued over time, can increase the content of vitamin E in the RBC membranes of the fetus.[120,121] Presumably, this would apply to other membranes as well, in particular those of the brain and spinal cord.

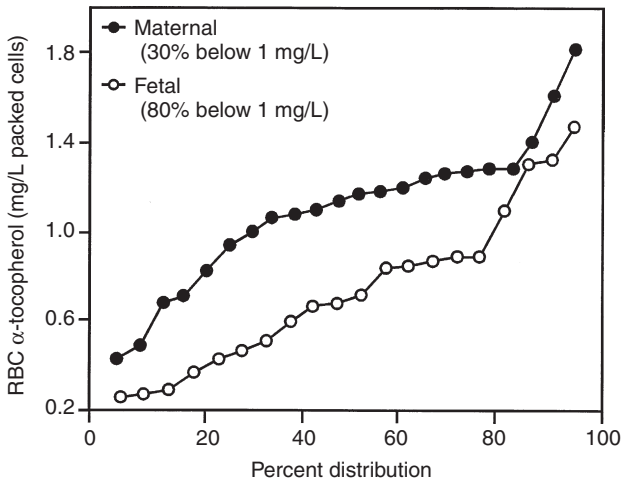


Figure 39-7 Frequency distribution of vitamin E content of centrifugation-packed red blood cells (RBCs). Maternal and fetal samples were obtained by percutaneous umbilical blood sampling between 20 and 40 weeks of gestation for diagnosis of malformations, intrauterine growth delay, toxoplasmosis, and other viral or parasite infections. The mean RBC α-tocopherol level in these fetuses was 0.74 ± 0.16 mg/L, which is less than the critical level for oxidant protection in the H_2O_2 hemolysis assay. The mean maternal RBC α-tocopherol level was 1.08 ± 0.14 mg/L (i.e., 108 μg/dL). Maternal and fetal levels were significantly correlated ($r = .66$; $p = .002$). The study population was from the Montpellier area of France. *(Modified from Cachia O, Léger CL, Boulot P, et al: The red blood cell vitamin E concentrations in fetuses are related to but lower than those in mothers during gestation: a possible association with maternal lipoprotein(a) plasma levels,* Am J Obstet Gynecol *173:42, 1995.)*

As reported by Mino and colleagues,[123] who studied premature and sick term infants for the first 6 months of life, the minimum concentration of RBC α-tocopherol to prevent hemolysis in the peroxide hemolysis test was 100 to 125 μg/dL of packed cells (see Figure 39-8). Based on these figures, the RBC tocopherol concentrations of 85% of fetuses and 15% of mothers in the study by Cachia and associates[120] mentioned earlier (see Figure 39-7) were lower than this critical level. As pointed out by these authors, because of the close correlation of the maternal and fetal RBC tocopherol content, it is reasonable to assume that dietary supplementation in most of these mothers would have benefited the antioxidant defenses of their soon-to-be-born infants.[120]

ESTIMATES OF OXIDANT RISK

In the oxygen-rich, relatively unstable extrauterine environment, antioxidant reserve of the rapidly growing newborn is precarious, even in the absence of perinatal and neonatal complications. For example, in a population of newborns characterized by low plasma and RBC concentrations of vitamin E,[31,32] significant spontaneous peroxidative membrane lipid damage was demonstrated in fresh, untreated RBCs, not exposed to an artificial environmental stress. This peroxidative membrane damage was evidenced by thiobarbituric acid reactivity, lipid fluorescence, and phospholipid-malonyldialdehyde (MDA) adduct formation at twice the magnitude of those observed in adult cells under the same conditions. Additional evidence indicates that at birth, the blood of newborn infants has increased coagulability owing to a marginal to deficient content of vitamin E in RBC membranes.[31,120] Even in adults, with adequate vitamin E nutrition, decreasing platelet adhesiveness is highly correlated with increasing plasma levels of vitamin E within the physiologic range.[131] These observations may have pertinence to the hypercoagulability of the blood of newborn infants, especially sick neonates.[32,120]

The functional requirement for α-tocopherol in the brain is thought to be at least as great as that of the RBC and probably greater. Membranes of the brain and retina, especially during fetal life and early infancy, have a high lipid content and an even higher complement of highly unsaturated fatty acids, particularly docosahexaenoic acid. The lipids of retinal photoreceptors, for example, are made up of 40% to 50% docosahexaenoic

TABLE 39-5

Effect of Oxidant Stress on Neonatal as Compared With Adult Red Blood Cell Ghosts*

	H_2O_2 AS OXIDANT STRESS INDUCER (mmol/L)				
Stress Indicator	**0.0**	**0.625**	**0.75**	**1.0**	**1.5**
α-Tocopherol (μg/mL)					
Neonate	2.27 ± 0.36	2.01 ± 0.33	1.46 ± 0.43†	0.60 ± 0.32†	0.22 ± 0.30
Adult	2.34 ± 0.34	2.08 ± 0.30	1.95 ± 0.40†	0.97 ± 0.31†	0.97 ± 0.31
α-Tocopherol-quinone (μg/mL)					
Neonate	0.0	0.2 ± 0.09‡	0.34 ± 0.10‡	0.72 ± 0.14†	0.40 ± 0.01
Adult	0.0	0.08 ± 0.07†	0.18 ± 0.07†	0.45 ± 0.14†	0.75 ± 0.20
Malonyldialdehyde (nmol/mL)					
Neonate	0.0	37 ± 9‡	37 ± 6‡	40 ± 8‡	59 ± 7§
Adult	0.0	14 ± 4†	14 ± 4†	14 ± 5†	16 ± 7§
% hemolysis at 8 hours					
Neonate	5% ± 3%	12% ± 4%‡	16% ± 7%§	21% ± 10%§	100% ± 0%§
Adult	4% ± 1%	4% ± 1%†	4% ± 2%§	4% ± 10%§	6% ± 2%§

*Washed red blood cells (RBCs) (5% hematocrit) in phosphate-buffered saline were incubated with increasing concentrations of hydrogen peroxide. Analyses were carried out after 8 hours for percentage of (%) hemolysis, concentration of α-tocopherol, α-tocopherolquinone, and malonyldialdehyde. Although RBC α-tocopherol levels were comparable in neonates and adults, the susceptibility of neonatal cells to oxidant challenge was much greater. The study population is from Brussels, Belgium.

†$p < .05$.

‡$p < .01$.

§$p < .001$.

Modified from Vanderpas J, Vertongen F: Erythrocyte vitamin E is oxidized at a lower peroxide concentration in neonates than in adults, *Blood* 66:1272, 1985.

acid.[79,111,132] Even a small decrement in membrane supply of vitamin E places such tissues at risk for oxidant damage. This risk is present during fetal life and the birth process and in the perinatal and newborn period because hypoxic or ischemic episodes followed by reoxygenation result in excessive production of oxidant radicals regardless of initial oxygen tensions.[2,4,133,134] In the rat, a number of studies have shown a protective effect of previous supplements of vitamin E on the outcome of episodes of umbilical cord clamping and release in utero.[135,136] A similar protective effect against hypoxia has been demonstrated in newborn rats.[137] These data are of importance to elucidating the ongoing occurrence of severe retinopathy of prematurity and cerebral palsy among very premature infants, especially those born at 23 to 27 weeks of gestation, who are now surviving as a result of sophisticated care in neonatal intensive care units. Almost all of these infants have been exposed to alternating exisodes of hypoxia and hyperoxia, periods of pulmonary and gastrointestinal failure, repeated systemic infection, and weeks of suboptimal nutrition before reaching a gestational age that even approaches term.[2,4,138-143]

TABLE 39-6

α-Tocopherol Levels of Red Blood Cells and Plasma in 26 Paired Mothers and Newborns at Delivery*

	Mothers	Newborns	*p* Value
RBC vitamin E: nmol/mL	2.95 ± 0.13	2.77 ± 0.14	.16
Packed cells (26)†: nmol/μmol total lipids (25)‡	0.64 ± 0.04	0.56 ± 0.03	.03
Plasma vitamin E: nmol/mL (26)‡	26.13 ± 1.14	5.46 ± 0.41	.0001
nmol/μmol total lipids (24)	2.57 ± 0.11	1.87 ± 0.12	.0001

From Jain SK, Wise R, Bocchini JJ Jr: Vitamin E and vitamin E-quinone levels in red blood cells and plasma of newborn infants and their mothers, *J Am Coll Nutr* 15:14, 1996.

*The low plasma α-tocopherol levels in these New Orleans newborns were significantly below those of their respective mothers *after* as well as *before* normalization for their minimal plasma lipid concentrations. Even in these infants, however, the level of tocopherol in the red blood cell (RBC) was not significantly below that of the mother unless the greater lipid content of the newborn RBC membrane was taken into account (i.e., *after* lipid normalization). It is the greater membrane content of lipids, rich in unsaturated fatty acids, in newborn RBCs that makes them more susceptible to lipid peroxidation than adult RBCs at any given concentration of tocopherol per mL of packed cells. Therefore physiologic characteristics as well as tocopherol concentration determine functional adequacy of vitamin E at the tissue level.

†Number of samples given in parentheses. Values are means ± SE (5.46 nmol/mL = 2.38 μg/mL = 238 μg/dL).

‡Numbers in parentheses are number of subjects in study.

FUTURE DIRECTIONS AND ONGOING STUDIES

Functional Requirement for Vitamin E

An absolute membrane requirement for α-tocopherol that varies from tissue to tissue has been documented and depends to a significant extent on composition of the cellular membrane, stage of development and metabolic, constitutional, and environmental stress and on composition of the diet. In mammals, this requirement cannot be met by any other antioxidant or antioxidant system.[39-43,66-68,74] In humans, in addition to its essentiality for reproduction, the most critical requirement is that for the central nervous system, in which the α-tocopherol content of the cerebral hemispheres, medulla, and pons is tightly guarded.[41] Deterioration of mental function[144] and of the retina[51] occurs later than deterioration of spinocerebellar function in patients with vitamin E deficiency associated with childhood cholestasis and abetalipoproteinemia.[53-65] Long-term, high-dose vitamin E supplementation decreases clinical evidence of deficiency in these and related conditions, but successful pregnancy has not been reported.

The Complexity of Early Stages of Reproduction, Both Normal and Abnormal

Recent work[145-220] suggests that a critical balance of prooxidant and antioxidant elements in tissues and cells of the fetoplacental unit is necessary for normal differentiation and development and confirms an important role for vitamin E in its achievement and maintenance. Other closely linked factors important

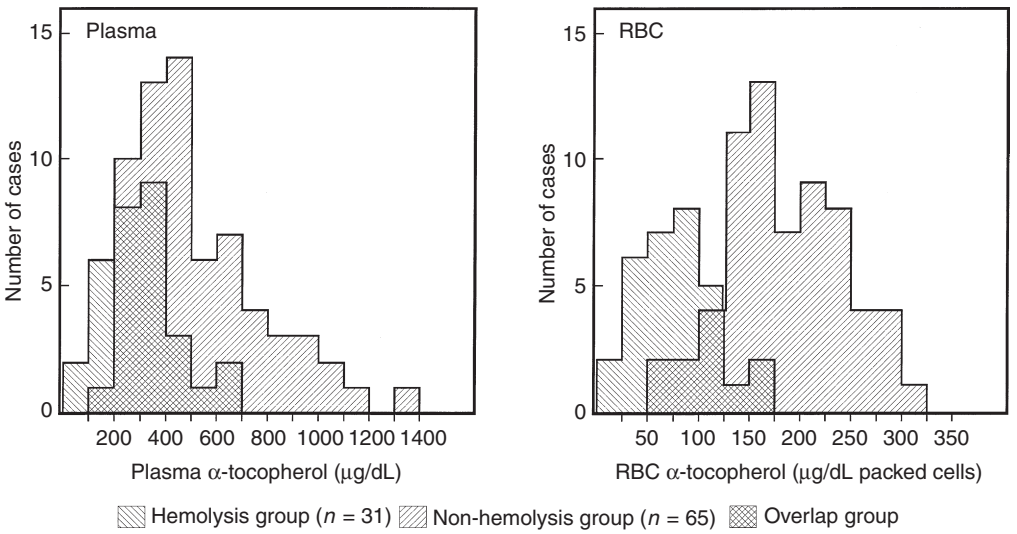


Figure 39-8 Plasma and red blood cell (RBC) α-tocopherol levels as determinants of susceptibility to oxidant stress. Samples were obtained from 96 premature and sick infants ranging in age from birth to 6 months. Under standard conditions, RBC α-tocopherol level predicts H_2O_2-induced hemolysis better than plasma α-tocopherol level (11.5% versus 25% overlap of RBC versus plasma tocopherol levels associated with presence or absence of hemolysis). The RBC level of α-tocopherol in 90% of infants in the hemolysis group was less than 125 μg/dL. Hemolysis did not occur if the RBC tocopherol level was higher than 175 μg/dL. The study population was from the Osaka area of Japan. *(From Mino M, Nakagawa S, Tamai H, et al: Clinical evaluation of red blood cell tocopherol,* Ann N Y Acad Sci *393:175, 1982.)*

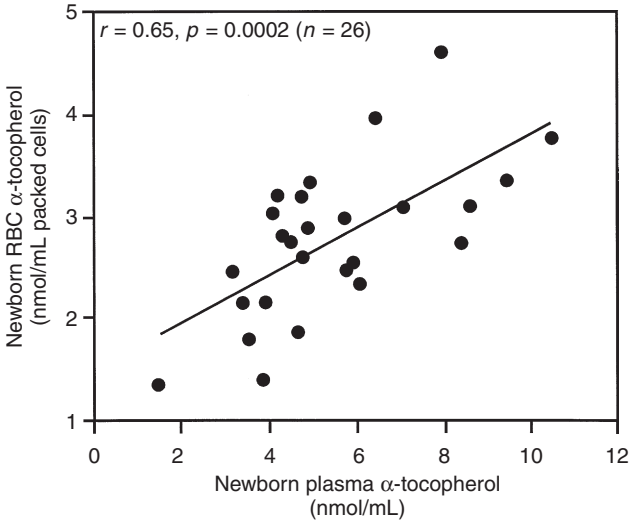


Figure 39-9 Correlation of α-tocopherol concentrations in plasma and red blood cells (RBCs) of 26 term newborns. In these newborns, with a mean RBC α-tocopherol level of only 1.18 μg (2.77 nmol) per mL of packed cells, good correlation was found between plasma and RBC α-tocopherol without normalization for plasma and RBC lipid level as shown, as well as with lipid normalization (RBC α-tocopherol as nmol/μmol RBC total lipid in relation to plasma α-tocopherol as nmol/μmol plasma total lipid; $r = .63$). The study population was from the New Orleans, Louisiana, area. *(From Jain SK, Wise R, Bocchini JJ Jr: Vitamin E and vitamin E-quinone levels in red blood cells and plasma of newborn infants and their mothers,* J Am Coll Nutr *15:44, 1996.)*

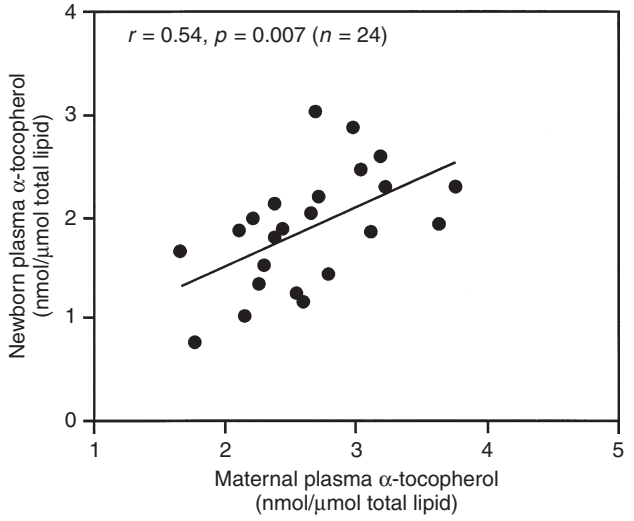


Figure 39-10 Plasma α-tocopherol concentration in 24 mother-infant pairs at term delivery. Good correlation was found between maternal and infant plasma α-tocopherol levels when expressed as nmol tocopherol/μmol total lipid. No correlation was found when levels were expressed as nmol tocopherol/mL plasma ($r = .09$; data not shown). The study population was from the New Orleans, Louisiana, area. *(Modified from Jain SK, Wise R, Bocchini JJ Jr: Vitamin E and vitamin E-quinone levels in red blood cells and plasma of newborn infants and their mothers,* J Am Coll Nutr *15:44, 1996.)*

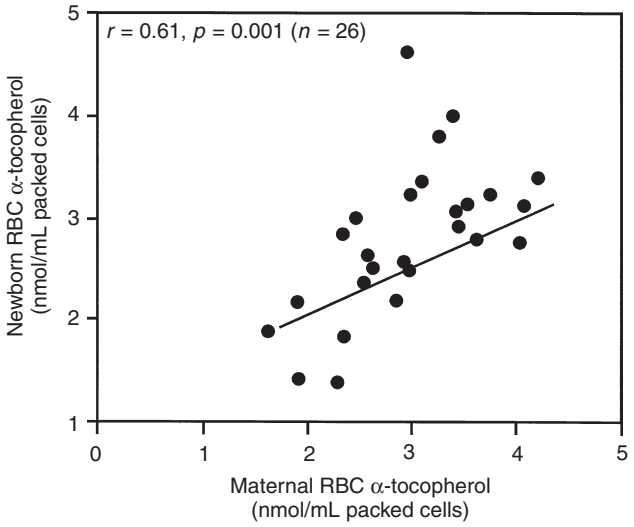


Figure 39-11 Red blood cell (RBC) α-tocopherol concentration in 26 mother-infant pairs at term delivery. As evident in this figure, a good correlation was present between newborn and maternal RBC γ-tocopherol levels expressed as nmol/mL of packed cells. The correlation also was present when levels were expressed as nmol/μmol of total lipid ($r = 0.46$; $p = .02$). The study population was from the New Orleans, Louisiana, area. *(Modified from Jain SK, Wise R, Bocchini JJ Jr: Vitamin E and vitamin E-quinone levels in red blood cells and plasma of newborn infants and their mothers,* J Am Coll Nutr *15:44, 1996.)*

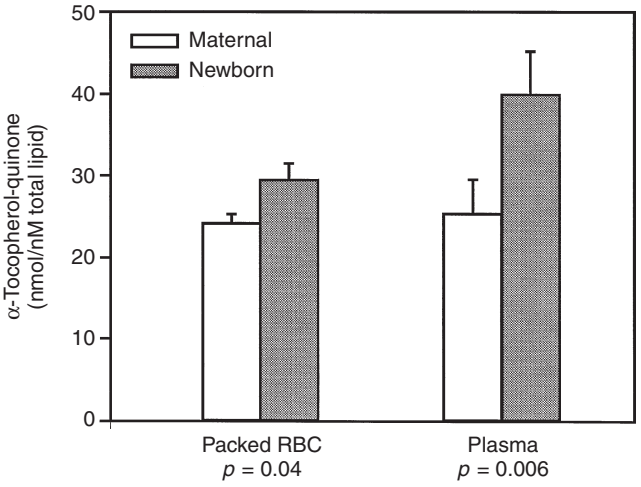


Figure 39-12 α-Tocopherol-quinone levels in 26 mother-infant pairs. Red blood cell (RBC) and plasma levels of α-tocopherol-quinone, the oxidation product of α-tocopherol, were measured in mother-infant pairs at birth and were found to be significantly higher in the infant. The mean newborn RBC α-tocopherol level was 2.77 ± 0.14 nM (1.19 μg/mL); in the mothers, it was 2.95 ± 0.13 nM (1.29 μg/mL). Quinone levels in the infant did not correlate with quinone levels in the mother. Blood specimens were protected against oxidant exposure throughout the analytic procedure. The study population was from the New Orleans, Louisiana, area. *(Data from Jain SK, Wise R, Bocchini JJ Jr: Vitamin E and vitamin E-quinone levels in red blood cells and plasma of newborn infants and their mothers,* J Am Coll Nutr *15:44, 1996.)*

to redox balance include vitamin C and carotene; the antioxidant enzymes superoxide dismutase, glutathione peroxidase, and catalase; and the metabolic intermediaries reduced glutathione and cysteine; saturated and unsaturated lipids; and intra- and extracellular calcium ions.[145,146] The expanding number of markers of oxidant stress now being identified and measured during human pregnancy and in animal and in vitro models testifies to the complexity of the phenomena under investigation. Currently these markers include leptin, placental growth factors, indices of placental endothelial dysfunction of which the most important is the plasminogen activator-inhibitor ratio (PAI-1/PAI-2), a range of pro- and antiinflammatory cytokines such as the T_H1 helper cell profile (interferon-γ, tumor necrosis factor-α, and the interleukins IL-l l and IL-12), the T_H2 helper cell profile (IL-4 and IL-10), and nuclear factor-κ B, 8-isoprostane, 8-epi-prostaglandin, IL-6 and IL-8, leukemia inhibitory factor, peroxynitrites, and uric acid.[148-221]

Presented next are selected quotes, followed by the abstract from the paper by Allen and Venkatraj, "Oxidants and antioxidants in development and differentiation,"[145] that are pertinent to the ongoing search for safe, effective treatment interventions for management of high-risk pregnancies and for selection of the readily available, easily administered and inexpensive antioxidant vitamins E and C as early choices for study in controlled clinical trials in humans.

"The redox status of cells is the ratio of oxidizing equivalents to reducing equivalents present in the cell. It can be regarded as the net balance of the interaction of all cellular oxidants and antioxidants. Redox state is strongly modulated by a variety of environmental factors, including nutritional status. For example, increasing dietary antioxidants such as ascorbate, alpha tocopherol or beta carotene tends to depress the synthesis of GSH (reduced glutathione) as well as the activity of the enzymic antioxidant defenses.

"Both cell cycle and development are associated with enormous changes in redox balance.

"Cellular environments enriched with antioxidants appear to impede differentiation.

"The underlying cause of these effects appears to stem from the influence of redox status on calcium metabolism.

"Metabolic gradients are known to influence cellular differentiation at critical junctures of development. The existence of these gradients will stimulate variations in the rate of generation of active oxygen metabolites by metabolic pathways. Recent evidence suggests that cellular oxidation can induce changes in gene expression during normal development. Conversely, antioxidants such as ascorbate, alpha tocopherol and beta carotene are inhibitory to differentiation in many types of cells. One basis of oxidative influence on gene expression stems from changes in cellular calcium distribution."

Ongoing Evidence of the Essentiality of α-Tocopherol for Reproduction

An exciting body of ongoing research[146-220] is investigating the need for vitamin E during implantation, early, middle, and late stages of gestation as modulated by genetic, imunologic, nutritional, and environmental factors.[192,198,209-211,215-221] Isotopic and immunochemical studies of α-tocopherol transfer protein (α-TTP) in human placental tissues, and in α-TTP–null and –heterozygous and wild-type mice, support the essentiality of vitamin E for implantation and placental development.[212-217] Vitamin E has been identified in the syncytiotrophoblast, the villous and extravillous invading cytotrophoblast, basal decidual cells, and amnion and fetal capillaries.[215-218] Among five human tissues, including brain, the quantified placental expression of α-TTP mRNA by 5′-nuclease assay is second only to that of the liver.[215]

The gestational sac in the early first trimester limits, rather than facilitates, oxygen transfer. Oxygen tensions are in the clearly hypoxic range, metabolism is essentially anaerobic, and nutrition is accomplished by means of uterine glandular secretions.[216-221] Antioxidant molecules, are limited to vitamin C, uric acid, and α- and γ-tocopherol and are made available by diffusion through the exocelomic cavity, rather than by a yet-to-be-developed placental vasculature. Antioxidant enzymes are essentially undetectable.[144,218,219] During this initial trimester, the fetus is most vulnerable to oxidant stress and in need of antioxidant protection if exposure to an excess of oxidant radicals occurs.[189-193,201-203,209-212]

Studies of Antioxidant Supplements During Pregnancy

From many studies in animals and humans, it is known that overproduction of reactive oxygen species occurs in diabetic pregnancies and pregnancies characterized by preeclampsia and pregnancy-induced hypertension,[135-137,148-155,190,205] intrauterine growth restriction,[174,184,188,190,205] diabetes,[156-166,205] maternal smoke inhalation,[167-169] maternal ethanol ingestion,[170-173] premature rupture of membranes,[146,175] and maternal nutritional deprivation.[176,180,184,185] In addition to the recognized adverse neonatal outcomes associated with such pregnancies, they are implicated as contributors to the fetal origins of pediatric and adult diseases.[143,174-184,202]

In vitro studies and animal models of abnormal pregnancy have shown consistent, clearly beneficial results in association with preventive or corrective treatment with vitamins E and C.* For example, in a recent study of human placental villous explants exposed to hypoxia-reoxygenation, vitamin E– and vitamin C–blocked activation of the signaling pathways of nuclear factor κB, p38, and stress-activated and mitogen-activated protein kinase, which regulate downstream release of proinflammatory cytokines. Nevertheless, results of randomized, placebo-controlled, clinical trials of vitamin E and C treatment in human pregnancies classified as at high risk for pathologic oxidant stress have shown inconsistent benefit[187-190,208] and sometimes unexpected, possibly harmful side effects. Such side effects were most evident when normal pregnancies made up a major proportion of the study population and when doses of vitamin E in excess of 400 IU of all-racemic α-tocopherol were used.[195,196] The recommended dietary supplement of vitamin E for normal pregnancies is 22 to 30 mg/day.

Accurate early identification of clinically significant oxidant stress, with provision of prompt, targeted treatment in the first trimester, is not yet possible in humans. Unrestricted access to maternal, fetal and placental blood and tissues, as well as control of absence or presence of disease and its severity, can be achieved only in animal models of pregnancy complicated by diabetes, maternal nutritional deprivation, and excessive smoking and ethanol intake.[157-173] No such animal models for preeclampsia and pregnancy-induced hypertension have been described.

Diabetic Pregnancies

Oxidant stress and overproduction of oxidant radicals, with a range of abnormal outcomes, occur in all human diabetic pregnancies, including those involving gestational diabetes as well as diabetes type 1 and type 2.[201-203,206,211] The risk of preeclampsia in diabetic pregnancies is two to four times higher than in nondiabetic pregnancies.[201,206] Without antioxidant treatment, strict maintenance of normoglycemia is remarkably effective in decreasing all diabetes-related abnormalities, including preeclampsia, but initiation and maintenance of strict normoglycemia must begin

*References 135-137, 148, 157, 159-166, 206, 207, 221.

very early in pregnancy to be effective.[205-207] The potential for antioxidant therapy with vitamins E and C to improve outcome in diabetic pregnancies was investigated in 20 metabolic antenatal units in the United Kingdom taking part in DAPIT (the Diabetes and Pre-eclampsia Intervention Trial).[200]

The DAPIT trial enrolled women with a diagnosis of type 1 diabetes, monitored for glycemic control, who presented between 8 and 22 weeks gestation from April 2003 to June 2008. Women were randomly assigned to receive 1000 mg of vitamin C and 400 units of α-tocopherol (presumably dL racemic) or placebo until delivery.

By intention-to-treat analysis of the 379 vitamin and 383 placebo women who completed the study, no differences in the primary outcome of incidence of preeclampsia were found. In two of 11 predefined subgroups that categorized enrollees by baseline antioxidant status, however, the risk of preeclampsia was significantly reduced in women with low baseline antioxidant criteria (<10 μmol/L plasma ascorbate or serum α-tocopherol ≤5 μmol/mmol cholesterol) who received vitamin treatment as compared to placebo during pregnancy. No adverse side effects of antioxidant supplementation were found for either mother or infant in the study population as a whole or in any of the 11 subgroups.

The authors concluded that this finding deserved further investigation and suggested that vitamin supplementation may be beneficial in preventing preeclampsia in diabetic pregnancies only in women with vitamin depletion. The finding and its interpretation was consistent with earlier clinical trials assessing the effect of antioxidant supplements for prevention of preeclampsia in women with higher versus lower estimates of oxidant stress, antioxidant depletion, and risk for disease.[188-190,195-199]

Pregnancies Complicated by Preeclampsia

Worldwide, preeclampsia occurs in 2% to 3% of all pregnancies.[205,220] The effect of antioxidant therapy with vitamins E and C for prevention of preeclampsia (proteinuric hypertension) in nondiabetic pregnancies associated with high risk for the disease has been reported in a number of controlled clinical trials.[†] In these trials, the first-trimester predictive markers used to identify a high-risk study population were associated with a placebo incidence of preeclampsia of 15% to 20%.[188,195,208] An additional comparable percentage of pregnancies were associated with intrauterine growth restriction (IUGR) without preeclampsia.[190,195,199] Therefore, at least in relation to these pathologic conditions, 50% to 60% of the study population randomized for treatment had no recognizable clinical disease and may not have had clinically significant overproduction of oxidant radicals in the first trimester. Additionally, treatment did not begin until the second trimester. Improved early prediction would allow more targeted, timely antioxidant therapy,[191] clarify treatment results, and mimize possible deleterious effects of treatment in the absence of clinically significant overproduction of oxidant radicals.[189-191,195,196]

One controlled clinical trial in South Africa[187] involved 56 women with already diagnosed severe preeclampsia at 24 to 32 weeks of gestation. Only minimal benefit was obtained from treatment with allopurinol as well as vitamins E and C initiated at diagnosis. These results emphasize the need for initiating therapy early in the pathologic process. One of the trials[208] was terminated early because interim analyses indicated that the sample size was likely to be inadequate.

Three completed controlled clinical trials[188,195,199] in which treatment was initiated in the mid–second trimester and a small case-control study in "normal" pregnancies[196] in which treatment was started in the early first trimester are summarized here because of the insights provided for improved protocol development and direction of future research.

Summary of Antioxidant Clinical Trials

Clear benefit from antioxidant treatment was reported in 1999 for a controlled clinical trial from the Guy's, King's College, and St. Thomas Hospitals in London,[188-190] which included serial measures of oxidant stress in both treatment groups after randomization. The study population was confined to pregnancies classified as being high risk for preeclampsia because of a previous history of preeclampsia or abnormal findings on two-stage uterine artery Doppler analysis. Serial measures of oxidant stress were measured until delivery in the 142 placebo- and 141 vitamin-treated women enrolled in the study. Oral treatment was begun at 16 to 22 weeks gestation and continued until birth of the infant. Treatment consisted of 400 IU α-tocopherol (presumably all-racemic) and 1000 mg of vitamin C or placebo. Of the 283 enrollees, 160 (81 placebo and 79 vitamin) completed all aspects of the study, including monthly plasma assessments of oxidant stress and placental function.

In the intention-to-treat analysis, there was a decrease in incidence of preeclampsia ($p = 0.02$), from 17% in placebo-treated mothers to 8% in vitamin-treated mothers ($n = 283$). In the cohort who completed the study, the decrease in incidence of preeclampsia was at the $p = 0.002$ level. There also was a 21% decrease ($p = 0.015$) in the ratio of plasminogen-activator inhibitor (PAI)-1 to PAI-2 (placental dysfunction) in vitamin- versus placebo-treated mothers.

In further analysis, other measures of oxidative stress and placental dysfunction were found to be abnormal in placebo-treated compared with vitamin-treated enrollees and compared with a group of untreated, low-risk mothers who received perinatal care during the same period and were similarly monitored for oxidative stress and placental function. These measures included decreased concentrations of ascorbic acid, PAI-2, and placental growth factor; increased concentrations of 8-epi-prostaglandin and leptin; and decreased PAI-1 to PAI-2 ratio. The best predictors of preeclampsia in the 17% of women ($n = 142$) in the placebo group in whom preeclampsia developed were leptin, placental growth factor, uric acid, and the PAI-1 to PAI-2 ratio.[189]

No treatment benefit but possible deleterious side effects were reported in the large controlled clinical trial, the Vitamins in Preeclampsia (VIP) trial, organized by the Maternal and Fetal Research Center of Kings College and St. Thomas Hospital (London). It was conducted in 25 participating U.K. hospitals[195] and enrolled a study population ($n = 2404$) of pregnant women classified as being at high risk for preeclampsia on the basis of "a range of clinical risk factors." Treatment consisted of placebo or 400 IU of RRR α-tocopherol plus 1000 mg of vitamin C per day from the second trimester until delivery; after randomization, 1205 women received placebo and 1199 received vitamin supplementation. RRR-α-tocopherol has 1.36 times the biologic potency of the all-racemic preparation on the basis of older biologic assays (see Table 39-2) and a potency approaching 2 times that on the basis of recent more sophisticated studies.[214-216] Despite this larger dose of vitamin E, no significant difference in incidence of preeclamspsia was found between treatment groups (16% for the placebo group versus 15% for the vitamin supplementation group); significantly more babies had lower birth weights (within the normal range) in the vitamin treatment group (28%, versus 24% in the placebo group), a possibly deleterious side effect. No difference in incidence of small-for-gestational age (SGA) status among infants born to these women was noted between the placebo group and the vitamin treatment group: 19% versus 21%, respectively. Data from 99.6% of patients were analyzed.

No treatment benefit and no untoward side effects were reported in a third controlled clinical trial in 1877 nulliparous women receiving care in the Australia and New Zealand

[†]See references 187, 188, 195, 196, 199, 208.

Neonatal Network,[199] who were randomized without further risk assessment to receive placebo or 400 IU of (presumably) all-racemic α-tocopherol and vitamin C at 14 to 22 weeks of gestation. The placebo group included 942 women, with 935 women in the vitamin treatment group. The study investigators reported no significant between-group differences in outcome variables including the incidence of preeclampsia (5.0% in the placebo group versus 6.0% in the vitamin treatment group; r = 1.20), SGA in infants born to these women (9.9% in the placebo group versu 8.7% in the vitamin group; r = 0.87), and death or other serious outcome (12.1% with placebo versus 9.5% with vitamins; r = 0.79).

In addition to these three controlled clinical trials, a case-control study also evaluated the effect of vitamin E supplementation on preeclampsia risk: No treatment benefit but possible deleterious side effects were reported in a nonplacebo, nonrandomized trial in normal pregnancies in 82 women managed in the Motherisk program of Toronto, Canada.[196] Women were enrolled, without risk assessment, early in the trimester and started on treatment with daily doses of 400 to 1200 IU of vitamin E (presumably all-racemic α-tocopherol) without vitamin C. Treatment was continued only during the first trimester. The rates for live births, premature deliveries, miscarriages, and stillbirths were compared with those in a matched control group from the same Motherisk program. Of note, preeclampsia was not diagnosed in either group. (The incidence of preeclampsia worldwide[205,218] is estimated at 2% to 3%.) One case of omphalocele was diagnosed in the vitamin treatment group. Of importance, and also unexpected, was the finding of a highly significant (p = .0015) decrease in mean birth weight (within the physiologic range) associated with the vitamin E supplementation regimen: The mean birth weight of infants born to vitamin-treated mothers was 3173 ± 467 g, versus 3417 ± 565 g for infants of control (untreated) mothers.

Discussion of Results

Differences in outcome among the three controlled clinical trials as summarized may have been related, in terms of biologic potency, to the relatively higher dose of vitamin E used in the second trial[195] and to differences in the high-risk classification criteria. All three trials initiated treatment in the second trimester rather than the first. The placebo-related incidence of preeclampsia was similar at 17% in the first[188] and 16% in the second[195] of these trial and, as expected, lower (at 5%) in the study population of nulliparous women without risk assessment in the third.[199] The second trial[195] specified use of RRR α-tocopherol, the naturally occurring form, which has close to twice the biologic potency of the all-racemic α-tocopherol[213,214] used in the other two. As in the case of brain cells, it is preferentially transferred across the placenta.[212] The much larger sample size of the second trial would be expected to ensure a more equal distribution of preeclamptic risk between treatment arms and a greater power to detect beneficial as well as harmful effects of the higher antioxidant dosage in pregnancies without preeclampsia. The third trial,[199] with its larger proportion of normal pregnancies and the 400-IU dose of all-racemic α-tocopherol, showed results paralleling those in the larger second trial in regard to lack of treatment benefit and those of the first in regard to absence of possible harmful side effects. The fourth nonrandomized trial, with an apparently normal study population, used a much higher dose of vitamin E, 400 to 1200 IU (presumably all-racemic α-tocopherol), which was started early in the first as opposed to the second trimester and was continued through only the first trimester.[199] The potential for deleterious side effects was most clearly suggested in this trial.

The possibility that untoward side effects can result from antioxidant treatment in "normal" pregnancies is an important observation and indicates that the safe but effective antioxidant dosage range for prophylaxis and treatment is more narrow than had been anticipated, especially in the absence of oxidant radical excess.[196,198,209]

Clinical and Laboratory Studies Pertinent to Trials of Treatment Interventions

That antioxidants can be harmful was reported in a study of pregnant diabetic rats (n = 166) treated at daily doses of 25, 50, 100, 150, and 500 IU of vitamin E (presumably all-racemic α-tocopherol). Increasingly greater benefit in decreasing malformations, increasing maturation and size of offspring and decreasing rate of reabsorption was associated with increasing doses up to 150 IU per day. At this dosage the reabsorption rate in treated vs untreated diabetic rats was 12.5% versus 24.7% and the malformation rate 13.9% versus 50%, respectively. By contrast, at the 500-IU dose, a clearly deleterious effect was found, with a reabsorption rate of 43.9% in treated rats versus 24.7% in untreated rats. The malformation rate at the 500-IU dose was 10.8% in treated rats versus 50% in untreated rats.

Recent studies have suggested that, especially in human pregnancies without increased oxidant stress, more vitamin E than is strictly needed for metaboloic efficiency may interfere with the immunologic switch from T_H1 to T_H2 cytokine profiles that is essential to normal placental physiology in early (versus late) pregnancy.[198,209] Of great interest in this regard is the report by British investigators that this immunologic transition from early to late placental cytokine profiles does not seem to occur in pregnancies characterized by first-trimester oxidant stress and subsequent preeclampsia and IUGR.[209] In these pregnancies, interferon-γ (IFN-γ) R2 protein expression in trophoblasts at the maternal-fetal interface was found to be severely downregulated at 25 to 40 weeks of gestation and was similar to that present in normal placentas at 7 to 10 weeks developing under optimally low oxygen tesions. These findings are supported by those of Swedish investigators, who suggest that maladaptation of immune responses secondary to excessive maternal inflammatory response directed against foreign fetal antigens in the initial stages of pregnancy results in defective trophoblast invasion and a chain of pathologic events leading to clinical preeclampsia.[210]

The current hypothesis is that normal trophoblastic control of maternal immune effectors and cytokine profiles at the fetal-maternal interface is compromised by exposure to abnormal oxidative stress in the initial stages of preeclampsia and may be benefited by treatment directed at maternal-fetal imunologic incompatibilities as well as antioxidant treatment to minimize inflammation.[209,210] In related work,[193] impaired redox regulation was found in umbilical vein endothelial cells and RBCs at 14 to 20 weeks of gestation in pregnancies associated with preeclampsia as compared with those in which preeclampsia did not develop in the study population enrolled in the large VIP trial summarized earlier.[195] Impaired redox regulation was associated with decreased hexokinase and glucose-6-phosphate dehydroxylase (G6PD) activity, suggesting that deficiency in G6PD activity may explain the relative ineffectiveness of vitamin E in preventing preeclampsia in this trial.[193]

Unanswered Questions: Direction of Future Research

As noted in the earlier summary of the paper by Allen and Venkatraaj,[145] the physiologic role of vitamin E in the low-oxygen environment of the normal first trimester may relate to its actions as a cofactor in functions not strictly antioxidant in nature but required for achieving maximum metabolic efficiency for development and differentiation.[145] In excess of this amount, a real concern is that vitamin E, under certain circumstances, may potentially act as an IFN-γ mimic to promote

proinflammatory reactions at the fetal-maternal interface.[198] Investigators are now in general agreement that the importance of vitamin E for reproduction is underestimated and misunderstood if only its antioxidant properties are considered.[191-193] Properties associated with antiinflammation and inhibition of NAD(P)H oxidase activation need to be investigated,[191-193] as do also the role and importance of tocopherol forms other than α-tocopherol, in particular, γ-tocopherol; the role of the tocotrienols (see also under "Non-Antioxidant Functions of Vitamin E," earlier); and the novel mechanisms of antiatherosclerotic and anticarcinogenic properties of vitamin E involving modulation of cellular signaling, transcriptional regulation, and induction of apoptosis.[192]

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Reidar Wallin and Susan M. Hutson

Vitamin K Metabolism in the Fetus and Neonate

VITAMIN K AND BLOOD COAGULATION

Since the discovery of the pathogenesis of vitamin K deficiency in the 1950s,[1] the importance of prophylactic vitamin K in prevention of hemorrhagic disease in the fetal and neonatal periods has been debated. However, the development of sophisticated high-performance liquid chromatography systems, which can measure low levels of vitamin K and its metabolites in blood and tissues, has provided valuable new information on this subject. These measurements have shown that placental passage of the vitamin from maternal to fetal blood is restricted.[2-5] Thus the fetus can acquire a state of vitamin K deficiency.

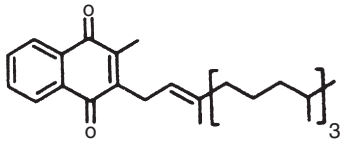
It is well documented that premature infants and infants at term are invariably born with reduced levels of many of the essential protein factors of the hemostatic system.[6,7] Among these factors are the vitamin K–dependent clotting factors—prothrombin; factors VII, IX, and X; and protein S and protein C.[7]

Because classic hemorrhagic disease of the newborn has been treated successfully with vitamin K, it has been suggested that neonates begin extrauterine life with a vitamin K deficiency, so that vitamin K administration is necessary to correct the resulting imbalance of the hemostatic system.[4] Several studies contradict this assumption. Using a procedure for blood sampling in utero, Forestier and colleagues[8] found good correlation between procoagulant activity of the vitamin K–dependent clotting factors and the respective antigens in midtrimester fetal blood. Further evidence was the absence of PIVKA (*p*rotein *i*nduced in *v*itamin *K a*bsence) forms of the vitamin K–dependent clotting factors in their samples, arguing against vitamin K deficiency in the fetus.[8] Indeed, in most studies performed on newborns, PIVKA factors have been found in minimal concentrations or are totally absent, even in low-birth-weight infants.[5,8-12] Thus classic hemorrhagic disease of the newborn is likely to result from (1) malnutrition by the mother during pregnancy, (2) use of vitamin K antagonists during pregnancy, (3) exhaustion of the liver stores of vitamin K in the newborn, and (4) undefined causes of vitamin K deficiency.

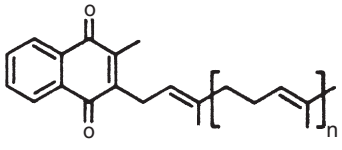
Production of the vitamin K–dependent clotting factors by the liver is related to gestational age.[7,13-15] The blood concentrations of these factors in the neonate are approximately 30% to 60% of their concentrations in the adult; these low values gradually increase until they reach normal adult values by the age of 6 weeks.[16] Thus the lower concentrations found in the developing neonate apparently result from immaturity in the system that produces these proteins. The hemostatic system requires a fine balance between coagulation on one side and fibrinolysis on the other, so abnormal development of the liver system that produces the vitamin K–dependent clotting factors presumably can contribute to hemorrhagic and thrombotic problems in the fetal and neonatal periods. Because vitamin K metabolism and vitamin K function are linked processes in the liver, this chapter focuses on maturation of the vitamin K–dependent γ-carboxylation system in the fetal and neonatal periods.

FUNCTION AND METABOLISM OF VITAMIN K IN LIVER

The essential steps in vitamin K metabolism and the steps targeted by the anticoagulant drug warfarin were investigated by several laboratories (reviewed by Suttie[17]), including ours,[18-23] in the 1970s and 1980s. Structures of the naturally occurring forms of vitamin K are shown in Figure 40-1. Vitamin K_1, or phylloquinone, is found in plants. The plant vitamin is a naphthoquinone with a saturated polyisoprenoid side chain in the 3 position on the napthoquinone ring system. Vitamin K_2, which comprises the members of the menaquinone family, is found in bacteria and has an unsaturated side chain of variable length in the 3 position. Vitamin K is absorbed in the small intestine and enters the lymphatic system by a process that requires the presence of both bile and pancreatic juice for maximum absorption.[17,24] After uptake, the vitamin is distributed ubiquitously among body tissues, but significant quantitative differences have been noted, the liver being the major organ for uptake. Intracellularly, the vitamin has been found in membranes of mitochondria, the endoplasmic reticulum, and the Golgi apparatus.[25] Although menaquinones have been shown to promote biosynthesis of vitamin K–dependent clotting factors by the liver,[26] the bacterially produced vitamin does not contribute to fetal biosynthesis of vitamin K–dependent clotting factors. Some evidence indicates that the infant is born with essentially no liver stores of menaquinones.[27,28]



Phylloquinone (vitamin K_1)



Menaquinones (vitamin K_2)

Figure 40-1 Chemical structures of phylloquinone, or vitamin K_1, and menaquinones, collectively termed vitamin K_2. Vitamin K_1 is found in plants. Vitamin K_2 is synthesized with a side chain of variable length by bacteria.

Several theories on the biologic action of vitamin K were proposed before the finding that vitamin K was involved in posttribosomal modification of proteins. Stenflo and associates[29] identified the modification as γ-carboxyglutamic acid carried out by an enzyme in the endoplasmic reticulum. Independently, Nelsestuen and co-workers[30] found the modification in a dipeptide (Glu-Ser) from prothrombin. Magnusson and colleagues[31] later showed that the first 10 amino-terminal (N-terminal) glutamic acid (Glu) residues in prothrombin are γ-carboxylated. The new amino acid formed as a result of vitamin K action was called γ-carboxyglutamic acid, abbreviated Gla. Its structure is shown in Figure 40-2. The Gla residues bind calcium, and calcium binding by the fully γ-carboxylated clotting factors is essential for optimal activation or activity of these factors in blood.[32]

Vitamin K–dependent γ-carboxylation has been shown to require molecular oxygen, carbon dioxide, and the fully reduced form of vitamin K, vitamin K hydroquinone[17] (Figures 40-3 and 40-4). Because the normally occurring forms of vitamin K are quinones (see Figure 40-3), the vitamin must be reduced by liver enzymes before the γ-carboxylation reaction is triggered. As illustrated in Figure 40-4, γ-carboxylation of one Glu residue is coupled stoichiometrically to formation of one molecule of the vitamin K metabolite, vitamin K 2,3-epoxide (vitamin K>O). Vitamin K>O is reduced by liver enzymes back to the hydroquinone form, which establishes a redox cycle for vitamin K in liver, known as the vitamin K cycle (see Figure 40-4).[17,18] Two pathways for vitamin K reduction are associated with the cycle. Pathway I

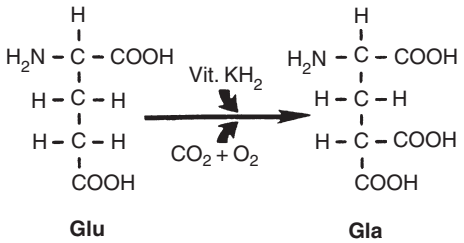


Figure 40-2 Vitamin K–dependent synthesis of γ-carboxyglutamic acid (Gla).

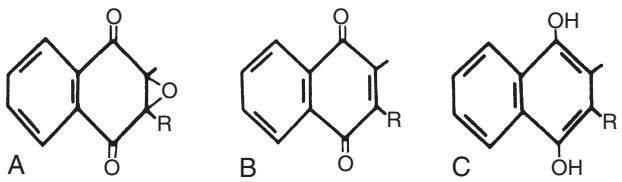


Figure 40-3 Chemical structures of vitamin K metabolites produced by the vitamin K cycle. A, Vitamin K 2,3-epoxide (K>O). **B,** Vitamin K quinone (K). **C,** Vitamin K hydroquinone (KH_2).

is physiologically the more important pathway and operates at normal and low liver concentrations of vitamin K.[22] The enzyme involved, vitamin K epoxide reductase, catalyzes reduction of vitamin K>O and vitamin K to the active vitamin KH_2 cofactor (KH_2). The reduced thiol compound dithiothreitol is an excellent source of electrons for the enzyme-catalyzed reaction (see Figure 40-4). However, the thiol compound, which is active in vivo, has not yet been identified. It has been documented that vitamin K epoxide reductase is the target for the anticoagulant drug warfarin.[17] Thus warfarin mediates its anticoagulant effect by preventing production of reduced vitamin KH_2 by pathway I. Inhibition of pathway I by warfarin is essentially irreversible.[33] However, vitamin K commonly is used clinically as an antidote to overcome intoxication with this drug. The antidotal effect of vitamin K is mediated by pathway II,[22] which is operative only at high liver concentrations of vitamin K. Pathway II, which is insensitive to warfarin, has been shown to consist of more than one dehydrogenase that are dependent on the reduced form of nicotinamide adenine nucleotide (NADH) to reduce the quinone form of the vitamin.[19,20]

Since Bell and Matchiner[34] discovered the warfarin-sensitive vitamin K epoxide reductase (VKOR) of the vitamin K cycle in 1970, much research has focused on this important enzyme. Attempts to purify the enzyme were undertaken by many laboratories (as reviewed by Suttie[33]), but successful research has now been accomplished on the VKOR gene and the VKOR cDNA has been expressed in vitro.[34a] The carboxylase, a 92-kDa membrane protein of the endoplasmic reticulum membrane, has been purified[36,37] and cloned.[38] The successful isolation of the carboxylase has contributed significantly to current understanding of the mechanism by which the carboxylase participates in biosynthesis of vitamin K–dependent clotting factors. Because VKOR has now been purified, knowledge of vitamin K metabolism and the mechanism of action of the anticoagulant warfarin on the vitamin K–dependent γ-carboxylation system and how it is linked to operation of the γ-carboxylase has improved immensely. Our laboratory has provided evidence that VKOR is a multicomponent enzyme complex in the endoplasmic reticulum membrane and that the activity is regulated by the chaperone calumenin.[39]

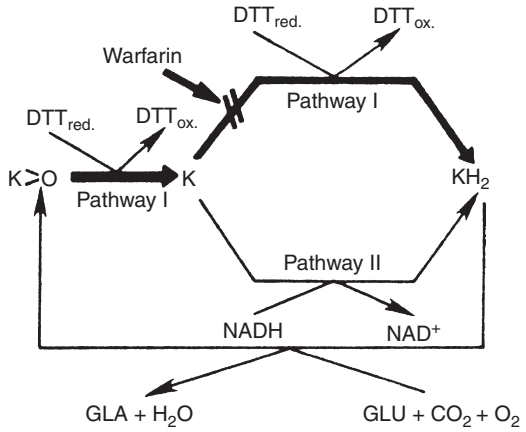


Figure 40-4 Vitamin K metabolism and vitamin K function in liver. Reduction of vitamin K epoxide (K>O) and vitamin K (K) by pathway I is catalyzed by vitamin K epoxide reductase, which requires reduced thiols (dithiothreitol- [DTT]) for the reaction. Pathway I is inhibited by the anticoagulant drug warfarin. Reduction of vitamin K by pathway II is catalyzed by NAD(P)H-dependent dehydrogenases. This pathway is insensitive to warfarin and mediates the antidotal effect of vitamin K. Reduced vitamin K (KH_2) triggers γ-carboxylation of glutamic acid (GLU) residues and converts them to γ-carboxyglutamic acid (GLA) residues. Formation of GLA is coupled to formation of K>O.

MATURATION OF THE VITAMIN K–DEPENDENT γ-CARBOXYLATION SYSTEM IN THE FETAL AND NEONATAL PERIODS

The available data on vitamin K metabolism and γ-carboxylation in the fetal and neonatal periods are derived primarily from the rat model. Nevertheless, in one human study,[5] plasma levels of vitamin K_1 and vitamin Ko were measured in 133 term and 26 preterm neonates. The data indicate that the ratio of epoxide to vitamin K increases with gestational age. One possible explanation for this observation is that maturation of pathway I takes place toward term, potentially leading to underproduction of reduced vitamin KH_2 cofactor and incomplete γ-carboxylation of the vitamin K–dependent clotting factors. Figure 40-5 shows the specific activity profile of the warfarin-sensitive pathway I enzyme, vitamin K epoxide reductase, in 16- to 20-day fetal rats, at birth (day 21), and in 2- and 7-day neonatal rats.[39] As shown, warfarin sensitivity was detected from day 16 in the fetal period, the earliest point in gestation studied. None of the fetal livers had less than 50% of the specific activity measured in 7-day neonatal rats. These studies indicate that in the rat, significant amounts of

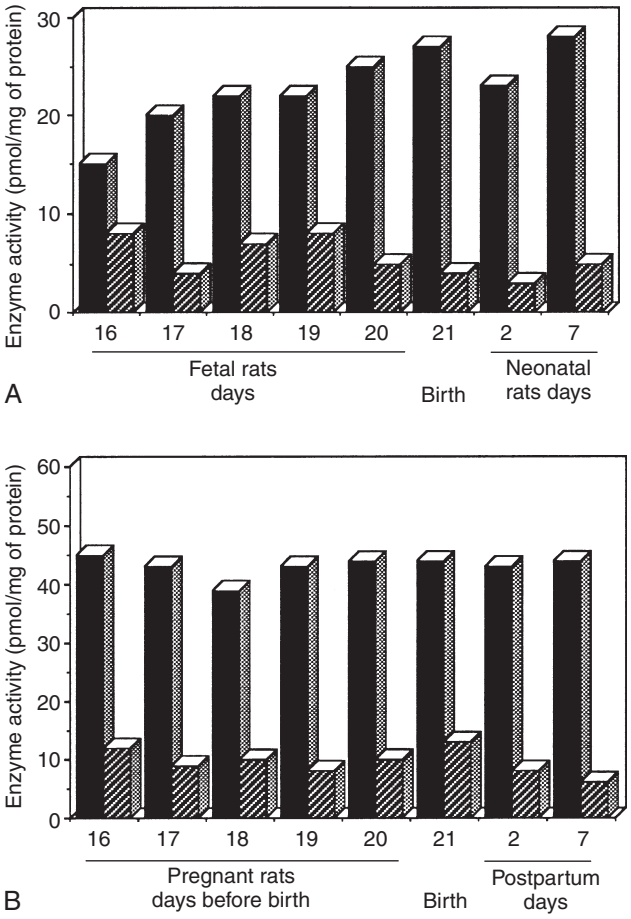


Figure 40-5 Vitamin K epoxide reductase activity in fetal and neonatal **(A)** and maternal **(B)** livers. Before determination of enzyme activity, pregnant rats were injected with saline *(solid bars)* or warfarin *(cross-hatched bars)*. *(From Wallin R: Vitamin K–dependent carboxylation in the developing rat: Evidence for a similar mechanism of action of warfarin in fetal and adult livers,* Pediatr Res *26:370, 1989.)*

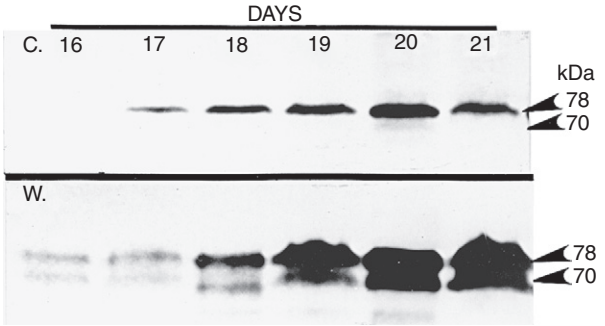


Figure 40-6 Vitamin K–dependent carbon 14 labeling of precursors of prothrombin and factor X in fetal livers. Fetal livers were isolated from day 16 to day 21 in pregnant rats injected with saline (C), and warfarin (W.). The fluorograms show the prothrombin (78 kDa) and the factor X (70 kDa) precursors. *(From Wallin R: Vitamin K–dependent carboxylation in the developing rat: evidence for a similar mechanism of action of warfarin in fetal and adult livers,* Pediatr Res *26:370, 1989.)*

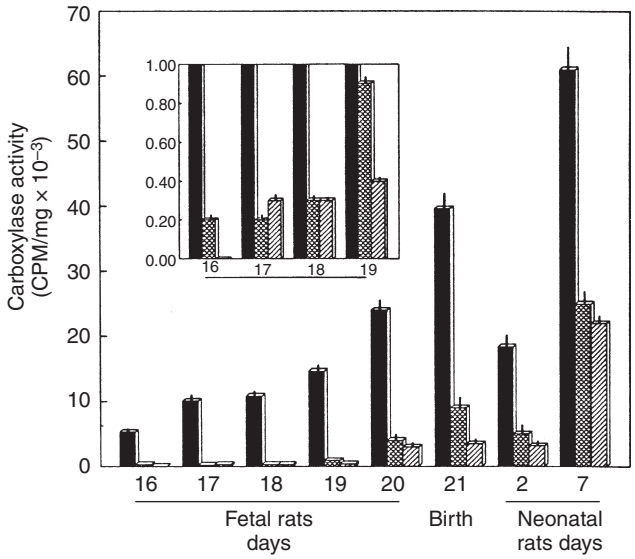


Figure 40-7 Vitamin K–dependent carboxylase activity in fetal and neonatal rat liver. Carboxylase activity, measured in counts per minute (cpm)/mg, was triggered with chemically reduced vitamin K_1H_2 *(solid bars)* or when supported by pathway I *(cross-hatched bars)* and by pathway II *(diagonal bars)*. *(From Wallin R: Vitamin K–dependent carboxylation in the developing rat: evidence for a similar mechanism of action of warfarin in fetal and adult livers,* Pediatr Res *26:370, 1989.)*

vitamin K epoxide reductase are expressed in midterm fetal life (see Figure 40-5, *A*). Figure 40-5, *B*, shows this activity profile in the mothers. The prediction that emerged from the data in Figure 40-5 was that administering warfarin to the mother would affect the developing fetus. This was demonstrated using vitamin K–dependent carbon 14 labeling of prothrombin (78-kDa) and factor X (70-kDa) precursors in fetal rat livers. As shown in Figure 40-6, only the livers of pups from mothers that had been injected with warfarin demonstrated labeling of the factor X precursor (the 70-kDa band on the "W." fluorogram); this observation provides direct evidence for the effect of warfarin on the vitamin K–dependent carboxylation system.[40] Indeed, the data in Figure 40-6 demonstrate that warfarin passes the placental barrier and anticoagulates the fetus. This finding is consistent with its known effect on human fetal development, wherein warfarin intake during pregnancy has been shown to result in an embryopathy characterized by stippled epiphyses, central nervous system abnormalities, optic atrophy, deafness, hypoplasia of the nasal bridge, and distal digital hypoplasia.[41]

Ontogeny of the vitamin K–dependent carboxylase and of its supporting enzymes involved in vitamin K metabolism in the rat is presented in Figure 40-7.[30] A dramatic (12-fold) increase in the specific activity of the vitamin K–dependent carboxylase occurs from day 16 in the fetal period to day 7 in the neonatal period. Of interest, and consistent with our data, Romero and colleagues[42] found, by in situ hybridization, that γ-carboxylase messenger RNA (mRNA) could not be detected before day 16 of fetal life. The activity increases steadily during the fetal period toward birth. At birth, the activity drops, but it increases again to reach adult enzyme activity levels in 7-day neonatal rats. These findings may be of relevance to the prolonged prothrombin time often observed in human neonates 2 to 3 days after birth.[14] Jamison and Degen[43] noted a "dip" in rat prothrombin mRNA at birth. Taken together, these data may reflect the dramatic changes in hormonal control that occur around birth,[44] and they may indeed indicate a critical point in development of the coagulation system. Figure 40-7 also shows the carboxylation "capacity" of the vitamin K–dependent carboxylation system during development, when the system is dependent on vitamin K–metabolizing enzymes to provide the reduced vitamin KH_2 cofactor. The cross-hatched bars in Figure 40-7 show carboxylation activity when triggered by pathway I (the warfarin-sensitive pathway), and the diagonal bars show this activity when triggered by the warfarin-insensitive pathway II. The activity of pathway II, which mediates the antidotal effect of vitamin K, could not be measured in livers from 16-day rat fetuses. This finding is supported by the observations of Wallin and Martin,[18] who demonstrated that the hepatic enzyme DT-diaphorase (Enzyme Commission [EC] number 1.6.99.2), one of the pathway II enzymes, appears late in gestation. These data suggest that vitamin K will not work as efficiently in the fetal liver as in the mature liver for treatment of warfarin intoxication. The vitamin K–dependent carboxylation system has been shown to be similar in rat liver and in human liver.[18]

REGULATION OF DEVELOPMENT OF THE VITAMIN K–DEPENDENT γ-CARBOXYLATION SYSTEM

The stepwise emergence of many enzymes in the developing organism has been shown to be closely related to changes in the hormonal environment, and it is reasonable to assume that the vitamin K–dependent carboxylation system also is under the influence of such control. The appearance of enzymes during late fetal life in the rat is believed to be influenced significantly by glucocorticoid secretion and the appearance of the glucocorticoid receptor on day 18 of gestation.[45] The next profound change in the hormonal milieu takes place around birth. Shortly after birth, the insulin-glucagon ratio, which is high in the fetus, drops significantly; this change induces another cluster of enzymes.[43] Because output of vitamin K–dependent clotting factors from liver to blood is dependent on their γ-carboxylation status in the endoplasmic reticulum,[46] factors that modulate the activities of the carboxylase and the vitamin K–metabolizing enzymes presumably may be used to control development of the hemostatic mechanism. The only information that is available on this issue has been obtained from studies in the rat.[47] As shown in Figure 40-8, fetal rat hepatocytes treated in vitro with dexamethasone express significantly higher carboxylase activity than that noted in untreated cells. An effect of insulin on the activity could not be demonstrated. These cell culture data are supported by in vivo data derived in dexamethasone-injected rats, which documented stimulation of γ-carboxylase activity in both liver[39] and lung.[48]

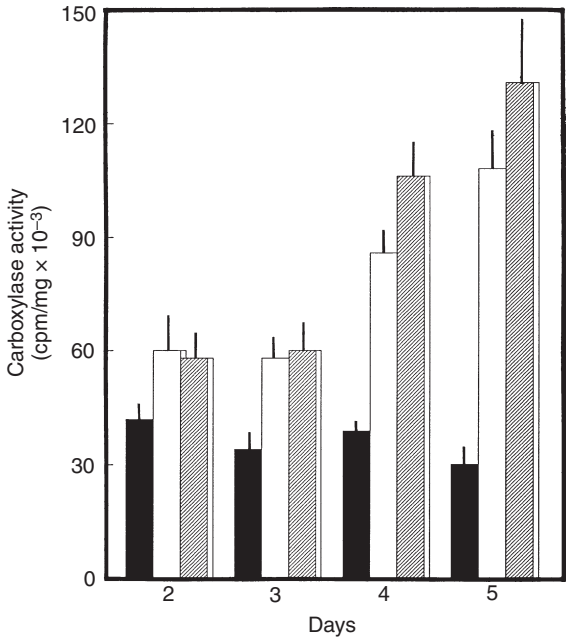


Figure 40-8 Vitamin K–dependent carboxylase activity, measured in counts per minute (cpm)/mg, in cultured fetal hepatocytes treated with dexamethasone. Hepatocytes from day 18 rat fetuses were cultured for 5 days in the absence *(solid bars)* and presence (0.1 μM, *open bars*; 1 μM, *diagonal shaded bars*) of dexamethasone. Fresh medium with hormones was added to the cell each day. *(From Wallin R, Hutson SM: Dexamethasone stimulates vitamin K–dependent carboxylase activity in neonatal rats and cultured fetal hepatocytes,* Pediatr Res *30:281, 1991.)*

Studies demonstrating dexamethasone stimulation of γ-carboxylase activity in fetal life should be extended to include studies on the effect of the drug on the activities of the vitamin K–metabolizing enzymes. The lifesaving effect of dexamethasone on maturation of the lung surfactant system in premature infants (reviewed by Ballard[49]) should stimulate research on the potential use of this agent to control maturation of the vitamin K–dependent γ-carboxylation system in premature neonates with bleeding problems that cannot be corrected by vitamin K administration.

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SECTION VI

Lipid Metabolism

41

Emilio Herrera and Miguel Angel Lasunción

Maternal-Fetal Transfer of Lipid Metabolites

Changes in maternal lipid metabolism during gestation control the availability of lipid metabolites to the fetus, even though some components do not directly cross the placental barrier. This is the case with maternal plasma lipoproteins, the profile of which during pregnancy differs markedly from that seen in nonpregnant subjects. Although no evidence exists for their transfer to the fetus, placental cells have lipoprotein receptors that allow the uptake and release of their lipid components to the fetus. Other products of maternal lipid metabolism, however, such as free fatty acids (FFAs), glycerol, and ketone bodies, are able to cross the placenta and become available to the fetus without previous modification. Although the efficiency of transfer across the placenta differs for each of these metabolites, the major force controlling their actual transfer is the maternal-fetal concentration gradient.

HYPERLIPOPROTEINEMIA IN PREGNANCY AND ITS ROLE IN PROVIDING FATTY ACIDS FOR THE FETUS

Maternal hypertriglyceridemia is one of the most striking changes that takes place in lipid metabolism during gestation. The increase in plasma triglycerides during pregnancy is greater than the increases in phospholipids and cholesterol,[1,2] and more triglycerides are found in all of the lipoprotein fractions.[3,4] As shown in Figure 41-1, although both triglycerides and cholesterol in very-low-density lipoproteins (VLDLs), low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs) are higher in pregnant women in the third trimester of gestation than in the same women during the postlactation period, the triglyceride-cholesterol ratio remains stable in VLDL despite significant increases in both LDL and HDL. An examination of different HDL subclasses indicates that the rise in triglyceride-enriched HDL_{2b} is mainly responsible for the changes in HDL levels, whereas the small HDL_3 fractions become less abundant.[5] The increase in triglycerides per LDL particle is accompanied by a decrease in cholesterol and phospholipid content,[6] which causes reductions in LDL particle size,[7] although increases in the most buoyant of the LDL subfractions, LDL-1, have been reported with advancing gestation.[6]

The mechanisms responsible for these changes in the maternal lipoprotein profile during pregnancy are summarized in Figure 41-2. The increased adipose tissue lipolytic activity during late gestation[8,9] (which is mediated by an insulin-resistant condition[10]) enhances the availability of substrates for triglyceride synthesis in the liver. This action, together with the stimulating effect of estrogen on VLDL production[11] and the decreased extrahepatic lipoprotein lipase (LPL) activity,[5,12] is responsible in part for the augmented circulating maternal levels of VLDL in late pregnancy. This change in LPL activity corresponds to its decrease in adipose tissue, which in the rat is the body tissue that normally has the highest LPL activity and is the only tissue that shows a marked decrease during late gestation.[13,14] The decreased adipose tissue LPL activity also is a consequence of the insulin-resistant state characteristic of late pregnancy.[10,15] Although the abundance of VLDL could justify an enhanced conversion to lipoproteins of higher density, the specific enrichment in triglycerides of the latter seems to be the result of two additional mechanisms (Figure 41-2): (1) augmented activity of the cholesteryl ester transfer protein (CETP),[5,16] which mediates the transfer of triglycerides from triglyceride-rich lipoproteins such as VLDL to the higher-density lipoproteins LDL and HDL, and (2) decreased activity of hepatic lipase (HL),[5,12] which reduces the conversion of triglyceride-rich HDL_{2b} into the lipid-poor HDL_3. The decreased HL activity also has been proposed to contribute to the reductions in LDL size.[7] In addition, the decreased HL during late gestation may be a response to the increase in estrogens, because these hormones are known to inhibit both HL activity and messenger RNA (mRNA) expression.[12,17,18]

The events just summarized are responsible for the sustained hyperlipoproteinemia in the mother during gestation. Despite the impermeability of the placenta to lipoproteins, their lipidic components become available to the fetus; therefore those changes actively contribute to fetal development. In fact, reductions in maternal hyperlipoproteinemia in animals by treatment with hypolipidemic drugs has negative effects on fetal development.[19,20]

Essential fatty acids (EFAs) and long-chain polyunsaturated fatty acids (LC-PUFAs) derived from maternal diet are transported mainly in their esterified form in maternal plasma lipoproteins, rather than in their FFA form,[21,22] and must become available to the fetus despite the lack of a direct placental transfer of maternal lipoproteins. This transfer occurs thanks to the presence of lipoprotein receptors in the placental trophoblast cells that lie at the interface with maternal blood. These cells are therefore positioned to bind maternal lipoproteins and mediate their metabolism and subsequent transfer of the fatty acids they deliver to the fetal circulation. As described in more detail later, VLDL/apolipoprotein E (apo E) receptor (VLDLR) and LDL, HDL, and scavenger receptors are expressed in human placental tissue.[23-31]

Placental tissue expresses LPL activity[32-34] as well as phospholipase A_2[35-37] and intracellular lipase activities.[38,39] Esterified fatty acids in maternal plasma lipoproteins are therefore hydrolyzed and taken up by the placenta, where their reesterification and intracellular hydrolysis facilitate the diffusion of released fatty acids to the fetus and their subsequent transport to fetal liver. Using cultured placental trophoblast cells, it has been shown that esterified lipids provide a reservoir of fatty acids that can be released into the experimental medium.[40]

Once released into fetal plasma, placental transferred fatty acids bind to a specific oncofetal protein, the alpha

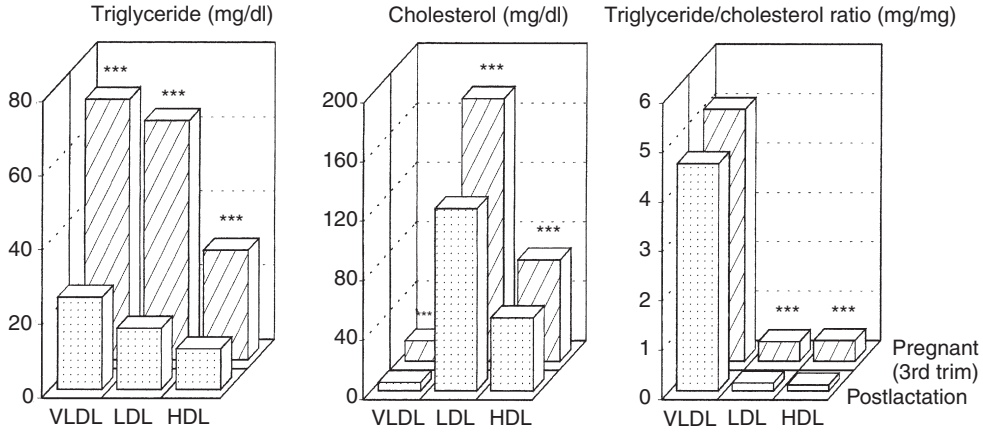


Figure 41-1 Plasma lipoprotein lipids in women in the third trimester of pregnancy and at postlactation. *Asterisks* indicate significant differences between the two groups. *HDL,* High-density lipoprotein; *LDL,* low-density lipoprotein; *VLDL,* very-low-density lipoprotein.

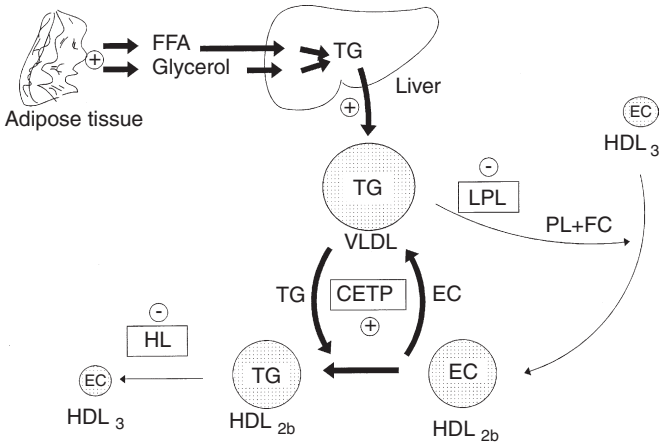


Figure 41-2 Proposed control of major pathways of very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) metabolism during late pregnancy. *CETP,* Cholesteryl ester transfer protein; *EC,* esterified cholesterol; *FC,* free cholesterol; *FFA,* free fatty acids; *HL,* hepatic lipase; *LCAT,* lecithin cholesterol acyl transferase; *LPL,* lipoprotein lipase; *PL,* phospholipids; *TG,* triglycerides.

fetoprotein,[41-43] and are rapidly transported to fetal liver. Those fatty acids taken up by fetal liver are esterified and released back into circulation in the form of triglycerides. This observation is consistent with the significant linear correlation found for certain PUFAs between maternal plasma and cord plasma triglycerides during late gestation in humans.[44] A linear correlation also has been found between maternal and fetal plasma triglycerides and PUFAs in the rat.[45,46] This relationship may have important implications for newborn weight, because a direct relationship between maternal triglycerides and newborn weight has been found in humans.[47-49] It has been proposed that the activation of the placental phospholipase A_2, resulting in an accumulation of lipid mediators in the fetoplacental unit, facilitates excess fetal fat accretion, thereby programming in utero obesity.[37]

MATERNAL LIPID METABOLISM AND PLACENTAL TRANSFER OF FREE FATTY ACIDS, GLYCEROL, AND KETONE BODIES TO THE FETUS

During the first part of gestation, the maternal body accumulates fat[50,51] as the result of combined effects of hyperphagia,[52] enhanced lipogenesis,[53] and unmodified or even increased extrahepatic LPL activity.[5,14,54] The tendency to accumulate fat ceases during late gestation[51,55] because maternal lipid metabolism changes to a catabolic condition. This is evidenced by increased adipose tissue lipolysis[9,14] and reduced uptake of circulating triglycerides,[56] secondary to the reduction in adipose tissue LPL activity,[5,12-14] as reviewed earlier. These changes, together with hepatic overproduction of triglycerides[57,58] and the enhanced absorption of dietary lipids,[59] are responsible for the marked progressive increase in maternal circulating triglycerides occurring during late gestation.[3,5] The major changes in the maternal lipid metabolism are summarized in Figure 41-3, which diagrams the changes in adipose tissue, liver, and intestinal activity that are responsible for the physiologic increase in circulating FFAs, glycerol, and triglyceride-rich lipoproteins (VLDL and chylomicrons). Under fed conditions, the frequency of maternal ketosis is no different from that in nonpregnant subjects, but it increases markedly under fasting conditions.[60,61]

With the exception of glycerol used in gluconeogenesis[62,63] and the LPL-mediated circulating triglyceride uptake by the mammary gland before labor,[59,64,65] no part of the increase in circulating lipid components in the fed mother during late gestation seems to benefit her metabolic needs directly. This increase, however, may benefit the fetus because this gestational period coincides with the rate of maximal fetal accretion, a time when the substrate, metabolic fuel, and essential component requirements of the fetus are greatly enhanced. The lipid component also may constitute a "floating" fuel store for both mother and fetus, easily accessible under conditions of food deprivation; this may explain the enhanced ketogenesis in the mother seen under fasting conditions.[60,66]

The enhanced availability of ketone bodies to maternal tissues under fasting conditions allows them to be used as metabolic fuels, with sparing of other, more limited and essential substrates, such as amino acids and glucose, for transport to the fetus. The fetus also receives maternal ketone bodies through the placenta, and their use plays an important role in the fetal metabolic economy under conditions of maternal food deprivation. Augmented lipolytic activity also increases maternal circulating glycerol levels.[13,62] Glycerol can be used as an efficient gluconeogenic substrate,[62,63,67] contributing to the maintenance of glucose production for fetal and maternal tissues. Metabolic adaptations found in the mother during starvation are summarized in Figure 41-4. The transfer of glucose, ketone bodies, and amino acids is emphasized in this figure because quantitatively, they are the major substrates crossing the placenta in this condition.

An understanding of fatty acid, glycerol, and ketone body placental transfer as well as their respective metabolic fates in the fetus provides insight into the effect on the fetus of these

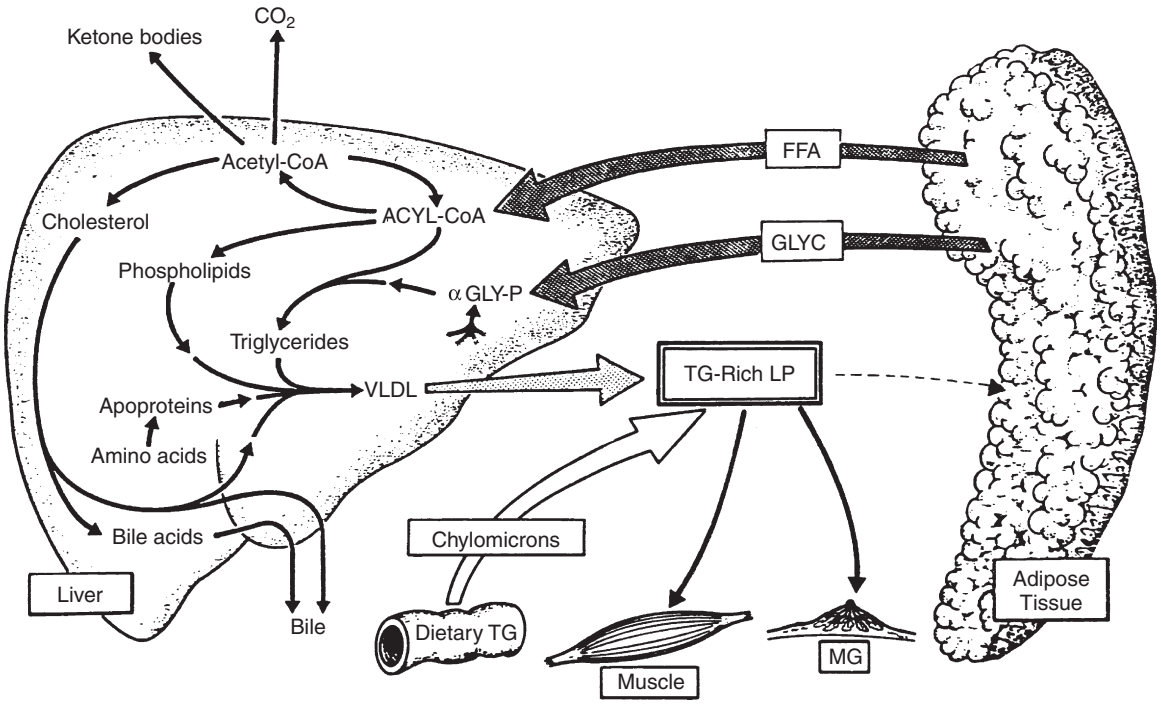


Figure 41-3 Summary of major changes in maternal lipid metabolism at late gestation. *CoA,* Coenzyme A; *FFA,* free fatty acids; α-GLY-P, alpha glycerolphosphate; *Glyc,* glycerol; *MG,* mammary gland; *TG,* triglycerides; *TG-RICH LP,* triglyceride-rich lipoproteins; *VLDL,* very-low-density lipoprotein. *(Modified from Herrera E, Gómez-Coronado D, Lasunción MA: Lipid metabolism in pregnancy,* Biol Neonate *51:70, 1987.)*

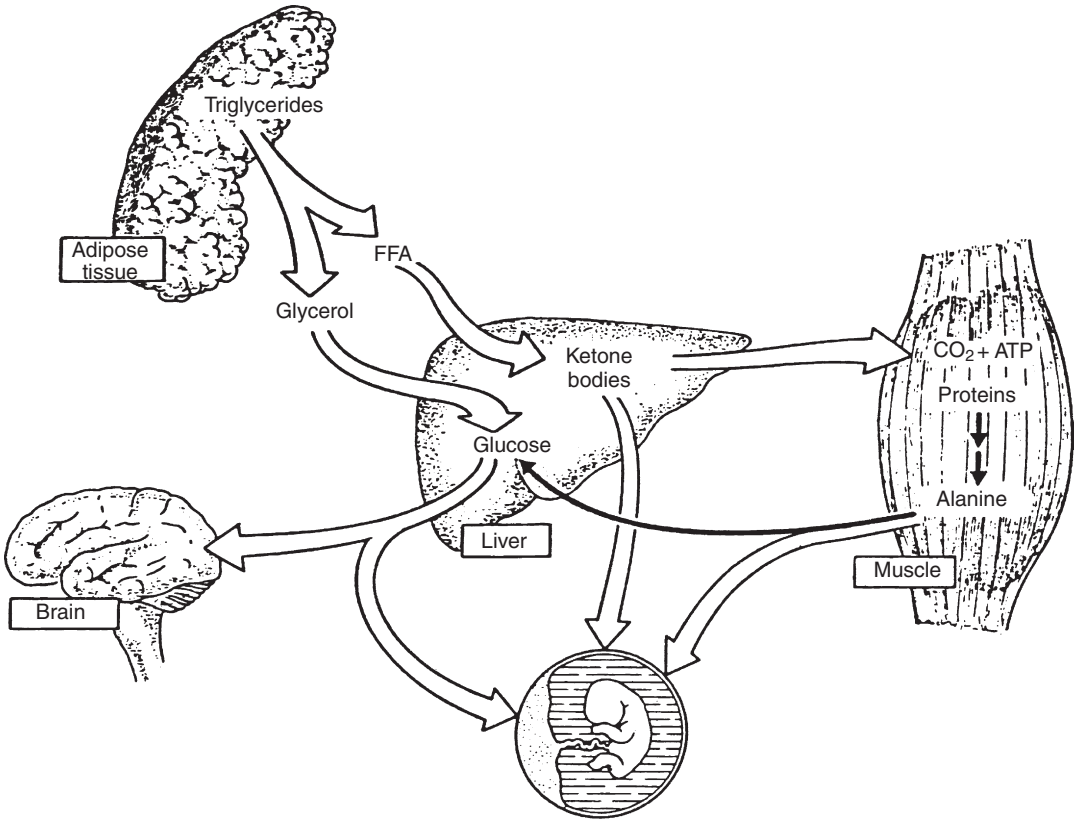


Figure 41-4 Maternal response to starvation. Enhanced adipose tissue lipolysis increases the availability in the liver of glycerol to be used as a preferential substrate for gluconeogenesis and of free fatty acids (FFA) for ketone body synthesis. By this mechanism, the mother conserves other gluconeogenic substrates, such as alanine, and ensures the adequate availability of fuels and metabolites to the fetus. *ATP,* Adenosine triphosphate. *(From Herrera E, Gómez-Coronado D, Lasunción MA: Lipid metabolism in pregnancy,* Biol Neonate *51:70, 1987.)*

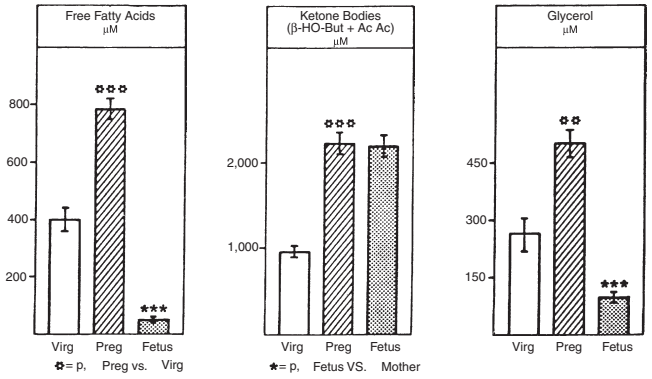


Figure 41-5 Concentrations of free fatty acids, ketone bodies, and glycerol in plasma of starved virgin rats and starved 19-day pregnant rats and their fetuses measured at 48 hours. *AcAc,* Acetoacetate; β-*HO-But,* beta hydroxybutyrate. *(From Herrera E, Gómez-Coronado D, Lasunción MA: Lipid metabolism in pregnancy,* Biol Neonate *51:70, 1987.)*

persistently elevated maternal circulating lipid levels. Figure 41-5 compares plasma levels of these metabolites in virgin and in 48-hour–fasted late-pregnancy rats and their fetuses. It can be seen that although fetal FFA and glycerol levels are much lower in the fetuses than in their mothers, the concentrations of ketone bodies are similar. These maternal-fetal concentration differences probably reflect the efficiency or magnitude of the placental transfer process.

Maternal-fetal nutrient transfer through the placenta may be accomplished by means of different mechanisms, including facilitated diffusion, active transport, and simple diffusion.[68,69] Transfer by simple diffusion seems to be a common mechanism for fatty acids and related moieties. The rate of transfer is a direct function of the concentration gradient and decreases with the molecular size and hydrosolubility.[70] In the case of placental transfer, however, other factors also participate[71,72]: uterine and umbilical blood flows, intrinsic placental metabolism, and structural characteristics. As might be expected, some of these factors, such as blood flow, contribute analogously to the transfer of any nutrient, but other factors differ with each nutrient and require specific consideration.

Free Fatty Acids

The fetus requires not only EFAs from the mother, to support growth[73] and brain development,[74] but also nonessential fatty acids, which, stored in fetal body fat, constitute an important substrate during early postnatal life.[75] This storage pool is especially important in species such as the guinea pig and human, in which body fat at term represents a substantial percentage of body weight (10% in guinea pigs and 16% in humans),[76] and de novo fatty acid synthesis by fetal tissues cannot fulfill fetal requirements.

Either FFAs bound to albumin or esterified fatty acids transported in lipoproteins are the potential sources of the fatty acids in the maternal side that cross to the placenta. Early studies in sheep[77] that measured venous-arterial differences across the umbilical circulation of the fetus in utero and across the maternal uterine circulation showed no significant passage of FFA to the fetus and led to the conclusion that FFAs did not appear to constitute a significant part of the metabolic fuel supplied there by the mother.[77] Later studies demonstrated, however, that the net flux of fatty acids from mother to fetus across the placenta varies greatly among species. For example, in species with both maternal and fetal layers in the placenta, such as the sheep, pig, and cat, the net transfer of fatty acid to the fetus generally is small.[78-80]

In humans, although maternal plasma FFAs are in a smaller proportion than esterified fatty acids in plasma lipoproteins, they are an important source of PUFAs to the fetus.[81,82] Current evidence suggests that cellular uptake of FFA occurs through a process of facilitated membrane translocation involving a plasma membrane fatty acid–binding protein ($FABP_{pm}$).[83,84] It has been shown that $FABP_{pm}$ is present in human placental membranes[85,86] and is responsible for the preferential uptake of LC-PUFAs by the human placenta. The preference for human placental fatty acid transfer from the maternal to the fetal circulation has been reported as docosahexaenoic ⇒ α-linolenic ⇒ linoleic ⇒ oleic ⇒ arachidonic acid.[87] Arachidonic acid was, however, the fatty acid with the highest accumulations in the placenta,[87] and it has been shown that this process of arachidonic uptake by placental syncytiotrophoblast membranes is highly dependent on adenosine triphosphate (ATP) and sodium,[88] implying an active transport mechanism for this fatty acid. A selectivity in the LC-PUFA placental transfer also may be exerted at the level of cellular metabolism: A certain proportion of arachidonic acid is converted to prostaglandins,[82] a selective incorporation of certain fatty acids into phospholipids has been found in the ovine placenta,[89] and even selective placental fatty acid oxidation[90,91] and lipid synthesis[92,93] may occur.

The combination of all of these processes determines the actual rate of placental fatty acid transfer and its selectivity. Through these mechanisms, the placenta selectively transports arachidonic acid and docosahexaenoic acid from the maternal to the fetal compartment, resulting in a proportional enrichment of these LC-PUFAs in circulating lipids in the fetus.[94,95] This transport occurs during the third trimester, when the fetal demands for neural and vascular growth are greatest.[96,97]

Although, as commented on earlier, current evidence indicates that fatty acids are selectively transferred across the placenta, EFAs and nonessential fatty acids also may use a common transfer mechanism. Using in situ perfused guinea pig or rabbit placentas, several investigations have demonstrated that within the physiologic range, the net FFA transfer to the fetus correlates with maternal plasma levels of FFA and that this transfer is regulated by the transplacental concentration gradient.[98,99] Furthermore, during maternal fasting, increased amounts of maternal FFA cross the placenta into fetal circulation and are incorporated into fetal stores.[100] These observations suggest that the transfer of several FFAs across the placenta is mainly by diffusion. Other factors affecting this transfer process are the uterine and umbilical blood flow rates[98,99] and the fetal plasma albumin concentration.[98,101,102] In this respect, the increase in albumin levels throughout the third trimester in the human fetus[103] may increase its FFA supply.

Glycerol

As a result of the enhanced lipolytic activity of maternal adipose tissue, plasmatic glycerol levels are consistently elevated during late gestation.[14,62,63] Values for plasma glycerol therefore generally are higher in the mother than in the fetus (see Figure 41-5), with some interspecies differences: The maternal-fetal glycerol gradient is greater in species with an epitheliochorial placenta (ruminants)[77,104] than in those with a hemochorial placenta.[105,106]

The available experimental data on placental glycerol transfer in any species are very limited. Although the molecular characteristics of glycerol (low weight and uncharged molecule) should facilitate easy placental transfer, its transfer rate is notably lower than for other metabolites with similar molecular characteristics such as glucose or L-alanine.[107-109] In contrast with the carrier-mediated process used for these two metabolites, placental

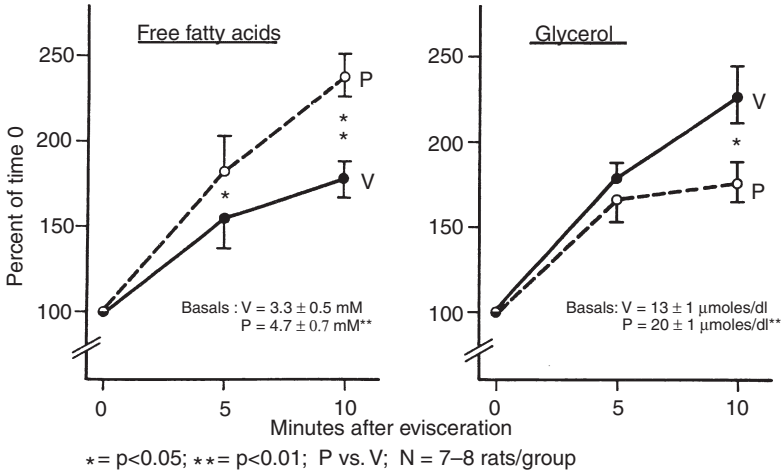


Figure 41-6 Effect of hepatectomy-nephrectomy on plasma free fatty acids and glycerol in virgin (V) and 20-day pregnant (P) rats. Experimental details are described in the original report by Yount and McNamara.[154] *(Data from Yount NY, McNamara DJ: Dietary regulation of maternal and fetal cholesterol metabolism in the guinea pig,* Biochim Biophys Acta *1085:82, 1991.)*

glycerol transfer is accomplished by simple diffusion.[104] In the sheep fetus, glycerol uptake is low, accounting for no more than 1.5% of the total oxygen consumption of the fetus.[77] In humans, it has not been possible to detect a transfer of glycerol from mother to fetus despite its favorable gradient.[110] By comparing different substrates, using the in situ infused placental technique in the rat, the rate of transfer of glycerol has been found to be much lower than that of glucose and alanine and similar to that of FFAs.[107] In addition, the fetal-placental unit has been demonstrated to convert glycerol into lactate and lipids[106]; this rapid use may actively contribute to maintaining the high glycerol gradient consistently found between maternal and fetal blood.[108-110]

Accelerated turnover of maternal glycerol seems to be influenced by the high liver glycerol kinase activity, which facilitates its rapid phosphorylation and subsequent conversion into glucose.[62,63,67] Although this mechanism indirectly benefits the fetus by providing glucose (see Figure 41-4), it may limit the availability of sufficient glycerol molecules for transfer to the fetus. Figure 41-6 summarizes studies that support this hypothesis. Hepatectomy normally results in increased plasma glycerol levels because of a reduction in glycerol use secondary to absence of the liver, the major receptor organ for this metabolite.[111] In pregnant rats, hepatectomy and nephrectomy produce significant but smaller increases in plasma glycerol levels than in nonpregnant animals. This difference cannot be interpreted as reduced lipolytic activity in the pregnant rat because plasma FFA, the other lipolytic product, increases more than in nonpregnant animals. The smaller increase may, however, reflect an augmented transfer of glycerol to the fetus, because glycerol levels in fetal plasma increase significantly after maternal hepatectomy and nephrectomy.[112]

Therefore placental glycerol transfer seems to be limited by the effective, rapid use of this substrate for gluconeogenesis by the liver and kidney cortex of the mother. Although the fetal-placental unit actively uses glycerol (which helps to maintain a favorable transfer gradient), its quantitative and physiologic roles in the fetus, except as a preferential substrate for fetal liver glyceride glycerol synthesis,[106] seem to be limited under normal conditions. Under conditions of markedly elevated maternal glycerol levels, however, the placental transfer of glycerol could become an important mechanism for providing substrates for the fetus.

Ketone Bodies

Although plasma levels of ketone bodies in the fed pregnant mother late in gestation are unchanged under physiologic conditions, under fasting[60,66,113,114] or diabetic[3,115] conditions, they are greatly elevated as a result of increased adipose tissue lipolytic activity and enhanced delivery of FFA to the liver. As noted earlier, when the supply of glucose is limited (e.g., in hypoglycemia or reduced insulin levels or sensitivity, or both), ketone bodies are used by some maternal tissues (e.g., skeletal muscle) as alternative substrates. Ketone bodies also can cross the placental barrier and be used as fuels and lipogenic substrates by the fetus.[116-118]

Maternal ketonemia in the pregnant patient with poorly controlled diabetes, with or without acidosis, has been associated with an increased stillbirth rate, an increased incidence of congenital malformations, and impaired neurophysiologic development in the infant.[117,119,120] These effects are thought to be secondary to placental transfer of maternal ketone bodies to the fetus.[121]

In addition to size and lipid solubility, molecular charge has an important effect on placental membrane permeability. At pH 7.4, most molecules of the two main ketone bodies, β-hydroxybutyrate and acetoacetate, are present in dissociated or ionized form, which retards their diffusion across the placenta. Nevertheless, in all species studied (human,[113,114,122] rat,[66,123] and sheep[121]), increments in maternal ketone bodies are accompanied by increments in fetal plasma levels, indicating efficient placental transfer; fetal liver ketogenesis is practically negligible.[124]

Placental transfer of ketone bodies occurs either by simple diffusion or by a low-specificity carrier-mediated process[104,125]; the efficiency of which varies among species. Although the maternofetal gradient for ketone bodies is higher than 10 in sheep,[121] in humans it is approximately 2,[110] and in rats it is close to 1[66,123] (see Figure 41-5), indicating that the amount of ketone bodies crossing the placenta is much smaller in ruminants than in nonruminant species. It has even been proposed that in the fasting condition, the contribution of ketone bodies to the fetal oxidative metabolism accounts for only 2% to 3% of total oxygen consumption in the case of sheep.[126] In the rat, β-hydroxybutyrate adequately replaces the glucose deficit in the placenta, fetal brain, and liver during fasting hypoglycemia.[118] This finding suggests a much greater contribution of ketone bodies to the fetal oxidative metabolism in the fasted nonruminant.

Key enzymes for ketone body utilization—3-hydroxybutyrate dehydrogenase (Enzyme Commission [EC] 1.1.1.30), 3-oxoacid-CoA transferase (EC 2.8.3.5), and acetyl-CoA acetyltransferase (EC 2.3.1.9)—have been found in the brain and other tissues in both the human and the rat fetus.[116,127] Both the human[117] and the rat brain[128] oxidize β-hydroxybutyrate in vitro in a form that is dependent on substrate concentration and not on the maternal nutritional state. Other fetal tissue

types known to oxidize ketone bodies are kidney, heart, liver, and placenta.[128,129] Some tissues are even known to use ketone bodies as substrates for fatty acid and cholesterol synthesis, as has been shown in the rat brain, liver, placenta, and lung after in vivo administration of ^{14}C-β-hydroxybutyrate to pregnant animals.[130] The activity of ketone body–metabolizing enzymes in fetal tissues (brain, liver, and kidney) can be increased by conditions that result in maternal hyperketonemia, such as starvation during the last days of gestation[131] or high-fat feeding.[132] Such a change is especially evident in the fetal brains from starved late-pregnancy rats[131] and may represent an important fetal adaptation to guarantee brain development under these conditions because fetal brain weight is better preserved than other fetal organ weights.

In conclusion, evidence obtained in nonruminant species is suggestive of efficient placental ketone body transfer, and of fetal use of these materials as substrates for both oxidation and lipogenesis, even in preference to other substrates (glucose, lactate, and amino acids). Because both the placental transfer and the use of ketone bodies are concentration-dependent, the quantitative contribution to fetal metabolism is important only under conditions of maternal hyperketonemia (e.g., starvation, high-fat diet, diabetes).

CHOLESTEROL IN THE FETUS

Role of Cholesterol and Related Compounds in Development

Cholesterol plays an important role in fetal development as well as in the general physiology of the organism. First, it is an essential component of cell membranes. By interacting with phospholipids and sphingolipids, cholesterol contributes to the characteristic physicochemical properties of membranes, mainly fluidity and passive permeability.[133] Cholesterol is not homogeneously distributed in the membrane; rather, it is concentrated in structures such as rafts and caveolae, where it modulates the function of different integral proteins and receptors. Cholesterol is the precursor of both bile acids and steroid hormones; in the fetus, glucocorticoids are intensely synthesized by the adrenal gland in the last part of development. Cholesterol and its oxidized derivatives—oxysterols—are key regulators of different metabolic processes, both by modulating the proteolytic activation of sterol response element–binding protein (SREBP) and by acting as ligands of nuclear receptors, such as LXR (liver X receptor).[134,135] Active SREBP and LXR are transcription factors that regulate the expression of multiple genes implicated in intracellular lipid homeostasis and lipoprotein metabolism.[134,136]

Other important actions of cholesterol also have become apparent; these actions have special relevance for the fetus because they are related to development, embryogenesis, and differentiation. Cholesterol is required for cell proliferation, not only for membrane formation, but also for the activation of regulatory proteins involved in cell cycle progression—specifically, in the transition from the G_2 phase to mitosis.[137,138] Cholesterol plays important roles in differentiation and cell-to-cell communication; in fact, it has been demonstrated to be a key factor in synaptogenesis.[138] Finally, cholesterol is essential in embryonic patterning in both vertebrates and invertebrates.[140] This patterning is attained mainly by activation of hedgehog proteins (i.e., sonic hedgehog [Shh] in humans), which are involved in cell differentiation.[141]

It is thus conceivable that defects affecting cholesterol availability will have deleterious consequences in fetal development. In fact, congenital defects in cholesterol biosynthesis or the reduction of cholesterol synthesis with xenobiotics result in severe malformations and dysfunctions, mainly affecting the craniofacial organs and the central nervous system (CNS), respectively, alterations that are similar to those caused by Shh deficiency.[140,142,143]

It has been observed in pigs that fetal weight is directly correlated with plasma cholesterol concentration in the fetus at late gestation.[144] Similarly, neonatal pigs from lines with genetically low cholesterol levels are smaller at birth and grow more slowly, but the growth rate improved when they were fed with cholesterol.[145,146] An increase in neonatal survival is noted with supplementary dietary fat for the peripartal sow.[147] Thus cholesterol is essential to body growth and the development of the CNS, and fetal requirements must be met either by efficient endogenous cholesterol biosynthesis or transfer from the mother.

Cholesterol Biosynthesis and Congenital Defects

Cholesterol biosynthesis is a multienzymatic pathway that can be separated into three segments according to the type of compounds that are synthesized in each one, that is, mevalonic acid, isoprenoids, and sterols, respectively. In the first, also called the *mevalonate pathway,* three molecules of acetyl-coenzyme A (CoA) are successively condensed by the action of acetyl-CoA acetyltransferase and cytosolic 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase to form HMG-CoA, which is then reduced with the loss of CoA, generating mevalonate, a six-carbon compound[148] (Figure 41-7). This complex reaction is catalyzed by HMG-CoA reductase, which is present in the endoplasmic reticulum and is the rate-limiting enzyme in cholesterol biosynthesis. In the next series of reactions, mevalonate is converted to squalene (see Figure 41-7), which is the immediate precursor of sterols. The first sterol formed is lanosterol, which contains 30 carbons (see Figure 41-7). The transformation of lanosterol into cholesterol occurs in the endoplasmic reticulum and involves at least seven different enzymes (Figure 41-8).

In humans, six different genetic defects in the cholesterol biosynthesis pathway have been identified. Mevalonic aciduria (i.e., Mendelian Inheritance in Man designation [MIM] 251770) is caused by missense mutations in mevalonate kinase, which impair the formation of both isoprenoids and sterols (see Figure 41-7). The patients show dysmorphias and failure to thrive. Milder mutations of the enzyme also underlie hyperimmunoglobulinemia D and periodic fever syndrome (MIM 260920). The rest of the disorders are due to defects in the postsqualene segment of the pathway (see Figure 41-8). Greenberg skeletal dysplasia (MIM 215140), which is associated with short-limb dwarfism, probably is caused by mutations in Δ^{14}-reductase, but confirmation at the molecular level has yet to be achieved. The CHILD (*c*ongenital *h*emidysplasia with *i*chthyosiform erythroderma and *l*imb *d*efects) syndrome (MIM 308050) and Conradi-Hünermann-Happle syndrome (CPDX2; MIM 302960) are caused by deficiencies of C4-demethylase and Δ^8-Δ^7-isomerase, respectively; these two disorders are of X-linked dominant inheritance, so carrier males are lethally affected and females present with several skeletal and skin abnormalities. Desmosterolosis (MIM 602398) is an extremely rare disorder, probably of autosomal recessive inheritance, that is due to the deficiency of Δ^{24}-reductase; the infants affected died shortly after birth and suffered from multiple malformations and dysmorphias. The last of these disorders, and probably the best known and most widely studied, is the Smith-Lemli-Opitz syndrome (SLOS) (MIM 270400), which is caused by mutations in Δ^7-reductase. The phenotypic expression is highly variable; the most prominent anomalies are microcephalia and facial dysmorphias. All affected patients accumulate 7-dehydrocholesterol in plasma and tissues, but the clinical severity of this syndrome correlates best with its relative level to plasma cholesterol. This observation suggests that the availability of cholesterol during development is one of the major determinants of the phenotypic expressions in SLOS. In animals, several experimental models confirm the molecular basis of the aforementioned disorders and extend the current knowledge on the significance

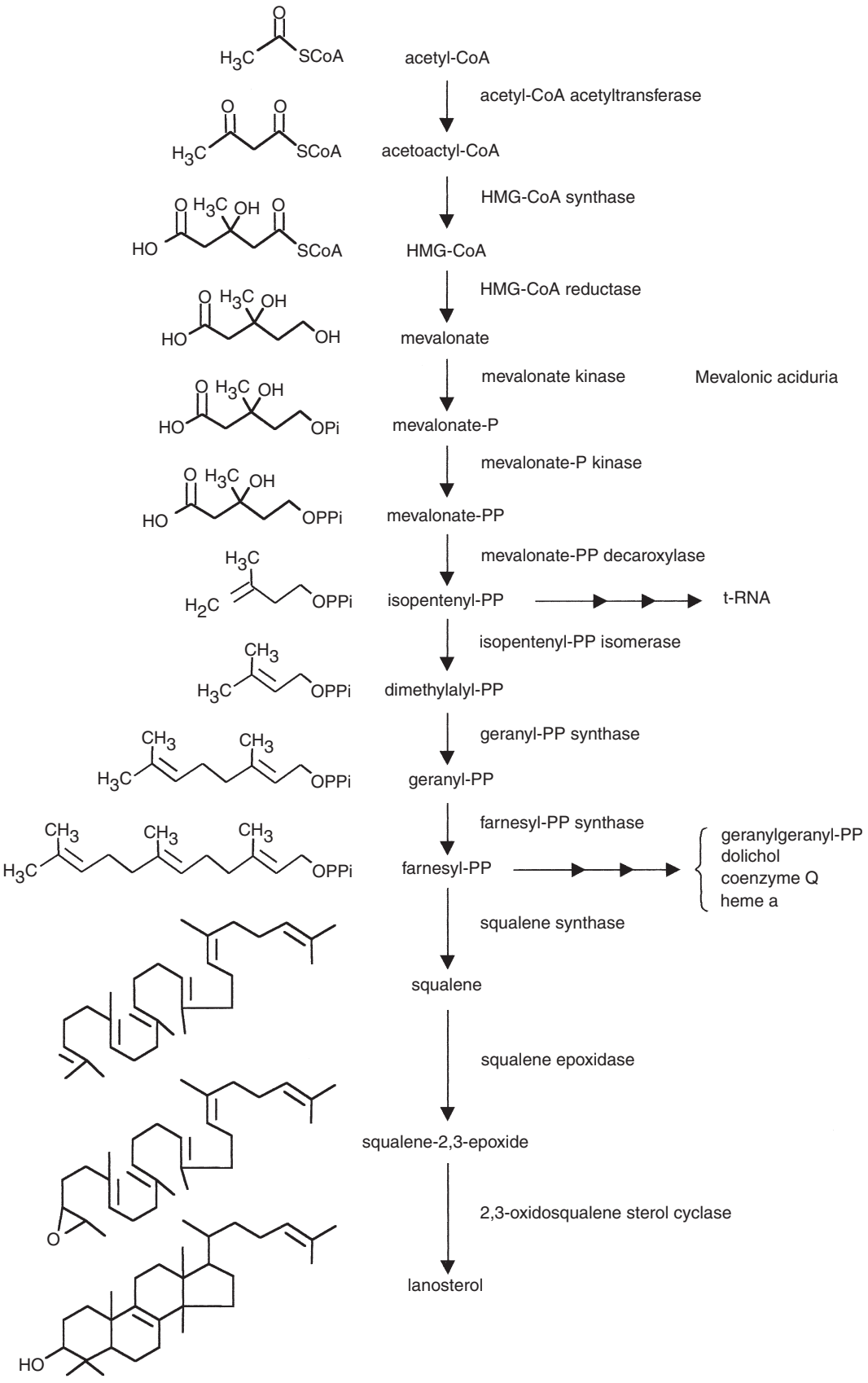


Figure 41-7 Biosynthesis of lanosterol from acetyl-CoA. Aside from the route leading to the formation of lanosterol, the first sterol in the cholesterol biosynthesis pathway, the alternative use of isopentenyl-PP for the derivation of certain types of transfer RNA (tRNA) and farnesyl-PP for several isoprenoids is shown. *Multiple-headed arrows* indicate several reactions. The name of a human inherited disorder is shown beside the affected enzyme. *CoA,* Coenzyme A; *HMG,* 3-hydroxy-3-methylglutaryl; *PP,* pyrophosphate; *tRNA,* transfer RNA.

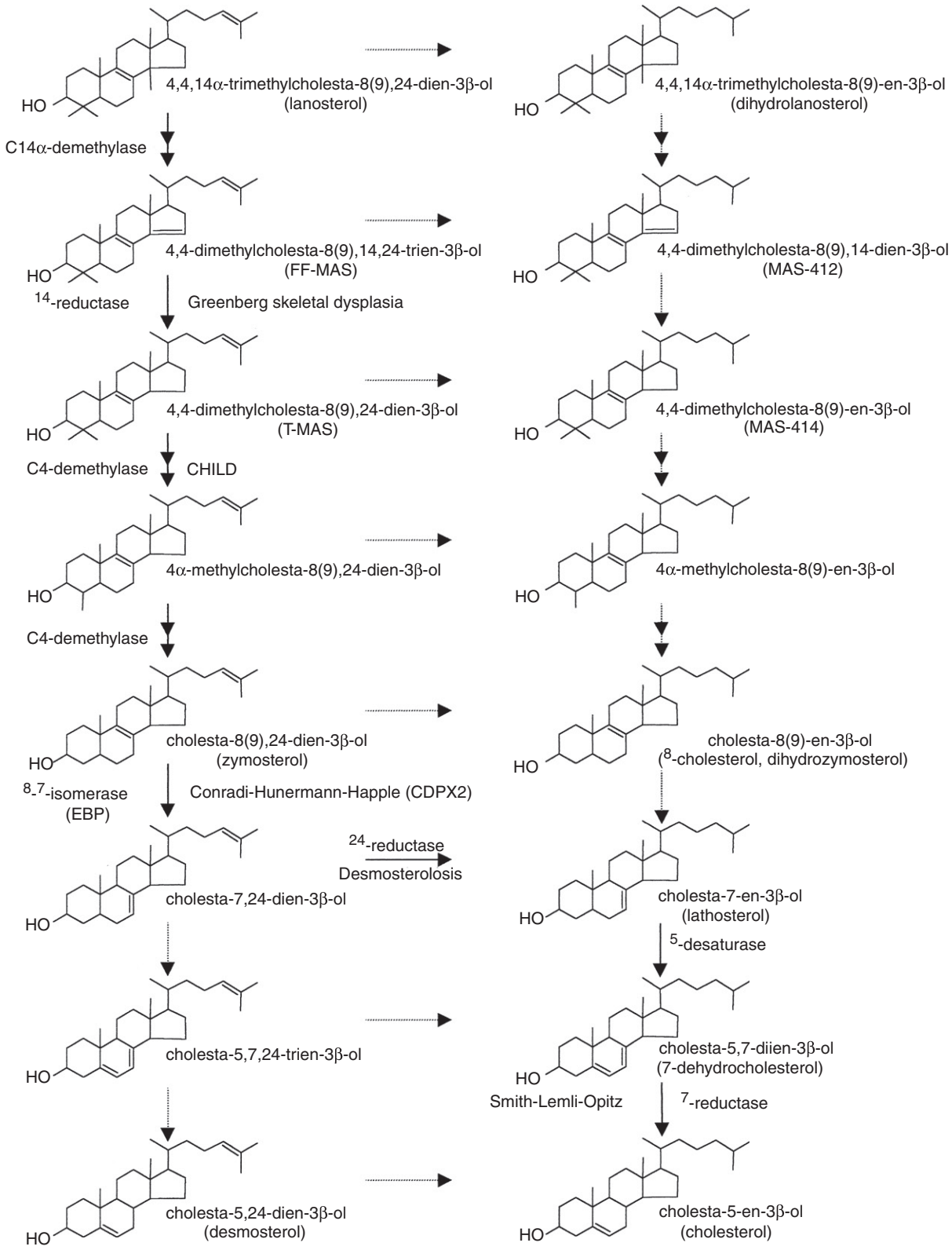


Figure 41-8 Biosynthesis of cholesterol from lanosterol. The main route is indicated by the *solid arrows* (see text for comments). *Double-headed arrows* indicate several reactions.The names of the human inherited disorders are shown beside the affected enzymes. *CHILD, c*ongenital *h*emidysplasia with *i*chthyosiform erythroderma and *l*imb *d*efects; *EBP,* emopamil-binding protein; *FF-MAS,* follicular fluid-MAS; *MAS,* meiosis-activating sterol; *T-MAS,* testis-MAS.

of cholesterol biosynthesis defects on pathogenesis. In this regard, a null mutation of lanosterol synthase and combinations of hypomorphic mutations of lanosterol synthase and farnesyl diphosphate farnesyltransferase-1, both of which conditions lead to decreased cholesterol synthesis, have been associated with cataract formation in the Shumiya rat, a hereditary cataractous strain.[149] In general, these congenital alterations show the important role of cholesterol and its immediate precursors in morphogenesis and fetal development. Excellent reviews on this subject are available.[142,143]

Sources of Fetal Cholesterol

The demands for cholesterol in the fetus are relatively high, especially during the last third of gestation, when fetal growth is extremely rapid. In principle, the fetus may obtain cholesterol from both endogenous biosynthesis and from the yolk sac and placenta. A comprehensive review on this subject has recently been published.[150] By tracking the appearance of radioactivity in the fetus, early experiments demonstrated the placental transfer of maternal cholesterol to the fetus in different species, such as the rat,[151] guinea pig, rabbit,[152] and rhesus monkey.[153] In those studies, the estimated contribution of maternal cholesterol to the fetus varied widely. An accurate approach to estimate the needs for extra sources of cholesterol in the fetus is to compare cholesterol accretion with the absolute rate of cholesterol biosynthesis. Measurements of [^{3}H]water incorporation into cholesterol revealed that cholesterol biosynthesis in fetal tissues is highly active; when calculated per mass unit, the rate of cholesterol synthesis in fetal tissues is several times higher than in maternal tissues in different species.[154-159] This increased level of synthesis is especially high in the fetal brain, which appears almost completely autonomous in cholesterol accretion, and the liver, for which cholesterol biosynthesis proceeds at a rate exceeding the need for cholesterol accretion, resulting in an excess that is secreted into the plasma for uptake by other developing fetal organs.[157,158] These findings are consistent with the near-maximal expression at the level of mRNA of different enzymes involved in cholesterol biosynthesis[159] and the high activity of HMG-CoA reductase—the rate-limiting enzyme of cholesterol biosynthesis—in fetal tissues.[160,161] Important differences among species have been recognized, however.

In the rat, endogenously synthesized cholesterol appears to account for practically all fetal cholesterol,[157,158] meaning that the other potential sources are insignificant, at least during the later stages of gestation. In fact, Belknap and Dietschy[155] found that although the rat placenta did take up ^{125}I-cellobiose–labeled lipoprotein from maternal circulation, none of the apolipoprotein or cholesterol was transferred in any appreciable amount to the fetus. These studies indicate that the rat fetus receives little or no cholesterol from the mother but, rather, satisfies its need for cholesterol during fetal development through local synthesis. Maneuvers directed to modify cholesterol homeostasis in the mother had no significant effects on cholesterol levels or cholesterol synthesis rates in the fetus. Thus feeding pregnant rats with cholesterol, which resulted in an increase in plasma cholesterol concentration and reduced cholesterol synthesis in the maternal compartment, did not affect any of these parameters in the fetus.[154,155,162] Conversely, treatment of pregnant rats with cholestyramine—a bile acid sequestrant that interferes with intestinal cholesterol absorption and consequently stimulates cholesterol biosynthesis in maternal tissues—did not alter cholesterol accretion in the fetus.[163] All of these findings affirm that in the rat, fetal cholesterol originates mainly from endogenous de novo synthesis, rather than from placental transfer.

In the early stages of gestation in the rat, however, maternal cholesterol may make a significant contribution to the fetal cholesterol pool. For instance, treatment of pregnant rats with AY 9944—an inhibitor of Δ^7-reductase—results in fetal teratogenesis, but the simultaneous oral administration of cholesterol early in gestation completely prevents the characteristic holoprosencephalic brain malformations.[140,164,165] By contrast, the anomalies of fetal masculinization caused by AY 9944, when administered late in gestation, are not prevented by the compensatory administration of cholesterol to the mother.[140] These results firmly suggest that maternal cholesterol reaches the fetal compartment at least early in gestation and is of significant physiologic relevance in the rat. In mice, by injecting ^{13}C-cholesterol into the maternal circulation at different stages and following its appearance in fetal tissues, it has been demonstrated that maternal cholesterol is transferred through the yolk sac and the placenta to the embryo and fetus, respectively.[166]

In other species, exogenous cholesterol may constitute an important, quantitative source of cholesterol for the fetus. In the golden Syrian hamster, Woollett found that endogenous biosynthesis accounted for only 40% of the fetal cholesterol, suggesting that the placenta or the yolk sac, or both, contributed the remainder.[156] Actually, in hamsters fed increasing amounts of cholesterol, the cholesterol concentration in the fetal tissues was found to be linearly correlated with the maternal plasma cholesterol concentration, whereas cholesterol synthesis decreased in the reverse way.[166] These studies in hamsters demonstrated that fetal cholesterol homeostasis is affected by maternal plasma cholesterol concentration in gradient fashion.[166] In the guinea pig, fetal cholesterol homeostasis was found to be relatively insensitive to dietary cholesterol manipulations in the mother throughout gestation.[154] Nevertheless, feeding cholestyramine to the mothers, although producing the expected stimulation of [^{3}H]water incorporation into sterols in maternal liver, also resulted in a 1.4-fold increase in fetal carcass cholesterol synthesis at 60 days gestation, which demonstrates that fetal cholesterol homeostasis may respond to induction by maternal hypocholesterolemia during the late gestation period.[154]

Data in humans are more limited, and cholesterol biosynthesis in fetal tissues has not been evaluated for obvious reasons. In deliveries at term, Parker and associates[167] measured cholesterol levels in the umbilical venous and the umbilical arterial plasma and found a highly significant difference between paired HDL, LDL, and total cholesterol concentrations, venous levels being 7.7% to 12.8% higher than those in arterial plasma. These data were suggestive of the delivery of cholesterol from the placenta to the fetus, which could either be synthesized in the placenta or derived from the maternal plasma. The same investigators, however, estimated that the contribution of such cholesterol to the fetal plasma cholesterol pool was of minimal quantitative importance in term newborns of women experiencing uncomplicated pregnancies.[167] Several observational studies have addressed this issue by comparing maternal lipoprotein-cholesterol levels with those in mixed umbilical cord blood, reporting either a positive correlation[168,169] or no correlation between these values.[167,170,171] The gestational stage, however, could influence these results, because cholesterol plasma concentration has been reported to be significantly higher in premature than in full-term newborns.[172,173] In fact, fetal cholesterol levels show a strong inverse correlation with fetal age, being two-fold higher in 5-month than in 7-month fetuses.[174] This finding has been interpreted as an indication of the greater requirements of cholesterol in the younger, more immature fetuses.[174] Of interest, in fetuses younger than 6 months, although not in older fetuses, plasma cholesterol levels are significantly, directly correlated with maternal levels.[174] Therefore available results in humans strongly suggest that maternal cholesterol substantially contributes to fetal cholesterol accretion early in gestation.

Both the placenta and the yolk sac are able to synthesize and remove cholesterol from the maternal circulation and to secrete it into the fetal circulation. In the pregnant rat, it was determined

that placenta takes up LDL at rates approximately one-third those seen in the maternal liver.[155] In the hamster, LDL clearance rates in the placenta and yolk sac were similar to those in the liver and higher than those in the decidua when measured at midgestation (on day 10.5).[175] In the same study, it was found that clearance rates for HDL apo A-I and HDL cholesteryl ether were similar to those for LDL in the placenta and decidua, whereas rates in the yolk sac were dramatically higher. As gestation progressed to day 14.5, LDL and HDL clearance rates decreased in all three tissues.[175]

Regarding the receptors responsible for the uptake of lipoprotein cholesterol, multiple possibilities have been examined. Both the placenta[23-29] and, to a lesser extent, the yolk sac[175] express LDL receptors in their membranes. In correlation with this, several groups of investigators documented the use of LDL cholesterol for progesterone synthesis by trophoblastic cells in vitro.[176,177] Of interest, it was found that HDL-2 cholesterol stimulated placental progesterone secretion to a greater extent than LDL did, by a mechanism that did not involve the LDL receptor.[177] Further evidence on the role of maternal HDL as an exogenous source of fetal cholesterol comes from studies in apolipoprotein A-I (apo A-I)–deficient mice. These animals have markedly reduced HDL cholesterol levels in plasma, and cholesterol accretion in the fetus was diminished, although fetal cholesterol synthesis was not affected.[178] These results were in line with previous observations by Knopp and associates,[179] describing apo A-I concentration in maternal plasma as a significant positive predictor of birth length. It appears that HDL may potentially contribute a significant proportion of the cholesterol required for fetal development.

Several lipoprotein receptors, different from the LDL receptor, that can mediate the uptake of lipoprotein cholesterol have been detected in placental preparations. These include CLA-1/SR-BI (*C*D36 and *L*IMPII *a*nalogous-*1*/*s*cavenger *r*eceptor type *B* class *I*),[175,180-182] an HDL receptor; megalin/glycoprotein 330,[175,183,184] a protein related to the LDL receptor; cubilin,[185] a protein that binds HDL and acts in conjunction with megalin to mediate HDL endocytosis; and LDL receptor–related protein (LRP)[183] and apo ER2,[186,187] which bind to and mediate the uptake of VLDL . Although the yolk sac is vestigial in humans, in rodents it plays an important role in fetal nutrition. Epithelial cells of the visceral yolk sac were shown to express several lipoprotein receptors, including LDL receptor,[175] CLA-1/SR-BI,[175,188] megalin,[183] and cubilin.[189,190]

A final question is how trophoblast or yolk sac cholesterol reaches fetal circulation. One possibility is that these organs synthesize and secrete apo B–containing lipoproteins, similar to the liver or the intestine in the adult. A main issue is the expression of apo B and microsomal triglyceride transfer protein (MTP), which are mandatory for assembly and secretion, respectively, of apo B–containing lipoproteins. In biopsy specimens from human term placentas, both apo B and MTP mRNAs were detected, and on incubation in vitro, secretion of newly formed apo B–containing lipoproteins was demonstrated.[191] Similar findings were reported for the yolk sac in rodents.[192,193] Whether these lipoproteins efficiently reach the fetal circulation is elusive. It is intriguing that no abnormalities of conception and embryonic lethality have been reported in humans who inherit genetic deficiencies of either of these proteins, whereas in homozygous mice bearing knockouts of either apo B[194,195] or MTP,[196] early embryonic lethality is characteristic. This difference may reflect a greater dependence of the mouse embryo on lipoproteins from placental or yolk sac origin for development, in comparison with the human embryo. On the other hand, other mechanisms for the efflux of cholesterol also are present in both the placenta and the yolk sac.[150] Actually, in monolayer cultures of human coriocarcinoma BeWo cells, it has been shown that either radiolabeled free cholesterol or LDL cholesteryl esters given to the apical side are transported to the basolateral surface of the cells and then effluxed to lipid acceptors present in the fetal side.[197] Recently, a transplacental transfer of lipoprotein-derived cholesterol to the fetus, mediated by endothelial ABCA1 and ABCG1, has been proposed.[198]

Taken together, these data confirm the ability of both the placenta and the yolk sac to take up cholesterol from maternal lipoproteins and to deliver it to the fetus, but the extent to which it is exported and the factors that regulate this process remain to be clarified.

SUMMARY

During gestation, both triglyceride and cholesterol increases in all lipoprotein fractions and are associated with an increase in the triglyceride-cholesterol ratio in LDL and HDL. The increase in HDL corresponds mainly to triglyceride-enriched HDL-2. The presence of lipoprotein receptors in the placenta ensures the availability of essential lipoprotein components to the fetus and provides a teleologic reason for maternal hyperlipoproteinemia.

Sustained maternal hyperlipidemia during late pregnancy is of pivotal importance in fetal development, especially during the stage of maximal fetal accretion. Besides using transferred fatty acids, the fetus also benefits from two other products of maternal lipid metabolism: glycerol and ketone bodies. Although only a small proportion of maternally derived glycerol crosses the placenta, it is quantitatively important as a substrate for maternal gluconeogenesis. Because fetal oxidative metabolism is preferentially sustained by maternal glucose crossing the placenta, the use of glycerol for glucose synthesis actively contributes to the fetal glucose supply.

In nonruminant species, maternal ketone bodies are readily transferred to the fetus, where they can be efficiently used as carbon fuels for oxidative metabolism or as lipogenic substrates. Because all of these processes are concentration-dependent, they become relevant only under conditions of maternal hyperketonemia. Under healthy physiologic conditions, they constitute an important support for fetal metabolism when the availability of other substrates is more limited (e.g., during periods of maternal starvation). Under conditions of sustained maternal hyperketonemia, such as high-fat feeding, fetal metabolism also adapts to an enhanced consumption of ketone bodies.

Although the contribution of maternal cholesterol to fetal cholesterol appears to be important during early gestation, it seems to be of minimal quantitative importance during late gestation. This is consistent with the high capacity of all fetal tissues to synthesize cholesterol. In humans, several congenital defects in the cholesterol biosynthesis pathway have been identified, showing the important role of cholesterol in morphogenesis and fetal development.

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John E. Van Aerde, Michaelann S. Wilke, Miguel Feldman, and M. Thomas Clandinin

Accretion of Lipid in the Fetus and Newborn

FETAL FAT METABOLISM

The nutrient and energy requirements of the developing mammalian fetus include the fuels to provide energy for tissue synthesis and the basic nutrients for synthesis of new tissues.[1] From weeks 26 through 40 of gestation, human fetal weight increases more than four-fold. Part of this increase in weight is water, but the rate of accretion of fat and nonfat components is even greater.[2] Between 26 and 30- weeks, nonfat and fat calories contribute equally to the energy content of the body[2]; however, beyond that time, fat accumulation considerably exceeds that of the nonfat components.[3] At 36 weeks of gestation, 1.9 g of fat accumulates for each gram of nonfat daily weight gain, and by term gestation, the deposition of fat accounts for more than 90% of the calories accumulated by the fetus.[1] The rate of fat accretion is approximately linear between 36 and 40 weeks of gestation, and by the end of pregnancy, fat accretion ranges from 1.6 to 3.4 g/kg/day.[1] At 28 weeks, it is slightly less, ranging between 1 and 1.8 g/kg/day.

The main phospholipids of cell membranes contain two of a variety of fatty acids and a substituted (amino) alcohol attached to a glycerol phosphate backbone. The nature of the alcohol head group and that of the attached fatty acids have major effects on the biologic function of that membrane.[4]

The nature and chain length of fatty acidsassociated with complex lipids are of particular significance because they determine the structural and functional roles of these lipids in neural membranes.[5] Fatty acids can be chemically characterized according to presence and number of double bonds: Those with no double bonds are termed *saturated;* those with one double bond, *monounsaturated;* and those with two or more double bonds, *polyunsaturated* fatty acids [PUFAs]). Metabolism of PUFAs results in formation of long-chain PUFAs (LC-PUFAs) (i.e., fatty acids with more than 18 carbon atoms and multiple double bonds), which are incorporated into complex structural membrane lipids or used for production of biologically active substances (eicosanoids). Biosynthetic pathways producing LC-PUFAs consist of a series of desaturation and chain elongation reactions in which there is no direct cross-over between unsaturated metabolites from one sequence to the other. The type and amount of any given unsaturated fatty acid found in tissue lipids are determined by factors that regulate the synthesis of unsaturated fatty acids, as well as by control mechanisms dictating the incorporation of a given fatty acid into a lipid.[6]

Eicosanoids are potent compounds that display many different biologic effects.[7] In response to various stimuli, eicosanoids are generated by de novo synthesis, are not stored, and are quickly inactivated.[8] The precursor fatty acids, predominantly arachidonic acid (AA) (C20:4*n*-6), are esterified in the second position of cellular phospholipids and do not exist as free acids to any significant extent. Thus the generation of free AA from phospholipids is a rate-limiting step for formation of biologically active eicosanoids.[8]

Brain Development

Biochemical Development and Myelination

Brain development is a sequential anatomic process characterized by specific, well-defined stages of growth and maturation.[9] Biochemical development of the brain also appears to be sequential, correlating with anatomic development.[10] The role of lipids during brain development is the subject of a published text.[11] Any defect in the course of brain development can easily result in gross abnormalities of brain function and can cause serious and irreparable damage in the regulation of body activities and expression of behavior.

Lipids are extremely important constituents of the brain. Not only are they essential for the structure and function of the neuronal and glial membranes, but they also constitute the main components of the myelin sheath, which endows the mature axon with its rapid conduction capacity. In myelin, lipids constitute 70% to 75% of dry weight. All of the major lipids found in whole brain also are present in myelin, and no lipid components are present exclusively in myelin. However, cerebroside is the most typical lipid found in myelin, and its concentration in brain is proportional to the myelin content.[12] In the adult brain, lipids are the most abundant compounds, constituting approximately 60% of the total solids or 10% to 20% of the fresh weight of the brain. Significant regional variation in brain lipid composition has been demonstrated: The number of lipid types, as discussed next, is lower in gray matter and higher in white matter. In cortical gray matter, lipids make up only 7.5% of fresh weight, but in the subcortical white matter, they constitute more than 20%.[13]

The major classes of brain lipids are cholesterol, phospholipids, cerebrosides, sulfatides, and gangliosides. Cerebrosides and sulfatides are found primarily in myelin; the gangliosides

are associated with the structure of the external surface of the neuronal membrane, especially in the synaptic region.[14] In myelin, the fatty acids are predominantly saturated or monounsaturated, whereas in neuronal membranes, particularly synaptosomes, the PUFAs predominate. Each neuron in the cerebral cortex gray matter has more than 10,000 discrete areas of membrane responsible for neurotransmission to other nerve cells through the synaptosomes, which have high concentrations of polyunsaturates, particularly docosahexaenoic acid (DHA) (C22:6*n*-3). Phosphatidylcholine is quantitatively the most abundant phospholipid in the immature brain, but the major DHA-containing phospholipids in the synaptosomes are phosphatidylserine and phosphatidylethanolamine.[15] In the developing brain, the lipid composition undergoes very significant qualitative and quantitative changes with maturation. During the early stages of development, only those lipids that are part of the general structure of the cell membrane increase. With the onset of myelination, special lipids accumulate in brain tissue and drastically alter its lipid composition, both qualitatively and quantitatively.

In the human brain, myelination begins during the perinatal period, demonstrates a rapid increase during the first year, and continues into the second decade of life. The first structures in the human brain to be myelinated are the motor and sensory roots; the last ones are the reticular formation, nonspecific thalamic projections, and the neocortex.[16,17] Phylogenetically older structures (e.g., the spinal cord) are myelinated earlier, and the more recently acquired structures follow later.

The total quantity of all classes of lipids increases during brain development. However, not all lipids show the same temporal pattern of increase. For example, phospholipids tend to decrease during brain maturation, whereas others, such as cholesterol, cerebrosides, and sulfatides, are found to increase.[18] These observations reflect the deposition of myelin in the developing brain and also changes in myelin composition at different ages. The relative proportions of different brain phospholipids and the fatty acid composition of phospholipids in gray and white matter also change with age throughout prenatal life, particularly in the last trimester of pregnancy.

Fatty Acid Deficiency

Prenatal and postnatal essential fatty acid deficiency reduces body and brain weights of the developing progeny even though the mothers are asymptomatic. The extent of these changes in the brain depends on the timing of the deficiency. Compositional changes in myelin parallel or even exceed those observed in whole brain[19] and can be manipulated by dietary fatty acid composition.[20,21]

The increasing survival of very-low-birth-weight infants has led to study of the development of accretion of essential fatty acids during the last trimester of fetal development through the early weeks of life. The intrauterine requirements for *n*-6 and *n*-3 fatty acids are estimated to be 400 and 50 mg/kg/day, respectively. For the high-risk, low-birth-weight infant weighing approximately 1300 g, extrauterine tissue synthesis would use approximately 280 mg/kg/day of *n*-6 fatty acids and approximately 35 mg/kg/day of *n*-3 fatty acids (based on a projected growth rate that is similar in composition and amount to the intrauterine rate)[22] (Figure 42-1).

Clandinin and colleagues[23] determined that during the last trimester of fetal growth, accretion of major tissue fatty acids occurs earlier and faster in cervical segments than in lumbar regions. Minimal postnatal accretion of fatty acids occurs in cervical regions of the spinal cord, with the exception of moderate increases in total *n*-9 and saturated fatty acids, reflecting myelination. Postnatal accretion of fatty acids in lumbar regions of the spinal cord occurs for approximately 4 weeks, at rates significantly in excess of in utero rates.

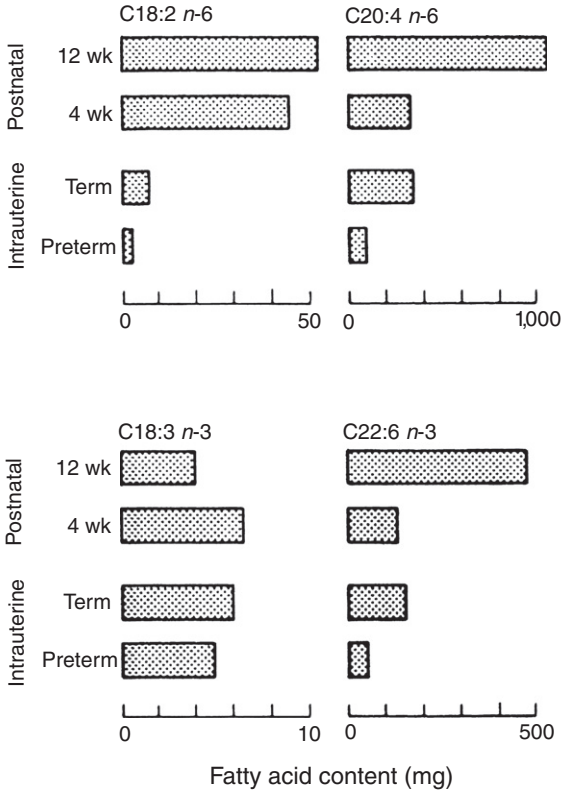


Figure 42-1 Accretion of major essential fatty acids in human brain during intrauterine and early postnatal development.

Changes in Composition of the Brain During Development

Changes in the lipid composition of the brain during development principally reflect accumulation of lipids in myelin sheaths.[24] As a specialized plasma membrane of the myelinating cell, myelin contains large amounts of cholesterol.[25] Cholesterol and sphingolipids (cerebroside, sulfatide, and sphingomyelin) are found mainly in myelin and are the major constituents of total cerebral lipids in the mature brain. On a dry weight basis, approximately 70% of cerebral lipids are contained in myelin. Cerebroside contributes to the stability of the structure of the myelin sheath. In the immature brain, before myelination has occurred, the lipid composition is similar to that in other tissues.[26] At that time, lipid droplets of cholesterol esters are present in glial cell bodies,[27] which rapidly disappear as myelination progresses. Cerebrosides and sulfatides are virtually absent from the brain until myelination begins. At 4 months of gestation, the human fetal brain (which lacks myelin) contains very small amounts of cerebroside and sulfatide, in addition to other glycolipids. The changes in the relative proportions of lipids and the content of individual brain lipids reflect differences in the distribution of lipids in the various cell membranes of the central nervous system. Early in gestation, most cells are devoid of cell processes, and therefore, the lipid content is mainly related to the cell body. Oligodendroglial cells from the mature brain are characterized by high cerebroside and cholesterol concentrations, but neurons and astrocytes do not change in lipid composition during development.[27]

Ethanolamine phospholipids contain most of the PUFAs esterified in cerebral lipids.[28] Essential fatty acid deficiency in the rat, if imposed during pregnancy, results in significant alterations in the content of LC-PUFAs.[29,30] Conservation of essential fatty acids in nervous tissue by recycling processes may contribute to stability of the fatty acid composition of cerebral lipids.

LC-PUFAs with more than 22 carbon atoms are absent from the human fetal brain but appear in sphingomyelin during the late fetal period, when C24:1 predominates.[31] Two classes of sphingomyelin exist: One possesses long-chain fatty acids, with low turnover and localized to myelin; the other is present in non-myelin membranes. The fatty acid composition of cerebroside also changes with development, as does that of individual phospholipids (i.e., phosphatidylcholine) from whole brain and from myelin. Phospholipids exhibit an increase in chain length and a greater proportion of unsaturated fatty acids during maturation. Throughout development, lipid components of the cell membrane undergo continuous turnover, and the lipid components of plasma membranes, including the myelin sheath, are in dynamic equilibrium with intracellular membranes. The enzymes required for lipid biosynthesis exhibit a temporal increase in activity that parallels the rates of accumulation of cerebral lipids.

The ganglioside composition of the brain was reviewed by Ramsey and Nicholas.[24] Gangliosides are important constituents of membranes and are involved in cell differentiation, proliferation, neuritogenesis, growth, inhibition, signaling, and apoptosis.[32-35] The major types of brain gangliosides are fairly consistent across species and include GM_1, GD_3, GD_{1a}, GT_{1a}, GD_{1b}, and GT_{1b}.[36] Gangliosides are present in highest amounts in gray matter and are particularly enriched in synaptic junctions. Rapid changes in the ganglioside composition of the human fetal cortex occur in the latter half of gestation. Vanier and associates[37] revealed that white matter is similar to that of gray matter ganglioside composition of the cerebellum until term, after which GM_1 composition increases to approximately 60% of total gangliosides. Maximal increase in brain ganglioside content, approximately three-fold in the cerebral cortex from fetal week 15 to the age of 6 months, corresponds to rapid increases in development of neuronal connections at these times. This pattern of increase includes increases in GM_1 and GT_1, with GD_{1a} fractions.[37] Severe maternal protein-calorie deficiency that lasts throughout gestation and lactation does not alter prenatal and early postnatal ganglioside accumulation.[38] However, a deficit in postnatal ganglioside accumulation develops after postnatal day 10. Stage of development and thus ganglioside amount and composition may relate to membrane ganglioside function. Research by Park and associates[39] showed that in neonatal rat brain, postnatal ganglioside intake seems to influence ganglioside amount but not composition. Although the ganglioside composition of the diet and enterocyte changed, the plasma and brain composition of the major types of gangliosides measured did not. The functional significance of this preservation of composition is not known.

The fatty acyl-coenzyme A synthetase of cerebral mitochondria is already at maximum activity before birth.[40] A correlation has been found between the main fatty acid changes occurring in the brain and those occurring in the liver during early human development. Linoleic acid (LA) (C18:2*n*-6) and α-linolenic acid (ALA) (C18:3*n*-3) represent a small proportion of the fatty acyl components of the phosphoglycerides of the fetal brain. By contrast, AA and DHA are readily incorporated into the structural lipids of the developing brain.[41]

Intrauterine Fatty Acid Accretion

Development of the infant brain is accompanied by a rapid rise in cellularity and a declining water content.[42] These changes are associated with an increase in the brain and cerebellar content of LC-PUFAs secondary to an increase in the number of plasma membranes.[43] Quantitative analysis of the fatty acid composition of brain lobes indicates rapid accretion of chain elongation and desaturation products during the last trimester of brain growth. Similar analysis of the essential fatty acid composition of cerebrum and cerebellum has demonstrated the vulnerability of the very-low-birth-weight infant to essential fatty acid deficiency at birth.[43] For example, analysis of the chemical composition of the developing fetus indicates that the total fat content of a 1000-g infant is only 28 g.[44] Levels of LA and ALA are consistently low in brain during the last trimester of development, although a marked accretion of long-chain desaturation products, AA and DHA, has occurred by that time (see Figure 42-1).[43]

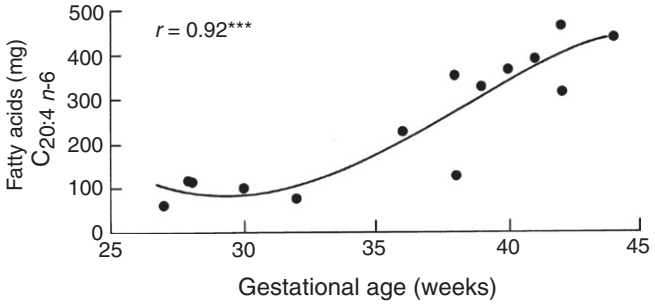


Figure 42-2 Fatty acid content of infant brain expressed as a function of gestational age. Values of *r* and significance levels are indicated (degrees of freedom = 13). Totals for each fatty acid (mg) are calculated for infants of various gestational ages (in weeks).

During the last trimester of pregnancy, a steady increase is seen in the cerebellar content of all fatty acids, reflecting a general increase in fatty acid content. However, *n*-3 fatty acids represent an exception in that absolute accretion rates, particularly DHA, are higher in the antenatal than in the postnatal period. This finding suggests that the accretion of long-chain *n*-3 fatty acids during the third trimester may be particularly important for the normal rise in brain cellularity.[43]

Examination of intrauterine accretion of *n*-6 and *n*-3 LC-PUFAs has indicated rapid accretion of these components in the fetal brain.[43] In the third trimester, accumulation rates are approximately 43 mg of *n*-6 and 22 mg of *n*-3 PUFAs per week[22] (Figure 42-2). Thus, during the last trimester, the major fatty acids to accrue are the chain elongation–desaturation products; only 2.6% of the brain fatty acid content is LA. In term infants during early postnatal brain development, the LA content increases significantly, whereas little increase occurs in brain levels of chain elongation–desaturation products in the first several weeks after birth.[45] In humans, the brain undergoes an accelerated growth phase during the last trimester of pregnancy and the first 18 months of postnatal life (Figure 42-3). During this vulnerable period, essential fatty acids are required for structural expansion of the brain.

PLACENTA

Placental Metabolism and Transport of Lipid

The maternofetal transfer of substances across the placenta involves any of five potential mechanisms (passive diffusion, facilitated diffusion, active transport, ultrafiltration, and pinocytosis); however, fatty acids, some amino acids, and lipid-soluble vitamins are transferred by a passive diffusion process.[46,47] Interspecies comparison of fetal fat accretion, with placental permeability for free fatty acids, demonstrates that the human neonate (who has a high body fat content) has a placenta relatively permeable to free fatty acids. Most observations suggest that embryonic and fetal lipids in early gestation are derived from maternal fatty acids that cross the placenta, followed by a gradual shift to de novo synthesis from glucose in fetal tissue with advancing gestation.[48]

Free fatty acids and cholesterol are the only lipid fractions present in maternal blood that are known to cross the placenta.[49] Free fatty acids are released to the fetus, but the mechanism of

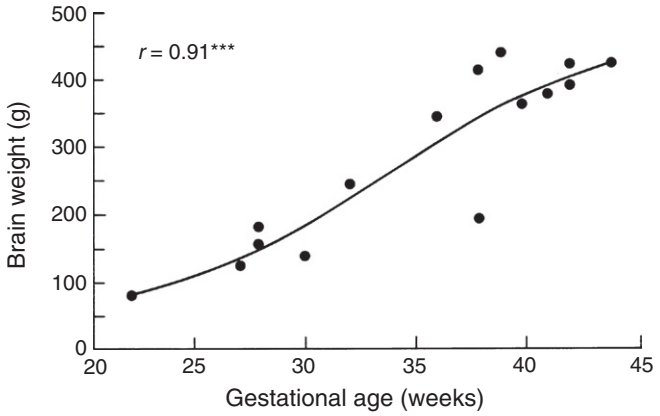


Figure 42-3 Brain weight as a function of gestational age.

release is not known. Maternal free fatty acids enter the placenta and pass into the fetal circulation[47]; however, the placental permeability for free fatty acids is low. Although intact complex maternal lipids may not be transported as triacylglycerol, partial glycerides or phospholipids may be synthesized within the placenta and then released into the fetal circulation.[50] Triglycerides and phospholipids are taken up by the placenta but are metabolized to such an extent that neither can be considered a source of free fatty acids for the fetus.[49] In humans, the levels of free fatty acids are higher in maternal blood than fetal blood, and their concentrations are highly correlated.[49] Under normal circumstances, the fetal/maternal ratio of free fatty acids is less than 0.5.[1] "The fetus appears to obtain [fatty acids] from a combination of de novo synthesis, a passive gradient–dependent transplacental passage of nonesterified [fatty acids] and a selective maternofetal placental transport for certain [fatty acids], such as physiologically important LC-PUFAs."[51] Preferential uptake of LC-PUFAs by the fetal side of the placenta has been suggested as the main mechanism of incorporation of LC-PUFAs in the fetus.[52]

Fatty acids are required by the fetus and the placenta for synthesis of complex lipids.[50] Because fetal tissues can actively synthesize fatty acids, controversy has arisen concerning the relative amounts of fatty acids synthesized by the fetus and the amounts contributed by the mother.[53] An inverse correlation exists between chain length and transfer rate for fatty acids. Interpretation of this information is difficult because many factors change coincident with a reduction in chain length, including a decrease in the association constant of the albumin-free fatty acid complex. Observations in animals and estimates of placental transfer of fatty acids, derived from in vitro studies, suggest that approximately 30% of the daily lipid accumulation by the fetus is transported from the maternal circulation[54]; the remainder is synthesized by the fetus. Thus approximately 1.7 g/day of fatty acids (including essential fatty acids) is transferred each day.

Evidence in humans indicates a placental role in preferential accumulation of LC-PUFAs in fetal tissues.[55] Selective transfer of monounsaturated and polyunsaturated fatty acids into fetal circulation seems to occur as a product of a physiologic mechanism, rather than only as a function of free concentration in the maternal perfusate.[56] Selectivity of placental uptake was proposed previously as the result of brush border and basal membrane concentrations of fatty acid–binding proteins in syncytiotrophoblasts,[57] as well as LC-PUFAs specificity of and competition for fatty acid–binding sites.[55] Human placental tissue lacks both the δ^6 and δ^5 desaturase activities, so any LC-PUFAs in the fetal circulation must come from the maternal plasma.[51,55,58] The presence of desaturase and elongase activity in developing fetal liver[58] makes it more difficult to determine the sources of these fatty acids in the developing fetus. Isotope tracer studies may help to elucidate these processes in vivo.

Examination of intrauterine accretion of *n*-6 and *n*-3 LC-PUFAs has indicated rapid accretion of these components in the fetal brain.[43] In the third trimester, they accumulate at rates of approximately 43 mg of *n*-6 and 22 mg of *n*-3 PUFAs per week.[22] Thus, during the last trimester, the major fatty acids to accrue are the chain elongation–desaturation products; only 2.6% of the brain FA content is LA. In term infants during early postnatal brain development, the LA content increases significantly, whereas little increase occurs in brain levels of chain elongation–desaturation products for several weeks after birth.[45]

The transport of essential fatty acids derived from the maternal diet appears to be affected by maternal nutrition and varies during the course of gestation. Crawford and associates[59] demonstrated that the proportion of LC-PUFA derivatives of parent-essential fatty acids is greatest in fetal brain, less in fetal liver, and further reduced in cord blood. Maternal blood has an even lower proportion of PUFAs. This biomagnification in the fetal brain indicates that the composition of phospholipids in the fetoplacental unit is the result of alterations designed to achieve a high proportion of LC-PUFAs necessary for structural components of the developing brain.[52] Placental prostaglandin synthesis has been assessed in several cellular and subcellular preparations, and it appears that the placenta is capable of producing numerous AA metabolites including prostaglandin D_2, prostaglandin I_2 (i.e., prostacyclin), and several hydroxyacids.[52] Research done with perfused human placentas indicates that the biomagnification of AA may result from fetal synthesis, whereas selective placental transport may be the case for DHA.[56]

Free fatty acids may cross the placenta in either direction, but the movement of phosphoglycerides and triglycerides generally is restricted or absent. This is especially relevant when one considers that maternally derived LA has been found mostly in the free fatty acid fraction of fetal circulating lipids, whereas AA is selectively compartmentalized into phosphoglycerides by the placenta and is exported to the fetus mostly in that form. Thus, the selectivity with which the placenta distributes LC-PUFAs into a lipid fraction impermeable to the placental barrier may allow those fatty acids to be preferentially retained in the fetoplacental unit, in relation to shorter chain homologous essential fatty acids. It also is possible that this mechanism serves to provide the fetus with preformed structural components for inclusion in developing membrane systems.[52] The placenta is able to take up lipids associated with chylomicrons and very-low-density lipid fractions in maternal plasma through the action of lipoprotein lipase. Triacylglycerol hydrolase may be important for releasing free fatty acids from intracellular triacylglycerol stores in the placenta.[55] These lipids are subsequently released into the fetal circulation as both esterified and nonesterified fatty acids.

Although little is known about the processing of fatty acids accumulated in fetal liver, it has been suggested that they are incorporated into lipoprotein for transport to sites of utilization (i.e., adipose tissue).[60] High rates of lipogenesis have been observed in fetal liver (as compared with lipogenesis after birth), suggesting an important role for the liver in the synthesis of endogenous fatty acids and indicating that not all of the fetal need for fatty acids can be met by placental transfer.[61]

Placental Transfer and Cord Polyunsaturated Fatty Acids

Normal growth of infants depends on an adequate supply of essential fatty acids. The human fetus is unable to synthesize essential fatty acids, which must be delivered from the maternal circulation via the placenta.[12] Previous reports indicated that maternal plasma lipids are elevated during pregnancy and that levels of total lipids, triglycerides, cholesterol, and phospholipids

are also significantly higher than those in the neonate.[62] However, the relationship of maternal nutrition during pregnancy with fetal plasma lipid levels remains controversial.

Friedman and colleagues[63] analyzed the phospholipid, cholesterol ester, triglyceride, and free fatty acid levels in maternal plasma and in cord venous and arterial blood of infants at 28 to 44 weeks od gestation. In that study, no difference was found in fatty acid composition of phospholipids, cholesterol esters, triglycerides, and free fatty acids between cord venous and arterial plasma samples obtained at birth.[63] The concentration of LA in cord plasma phospholipid was less than 40% of the maternal value. Lowest values were noted before 34 weeks gestation. By contrast, venous and arterial cord plasma contained a higher concentration of AA than maternal plasma in various lipid fractions (phospholipids, cholesterol esters, triglycerides, and free fatty acids).[62,63] Similarly, the concentration of DHA was higher in cord plasma at term as compared with levels in maternal plasma.[12] The total levels of fatty acids of the ALA and LA series in cord plasma phospholipid increased from 25.5% at 24 to 33 weeks to 33.7% at 34 to 37 weeks and 36% at term. By comparison, the maternal plasma level of these fatty acids was 39.9% at term.[64] Factors that may be responsible for the increased concentrations of AA and DHA in the fetal circulation include preferential transfer of these fatty acids in later gestation and the presence of enzymatic activity in the fetus, resulting in desaturation and elongation of LA to form AA.

Maternal exposure to *trans* fatty acids may be a factor that influences essential fatty acid metabolism in term and preterm infants at birth. Maternal plasma and infant plasma have been found to contain similar percentages of *trans* fatty acids.[65] Humans cannot synthesize *trans* fatty acids; presumably, then, these fatty acids must originate from the maternal diet,[66] and they appear in fetal circulation via placental transport.[55] An inverse relationship has been indicated between *trans*-isomeric fatty acids and LC-PUFAs in cord blood lipids. Research thus far has indicated that total *trans* fatty acids in plasma lipid fractions are not related to LA and ALA but are inversely correlated to *n*-3 and *n*-6 LC-PUFAs and to the product/substrate ratios of long-chain polyunsaturated biosynthesis.[67] *Trans* fatty acids may inhibit the transport of LC-PUFAs to the placenta by competing for fatty acid–binding sites.[55] Existing evidence has not yet shown whether these effects on fatty acid metabolism alter fetal growth or length of gestation.

The fatty acid composition of adipose tissue triglycerides in the newborn infant differs from that of the mother, similar to the observed differences in plasma lipid composition. Body stores of essential fatty acids are particularly low in low-birthweight infants. The concentration of LA in fetal tissue is less than in adults, and the proportion of LA in muscle phospholipids increases with advancing gestational age. Because of the low concentration of LA in fetal adipose tissue, the contribution of LA to the pool of plasma-free fatty acids during lipolysis is also small.[68] Cord plasma levels of LA are also diminished, a finding reflecting the reduced tissue level of this fatty acid. The low plasma concentrations of LA may have a beneficial effect by increasing the maternofetal gradient for LA and facilitating transfer across the placenta. As noted earlier, the relatively high concentration of AA in fetal tissue could result either from preferential transfer of this fatty acid or from desaturation and elongation of LA in the fetus to produce AA. Tissue enrichment in AA plays an important role during fetal life by maintaining the normal function of biologic membranes and perhaps by providing a substrate for prostaglandin biosynthesis. Postnatal brain development is characterized by an increase in LA content; however, chain elongation–desaturation products do not increase for several weeks postnatally. This finding suggests that placental transfer of LC-PUFAs is of primary importance in accretion of these fatty acids in the fetus.[69]

POSTNATAL LIPID ACCRETION

Fatty Acid Utilization

For most mammalian species, birth represents an abrupt transition from a low-fat to a high-fat diet. The endocrine changes that occur at birth, particularly the surge in the plasma concentration of glucagon, favor mobilization of lipids from peripheral tissues. Blood concentrations of free fatty acids and glycerol increase, resulting in a higher rate of fatty acid oxidation and increased ketone body production. After birth, milk provides a relatively high-fat diet compared with that consumed by the fetus, and it is clear that lipids play an important role both as metabolic fuels and in fat deposition. Fat is the main energy source for the newborn infant. In addition to providing 40% to 50% of the total calories in human milk or formula, fats are essential to normal development because they provide fatty acids necessary for brain development; they are an integral part of cell membranes, and they are the sole vehicle for fat-soluble vitamins and hormones in milk.[70]

Using the accretion rate of essential fatty acids during normal growth of the human fetus and newborn as a reference point, Clandinin and associates[43,71,72] demonstrated a lag in accretion of LC-PUFAs in the brain and liver during fetal development and mobilization of PUFA deposits in the liver for a varying period after birth. From these studies, it was concluded that chain elongation products do not accrue in the neonatal brain for several weeks postnatally. In fact, chain elongation products are not synthesized at maximal rates for several weeks after birth in the term infant, further supporting the hypothesis that placental transfer of LC-PUFAs is of primary importance in their accretion.[72]

Although the composition of fatty acids contained in brain total lipids and phosphoglycerides demonstrates a characteristic pattern, small alterations in composition of dietary fat intake change the composition of brain membranes during development. When animals are fed n-3–deficient diets, docosapentaenoic acid (DPA) (C22:5*n*-6) replaces DHA (C22:*n*6*n*-3) in neural membranes.[73] The functional effects of this replacement are not yet clear. Even though DPA is a precursor for AA, this substitution does not seem to affect brain AA levels in *n*-3–deficient rats.[74] The *n*-6–*n*-3 balance also is important; hence, maternal diets providing a high ratio of LA to ALA (10% corn oil) result in higher levels of DPA and lower levels of DHA in the brains of newborn rats.[75] In similar experiments,[76] weanling rats fed diets supplemented with oil rich either in *n*-3 (fish oil) or *n*-6 (safflower oil) fatty acids underwent reciprocal replacement of *n*-6 and *n*-3 families of fatty acids in brain ethanolamine phosphoglycerides according to the dietary levels. Jumpsen and associates[77] examined the effects of feeding ALA, AA, and DHA on brain development in neonatal rats. The *n*-3–*n*-6 ratio at amounts within the recommended limits significantly altered the fatty acid content of the developing rat brain. The amount and rate of AA or DHA accretion into glial and neuronal membranes varied, and ethanolamine phosphoglycerides exhibited the most distinct changes when LC-PUFAs were fed.[77] Thus fatty acid constituents and synthesis of structural lipid in brain membranes that undergo turnover can be altered by changes in the composition of dietary fat intake.[78]

Inclusion of LC-PUFAs is currently implemented in infant formulas. The addition of DHA or AA alone is inappropriate because the balanced addition of both *n*-6 and *n*-3 LC-PUFAs (AA and DHA) is required to achieve optimal accretion. The balance between AA and DHA in the diet is a powerful determinant of the level of these fatty acids in the developing brain. It is becoming increasingly evident that the addition of LA and ALA does not provide optimal *n*-3 and *n*-6 LC-PUFAs, and optimal growth and development require the addition of both AA and DHA. Previous studies had difficulty showing an effect of AA- and DHA-supplemented formulas on growth; however, this difficulty probably was related to a lack of statistical power resulting from insufficient length or size of study

or narrow inclusion criteria that eliminated detection of difference. In a double-blind, controlled multicenter study,[79] Clandinin and associates demonstrated that feeding AA and DHA is essential to enhance growth and neurodevelopment in premature infants (less than 35 weeks of postmenstrual age with a birth weight less than 1500 g). Infants fed standard formula supplemented with 34 mg/100 kcal AA and 17 mg/100 kcal DHA from single-cell oils (n = 95) had significantly greater mean body weights than those of infants in the control group (unsupplemented formula) (n = 98) at every study time point from 66 weeks of postmenstrual age until the study's end, and a length similar to or greater than that in the control group from 40 to 100 weeks; by 118 weeks (n = 72) they also had a mean length and weight comparable with those for a reference term group of infants (n = 97). In addition, mental and psychomotor scores were higher in the supplemented formula group than in the control group. It was concluded that formulas supplemented with DHA and AA at median levels found in human milk are safe for very-low-birth-weight premature infants and are essential for optimal growth and development.

Intake

Human Milk

Many physiologic factors influence milk composition, such as stage of lactation, length of gestation, age of the mother, intrafeed regulation of milk release, and the baby's demand for milk.[80] Throughout early lactation, "preterm" milk is 40% to 50% higher in its total fat content than milk from mothers whose babies were born at term. As the period of gestation increases and approaches term, differences in the nutrient content between preterm and term breast milk diminish. The stage of lactation may also affect lipid content: Colostrum has 1.19% (weight for weight) fat, transitional milk has 3% fat, and mature milk has 3.3% fat.[81] Fat provides approximately 50% of the energy content in human milk.

When compared with cow's milk, the fat content of human milk is characterized by a relatively high proportion of oleic acid, a higher level of essential fatty acids, and a relatively lower level of short-chain fatty acids such as butyric, caproic, caprylic, and capric acids.[80] Also notable are the differences in the ganglioside content of human colostrum, later milk, formula, and cow's milk. Human milk and colostrum contain almost twice the total lipid-bound sialic acid found in cow's milk or formula.[82] The major ganglioside fractions identified in colostrum were GD_3 and GX_1, and later milk also contained a high proportion of GM_3, whereas cow's milk and formula consisted mostly of GD_3.[82] The biologic significance of these differences has not yet been fully delineated.

Limited quantitative information is available on the actual amounts of fatty acids consumed by breast-fed newborn infants. Although better equipment has become available for analysis of fatty acid composition of milk samples, most studies lack basic information, such as lipid content and volume of the milk consumed.[83] Alterations in maternal intake of PUFAs are reflected in milk fatty acid composition; as a consequence, newborn tissue composition is affected by maternal diet in both animals and humans.[84-87] The LC-PUFA status at birth is one of the major determinants of postnatal change in LC-PUFA status, with breast milk the most effective diet for maintaining these levels.[88] Consequently, lower LC-PUFA status in formula-fed infants compared with breast-fed infants may persist at least until the first year of life.[89]

In well-nourished women (who are in energy balance), the fatty acid composition of breast milk shows a strong relationship with the distribution of fat and carbohydrate calories in the diet. For example, a carbohydrate-rich diet providing 75% of energy requirements may result in an increase in the content of saturated fatty acids such as lauric acid (C12:0) and myristic acid (C14:0) and a decrease in the LA content.[80] Maternal diet in well-nourished women affects milk lipid constituents, but not the total amount of fat present in milk. Milk from vegetarian mothers exhibits levels of milk fat similar to those in milk from women who consume an omnivorous diet; however, it has a higher proportion of PUFAs derived from increased consumption of vegetable fats, with the primary effect on the milk LA content.[90] In women who are vegetarians, the higher concentration of LA is accompanied by lower concentrations of DHA in the erythrocyte membranes of their breast-fed infants.[90] It is estimated using isotope tracers that approximately 30% of milk LA is directly transferred from maternal diet, whereas conversion of dietary LA to AA and other longer-chain LA homologues (dihomo-γ-linolenic acid) (20:3n-6) occurs at approximately 1.2% and 11%, respectively.[91] However, the contribution of dietary LA to milk AA may be slightly underestimated because of isotope dilution. Human milk supplies significant amounts of C20 and C22 fatty acids, which are more effective in supporting tissue AA and DHA accretion than are similar amounts of LA and ALA.[92] A review of the research on human milk fatty acid composition of milk lipids detailed the differences in DHA content of human milk between Western and non-Western women, with content averaging 0.45% and 0.88% of total fatty acids, respectively, although a wide variation in Western diet was reported.[93] The largest changes in fatty acid composition occur when maternal diet is supplemented with fish oil.[85] For example, high levels of fish consumption by Inuit women increase the breast milk content of n-3 LC-PUFAs.[94] Levels of DHA in human milk have been reported to range from 0.2% to 1.4% of total fatty acids.[85,94] Supplementation of maternal diet with DHA also results in markedly increased DHA content in human milk, directly attributable to dietary increase rather than to preferential secretion.[95] Levels of AA remain fairly constant despite wide variation in maternal intake of LA or DHA.[85,94,96]

Because maternal diet affects the fatty acid composition of milk so easily, a good question is whether the drastic change in diet over the last few centuries also has modified human milk fatty acid composition. For instance, the dietary n-6/n-3 ratio has changed from 1:1 to 10:1 or 20:1, resulting in a higher ratio of n-6/n-3 than evolution would have allowed without human interference.[97,98] Moreover, the proportion of polyunsaturated to saturated fatty acids present in human milk has been shown to correspond with the ratio of polyunsaturated to saturated fatty acids in the maternal diet.[84] *Trans* consumption also is noteworthy because the *trans* fatty acid content of milk reflects short- and long-term maternal diet.[84]

Fatty acids appear in milk not only as a result of maternal dietary intake but also from factors such as mobilization from maternal fat deposits and endogenous synthesis by the mammary gland.[99] LA and ALA are not synthesized by the mammary gland and are dependent on dietary intake, although their long-chain derivatives may be synthesized partly in the mammary gland, independent of the length of gestation.[100] Long-term maternal diet is important because mobilization of maternal adipose tissue stores is a major source of milk fatty acids, including LA, AA, and DHA.[101,102] Consequently, gestational age and lactational stage influence milk *trans* fatty acid level, but the rate of maternal postpartum weight loss has the most significant effect.[84]

Interest in human milk and the lipid composition of formulas has focused primarily on "humanized" formulas for premature infants, which have been designed to ensure adequate absorption and metabolism of fat and to promote a satisfactory essential fatty acid status. In this regard, the changes in fat content of milk from mothers of term and preterm infants have been investigated. Chappell and colleagues[103] found that milk from mothers who delivered preterm infants has a higher content of LA and ALA and their LC-PUFA derivatives. The prevailing consensus is that early milk produced by women who deliver prematurely is more appropriate for very-low-birth-weight infants than donor

milk from the later stages of lactation.[104] LC-PUFAs are present in the milk of mothers of premature infants. Furthermore, the level of these fatty acids is significantly higher in colostrum and the milk of mothers of premature infants than in the milk of mothers of full-term infants.[105] Finally, whereas the supply of DHA and AA remains fairly constant over 26 weeks of lactation in well-nourished, omnivorous mothers of premature infants, the content of these fatty acids decreases in the milk of mothers of infants born at term. As a result, after 26 weeks of lactation, the relative concentration of AA is 1.5 times higher and that of DHA is twice as high in preterm than in term human milk.[106] Thus human milk apparently can meet the need for LC-PUFAs in preterm infants and can efficiently make up for the diminished supply of fatty acids to the fetus.

Studies of energy metabolism in low-birth-weight infants fed mother's own milk indicate that for a net energy intake of 120 kcal/kg/day, approximately 20 kcal/kg/day of fat will be deposited in tissues (i.e., some 2.2 g fat/kg/day). Because human milk contains 1.08% to 2.65% *n*-6 fatty acids and 1.09% to 1.47% *n*-3 fatty acids synthesized from the C18 precursor, it can be estimated that 23.6 to 58.3 mg of *n*-6 and 23.9 to 32.3 mg of *n*-3 LC-PUFAs will be deposited in tissue each day in a low-birth-weight infant fed adequate amounts of human milk. It also is apparent that human milk contains levels of LC-PUFAs sufficient to meet the requirements of neural tissues for these fatty acids.[45]

Formula for Preterm Infants

Special formulas have been designed to provide adequate intake of calories, fluids, and micronutrients to support the rapid cell division and synthesis of new tissue in the infant.[107,108] The current trend in infant formula preparation is to use a fatty acid profile that more closely reflects the fatty acid composition of human milk, partly owing to its digestibility by the infant. This is the result of many factors, including the fatty acid composition of human milk fat, the triglyceride positional specificity, and complementary enzymatic activities that originate in the breast milk and in the infant's gastrointestinal tract.[107] For instance, the structure of human milk triglyceride is recognized to be unique with regard to the position of saturated and short-chain fatty acids, the presence of phospholipids containing LC-PUFAs, and a triglyceride-to-phospholipid ratio that undergoes a continuous evolution from birth through weaning at 1 year.[109]

Infant formulas and feeding regimens that differ markedly from the composition of human milk fats are prevalent. In some instances, these formulations have been designed to promote utilization of fats with chain lengths of 8 and 10 carbons for maximal absorption.[110] Quantities of each nutrient in formulas for low-birth-weight infants have been adjusted to account for the average absorption of that particular nutrient.[111] Fat accretion in the growing formula-fed premature infant is markedly increased in comparison with that in the fetus of similar weight, rate of weight gain, and postconceptional age. This finding suggests that the weight gain and subsequent body composition of the premature infant differ from those of the comparable placentally nourished fetus.[108]

Interest has focused on the essential fatty acid requirements of preterm infants and the quantities of ALA, AA, and EPA that should be added to formulas in addition to LA. Body stores of essential fatty acids are diminished in low-birth-weight infants, and therefore, they theoretically should be at increased risk of developing clinically apparent essential fatty acid deficiency when they are fed a deficient diet.[112] In that regard, Combes and co-workers[113] did not observe gross abnormalities of the skin in premature infants who were fed milk mixtures low in LA; however, they did note histologic features of the skin compatible with LA deficiency and serum dienoic, trienoic, and tetraenoic acid levels that were significantly different from those in infants fed 4% of their calories as linoleate. Similarly, Hansen and associates[114] demonstrated that many babies younger than who were fed milk diets deficient in LA for 3 months beginning before 6 weeks of age exhibited dry, thick, desquamated skin and retarded growth. Clinical manifestations apparently disappeared after the feeding of diets that provided 1% or more of calories as LA.

For term infant formula, the American Academy of Pediatrics recommends a minimum of 3.3 g of fat per 100 kcal (30% of calories) and 300 mg of LA (approximately 2.7% of calories).[115] For preterm infants, the consensus among Europe, the United States, and Canada is 4.4 to 6.0 g of fat per 100 kcal.[116] Several of the most widely used formulas are based on cow's milk protein and also contain lactose. All contain vegetable oils of one type or another, and most contain more LA than human milk. In these vegetable fat blends, palmitic acid is esterified predominantly to the *sn*-1 and *sn*-3 positions of the triglycerides, resulting in the release of free palmitic acid after intraluminal hydrolysis by lipases. The absorption of palmitic and stearic acids is low, and their presence in the intestinal lumen results in the formation of soaps with calcium and other divalent cations.[117,118] To minimize this problem, medium-chain triglycerides (MCTs) or coconut oils sometimes are used to replace part of the oils containing a large amount of palmitic and stearic acids. Oleo oils are used to simulate the saturated and monounsaturated fatty acid content of human milk. In this type of oil, most of the palmitic and stearic acids are esterified in *sn*-1,3 of the oleo triglycerides. The long-term effects of these varied dietary fatty acid regimens used in feeding infants are unknown, but in animal models, physiologic variations in dietary fat intake are known to alter membrane structure and many related membrane functions.

Research has focused on the requirements for LC-PUFAs.[4,83,119,120] Preterm and term infants fed formula without AA and DHA have lower levels of those fatty acids in plasma and erythrocyte phospholipids than do infants fed human milk.[92,121] A clear dose response is evident in the levels of AA and DHA found in erythrocyte membrane phospholipids when infant formula is supplemented with increasing levels of AA and DHA.[122] The dietary intake of LA and ALA in infant formulas lacking longer-chain homologues of these fatty acids results in reduced levels of AA and DHA in membrane phospholipids compared with those of infants fed human milk.[121] The currently available formulas with high concentrations of LA or high LA-ALA ratios may be contributing to the low incorporation of DHA, both by providing insufficient substrate (i.e., ALA) and by inhibiting the incorporation of endogenously formed DHA into the membrane. ALA does not seem to act as a competitive inhibitor of the incorporation of AA into membranes,[123] unless the content of ALA is increased to unphysiologic levels.[124,125] Energy intake may also be an important variable because erythrocyte phosphatidylethanolamine DHA decreases rapidly in preterm infants who receive less than 100 kcal/kg/day, and a similar decrease is seen for AA in erythrocyte phosphatidylethanolamine and phosphatidylcholine.[92] When preterm infants receiving human milk are in energy balance, the levels of erythrocyte DHA remain constant, but they decline in premature infants fed vegetable oil formulas containing ALA but no DHA.[121,126,127] This decline is rapid and is associated with electroretinographic changes when the formula contains less than 0.37% kcal of ALA; however, both the visual development and the DHA levels in plasma and erythrocytes are comparable to those of breast-fed preterm infants if the formula contains at least 1% kcal of ALA.[121,126,127] Evidence from autopsy material indicates that formula-fed infants experience a similar decrease in DHA content in the brain cortex, and this correlates with a similar decrease in the erythrocyte DHA content.[128] Similarly, results in term infants indicate that the lower DHA level in erythrocytes in formula-fed as compared with breast-fed infants correlates with decrease in visual acuity.[129] As in preterm infants, a higher intake of ALA (1% kcal or greater) should result in visual acuity similar to that in breast-fed infants.[130]

Supplementing marine oil in formula-fed infants also alters visual acuity, as compared with nonsupplemented formula-fed infants.[131] Weak clinical evidence supports a higher intelligence test score in breast-fed than in formula-fed infants. In a nonrandomized study, the advantage in intelligence quotient with human milk feeding at 7 to 8 years of age was 8 points, which is half of one standard deviation in the sample of infants studied.[132] These results, however, also could be explained by group differences in parenting skills and genetic potential.[133] Feeding preterm infants formulas with DHA and arachidonic (ARA) from algal and fungal oils resulted in enhanced growth and provided better developmental outcomes than those documented with unsupplemented formula feeding.[134] The clinical evidence regarding factors such as fatty acid status, growth, and visual and cognitive functions on dietary PUFAs for preterm and term infants has been reviewed by Fleith and Clandinin.[135]

In the foregoing discussion, several aspects of fatty acid metabolism and functional implications were compared for breast milk and several types of commercial formula. Until recently, however, no commercial formula with essential fatty acids of chain length designation greater than C20 was available in North America. For premature (and term) infants fed synthetic formulations in place of human milk fats, it is important to establish whether such formulas possess the enzymes capable of elongating and desaturating LA and ALA and whether they are capable of producing sufficient amounts of LC-PUFAs for optimal synthesis of neural tissues and prostaglandins. This is an especially important question because these infants possess adipose tissue stores adequate for only a few days before essential fatty acid deprivation occurs.[136] Synthesis of AA and DHA therefore depends on adequate desaturase-elongase activity, as well as an adequate supply of dietary LA and ALA and energy. Because specific information on desaturase activity in infants is lacking and because human milk contains a significant amount of AA and DHA, formulas probably need to be supplemented with those fatty acids as well. Some evidence indicates that term infants fed formulas without LC-PUFAs synthesize sufficient AA and DHA from dietary precursors, although the consensus is that this process may not provide enough DHA to meet accretion demands during rapid brain development.[137-139] Furthermore, in preterm infants, evidence indicates that formula supplemented with AA and DHA is essential for optimal long-term growth and development.

MCTs make up half the fat content of many formulas for preterm infants. These nutrients have been added because it is thought they are better absorbed by the premature gut than long-chain triglycerides. Although earlier studies reported improved growth, further studies did not demonstrate an increased gain in weight, head circumference, or linear growth in preterm infants fed a formula containing medium- rather than long-chain fatty acids.[140-143] Energy wastage associated with oxidation followed by biosynthesis, compared with direct tissue incorporation of long-chain fatty acids, may explain the absence of improved energy or weight gain in infants fed formulas with MCTs.[144,145] At higher dietary levels of MCTs, the essential fatty acid balance in the infant may possibly be adversely affected. Lower plasma levels of AA and DHA associated with formula feeding may be exacerbated when MCTs rather than palmitic acid are used as the source of saturated fatty acids in formula.[146] Although high doses of MCTs do not seem desirable, no overwhelmingly clear evidence has shown that they are harmful. Accordingly, more extensive studies are needed to determine the optimal fat blend to use in formulas for low-birth-weight infants.

In summary, the fat in commercial infant formula is different in many aspects from that in human milk: The content of monounsaturated fatty acids, LA, and ALA varies with the oil blend used, the absence of C20/C22 *n*-6/*n*-3 fatty acids, the abundance of MCTs, and the triglyceride fatty acid stereoconfiguration. High levels of LC-PUFAs are present in milk produced by mothers of low-birth-weight infants, whereas current "humanized" formulas are devoid of essential fatty acids with greater than 20 carbons in chain length.[72] Supplementation of infant formulas with very-long-chain PUFAs at levels and ratios similar to those of human milk increases the incorporation of these components into membrane lipids in the developing infant,[119] in a dose-dependent manner.[147] At these physiologic levels, AA and DHA from marine oil sources[148,149] and from single-cell oils[147] maintain AA and DHA status in formula-fed infants similar to that in breast-fed infants.

Absorption

Physiologic Phases

Fat absorption improves with gestational age. Most of the dietary fats are triglycerides, and approximately 2% are phospholipids, free fatty acids, monoglycerides and diglycerides, cholesterol, and other substances.[150] The absorption of triglycerides is dependent on fatty acid chain length. MCTs can be absorbed directly into the circulation without being hydrolyzed by pancreatic lipase. All other fats, however, are hydrolyzed and are then reesterified to form triglycerides within the mucosa. Absorption coefficients for unsaturated fats have been demonstrated.[151]

In the newborn infant, pancreatic lipase and bile salt concentrations are diminished, thus impairing emulsification and breakdown of triglyceride.[152] Efficient fat absorption in the newborn depends on lingual and gastric lipases. These have a special role in the hydrolysis of milk fat and can account for hydrolysis of 60% of the ingested fat. Lingual lipase hydrolyzes medium- and short-chain triglycerides,[152] as well as monounsaturated and polyunsaturated, but not saturated, long-chain triglycerides.[100,153,154] Animal studies have shown that short- and medium-chain fatty acids can be absorbed directly through the gastric mucosa, suggesting that these products of intragastric lipolysis appear rapidly in the circulation. Free fatty acids and monoglycerides, the products of intragastric lipolysis, are relatively polar. Therefore they can locate in the surface layer of the fat globule (dislocating phospholipids and proteins), making the core triglyceride more accessible to pancreatic lipase and human milk bile salt–stimulated lipase. The latter enzyme hydrolyzes milk fat at pH 7.0 to 8.0, in the presence of bile salts, thereby acting in the intestine to complete the digestive process initiated in the stomach by lingual and gastric lipases.[152]

The breast-fed newborn infant depends on an additional compensatory enzyme, the bile salt–stimulated lipase of human milk.[152] This lipase is present in milk beginning at 26 weeks of gestation, has no positional specificity for triglycerides, and hydrolyzes various triglycerides to free fatty acids and glycerol in the presence of bile salts.[155,156] The major differentiating characteristics of milk lipase and other lipases in gastric aspirates are shown in Table 42-1. It is estimated that milk bile salt–stimulated lipase concentrations are sufficient to hydrolyze 30 to 40% of available triglyceride in 2 hours.[157] Addition of this lipase to formula for preterm infants may be feasible.[158] Because bile salt–stimulated lipase does not discriminate between fatty acids and completely hydrolyzes triglycerides, this modification may aid in efficient release of LC-PUFAs, especially in the presence of pancreatic colipase-dependent lipase.[158] The combined action of intragastric lipolysis and intestinal hydrolysis of fat by the bile salt–stimulated lipase of human milk can effectively substitute for low pancreatic lipase activity and low levels of bile salts in the newborn.[152]

It is apparent that a dynamic interrelationship must exist among the products of lipid digestion. The triglyceride emulsion particle, stabilized by the products of intragastric lipolysis and phospholipids, undergoes hydrolysis by pancreatic lipase-colipase and phospholipase A_2. The accumulated lipolytic products are then extruded as unilamellar liposomal vesicles that are in

TABLE 42-1

Compensatory Digestive Lipases Versus Pancreatic Lipase in the Newborn

Feature	Lingual	Gastric	Milk (BSSL)	Pancreatic
Origin	Serous glands	Gastric mucosa	Mammary gland	Acinar cells
TG site (sn)	sn-3	sn-3	sn-1,2,3	sn-1,3
End products	FFA, DG, MG	FFA, DG, MG	FFA, glycerol	FFA, MG
pH optimum	2.2-6.0	3.5-6.5	7.0-8.5	6.5-8.0
Bile salts	No	No	Obligatory	Obligatory
Stability (at pH 2.5-3.0)	Yes	Yes	Yes	No

Modified from Hamosh M, Iverson SJ, Mehta NR, et al: Lipids in human milk and their digestion by the newborn infant. In Ghisolfi J, Putet G (eds): *Essential Fatty Acids and Infant Nutrition,* Paris, 1992, J Libbey Eurotext, pp 119-137.

BSSL, Bile salt–stimulated lipase; *DG,* diglyceride; *FFA,* free fatty acid; *MG,* monoglyceride; *sn,* stereospecific numbering; *TG,* triglyceride.

equilibrium with large bile salt mixed micelles. The mechanism of lipid exchange between these phases, particularly for lipids of differing aqueous solubility or chemical composition, is not completely known.[157] (Chapter 115 describes digestive and absorptive processes in infants in further detail.)

Medium- and Long-Chain Triglycerides

The efficiency of fat absorption in preterm infants is determined not only by maturity of the gastrointestinal tract and pancreas but also by the dietary fat composition. Preterm infants fed fresh human milk can absorb fat as well as term infants, at an efficiency of 90% to 95% of intake.[159] Only 85% to 92% of the fat in formulas that do not contain MCTs is absorbed by preterm infants.[155] Feeding breast milk also has been shown to promote better fat absorption than that observed in formula-fed infants,[151] not only because of the presence of bile salt–stimulated lipase, but also because of differences in the structure of the milk fat globule and the stereoconfiguration of triglycerides. Feeding of pasteurized or stored human milk eliminates some of these factors.[160] For instance, lipases are completely destroyed by pasteurization, whereas fatty acid levels seem to be unaffected.[161] The stereoconfiguration of triglyceride is important in piglets, in which palmitic acid is absorbed from sow's milk and from formula with rearranged triacylglycerols as an *sn*-2 monoacylglycerol.[162] Similarly, in full-term infants who receive human milk, palmitic acid is absorbed as *sn*-2 monopalmityl-glycerol.[163] Positional distribution seems to affect digestion, absorption, and chylomicron triglycerides and has an effect on infant lipoprotein metabolism.[164] However, in preterm infants, randomizing palmitic acid in infant formula triglycerides to improve absorption of fat remains controversial, and no net physiologic benefit may result from an increased level of palmitic acid in the *sn*-2 position of the triglyceride. Previous observations indicated that the coefficient for absorption of palmitic and stearic acids is 20% to 30% lower for premature infants fed formula versus infants fed human milk.[151] Some findings suggest that a palmitic acid configuration similar to that of human milk (with C16:0 esterified in the *sn*-2 position) confers better absorption than when C16:0 is esterified in the *sn*-1 or *sn*-3 position.[165] Thus it is reasonable to expect that if the presence of palmitic acid in the *sn*-2 position confers improved absorption of palmitic acid, then increasing the content of saturated fatty acid in the *sn*-2 position of the fed triglyceride could significantly improve absorption of palmitic acid by up to 10%.

Significant improvement of net absorption of total fatty acid probably would be difficult to demonstrate, because total fat absorption would be likely to increase by only a small amount. In a feeding experiment, the proportion of saturated fatty acids in the *sn*-2 position of palm oil was randomized to simulate the positional distribution of fatty acids in human milk triglyceride closely. Fatty acid absorption was assessed in preterm infants fed a commercial formula or a similar formula in which the palmitic acid was randomized into all positions of the triglyceride molecule. Incorporation of approximately one third of the palmitic acid into the *sn*-2 position of the fed triglyceride improved absorption of fat, but no effect of this triglyceride structure on the net absorption of palmitic acid was observed. Alternatively, other modifications in the triglyceride structure may occur during randomization.[166]

The MCTs are relatively more water-soluble and are more completely hydrolyzed by pancreatic lipase. They also can be absorbed directly into mucosal cells. C8:0 and C10:0 are almost 100% absorbed, despite the low lipase and bile salt concentrations. Efficiency of fat absorption increases with the degree of unsaturation, so fatty acids with more double bonds are absorbed more efficiently (C18:3 > C18:2 > C18:1).[151,167] Several previous studies[168] demonstrated improved fat absorption but only small differences in weight gain with infant formulas containing MCTs.[169] The effect of MCT diet on energy and macronutrient utilization in very-low-birth-weight infants was studied by Van Aerde,[145] who concluded that the growth of infants fed a formula containing 50% MCTs (at an energy intake of 124 kcal/kg/day) will mirror the intrauterine gain in length, head circumference, and weight. Body composition cannot be simulated because of the higher rate of fat deposition. Further studies could not demonstrate an increased gain in weight, head circumference, or linear growth of preterm infants fed a formula containing medium-chain rather than long-chain fatty acids.[140-143] Energy wastage associated with oxidation followed by biosynthesis, compared with direct tissue incorporation of long-chain fatty acids, may explain the absence of improved energy or weight gain in infants fed formulas with MCTs. Premature infants oxidize one third to two thirds of the medium-chain fatty acids, indicating that the remaining medium-chain fatty acids are chain-elongated.[144,145] Production of ketones and dicarboxylic acid increases with an increasing amount of MCTs in the preterm infant formula.[170,171] The rapid oxidation of medium-chain fatty acids probably leads to accumulation of acetylcoenzyme A, exceeding the capacity of the tricarboxylic acid cycle. Dicarboxylic acids are the products of ω-oxidation of C8:0 and C10:0.

The absorption coefficient of oleic and LA from vegetable triglycerides is greater than 90%.[118,151] (For myristic acid, it is only 89%; for palmitic acid, it is 75%; and for stearic acid, it is 62%.) The absorption of oleate and linoleate (unsaturated C18 fatty acids) is highest in infants fed a formula with a linoleate-to-oleate ratio similar to the ratio provided by human milk. Therefore some infant formulas have been designed to provide high linoleate-oleate ratios.[172] In general, agreement in the literature that infant formulas containing cow's milk fat as the sole or principal form of fat also are less well digested by the preterm infant than those containing vegetable oils or MCTs.

Gastric lipolysis,[173] nonnutritive sucking,[174] and a "humanized" lipid formulation for infant feeding can partially compensate for the low-birth-weight infant's intraluminal immaturity.[151] Human milk triglyceride structure,[175] fat globule integrity,[176] inherent lipase activity,[177] and a substance stimulating expansion of the bile salt pool[178] have been suggested as additional factors responsible for relative efficiency of fat absorption in infants fed human milk. The influence of the free fatty acid composition of human milk on fat absorption also should be considered a variable that contributes to efficient absorption of milk fat.[151] Total absorption of fatty acids and the coefficient of fatty acid absorption are significantly higher in very-low-birth-weight infants fed their mothers' milk than in infants receiving formula.[119]

In the premature infant, the fecal fatty acid pattern generally resembles the fatty acid profile fed; however, an inverse relationship has been found between increasing chain length in

saturated fatty acids and absorption.[151] The marked difference in absorption of C18:0 in preterm mothers' milk and formula-fed infants may indicate that factors in addition to position of fatty acids on the triglyceride molecule are responsible for the efficient absorption of fatty acids from preterm mothers' milk.[151] The absorption of specific saturated fats may be low and decreased by the form or perhaps by the method of mineral supplementation.[151] Clandinin and colleagues[119] demonstrated that the overall distribution of individual fatty acids within an insoluble bound fraction is similar for infants fed breast milk and those fed formula. Insoluble bound C16:0 and C18:0 account for more than 65% of fatty acids. Calcium supplementation resulted in an unexpected relative increase in fecal C12:0 and C14:0 in formula-fed and breast milk–fed infants and a decrease in C18:0 in the feces of a breast milk–fed group. Providing palmitic acid appropriately esterified in *sn*-2 of the infant formula triglycerides seemed to improve mineral balance in formula-fed infants.[179]

It is apparent that some fatty acids (i.e., PUFAs) are readily and efficiently absorbed[119]; however, as much as 25% of dietary LC-PUFAs can be lost in the feces of formula-fed and breast-fed preterm infants.[180] In general, longer-chain fatty acids are less well absorbed, possibly because of solubilization and rate limitations of uptake and processing in the enterocyte.[181] Supplementation of formula with AA and DHA has resulted in decreased absorption of these fatty acids,[182] possibly resulting in variable results in clinical supplementation studies. As previously mentioned, human milk bile salt–stimulated lipase has a role in the utilization of LC-PUFAs,[158] so its absence in infant formula also may have an effect. However, it appears that a single-cell oil source AA and DHA in formula may be as efficiently absorbed as these fatty acids are from breast milk.[180] This evidence indicates that the absorption coefficients are not identical for all fatty acids, with a documented difference between human milk–fed and formula-fed very-low-birth-weight infants.[119]

Metabolism

Oxidation

Current understanding of the utilization of individual dietary fatty acids for energy is vague.[119] Because more than 50% of dietary calories of the newborn are derived from fat, metabolic adaptation in the oxidation of long-chain fatty acids and ketone body utilization must occur at the time of birth.[183] It is evident that infants deprived of sufficient fat stores at birth (i.e., premature or growth-restricted infants) lack an important fuel source.[184] In the immediate postnatal period, serum levels of free fatty acids rise dramatically, indicating active lipolysis of adipose tissue.[185] After 12 to 24 hours, the rate of lipolysis falls rapidly as feeding or intravenous nourishment is initiated. Rates of lipolysis in the first few hours of life appear to be regulated by catecholamine release, resulting in production of cyclic adenosine monophosphate and increased protein kinase C activity. Ultimately, this process leads to activation of adipose tissue lipoprotein lipase.[185,186] Lipolysis in neonatal adipose tissues may depend more on mobilization of glycogen stores than on catecholamine stimulation. Insulin, growth hormone, thyroxine, sex hormones, and glucagon also have a role in stimulating free fatty acid production in the neonate by increasing lipogenesis.[187] With typical fecal fat losses of approximately 10% to 15%, some 40% to 50% of the fatty acids consumed would be expected to be available for new tissue synthesis. However, selective β-oxidation of individual dietary acids has been shown in adults.[119] Whether or not this selective β-oxidation of PUFAs also occurs in infants is unknown. Therefore it is difficult quantitatively to assess or estimate the net oxidation rate for dietary LC-PUFAs on the basis of available data.[119]

Relative proportions of major dietary fatty acids consumed may potentially affect the net contribution of fat oxidation to total energy production. The metabolizable energy of saturated triglycerides has long been known to be less than that observed for unsaturated fats.[188] Studies in animals and humans suggest that the degree of unsaturation of dietary LC-PUFAs influences the partitioning of fat between oxidation and storage. Several reports have indicated that LC-PUFAs undergo faster oxidation as compared with their saturated counterparts. Thus, as expressed earlier, human metabolism of dietary fat is not independent of the long-chain fatty acid composition of the fat consumed.[189-191] From nutrient balance and energy balance studies, it appears that only a small amount of the absorbed fat is oxidized, and most fat is stored. A faster clearance and a higher rate of oxidation of fats have been observed in hypermetabolic patients than in physiologically normal subjects. By contrast, fat clearance is faster in patients receiving total parenteral nutrition (TPN), but the rate of oxidation is much lower.[192]

In the past, it was generally assumed that medium-chain fatty acids are preferentially oxidized, but more recent studies estimated that preterm infants oxidize only one third to two thirds of dietary C8:0.[144] Medium-chain fatty acids, particularly C12:0, are incorporated into adipose tissue of preterm infants in proportion to the formula content.[193] It is unclear whether this is of clinical benefit.

Accretion

The lipid content of the brain is fairly constant until 7 months of gestation, when lipid deposition gradually increases, reaching a peak in gray matter at 3 months of postnatal age. In white matter, the process is slower. By 2 years of age, 90% of the adult lipid content has been deposited, and by 10 years of age, 100% has been deposited. White matter accounts for most of the brain lipid, mainly in the form of myelin. Myelin itself contains 50% of the total lipid content and represents 25% of the weight of the mature brain.[194]

Preterm infants born at a weight appropriate for gestational age (AGA) (approximately 1300 g) have a total-body fatty acid content of approximately 30 g, whereas term AGA infants have a total-body fatty acid content more than 10 times greater (340 g). Quantitative analyses of the fatty acid content of liver and neural tissues have demonstrated a combined fatty acid content of 1.55 g in preterm infants and 4.63 g in term infants. Based on dissection data, the total amount of fatty acids in white adipose tissue of preterm and term infants can be estimated at approximately 3.45 g and 255.6 g, respectively.[119] During the first 5 weeks of postnatal life, an apparent mobilization of total fatty acids from the liver is seen. Of the essential fatty acids, only LA and ALA accrue during the initial weeks of life. Significant postnatal accretion of chain elongation–desaturation products does not occur immediately after birth. This finding suggests that early on, either synthesis in the liver may be limiting or mobilization of LC-PUFA homologues of LA and ALA exceeds the capacity of liver to synthesize them from dietary LA and ALA.[119]

Mammalian tissues contain *n*-3 fatty acids primarily in the form of DHA and are mostly concentrated in phospholipids.[195] Although DHA accounts for a small percentage of the fatty acid content of most tissues, very high levels are found in the retina, cerebral cortex, testes, and sperm.[196] Across mammalian species, the levels of DHA in brain and retina phospholipids are remarkably similar despite wide variations in dietary intake. This comparative constancy suggests an important role for DHA in brain and retinal function.[197] DHA occurs almost entirely in the 2-position of phospholipids. The 1-position is characteristically occupied by saturated fatty acids (stearic and palmitic acids). The specific role of DHA is not understood, but its chain length and uniquely high degree of unsaturation may help to create a membrane with high fluidity, flexibility, and permeability. However, some studies of model membranes fail to support the long-standing hypothesis that DHA increases membrane fluidity.[198] The role of DHA may

be more specific in that it may involve modulation of the kinetics of carrier-mediated transport systems, the activity of membrane-bound enzymes, or the properties of membrane receptors.[195]

These characteristics of DHA seem to be necessary for the dynamic behavior of rhodopsin (the photosensitive pigment of rod photoreceptors) in the photoreceptive process.[198] Within the retina, DHA is concentrated in specialized membranes, which make up the photoreceptor outer segments. However, it has been shown that visual cell membrane fatty acid composition and thus early retinal development are influenced by small dietary changes in DHA and AA. A diet containing a balanced mixture of both fatty acids within the physiologic intake range is required to support an *n*-3–*n*-6 balance necessary for developing the highest rhodopsin content in the rod outer segments, with potentially important functional implications.[199] Research has begun to delineate a possible role of DHA in promoting optimal G-protein–coupled signaling systems in visual transduction.[200] It has been suggested that early visual experience and input during infancy may be critical to later visual function and may result in lasting consequences.[201] "More generally, delays in the early stages of visual, cognitive, and motor development can have a cascade of effects on later-maturing processes."[201]

In summary, high levels of DHA are found in the photoreceptor outer segment membranes of the retina and in the gray matter of the cerebral cortex. Some evidence, discussed extensively in Chapter 44, suggests a relationship between essential fatty acid intake and the development of visual functions.[127,130,131,202,203] Therefore, it is important to determine whether optimal extrauterine accretion of LC-PUFAs requires dietary supply of these fatty acids (perhaps originating as normal constituents of human milk) or arises sufficiently in vivo from chain elongation–desaturation of shorter-chain precursors (i.e., LA and ALA). Important research on this aspect of lipid metabolism, along with its clinical implications, is reviewed next.

APPROPRIATE BALANCE OF DIETARY FATTY ACID INTAKE FOR PRETERM INFANTS

Fat Requirements

"The human neonate and its mammalian counterparts have for millions of years received balanced nutrition during early infancy exclusively through breast-feeding."[204] Unfortunately, with replacement of human milk by cow's milk or formula, the nutritional requirements of the infant have become less clear.[205] The caloric requirement of the full-term infant younger than 1 year of age is 100 to 120 kcal/kg/day. For each 100 kcal of energy intake, the term infant requires a minimum of 1.8 g protein, 3.3 to 6 g of fat, 300 mg of essential fatty acid, and 11 to 17 g of carbohydrate.[206] The fat content of preterm infant formulas should be 4.0 to 6.0 g/100 kcal, which would provide 4.8 to 7.2 g/kg/day to infants fed 120 kcal/kg/day.[207] Regardless of the fat content of a particular formula, the fat intake of the infant is generous. A premature infant who receives 180 mL/kg/day of 20 kcal/oz formula will consume 6.2 to 6.8 g/kg/day of fat. By contrast, only 2 g/kg/day of fat must be deposited to simulate the intrauterine fat accretion rate. Some of the excess fat is subjected to oxidation and fecal excretion.[205] Studies of energy requirements indicate that the low-birth-weight infant requires approximately 80 kcal/kg/day for maintenance. Thus, in the low-birth-weight infant with an energy intake of 120 kcal/kg/day, some 34% of the calories fed will be used for tissue synthesis or growth. Because approximately half of the available calories in human milk are fat, then 36% of fat intake will be used for growth in the preterm infant (assuming 67 kcal/100 mL of milk and 90% fat absorption). Under these metabolic conditions (if all fatty acids are used for energy to a similar degree, if fat absorption coefficients are greater than 0.9, and if adipose tissue is synthesized in amounts and composition similar to those of intrauterine growth), the maximum requirements for essential fatty acids in infants weighing less than 1300 g can be estimated at 1100 mg for *n*-6 fatty acids and 140 mg for *n*-3 fatty acids per day.[208]

In human milk, *n*-6 and *n*-3 fatty acids normally represent a consistent percentage by weight of total fatty acids. Therefore it has been recommended that these values should be used as a guideline for the composition of infant formulas.[119] Quantitative and qualitative analyses of the composition of milk from mothers of preterm infants indicate that it provides levels of *n*-6 and *n*-3 essential fatty acids approximating the predicted requirements for day 16 of life, at intake levels of 120 kcal/kg/day. As the infant's body weight and milk intake increase, the net intake of essential fatty acid will approach and rapidly exceed the predicted requirements.[208] Levels of LC-PUFAs in human milk suggest that adequate intakes of mother's own milk also provide quantities of these membrane structural precursors in excess of their reported intrauterine rates of accretion in neural and liver tissues. If the rate of adipose tissue synthesis is projected for the extrauterine growth of a 1300-g low-birth-weight infant gaining 17 g of body weight per day, it can be estimated that synthesis of adipose tissues would use 185 mg/day of C20*n*-6 and C22*n*-6 fatty acids and 30.8 mg/day of C20*n*-3 and C22*n*-3 fatty acids. An adequate intake of mother's own milk provides 90 to 130 mg of C20*n*-6 and C22*n*-6 fatty acids per day and 55 to 75 mg of C20*n*-3 and C22*n*-3 fatty acids per day.[208] It thus seems prudent to feed the preterm infant human milk or formulas with a fatty acid balance similar to that of human milk, containing longer-chain homologues of LA and ALA.[171,209] Liu and colleagues,[210] using fish oil as a source of DHA (in a dose of 11 mg/kg/day), maintained red blood cell DHA concentrations in phospholipid in the range observed for human milk–fed infants. The effect of adding marine oil to infant formula has since been investigated in several species and under many different test conditions.[127,211-213] In general, this dietary maneuver causes a reduction of plasma and membrane levels of AA.[213,214] Supplementation of premature infants with long-chain homologues of ALA in the absence of long-chain homologues of LA not only leads to membrane abnormalities[215] but also decreases circulating levels of AA, resulting in a reduction in growth potential during the first year of life.[216,217]

Clandinin and co-workers[119,147] recommended that formulas for premature infants contain the appropriate balance of *n*-6 and *n*-3 metabolites of LA and ALA at concentrations that reflect normal human milk levels of these constituents and that will provide a ratio of *n*-6 to *n*-3 (C20 and C22) fatty acids of 1 to 1.4:1.[119] A clear dose response is produced in the levels of AA and DHA found in erythrocyte membrane phospholipids when preterm infant formula is supplemented with increasing levels of AA and DHA. It appears that a formula with 0.32% to 1.1% AA and 0.24% to 0.76% DHA provides sufficient levels of these fatty acids to achieve a membrane phospholipid fatty acid composition similar to that of infants fed human milk. Approximately 0.6% AA and 0.4% DHA provide sufficient and perhaps optimum levels of these fatty acids in formula-fed preterm infants.[147]

Composition of Fat

Postnatal growth seems to differ from fetal growth, especially with respect to fat deposition, and net absorption of fat improves with increasing gestational age. In animal models, the brain is sensitive to alteration of dietary lipid intake, even with a nutritionally complete diet. It is therefore reasonable to postulate that dietary lipids fed during the early postnatal period are important determinants of structural and functional components of developing brain tissues.[218]

Although the overall fat content of human milk and that of cow's milk appear to be similar, detailed analyses show marked differences in lipid composition and fatty acid pattern. Compared with human milk, cow's milk contains more saturated

fatty acids and many fewer essential fatty acids. Human milk contains somewhat more monounsaturated fatty acids and LC-PUFAs.[205] Current formulations have attempted to simulate the energy and fat content of human milk, resulting in various calorie-dense and fat-dense formulas on the market.[205] To correct dissimilarities in fatty acid composition, bovine milk fat must be augmented or replaced with various vegetable oils to provide mixtures that offer a low saturated fatty acid content and a higher unsaturated fatty acid content, including LA and ALA.

Formulas for premature infants have been further modified by incorporation of MCTs. The result has been that some formulas have fatty acid profiles that are very dissimilar to that of human milk, especially with respect to medium-chain fatty acids (considerably increased) and long-chain saturated fatty acids (decreased). In the newborn infant, synthesis of AA and DHA depends not only on adequate desaturase and sufficient supply of dietary LA and ALA but also on calories to support energy metabolism. Based on the composition of human milk, changes in blood fatty acid levels in preterm infants in energy balance, and studies of other species, it is possible to estimate fatty acid requirements for preterm and term infants. The European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) committee recommended an LA-ALA ratio ranging from 5:1 to 15:1,[219] which is similar to the Canadian recommendation (which ranges from 4:1 to 16:1) when no C20 and C22 fatty acids are added.[220] This ratio probably should be closer to the lower than to the upper limit, and this goal can be accomplished by increasing the ALA and decreasing the LA content of commercial formulas.[214] For LA, the recommended intake is 4% to 6% of energy, and for ALA, a minimum of 1% of energy for preterm and term infants fed formulas not containing any AA or DHA.[214] Although the addition of 0.25% to 0.5% kcal of long-chain *n*-3 fatty acids is sufficient to maintain DHA levels and to keep activities such as retinal function at the same level as observed in breast-fed infants, it also reduces the accretion of AA. Therefore, when *n*-3 LC-PUFAs are added, *n*-6 LC-PUFAs should be added simultaneously.[72,119,214,219,221] Preliminary evidence suggests that the long-chain *n*-6–*n*-3 ratio should be approximately 1.1:1.4.[148,159] The Child Health Foundation Expert Consensus Workshop[222] recommended that infant formulas for term infants should contain at least 0.2% of total fatty acids as DHA and 0.35% as AA. For preterm infants, because of their lower body stores, formulas should include at least 0.35% DHA and 0.4% AA.

The inclusion of high amounts of medium-chain fatty acids in formulas may not be as important as was previously indicated, because use of such formulas, while conferring no benefit on energy balance and growth, may be associated with abnormal fatty acid metabolism.[140-143,146] Although high doses of MCTs do not seem desirable, no convincing evidence has emerged to show that they are harmful. Consequently, more extensive studies are needed to determine the optimal fat blend to use in formulas for low-birth-weight infants.

Previous assessments of the essential fatty acid status of the neonate focused on measures of visual function that are different and do not enable analysis of the site of the functional deficit or mechanism involved. Measures of immune function have similar difficulties; however, in neonates, the peripheral immune system is available for analysis. Human milk is known to contain many immunologic factors that reduce the incidence of infection and aid in immune development and maturation. One review[223] briefly outlined some of the effects of *n*-3 fatty acids on the immune system, involving the suppression of inflammatory response through effects on eicosanoids, although it is suggested that *n*-3 PUFAs have many noneicosanoid effects on the immune system as well. In rats, dietary saturated fatty acids have little effect on cytokine production, whereas *n*-3 PUFAs are potent inhibitors of the T_H1-type (proinflammatory) cytokines.[224,225] As determined by Field and colleagues,[226,227] feeding infants with or without AA and DHA alters the composition of functional cells related to maturity of the immune system. By linking changes in the specific cell types with release of subcellular messages (exocytokines) and their receptors, it is possible to develop dynamic measures of cell function that can be used to link known physiologic functions through biochemical mechanisms altered by changes in the essential fatty acid status. The addition of AA and DHA to preterm formula, at levels similar to those in human milk, can influence the proportion, maturation, and cytokine production of peripheral blood lymphocytes. Improvement of the ability of peripheral mononuclear cells to produce interleukin-10 by supplemented preterm infants as compared with standard formula–fed infants suggests that these dietary modifications may aid induction of tolerance to oral antigens. Further studies will elucidate other aspects of immune development, including antigenic response.[227]

Parenteral Lipid Emulsions and Potential Complicating Factors

TPN frequently is considered when the gastrointestinal tract is not capable of digesting and absorbing nutrients. The basis for calculating nutrient requirements in infants receiving TPN is similar to that for the enterally fed neonate, except malabsorption and the energy cost of the digestion and absorption of food need not be considered. Current research, however, indicates that the enterocyte possesses significant fatty acid desaturation activity.[228] Thus parenteral feeding of fats may bypass this physiologic control over fatty acid metabolism.

The fat particles in emulsions are similar in size to chylomicrons and, like the latter, are cleared from the circulation by lipoprotein lipase[229] and by hepatic lipase.[230] As compared with the use of 10% lipid emulsions, the use of a 20% lipid emulsion does offer the advantage of more efficient triglyceride clearance with lower cholesterol and phospholipids in low-density lipoproteins.[231,232] This finding suggests that excessive phospholipid liposomes in 10% lipid emulsions, as opposed to the liposome-poor 20% emulsion, impede the removal of triglycerides from plasma. Friedman and colleagues[233] studied sick newborns who were maintained on fat-free intravenous alimentation. These infants demonstrated very rapid biochemical changes in plasma that were compatible with essential fatty acid deficiency during the first week of life and were reversible with oral feedings containing essential fatty acids. Infusion of lipid emulsions rapidly reversed clinical symptoms of essential fatty acid deficiency and returned plasma phospholipid *n*-6 fatty acid levels to normal. However, the erythrocyte and liver phospholipid *n*-6 fatty acid content and adipose tissue reserves of *n*-6 fatty acids normalized more slowly. Similar findings have been observed in experimental animals.[234] Innis[235] studied the effect of TPN with LA-rich emulsions on tissue *n*-6 and *n*-3 fatty acids in the rat and concluded that intravenous administration of large amounts of LA in parenteral lipid reduces desaturation and elongation of essential fatty acids but does not competitively inhibit esterification of other fatty acids into phospholipid. Innis also reported that after 3 days of fat-free parenteral nutrition, not only are DHA and AA both diminished in the red cell membrane phospholipids,[236] but some biochemical signs of essential fatty acid deficiency develop as well.[237] Essential fatty acid deficiency is avoided by intravenous lipid infusions of 0.5 to 1.0 g/kg/day.[238]

Energy metabolism, fat accretion, and oxidation in parenterally fed infants are determined not only by total energy intake[239] but also by the composition of the nonprotein calories.[240-243] The proportion of nonprotein calories supplied as intravenous fat probably should range between 30% and 50%. Commercial lipid emulsions contain excessive amounts of LA and no long-chain derivatives of either LA or ALA in the triglyceride fraction. In adults, the use of medium- and long-chain triglyceride mixtures may alter LC-PUFA synthesis, but no similar evidence is available

for neonates.[244] It also is not known whether this alteration in long-chain fatty acid metabolism results from a reduction in LA content or from the addition of MCTs to the intravenous lipid emulsion. Research involving structured lipid emulsions in which both medium- and long-chain fatty acids are esterified to the same glycerol backbone indicates that use of such emulsions could be a safe and well-tolerated approach to TPN.[245]

Delineation of the physiologic and pharmacologic effects of the LC-PUFAs in disease states may lead to development of therapeutic modalities directed at inflammation, endotoxin effects, liver cholestasis, and pulmonary hypertension.[246-248] Some concern has been raised regarding the occurrence of hepatic injury with intravenous lipid administration. The current belief is that TPN-associated cholestasis is increased in infants receiving fat emulsions.[250,251] The inclusion of *n*-3 PUFAs in intravenous lipid emulsions may be a way to decrease the incidence of TPN-induced cholestasis by improving the bile flow, which is reduced by intravenous soybean oil emulsions.[248] Very significant changes have been found in the fatty acid composition of liver ethanolamine phosphoglycerides and choline phosphoglycerides in infants receiving TPN with a soybean oil emulsion as the lipid source. These alterations have included an increase in LA, a decrease in linoleate elongation-desaturation products, and a decrease in the level of DHA in liver phosphatidylethanolamine and phosphatidylcholine. In the brain, an increase in the LA content of choline phosphoglyceride has been observed; however, it has not been accompanied by any increase in the *n*-6 LC-PUFAs. These findings demonstrate a potential for adverse effects of high doses of LA on the tissue levels of LC-PUFAs.[252] Conversely, fish oil–enriched emulsions reduced the impairment of bile flow seen in a newborn piglet model of TPN cholestasis.[253] Although feeding the fish oil emulsion resulted in alterations in canalicular and sinusoidal fatty acid composition of membrane phospholipids, the mechanism responsible for the prevention of cholestasis is not known. Changes in eicosanoid metabolites may be partly responsible. More research is required to determine clinical applications of fish oil–enriched emulsions in human neonate TPN.[253]

The necessity of carnitine supplementation for the metabolism of intravenous lipid metabolism remains uncertain, because some studies have demonstrated beneficial effects, which could not be confirmed by others. Comparisons are difficult to make, however, because the studies used different doses of carnitine.[254] Carnitine is present in human milk[255,256] and is essential for fatty acid metabolism, but high doses may have an undesirable effect.[257] No convincing data have documented physiologic or metabolic benefit of giving carnitine to infants receiving intravenous lipid. If carnitine is given, the dose should not exceed the amount a breast-fed infant receives (i.e., 15 μmol/100 kcal).

In summary, the fatty acid composition of commercial lipid emulsions is inappropriate in that it is unphysiologic and results in abnormal composition of circulating and tissue membrane lipids. For prevention of essential fatty acid deficiency, 0.5 to 1 g/kg/day of intravenous lipid is required, preferably as a 20% emulsion. To maintain an acceptable energy balance, 0.5 to 1 g/kg/day of intravenous lipid as part of the TPN solution should be introduced by the postnatal age of 24 to 48 hours. This dose can be progressively increased (by 0.5 to 1 g/kg/day) in incremental daily or alternate-day steps, as tolerated, up to 3 to 4 g/kg/day, while triglyceride clearance is monitored.

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Jan Nedergaard and Barbara Cannon

Brown Adipose Tissue: Development and Function

Mammals such as humans are exposed to their greatest temperature-related shock at birth. Coming from a protected and thermoneutral environment, the newborn infant is suddenly exposed to "cool" surroundings, where survival depends on self-generation of sufficient heat to keep warm.

The development of the ability to regulate body temperature regardless of the temperature of the surroundings (and through this to ensure that the activity of the organism is constant and high) apparently was a necessary step in the evolution of so-called higher animals. A requirement for this development was that newborns be endowed with a physiologic ability to produce the heat essential for survival. To accomplish this, an organ was developed without parallel in cold-blooded animals, an organ endowed with the function of producing heat exactly when required, especially around the time of birth. This organ is brown adipose tissue.

Thus, in evolutionary terms, brown adipose tissue is a rather new organ. Even within the scientific community, brown adipose tissue is rather "new." Its function as a heat producer in the cold[1] and in newborns[2,3] was first described less than 50 years ago. However, during subsequent years, understanding has grown about the events responsible for the heat production in that tissue,[4-10] and at present, researchers are actively elucidating mechanisms underlying the recruitment processes in brown adipose tissue (i.e., those processes that, during perinatal development, are responsible for the growth and differentiation of the tissue).

One of the most important accomplishments in research on brown adipose tissue thermogenesis has been the recognition that the ability to produce heat resides in the fact that brown adipose tissue mitochondria are endowed with a unique protein—the original uncoupling protein (i.e., UCP1), thermogenin. This protein functions as a regulated protonophore through the mitochondrial membrane. The identification of this protein has in many ways consolidated brown adipose tissue research, so a description of the function and fate of this protein must be a central issue in a review on the perinatal recruitment of brown adipose tissue.

DEVELOPMENT OF BROWN ADIPOSE TISSUE IN HUMAN INFANTS AND OTHER NEWBORNS

Significant differences in perinatal development of brown fat (and many other characteristics) have been documented among newborns of different species.[11-15] Broadly speaking, three different groups of newborns can be distinguished: altricial newborns, so-called true immature newborns, and precocial newborns.[11]

The *altricial* (literally, nest-dependent) newborns often are whelped as members of litters of more than five pups. They are poorly developed at birth, have no fur, are blind, and move poorly if at all. To keep warm, they huddle together. As discussed later on, development of brown adipose tissue in these newborns is a slow process that starts just before birth and reaches its

maximum some days after birth. The main driving force for this recruitment is probably the (comparatively) low environmental temperature coupled with the attempt by these newborns to activate processes to oppose hypothermia. Rat and mouse pups belong to this group. The morphologic and ultrastructural development of the tissue in these animals has been described in several papers.[16-21]

The *immatures* (not to be confused with prematures) are not able to respond to the environmental temperature at birth and are truly poikilothermic. In species with immature newborns, the recruitment process in brown adipose tissue starts only when the central control system has developed, after which processes occur that seem similar to those elicited immediately after birth in the altricial newborns. Hamster pups[22] belong to this group.

The *precocial* newborns normally are born singly or in small litters. They are very well developed, their eyes are open, they are furred, and they can walk around shortly after birth. These newborns are born with well-developed brown adipose tissue, which tends to atrophy postnatally. It is unknown how this uterine recruitment of brown adipose tissue is accomplished. Certain small newborns, such as the guinea pig,[25] belong to this group, but more obvious members are the newborns of many larger species, such as lambs,[26,27,28] goats,[29] calves,[30,31] and musk oxen.[32]

Classification of the human infant (or other primate infants[33]) as either altricial or precocial is not self-evident. Although born in a somewhat developed state, the human infant has several traits of immaturity and probably is most akin to the altricial newborns—specifically, human perinatal brown adipose tissue development has similarities to that found in the newborn rat pup.

HOW MANY ADIPOSE TISSUES EXIST?

Experiments concerning the function of most body organs are helped from a practical standpoint by the fact that most organs are anatomically well-defined entities with a single localization in the body and with structurally homogeneous and stable cells. Unfortunately, such characteristics do not pertain to brown adipose tissue.

In the human infant, the major depots are in the neck, under the clavicles, around the intercostal arteries, and around the kidneys and adrenals. Only a thin sheet of interscapular brown adipose tissue is found.[34,35,35a]

Distinguishing between white and brown fat is not straightforward. When activated, brown adipose tissue can be distinguished from white by its ability to express UCP1. However, because the potential ability to express UCP1 in a given cell (in a given physiologic situation) may not have been invoked at the moment of study, it is likely that each anatomically defined depot contains cells that are genuinely "white," as well as cells that are only visually disguised as white but that still carry the potential to be brown. Leptin is well expressed in white adipose tissue, but it is not expressed in recruited (i.e., activated) brown adipose tissue.[36] It is, however, expressed in nonactive brown adipose tissue, and its expression in both adipose tissues is inhibited by sympathetic stimulation.[37]

Morphologically, even epididymal white adipose tissue can be altered by an intense cold stress to visually resemble brown adipose tissue and to have brown fat–like mitochondria.[38,39] Not even under these extreme circumstances, however, has it been possible to detect UCP1 as a protein immunologically or to identify UCP1 messenger RNA (mRNA) in this tissue.[39] Thus, within this depot, apparently only truly white fat cells are present. By contrast, in the parallel female tissue, the parametrial fat pad, good evidence has emerged for the presence of genuine brown fat cells (i.e., those containing UCP1).[40] Additionally, UCPI may be detected in small amounts in other depots of seemingly white adipose tissue.[40a,40b]

In certain species, even the subcutaneous adipose tissue (which generally is believed to have storage and insulatory properties) can be brown. As indicated by a functional analysis of the mitochondria in the subcutaneous adipose tissue of newborn seals[41] and in dog pups treated with sympathomimetics,[42] the presence of UCP1 in subcutaneous tissue can be discerned.[43] In these species, the subcutaneous adipose tissue works not only as a blanket but also as an "electric blanket." Thus, although certain fat depots apparently lack any capacity for conversion to brown fat, most depots have this potential, and UCP1 may be expressed under certain physiologic or pharmacologic circumstances.

As a general rule, more visible brown adipose tissue depots are present in the newborn than in the adult, even the cold-acclimated adult. It has been stated that in some newborn species, all adipose tissue is brown, and these depots are converted to white adipose tissue in the adult. In cell biology, however, the meaning of this statement is vague,[44] and no strict lineage relationship between brown and white adipose cells has been clarified. Recent studies indicate that brown and white adipose tissues are of distinct lineages, with the brown having a close relation with a skeletal muscle lineage.[40b,45,46,] Certainly, the behavior of certain depots that look white in the adult is functionally close to that of brown adipose tissue in the newborn, and the fat in these depots retains some potential to again become brown.[47]

UCP1 AND THERMOGENESIS

Heat production may result from shivering or nonshivering thermogenesis. In the newborn infant and in the adult animal, the first resource used for extra heat production is nonshivering thermogenesis. Only after this capacity is fully utilized does shivering begin. In an adult living in a warm environment, with little active brown adipose tissue, shivering is the dominant source of extra heat production initially. In the infant, however, the ability to shiver sufficiently and intensively enough to produce substantial amounts of heat does not seem to be fully developed, so brown adipose tissue is the sole source of heat production.

Heat produced during nonshivering thermogenesis results directly from the activity in brown adipose tissue of the unique mitochondrial UCP1; in the absence of this protein, no heat is produced in brown fat cells.[48,49] Heat production is governed by signals from the hypothalamus. These signals are relayed through the sympathetic nervous system and transmitted to the cell as a norepinephrine stimulus leading to a series of events within the cell (for overview, Figure 43-1). Sympathetic innervation to the tissue is dense. One system of fibers, which contains the costored neuropeptide Y (NPY), innervates the numerous blood vessels entering the tissue, and another system innervates the adipocytes.[50] The neuropeptides calcitonin gene–related peptide (CGRP) and substance P also are found in the tissue, the latter in afferent nerves; however, the localization of CGRP currently is not completely clarified, and the functions of these neuropeptides are unknown.[51]

THE UNCOUPLING PROTEIN UCP1 (THERMOGENIN)

Considerable research by several groups of investigators led to the identification of UCP1 as the key enzyme in nonshivering heat production. Much is known today about this protein (Figure 43-2, *A*). It has been sequenced both as a protein and from complementary DNA (cDNA) clones corresponding to the UCP1 mRNA. The amino acid sequence is known for many species of mammals.[52-55] The amino acid sequence of the human protein is 79% homologous to that of the rat protein, allowing considerable immunologic cross-reactivity between human UCP1 and rodent UCP1.[56] The human UCP1 gene has been isolated[57,58] and its organization analyzed.

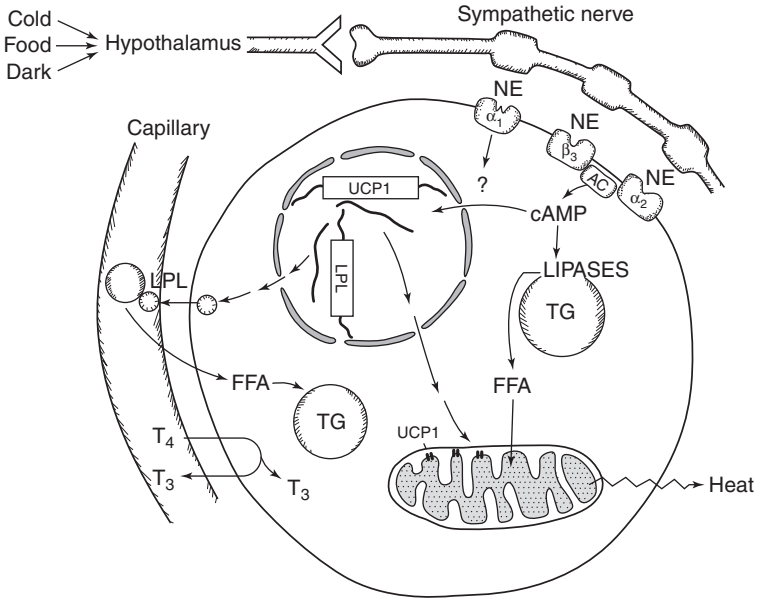


Figure 43-1 Function of the brown fat cell: An overview. When postnatal cold is perceived, the sympathetic nervous system is activated. Norepinephrine (NE) is released and interacts with adrenergic receptors, leading to release of fatty acids, which undergo combustion in the mitochondria. This combustion is possible as a result of the presence of uncoupling protein-1 (UCP1). Further substrate is provided by the action of lipoprotein lipase. Brown adipose tissue also may produce triiodothyronine (T_3) from thyroxine (T_4), leading to local and systemic effects. Dietary factors and darkness also activate centers in the hypothalamus in a manner analogous to that of norepinephrine. *AC,* Adenylyl cyclase; *cAMP,* cyclic adenosine monophosphate; *FFAs,* free fatty acids; *LPL,* lipoprotein lipase; *TG,* triglycerides.

UCP1 is a member of the superfamily of mitochondrial carrier proteins (see Figure 43-2, *B*).[59-61] Most closely related to UCP1 are two more recently identified but evolutionarily more ancient proteins known as UCP2 and UCP3 because of their extensive homology with UCP1.[62] The functions of these proteins remain unclear. They are, however, not responsible for any thermoregulatory thermogenesis.[49] Other proteins (e.g., those termed UCP4, UCP5) are not closely related to UCP1; therefore functional relationships cannot be expected.

From analysis of the amino acid sequence, it has been proposed that UCP1 may constitute three membrane-spanning Us, as shown in Figure 43-2, *A*. When functionally inserted into the brown fat mitochondrial inner membrane, UCP1 endows these mitochondria with a series of unique properties, including a high rate of respiration in the absence of adenosine diphosphate (ADP) or uncoupler addition, a high proton conductivity, and a high Cl^- conductivity.

THE *UCP* GENE

The *UCP1* gene is found on chromosome 8 in mice[63] and on chromosome 4 in humans.[64] Only one copy of the gene is present.[57]

An interesting functional and evolutionary connection between the exon-intron pattern of the *UCP1* gene and the suggested transmembrane structure of the protein has been described: Each of the six proposed membrane-spanning segments is represented by one exon.[55,64,65] This pattern also is found in the other *UCP* genes. A gene for a proto UCP1 exists in fish,[65a] and a rapid evolution led to this proto UCP1 gaining its thermogenic function in mammals.[65b]

ACUTE REGULATION OF UCP1 ACTIVITY

The function of UCP1 in the mitochondrial membrane is depicted in simplified form in Figure 43-3. UCP1 acts as the equivalent of a proton translocator, allowing dissipation of the proton gradient that has arisen from the functioning of the respiratory chain.

UCP1 cannot be constantly active, because such activity would lead to constant high heat production in the tissue, irrespective of the environmental (or ambient) temperature. In experiments with isolated mitochondria, it has been observed that the activity of UCP1 can be inhibited by the addition of purine nucleotides.[66,67] Traditionally, GDP has been the nucleotide of choice for such experiments, and thus, UCP1 has been known as the GDP-binding protein. This protein is not related to the G-proteins in signal transduction, nor to the ribosomal guanosine triphosphate (GTP)-binding proteins, and the term may be considered to be physiologically misleading, because adenosine triphosphate (ATP) is as good as or better than GDP both for binding and in inhibiting respiration.[68,69] Because ATP is present in much higher concentrations in the cytosol of brown fat cells than is GDP,[70] ATP is the physiologically relevant nucleotide that binds to UCP1 and inhibits thermogenesis when thermogenesis is not physiologically required.

The affinity of UCP1 for ATP is so high that the binding site would probably always be saturated with ATP and thermogenesis inhibited if this were the only agent that interacted with UCP1. Thus it is necessary to postulate the existence of a physiologic activator of UCP1.[67] The nature of this "positive" modulator continues to be investigated. A detailed analysis of the problem is outside the scope of this chapter, but a few main points can be summarized here.

Among the many suggestions, the most simple hypothesis for the nature of this modulator is that it is produced during the release of fatty acids, occurring as an effect of stimulation of the cell. Within this framework, several alternatives have been proposed. The positive modulator could be the free fatty acids themselves.[67,71,72] The most telling experiments that indicate a direct effect of free fatty acids are those of Rial and colleagues.[73] More recent experiments support this theory.[74] Alternatively, the fatty acids have been suggested to have a catalytic function, although in this case a positive modulator may be involved.[75,76] Another hypothesis along the same line has suggested that palmitoyl-coenzyme A (CoA) may interact directly with UCP1.[77,78] Each proposal has advantages and problems. Although conclusive experiments have been published that the modulator is downstream of lipolysis, general agreement on the mechanism has not yet been reached.

POSSIBLE SEMIACUTE REGULATION OF UCP1 ACTIVITY

Under certain conditions, apparently no direct correlation has been established between the amount of UCP1 (e.g., as measured immunologically) and the activity of UCP1 (as estimated by GDP binding or proton or Cl^- translocation). When the level

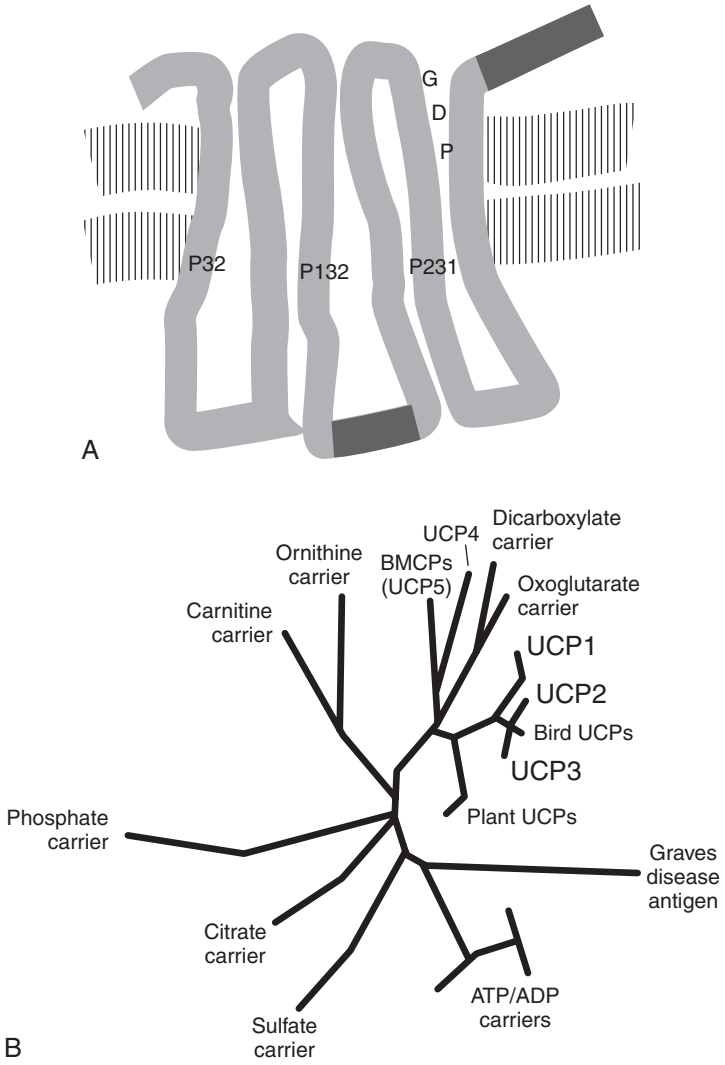


Figure 43-2 A, Suggested structure of uncoupling protein-1 (UCP1). The tripartite transmembrane structure is common to members of the mitochondrial carrier protein superfamily. P32, P132, and P231 indicate proline residues conserved among all family members, and GDP denotes the general area in which nucleotide binding occurs. **B,** Rootless dendrogram showing similarity between members of the mitochondrial carrier protein superfamily. *ADP,* Adenosine diphosphate; *ATP,* adenosine triphosphate; *BMCP1,* brain mitochondrial carrier protein 1; *GDP,* guanosine diphosphate; *UCP,* uncoupling protein. *(***A,** *Modified from Nedergaard J, Golozoubova V, Matthias A, et al: UCP1: The only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency,* Biochim Biophys Acta *1504:82, 2001;* **B,** *Modified from Borecký J, Maia IG, Arruda P: Mitochondrial uncoupling proteins in mammals and plants,* Biosci Rep *21:201, 2001.)*

of activity is found to be lower than expected, the phenomenon has been termed *masking* of UCP1.[79] UCP1 thus typically is masked when it is inactive (as is probably the case in utero) but becomes *unmasked* when called into function. Accordingly, it is reasonable to speculate that the rapid increase in GDP-binding capacity seen shortly after birth in guinea pigs[25] may be due to this unmasking mechanism.[80]

ADAPTIVE REGULATION OF UCP1 AMOUNT

The total amount of UCP1 in the newborn is expected to be the rate-limiting factor for nonshivering thermogenesis. In newborns and adults, the amount of UCP1 is under precise regulation. Although a significant temporal delay between a change in UCP1 mRNA and the ensuing increase in amount of UCP1 has been documented,[81] the simplest explanation is that the mRNA level determines the protein level.

Receptors Involved

The major stimulus for UCP1 gene expression is from the sympathetic nervous system through the release of norepinephrine. β-Adrenergic receptors are well recognized to be involved in this regulation.[82-84] Furthermore, some evidence indicates that simultaneous α-stimulation is necessary in vivo.[83] Perhaps this α-stimulation is of a permissive nature.

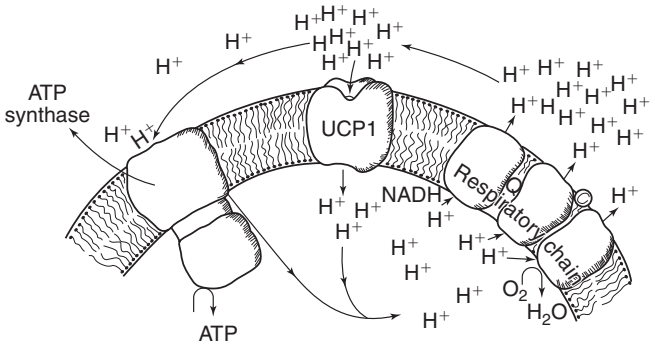


Figure 43-3 Suggested function of uncoupling protein 1 (UCP1) in the brown fat cell. UCP1 functions as a regulated translocator of protons (or proton equivalents) through the mitochondrial membrane, thereby allowing dissipation of the proton gradient built up by the respiratory chain, as a consequence of the oxidation of substrate, such as nicotinamide adenine dinucleotide *(NADH).* Thus respiration can proceed unlimited by the turnover of adenosine triphosphate *(ATP). (Based on descriptions in Nicholls DG: The bioenergetics of brown adipose tissue mitochondria,* FEBS Lett *61:103, 1976.)*

Perinatal UCP1 Expression

Altricial Species

The amount of UCP1 increases around the time of birth (in mice and rats).[21,85] This increase is caused by a concomitant increase in the amount of UCP1 mRNA[84,86,87] (Figure 43-4, *A* and *B*). The cause of the postnatal recruitment in brown adipose tissue (and increase in UCP1 mRNA) could be either ontogenic or an effect of the cold stress experienced by the pups at birth.[11] No postnatal increase in UCP1 mRNA occurs in the absence of a postnatal cold stress (see Figure 43-4, *C*); presumably, therefore, in these species, the postnatal brown adipose tissue recruitment is a response to the cold stress experienced by the newborn.[87] This correlation indicates that the increase is mediated by sympathetic activation as in adults.

Immature Newborn Species

In immature-type newborns, the increase in UCP1 occurs rather late.[22,23] It is conjectured that when the ability to react to environmental cues has developed in these animals, the recruitment of brown adipose tissue takes place in the same way as already described for altricial species.

Precocial Newborn Species

In species with precocial newborns, UCP1 is expressed in the fetal state.[25,30,88] Because fetal production of excessive heat would be potentially lethal, it follows that a uterine inhibitor of thermogenesis must exist, which still allows transmission of the signal for gene expression to occur but inhibits a thermogenic response. Without such an inhibitor, the activation signal presumably would trigger fetal heat production in utero. In experiments in which the umbilical cord has been sectioned, some indications for the existence of such an inhibitor have been observed.[89-92]

The central signal that leads to sympathetic stimulation of the tissue in utero is unknown. It cannot be a cold stimulus and could hardly be a food stimulus. The only other documented pathway for sympathetic stimulation of brown fat recruitment is through short day length,[93] probably mediated by the pineal gland and melatonin. It could be argued that the fetus lives its life in darkness, and that newborns are more sensitive to the day's elapsed length.[94] Whether such a mechanism could function in precocial species is doubtful, and most available data indicate that it is merely the dam's level of melatonin that is reflected in the fetal blood level.

Alternatively, another mechanism of recruitment, independent of sympathetic stimulation, may be postulated: Specifically, it is possible that activators of peroxisome proliferator–activated receptor-gamma (PPAR-γ) are formed and stimulate UCP1 expression without concomitant thermogenesis.[94a]

The Human Newborn

Because the classification of the human newborn as precocial or altricial is not fully clear, it is not evident which animal neonate is the best for experimental models to make determinations that may be considered applicable to the human infant. Human brown adipose tissue is present in considerable quantities at term gestation but also demonstrates a postnatal increase.[95] The amount of UCP1 is high in children, and brown adipose tissue containing UCP1 is found in a significant fraction of adults.[96-100,100a-100f]

SUBSTRATES FOR THERMOGENESIS

Intracellularly Derived Fatty Acids

During the initial acute phase of thermogenesis, the supply of fatty acids for combustion comes from triglyceride droplets found within the tissue. The release of fatty acids for combustion is caused by a series of events similar to those known to occur in white fat cells, leading to a release of fatty acids in the circulation. Thus stimulation of β-receptors leads to an increase in intracellular levels of cyclic adenosine monophosphate (cAMP),[101] which in turn leads to activation of the cAMP-dependent protein kinase (see Figure 43-1). Both of these events can be observed during postnatal development: cAMP is elevated in brown adipose tissue,[102,103] and the activity ratio of cAMP-dependent protein kinase increases, from 0.3 in fetal tissue to 0.7 after birth.[104]

Different Adrenergic Receptors

A β-adrenergic receptor is the adrenergic receptor type involved in the increase in cAMP in fully differentiated brown adipocytes. In rodents, it is the β_3-receptor that is predominantly involved, but the situation in human brown fat is less clearly understood.[105-110] The effects of β-receptor activation on cAMP levels may be counteracted by α_2-adrenergic receptors, which also are found in brown adipose tissue of fetuses and newborns.[111-113] Adenosine receptors also may counteract β-stimulation.[114]

Brown fat cells also are endowed with α_1-adrenergic receptors.[115] Although some of their intracellular actions are known,[116-118] their significance for thermogenesis is not fully understood. In in vitro experiments, only a small fraction of thermogenesis resulting from adrenergic stimulation can be

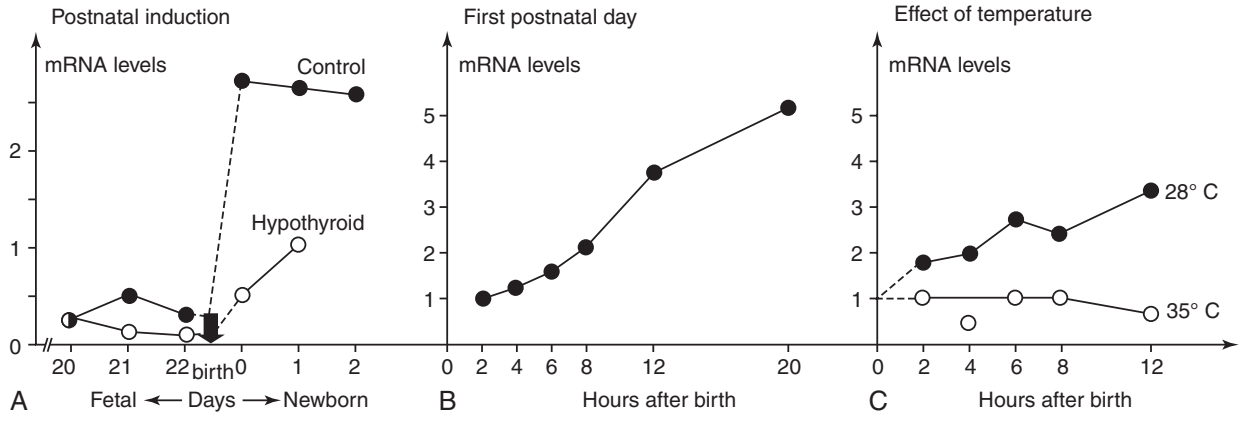


Figure 43-4 Postnatal increase in UCP1 messenger RNA (mRNA). A, Prenatal to postnatal transition; note the effect of hypothyroidism. **B,** Time resolution of the first postnatal day. Pups remained with their dams. **C,** Effect of temperature on the postnatal increase in UCP1 mRNA. Pups were exposed individually to the indicated environmental temperatures. *UCP1,* Uncoupling protein-1. *(***A** *to* **C,** *Modified from Obregón MJ, Jacobsson A, Kirchgessner T, et al: Postnatal recruitment of brown adipose tissue is induced by the cold stress experienced by the pups: An analysis of mRNA levels for thermogenin and lipoprotein lipase,* Biochem J *259:341, 1989.)*

demonstrated to result from stimulation of α_1-adrenergic processes. Nonetheless, a large fraction of nonshivering thermogenesis can be inhibited in vivo by α_1-blockade[119] in the newborn rabbit. This effect may be secondary to cardiovascular phenomena. In isolated cells, α_1-adrenergic stimulation may enhance the effectiveness of cAMP.[120]

Protein Kinase and Hormone-Sensitive Lipase

One substrate for the activated cAMP-dependent protein kinase is the hormone-sensitive lipase found in brown adipose tissue.[121] Activation of this lipase leads to the breakdown of diglycerides formed by the action of adipose tissue triglyceride lipase (ATGL) on the stored triglycerides; how ATGL is stimulated is still unclear.[121a] The fatty acids released probably undergo immediate combustion in the mitochondria, whereas glycerol probably is released to the circulation. The decrease in triglyceride droplet size after birth can be seen on electron micrographs.[18]

Whether a physiologically significant release of fatty acids from brown adipose tissue into the circulation occurs is still unconfirmed. It originally was suggested that brown fat (unlike white fat) did not release any fatty acids to the circulation,[122] but isolated brown fat cells break down more triglycerides to fatty acids than can be subject to combustion within the cells and release fatty acids in vitro.[123] Investigations of the effluent blood from brown adipose tissue have yielded somewhat variable results,[124,125] but a capacity for export is likely.

Also unknown is whether increases in plasma levels of fatty acids observed in cold-stressed animals may be due to an increased release from brown fat and thus reflect the activity of this tissue. Tissue ablation experiments have indicated that this may be the case,[126] especially in newborns in whom a large fraction of the adipose tissue found is in the form of brown adipose tissue.

Fatty Acids From the Circulation

The brown fat cell is endowed with a store of triglycerides that can be used in any situation in which thermogenesis is required. This store is limited, however; in newborn rabbits, the amount of stored triglycerides is sufficient for only 3 days of heat production. After this time, unfed rabbits die as a result of hypothermia.[127] Similar observations can be made in premature human infants.[128] To ensure a constant supply of substrate for combustion, additional substrate must be supplied by the circulation. Only under conditions of starvation before cold stress has it been reported that the uptake of circulating free fatty acids is significant.[129] The quantitatively most important source of lipid substrate is that found in chylomicrons, which are formed from ingested food. Fatty acids in chylomicron triglycerides are released to the brown fat cells by the enzyme lipoprotein lipase.

Regulation of Lipoprotein Lipase

In contrast with the situation in white adipose tissue, brown adipose tissue lipoprotein lipase activity is increased by adrenergic stimulation.[130,131] (In this respect, the tissue is similar to that of the heart.) This stimulation is mediated by β-adrenergic receptors, resulting in an increase in cAMP and an ensuing increase in the expression of the gene for lipoprotein lipase.[131-134] This is a comparatively slow process, requiring several hours to reach full effect, because the lipase must be synthesized and transported to the capillaries. Thus the tissue cannot rely on this process to generate an acute thermogenic response.

Insulin also can stimulate lipoprotein lipase activity.[135-137] This insulin stimulation does not seem to be mediated through central effects and the sympathetic nervous system; instead, it seems to be a direct effect of insulin on tissue in states of proper nutrition. Insulin stimulation also is mediated through an increased expression of the lipoprotein lipase gene.[133,134]

Perinatal Recruitment of Lipoprotein Lipase

Lipoprotein lipase activity is low in the fetus but increases postnatally[138] (Figure 43-5, *A*). This postnatal increase also can be seen in premature pups. It is not known whether the increased activity is an effect of suckling, temperature change, or release of an inhibitor transferred through the umbilical cord.

When this "birth-induced" increase in activity is evaluated in the context of measurements of lipoprotein lipase mRNA, an

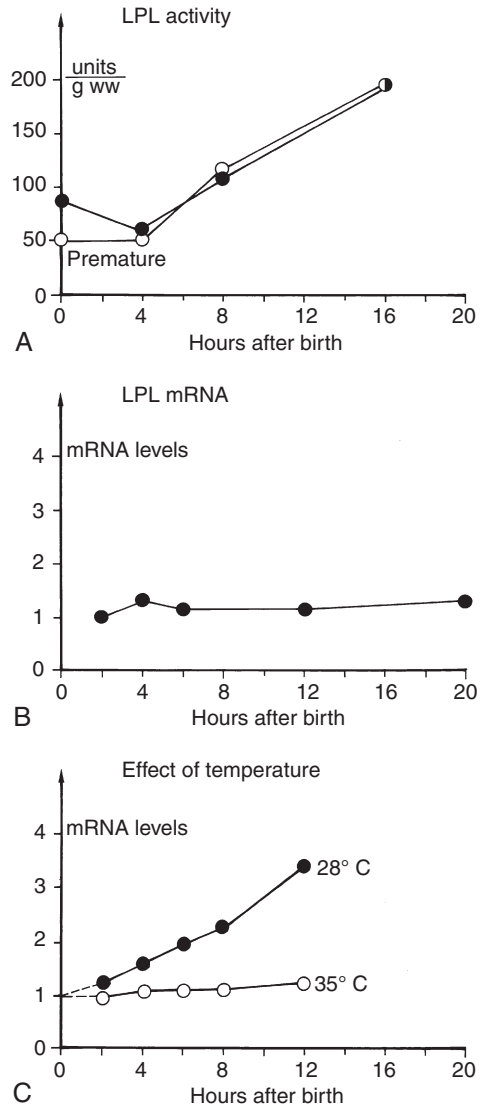


Figure 43-5 Lipoprotein lipase *(LPL)* activity and gene expression. A, Lipoprotein lipase activity in newborns staying with their dams. **B,** Lipoprotein lipase messenger RNA (mRNA) levels in newborns staying with their dams. **C,** Lipoprotein lipase mRNA levels in individual pups exposed to the indicated environmental temperatures. *(***A,** *Modified from Cryer A, Jones HM: Developmental changes in the activity of lipoprotein lipase (clearing factor lipase) in rat lung, cardiac muscle, skeletal muscle and brown adipose tissue,* Biochem J *174:447, 1978;* **B** *and* **C,** *modified from Obregón MJ, Jacobsson A, Kirchgessner T, et al: Postnatal recruitment of brown adipose tissue is induced by the cold stress experienced by the pups: an analysis of mRNA levels for thermogenin and lipoprotein lipase,* Biochem J *259:341, 1989.)*

enigma arises, however (see Figure 43-5, *B*): Lipoprotein lipase mRNA is not increased (possibly owing to an inhibitory factor in the colostrum).[139] To explain the discrepancy between lipoprotein lipase mRNA level and lipoprotein lipase activity, a transfer of lipoprotein lipase across gut membranes has been suggested.[87] This hypothesis requires further investigation. Under conditions of mild cold stress in the absence of the dam, however, lipoprotein lipase mRNA level within brown adipose tissue increases, apparently through processes similar to those described in the adult (see Figure 43-5, *C*).

One effect of this postnatal activation of lipoprotein lipase on the fatty acid composition of tissue triglycerides is that fatty acids become more unsaturated and in this respect reflect the composition of the diet (i.e., the mother's milk).[140-142]

Pathology

No pathologic state that involves activity of lipoprotein lipase in human brown adipose tissue is known, but a fatal genetic disease in mice, combined lipase deficiency (the mutation is therefore designated cld/cld), leads to the synthesis of an inefficient lipoprotein lipase.[143] The absence of lipoprotein lipase activity has the expected effect of reducing the amount of fat droplets in brown adipose tissue.[144]

Glucose

Uptake of glucose was early documented in brown adipose tissue in the newborn rabbit,[129] but the identification of brown adipose tissue through intense glucose uptake in adult man demonstrates that this is a general process.[100,100a-100d,100f] The glucose probably is used mainly as a substrate for thermogenesis, but on the basis of in vitro experiments, it may be envisaged that this uptake of glucose may also function to increase the catabolic capacity of the citric acid cycle through the action of pyruvate carboxylase.[145] Brown adipose tissue also shows insulin-sensitive glucose transport through redistribution of the GLUT4 glucose transporter from intracellular vesicles to the plasma membrane.[146] Glucose uptake in brown adipose tissue is markedly stimulated by cold exposure; this stimulation is mediated through norepinephrine rather than insulin. Although it apparently does not alter GLUT4 distribution, norepinephrine may increase either GLUT1 or GLUT4 activity and, correspondingly, glucose uptake rates.[147-149]

Fatty Acid Synthesis

The significance of lipogenesis in brown adipose tissue has been discussed. The capacity for lipogenesis in brown adipose tissue is high.[150,151] Lipogenesis also has been observed in human infants.[152] However, strong evidence suggests that this lipogenesis is not of quantitative or qualitative significance for thermogenesis. Rather, it could be considered to represent a "refilling" reaction that occurs in tissue after cessation of the thermogenic stimulus. Thus, during the perinatal period, the activity of lipogenesis is low when thermogenesis is high. This inverse correlation probably is due to the effect of suckling (i.e., a change from a high-carbohydrate "diet" in the fetal state to a high-lipid diet). A direct inhibitory effect of increased sympathetic stimulation on lipogenesis also has been postulated.[11] Weaning, which reintroduces a high-carbohydrate diet but also coincides with a reduced demand for thermogenesis, is associated with an increase in lipogenesis.

The so-called lipogenic enzymes (in addition to the fatty acid synthase complex) include pyruvate dehydrogenase, the citrate cleavage enzyme (ATP citrate lyase), acetyl-CoA carboxylase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzymes.

All of these enzymes generally follow the same perinatal pattern: Levels are high in the fetal state and much reduced during suckling, returning toward high fetal values as suckling ends.[11] For malic enzyme, it has been demonstrated that this phasic pattern is due to a change in the amount of enzyme (not a change in activity of existing enzyme).[153]

The activities of the glycolytic enzymes increase after weaning,[154] indicating that during the time when the diet consists of milk alone, it is those fatty acids that supply brown adipose tissue and are spent in combustion. Only after weaning to a diet rich in carbohydrates does glycolysis contribute to fatty acid synthesis.

Amino Acid Uptake

The possibility that amino acid uptake occurs has been investigated. It generally is believed that this pathway is of minor importance for the functioning of brown adipose tissue.[155] Amino acid uptake could account for approximately one third of the uptake of glucose. Several amino acids have propionyl-CoA as the end product of oxidation. This three-carbon fatty acid cannot be degraded through the normal β-oxidation pathway for fatty acid derivatives, so detrimental levels of this fatty acid could accumulate, resulting in a sequestration of all mitochondrial CoA, with consequent inhibition of thermogenesis. This effect may explain why the mitochondria have a high activity level of a propionyl-CoA hydrolase with unusual regulatory properties.[156-158] This hydrolase also could function to recover the CoA otherwise bound up in the propionyl-CoA.

5′-DEIODINASE AND THYROID

In brown adipose tissue, high activity of thyroxine 5′-deiodinase, which produces the active thyroid hormone triiodothyronine (T_3), has been described[129] (Figure 43-6). The 5′-deiodinase found in brown adipose tissue has been classified as a type II deiodinase (distinguished from type I by its insensitivity to propylthiouracil in vitro[159] and by its being induced by hypothyroidism in vivo[160]). The gene has been cloned.[161] The enzyme probably is localized to the endoplasmic reticulum. The presence of 5′-deiodinase has been demonstrated in human, lamb, and rodent brown fat.[159,162,163]

Internal Action

It is thought that the activity of the 5′-deiodinase is important both for the normal development of brown adipose tissue and for the rapid increase in brown fat during the perinatal period. The T_3 produced probably binds to T_3 receptors found within the tissue[164,165] (Figure 43-6) and in this way influences expression of the *UCP1* gene through binding to sequences in the promoter region.[166] Indeed, hypothyroidism leads to a decreased expression of UCP1 in both rats and lambs (see Figure 43-4, *A*).[86,162] However, in mice in which the nuclear thyroid hormone–binding receptors are ablated, UCP1 expression is high, confirming that the hormone is required primarily to relieve a repression, rather than to cause activation.[167,168]

Systemic Action

The possibility also has been considered that brown adipose tissue 5′-deiodinase is of systemic physiologic significance in that it could release T_3 from brown fat into the circulation.[169] Indeed, such release has been substantiated in the newborn rat.[170]

T_3 could be responsible for the small increase in basal metabolism sometimes found in conditions in which brown fat is stimulated, but no experiments have been performed in which brown fat has been removed to investigate this possibility.

In addition to the metabolic effects of T_3, thyroid hormone also may have an important role in promoting differentiation. Some interesting but as-yet unconfirmed observations suggest that

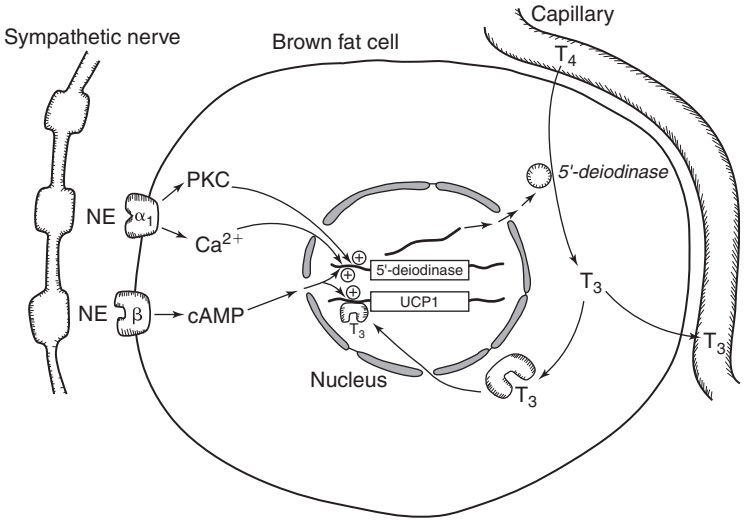


Figure 43-6 The regulation and action of 5′-deiodinase in brown adipose tissue. *cAMP,* Cyclic adenosine monophosphate; *NE,* norepinephrine; *PKC,* protein kinase C; T_3, triiodothyronine; T_4, thyroxine; *UCP1,* uncoupling protein-1.

removal of brown adipose tissue at a young age affects development of other bodily functions in later life. Thus, in several studies, Jankovic and colleagues have implied the presence of such a brown adipose tissue effect on the development of the immune response,[171-173] but so far these observations have not been independently verified.

Regulation of Deiodinase Activity

The activity of 5′-deiodinase is rapidly induced under conditions in which brown adipose tissue is recruited. This increase in activity is due to an increased synthesis of the enzyme.[174,175]

Adrenergic Regulation

It originally was suggested that the increase in deiodinase activity was mediated through an α_1-adrenergic mechanism.[176] Apparently, although an α_1-stimulus presumably is necessary to produce a significant stimulation, α- and β-adrenergic stimulation interact synergistically to induce maximal induction of 5′-deiodinase activity. From in vitro experiments with isolated cells, it has been suggested that this interaction occurs at the cellular level, apparently at a post-cAMP step in the activation pathway.[177,178]

Insulin

Insulin can increase 5′-deiodinase activity when injected into animals[175] and when added to isolated brown fat cells.[179] Neither the physiologic significance of this phenomenon nor the specific intracellular pathways involved in the gene activation events have been characterized.

Perinatal Recruitment of 5′-Deiodinase

The perinatal pattern of development of 5′-deiodinase is unusual: The deiodinase activity rises markedly before birth, after which it spontaneously declines[180-182] (Figure 43-7). The regulation of this activity and its significance for brown fat recruitment are of interest, but no experimental evidence concerning these questions is available at present.

REGULATION OF RECRUITMENT OF THE TISSUE

The capacity of brown adipose tissue thermogenesis is limited by the amount of UCP1 in the tissue, and this level is governed by the activity of the sympathetic nervous system and norepinephrine. To increase the amount of UCP1 in the tissue, two principal

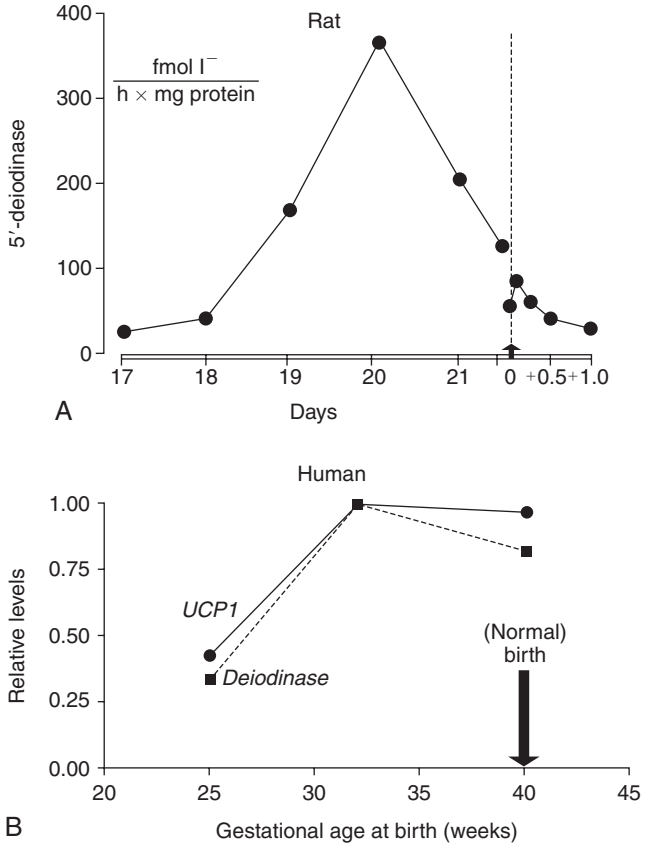


Figure 43-7 5′-Deiodinase activity in the perinatal period. *(Data based on rat studies from Giralt M, Martin I, Mampel T, et al: Evidence for a differential physiological modulation of brown fat iodothyronine 5′-deiodinase activity in the perinatal period; supported by similar data from Obregón MJ, Ruiz de Oña C, Hernandez A, et al: Thyroid hormones and 5′-deiodinase in rat brown adipose tissue during fetal life,* Am J Physiol *257:E625, 1989. Human data from Houstěk J, Vízek K, Pavelka S, et al: Type II iodothyronine 5′-deiodinase and uncoupling protein in brown adipose tissue of human newborns,* J Clin Endocrinol Metab *77:383, 1993.)*

strategies may be used. In the first, the amount of UCP1 per brown adipocyte is increased. In the second, an increase in cell proliferation (i.e., in the number of adipocytes) occurs, but each individual adipocyte maintains an unchanged capacity. Although different species appear to use more or less of either the first or the second modality, both seem to be operative in all species studied. The situation has not been studied for the human neonate.

The tissue retains a potential proliferative capacity, even in adulthood. Preadipocytes in culture can be stimulated to increase their proliferation by the addition of norepinephrine, and the stimulation is through β_1-receptors and cAMP-coupled mechanisms.[183] This mechanism is in contrast with the norepinephrine stimulation of *UCP1* gene expression in mature brown adipocytes, which, while also using cAMP as the intracellular messenger, couples to the adenylyl cyclase through β_3-receptors.[184]

Various transcription factors might be expected to be involved in the control of cell proliferation, and many are currently being studied. Of note, an important adipose-related factor, CCAAT/enhancer binding protein alpha (C/EBPα), is transiently decreased as proliferation begins.[185] Other fat-related transcription factors, such as members of the PPAR family, and the coactivator PGC1α (PPAR-gamma coactivator 1 alpha), also appear to be of importance.[186] A more comprehensive study of the roles of these transcription factors is required to elucidate the complex control of cell proliferation, both in utero and postnatally. It is evident that in the fetal state, growth factors such as insulin-like growth factor-I (IGF-I)[187,188] are important for cell growth.

CLINICAL IMPLICATIONS

Considerable evidence suggests that one of the major problems with temperature regulation in small premature infants is that the lipid supply to brown adipose tissue and its mechanism of recruitment are not sufficiently developed. This deficiency, combined with the larger surface-to-volume ratio characteristic of the small premature infant promoting rapid heat loss, places them at increased risk for hypothermia. Therefore the main problem resulting from the incomplete development of brown adipose tissue in premature newborns was solved through the introduction of the incubator.[189]

Effects of Environmental Temperature

Convincing evidence from animal experiments indicates that the postnatal environmental temperature has a substantial effect on brown adipose tissue development (see Figures 43-4 and 43-5).[11] Indeed, some anecdotal evidence suggests that children who live in relatively cold environments will acquire or maintain an improved cold tolerance, probably through persistent brown adipose tissue function.

Anesthesia in Infants

During general anesthesia, body temperature regulation is disturbed, and this effect is more marked in young infants. Although effects on central mechanisms controlling body temperature also may be observed, it is now apparent that the commonly used inhalation anesthetic halothane and its derivatives are potent inhibitors of brown adipose tissue thermogenesis.[190-193] This effect is of particular significance in young infants, in whom the activity of brown adipose tissue is very important for body temperature regulation.

Effects of Malnutrition

The effects of prenatal or postnatal malnutrition have been investigated in experimental animals. In general, food restriction reduces the function of brown adipose tissue,[194,195] although paradoxical observations have been reported.[196] Adverse effects of more specific dietary restrictions (e.g., riboflavin, protein) also have been reported.[197,198] In scattered reports in the literature, malnutrition has been implicated as a cause of hypothermia in human newborn infants, presumably owing to a lack of substrate being available for thermogenesis.[199]

Overfeeding

The presentation of a palatable, so-called cafeteria diet to adult rats results in overfeeding. However, these animals do not gain weight to the extent anticipated by the degree of overfeeding. The brown adipose tissue of such animals is markedly recruited, and it has been suggested that the decrease in the animals' whole-body efficiency (as indicated by lower weight gain than anticipated) could result from increased thermogenesis in brown adipose tissue.[200,201] Postnatal overfeeding in rats (induced by a reduction in the number of pups per litter) also leads to activation of brown adipose tissue.[202,203] Postnatal overfeeding leading in the adult to decreased brown fat thermogenesis may have long-lasting efects.[204]

It can be posited that in the human infant, overfeeding may cause an activation of brown adipose tissue, leading to an increased thermogenesis, which could counteract the effect of the increased food intake.

Genetically Obese Animals

In genetically obese animals (the fa/fa rat and the ob/ob and db/db mice, all of which have problems in the leptin system), the postnatal recruitment process in brown adipose tissue is blunted, before any signs of obesity become evident.[205-207] This inactivity of brown adipose tissue appears to constitute at least one cause of developing obesity. This correlation was apparently confirmed by studies in which brown adipocytes were molecularly ablated from mice.[58] For these experiments, transgenic animals were produced in which the UCP1 promoter region was coupled to the gene for the diphtheria toxin A chain. This A chain inhibits protein synthesis, thereby killing the cells in which it is expressed (e.g., primarily brown adipocytes). The transgenic animals developed obesity and other manifestations of pathophysiology associated with obesity, such as insulin resistance. However, mice with an ablation in the UCP1 gene and thus possessing thermogenically incompetent brown fat initially did not appear to become manifestly obese.[208] However, if animals are kept at thermoneutral temperatures, at which thermogenesis is not needed to defend body temperature, the absence of UCP1 leads to increased adiposity.[209,209a]

It is unclear whether some humans may be subject to the same forces as in these genetically obese animals. Although the potential to develop obesity is known to have a strong genetic component, to what extent brown adipose tissue inactivity contributes to the development of obesity in children remains undetermined.

Diseases Affecting Brown Adipose Tissue

In fever, some of the heat necessary for the increase in body temperature comes from brown adipose tissue, at least in young experimental animals.[210,211] It also is clear that brown adipocytes not only are responsive to cytokines released during infections that cause fever but also can synthesize such cytokines.[212] A relatively rare benign tumor, the hibernoma, can occur, even in neonates.[213] Also of note is that treatment with prostaglandin E_1 for palliation of neonatal congenital heart disease results in calcific brown fat necrosis, which can resolve over time.[214,215] In a number of relatively rare diseases such as Duchenne progressive muscular dystrophy,[216] subcutaneous fat necrosis of the

newborn,[217] and sudden infant death syndrome (SIDS),[218-220] a role for brown adipose tissue has been suggested. In the case of SIDS, a link with gestational magnesium deficiency has been proposed.[221] At present, however, no direct evidence has emerged to show causative involvement of brown adipose tissue in any of these syndromes.[222]

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44 Lipids as an Energy Source for the Premature and Full-Term Neonate

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Lipids and carbohydrates are the major sources of energy for the neonate. Together with carbohydrates, lipids provide the dietary energy necessary for optimal utilization of protein. Lipids also supply the essential omega-6 and omega-3 fatty acids and are a vehicle for fat-soluble vitamins. In human milk and in formulas designed for preterm and full-term infants, fat provides approximately 40% to 50% of the total calories and accounts for more than 50% of nonprotein energy. Most dietary lipids are triglycerides. Long-chain triglycerides provide about 9 kcal of energy per gram; medium-chain triglycerides (MCTs), because of the shorter chain length, have been reported to have an energy value of 8.3 kcal per gram as determined by bomb calorimeter[1] and a net (usable) energy value of 6.8 kcal per gram as determined from animal studies and calculations of molecular composition.[2] The quantity and quality of dietary lipids have an effect on the absorption, metabolism, and tissue deposition of fat. When fed currently available formulas and human milk, very-low-birth-weight (VLBW) infants are reported to have efficiency of fat absorption greater than 85% and that of carbohydrate exceeding 95%.[3]

Substrate oxidation in neonates receiving enteral and parental nutrition has been estimated from gaseous metabolism as measured by indirect calorimetry and by stable isotope methodology. Concentrations of plasma metabolites have also been used in determining the type of substrate being oxidized in the infants. In this chapter the importance of lipids as a fuel for oxidative metabolism in term and preterm infants is discussed. This review is followed by a brief discussion of effects of quality of energy (fat versus carbohydrate) on substrate oxidation.

LIPIDS AS A FUEL FOR OXIDATIVE METABOLISM IN UTERO AND AT BIRTH

Glucose is the major energy source for the human fetus. Other substrates available for oxidative metabolism are amino acids, lactate, and free fatty acids (FFAs). Although triglycerides do not cross the placenta, FFAs cross by facilitated diffusion.[4,5] During fetal life approximately 80% of the energy expended is derived from carbohydrate oxidation.[6-9] If one assumes that energy expenditure of a fetus averages 50 kcal/kg/day,[10] the fetus should oxidize about 10 g/kg of carbohydrate each day. The major portion of the remaining energy needs is derived from amino acid oxidation. Thus, in the human fetus, lipids do not seem to be a major source of energy; however, they do play an important role in fetal development.

At birth, an abrupt change occurs in the nutrient supply after the umbilical cord is clamped and divided. In newborn term infants who have not received any feedings, the respiratory quotient (RQ) has been reported to drop from approximately 0.9 to less than 0.8 in the first 12 hours of life,[11] indicating increasing use of lipids as energy source. At the low RQ, the contribution of energy from fat is approximately 60% to 70% of the energy expenditure. During the first few hours after birth, an increase in plasma FFAs, glycerol, and ketone bodies has also been reported, indicating that lipolysis is actively occurring.[12,13] In the next few days, the RQ of infants increases as enteral feedings increase.[11]

Preterm infants are provided with glucose immediately after birth. On the first day of life, contribution of energy from the various substrates to the energy expenditure of these infants can be calculated from substrate oxidation rates that have been determined by stable isotope methodology. The energy derived from glucose oxidation has been reported to be approximately 16.4 kcal/kg/day[14] and that from protein oxidation, 6.7 kcal/kg/day.[15] With an estimated energy expenditure in these infants of approximately 30 to 45 kcal/kg/day on day 1 of life,[16] the contribution of energy from glucose is approximately 35% to 50% of the energy expenditure, that of protein approximately 15% to 20%, and the remaining energy is provided from fat oxidation.

LIPID AS A FUEL FOR OXIDATIVE METABOLISM FOR GROWING INFANTS

For full-term infants with adequate enteral intakes after the first week of life, fat is provided in abundance. Minimal data, however, are available on the RQ and substrate utilization in the normally fed and growing full-term infant between weeks 1 and 4 of postnatal life. The most careful estimates of RQ place it at around 0.85 or greater.[11] Based on those estimates and an oxygen consumption that has been measured at between 7 and 8 mL/kg/min, one can calculate that between 2 and 3 g/kg/day of fat is oxidized. This means that between 40% and 50% of calories oxidized after the first week of life in term infants are derived from fat. (This percentage would range between 30% and 40% for an RQ estimated at 0.9.)

For the growing preterm infant fed either human milk or a preterm formula, considerably more data on energy balance are available. Lipids provided 40% to 50% of total energy intake in these diets. Table 44-1 summarizes studies from the literature, in which data on metabolizable energy (energy intake minus losses in stools), energy expenditure, nutrient balances, and RQ have been precisely determined. Few published reports have provided sufficient data to permit a calculation of the amount of fat oxidized[17-23]; however, several conclusions can be reached based on the data in Table 44-1:

1. Almost all the studies listed in Table 44-1 have an observed RQ greater than 0.9. These values for RQ are similar to values from other studies[24-28] from which data were not precise enough to be included in this table, but the RQ values were measured in growing infants receiving full oral feedings (lower RQ values have been reported in nongrowing infants.[29]
2. The amount of fat oxidized averages 1.5 g/kg/day (i.e., approximately 14 kcal/kg/day). This represents approximately 30% of the fat absorbed and 20% (range, 10% to 35%) of the calories oxidized.
3. As listed in the table, although the values for RQ are noted to be high, they are slightly lower with human milk than with preterm formula. A high RQ can result from either a high rate of carbohydrate oxidation (fat being almost

TABLE 44-1

Respiratory Quotients (RQs), Energy Balances, and Fat Oxidation Rates in Low-Birth-Weight Infants Fed Human Milk or Preterm Formulas

				FAT					% ENERGY OXIDIZED FROM		AGE AT STUDY	
Type of Milk*	Met EI (kcal/kg/day)	Energy Expenditure (kcal/kg/day)	RQ	Intake (g/kg/day)	Absorbed (g/kg/day)	% Met EI	Oxidized (g/kg/day)	% Absorbed	Fat	Carbohydrate	GA (weeks)	Postnatal (days)
HM[17]	95	49	0.88	5.6	4.6	45	1.6	35	29	67	34.4	31
HM[18]	100	56	0.91	4.7	3.8	35	1.6	42	27	66	33.3	21
HM[19]	87	46	0.91	5.3	3.8	40	1.3	34	26	68	33.2	21
HM[19]	102	51	0.88	5.7	5.2	47	1.9	36	35	61	36	45
PF[20]	110	68	0.96	5.7	4.7	40	0.7	15	10	79	36	21
PF[19]	118	57	0.92	5.3	4.6	36	1.4	30	23	73	33.3	29
PF[19]	123	63	0.92	5.5	5.0	38	1.5	30	22	73	36	46
PF[21]	130	63	0.96	7.5	6.0	43	0.6	10	9	85	32.3	21
PF[22]	102	62	0.95	6.6	4.7	43	1.1	23	16	77	34.1	29
PF[22]	82	58	0.92	5.3	3.4	38	1.7	50	27	66	34.0	28
PF[23]	116	63	0.93	6.1	5.1	41	1.1	22	17	77	≥32.4	≥15

*Data from studies in cited references.
GA, Gestational age; *HM,* human milk; *Met EI,* metabolizable energy intake; *PF,* preterm formula.

nonutilized as a source of energy) or increased storage of carbohydrate as triglyceride.[30,31] With human milk, 30% to 35% of the calories oxidized are derived from fat (1.6 to 1.9 g/kg/day) compared with 10% to 25% with preterm formula (0.6 to 1.6 g/kg/day). It is difficult to assess whether this difference in fat oxidation rate is the consequence of a lower total metabolizable energy intake or of differences in the quality of the human milk. However, if studies that have used only preterm formula are analyzed, it appears that the highest RQ values are consistently observed in infants with the highest metabolizable energy intakes. Thus the lower oxidation rate of fat seen with formula is more likely to be due to the higher caloric intake provided as carbohydrate rather than a difference in the quality of milk. The higher carbohydrate intake in infants receiving formula could also explain the higher urinary C-peptide levels observed in formula-fed infants.[32]

Relatively few studies in the literature have made repeated measurements of energy expenditure in infants. Of interest is the observation by Gudinchet and colleagues[33] who demonstrated an increased participation of fat in oxidative metabolism with advancing age in the premature infant fed a constant energy intake of 130 kcal/kg/day (Table 44-2). Decreasing RQ, indicating increasing fat oxidation, has also been reported in extremely LBW infants in the first 5 weeks of life.[34]

TABLE 44-2

Evolution of Energy Expenditure and Respiratory Quotient (RQ) in Preterm Infants During the First 6 Weeks of Life*

	AGE (WEEKS OF LIFE)		
Component	1	3	6
Weight (g)	1153	1334	1793
RQ	0.97	0.91	0.90
Energy expenditure (kcal/kg/day)	40	64	60
% Energy derived from:			
Fat oxidation	14%		30%
Carbohydrate oxidation	80%		65%

*With advancing postnatal age, an increase in energy expenditure and fat oxidation is observed.
From Gudinchet F, Schutz Y, Micheli JL, et al: Metabolic cost of growth in very low birth weight infants. *Pediatr Res* 16:1025, 1982.

Medium-Chain Triglycerides as Fuel

MCTs (C8 and C10) were introduced in premature infant nutrition to enhance fat absorption. Several studies[28,35,36] demonstrated an improved total fat absorption rate when part of the long-chain triglycerides are replaced by MCTs, but others[37] have not. Formulas designed specifically for preterm infants contain 25% to 50% of total fatty acids as MCT. In human milk, approximately 8% to 10% of the total fatty acids is available as MCT.[38] Although no evidence shows that MCTs are harmful, storage or incorporation of MCTs into structural lipids may be of concern.

In the adult, MCTs appear to be entirely oxidized, but little is known about the metabolism (oxidation and storage) of MCTs in premature infants. Increases in the plasma concentrations of β-hydroxybutyrate and acetoacetate in infants receiving formula containing these fats[39,40] demonstrates that oxidation of MCTs does occur. However, when the total amount of fat oxidized by a preterm infant (range 1.3 to 1.5 g/kg/day, see Table 44-1) is calculated, it appears that an infant fed 160 mL/kg/day of a formula containing more than 20% of its fat as MCTs has either to oxidize all the MCTs and no other fat, or store some of the MCTs if longer-chain fatty acids are to be oxidized simultaneously.

Quantitation of MCT oxidation in preterm infants fed formula containing 40% of its fat content as MCTs has been determined by stable isotope tracer methodology with simultaneous determination of substrate utilization using indirect calorimetry.[41,42] The results of the energy expenditure, RQ, and fat oxidation as determined by indirect calorimetry and MCT oxidation as determined by isotopic measurement of expired CO_2 of the labeled octanoic acid are shown in Table 44-3. The MCT oxidation was more than 80% of the calories derived from fat oxidation, indicating a preferential utilization of C8 and C10 triglycerides. The high rate of utilization of these fatty acids is likely to be related to their fast absorption; they are the first fats available for oxidation after a meal. MCTs that are not oxidized can either be stored as such in adipose tissue or be elongated to longer-chain fatty acids. Sarda and associates[43] have reported C8:0, C10:0, and C12:0 storage in adipose tissue in association with MCT dietary intake in infants.

No difference has been observed in carbohydrate utilization by preterm infants fed formulas containing 38% MCTs compared with 6% MCTs in the fat blend. However, the glucose oxidation in infants fed the high MCT formula was reported to

TABLE 44-3

Energy and Fat Oxidation Values*

Component	DATA SOURCE	
	Putet et al (n = 7)	Sulkers et al (n = 8)
Intake (kg/day)		
Energy (kcal)	116	120
Total fat (g)	5.4	6.7
MCTs (g)	2.2	2.7
Other fat (g)	3.2	4.0
Oxidized (kg/day)		
Energy (kcal)	62	58
Total fat (g)	1.3	1.4
MCTs (g)	1.2	1.2
Other fat (g)	0.1	0.2

Data from Putet G, Thelin A, Philippossian G, et al: Medium-chain triglycerides as a source of energy in premature infants. In Horisberger M, Bracco U (eds): *Lipids in Modern Nutrition,* New York, Raven Press, 1987, pp 43-49; and Sulkers EJ, Lafeber HN, Sauer PJ: Quantitation of oxidation of medium-chain triglycerides in preterm infants, *Pediatr Res* 26:294, 1989.

*According to the oxidation rate of the labeled octanoic acid, 1.2 g/kg/day of the ingested medium-chain triglycerides (MCTs) is oxidized; because indirect calorimetry measurements give a total fat oxidation of 1.3 and 1.4 g/kg/day, the amount of long-chain fatty acids oxidized is assumed to be the difference (i.e., 0.1 and 0.2 g/kg/day).

TABLE 44-4

Difference in Intravenous Fat Oxidation Rates in Neonates (Preterm and Term) on Total Parenteral Nutrition at Two Different Levels of Energy Intake*

Total Nonprotein Energy Intake (kcal/kg/day)	Fat Intake (g/kg/day)	Energy Expenditure (kcal/kg/day)	Fat Oxidation (g/kg/day)	% Energy Expenditure Derived from Fat
102 ± 12 (n = 9)	4.6 ± 1.6	63 ± 12	1.4 ± 0.9	21
73 ± 4 (n = 8)	2.5 ± 0.5	52 ± 3	1.7 ± 0.5	30

*Note that an increase in fat intake (which accounts for almost 70% of the increase of the nonprotein energy intake) is not followed by an increase in fat oxidation rate.

Calculated from data of Putet G, Verellen G, Heim T, et al: Energy intake and substrate utilisation during total parenteral nutrition in newborn. In Wesdorp RIC, Soeters PB (eds): *Clinical nutrition '81,* Edinburgh, Churchill Livingstone, 1982, pp 63-70.

be significantly decreased,[44] suggesting that a larger portion of carbohydrate was used in the nonoxidative pathway (i.e., lipogenesis). MCTs have also been reported to effectively reduce polyunsaturated fatty acids and long-chain polyunsaturated fatty acids oxidation in enterally[45] and parenterally[46] fed preterm infants.

A consequence of MCT oxidation is an increased urinary excretion of dicarboxylic acids.[47] The clinical significance of this remains to be determined. No evidence, however, suggests that the presence of these oxidation products is harmful.

FAT AND TOTAL PARENTERAL NUTRITION

Intravenous fat emulsions have been given to newborn infants for several years. Some of the intravenously administered fats are used as a source of energy, as manifested by changes in the concentrations of FFAs and ketone bodies[48,49] and alterations in the RQ.[49-51] Even when lipoprotein lipase activity is impaired during the first days of life (especially in VLBW and small-for-gestational-age infants), the increase in ketone bodies[48] indicates that fat oxidation occurs during total parenteral nutrition. Currently it is recommended that intravenous fat be administered initially at a dose of 1 to 2 g/kg/day given continuously during 18 to 24 hours and increased as tolerated to a maximum of 3 g/kg/day.[52] Providing neonates approximately 30% of energy intake as lipids in total parenteral nutrition (TPN) regimens has been reported to improve their nitrogen utilization.[53,54]

Some evidence shows that oxidation of intravenous lipids may be correlated with total energy intake. The portion of energy expenditure derived from fat oxidation and the net amount of fat oxidized are reported to decrease with increasing energy (even as fat) intake.[54] (Table 44-4) This observation also correlates with data from adults, demonstrating that an increased oxidation rate of glucose is followed by a decreased oxidation rate of concomitantly infused lipids.[55] When an important part of energy intake is given as glucose, it is likely that glucose oxidation will impair utilization of lipid as a fuel during TPN. Similar observations have been made in enterally fed preterm infants[56] and in adults.[57]

The observation that an increase in fat intake is not followed by a higher rate of fat oxidation when total energy intake is high means that it may not be appropriate to increase the amount of intravenous lipid during total parenteral nutrition (at least when nonprotein energy intake exceeds 80 to 90 kcal/kg/day). When the total nonprotein energy intake is lower, it is possible that more fat will be oxidized and, therefore, more energy will be provided. Indeed, Rubecz and colleagues[51] have demonstrated a higher rate of fat oxidation when intravenous fat was the only energy provided, and in this experimental design 85% of the fat infused was oxidized.

EFFECTS OF QUALITY OF ENERGY (FAT VERSUS CARBOHYDRATE) ON PROTEIN ACCRETION AND SUBSTRATE OXIDATION

The effect of energy intake on protein utilization has been studied in animals,[58] human adults,[59,60] and human infants.[61-63] Although greater nitrogen retention with increasing energy intake has been reported in enterally fed[62,63] and parenterally fed[64] preterm infants, little is known about the differential effects of carbohydrate versus fat on substrate utilization in these infants. In adults substrate oxidation is dependent on the relative proportions of carbohydrate and fat in the diet.[57,65] Evidence shows that, calorie for calorie, carbohydrate used as an energy source is more effective than fat in supporting protein accretion.[59,66-68] Increased protein retention has been observed when carbohydrate is the primary energy source in adults with both enteral[65,66] and parenteral[67] diets and in preterm infants on enteral[69] and parenteral[68] diets. Although protein oxidation has been reported to decrease with increasing carbohydrate oxidation in preterm infants, no correlation was observed with fat oxidation.[69] Increasing carbohydrate intakes has been associated with increasing carbohydrate oxidation in adults[65,70] and in enterally[56] and parenterally fed infants.[71,72] Decreasing fat oxidation has also been associated with increasing carbohydrate intake.[56,57] Although the association of increasing fat intake with increasing fat oxidation has been reported by some investigators,[53,57] others have not observed this association.[54,73,74]

Because de novo lipogenesis from carbohydrate is an energy-consuming process, metabolic efficiency is improved if the nonprotein energy is provided as a mixture of carbohydrate and lipids rather than predominantly carbohydrate. Although the recommendation for enteral lipid intake of at least 30% of total energy intake has been made,[75] it is unclear if this intake would be adequate for neonates. Some evidence supports that preterm infants fed diets providing 35% of nonprotein energy as fat grow normally.[69] Various committees and expert panels have recommended that the total fat energy intake be 36% to 55% of the total energy intake for a preterm infant.[52,76-79]

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45

†*Dermot H. Williamson and Paul S. Thornton*

Ketone Body Production and Metabolism in the Fetus and Neonate

AVAILABILITY OF KETONE BODIES

Under physiologic conditions, the rate of utilization of ketone bodies is directly proportional to their concentration in the circulation, which in turn represents a balance between production (ketogenesis) by the liver and removal by peripheral tissues. Ketone bodies are excreted in the urine (ketonuria) when the renal threshold is exceeded. The relationship between production and utilization can be disturbed if the utilization of ketone bodies is inhibited by drugs,[1,2] with congenital absence of key enzymes required for ketone body utilization (e.g., 3-oxoacid–coenzyme A [CoA] transferase),[3] or in insulin-deficient states secondary to a metabolic defect in utilization.[4]

The concentration of ketone bodies in the blood is extremely sensitive to alterations in the physiologic state. *Normoketonemia* in humans has been defined as a blood ketone body concentration less than 0.2 mmol/L; *hyperketonemia,* greater than 0.2 mmol/L; and *ketoacidosis* (by analogy to the definition of lactic acidosis), greater than 7 mmol/L.[5] In adult humans, small but characteristic diurnal changes in blood ketone body concentrations have been noted.[6] Larger increases in concentration occur with fasting (in both humans and rats), with consumption of a high-fat diet, after exercise, in late pregnancy, and during suckling (Table 45-1). In pregnancy, blood ketone bodies will be available to the fetus in prevailing concentrations, because the placenta appears to be freely permeable to acetoacetate and 3-hydroxybutyrate.[7,8]

Ketonemia in Pregnancy

The major substrate of the mammalian fetus is considered to be glucose supplied by the mother.[8] However, the concentration of ketone bodies in maternal blood is increased in the last trimester of pregnancy,[5,7] and high concentrations are attained during delivery, particularly if it is prolonged (ketosis of labor).[7,8] This increase in blood ketone bodies may be related in part to the decrease in food intake around parturition, because hyperketonemia develops more rapidly with fasting in women in the second trimester of pregnancy than in nonpregnant control subjects.[9,10] Poorly controlled diabetes during pregnancy (including gestational diabetes) also is likely to lead to wide fluctuations in blood ketone bodies.[11]

Ketonemia in the Neonatal Period

During the suckling period, the neonate is presented with a relatively high-fat and low-carbohydrate diet. Both humans and rats have marked hyperketonemia in the early suckling period, compared with the respective adult fed values (see Table 45-1). By contrast, ketone bodies are not increased to the same extent in the blood of puppies,[12] piglets,[13] and lambs.[14] Nonesterified fatty acids (both long- and medium-chain) are the major precursors of ketone bodies, and a possible reason for the species differences in neonatal ketonemia may be the fat content of the maternal milk (rat milk contains 14.8 g fat/100 g; sheep milk contains 5.3 g fat/100 g). In addition, the brains of sheep and dogs have a low rate of ketone body utilization, so the demand for ketone bodies may be less than that in humans or rats.

The hyperketonemia of the neonatal period in humans is a physiologic event; therefore any change in blood ketone body concentration (decrease or increase) is likely to indicate a pathologic process. Increased ketone bodies may accompany the hypoglycemia associated with certain inborn errors of metabolism, and a decrease may occur in hyperinsulinism. It is therefore advisable to measure blood ketone bodies by a specific enzymatic method[15] in neonates presenting with hypoglycemia or any other abnormality in the concentration of circulating substrates.

†Deceased

TABLE 45-1

Range of Blood Ketone Body Concentrations in Humans and Rats

	KETONE BODY CONCENTRATION (mmol/l)*	
Clinical/Experimental Condition	**Human**	**Rat**
Fed normal diet	~0.1	Up to 0.3
Fed high-fat diet	Up to 3	4-5
Fasted: 12-24 hours	Up to 0.3	1-2
Fasted: 48-72 hours	2-3	2-3
Postexercise	Up to 2	Up to 2
Late pregnancy	Up to 1	Up to 0.3
Late pregnancy after 48-hour fast	4-6	6-15
Neonate: 0-1 days	0.2-0.5	0.2
Neonate: 5-10 days	0.7-1.0	0.5-1.1
Hypoglycemia	1.5-5	—
Untreated diabetes mellitus	Up to 25	Up to 10

*Concentrations of total ketone bodies (acetoacetate + 3-hydroxybutyrate measured by enzyme assay) in whole blood.

For example, infants born small for gestational age (SGA) have increased blood concentrations of gluconeogenic substrates, in association with decreased blood ketone body concentrations.[16] The latter may be a result of defective development of the ketogenic capacity of the liver.

REGULATION OF KETOGENESIS

Current understanding of the regulation of ketogenesis is based mainly on studies in rats—in particular, during the fed-to-starved transition, when a large increase (three- to four-fold) in ketogenesis occurs. In addition, a high proportion of the experimental evidence comes from work on perfused livers or isolated hepatocytes from adult rats. Consequently, in the review of regulation of ketogenesis in the adult presented in this section, comparison is made with the neonate or fetus whenever information is available. Further details are available in more comprehensive reviews.[17-21]

Extrahepatic Regulation

It is convenient to discuss the regulation of ketogenesis in two parts: extrahepatic and intrahepatic. The major precursors of ketone bodies in the postabsorptive state are long-chain fatty acids (LCFAs), and in all physiologic situations associated with hyperketonemia, including those of the neonatal period, the plasma concentrations of LCFAs are increased (see Table 45-1).[22-25] Extraction of LCFAs by the liver is concentration-dependent, and a direct relationship between their concentration and the rate of ketogenesis has been shown in rat and human.[26]

Adipose Tissue Lipolysis

In the adult, a key factor in determining the supply of LCFAs to the liver is their rate of release (lipolysis) from adipose tissue triacylglycerol stores. Lipolysis is initiated by activation of hormone-sensitive lipase; glucagon, epinephrine, norepinephrine, and thyroxine increase enzyme activity, whereas insulin (as well as some prostaglandins) has the opposite effect.[27] Isolated human adipocytes from neonates are very sensitive to the lipolytic effects of thyrotropin,[28] which increases in concentration immediately after birth and may therefore be involved in the regulation of lipolysis in the perinatal period. A decrease in blood glucose (e.g., in starvation, fat feeding) along with a concomitant decrease in plasma insulin leads to an increase in lipolysis and efflux of nonesterified fatty acids from adipose tissue. When carbohydrate is then supplied, the increases in glucose and insulin inhibit

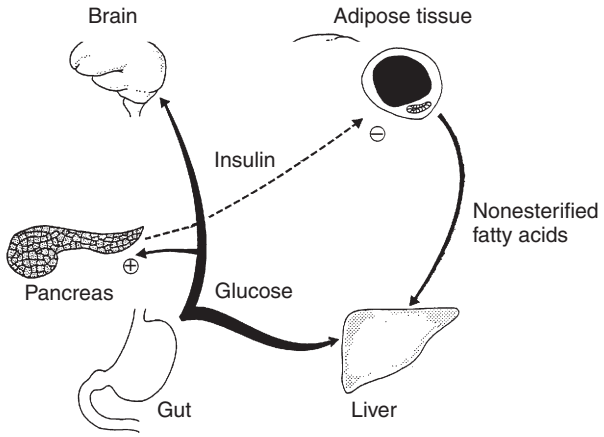


Figure 45-1 Intertissue fluxes in the fed state. Relative rates of flux are reflected in thickness of *arrows*.

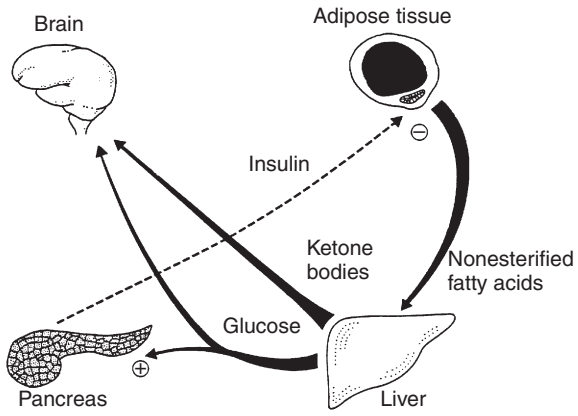


Figure 45-2 Intertissue fluxes in the starved state. Relative rates of flux are reflected in thickness of *arrows*.

lipolysis. The rate of ketogenesis can therefore respond in a reciprocal way to the availability of glucose in the circulation, so that an alternative fuel for the brain is provided when needed (Figures 45-1 and 45-2). During suckling in the rat, the plasma insulin-to-glucagon ratio is decreased, thereby favoring lipolysis.[29,30]

Ketone bodies apparently can regulate their own formation by feedback mechanisms on adipose tissue (Figure 45-3) to decrease lipolysis. Current evidence suggests two mechanisms: direct inhibition of lipolysis by ketone bodies and an indirect effect mediated by stimulation of insulin secretion[31,32] (see Figure 45-3). Work with the perfused rat pancreas suggests that ketone bodies increase insulin secretion only at concentrations above 1 mM.[33] These feedback mechanisms have not been investigated in neonates.

In the suckling neonate, another important source of precursors for ketogenesis is the medium-chain fatty acids (MCFAs) (12 or fewer carbons) of maternal milk.[34] These are in the form of triacylglycerols (one MCFA and two LCFAs per triacylglycerol) and are hydrolyzed by the action of lingual lipase. They are rapidly absorbed from the stomach into the portal venous system[35] and are therefore directly available to the liver, unlike LCFAs derived from milk lipids, which are transported as triacylglycerols (chylomicrons) by way of the lymphatic system and initially enter the peripheral circulation (Figure 45-4).

In the neonatal period, chylomicrons may provide a direct source of LCFAs for the liver, as suggested by the presence of lipoprotein lipase (responsible for the hydrolysis of triacylglycerols contained in chylomicrons to LCFAs) in neonatal rat liver[36] (see Figure 45-4). However, the total activity in liver constitutes

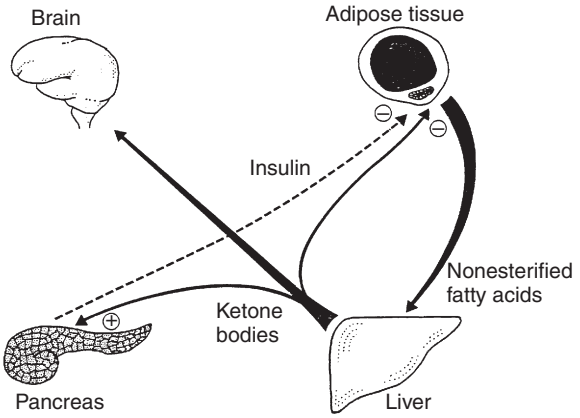


Figure 45-3 Role of ketone bodies as feedback regulators. Relative rates of flux are reflected in thickness of *arrows.*

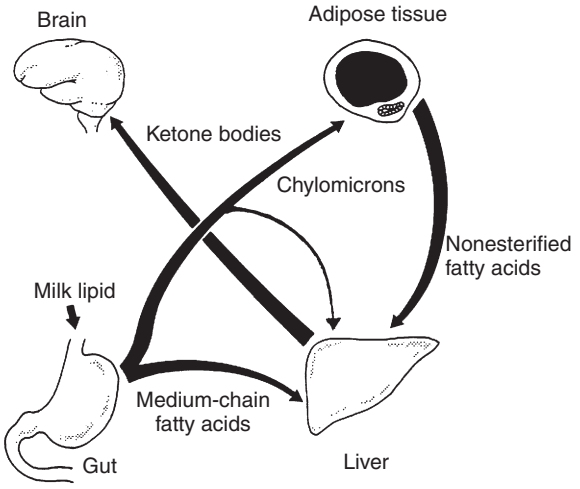


Figure 45-4 Intertissue fluxes in the suckling neonate. Relative rates of flux are reflected in thickness of *arrows.*

only a small percentage (3%) of that in the carcass,[34] presumably mainly muscle and adipose tissue.

Intrahepatic Regulation

It is generally considered that liver is the sole tissue capable of synthesis and release of ketone bodies to the circulation. However, ketone bodies can be formed by the intestinal mucosa of neonatal rats; this is due to the presence of an active hydroxymethylglutaryl (HMG)-CoA pathway (Figure 45-5) during suckling.[37,38] Research has shown that the expression of the key enzyme, HMG-CoA synthase, in intestinal mucosa is suppressed in weaning suckling rats.[39,40] The rate of intestinal ketogenesis is less than 10% of that in suckling liver, but this mechanism does provide an additional strategy for supplying ketone bodies to developing tissues.[34]

Pathway of Fatty Acid Catabolism

LCFAs derived from adipose tissue are transported in the plasma bound to albumin, cross the cell membrane as free fatty acids, and then bind again to cytosolic binding proteins.[32] Within the liver, the LCFAs either can be reesterified to form triacylglycerols (for subsequent secretion as very-low-density lipoprotein) and phospholipids or can enter the mitochondria by way of the carnitine acyltransferase system to undergo β-oxidation. The resultant acetyl-CoA can be converted to ketone bodies (acetoacetate and 3-hydroxybutyrate) by the (HMG-CoA pathway, or it can undergo complete oxidation in the tricarboxylic acid cycle (see Figure 45-5). By contrast, MCFAs are not converted to triacylglycerols in mammalian liver and can directly cross the inner mitochondrial membrane, thereby bypassing the carnitine acyltransferase system. Within the mitochondrial matrix, the MCFAs are converted to the corresponding acyl-CoA derivatives by acyl-CoA synthetases and then undergo β-oxidation (see Figure 45-5).

Changes in Neonatal Liver

As might be expected because of the relative impermeability of the placenta to LCFAs,[41] the concentration of the cytosolic fatty acid–binding protein is very low in fetal liver and gradually increases (approximately 20-fold) to reach adult values; evidence is lacking for a peak concentration of the protein in livers from suckling rats.[42]

Liver of fetal rats has a low capacity for fatty acid catabolism and ketogenesis, and this is rapidly increased after birth. The signals and mechanisms regulating the maturation of ketogenesis in the perinatal period are not completely understood[43] (as reviewed by Girard and colleagues[44]). Available evidence indicates that the capacity of the liver for LCFA catabolism is increased in the suckling period. A number of the key enzymes show higher activities,[45-47] particularly carnitine acyltransferase I [Enzyme Commission (EC) number 2.3.1.21], which increases at birth and attains values three to six times higher than those of the adult within a few days of parturition; it then declines rapidly on weaning.[47] These increases in enzyme activity[45-47] are paralleled by higher rates of ketogenesis from LCFAs in isolated hepatocytes from suckling rats than in cells from adult rats.[48,49]

The rate of ketogenesis from MCFAs is not increased in hepatocytes from suckling rats compared with that in cells from adult animals.[48] Presumably, then, the increased ketogenesis from LCFAs during the neonatal period is connected with either their esterification or entry into the mitochondria by way of the carnitine acyltransferase transport system.

Role of Malonyl Coenzyme A

In adult liver, carnitine acyltransferase I is regulated by short-term changes in the concentration of carnitine (a co-substrate) and malonyl coenzyme A (malonyl-CoA), which is a potent inhibitor of carnitine acyltransferase I.[17] Malonyl-CoA is a key intermediate in the conversion of carbohydrate into fat, and the hepatic concentration is directly correlated with the rate of lipogenesis (de novo fatty acid synthesis).[50] In liver, the major lipogenic precursor is pyruvate, formed from lactate returning to the liver as a product of glycolysis in peripheral tissues, or from hepatic glycogen through glycogenolysis and glycolysis (Figure 45-6). Therefore the rate of lipogenesis and the concentration of malonyl-CoA indicate the carbohydrate status of the liver: A high rate of lipogenesis is associated with an elevated malonyl-CoA, inhibition of carnitine acyltransferase I, and a decreased rate of ketogenesis. Conversely, a decrease in lipogenesis secondary to lack of substrate or hormonal inactivation of the key enzyme[51] acetyl-CoA carboxylase [EC 6.4.1.2] results in a decrease in malonyl-CoA and a stimulation of ketogenesis owing to increased entry of long-chain acyl-CoA into the mitochondria (see Figure 43-6). In addition, it appears that the sensitivity of carnitine acyltransferase I to inhibition by malonyl-CoA is affected by change in the physiologic state[52,53]; it decreases during suckling.[54,55]

In suckling liver, the rate of lipogenesis in isolated hepatocytes[48] and in vivo[56] is low, owing mainly to the decrease in the activities of key lipogenic enzymes (e.g., acetyl-CoA carboxylase,[57] fatty acid synthetase[58]); this pattern is rapidly reversed on weaning. It is therefore reasonable to assume that hepatic malonyl-CoA concentration is very low during suckling and cannot be altered acutely. This fact, together with the decreased sensitivity of carnitine acyltransferase I to inhibition by malonyl-CoA,[54,55]

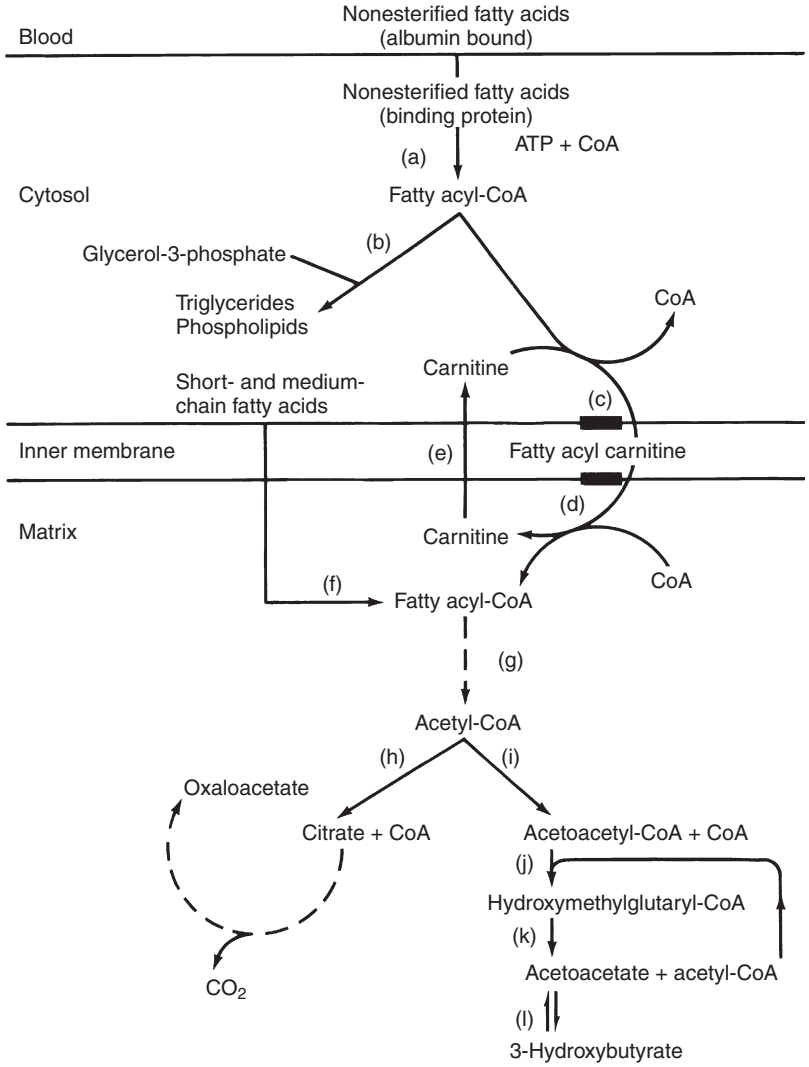


Figure 45-5 Pathway of fatty acid catabolism in liver. Enzymes involved: *(a)* long-chain fatty acyl-CoA synthetase; *(b)* glycerol-3-phosphate acyltransferase; *(c)* carnitine acyltransferase I; *(d)* carnitine acyltransferase II; *(e)* carnitine exchange; *(f)* short- and medium-chain fatty acyl-CoA synthetase; *(g)* fatty acid oxidation complex; *(h)* citrate synthase; *(i)* acetoacetyl-CoA thiolase; *(j)* hydroxymethylglutaryl-CoA synthase; *(k)* hydroxymethylglutaryl-CoA lyase; *(l)* hydroxybutyrate dehydrogenase. *ATP,* Adenosine triphosphate; *CoA,* coenzyme A.

suggests that in the suckling neonate, regulation of ketogenesis (once maturation has occurred) depends on both substrate supply and the increased capacity of the mitochondria for fatty acid catabolism, particularly the entry of long-chain acyl-CoA. Still to be determined is the nature of the signal or signals that bring about the stimulation of ketogenesis immediately after birth.[43,44,59] One possibility is the acute decrease in the insulin-glucagon ratio.[30,44]

Role of Insulin and Glucagon

The role of hormones in regulating the supply of LCFAs to the liver has already been discussed. It might be expected that insulin and glucagon also would act directly on the liver. Glucagon or dibutyryl cyclic adenine monophosphate (cAMP)—the so-called second messenger—stimulates ketogenesis from LCFAs in perfused livers[60,61] and isolated hepatocytes[48] from adult rats. Dibutyryl cAMP or glucagon does not increase ketogenesis from MCFAs,[62,63] suggesting that the site of action of glucagon is at the disposition of long-chain acyl-CoA between the pathways of esterification and β-oxidation (see Figure 45-6). A possible mechanism for the effects of glucagon on ketogenesis is suggested by the finding that the hormone (or cAMP) inhibits hepatic lipogenesis in vivo[64] and in vitro[64,65] and, consequently, decreases the concentration of malonyl-CoA.[50] The site of inhibition is acetyl-CoA carboxylase, which is inactivated by glucagon through increased phosphorylation of the protein.[51] Other sites of action of glucagon cannot be ruled out, however, because cAMP still stimulates ketogenesis and decreases esterification of LCFAs in isolated hepatocytes when an inhibitor of lipogenesis is present,[48] and malonyl-CoA presumably is low.

Decreased esterification of LCFAs by microsomal fractions isolated from livers of fed rats perfused with dibutyryl cAMP has been reported.[66] Activation of carnitine acyltransferase I by glucagon (or cAMP) has been described.[67] In hepatocytes from suckling rats, dibutyryl cAMP has no effect on ketogenesis.[48] This lack of effect may be a result of the elevation of cAMP in neonatal hepatocytes[8] and the consequent maximal stimulation of ketogenesis. Alternatively, it can be argued that because of the low rate of lipogenesis in neonatal hepatocytes, glucagon (cAMP) addition does not lead to a further decrease in the already low concentration of malonyl-CoA. Additional evidence that hepatocytes from neonatal rats are maximally stimulated is the finding that starvation of neonatal rats, in contrast with adult rats, does not further increase the rate of ketogenesis from LCFA in vitro.[49]

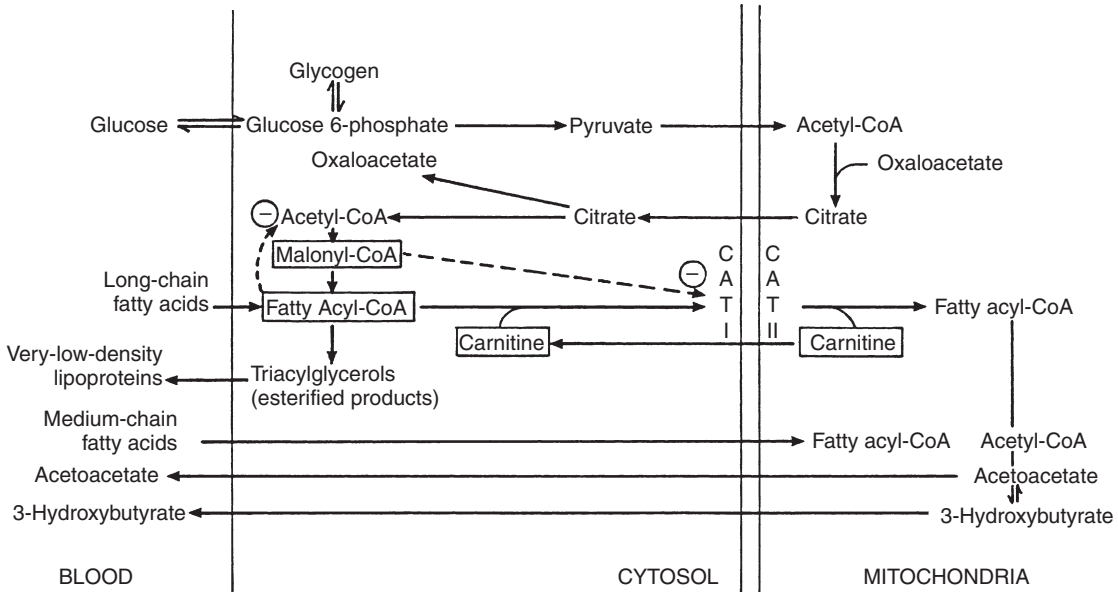


Figure 45-6 Interrelationship of hepatic carbohydrate metabolism, lipogenesis, and ketogenesis. *Circled minus signs* indicate sites of inhibition by metabolite. *CAT-I, CAT-II,* Carnitine acyltransferase I and II; *CoA,* coenzyme A.

By analogy to its potent antilipolytic effect on adipocytes, insulin might be expected to exert a direct antiketogenic effect on the liver. However, unlike with glucagon, demonstration of direct hepatic effects of insulin has proved comparatively elusive.[63] The general view is that in adult liver, insulin stimulates the de novo synthesis of fatty acids, in part by activation of acetyl-CoA carboxylase,[51] which in turn increases malonyl-CoA. It is therefore not surprising that in perfused livers of adult rats, insulin decreases the oxidation of LCFA and increases the rate of their esterification.[68] Investigation of the effects of insulin on LCFA catabolism in neonatal liver has been limited. Administration of insulin to suckling rat pups decreases blood ketone body concentrations without altering the concentration of plasma LCFAs, suggesting a direct antiketogenic effect of the hormone not mediated by a change in LCFA supply to the liver.[69] Livers of suckling rats[31] and human neonates[70] are resistant to the suppressive effects of insulin on glucose production, and this resistance also may extend to its effects on lipid metabolism. Of note, none of the putative direct hepatic actions of glucagon or insulin discussed earlier are likely to affect MCFA metabolism, which appears to be favored over LCFA metabolism in suckling liver.[71] This predilection may be important in the formulation of infant feedings.[72]

Thus a number of changes favor a high rate of ketogenesis in the suckling neonate (Table 45-2). All of these changes are reversed on weaning to a high-carbohydrate diet.

Fatty Acid Catabolism in Fetal Liver

LCFAs cannot readily cross the placental barrier,[41] and the capacity of fetal liver to oxidize them and form ketone bodies is very limited.[45,73] Ketone body formation by the liver in early human fetuses has been demonstrated.[74]

KETONE BODY UTILIZATION

With availability of sufficient ketone bodies in the circulation, their rate of utilization is to a large extent regulated by the prevailing concentration.[75] Transport of ketone bodies through the plasma membrane or mitochondrial inner membranes is not considered to limit their utilization in muscle, because the tissue concentration is linearly related to the plasma concentration.[76]

TABLE 45-2

Changes That Favor Ketogenesis in Suckling Rats

Factor	Change*
Plasma insulin[29,30,44]	Decreased
Plasma glucagon[29,30,44]	Increased
Insulin-glucagon ratio[29,30,4 4]	Decreased
Plasma LCFAs[24]	Increased
Hepatic carnitine acyltransferase I activity[47]	Increased
Sensitivity of carnitine acyltransferase I to malonyl-CoA inhibition[54,55]	Decreased
Hepatic [carnitine][143]	Increased
Hepatic lipogenesis[48,56]	Decreased
Hepatic [malonyl-CoA]	Decreased†

*Reported changes are relative to determinations in the adult fed rat, which has a low rate of ketogenesis.

†Malonyl-CoA has not been measured in neonatal rat liver, but a reasonable assumption is that it is low.

CoA, Coenzyme A; *LCFAs,* long-chain fatty acids.

Data from cited references.

This may not be true for brain, however, because the ketone body concentrations in rat brain[77] and human cerebrospinal fluid[78] are considerably less than those in blood in hyperketonemic states. Within the cell, the metabolism of ketone bodies depends on the activities of the "initiating" enzymes, which allow their entry into the metabolic pathways of the cell. The acetyl-CoA formed from ketone bodies either is completely oxidized in the tricarboxylic acid cycle to provide energy (adenosine triphosphate [ATP]) or is used as a precursor of fatty acid or sterol (cholesterol) synthesis. The pathways of ketone body utilization are shown in Figure 45-7. (A more detailed review of ketone body metabolism in peripheral tissues is available.[32])

Pathways of Ketone Body Utilization

Mitochondrial Pathway

As might be expected in view of its role as an energy-yielding substrate, the major site of ketone body utilization is the mitochondria. The initiating enzyme for acetoacetate metabolism is

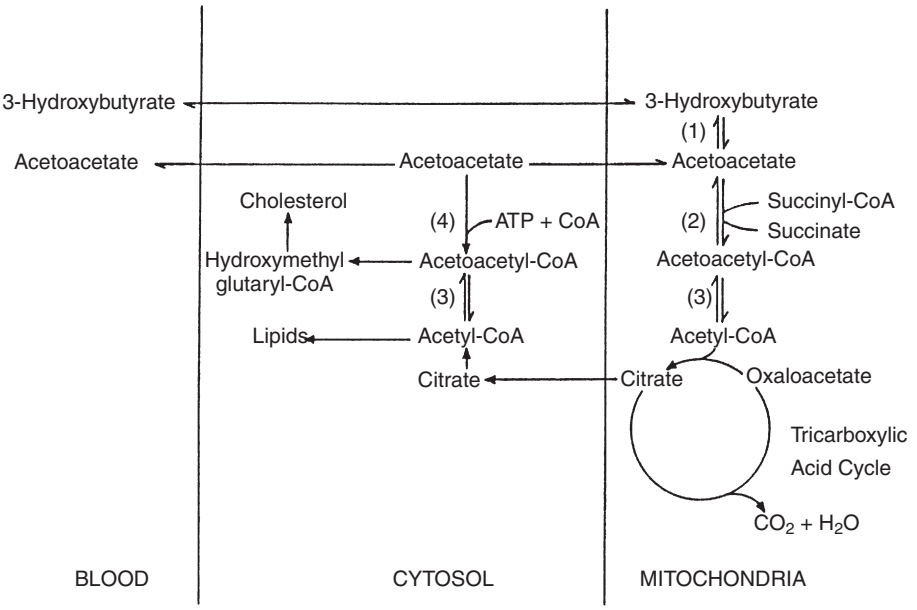


Figure 45-7 Pathways of ketone body utilization in peripheral tissues: *1,* hydroxybutyrate dehydrogenase; *2,* 3-oxoacid–CoA transferase; *3,* acetoacetyl–CoA thiolase; *4,* acetoacetyl–CoA synthetase. *ATP,* Adenosine triphosphate; *CoA,* coenzyme A.

3-oxoacid–CoA transferase [EC 2.8.3.5], which carries out the following reversible reaction:

$$\text{Acetoacetate} + \text{succinyl-CoA} \rightleftharpoons \text{acetoacetyl-CoA} + \text{succinate}$$

The succinyl-CoA is derived from the tricarboxylic acid cycle and as succinate is returned to the cycle; no loss of cycle intermediates occurs. The equilibrium of the reaction is in favor of acetoacetate formation. The K_m value (around 0.2 mM) of 3-oxoacid–CoA transferase for acetoacetate and its kinetic properties are similar in different rat tissues.[79] The transferase of rat tissues is inhibited by acetoacetate at concentrations in excess of 5 mM; this inhibition may depress ketone body utilization in severe hyperketonemic situations.[80]

The acetoacetyl-CoA formed is cleaved by acetoacetyl-CoA thiolase [EC 2.3.1.9] to yield two molecules of acetyl-CoA:

$$\text{Acetoacetyl-CoA} + \text{CoA} \rightleftharpoons 2 \text{ acetyl-CoA}$$

The equilibrium of this reaction is strongly in favor of acetyl-CoA formation, and when this reaction is coupled with that of the 3-oxoacid–CoA transferase, utilization of acetoacetate is favored. Acetoacetyl-CoA thiolase, unlike the transferase, is present in both the mitochondrial matrix and the cytosol.[81]

D(–)-3-Hydroxybutyrate is oxidized to acetoacetate by 3-hydroxybutyrate dehydrogenase [EC 1.1.1.30].

$$\text{D(–)-3-Hydroxybutyrate} + \text{NAD}^+ \rightleftharpoons \text{acetoacetate} + \text{NADH} + \text{H}^+$$

where NAD^+ is nicotinamide adenine dinucleotide and NADH is its reduced form. The reaction is reversible, but the equilibrium is in favor of acetoacetate reduction. The dehydrogenase is tightly bound to the inner mitochondrial membrane.

Unlike the hepatic HMG-CoA pathway for synthesis of ketone bodies (see Figure 45-5), the mitochondrial utilization pathway is freely reversible. So long as the cell contains sufficient activity of the three enzymes to catalyze near-equilibrium among the three reactions, the utilization of ketone bodies depends on the prevailing concentrations of 3-hydroxybutyrate and acetoacetate and the rate of removal of acetyl-CoA. The latter depends on the activity of the tricarboxylic acid cycle and on the rate of acetyl-CoA formation from other substrates, mainly, oxidation of fatty acids. This alternative acetyl-CoA source explains why it is possible to demonstrate net formation of ketone bodies in vitro when kidney slices are incubated with fatty acids.[82] Thus, in certain tissues, the free reversibility of the pathway can be viewed as means of "buffering" the mitochondrial acetyl-CoA pool. Of course, the concentrations of co-substrates (succinyl-CoA, succinate) and cofactors (CoA, NAD^+, NADH) also may influence the utilization of ketone bodies.

As already stated, the major role of the mitochondrial pathway is to allow complete oxidation of ketone bodies for energy supply. in tissues with active lipogenesis, however, a portion of the acetyl-CoA is transported to the cytosol in the form of citrate to act as a precursor for lipid synthesis (see Figure 45-7).

Cytosolic Pathway

The cytosol of all lipogenic tissues—brown and white adipose tissue, developing brain, lactating mammary gland, and liver—contains an enzyme, acetoacetyl-CoA synthetase, that converts acetoacetate into acetoacetyl-CoA[83-85]:

$$\text{Acetoacetate} + \text{ATP} + \text{CoA} \rightarrow \text{acetoacetyl-CoA} + \text{AMP} + \text{pyrophosphate}$$

This reaction is virtually irreversible, and its activity is, at most, 10% of that of the 3-oxoacid–CoA transferase. However, the enzyme has a high affinity (K_m = 50 μM) for acetoacetate and is therefore probably saturated with substrate even in the fed state.

The presence of 3-hydroxy-3-methylglutaryl-CoA synthase [EC.4.1.3.5] and acetoacetyl-CoA thiolase in the cytosol of the lipogenic tissues allows the acetoacetyl-CoA from acetoacetate to enter the pathway of sterol synthesis (see Figure 45-7). Experiments in suckling rat brain in vivo[86,87] and in adult rat liver in vitro[88] have yielded evidence for the preferential conversion of acetoacetate to sterols rather than lipids The activity of acetoacetyl–CoA synthetase is very low in suckling rat liver and increases on weaning.[84,85] Although of relatively low activity compared with that of the mitochondrial pathway, the cytosolic pathway allows the specific direction of acetoacetate to lipid or sterol synthesis (see Figure 45-7). The fact that the cytosolic pathway is irreversible means that the flux is unidirectional—which, together with the delivery of acetoacetyl-CoA and acetyl-CoA at the site of utilization, may be the physiologic advantage of this pathway over the mitochondrial pathway.

Utilization of L(+)-3-Hydroxybutyrate

It is generally accepted that D(−)-3-hydroxybutyrate, the isomer present in biologic fluids, is the physiologic substrate. However, the L(+) isomer also can be used by mammalian tissues,[88-91] although its presence in the circulation has not yet been demonstrated. A comprehensive study of the enzymes of L(+)-3-hydroxybutyrate metabolism in the adult rat has been reported.[92] In suckling rat brain, the L(+) isomer can be effectively used for synthesis of fatty acids and sterols.[91,92] These findings may be important when DL-3-hydroxybutyrate is administered to humans.

Acetone as a Gluconeogenic Precursor

Acetone is produced by decarboxylation of acetoacetate:

$$\text{Acetoacetate} \rightarrow \text{acetone} + CO_2$$

and it can reach high concentrations in blood in states of prolonged hyperketonemia (starvation, diabetic ketoacidosis). The rate of chemical decarboxylation is insufficient to explain the rate of acetone formation; therefore tissue acetoacetate decarboxylases have been implicated.[93] A rat kidney enzyme has been studied that has a K_m for acetone around 1 mM and is competitively inhibited by its substrate.[93]

Although early radioactive tracer experiments demonstrated that [2-^{14}C]-acetone can be converted to liver glycogen,[94] only recently has this mechanism been investigated in detail,[93,95] and net conversion of acetone to glucose has been measured in rat hepatocytes.[96] The pathways for acetone conversion to glucose are shown in Figure 45-8. Current evidence suggests that at low acetone concentrations (physiologic conditions), most of the acetone is converted to glucose, but that at high concentrations (hyperketonemia), the acetone is converted mainly to acetate[95] (see Figure 45-8). A study in human diabetic ketoacidosis with [2-^{14}C]acetone indicated that some 10% of the newly gained glucose carbon atoms were derived from acetone.[97] This rate of conversion of fat to carbohydrate (fatty acid oxidation → acetoacetate → acetone → glucose) is not insignificant and merits investigation in neonatal liver.

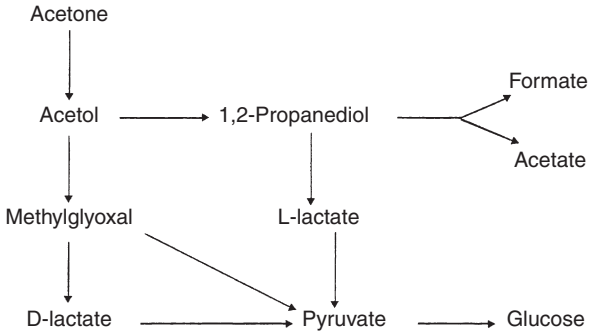


Figure 45-8 Pathways in the conversion of acetone to glucose.

Enzyme Activity

Adult Tissues

The relative activities of the enzymes of ketone body utilization in adult rat tissues are given in Table 45-3. The mitochondrial pathway is most active in kidney, heart, brown adipose tissue, and adrenal gland, followed by submaxillary gland, lactating mammary gland, and brain (see Table 45-3). The activities do not change appreciably during short-term fat feeding, starvation, or alloxan-induced diabetes.[22,98,99] By contrast, the cytosolic pathway enzyme acetoacetyl-CoA synthetase is more sensitive to alterations of hormonal and nutritional status.[83,85,100]

Neonatal Tissues

In contrast with those in the adult, the enzymes of ketone body utilization show marked changes in activity during development of the rat, and the pattern is tissue-specific. The activities of 3-hydroxybutyrate dehydrogenase and 3-oxoacid–transferase increase after birth to reach adult levels by approximately 5 days post partum and then continue to rise until just before weaning, when the values are two to three times higher than those in the adult.[22,24,47,101-103] Experiments indicate that the amount of 3-oxoacid–CoA transferase mRNA is three times higher in suckling rat brain than in fetal or adult brain.[104] Experiments with a modified milk formula low in fat have shown that the physiologic hyperketonemia in the neonatal period is not essential for the normal development of 3-oxoacid–CoA transferase in rat brain.[105]

By contrast, in the two adult tissues that have the highest activity of these enzymes, namely, kidney and heart (see Table 45-3), the activities remain below the adult values throughout the suckling period. If the changes in tissue mass during development also are taken into account, it is clear that neonatal brain is the major site for ketone body utilization.

The activities of the cytosolic pathway enzymes (see Figure 45-7) also are higher in neonatal rat brain.[84,106] These findings support their postulated role in lipogenesis (myelinization).

This pattern of increased capacity for utilization of ketone bodies in neonatal rat brain is not shown in other species. In neonatal pigs, the concentration of blood ketone bodies is low,[13,107] and the brain activity of 3-oxoacid–CoA transferase is barely detectable.[108] In contrast with the findings in rat, the transferase activity in neonatal pig heart and kidney is considerably higher than that in neonatal pig brain.[108] Similarly, the activities of 3-oxoacid–CoA transferase and 3-hydroxybutyrate dehydrogenase remain low throughout the neonatal period in guinea pig brain.[109] Data are lacking on the activities of the enzymes of ketone body utilization in human neonatal tissues. The available information derived from postmortem material suggests that in brain, the activities of the mitochondrial pathway enzymes remain comparatively constant throughout the postnatal period.[110,111]

Effects of Ketone Bodies on Glucose Metabolism

Glucose Utilization

It is well established that physiologic concentrations of acetoacetate and 3-hydroxybutyrate decrease glucose utilization in a number of adult rat tissues, including heart, soleus muscle, kidney, brain, and lactating mammary gland.[32] These tissues all exhibit high rates of glycolysis and utilization of the pyruvate formed from glucose-6-phosphate. Consequently, if glucose metabolism was not inhibited in situations associated with carbohydrate lack, the animal would rapidly become hypoglycemic. The mechanism for the inhibition of glucose metabolism by ketone bodies was established in studies by Randle and colleagues[112] and involves inhibition of the enzymes phosphofructokinase [EC 2.7.1.11] and hexokinase [EC 2.7.1.1] and inactivation of pyruvate dehydrogenase [EC 1.2.4.1]. The overall effect is decreased glucose utilization. In addition, a higher proportion of any glucose that still undergoes glycolysis leaves the tissue as lactate and pyruvate and returns to the liver for gluconeogenesis. No systematic investigation of this "sparing" of glucose by ketone bodies appears to have been made in neonatal tissues. In normal children, however, an inverse correlation has been noted between glucose flux and the degree of ketonemia.[113] This observation supports the view that glucose and ketone body interactions operate at the whole-body level.

Glucose Production

Ketone bodies can alter hepatic glucose production by their effects on the supply of two important precursors, glycerol and alanine. Ketone bodies decrease flux of glycerol to the liver by

TABLE 45-3

Relative Activities of Enzymes of Ketone Body Utilization in Adult Rat Tissues

Tissue	RELATIVE ENZYME ACTIVITY*			
	3-Oxoacid-CoA Transferase	Acetoacetyl-CoA Thiolase	3-Hydroxybutyrate Dehydrogenase	Acetoacetyl-CoA Synthetase
Adipose tissue—white	0.14	0.05	—	1.0
Adipose tissue—brown	6.9	10.0	—	—
Adrenal gland	2.7	14.8	4.2	—
Brain	1.0	1.0	1.0	1.0
Heart	12.4	4.2	4.2	—
Kidney	12.4	6.9	6.7	—
Liver	0.2	12.9	20.7	6.7†
Lung	0.8	0.3	—	—
Mammary gland	2.7	1.2	1.3	10
Muscle (hind limb)	0.4	0.3	1.0	—
Submaxillary gland	3.2	1.1	—	—

*The respective enzyme activities (in mol/min per g fresh wt. at 25° C) for adult rat brain were set at unity (transferase: 2.1; thiolase: 2.1; dehydrogenase: 0.6; synthetase: 0.01). For further details, see references 32, 141, 142, 144.

†Higher values are reported during dark phase.[48]

their antilipolytic action on adipose tissue and apparently also on muscle triacylglycerol stores.[114] Hypoalaninemia is present in a number of conditions associated with hyperketonemia, including starvation, diabetes, and ketotic hypoglycemia of childhood, and can be induced by infusion of 3-hydroxybutyrate.[115] The mechanism by which ketone bodies decrease muscle release of alanine has not yet been established. Two possibilities are recognized: (1) inhibition of muscle glycolysis decreases pyruvate availability to form alanine by transamination or (2) a direct inhibition of muscle proteolysis.

Roles of Ketone Bodies in Neonatal Tissues

Only three neonatal tissues of the rat—brain, lung, and brown adipose tissue—have been examined in any detail with regard to ketone body metabolism. The findings are briefly discussed next.

Brain

Arteriovenous difference measurements across the brain of adult and suckling rats have shown that for a given arterial concentration of ketone bodies, the extraction by suckling brain is three to four times higher than by adult brain.[77,116] This finding is in agreement with the higher activities of the enzymes of ketone body utilization in suckling brain. A similar increase in ketone body extraction by brain has been demonstrated when human neonates and adults have been compared.[117,118] However, no appreciable increase in the activities of the enzymes of ketone body utilization has been demonstrated in human neonatal brain.[110,118] Therefore the increased utilization in the human neonatal brain is likely to be due to another factor. A possibility is increased cerebral permeability to ketone bodies, as demonstrated in neonatal rat brain.[119]

The major portion (90%) of the ketone bodies used by suckling brain is likely to be completely oxidized to provide energy,[120] thereby sparing glucose. However, it is now well established that a portion of the carbon (5% to 10%) is converted to fatty acids and cholesterol.[86,106,111,120,121]

Ketone bodies inhibit glucose utilization in a number of rat tissues, but such inhibition does not occur in brain.[122,123] They do, however, decrease glucose oxidation and its conversion to lipid,[122,124] suggesting inhibition of pyruvate dehydrogenase. Support for this hypothesis is the correlation between ketone body extraction by the brain and the proportion of extracted glucose released as lactate and pyruvate in rat[123] and humans.[125]

A point worthy of emphasis is that measurements of arteriovenous differences or enzyme activities in whole brain give no information on the regional utilization of ketone bodies within the brain. This question has been addressed in some elegant experiments,[126] and it appears that the telencephalon uses the most ketone bodies and the hind brain the least.[126,127]

Lung

Like neonatal brain, the developing lung has a specific requirement for the synthesis of complex lipids, that is, pulmonary surfactant. In vivo synthesis of lipids is more rapid from acetoacetate than from glucose, and the incorporation of ketone bodies appears to occur through direct generation of cytosolic acetyl-CoA.[128] The presence of cytoplasmic acetoacetyl-CoA synthetase and acetoacetyl-CoA thiolase and their developmental pattern, which parallels that of acetoacetate incorporation into fatty acids, indicates that the cytoplasmic pathway (see Figure 45-7) is as important in this tissue in the immediate postnatal period[129] as it is in developing brain.

Fetal Tissues

As already discussed, the fetus is exposed to the concentrations of ketone bodies present in maternal blood. The utilization of ketone bodies as oxidizable substrates has been demonstrated in the fetal human brain[130] and in several tissues of the fetal rat,[131] including liver.[132] The interpretation of these findings is not clear, however, because in some experiments, the tracer used was DL-[3-^{14}C]hydroxybutyrate, so the metabolism of the nonphysiologic L isomer may have made a contribution to $^{14}CO_2$ production. Ketone bodies also can act as lipogenic substrates in the fetus.[133] Injection of D-[3-^{14}C]hydroxybutyrate into the femoral vein of pregnant rats in late gestation results in significant incorporation into the lipids of a number of tissues,[133] the greatest relative incorporation on a per gram basis being into brown adipose tissue.

Ketone Bodies and the Embryo

In contrast with fetal and neonatal tissues, the rates of $^{14}CO_2$ production by mouse conceptuses in vitro from DL-[3-^{14}C]hydroxybutyrate are higher than those from the D isomer, suggesting that utilization of the L isomer is greater.[134] Of some concern is the finding that DL-3-hydroxybutyrate has a teratogenic effect on rodent embryos in culture[135-137]; the malformations are similar to those observed in offspring of human diabetics.[138] One potential pathogenic mechanism appears to be inhibition of the

oxidative pentose phosphate pathway of glucose metabolism,[139] which supplies ribose moieties for pyrimidine synthesis. It may be relevant that DL-3-hydroxybutyrate also inhibits the synthesis of pyrimidines in fetal brain slices.[139] Ribose supplementation will maintain the rate of DNA synthesis in embryos exposed to DL-3-hydroxybutyrate,[140] which is consistent with the proposed mechanism of action of the hydroxybutyrate.

It thus appears that increased maternal blood concentrations of ketone bodies in the first weeks of pregnancy secondary to malnutrition or insulin deficiency (diabetes mellitus) may lead to malformations in the embryo and subsequent abortion. This sequence of events can be viewed as a mechanism for terminating pregnancy in adverse conditions.

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Carol L. Cheatham, John Colombo, and Susan E. Carlson

Long-Chain Fatty Acids in the Developing Retina and Brain

The brain and retina contain large quantities of long-chain (20 and 22 carbon) n-3 and n-6 polyunsaturated fatty acids (LCPUFAs), particularly docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (AA; 20:4n-6).[1,2] During the last intrauterine trimester[3,4] and the first 18 months of postnatal life, DHA and AA accumulate in neural tissue at a high rate supported by selective placental transfer of DHA and AA from mother to fetus[5,6]; transfer from human milk consumption to breast-fed infants[7,8]; and synthesis from the dietary essential fatty acids, α-linolenic acid (18:3n-3), and linoleic acid (18:2n-6),[9-12] respectively. In 2002, DHA and AA were added to infant formulas in the United States. (For a more extensive treatment of these topics, see Chapter 41[13] and Chapter 42.[14])

In animal studies, diets deficient in α-linolenic acid result in lower brain DHA level and a reciprocal increase in docosapentaenoic acid (DPA; 22:5n-6 or n-6 DPA) (Figure 46-1),[15] which has been related to atypical retinal physiology[16-18] and development of visual acuity,[19] as well as atypical learning abilities.[20-28] As first observed in the late 1970s and now well established, infants fed formulas without DHA have lower levels of DHA in red blood cell and plasma lipids than do breast-fed infants.[7,8] Even though early infant formulas contained essential fatty acids, these formulas lacked DHA or AA. It was hypothesized that lower DHA in the lipid profiles of formula-fed infants could indicate limited DHA accumulation in the brain and retina, thereby resulting in less than optimal sensory and cognitive function.

Randomized studies of DHA supplementation in infants were necessary to determine whether functional outcomes such as visual acuity could be improved by adding DHA to infant formula. The first small studies focused on infants born preterm because they were considered more likely than infants born at term to need supplemental dietary DHA; that is, they are born before maternofetal DHA transfer is complete[3,4] and before lactation is possible. The first preliminary reports of higher visual acuity[29] and more mature retinal physiology[30] as a function of supplementation were followed by full reports in 1992 and 1993.[31,32]

After Makrides and associates[33] reported an association between higher DHA status and visual acuity in breast-fed infants born at term, the first randomized trials in preterm infants were conducted to determine the potential benefits of adding DHA to all infant formulas.[34,35] Subsequently researchers have conducted a number of randomized trials to compare the growth and development of term and preterm infants fed formulas with varying levels of DHA and sources of DHA, as well as of infants born to mothers who received DHA supplementation during pregnancy or lactation. These randomized trials have been reviewed and reported elsewhere.[36-41]

Dietary sources of AA were not available for addition to infant formulas when the first randomized studies were conducted; when early studies showed slower growth in infants born preterm who were fed DHA and not AA,[42,43] it was speculated that this could be due to an imbalance of the n-3 and n-6 LCPUFA. On the basis of findings that AA is important for typical growth,[44] that dietary n-3 LCPUFA reduces red blood cell phospholipid AA,[45] and that the concentration of AA in circulating lipids is related to size at birth[46] and first-year growth achievement,[47] subsequent study formulas have included AA and DHA to provide a balance of n-3 and n-6 LCPUFA. A review of the randomized studies in which growth was assessed, however, failed to reveal any physiologically relevant difference between the growth rates of infants fed formulas containing both DHA and AA and growth rates of infants fed control formulas.[48] Nonetheless, it can be argued that AA should be a component of early milk because it is present in all human milk. As well, AA is a major component of brain phospholipids, which accumulate rapidly after birth.[4]

Both DHA and AA were added to commercially available term and preterm formulas in the United States in 2002 after the Food and Drug Administration gave generally recognized as safe (GRAS) status to specific single-cell oil sources of DHA and AA *(Federal Register).* After the addition of DHA and AA to infant formula, research in this area has focused on questions of the proper timing of supplementation for effective accumulation of DHA and the optimal level of supplementation. Work on animal models has continued to focus on the mechanisms in the retina and brain by which DHA influences functional performance.

HISTORICAL PERSPECTIVE

DHA constitutes 30% to 45% of the total fatty acids in some phospholipid classes (specifically ethanolamine and serine phosphoglycerides) in brain gray matter and the retina, respectively. In the

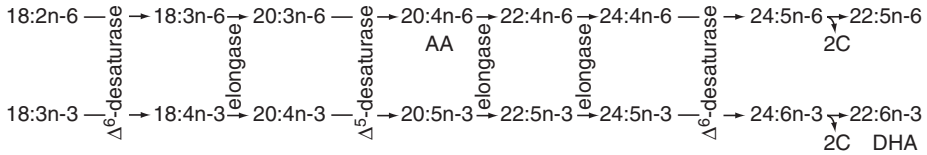


Figure 46-1 Elongation and desaturation of the essential fatty acids linoleic acid (18:2n-6) and linolenic acid (18:3n-3).

brain, DHA is especially concentrated in membranes surrounding synapses.[1] In the retina, DHA is concentrated in the synaptic regions and in the disk membranes of photoreceptors.[2] During the last intrauterine trimester, brain DHA increases exponentially and AA decreases linearly, such that the ratio of DHA to AA in the retina doubles between 24 and 40 weeks of gestation and increases further after birth.[49]

Sanders and Naismith[7] and Putnam and colleagues[8] were the first to show, in infants born at term, that those who were formula fed had lower erythrocyte phospholipid DHA and AA levels compared with breast-fed infants. Infants fed a formula that contained 5% of total fatty acids as α-linolenic acid had similar amounts of DHA in red blood cell phospholipids when compared with infants fed a formula with 1.2% α-linolenic acid, suggesting that α-linolenic acid intake was not the cause of lower levels of circulating DHA.[8] This observation was consistent with the observation of Sinclair and Crawford[50,51] that DHA and AA were more efficiently incorporated into rat brain than were their essential fatty acid precursors.

A study of rats fed an α-linolenic acid–deficient diet during development demonstrated that lower DHA accumulation in the brain was reflected in lower red blood cell phospholipid DHA.[52] From the human and rat studies,[7,8,52] it was inferred that DHA intake was the most likely reason for higher red blood cell DHA in breast-fed compared with formula-fed infants and that it was reasonable to conclude that red blood cell DHA accurately reflected brain DHA accumulation.

Clandinin and colleagues[3] first demonstrated that fetal brain DHA and AA accumulate during the third intrauterine trimester and that this accumulation continued after birth. A later report by Martinez[4] confirmed this finding of rapid postnatal DHA accumulation with a larger sample. Infants born preterm could miss as many as 18 weeks of intrauterine DHA transfer. Formula-fed compared with human milk–fed infants born preterm also had lower levels of erythrocyte phospholipid DHA and AA.[53] This finding was similar to α-linolenic acid–deficient rodents and nonhuman primates that exhibited behavioral effects of deficiency. It was hypothesized that formulas containing DHA relative to formulas containing α-linolenic acid as the only n-3 fatty acid could improve early visual and neural development of infants born at the beginning of the last trimester. As will be detailed, the experimental human studies compared outcomes in infants fed formulas that contained α-linolenic acid with or without a source of DHA. Later, infant and child development was studied for the effects of providing variable amounts of DHA during fetal or postnatal life by experimentally altering maternal DHA intake.

FACTORS INFLUENCING ACCUMULATION OF LONG-CHAIN POLYUNSATURATED FATTY ACIDS DURING DEVELOPMENT

The timing of brain fatty acid accumulation varies considerably among mammalian species. Humans can receive a preformed supply of DHA from the mother both before and after birth,[7,8,53-56] whereas guinea pigs receive most maternal LCPUFA during fetal life[57] and rats receive most maternal LCPUFA after birth.[50] Researchers have used these differences to create models for studying the temporal effects of low brain DHA levels on brain development. For example, newborn rats may be a model for human DHA accretion beginning with the third intrauterine trimester, whereas in newborn pigs postnatal DHA accretion resembles that in postnatal humans. We will describe the processes through which LCPUFA is accumulated by the body. Research in this area is necessarily reliant on animal models, and it is with caution that we extend results from the animal literature to the human.

Maternal Influences on Accumulation of Arachidonic Acid and Docosahexaenoic Acid in the Fetus and Infant

Arbuckle and Innis[58] found that feeding fish oil to pigs increased DHA levels in sow milk, as well as in the tissues of piglets. Connor and colleagues[59] and Harris and associates[60] published the first direct evidence in humans that consumption of fish oil by lactating women increased the amount of postnatal DHA transfer to their infants in milk and increased the DHA status of their infants: fish oil consumption by breast-feeding women increased erythrocyte and plasma phospholipid DHA in their offspring.

Researchers have shown that maternal DHA status is highly variable during pregnancy and lactation,[61-66] and maternal DHA status is related to the DHA status of the infant.[61,62,65,67] Two independent research groups showed that infants fed human milk compared with infants fed formulas without DHA had more brain DHA at autopsy.[68,69] Other observational studies associated higher maternal DHA status during pregnancy with higher infant development status.[33,70-73] This evidence suggests that prenatal and postnatal transfer of DHA can influence infant brain biochemistry and subsequent development.

Indeed, experimental studies have shown that maternal DHA status during pregnancy or lactation can enhance development.[74,75] Jensen and colleagues[74] randomly assigned women to consume DHA as single-cell oil during lactation. The toddlers of the women who received supplements had higher scores on a standardized test of motor function at 30 months of age. Similarly, Helland and colleagues[75] randomly assigned women to consume DHA in the form of fish oil with the goal of increasing DHA during pregnancy and lactation. They reported a higher intelligence quotient (IQ) at 4 years of age. Notably, no accelerated development was evident in either the Helland or Jensen samples during infancy (for discussion see Specific Measures of Cognitive Development section later in this chapter).[76,77]

Pregnancy itself alters the amount of DHA in circulating blood lipids and red blood cell phospholipids. Otto and colleagues[63] observed that phospholipid DHA in maternal plasma and red blood cells increased in early pregnancy, regardless of the initial levels of red blood cell DHA. These data suggest that DHA is mobilized during pregnancy for transfer to the fetus. However, the same group of investigators reported increased maternal n-6 DPA relative to DHA, from which they concluded that pregnant women's systems lag behind with respect to DHA production and transfer to the fetus.[64] Van Houwelingen and colleagues[78] observed similar relative increases in the ratio of n-6 DPA to DHA in infants born preterm, providing further evidence that an increase in n-6 DPA/DHA may be an indicator of inadequate DHA synthesis or accumulation. Thus, even though DHA is mobilized during pregnancy, it may not be sufficient for fetal development.

Several factors affect the amount of DHA available for mobilization. In a comparative study in pregnant women from the Netherlands, Hungary, Finland, England, and Ecuador, large differences were found in the percentage of fatty acids present in the blood as DHA.[61] These national differences most likely reflect differences in dietary DHA intake. The number of prior pregnancies has been shown to be inversely related to maternal DHA as well.[55] To our knowledge, no work has been undertaken to explore maternal DHA levels in women carrying multiple fetuses, but one might predict that multiple births would reduce maternal DHA transfer to each fetus.

Just as a woman's DHA status during pregnancy affects transfer of DHA to her fetus, a woman's DHA status also influences DHA transfer to her newborn in human milk. DHA content of human milk is quite variable within and among populations. The extremes of milk DHA that have been reported are, as a percentage of total fatty acids, 0.02% in vegans[79] and 2.4% in women from China who consume a diet high in fish.[80] Human milk DHA in most populations falls between 0.1% and 0.5% of total fatty acids.[55,56] In general, DHA content of human milk in the United States is within this range.[7,81-83] In a case study, the DHA content in the milk of a given woman consuming an American diet was relatively stable across two periods of lactation 9 months apart.[84] However, in a study in 12 fish-eating women, it was shown that DHA content in breast milk can vary 10-fold from day to day and is directly related to the timing of fish consumption.[85] As has been observed in other nutrients, some day-to-day variation would be expected. However, it is unlikely that such variability would be sufficient to have much impact on infant status, particularly among women whose DHA consumption is low.

As with DHA, the reported average amount of AA in human milk encompasses a wide range among cultural groups, from 0.2% to 1.2%.[83] However, the average AA in the milk of most populations falls within the range of 0.4% to 0.6%, and the linoleic acid content ranges from 10% to 17% of total fatty acids.[55,56,83]

Synthesis as a Source of Long-Chain Polyunsaturated Fatty Acids

Infants who do not receive dietary DHA must synthesize their DHA from α-linolenic acid (see Figure 46-1). Many clinical studies have asked whether infants who consume α-linolenic acid are able to accumulate enough brain DHA for optimal function or whether they would benefit from receiving additional dietary DHA. In a later section, the evidence of functional benefits of DHA is discussed. This section provides a brief review of DHA synthesis and the relative role that synthesis plays in tissue accumulation of DHA.

Voss and colleagues[86] followed stable isotope–labeled fatty acids and demonstrated that the likely pathway for conversion of α-linolenic acid to DHA involves two successive additions of two-carbon units to 20:5n-3 (20:5n-3 to 22:5n-3 to 24:5n-3) followed by a δ6-desaturation (24:5n-3 to 24:6n-3) and the subsequent removal of a two-carbon segment (24:6n-3 to DHA or 22:6n-3) (see Figure 46-1). Synthesis of DHA from 22:5n-3 by frog retinal pigment epithelium also generated 24:5n-3 and 24:6n-3.[87] The final step in synthesis of DHA occurs in peroxisomes. Important supporting evidence of the importance of peroxisomes in the supply of DHA comes from Martinez's studies in patients with peroxisomal disorders.[88,89]

DHA can be synthesized from α-linolenic acid by the liver, retina, and brain.[87,90-94] Synthesis in the retina has been localized to the retinal pigment epithelium,[87] and synthesis in the brain has been localized to astrocytes and cerebral endothelium.[93-95] Retinal photoreceptor cells and neurons do not synthesize DHA.[92-94] Chen and Anderson[96] found that DHA was preferentially incorporated into triglycerides in retinal pigment epithelium. Their observation suggests a role for triglycerides in the DHA enrichment of the retina or recycling of photoreceptor DHA between the retina and the retinal pigment epithelium. Electron microscopic studies of retinal DHA accumulation suggest that DHA is stored in lipid droplets of the pigment epithelium and is transferred to photoreceptors.[97] Moore and co-workers[93] suggested an analogous model for uptake of DHA by neurons. Either preformed DHA or n-3 fatty acid precursors of DHA could be taken up by the cerebral endothelium and incorporated into astrocytes, which could synthesize DHA and transfer it as well as preformed DHA to neurons. Innis and Dyer[95] showed that astrocytes release fatty acids in cholesterol esters.

Uauy and associates[30] generated early evidence that infants born preterm had the capacity to synthesize DHA from α-linolenic acid. These researchers showed that infants born preterm and fed formulas containing soybean oil had higher erythrocyte DHA levels than those fed formulas containing corn oil (a fat with much less α-linolenic acid than soybean oil). An early study showed δ6- and δ5-desaturase activity in the human liver at midgestation, suggesting that the fetus could metabolize essential fatty acids to more polyunsaturated forms.[98] Stable isotope studies in human infants born preterm and at term demonstrated whole body synthesis of DHA,[9-12] as well as high variability among infants.[11]

Only recently have stable isotope studies in humans permitted quantification of DHA accumulation from new synthesis relative to preformed DHA.[99] Using isotope ratios, Carnielli and colleagues[100] determined that whole body DHA synthesis of 1-month-old preterm infants was only 12.6 mg × kg^{-1} × day^{-1} of DHA, and DHA synthesis fell dramatically with time reaching only 3.2 mg × kg^{-1} × day^{-1} at 3 months of age. Not surprisingly, synthesis was also reduced by DHA feeding. Studies with nonhuman primate neonates are helpful for assessing the relative contribution to tissue DHA of newly synthesized versus preformed DHA. Studies with neonatal monkeys suggested that the amount of DHA derived from synthesis was relatively minor compared with the amount received from mother's milk.[101] Newborn baboons synthesized DHA from α-linolenic acid[102]; however, preformed DHA was eight times more likely than α-linolenic acid to accumulate in the brain. It has been estimated that the third-trimester fetus accumulates approximately 40 to 60 mg × kg^{-1} × day^{-1} of n-3 LCPUFA.[103] On the basis of the results of Carnielli and colleagues,[100] it is unlikely that synthesis of DHA from α-linolenic acid can meet the neonate's need for optimal brain DHA accumulation.

Although pigs[104,105] and possibly other species may have different relative capacities for synthesis of DHA compared with humans, studies with animals are valuable as a means of understanding the relative effects of different tissues and physiologic effects on DHA synthesis. In the rat, the activity of hepatic δ6- and δ5-desaturases falls after birth and then peaks after weaning, and brain activities are also maximal at birth and fall steadily through the first several weeks of life when animals are suckled.[106] Rat milk, like all mammalian milk, contains DHA. These changes in enzyme activity suggest a classic feedback inhibition of DHA on LCPUFA biosynthesis. The mouse exhibits a postnatal decline in brain δ6-desaturase activity similar to that in the rat.[107] After 3 months of age, mouse brain activity of this enzyme is extremely low, but hepatic activity remains fairly high through 9 months of age and then decreases dramatically at later ages.[107] In the fetal pig, δ5-desaturase activity increases by seven-fold in the brain and by 23-fold in the liver during the last half of gestation.[104,105]

Effects of Dietary Influences on Accumulation of Long-Chain Polyunsaturated Fatty Acids

From fatty acid analyses, it is known that diets deficient in α-linolenic acid decrease tissue DHA and increase n-6 DPA. Because of the recognized importance of retinal and neural DHA accumulation, most recent infant formulas in the United States

have contained a ratio of linoleic to α-linolenic acid of less than 10 and have provided at least 1.5% of total fatty acids (0.75% energy) as α-linolenic acid. Now that DHA and AA have been added to infant formulas in the United States, it may be time to reassess the high amount of linoleic acid and α-linolenic acid in some formulas. High levels of 24:2 have been reported to replace 24:1 in red blood cell sphingomyelin, especially in infants born preterm.[108] Transfer of essential fatty acids directly to the fetus during gestation appears to be limited, on the basis of low linoleic acid levels in circulating phospholipids of cord blood.[53] Maternal linoleic acid intake was inversely related to infant head circumference in one published study.[65]

A diet containing α-linolenic acid does not compensate for the DHA accumulation that occurs in red blood cells, plasma, and the brain of infants fed even small amounts of DHA.[32,45,68,69] In the months after birth, infants fed formulas that contain α-linolenic acid but not DHA have progressively lower amounts of circulating DHA.[45] Infants fed formulas with DHA have higher concentrations of red blood cell phospholipid DHA, an indicator of infant DHA status, than infants fed formulas with more than 0.75% energy from α-linolenic acid.[32,45]

Breast-fed human infants have higher brain,[68,69] but not retinal,[69] DHA accumulation compared with infants who were fed formulas without DHA. A functional study by Uauy and coworkers[109] found normal electroretinogram responses in infants at 4 months corrected age who were born preterm, even though they had been fed a formula low in α-linolenic acid from birth. Thus, although diets deficient in α-linolenic acid lead to reduced retinal DHA in monkeys[110] and piglets,[111] it appears that formulas that contain α-linolenic acid and no DHA may provide for sufficient retinal DHA.[112]

The many remaining questions include whether brain DHA in formula-fed infants eventually catches up to that of milk-fed animals, whether there is a critical period for accumulating retinal and brain DHA, whether there is a threshold of DHA accumulation in retina and brain for optimal function, and whether there is a level of DHA intake that might be harmful to the developing infant. In addition, it remains uncertain whether any of the answers to these questions interacts with gestational age, size at birth, or other maternal or infant factors.

EFFECTS OF ESSENTIAL FATTY ACID DEFICIENCY ON BEHAVIOR

The kinds of behavior in animals subjected to toxic insult or nutrient variation during development can provide useful information about affected behavioral domains, which, in turn, reflect neural function. Behavioral domains include sensory, motivational or arousal, learning, cognitive, and motor. Wainwright[113,114] has reviewed the principles governing animal models of behavior and the evidence from these models that lipids influence behavior. Carlson[115] and Mostofsky[116] have reviewed models and methods for studying behavior that have been or could be applied to studies of n-3 fatty acid–deficient animals.

The earliest studies of essential fatty acid deficiency restricted intake of dietary fat, thereby producing deficiency in both linoleic and α-linolenic acids. Diets deficient in both essential fatty acids led to reduced number of brain cells; lower LCPUFAs in the brain; and poor growth, probably from AA deficiency.[117] Diets containing linoleic acid but little α-linolenic acid (e.g., made with safflower oil or sunflower oil) reduce DHA accumulation in the retina and brain, with a proportional increase in n-6 DPA compared with diets that contain α-linolenic acid. Animals fed α-linolenic acid–deficient diets grow normally and have no evidence of disease; however, they have atypical retinal responses to light,[16,17,110] visual acuity,[19] and behavior.[20-28,36,118,119] Behavioral studies of α-linolenic acid–deficient animals differ from studies of DHA adequacy that have been done in human infants. In the animal models, DHA is low because dietary α-linolenic acid is deficient. In the infant studies, α-linolenic acid is not deficient. However, as noted above (and supported by the evidence from stable isotope studies), synthesis of DHA from α-linolenic acid may appear sufficient to support optimal brain DHA accumulation and function. Studies of n-3 fatty acid–deficient animals provide clues to the effects of lower brain DHA on behavior, regardless of why brain DHA is reduced. However, given that infants can synthesize DHA from its essential fatty acid precursor,[9-12] any effects of inadequate conversion would likely be less severe than those found in animal models of n-3 fatty acid deficiency.

Evidence of the Sensory Effects of n-3 Fatty Acid Deficiency

The physiologic response of the retina evoked by a brief flash of light (electroretinogram [ERG]) has components determined by the photoreceptor outer segments and the synaptic regions of the retina, the a wave and b wave, respectively. Diets deficient in n-3 fatty acid have a negative effect on the ERG in rats and monkeys. Wheeler and colleagues[17] reported a higher a-wave amplitude in rats fed α-linolenic acid compared with linoleic acid. Feeding α-linolenic acid to depleted animals only partially normalized the a-wave amplitude, evidence that the effects of n-3 fatty acid depletion on retinal physiology were not completely reversible.[16] Other investigators have also reported lower a-wave and b-wave amplitudes in n-3 fatty acid–deficient rats[24,120] compared with controls, but reports to the contrary exist in the rat and guinea pig.[121] Among ERG studies, the variability in results may be caused by differences in the duration of light stimulus, interstimulus interval, stimulus intensity, and the degree of n-3 fatty acid deficiency. For example, an effect on amplitude would be suggested in n-3 fatty acid–deficient animals with a long recovery latency if the retinas were not allowed to recover fully between stimuli.

In n-3 fatty acid–deficient monkeys, the ERG responses of retinal rods and cones are delayed compared with controls, and the time required for the dark-adapted retina to recover from an initial bright flash of light is significantly increased.[110,122] Monkeys deficient in n-3 fatty acid have also been found to have transiently lower a-wave and b-wave amplitudes compared with controls.[122] When n-3 fatty acid–deficient monkeys were fed fish oil, brain DHA increased to normal levels in both young and adult animals. However, the ERG peak latencies did not return to normative values.[122] In fact, even when n-3 fatty acid repletion was started at birth, membrane DHA was reduced, and recovery of the dark-adapted ERG was prolonged relative to controls. These results may be attributable to the finding that, at birth, the monkey retina is more developed than the human retina, and thus some critical developmental period and the opportunity for repleting the deficiency may have passed.[122]

Visual acuity requires a functional retina and a functional brain. Monkey infants whose mothers were fed an n-3 fatty acid–deficient diet made with safflower oil during pregnancy had only half as much DHA in phospholipids as those whose mothers were fed a diet with soybean oil.[19] The n-3 fatty acid–deficient animals had lower visual acuity (determined by forced choice preferential looking) at 4, 8, and 12 weeks of age compared with controls. Bogener and associates[123] and Radel and associates[124] studied brain cortical responses evoked by visual and auditory stimuli in a rat model, and the brain DHA depletion was observed to be similar to that occurring in human infants fed α-linolenic acid without DHA. In adult rats, visual and auditory brain cortical responses showed increased amplitudes in deficient animals[123,124] compared with controls, a finding suggesting decreased ability to inhibit responses to these stimuli. Evidence that the responses were cortical was suggested by the finding that both the ERG and auditory brain responses were within typical ranges. Half of

the animals in each litter were given a diet with DHA beginning at weaning, and their visual, but not auditory, cortical responses returned to normative levels as adults.[124] In addition, a lowered pain threshold with n-3 fatty acid deficiency has been demonstrated and may reflect an effect on sensory function.[125,126]

Evidence of the Cognitive Effects of n-3 Fatty Acid Deficiency

Some studies in rodents suggest that n-3 fatty acid–deficient animals may learn more slowly than n-3 fatty acid–sufficient animals. Differences in performance on tasks generally thought to reflect learning could be evidence of effects on cognition. However, as Wainwright[113,114] has pointed out, many studies did not control for what are known to be effects of n-3 fatty acid deficiency on visual function, reflex development, and exploratory activity, all of which could influence performance even if the ability to learn were unaffected by n-3 fatty acid deficiency.

Lamptey and Walker[20] noted that the offspring of rats fed safflower oil compared with soybean oil had diminished ability to discriminate in a black and white Y-maze that used food as reinforcement. Similarly, Yamamoto and colleagues[21,22] demonstrated that n-3–deficient animals had difficulty discriminating between levels of light intensity to obtain food and required longer to extinguish previously reinforced behavior in a new task. Yehuda and Carasso[126] also reported poorer ability to extinguish a previously learned behavior in n-3–deficient rats. These studies and many more provide the basis for the hypothesis that n-3 fatty acids have an effect on cognitive processes.

Further work with monkeys revealed an inverse relationship between brain DHA accumulation and look duration during a visual recognition task.[28] The observation could be interpreted as evidence of an effect of higher brain DHA on the speed of learning visual stimuli, because the duration of looking generally declines over age during infancy, and shorter look durations are generally associated with faster perceptual learning.[127] Alternative explanations for shorter look durations include increased ability to disengage attention[128] and lower reactivity.[129] Nevertheless, each of these possible explanations suggests a positive effect on cognition of higher brain DHA during development.[130]

Using mice, Frances and colleagues[131] studied the effects of n-3 deficiency on habituation, a form of learning, and found that habituation occurred more slowly in deficient mice relative to repleted mice. In a brightness discrimination task, n-3–deficient mice exhibited inferior learning that was not rectified by practice.[132] However, it has been shown that altered learning behavior in n-3 fatty acid–deficient mice can be reversed by supplementing with DHA after weaning,[133] and the altered learning patterns seen in deficient mice are not associated with substantial changes in the rate of protein synthesis in the brain.[134] Researchers who measured learning through tasks that did not require visual function, but rather tested olfactory function, found higher performance in rats fed α-linolenic acid compared with an n-3 fatty acid–deficient diet.[135-138] Thus, because olfactory function is not thought to be affected by DHA deficiency, these studies are indicative of an effect of DHA on cognition independent of sensory processes.

Evidence of the Effects of n-3 Fatty Acid Deficiency on Motivation and Arousal

Rats fed n-3 fatty acid–deficient diets were reported to have increased exploratory activity in a novel environment,[139] which Wainwright[113] suggested may reflect greater arousal. Differences in arousal may also explain the polydipsia and polyuria that have been observed in n-3 fatty acid–deficient monkeys that have normal renal function, growth, and physical health.[140] Reisbick and associates[119] reported evidence of a higher frequency of stereotyped behavior, higher locomotor activity, and higher general behavioral reactivity in n-3 fatty acid–deficient monkeys. Moreover, it has been reported that DHA and AA supplementation in the diets of infant rhesus monkeys markedly increased heart rate variability that persisted into adolescence.[141] According to these authors, dysregulation of behavior resulting in, for example, aggression, is related to a concomitant dysregulation of sympathetic responses such as heart rate variability. Thus proper levels of fatty acids are important for arousal and behavior regulation.

Changes in arousal and motivation may be related to catecholaminergic systems.[142] Levant and colleagues[143] used a model of physiologically relevant reduction in brain DHA content (i.e., modeled after reductions that have been observed in human infants[68,69]) to explore behavioral changes reflective of dopaminergic tone, in particular hyperactivity of the mesolimbic dopamine system.[144] They found higher basal locomotor activity in n-3 fatty acid–deficient adult rats that did not normalize in deficient rats fed a fish-oil source of DHA starting at 21 days of age. Rats that were n-3 deficient through adulthood exhibited significantly higher activity levels relative to controls in a novel environment consistent with dysregulation of the dopamine systems. Wainwright[114] showed that n-3 fatty acid–deficient rats had delayed matching-to-place abilities in the water maze compared with controls, a task associated with prefrontal dopamine function. As discussed later in this chapter, this finding suggests negative effects of n-3 fatty acid deficiency on higher order cognitions but also reflects altered states of motivation.

Possible Mechanisms for Changes in Behavior With Lower Brain Docosahexaenoic Acid Accumulation

Although it is clear from numerous studies that variations in brain DHA influence behavior, the underlying mechanism by which this occurs has not yet been definitively explained. It was hypothesized early on that a cyclooxygenase or lipoxygenase metabolite of DHA, but not n-6 DPA, played an important role in the function of the central nervous system, but a review by Salem and associates[145] found insufficient evidence to support this idea.

Another hypothesis, for which there is now good evidence, was that phospholipids containing DHA compared with those containing n-6 DPA affected cellular functions. Using a specific example of visual signaling, Salem and associates[145] reported evidence that the signaling pathways depended on the acyl side chains of phospholipids. Cellular studies demonstrated the importance of DHA for the synthesis and accumulation of phosphatidylserine, an aminophospholipid enriched in DHA. Other cellular studies showed that phosphatidylserine accumulation is related to the antiapoptotic effect of DHA.[145] Finally, biophysical studies of membranes constituted with DHA or DPA n-6 in the sn-2 position by these authors showed that membranes with DHA compared with DPA n-6 have lower-order parameters, a finding reflecting a change in bond geometry and an increase in chain dynamics.

The combined evidence for the effects of variations in DHA accumulation in membrane phospholipids suggests a unifying mechanism that could account for the effects reported for n-3 fatty acid deficiency on neurotransmitters and their receptors. DHA affected the neural *N*-methyl-D-aspartate response that relates to calcium influx into postsynaptic neurons.[146] Chalon and colleagues[144] and Delion and colleagues[142] reported an increase in dopaminergic function in the mesolimbic area of the brain and a decrease in the frontal cortex in adult rats. De la Presa Owens and Innis[147] also observed regional differences in piglet brain monoaminergic neurotransmitters after dietary variations that influenced brain DHA and AA accumulated during development. Presumably, all membrane proteins could be influenced by the DHA/DPA n-6 ratio of the membrane phospholipids, in which case functional effects would probably not be limited to effects on neurotransmitters.

LONG-CHAIN POLYUNSATURATED FATTY ACIDS AND THE DEVELOPING HUMAN INFANT

Human fetuses accumulate DHA during the last intrauterine trimester by maternal transfer of DHA across the placenta.[5,6] The process of maternal transfer continues after birth through breast-feeding: all human milk contains DHA, albeit in variable amounts.[7,8,54-56] With medical advances, infants born as early as 20 weeks of gestation, and thus before in utero brain DHA accumulation, now have a good chance of survival. Before the addition of DHA and AA to preterm formulas, these infants had to rely on synthesis for any additional postnatal DHA accumulation.

Breast-fed infants have more DHA and AA in red blood cells than do formula-fed term infants.[7,8,35] Regardless of gestational age at birth or diet (human milk or formula), mean red blood cell phospholipid DHA is higher at birth than in the months after birth in U.S. infants. The apparent "physiologic" decline in red blood cell DHA in breast-fed infants raises several questions, such as whether mothers transfer less DHA to their infants in milk than in utero, whether DHA decreases because of enhanced utilization by other tissues, and whether this postnatal decline is unique to infants in the United States, whose mothers have been shown to have milk DHA content among the lowest in the world.[8,53-56,83]

Differences in maternal DHA status reflected by milk DHA content also likely influence an infant's DHA accumulation in utero. The evidence of variable intrauterine DHA accumulation includes (1) the large range of normative values for DHA in cord red blood cell phospholipids of infants born preterm,[53] (2) the progressive increase in phospholipid DHA throughout the last intrauterine trimester,[148] (3) the decline in cord red blood cell phospholipid DHA with successive pregnancies,[84] and (4) the variability in brain DHA among infants born at the same gestational age.[3]

The addition of DHA to infant formula can prevent declines in red blood cell and plasma phospholipid DHA after birth compared with formulas without DHA. DHA from fish oil,[32,45,149,150] egg phospholipids,[35] and single-cell oils[151] has been shown to increase DHA in circulating phospholipids, a finding suggesting that these sources could increase DHA supply available for the brain. Conversely, infant formulas with α-linolenic acid but no DHA did not prevent the postnatal decline in DHA over several months, and DHA in circulating phospholipids remained low through follow-up to 12 months corrected age.[45,152]

After birth, AA declines in plasma and red blood cell phospholipids. To some degree, the decline appears to be physiologic. For example, AA is higher in preterm than in term cord blood, and even after 4 to 6 months of breast-feeding, infants born at term have much lower red blood cell AA than is seen at preterm delivery (8.8% vs. 15.7%).[8,12] AA in the brain and liver also declines during the last intrauterine trimester.[153] However, breast-fed infants have more AA in red blood cell and plasma phospholipids than do infants fed formulas without LCPUFAs, and those fed formulas with n-3 LCPUFAs have still lower phospholipid AA levels.[154]

AA is the source of metabolically important prostaglandins and leukotrienes. Researchers report significant correlations between AA levels with both intrauterine growth[46,148] and growth in the first year of life of infants born preterm.[47] DHA or total n-3 LCPUFAs added to infant formula reduces AA and alters the balance of n-3 and n-6 LCPUFAs in red blood cell phospholipids.[154] In three randomized trials, scientists found some reduction in growth of infants born preterm with the addition of fish oil DHA and no AA to infant formula.[42,43,155] In one study, the effect was limited to a lower weight for length at several ages in the first year.[155] In another study, the growth effects were found only in male infants.[43] Researchers have not found slower growth in either term infants fed formula containing DHA or in infants born preterm and fed formula containing AA as well as DHA (for review, see Lapillone and Carlson[48]). A recent meta-analysis concluded that the effects of DHA supplementation on growth were not clinically meaningful in term infants.[156]

Still, it is possible that long-term feeding of n-3 LCPUFAs without AA could decrease brain AA accumulation and thus affect the developing central nervous system. High concentrations of α-linolenate[157] and n-3 LCPUFAs from fish oil[158] decreased brain AA accumulation in rats. Arbuckle and associates[159] reported that piglets fed a diet with 4% of fatty acids as α-linolenic acid (but not one with 1% α-linolenic acid) had lower brain weights and lower levels of AA in membranes of synaptosomes compared with controls. Although experimental evidence for adding AA to the diet of formula-fed infants is lacking, it seems prudent to balance the addition of DHA to infant formulas with AA, as has happened with LCPUFA-supplemented formulas in the United States. This is a defensible practice because both these fatty acids are found in all human milk, albeit in variable amounts.

FUNCTIONAL EFFECTS OF DOCOSAHEXAENOIC ACID STATUS: OBSERVATIONAL AND EXPERIMENTAL STUDIES

Retinal and Visual Effects of Feeding Docosahexaenoic Acid to Preterm and Term Infants

Outcomes measured in infant studies of DHA supplementation were modeled after those found to be affected by n-3 fatty acid deficiency in nonhuman primates. Lower retinal and visual development were noted first in n-3 fatty acid–deficient primates,[19] and some aspect of visual development has been measured in most infant studies.[30-35] Retinal physiology is measured by ERG. Visual acuity has been assessed by both a behavioral procedure (Teller Acuity Card) and an electrophysiologic procedure (visual evoked potential [VEP]).[160] Although studies using both procedures have shown higher acuity with higher DHA status, two studies that used both procedures found significant effects only with the electrophysiologic procedure.[32,161]

The first interventions in which DHA was added to infant formula were designed for infants born preterm because, in the absence of in utero maternal transfer, these infants were the most likely to benefit from supplementation. Preliminary reports of higher visual and retinal function, respectively, with DHA supplementation were published in 1989,[29,30] and full reports soon followed.[31,32] Most of the trials of DHA supplementation in preterm formula were relatively small (number per group approximately 20 to 30). However, all the randomized studies of DHA-supplemented preterm formula that measured visual acuity found higher visual acuity with DHA at some age in the first year of life.[31,32,155,161,162] Unfortunately, one quite large study of DHA and AA supplementation did not include any measure of visual development.[163] Systematic reviews by SanGiovanni and associates[164] and by Simmer and Patole[39] concluded that supplementing formulas with DHA improves visual development of infants born preterm but noted the absence of long-term evidence. However, as discussed later, positive effects of an intervention at one age may be manifest in other outcomes at later ages. Relevant to the existence of continued higher grating visual acuity, several studies in term infants using electrophysiologic procedures for measuring visual acuity find persistent effects after postnatal DHA supplementation is discontinued.[151,165,166]

Compared with infants born preterm, the supplemental DHA need of infants born at term for optimal visual development has been more difficult to ascertain, because the results of the published studies are mixed.[37,38,167] The first suggestion that DHA status played a role in visual development of term infants came from a study by Makrides and colleagues,[33] who found higher blood levels of DHA in breast-fed infants to be associated with higher visual acuity. Observational studies by Jorgensen and

colleagues[70] and by Innis and colleagues[71] also showed associations between DHA status of lactating women and the visual acuity of their infants. Finally, results of a population-based cohort study suggested that maternal prenatal and postnatal intake of oily fish, a source of both DHA and eicosapentaenoic acid (EPA), was related to higher stereoacuity in their children at 3.5 years of age.[168]

Only experimental studies that increase DHA transfer to the infant can determine a causal relationship between DHA and optimal visual function in infancy. There have been several published intervention studies with some source of DHA (high-EPA fish oil, low-EPA fish oil, egg phospholipid, single-cell oils) with or without AA that measured some aspect of visual development in formula-fed infants born at term.* Makrides and colleagues found higher VEP acuity in one study but not in another.[34,169] Higher visual acuity with use of the Teller Acuity Card procedure has been observed at 2 months of age but not at 4, 6, 9, and 12 months of age.[35] Jorgensen and colleagues[170] did not find a significant difference between formula groups, although the visual acuity in the group fed formula without DHA was significantly less than in the breast-fed group, with use of the Teller Acuity Card procedure.

In a study of infants randomly assigned at birth to receive a DHA-supplemented formula until 4 months of age, Birch and colleagues[151] found higher VEP acuity at 6, 17, and 52 weeks of age but not at 26 weeks. More recently, the same group of investigators reported higher VEP acuity at 17, 26, and 52 weeks of age in infants randomly assigned to consume formula with, compared to without, DHA and AA after they were weaned from human milk at 6 weeks,[171] or approximately 6 months of age.[172] On the other hand, two large multicenter trials conducted in term infants by Ross Laboratories found no effect of DHA supplementation on visual acuity.[173,174] However, the formulas used in these studies contained much less DHA than the formulas used by Birch and co-workers[151] in their term studies. Recent systematic reviews[38,175] included all but the most recent Ross Laboratories study. Simmer and colleagues[38] concluded that there was little evidence that LCPUFAs conferred any visual development advantage in term infants. SanGiovanni and colleagues[175] concluded that significantly higher visual function was associated with LCPUFA intake at 2 and 4 months in both randomized and unrandomized studies.

Even though DHA and AA were added to term formulas in the United States in 2002, the report from the American Society for Nutritional Sciences Life Sciences Research Office did not recommend a minimum intake of DHA and AA for infants born preterm and cited some of the positive effects noted as being small or transient. The report did recommend a maximum concentration of 0.6% AA and 0.35% DHA and cited no adverse effects at these intakes and the possibility that functional changes noted in studies "may be important on a population basis or important for subsequent visual and/or cognitive function."[176]

Although it is true that only about half of the studies showed higher visual acuity in term infants with the addition of DHA to infant formula, it is difficult to draw any definitive conclusions because of the wide variation in the age and environments of the populations assessed and wide variation in the source and amount of fatty acids in the formulas studied. One review of these studies led to the conclusion that many lack sufficient statistical power to reject the null hypothesis because of uncontrolled variability.[177] Failure to control for variables other than background diet that potentially influence visual acuity could inflate or mask the effectiveness of an intervention. Factors such as DHA status at enrollment, head circumference at birth, paternal smoking, postconceptual age at assessment, and even paternal social status should be considered potential covariates of visual acuity.[178]

*References 34, 37, 38, 151, 165, 166.

Indeed, groups of researchers who controlled for gestational age at birth reported statistically significant differences in visual acuity, favoring DHA-supplemented groups.[35,154] Thus, with careful control of environmental variables, the results may more consistently show improved visual acuity with DHA supplementation.

Early increases in visual acuity with DHA may contribute to or be independent of accelerated maturation of other aspects of development over time. In two studies, infants born preterm and fed formulas with DHA had shorter duration looks to visual stimuli at 6, 9, and 12 months compared with infants fed formula without DHA. These results emerged even though differences in visual acuity that favored the supplemented group earlier in infancy did not persist at these later ages.[179,180] Another study found that higher reported intakes of oily fish during pregnancy were related to higher stereoacuity in childhood.[168] These observations suggest a possible effect of early differences in visual development on more complex tasks later in infancy and childhood.

Reviews by both Wainwright[114] and Colombo[181] include arguments against a static interpretation of development and suggest the consideration of developmental systems theory[182] in the interpretation of behavioral data arising from studies that vary LCPUFA status. The developmental systems perspective is transactional in nature: development occurs as a result of biologic and environmental bidirectional influences on the individual across time. Thus investigators need to consider environmental and metabolic factors that may interact with altered LCPUFA status.

Arousal in Infants

Cheruku and associates[72] reported an association between maternal plasma phospholipid DHA (another indicator of maternal status) and brain maturation in newborn infants based on differences in sleep patterns at birth. With maturation, there is an increase in quiet sleep, a decrease in active sleep, increased wakefulness, and a decrease in the transition from sleep to wake. These authors noted inverse relationships between maternal DHA status and both active sleep and the ratio of active sleep to quiet sleep. They also found that maternal DHA status was associated with more time awake and less time in transition between sleep and wake in the infants. This observation is evidence of more mature function with higher DHA status and adds to the evidence that intrauterine exposure to DHA affects development.

Docosahexaenoic Acid Effects on Language Development

The development of vocabulary proceeds concomitantly with motor, cognitive, and social development, and therefore it is reasonable to posit that a direct relation may exist between language development and supplementation with DHA. Innis and colleagues found that several indicators of DHA status (plasma phospholipid DHA, red blood cell phosphatidylcholine DHA, and phosphatidylethanolamine DHA) in breast-fed infants were significantly correlated with the ability to discriminate a nonnative language consonant at 9 months[71] and with a parent-report measure of vocabulary comprehension and production at 14 months of age.[183] O'Connor and colleagues[161] found higher vocabulary comprehension at 14 months in infants born preterm from English-speaking families who received formulas with versus without AA and DHA.

However, other researchers have reported apparent negative effects of DHA on vocabulary. In one study,[184] lower scores on a parent-report language inventory were observed at 14 months of age in a DHA-supplemented group relative to a control group. This may be attributed to an imbalance in the DHA to AA ratio.[185] Moreover, those differences were not detected in a follow-up at 3 years of age when language was assessed with use of an

age-appropriate standardized expressive language assessment (i.e., not parent report).[186] Similarly, Lauritzen and colleagues[187] found a lower language ability at 12 months of age in breast-fed infants whose mothers had received supplements, but this difference was no longer evident at 2 years of age. It is unclear how one would correctly interpret these findings. It may lie with the nature of the assessment of language comprehension and production at 12 to 14 months of age (i.e., parent report vs. a more objectively administered standardized test). More research is clearly needed to determine what variables are exacting an effect on language, whether or not that effect is transient, and what meaning it may have for future outcomes.

Visual Attention: Novelty Preference and Attention Duration in Preterm and Term Infants

Several studies compared novelty preference and attention duration in infants born preterm and receiving supplementation with DHA. Both novelty preference and shorter look duration in infancy were modestly correlated with higher cognitive function later in childhood in one study (for review see Carlson and Neuringer[130]). Compared with controls, DHA-supplemented infants had a greater number of discrete looks[188] and a significantly shorter look duration at 6, 9, and 12 months.[179] A replication confirmed these results at 12 months of age, when most infants were tested.[180] These data are analogous to those reported in n-3–sufficient compared with n-3–deficient monkeys (i.e., higher membrane DHA level correlated with shorter look duration).[28] As in the monkey studies, the results of the human studies suggest that DHA supplementation enhances either the speed of visual processing or the ability of the infant to disengage from the stimulus.

Other support for a relationship between DHA status and visual attention comes from observational studies. Look duration is a robust indicator of the development of attention, as it declines sharply across the first year of life. This decline was reported to have been accelerated in term infants whose mothers had red blood cell phospholipid DHA higher than the median level of the sample, relative to those whose mothers' levels fell below the median. This difference in look duration disappeared by 8 months of age but then reemerged at 12 months of age with the administration of a more sophisticated task.[73] Given that infants were not provided DHA supplementation after birth and background diets in the United States contain only small amounts of DHA, the data suggest that variable DHA accumulation during intrauterine life was the influential factor. Experimental studies that manipulate maternal DHA status during pregnancy are needed to test this hypothesis, and ongoing clinical trials should provide some information related to this question within the next few years.

Physiologically normal infants prefer novel stimuli; this is commonly used to assay visual recognition memory early in life. In two randomized trials, the Fagan Infantest (Infantest Corporation, Cleveland, OH) was used to measure visual novelty preference at 6, 9, and 12 months of corrected age of infants born preterm. Here, infants fed control and DHA-supplemented formulas had a similar novelty preferences.[179,180] In contrast, O'Connor[161] found a higher novelty preference but no effect on look duration in one group of infants fed AA and DHA (egg triglyceride and fish oil) compared with the control group at 6 months of age. In studies of term infants,[77,171,173] including one that provided supplemental DHA to women during pregnancy and lactation,[77] no effect on recognition memory was found.

Global Measures of Cognitive Development

Two tests of global neurodevelopment have been used in studies of DHA supplementation: the Bayley Scales of Infant Development (BSID-II),[189] which was developed in the United States, and the Brunet-Lezine Test,[190] which was developed in France. Both tests are standardized procedures that provide indices of mental and motor age relative to group norms. Standardized tests tend to be less-ambiguously interpreted and are familiar to pediatricians. As such, their use as outcome measures in studies of children receiving DHA supplementation has been encouraged.

However, researchers who have used global assessments of development have reported mixed results. Some found that supplementation with DHA resulted in higher standardized scores,[191-195] whereas others, including several larger and appropriately powered trials, found none.* Many explanations for these mixed results may be viable; differences in samples, supplementation, and administration could confound the results. Most important, the primary purpose of such standardized global assessments is to identify infants and children at risk for developmental delays or disabilities. Most are not sensitive enough to detect slightly different rates of development in typically developing infants and children. Thus caution should be exercised when interpreting the results of interventions by using these global assessments.

Specific Measures of Cognitive Development

Functional aspects of DHA suggest that its effects would be manifest in specific processes of cognitive development. Two specific processes underlie cognitive development: the acquisition of information (memory) and the speed with which that information is acquired (speed of processing).[199] DHA is concentrated at the synapses[200] and may act to improve synaptic efficiency and neuronal transmission speed. Moreover, DHA is implicated in processes that are integral to the laying down of a memory trace, such as long-term potentiation in the hippocampus.[201] Thus measurement of specific cognitive processes such as attention, processing speed, and memory is warranted in studies of the effects of DHA supplementation on the development of the child.

Researchers who have used specific measures of development have shown more consistent results relative to those who have used the global measures. Infants who have been fed formula supplemented with DHA reportedly are better able to detect novelty,[161] have faster processing abilities,[179,180,202] and are better problem solvers[203,204] relative to those fed unsupplemented control formula. As previously mentioned, infants whose mothers had DHA levels above the sample median at the time of their births are reported to have had enhanced information processing performance, as well as more mature orienting and attentional abilities.[73] Certainly, given these results, further research into the effects of DHA supplementation on specific cognitive abilities would be important.

We have argued elsewhere that the integration of memory and attention underlie and drive the development of the higher-order cognitions known as executive functions.[205] Executive functions include cognitive processes such as working memory, inhibitory control, and planning. It is likely that these developments are attributed to the development of the frontal lobes, which mature at a more protracted rate. Whereas such integrated cognition is initiated at the end of the first year, these abilities are not reliably evident until the early preschool period (e.g., 3-4 years of age).

Fatty acids are implicated in the higher-order cognitions. Frontal lobes accumulate significant amounts of DHA during their development.[206] Moreover, dopamine is an important modulator of the executive functions, specifically working memory and attentional control.[207] Dopamine has been shown to decrease in the frontal area in DHA-depleted rats,[208] and, relative to DHA-adequate rats, the DHA-depleted rats do not do as well on executive function types of tasks[26] (for review, see Wainright[114]). Thus it seems reasonable that the effects of DHA supplementation in infancy may also be apparent in the development of executive function.

*References 161, 169, 173, 174, 184, 196-198.

As children from the supplementation trials age, researchers are beginning to assess their developmental status. In a follow-up of 2½-year-olds whose mothers received supplementation with DHA during pregnancy, hand-eye coordination scores from a global assessment were significantly related to n-3 PUFA levels in cord blood erythrocytes.[209] Helland and colleagues[75] reported differential results on a global assessment of cognitive abilities at age 4 years in children whose mothers received supplementation during pregnancy and lactation, even though the infant assessments revealed no group differences: children of mothers who received DHA supplementation scored higher at 4 years of age on a standardized IQ test than those of mothers who did not receive supplementation. A multiple regression analysis showed that maternal DHA intake during pregnancy was the only variable related to IQ at 4 years of age. Similarly, in a small study of breast-fed infants, Gustafsson and colleagues[210] found that DHA levels in colostrum were significantly related to IQ at 6.5 years of age, further attesting to the importance of maternal DHA levels at birth.

SUMMARY

DHA and AA were added in 2002 to formulas for term infants and infants born preterm in the United States. The levels of DHA added to study formulas vary. Although the importance of DHA in infant development is becoming increasingly obvious from human and animal work, the optimal and maximum levels of DHA intake for pregnant women and infants have not yet been determined. Progress in these areas is to be expected in the future, because a number of large trials are currently ongoing around the world. However, it is clear that these studies are taking place at the same time as DHA intake from supplements, supplemented food, and formula may be changing the background DHA exposure of women, infants, and children. Variable DHA exposure in control groups will be a potential confounder of new studies of DHA supplementation. Studies of infant behavior will need to control for maternal prenatal and background diet postnatal DHA intake or use some biochemical measure of DHA exposure such as red blood cell or plasma DHA concentration.

Animal models continue to be used to answer questions about the effects of lower brain DHA accumulation on neural development. It seems likely that the results of these studies will aid in targeting new developmental outcomes, as well as explicate the effects of DHA on other aspects of physiology. Still other research is being directed toward understanding the mechanisms through which lower DHA accumulation in membranes affects physiologic functions. Readers wishing to have a more extensive treatment of the neural mechanisms of DHA are referred to a review by Salem and colleagues.[145]

As noted above, ongoing research is addressing maternal DHA status during pregnancy and its influence on development of the infant and child. Some experimental and observational studies report associations between more mature patterns of sleep, language, peak look duration, and global neurodevelopment scores with higher maternal DHA status. Experimental studies of infants whose mothers were provided different amounts of DHA during pregnancy are now in progress. It is hoped that, in addition to aiding our understanding of the timing and amount of DHA needed for the developing brain, these studies can incorporate novel outcomes that target specific behavioral domains. A positive effect of intervening with DHA during pregnancy and lactation has been found in children as old as 4 years of age, and DHA levels in breast milk have been related to positive outcomes in children at 6.5 years of age. The relation of DHA levels in infancy to child outcomes is not surprising given the involvement of polyunsaturated fatty acids in brain growth, neuroprotection, and neurotransmission. Thus research is needed to assess childhood outcomes in those who received supplementation in utero and/or in infancy. As supplemented populations grow, we can expect to see research in this area grow also.

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Glen E. Mott

Lipoprotein Metabolism and Nutritional Programming in the Fetus and Neonate

Studies of cholesterol and lipoprotein metabolism in the fetus and infant have dramatically expanded in the past few decades because of their relevance to the etiology and pathogenesis of atherosclerosis. Atherosclerosis, the process leading to coronary heart disease in adult life, is common in young adults and probably has its origins in the arterial fatty streaks found in infants and children. Plasma cholesterol and lipoprotein concentrations, which are associated with the rate of development of these arterial lesions, often track from infancy to adulthood. Furthermore, fetal undernutrition and the type of prenatal and postnatal nutrition can lead to metabolic "programming" for an increased risk of diabetes, the metabolic syndrome, and coronary heart disease in adulthood. Therefore prenatal and early postnatal nutrition has become the focus of intense investigation in preventive medicine. Because intervention can considerably slow the progression of atherosclerosis in adult life, identifying children at risk and providing follow-up and family counseling are important research goals.

All lipid and lipoprotein cholesterol values are given in milligrams per deciliter (mg/dL). In some instances, use of units of millimoles per liter (mmol/L) may be more convenient.

To convert *cholesterol* values from mg/dL to mmol/L, multiply by 0.02586.
To convert *triglyceride* concentrations from mg/dL to mmol/L, multiply by 0.01129.

Representative conversions are given in Table 47–1.

PRENATAL (FETAL) DEVELOPMENT

Serum Cholesterol, Triglyceride, and Lipoprotein Concentrations

Humans

Early in gestation, serum cholesterol concentrations average 85 ± 31 (SD) mg/dL[1] and decrease cyclically to 50 to 75 mg/dL in cord blood of normal term infants,[2-14] which is less than half the normal adult concentration (Figure 47-1). Serum cholesterol concentrations of preterm infants frequently are elevated and commonly exceed 100 mg/dL,[1,14-16] possibly because of a second serum cholesterol peak early in the third trimester.[1] Term babies who are small for gestational age (SGA) at birth may have slightly higher peak serum cholesterol levels[16]; those who are born near term with growth restriction have lower values.[17] Infants with familial hypercholesterolemia have cord blood cholesterol concentrations from 100 to 200 mg/dL.[18] Cord serum triglyceride concentrations average 30 to 50 mg/dL,* which are much lower than normal adult values. Some newborns infants, however, will have triglyceride levels above 200 mg/dL.[19] Most of the variation in plasma lipids at birth is due to genetic factors, gestational age, or disease.

Low-density lipoprotein (LDL) cholesterol (LDL-C) concentrations also decrease during gestation in the human fetus, from approximately 65 mg/dL in the first trimester[2] to 25 to 30 mg/dL at term,[2,3,6,25] which is approximately one-fifth the adult level (see Figure 47-1). Compared with serum cholesterol and LDL-C, high-density lipoprotein (HDL) cholesterol (HDL-C) concentrations in humans are stable during gestation,[2,3] although a decrease in HDL-C concentration was reported in premature infants.[9] The HDL-C concentration at term gestation is approximately 25 mg/dL (see Figure 47-1), compared with adult values of 40 to 50 mg/dL.[2,7,9,25,27-29] In contrast with the HDL in adults, HDL in cord blood contains higher levels of large HDL_2[30-32] and a broader range of particle sizes.[32-34] The very-low-density lipoprotein (VLDL) of cord blood carries less than one third of the total serum triglyceride concentration,[35,36] and its cholesterol content averages less than 10 mg/dL,[30,31,36] whereas in the adult, VLDL is the principal transport vehicle for triglycerides in fasting plasma. Although lipoprotein(a) concentrations at birth may be as high as 20 mg/dL, the average is 4 mg/dL,[8,37-39] compared with average adult values of 15 mg/dL.

The low serum cholesterol and lipoprotein concentrations in the human fetus and neonate compared with those in adults are consistent with the low apolipoprotein B (apo B)[23,25,28] and apolipoprotein A-I (apo A-I)[9,24,25,29,40] concentrations at birth. Although HDL-C concentrations in the human fetus are relatively stable, apo A-I concentrations double between 20 and 40 weeks of gestation and increase the apo A-I–HDL-C ratio.[40] Levels of cord serum apo E, which is principally associated with the lighter-density HDL classes,[32,36] are near or above adult concentrations of approximately 10 mg/dL.† Apo E polymorphisms affect the metabolism of apo B–containing lipoproteins such that infants with phenotype apo E 2/2 have lower apo B concentrations compared with other apo E phenotypes.[42] At birth, apo C-I and apo C-II levels also approach adult concentrations,[23] but apo C-III and apo A-II are only 40% to 60% of the adult level[23,25,43] (Table 47-2).

Ethnic and racial differences in serum cholesterol, lipoprotein cholesterol, and triglyceride concentrations at birth are small in contrast with those observed later in childhood.‡ It is not clear whether the reported ranges of cord serum cholesterol concentration (50 to 100 mg/dL) among different ethnic groups reflect methodologic variation, maternal diet, or genetic effects. Female infants have slightly higher cord serum cholesterol and lipoprotein concentrations than those reported for males[4,11,23,25,49] but similar triglyceride levels.[5,11]

Experimental Animals

As in humans, serum cholesterol, LDL-C, and apo B levels are low and decrease during gestation in fetal calves[50,51] and pigs.[52] HDL-C concentrations are low in fetal calves and do not change with fetal age. In contrast with other large animals, the fetal pig carries less than 1% of the total cholesterol in HDL early in gestation; HDL-C increases gradually until term gestation, when it reaches approximately 35% to 50% of the adult value.[52,53]

*References 5, 8, 10, 11, 14, 19-27.

†References 8, 23, 24, 28, 36, 41.

‡References 5, 25, 26, 29, 38, 39, 44-48.

TABLE 47-1

Corresponding Levels of Lipids in Milligrams per Deciliter and Millimoles per Liter

CHOLESTEROL		TRIGLYCERIDES	
mg/dL	mmol/L	mg/dL	mmol/L
35	0.9	150	1.7
50	1.3	250	2.8
110	2.8	400	4.5
130	3.4	500	5.6
170	4.4	1000	11.3
200	5.2		
240	6.2		

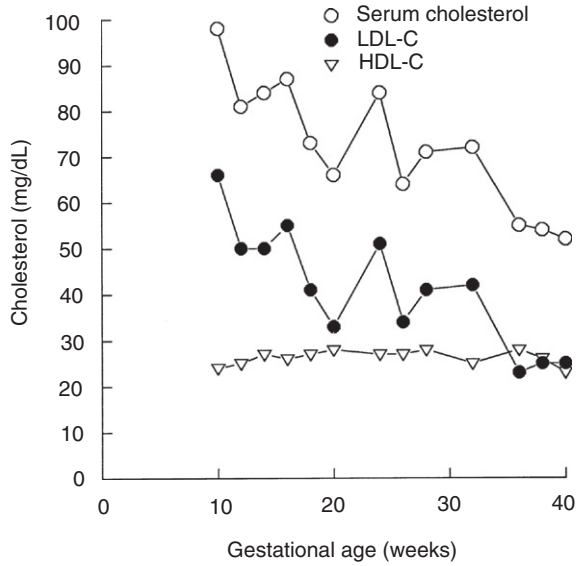


Figure 47-1 Serum cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) concentrations in 17 human fetuses by gestational age. *(Modified from Cai HJ, Xie CL, Chen Q, et al: The relationship between hepatic low-density lipoprotein receptor activity and serum cholesterol level in the human fetus,* Hepatology *13:852, 1991.)*

As in humans, the serum cholesterol concentration in newborn baboons averages 75 mg/dL,[54,55] but the ratio of LDL to HDL cholesterol[55] is 0.5, compared with approximately 1.0 in humans at birth.[2] As observed in newborn humans, apo A-I and apo B concentrations of neonatal baboons are only approximately half of adult values.[55]

In the rat, serum cholesterol measured late in gestation is higher than in the adult animal.[56] LDL is the principal carrier of cholesterol in the rat fetus, whereas in the adult rat, HDL is the major lipoprotein class.[15,56] HDL-C concentrations in fetal rats are approximately 60% and VLDL-C levels are approximately 10% of adult values.[57] In contrast with the neonatal rat, the adult rat has high concentrations of apo E–rich HDL, with low levels of LDL.[58] At birth, serum triglyceride levels in the rat are below adult values.[59] As in the rat, LDL predominates in the fetal rabbit, and HDL is the major lipoprotein class in the adult.[60] The guinea pig fetus has similar HDL-C and LDL-C levels, but in the adult animal, LDL predominates.[15] Newborn rabbits and guinea pigs have higher serum cholesterol levels than their dams unless the dams are fed a high-cholesterol diet.[61-64]

TABLE 47-2

Human Apolipoprotein Concentrations in Cord and Adult Serum

	CONCENTRATION* (mg/dL)		
Apolipoprotein	Cord	Adult	[Cord]/[Adult] Ratio
A-I	73.0 ± 1.5	134.0 ± 3.4	0.54
A-II	41.0 ± 1.0	68.0 ± 2.5	0.60
B	28.0 ± 0.6	98.0 ± 2.8	0.29
C-I	5.9 ± 0.2	7.0 ± 0.3	0.84
C-II	3.2 ± 0.3	3.7 ± 0.3	0.86
C-III	6.5 ± 0.2	13.0 ± 0.7	0.50
D	3.7 ± 0.1	10.0 ± 0.6	0.37
E	8.3 ± 0.3	10.0 ± 0.6	0.83

Modified from McConathy WJ, Lane DM: Studies on the apolipoproteins and lipoproteins of cord serum, *Pediatr Res* 14:757, 1980.
*Mean ± standard error.

Lipoprotein Metabolism: Humans and Experimental Animals

The development of the LDL receptor in humans has a major role in lowering LDL concentrations during gestation.[2] By midgestation, LDL receptor activity is higher in human fetal liver than in adult liver[2] (Figure 47-2), as in the fetal calf compared with the adult cow.[50] In the newborn infant, LDL receptor activity is suppressed by the absorption of biliary and dietary cholesterol and by the uptake of bile acids during fat digestion. In the fetus, the absence of these negative regulatory processes probably contributes to the sustained elevation of LDL receptor activity and low plasma cholesterol and LDL-C levels in humans during the third trimester. The inverse correlation of LDL receptor activity and serum cholesterol concentration during gestation is consistent with that rationale.[2] The lower level of plasma cholesterol esterification[24] (secondary to reduced lecithin-cholesterol acyltransferase activity [LCAT] in the fetus and newborn[9,41,65-67]) also may contribute to differences in lipoprotein composition in the neonate.

In the human fetus, hepatic and intestinal genes[68,69] that are essential for cholesterol and apolipoprotein synthesis are transcribed at high levels by 8 to 20 weeks of development.[69] Most cholesterol in tissues probably is derived from endogenous synthesis rather than from the maternal circulation. The placenta, however, contains the necessary cholesterol uptake and transport mechanisms for its transfer to the fetus, and a high cholesterol content in the yolk sac has been documented.[70] The positive association of serum cholesterol in human fetuses with the level of hypercholesterolemia in their mothers through the second trimester also suggests that maternal-fetal transfer of cholesterol occurs in humans, at least early in gestation.[71]

In pregnant rabbits, dietary-induced hypercholesterolemia increased neonatal serum cholesterol concentrations over levels in non–cholesterol-fed control animals by approximately 50% in one study[72] and minimally in several others.[73,74] Considerable maternal-fetal transfer of cholesterol occurs in both the rabbit and the guinea pig.[62] In the hamster, tissue cholesterol concentrations of the fetus increase with maternal cholesterol intake, but only with very-high-cholesterol diets.[75]

In fetal rats, LDL metabolism differs considerably from that in the adult. Late in gestation, the rat fetus greatly increases LDL production, leading to higher LDL-C levels and an LDL turnover rate more than 15 times that in the adult.[76] In contrast with that in humans, lipoprotein binding to hepatic LDL receptors in the rat fetus is reduced compared with that of the newborn or adult.[77] In the rat fetus, hepatic cholesterol synthesis[78] and hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase activity,[79] the

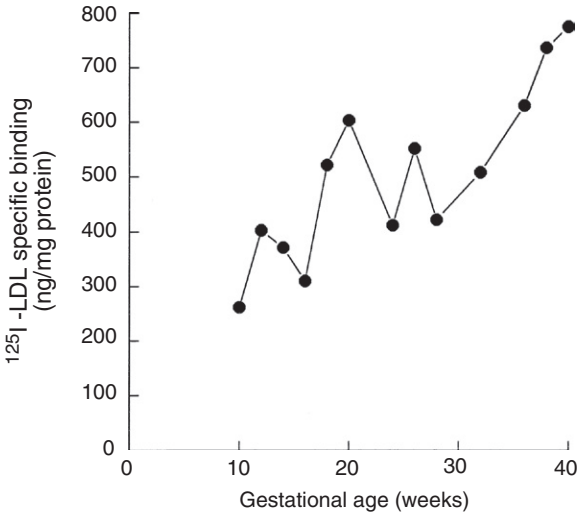


Figure 47-2 Specific binding of ^{125}I-labeled low-density lipoprotein (LDL) by hepatic LDL receptor preparations from 17 human fetuses by gestational age. *(Modified from Cai HJ, Xie CL, Chen Q, et al: The relationship between hepatic low-density lipoprotein receptor activity and serum cholesterol level in the human fetus,* Hepatology *13:852, 1991.)*

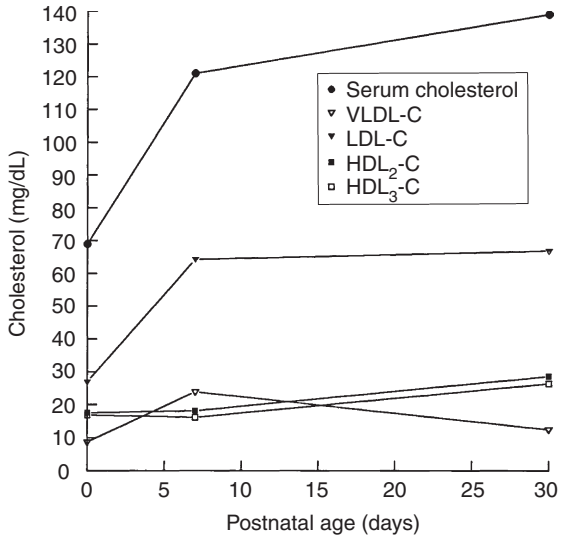


Figure 47-3 Serum cholesterol and lipoprotein cholesterol response of normal human infants who were exclusively breast-fed from birth to 30 days of age. *HDL_2-C, HDL_3-C,* High-density lipoprotein cholesterol subfractions 2 and 3; *LDL-C,* low-density lipoprotein cholesterol; *VLDL-C,* very-low-density lipoprotein cholesterol. *(Modified from Rosseneu M, Van Biervliet JP, Bury J, Vinaimont N: Isolation and characterization of lipoprotein profiles in newborns by density gradient ultracentrifugation,* Pediatr Res *17:788, 1983.)*

latter representing the rate-limiting step in cholesterol biosynthesis, markedly increase during the week before parturition but decrease somewhat before birth.[79,80] The fetal rat increases hepatic and intestinal messenger RNA (mRNA) concentrations for cholesterol metabolism or transport genes a few days before birth or at parturition.[69,81,82]

Summary of Fetal Lipoproteins

In the human fetus, the enzymes for cholesterol and lipoprotein metabolism develop early in gestation. As expression of the LDL receptor increases in gestation, serum cholesterol and LDL-C concentrations decrease to levels much lower than those observed in early postnatal life and adulthood. In the rat, the pathways for cholesterol and lipoprotein production develop rapidly late in gestation and are associated with higher serum cholesterol and LDL-C concentrations in the fetus than in the adult.

POSTNATAL DEVELOPMENT

Serum Cholesterol and Lipoprotein Concentrations

Humans

Serum cholesterol, LDL-C, VLDL-C, and triglyceride concentrations increase rapidly in normal breast-fed infants within a few days after birth.[30,31,66,83,84] Serum cholesterol concentration increases from an average of 50 to 60 mg/dL on day 1 to greater than 100 mg/dL by 1 week of age, and LDL-C from 25 mg/dL on day 1 to greater than 50 mg/dL by 1 week[36] (Figure 47-3). Serum cholesterol and LDL-C concentrations are approximately half the adult level.[28,36] At 1 month of age, serum cholesterol and LDL-C concentrations[30,83] are more than double the birth values but decrease after weaning to a typical Western diet.[13,85] By contrast, HDL-C concentrations increase only slightly during the first week of life[28] and thereafter increase to near adult levels (40 to 50 mg/dL) by the age of 1 month.[30] Throughout the first month of life, the ratio of the HDL subfractions HDL_2-C and HDL_3-C[31] remains approximately 1.0 (see Figure 47-3) but decreases to less than 0.5 later in childhood.[86] Apo B and apo A-I levels rise in association with increasing LDL-C and HDL-C concentrations during the first weeks of life[8] and then stabilize. By contrast, lipoprotein(a) concentrations increase steadily from low levels at birth and reach adult levels by the age of 1 year.[37,39] At birth, more than 80% of serum apo E is in the HDL fraction, but by 30 days of age, apo E decreases in HDL to approximately 60% of the total.[36] The apo E–enriched HDL-C is taken up rapidly by the LDL receptor, which may facilitate efficient delivery of cholesterol to tissues in the rapidly growing neonate.[87]

Part of the increase in serum cholesterol after birth is attributed to both cholesterol and saturated fat present in breast milk, although hemoconcentration during the first 24 hours may account for 10% of the increase.[88] Some of the postnatal increase in serum cholesterol is attributed to other, unidentified dietary factors or to metabolic maturation, because serum cholesterol and lipoprotein concentrations increase regardless of the diet composition.

Serum cholesterol, LDL-C, HDL-C, and apo B concentrations usually are higher in breast-fed infants than in those fed typical formulas low in cholesterol and containing fat with a higher ratio of polyunsaturated to saturated fatty acids (the P/S ratio) than that found in breast milk.* Feeding infant formulas prepared from cow's milk or evaporated milk that contain levels of cholesterol and saturated fat similar to those of human milk also increases serum cholesterol concentrations to levels comparable to those with breast-feeding.[4,44,95-100] The relative importance of cholesterol versus fat in breast milk in elevating the serum cholesterol concentration is not known, because most studies of neonatal dietary cholesterol intake in humans are confounded by differences in fat composition or other factors. Formulas high in saturated fat with half to three-fourths the level of cholesterol found in breast milk raise the serum cholesterol and apo B levels but have little effect on HDL-C or apo A-I concentrations compared

*References 13, 28, 43, 89, 90-94.

with the same formula low in cholesterol.[30,101,102] Infants fed formula containing cholesterol at twice the level in breast milk have slightly higher LDL-C concentrations but equivalent levels of HDL-C compared with those in infants fed a similar formula without cholesterol.[103] Generally, more of the lipoprotein response to a preweaning diet is attributed to the type of fat than to the cholesterol content. Serum cholesterol is inversely related to the dietary P/S ratio but is not significantly correlated with cholesterol intake among infants after weaning.[104]

The independent effects of types of fat in diets of the newborn infant have been more thoroughly studied than those of dietary cholesterol. Feeding a formula low in cholesterol with a fatty acid composition similar to that of breast milk increases the serum cholesterol and LDL-C concentrations to approximately two-thirds those levels in infants who are breast-fed.[90,105] Feeding low-cholesterol formulas containing higher proportions of coconut oil (and therefore more lauric [$C_{12:0}$] and myristic acids [$C_{14:0}$]) increases serum cholesterol,[106] LDL-C, and HDL-C concentrations to nearly the same levels as those observed with breast-feeding.[107] Infants fed formulas containing higher concentrations of corn or soy oil (P/S ratio greater than 3) have approximately 25% to 35% lower serum cholesterol and LDL-C concentrations[43,108] compared with those fed breast milk. Feeding formulas with more moderate P/S ratios (i.e., less than 2.0) lowers serum cholesterol and LDL-C concentrations by lesser amounts.[90,105,109] The HDL-C level is decreased very slightly with use of high or moderately high-P/S-ratio formulas.[43,107-109] Strong evidence indicates that dietary supplementation with long-chain polyunsaturated fatty acids is necessary for optimal growth and development, particularly for preterm infants, and that such supplementation of formulas possibly is indicated for to all infants.[110]

Experimental Animals

The serum cholesterol and lipoprotein responses of neonatal nonhuman primates are similar to those described for humans. Breast-fed rhesus monkeys[111] and baboons[54,55] have higher serum cholesterol concentrations than those in animals fed low-cholesterol formulas. In the rhesus monkey, however, breast-feeding principally increases the serum LDL-C fraction, whereas in the baboon, it increases HDL_1-C and HDL_2-C (and therefore HDL-C) levels.[55,112] In the infant rhesus monkey fed a low-P/S-ratio formula with a cholesterol content similar to that of breast milk, serum cholesterol levels increased to approximately 100 mg/dL higher than in breast-feeding.[113] In baboons, a high-P/S-ratio infant formula with a cholesterol content similar to that of breast milk increased the serum cholesterol concentration to the same extent as with breast-feeding.[54] Baboons fed formula with a low P/S ratio (similar to that of breast milk) had a higher LDL-C but lower HDL-C level compared with breast-fed baboons.[55] In the suckling pig, serum cholesterol levels increase to approximately 200 mg/dL by 1 month of age, primarily because HDL levels are more than two-fold higher than in the adult.[52,53] Breast-fed piglets have higher serum cholesterol and VLDL plus LDL-C concentrations than those measured in piglets fed low-cholesterol[114-116] or high-cholesterol formulas with unsaturated fat.[117] A palm oil–containing, low-cholesterol formula that was similar to sow milk in fatty acid composition raised the serum cholesterol in the piglet only to approximately 80 mg/dL, nearly half that in piglets fed sow milk; the serum VLDL plus LDL-C concentration increased to only 27 mg/dL, or approximately 25% of that in piglets fed sow milk.[116] In pigs, neither level of dietary cholesterol nor type of fat accounts for the serum cholesterol or lipoprotein differences between breast-feeding and formula-feeding.[118]

Suckling rabbits have serum cholesterol levels of 300 to 400 mg/dL,[61] predominantly as a result of greatly elevated VLDL-C and intermediate-density lipoprotein (IDL) concentrations.[119] In the suckling rat, total cholesterol increases from approximately 60 mg/dL at birth to 100 mg/dL at 10 days[120] and to 170 mg/dL at 3 weeks of age.[121] LDL is the principal carrier of cholesterol in the newborn rat,[122] and its concentration decreases after 2 to 3 weeks of age.[121-123] By contrast, serum HDL-C concentrations increase from approximately 20 mg/dL at birth in the neonatal rat to 40 mg/dL by 2 weeks of age.[121,123]

Lipoprotein Metabolism: Humans and Experimental Animals

Breast-feeding increases serum LDL-C concentration and decreases by three-fold the cholesterol synthetic rate in human infants[92,93] and by two-fold in piglets,[114] compared with rates observed in those fed low-cholesterol formulas. Consistent with those observations is a lower hepatic HMG-CoA reductase activity in the suckling rat[79,124] than that observed in the fetus.[79,80] The postpartum increase in serum LDL-C levels is associated with a rapid decrease in the number of LDL receptors during the suckling period in cattle,[50] but a similar decrease in the LDL receptor–binding capacity is not observed among suckling piglets compared with those fed a high-cholesterol versus a low-cholesterol formula.[118] The enzyme that controls the rate-limiting step in cholesterol degradation to bile acids (cholesterol 7α-hydroxylase) develops slowly in the fetus, but its activity increases rapidly and the bile acid pool size expands markedly late in gestation and in the early postnatal period (reviewed by various investigators[125-128]). The role of bile acid metabolism in the lipoprotein responses to early diet is not well understood.

Summary of Postnatal Lipoproteins

In most breast-fed animals, serum cholesterol and lipoprotein concentrations increase rapidly after birth. Although serum cholesterol and lipoprotein concentrations respond to the high cholesterol and saturated fat contents of breast milk, the particular lipoprotein that increases and the timing are species-specific. The biochemical and molecular mechanisms responsible for the dramatic neonatal increases in lipoprotein concentrations are only partially understood.

Perinatal Disease and Lipoprotein Concentrations

Familial hypercholesterolemia is associated with a several-fold increase in LDL-C concentrations in the newborn infant.[18,129] Most neonates with this disorder can be identified by a cut-off value for LDL-C of 41 mg/dL (95th percentile in control infants). Although no treatment for hypercholesterolemia generally is recommended for infants younger than 2 years of age, screening cord blood from infants at risk for the disorder should be done selectively in anticipation of later follow-up.[130] Aggressive treatment may be indicated for affected children.[131] Maternal diabetes,[132] hypertension,[133] or prenatal treatment with glucocorticoids[134] increases fetal serum cholesterol, LDL-C, HDL-C, or triglyceride levels. Fetal stress and hypoxia may elevate triglyceride and free glycerol concentrations, implying triglyceride mobilization and lipolysis in these conditions.[20] Premature infants with respiratory distress syndrome (RDS) and birth weight between 1 and 2 kg have lower levels of serum apolipoprotein B and arachidonic acid than in similar-sized neonates without RDS.[135] A causal relationship among these factors has not been demonstrated. In contrast with adult-onset hypothyroidism, congenital hypothyroidism has only small effects on serum cholesterol or lipoprotein concentrations during the first month of life,[136,137] but nevertheless an inverse association between triodothyronine (T_3) and LDL-C concentrations has been reported.[138]

LONG-TERM CONSEQUENCES OF FETAL AND NEONATAL NUTRITION ON LIPOPROTEIN CONCENTRATIONS

Humans and Experimental Animals

Human fetal undernutrition and low birth weight are associated with type 2 diabetes and the metabolic syndrome (syndrome X) in adulthood and result in hyperlipidemia.[139,140] The newborn infant with a birth weight less than 6.5 pounds has a 10-fold greater risk of developing the metabolic syndrome as an adult than that in an infant whose birth weight is greater than 9.5 pounds.[139] Furthermore, persons with a perinatal history of low birth weight have higher apo B and lower HDL-C concentrations as adults.[139] Maternal malnutrition in early gestation is associated with a higher LDL-C–HDL-C ratio in adult offspring at midlife.[141] Poor maternal nutrition late in gestation results in a smaller abdominal circumference in the neonate, a condition that may reflect impaired hepatic growth and, correspondingly, altered lipoprotein metabolism. For each 1-inch decrease in abdominal circumference at birth, serum cholesterol and LDL-C concentrations in older adults are increased by approximately 10 mg/dL.[142] Therefore body proportions at birth may reflect differential timing of gestational malnutrition, resulting in diverse metabolic consequences.

Restricted fetal growth without catch-up growth in 8- to 10-year-old children is associated with increased LDL-C levels over those in children who grow normally after intrauterine growth restriction.[143] Maternal hypercholesterolemia in humans has been positively associated with serum cholesterol concentration in the fetus through the second trimester,[71] and with the extent of arterial lesions (fatty streaks) in the fetus and also in children who died of multiple causes before the age of 13 years.[144] A surprising observation is that the small differences in serum cholesterol levels of children from mothers who were hyper- or normocholesterolemic do not explain the differences in atherosclerosis.The study investigators speculated that unidentified "atherogenes" may be programmed by gestational elevation of serum cholesterol concentration.[144]

In guinea pigs, maternal undernutrition lowered the birth weight and also resulted in a higher serum cholesterol and LDL-C in adult male offspring after a dietary cholesterol challenge.[145] Similarly, pregnant rats that are food-restricted produce progeny with higher levels of serum cholesterol than in offspring of normally fed mothers.[146] In rats, dietary protein restriction during either gestation or lactation produced offspring with lower serum cholesterol, HDL-C, and triglyceride concentrations than in control animals.[147] In pregnant rabbits, diet-induced maternal hypercholesterolemia does not increase serum cholesterol concentrations in the offspring at birth up to 1 year.[73] However, the frequency of arterial lesion development, which is possibly mediated by oxidative mechanisms, is significantly greater in the offspring of hypercholesterolemic dams than in those of normocholesterolemic dams studied during gestation.[73,74] These findings in humans and experimental animals support the concept that fetal growth restriction, perinatal malnutrition, and hypercholesterolemia during gestation permanently program plasma lipoprotein concentrations or enhance the risks of diseases that cause dyslipoproteinemia. The lipoprotein changes frequently are associated with an increased risk of cardiovascular disease in adult life.[148,149]

The type of neonatal nutrition also programs or imprints adult lipoprotein concentrations. Among elderly men who as infants were breast-fed or fed cow's milk–based formulas, serum cholesterol, LDL-C, and apo B concentrations were higher than among those who as infants were weaned before 1 year.[150] By contrast, women at 32 years of age who had been breast-fed had lower plasma cholesterol than those who were bottle-fed.[151] In a study of 6-year-old children, serum cholesterol and LDL-C concentrations increased with the duration of breast-feeding up to 6 months of age.[85] Similarly, breast-feeding duration was related to serum cholesterol and the apo B–apo A-1 ratio in adolescents.[152] Short-term prospective studies in children usually have not detected deferred effects of neonatal diet on total serum cholesterol concentrations.[153,154]

Studies of breast-fed versus formula-fed experimental animals also have reported long-term differences in serum cholesterol responses to a high-cholesterol challenge diet.[155-158] Juvenile and adult baboons that were breast-fed exhibited lower HDL-C levels and higher ratios of VLDL plus LDL-C to HDL-C than those in animals fed formulas as infants.[55,112,159-161] Variation in the type of dietary fat or the cholesterol content of formulas fed to infant baboons does not affect serum cholesterol or lipoprotein concentrations after weaning.[160,161] Therefore the long-term effects of breast-feeding versus formula-feeding on serum cholesterol and lipoprotein concentrations must be elicited by factors other than the level of cholesterol or the type of fat in the infant diet.

Many of the studies in humans and nonhuman primates were stimulated by results of experiments with rodents in which differences in neonatal diet or premature weaning permanently affected the serum cholesterol response of the adult animal.[155,154] The early studies with experimental animals tested the hypothesis that high cholesterol levels in breast milk affect development of cholesterol homeostasis in adult life (i.e., the cholesterol challenge hypothesis).[155,162,163] Most experiments, however, do not support that supposition.[154] No long-term studies of humans have been performed in which cholesterol intake in the newborn infant was controlled. One recent study of infants fed formulas with or without cholesterol for 6 months does not report a significant difference in serum cholesterol or LDL-C levels before or after a cholesterol challenge at 11 to 12 months. In a parallel study, cholesterol synthesis rates of both formula groups at 4 months of age were approximately four-fold higher than in a breast-fed group.[164]

Summary: Early Nutritional Programming

These studies provide compelling evidence that nutritional deficiencies during fetal life and the types of newborn feeding program plasma lipoprotein responses and other risk factors affecting the progression of coronary artery disease in the adult. However, the molecular or metabolic basis of these nutritional programming effects on lipoprotein concentrations has not been elucidated.

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SECTION VII

Carbohydrate Metabolism

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Metabolism of Glucose and Methods of Investigation in the Fetus and Newborn

The metabolism of glucose in the fetus and newborn has been studied extensively, both in animal species and in humans, and several reviews have been published. Our ability to study glucose metabolism has improved significantly because of the following: (1) the availability of the chronic fetal preparation in sheep, in which fetal blood sampling can be done without causing major changes in the physiologic state of the fetus; (2) the availability of stable, nonradioactive isotopic tracers; and, more recently, (3) the application of molecular biology techniques and transgenic animals. In this chapter, the metabolism of glucose in the fetus and newborn is discussed, and recent developments are emphasized. Throughout the chapter, emphasis is placed on the available information in humans, supplemented when necessary by animal data.

Since the early 1980s, numerous improvements in the synthesis of stable isotopically labeled tracers and sensitive mass spectrometric methods have allowed investigators to examine glucose metabolism in the human fetus and newborn. Such studies could not be performed in the past because of the lack of availability of these tracers and the risk associated with radioactive tracers. In addition, the development of newer synthetic techniques has increased the number of isotopic tracers, which can be used simultaneously to answer complex questions. By combining these tracer methods along with the measurements of energy consumption, the metabolic fate of substrates and their contribution to overall fuel economy can be quantified.

ISOTOPIC TRACER

Atoms, other than hydrogen, are made of protons (+), electrons (−), and neutrons. Atomic number (Z) represents the number of protons or the number of electrons in an atom; that is, the number of electrons is equal to the number of protons, and the atomic weight is equal to the sum of protons and neutrons. Isotopes are atoms of an element differing from each other only in the number of neutrons. Thus, for a given atomic number (Z), more than one value of atomic weight can exist. The stability of the nucleus of the atom is related to the ratio of neutrons and protons. If neutrons are too many or too few for the number of protons, the nucleus becomes unstable. To become stable, the nucleus may undergo rearrangement that involves the release of radiation—radioactive nuclei.

The stable, nonradioactive isotopes, by virtue of the lack of radioactivity, have been particularly useful for the study of metabolism in the mother and newborn infant. However, these isotopes also have limitations.

Cost

Initially, because of limited availability, the cost was prohibitive. At the same time, the instruments for their detection, mass spectrometers, were not easily available and were expensive. Now, with relatively easy accessibility and improved sensitivity of the instruments, and with changing attitudes of society toward radioactivity, the use of stable isotopic tracers has become popular, and the cost has continued to decrease.

Natural Abundance

The stable isotopes are naturally occurring nuclides and therefore have a certain background or natural abundance. This is in contrast to the radionuclides, the natural abundance of which is negligible. Because of this background abundance, and also in part related to the sensitivity of the mass spectrometry, the amount of tracer required for the study of metabolism is more than that used with radioactive tracer.

IDEAL TRACER

Although one could define an ideal tracer in many different ways, the following are some of the most important considerations. Its biologic behavior should be the same as that of the parent compound; that is, it is used in the same manner as the compound traced. This is important for both radioactive and nonradioactive stable tracers. Radioactive-labeled proteins, such as iodine-125 (^{125}I)–labeled albumin, are not necessarily metabolized in the same manner as for the native protein. In fact, the labeling may damage or denature the protein.

Stable isotopes, conversely, have their own chemical differences described as isotopic fractionation or isotopic effect. In this process, the isotope behaves differently from the native atom. Two types of isotopic fractionation are important:

1. Temperature: These fractionations are significant in geologic studies in which the changes in the carbon-13 (^{13}C)/^{12}C and oxygen-18 (^{18}O)/^{16}O ratio have been used to study the changes in the earth's temperature.
2. Kinetic and chemical fractionation: These fractionations are the result of isotopic discrimination by various enzyme systems; that is, the labeled compound is not as rapidly metabolized as the unlabeled compound. Such fractionation will result in the accumulation of the labeled compound in the metabolic pool and will potentially result in errors in measurements. In nature, isotopic fractionation results in differing isotopic composition of nutrients; for example, glucose obtained from sugar cane has different ^{13}C enrichment compared with glucose obtained from beet sugar.[1,2] The ^{13}C abundance of lipid fractions in several organisms is lower than that of carbohydrates and proteins.[3,4] The basis for these differences has been attributed to isotopic discrimination at certain key enzymatic steps involved in the synthesis of these compounds.[1] DeNiro and Epstein[5] showed that the low ratio of ^{13}C/^{12}C of lipids synthesized

by *Escherichia coli* grown in minimal media is the result of isotopic fractionation during the oxidation of pyruvate to acetyl coenzyme A.[5] It is important to recognize these isotopic fractionations when isotopic tracers are used to study metabolism. In general, it is accepted that more heavily labeled tracers such as those labeled with multiple isotopes are likely to be discriminated more than the lightly labeled tracers. In relation to stable isotopes, isotopic fractionation is probably more common with deuterium than with ^{13}C and nitrogen-15 (^{15}N).

The quantity of tracer used should be negligible; therefore it should have no or minimal pharmacologic effects. This requirement is easily met by the radiolabeled tracers, in which the background radioactivity is essentially zero. In contrast, stable isotope tracers that have a significant background natural abundance have to be infused in somewhat larger quantities that are not negligible. The amount of tracer infused is determined by the ability of the analytic instrument to measure the isotopic enrichment above the background in biologic samples. With the newer instruments and better analytic techniques, several of these problems have been resolved; therefore, in most instances, enrichments in the range of 0.5% to 1% above the background can be easily measured. Therefore the isotopic tracer can be infused at rates approximating 1% of the turnover rate of the "tracee." With multiple labeled compounds, in which the background abundance is much lower, even lower rates of tracer infusion can be used and still achieve excellent results.

Isotopic tracers are used in vivo to quantify the rate of appearance and disappearance of a substrate, such as glucose and amino acids, or to quantify the utilization and metabolic fate of the substrate, such as oxidation, or the contribution of a substrate to another compound: precursor-product relation.

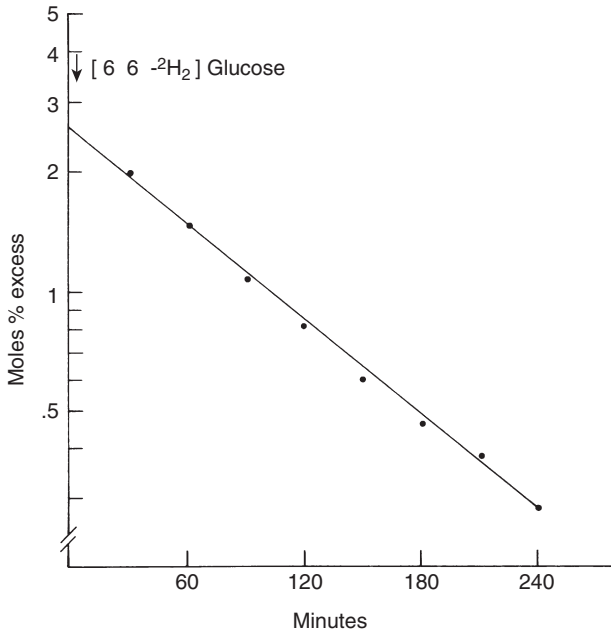


Figure 48-1 Changes in deuterium enrichment of plasma glucose after a single bolus injection of [6,6-$^2H_2$]glucose in a physiologically healthy man. The rate of glucose appearance is quantified from the rate of decrease (dilution) in the plasma glucose enrichment and the pool size. The latter is quantified from the concentration of glucose, and the volume of distribution is measured by extrapolating the disappearance curve to time zero.

Quantification of the Rate of Appearance and Disappearance of a Substrate

The usual methods involve the administration of the isotopically labeled tracer either as a single bolus (Figure 48-1) or as a constant-rate infusion (Figure 48-2). In both instances, the dilution of the tracer in the blood is estimated by measuring the enrichment or specific activity (in case of radioactive tracers) in the blood or plasma. The dilution of the tracer is the function of appearance of the substrate, and the disappearance or utilization of the substrate has no impact on the enrichment because the same proportion of the tracer and tracee will be used. This is a fundamental concept in the tracer studies. The following are the definitions of certain terms used in tracer studies.

Concentration: Amount of substrate per unit of blood or plasma, micromoles per milliliter or milligrams per milliliter.

V: Volume of distribution of the substrate or the space in which the substrate is distributed, milliliters per kilogram of body weight.

Q: Quantity (moles or grams) of the substrate in the system (body), that is, the size of the substrate pool or pool size, which is equal to the concentration of the substrate times

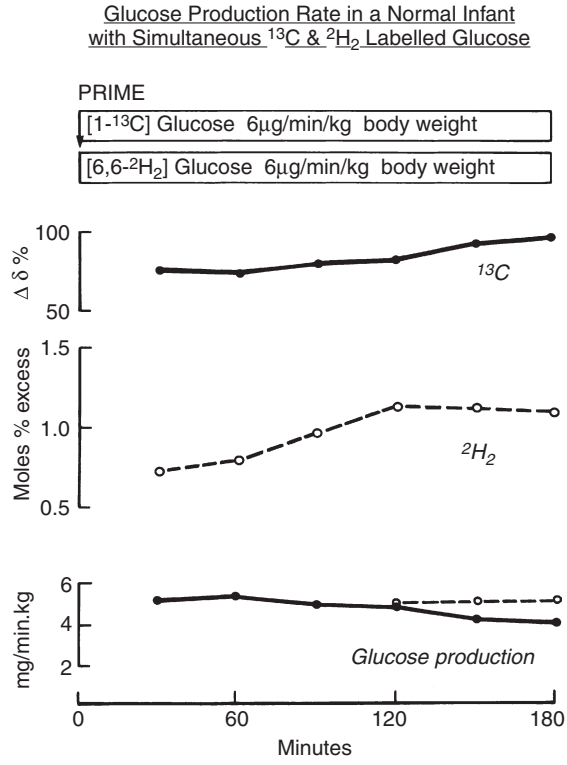


Figure 48-2 The constant-rate tracer infusion method. As shown, the deuterium-labeled glucose was infused at a constant rate whereas [1-^{13}C]glucose was administered as a prime–constant-rate infusion. Because of the tracer prime, an early plateau plasma glucose ^{13}C enrichment was achieved. However, a slow continuous increase in enrichment was also seen because of tracer carbon recycling. As shown, the measured rate of glucose production will decrease as the tracer recycling becomes more evident later in the study. *(From Kalhan SC, Bier DM, Savin SM, Adam PA: Estimation of glucose turnover and ^{13}C recycling in the human newborn by simultaneous [1-^{13}C]glucose and [6,6-$^2H_2$]glucose tracers,* J Clin Endocrinol Metab *50:456, 1980. Copyright The Endocrine Society.)*

volume of distribution, that is, moles (or grams)/milliliter × milliliters/kilogram = moles (or grams)/kilogram.

Ra: Rate of appearance of the substrate (tracee) in the compartment under study. In most instances, it is the plasma or extracellular fluid compartment, micromoles or milligrams per minute.

Rd: Rate of disappearance of the substrate from the compartment under study, micromoles or milligrams per minute. Disappearance is often used as synonymous with utilization. However, utilization is implied in the broad sense of overall utilization and does not necessarily imply a specific pathway.

Steady-state system: The system is said to be in a steady state when the input Ra is equal to output Rd and the concentration remains constant. Conversely, when these parameters are changing, it is called nonsteady state.

Turnover: Rate of turnover of the pool under steady-state conditions, that is, when the concentration is constant: Ra = Rd = Turnover, micromoles per minute or milligrams per minute.

Turnover time: Time it takes for the entire pool to turn over, which depends on the size of the pool and the turnover rate: Q (moles)/Rate of turnover.

MCR: Metabolic clearance rate, that is, the volume of plasma that is completely cleared of the substrate in a unit time: Rate of disappearance Rd (micromoles per minute)/Concentration (micromoles per milliliter) = Milliliters per minute. It should be called plasma clearance rate rather than metabolic clearance rate. It does not necessarily mean that the substrate has been *metabolized.*

Two more terms need to be known in relation to tracers:

Specific activity (SA): Radioactivity that is specific to a compound or substrate, expressed in relation to the weight or molecules of the substrate, that is, Radioactivity (disintegrations per minute, or dpm) per mole or gram of substrate = dpm/mole or gram.

Enrichment: Used in relation to stable isotopic tracers to describe the magnitude by which that particular isotope has been increased (enriched) above the background. Enrichment has also been used as synonymous with atom percent excess, that is, how many labeled atoms are present in excess of the background. For practical purposes, enrichment corresponds to specific activity with certain limitations. In relation to stable isotopic tracer, often the tracers are measured as molecules rather than atoms, and therefore they are described as moles percent excess, that is, the percentage of labeled molecules in excess of the background.

Further, the labeled molecules are designated by the number of labeled atoms added to the parent molecules (m), such as m+1, m+2, and m+3 (or m_0, m_1, m_2, m_3). Therefore enrichment is described as moles percent excess of these labeled molecules (m+3) enrichment or (m+1) enrichment.

Finally, a tracer is called a *reversible* or *recycling tracer* when, after its disappearance or utilization, it reappears in the plasma. The reappearance results in a second, not easily measurable, source of tracer, and it leads to higher enrichments and underestimation of kinetics. In contrast, when a tracer is lost during its utilization, it is called a *nonrecycling tracer*. In general, when the tracers are labeled with carbon, whether ^{13}C or ^{14}C, the carbon atoms are likely to be reincorporated (recycle) into newly synthesized substrate; in contrast, when the tracers are labeled with hydrogen ^{2}H, ^{3}H, the hydrogen atoms are lost mostly into water (H_2O) during the metabolism of the substrate and are termed *nonrecycling tracers*. Before selecting a tracer to study the kinetics of a tracee, a knowledge of the pathways of metabolism of the tracee can help in the selection of a particular type of label.

During a bolus injection technique, the labeled tracer is administered at zero time, and the rate of change in enrichment or specific activity is measured (see Figure 48-1). Extrapolation of the decay curve to zero time gives an estimate of the pool size and the volume of distribution of the tracer, and the turnover time is calculated from the rate constant of the disappearance curve.

The constant-infusion technique (see Figure 48-2), evaluated in detail by Altszuler and associates,[6] is a commonly used method to quantify tracee kinetics. Altszuler and colleagues further improved the technique by administering the priming dose of the tracers to achieve early plateau. Thus it should be called the prime–constant-rate technique. After a priming dose, the tracer is administered at a constant rate throughout the experiment. The size of priming dose depends on the pool size of the tracee and the rate of administration of the tracer. This relation is expressed as follows:

$$\begin{aligned}(\text{Prime}/\text{Pool}) &= (\text{Rate of infusion of tracer} \\ &\quad /\text{Rate of appearance of tracee Ra}) \\ &= \text{Enrichment or specific activity}\end{aligned}$$

The turnover rate or the rate of appearance and disappearance is calculated from the final asymptote of the enrichment curve and the rate of infusion of the tracer.[6,7]

The methods described earlier assume that the tracer mixes with the tracee in a single compartment instantaneously. Although such an assumption may hold true for a system that is turning over rapidly, it is not necessarily true for slow turnover systems, such as body bicarbonate pool, and it may lead to significant errors. Numerous multiple compartment systems have been used to fit the data most precisely under these circumstances.[8-10] However, at times the physiologic significance of such compartments is not easily understood.

Another problem studied in detail relates to the site of tracer administration and sampling in turnover studies.[11-14] This is particularly true in relation to compounds that are produced by multiple organs and tissues, such as lactate. Thus a venous sample obtained from a particular vein will not only represent the mixed blood from the body but will also be influenced by the metabolism of the tissues drained by that particular vein. Certain modifications have been proposed, such as retrograde venous sampling, arterial blood, or arterialized venous blood.[12,13] All these have their own limitations and must be recognized during data reduction and interpretation.

Finally, when the concentration of substrate is changing as a result of perturbations, the system is described as being in a *nonsteady state*. Under these conditions, a different set of assumptions and a different data reduction method are employed. The most commonly used method of data reduction is that described by Steele.[15] These equations have been subsequently modified and validated by several investigators.[16,17]

Quantification of Metabolic Fate of the Substrate

Oxidation to carbon dioxide (CO_2) and the contribution of a substrate to another: precursor-product relationship are two examples of reactions that can help quantify the metabolic fate of the substrate. This is estimated by the appearance of tracer isotope, usually carbon, in the product. However, the mere appearance of the isotope in the product does not necessarily imply net conversion of the precursor into the product.[18] It may simply represent an exchange of the isotope during biochemical reactions. Second, during the sojourn of the tracer from the precursor to the product, the tracer may be diluted by intermediary compounds. Thus there would be an underestimation of the contribution of the precursor to the product. The precursor-product relationship is quantified by measuring the turnover of the precursor and product by separate isotopically labeled tracers and by measuring the rate of appearance of the tracer in the product.[9] These

concepts of precursor-product relationship are best illustrated with use of glucose as a model system.

Glucose (precursor) is oxidized to CO_2 (product) by first conversion to pyruvate, which enters the tricarboxylic acid (TCA) cycle via acetyl coenzyme A. During oxidation of pyruvate to CO_2, the three carbons of pyruvate do not appear in CO_2 to the same magnitude. Carbon-1 of pyruvate appears in CO_2 100% during pyruvate's conversion to acetyl coenzyme A by pyruvate dehydrogenase. Carbon-2 and carbon-3 of pyruvate, which form carbon-1 and carbon-2 of acetyl coenzyme A, enter the TCA cycle, and neither of these carbons appears in CO_2 during the first spin of the cycle. It is only in the subsequent turns of the TCA cycle that carbon-2 and carbon-3 appear in CO_2 but not in equal amounts.[19,20] Wolfe and Jahoor[21] showed, in normal healthy adults by infusing labeled acetate, that only 80% of carbon-1 and 50% of carbon-2 of acetate appeared in CO_2 during isotopic steady state. On the basis of this and other studies, it can be concluded that during glucose-pyruvate oxidation, only 80% of the tracer carbon will appear in CO_2, whereas the remainder is lost via exchange in the TCA cycle. This loss of tracer or the lower appearance of tracer in CO_2 will result in underestimation of oxidation of glucose.

Another example of the loss or exchange of tracer is the synthesis of glucose from pyruvate via gluconeogenesis. During gluconeogenesis, pyruvate is first converted to oxaloacetate, a common intermediate between gluconeogenesis and the TCA cycle. Therefore when carbon-labeled precursor is administered for the estimation of gluconeogenesis, some of the tracer will be lost by exchange in the TCA cycle at the oxaloacetate level. Thus the enrichment or the specific activity of the immediate precursor of glucose, that is, phospho*enol*pyruvate, will be less than that of pyruvate, and it will result in a lower estimate of gluconeogenesis.[22] This problem was resolved by Landau and colleagues[23] by using deuterium-labeled H_2O, which, in turn, labels the pyruvate pool. Because this method results in constant labeling of pyruvate, and phospho*enol*pyruvate without loss of tracer, the appearance of deuterium in glucose can be used to quantify the contribution of gluconeogenesis to total glucose production accurately.

Finally, all these methods of quantification have their own limitations and are near approximations of the actual turnover rates. The assumptions involved in each experimental technique should be recognized when such estimations are done in biologic systems.

MEASUREMENTS OF ENERGY CONSUMPTION

Measurements of energy consumption rate and CO_2 production have become integral components of metabolic studies. Estimation of energy consumption and CO_2 production rate help in the partitioning of the contribution of various substrates to the overall energy utilization. In addition, most studies using carbon-labeled tracers also estimate the isotopic enrichment of CO_2 either in the blood or in the expired CO_2 to quantify the oxidation rate of the labeled substrate.[24,25] The major contribution of these estimates has been the ability to accurately quantify as many components of oxidative metabolism as possible and to develop as complete a picture as possible of the fuel metabolism in vivo. Two commonly used methods are briefly discussed here: open-circuit indirect respiratory calorimetry and the doubly labeled H_2O method.

Open-Circuit Indirect Respiratory Calorimetry

In this system, as shown in Figure 48-3, a plastic hood or canopy is placed over the subject's head, and a suction pump is used to draw air through the hood. The air exiting from the hood is mixed by passing through a baffled mixing chamber. The concentration of O_2 and CO_2 is measured in the mixed air by an appropriate device, either an O_2 and CO_2 analyzer or a mass spectrometer, such as the PerkinElmer medical gas analyzer MGA-1100 (PerkinElmer, Pomona, CA). The flow of air through the system is regulated by flow controllers and is measured by a flowmeter. Commercially available servocontrolled flow regulators are appropriate. The rate of O_2 consumption and CO_2 production is measured by multiplying the gradient of these gases across the face with the rate of flow of air and applying Haldane transformations.[26-29] The latter is based on the finding that the volume of air expired is less than the volume of air inspired and that nitrogen is not used or produced by the body. The transformations therefore use the concentration of nitrogen in the inspired and expired air to estimate the inflow of air into the system. The outflow is measured by the flowmeter. The open-circuit method has been validated over many years in large numbers of studies in adults, children, and newborn infants,[30-32] and its limitations have been examined.[33]

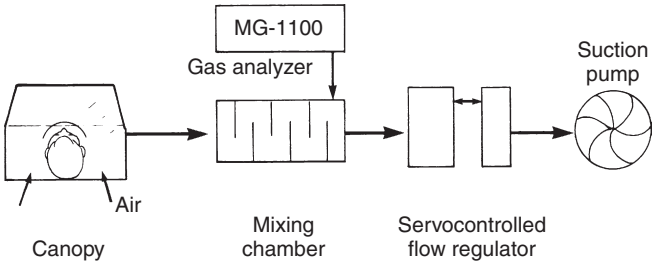


Figure 48-3 Open-circuit indirect respiratory calorimetry. A canopy is placed over the subject's head, and air is drawn through it by a suction pump. Between the canopy and the pump are placed a mixing chamber, a gas analyzer to measure the O_2 and CO_2 concentrations, and a flowmeter and regulator to measure and control the flow rate. For details, see text.

Doubly Labeled ($^2H_2{}^{18}O$) Water Method

The doubly labeled water method of measuring energy consumption is based on the estimation of the rate of CO_2 elimination from the body. It has become a popular method because of several distinct advantages:

1. It can be applied to free-living individuals; that is, the subject need not be restrained or confined to bed.
2. CO_2 elimination is measured over a prolonged period, hours to days, and thus it includes a composite measure of energy consumption throughout the entire period.
3. The method is simple and noninvasive. It requires administration of a single dose of labeled H_2O and obtaining a few samples of urine, or saliva, or blood to measure the changes in isotopic enrichment over several days. It can be used in adults, children, and infants with ease, without requiring any major modifications in equipment.

This technique was first described by Lifson and associates[34] to study energy metabolism in small animals and was extensively evaluated in humans, both adults and newborns, by Schoeller and colleagues[35-38] and by other investigators.[39-42] The method is based on the following principles: When H_2O labeled with deuterium (^2H) and ^{18}O is administered to a subject, it equilibrates in all the body H_2O compartments. Then the tracer is eliminated at the same rate as the body H_2O. The deuterium is eliminated from the body as H_2O (^2H_2O). Because oxygen (as well as the tracer ^{18}O) equilibrates rapidly between H_2O and CO_2 in the presence of carbonic anhydrase, the ^{18}O is eliminated both by CO_2 and H_2O:

$$CO_2 + H_2^{18}O \rightleftharpoons H_2CO^{18}O \rightleftharpoons H_2O + C^{18}O$$

Thus the rate of loss of ^{18}O represents the elimination rate of CO_2 plus H_2O, whereas the rate of loss of deuterium from body water represents only the loss of H_2O. Therefore the difference in the turnover rates of oxygen and hydrogen of H_2O is equal to the CO_2 elimination rate. The mathematical calculations, the limitation of the technique, and related analytic problems were described by Schoeller, and the reader is referred to the excellent review by him.[38]

The partitioning of fuel oxidation is estimated from the stoichiometry of the three oxidative reactions.[26,27]

$$1\ \text{Glucose} + 6\ O_2 = 6\ CO_2 + H_2O + 673\ \text{kcal}$$

$$\text{Respiratory quotient} = \frac{6\ CO_2}{6\ O_2} = 1.0$$

$$1\ \text{Palmitate} + 23\ O_2 = 16\ CO_2 + 16\ H_2O + 2398\ \text{kcal}$$

$$\text{Respiratory quotient} = \frac{16\ CO_2}{23\ O_2} = 0.70$$

$$1\ \text{Amino acid} + 5.1\ O_2 = 4.1\ CO_2 + 0.7\ \text{urea} + 2.8\ H_2O + 475\ \text{kcal}$$

$$\text{Respiratory quotient} = \frac{4.1\ CO_2}{5.1\ O_2} = 0.8$$

Because nitrogen is approximately 16% protein by weight, the amount of protein oxidized can be estimated from the urinary nitrogen excretion:

$$\text{Protein oxidized} = 6.25 \times \text{Urinary nitrogen}$$

The contribution of glucose and fat to oxidative metabolism can be estimated from the measured respiratory quotient corrected for protein oxidation.

The limitations in estimation of substrate oxidation by respiratory gas exchange have been discussed by numerous investigators.[20,26,27,43,44] Respiratory gas exchange measures net utilization or oxidation of the substrates, such as glucose or fat, irrespective of the intermediate steps. For example, if glucose is converted to fatty acids and the fatty acids are oxidized, it will be estimated as glucose oxidation only.[44] Respiratory gas exchange as commonly used and as described earlier does not include other metabolic processes such as gluconeogenesis and lipogenesis.[26,27] In addition, no net oxidation of fat is assumed to occur at respiratory quotients of less than 1.0.[44] At respiratory quotients greater than 1.0, a calculated negative rate of lipid oxidation is assumed to represent lipid synthesis.[44] Finally, respiratory gas exchange also assumes substrate homogeneity, that is, all fatty acids are represented by palmitate with a respiratory quotient of 0.72, and similarly all amino acids and proteins are assumed to have the same respiratory quotient. These limitations should be recognized when data from respiratory calorimetry measurements are interpreted.[19,20]

GLUCOSE METABOLISM IN THE FETUS

Maternal-Fetal Glucose Relationship

In most species studied, including humans, the fetal plasma glucose concentration has been observed to be significantly lower than that observed in a simultaneously obtained blood sample from the mother.[45,46] In addition, a significant linear relationship has been observed between the prevailing glucose levels in the mother and those in the fetus.[47,48] In humans, the fetal blood samples had been obtained mostly at term gestation from fetal scalp blood or at the time of delivery, by cesarean section, from a segment of doubly clamped cord.[47-49] However, because of the effects of labor and delivery on maternal and fetal circulation, these data had been questioned in regard to their representation of fetal metabolism. The technique of percutaneous umbilical blood sampling permits access to fetal circulation without causing any significant perturbations to the physiology of the fetus. Fetal blood samples have been obtained in midgestation (18 to 21 weeks) by fetoscopy or late in gestation by ultrasound visualization of the umbilical vessels.[45,46] Some interesting observations have been made. Bozzetti and colleagues[46] observed a significant correlation between maternal arterialized venous blood glucose and fetal umbilical venous glucose concentrations in 14 fetuses examined between 17 and 21 weeks gestation, with the fetal glucose concentration lower than that in the mother. Of interest were the findings that in 7 cases, the maternal glucose concentration was less than 4.4 mmol/L (79 mg/dL). These investigators found that, at maternal glucose concentrations lower than 4.4 mmol/L (4.12 ± 0.11 mmol/L), fetal umbilical venous glucose concentration may exceed the maternal glucose concentration (4.4 ± 0.06 mmol/L) and may be independent of it. Similar suggestions have been made by other investigators when fetal blood samples have been obtained early in gestation.[45] The significance of these findings remains to be defined. However, the higher levels observed were in the umbilical venous sample, whereas the umbilical arterial levels may have been much lower. Later in gestation, and as shown in Figure 48-4, most investigators have observed a close linear relationship between maternal and fetal glucose whether the samples were obtained by percutaneous umbilical blood sampling, by scalp sampling during labor, or from umbilical vessels at the time of elective cesarean section.[47-51] This linear relationship between maternal and fetal glucose concentrations has been observed during euglycemia, during hyperglycemia induced by glucose infusion to the mother, and during hypoglycemia induced by insulin infusion to the mother.

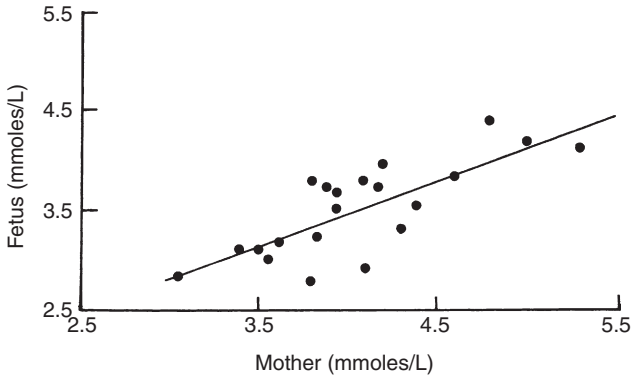


Figure 48-4 Relationship between maternal and fetal glucose concentrations. Simultaneous maternal venous and umbilical venous blood samples (via percutaneous umbilical blood sample) were obtained in the third trimester of pregnancy. *(Data from Ashmead GG, Kalhan SC, Lazebnik N, et al: Maternal-fetal substrate relationships in the third trimester in human pregnancy,* Gynecol Obstet Invest *35:18, 1993.)*

Glucose Transport Across the Placenta

On the basis of data of in vivo studies in animals and isolated perfused placenta, it can be concluded that glucose is transported across the placenta along a concentration gradient by a facilitated, carrier-mediated diffusion.[52,53] Furthermore, this process is stereospecific. Although the net transfer is from the mother to fetus, tracer isotope studies have demonstrated a bidirectional diffusion of glucose, that is, from the mother to the fetus and vice versa.[54] Studies of human placenta perfused in vitro have shown that the placental glucose uptake from the maternal perfusate and glucose transfer to the fetal perfusate is linearly correlated with maternal glucose concentration up to 20 mmol/L. Beyond this level, glucose is transferred to the fetal perfusate by simple diffusion.[55] Metabolically, the placenta is a very active organ, and glucose transfer to the fetus represents only 40% to 50% of the total glucose uptake by the placenta.[56] The remaining glucose is used by the placental tissue for oxidation, stored as glycogen, or

converted to lactate, which is then used by the mother and the fetus.[56-59] As gestation advances, an increase in uterine blood flow results in an increased delivery of glucose to the fetus.[60] Wilkening and colleagues[61] showed that, in the presence of a constant blood glucose concentration in the pregnant sheep, a reduction of uterine blood flow (from 600 to 300 mL/minute per kilogram of fetus) did not have any effect on fetal glucose uptake or fetal arterial glucose concentration. However, any further decrease in uterine blood flow resulted in decreased fetal glucose uptake and variable fetal arterial glucose concentration. Conversely, a reduction in umbilical blood flow by ligation of one of the umbilical arteries resulted in a decrease in fetal glucose uptake and fetal growth restriction.[62]

Sources of Glucose for the Fetus in Utero

Although simultaneous changes in fetal glucose concentration correspond to changes in maternal glucose concentration and are suggestive of fetal dependence on maternal glucose pool, they do not unequivocally indicate that the fetus in utero cannot mobilize hepatic glycogen; that is, the fetus does not release glucose from the glycogen stores. To determine whether the fetus in utero can regulate glucose metabolism independent of the mother under circumstances in which the maternal metabolism is compromised, two different methods have been applied. The first involves the infusion of glucose labeled with isotopic tracers, to either the mother or the fetus, and examines the isotopic enrichment or specific activity in the maternal and the fetal compartments.[50,54] In humans, this approach could be applied only in women undergoing elective cesarean section (and therefore minimal maternofetal perturbation) and examining the fetal compartment by obtaining an umbilical arterial and venous blood sample at the time of birth.[50] The second approach, commonly used in animal studies, combines the isotopic tracers with placement of catheters in fetal circulation. Using these techniques, in large as well as small animals, several groups have measured the umbilical blood flow, fetal glucose uptake, fetal glucose utilization, and oxidation.[54,63-68]

Studies in Humans

Only one study has examined the sources of glucose for the human fetus at term gestation.[50] Glucose labeled with ^{13}C was infused at a constant rate into pregnant women undergoing elective cesarean section for clinical indications after an overnight fast. At delivery, simultaneous blood samples were obtained from the maternal vein, cord artery, and cord vein. As shown in Figure 48-5, the ^{13}C enrichment of maternal vein, cord vein, and cord artery was similar in healthy women and women with gestational diabetes. These data indicate that the fetal glucose pool was in equilibrium with the maternal glucose pool and that maternal glucose was the only source of glucose for the human fetus at term gestation after a brief overnight fast. If the fetus had been producing (releasing) glucose, a lower [^{13}C]glucose enrichment would have been seen in the fetal blood. Whether such a relationship is also present early in gestation is not known. However, combining isotopic tracer infusion with percutaneous umbilical blood sampling permits an answer to this question at different gestations.

Marconi and colleagues[69] examined whether the fetus could produce glucose in nine patients with pregnancy complicated by intrauterine growth restriction between 29 and 35 weeks gestation. They administered glucose ^{13}C (ul) tracer to the mother and measured the isotopic enrichment in the maternal blood and in fetal blood obtained by cordocentesis. They observed no difference in tracer enrichment in the maternal and fetal compartments, again confirming that even in pregnancies with fetal growth restriction, the maternal glucose is the only source of glucose for the fetus.

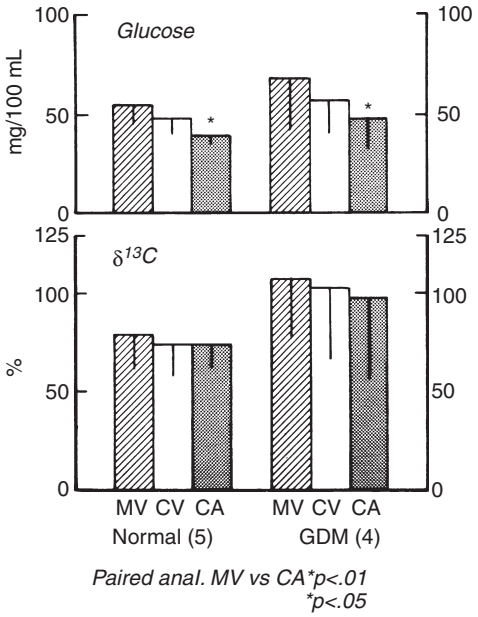


Figure 48-5 Plasma glucose and glucose ^{13}C enrichment (^{13}C) during [1-^{13}C]glucose infusion in maternal venous *(MV),* cord arterial *(CA),* and cord venous *(CV)* blood. Five healthy mothers and four mothers with gestational diabetes *(GDM)* were infused with [1-^{13}C]glucose tracer for at least 2 hours before elective cesarean section. Simultaneous maternal venous and umbilical blood samples were obtained at delivery. *(Reproduced from the* Journal of Clinical Investigation, *1979;63:388, by copyright permission of the American Society for Clinical Investigation.)*

Studies in Animals

Examination of the relationship between maternal and fetal glucose pool by measuring maternal and fetal blood glucose concentrations and by infusing isotopic tracer solely in the maternal compartment (as was done in human studies described earlier) provides only qualitative information regarding maternal and fetal glucose pools. To quantify the magnitude of glucose flux from the mother to the fetus, that is, umbilical glucose uptake and fetal glucose utilization and simultaneous tracer isotopic infusion to the mother and the fetus, measurements of umbilical arterial and venous gradient along with quantification of umbilical blood flow are required. The details of these techniques were described by Krauer and co-workers,[57] Anand and colleagues,[66] and Hay and associates.[67] Because acute perturbations, such as placement of vascular catheters, surgery, or anesthesia, result in major alterations in fetal metabolism, sufficient time must be allowed for the fetus to recover and return to a "normal" basal state. Such studies are not ethically possible in humans.

The sources of fetal glucose have been examined in animal species. The most commonly used animals have been the rat and the sheep. In the pregnant rat examined during the last 3 days of pregnancy (normal rat pregnancy is 21 days) and after a 16-hour fast and with the animal under anesthesia, the fetal glucose specific activity during a constant-rate maternal [^{14}C]glucose tracer infusion was consistently lower than that in the mother, a finding suggesting a fetal source of blood glucose.[63-65] However, such studies should be interpreted with caution because a 16-hour fast in a pregnant rat may be equivalent to a prolonged fast in larger animals.

In contrast to studies in rats, studies in sheep fetuses have shown that, under basal conditions during maternal euglycemia, maternal glucose is the only source of fetal glucose, and fetal

glucose production under these circumstances either does not occur or is negligible.[66,67] The only exception is the study of Hodgson and colleagues,[68] done with the animals in a fed state, which showed that the fetal uptake of glucose from the mother accounted for only approximately 70% of the fetal glucose pool. The reason for this discrepancy is not clear and may be related to feeding in a ruminant mammal. However, it is now accepted that, in most species during basal state, the fetal glucose pool is entirely derived from the maternal glucose pool, and, under these circumstances, the fetus late in gestation does not release endogenous glucose.

Gluconeogenesis in Utero

Because in certain animal studies, as stated earlier, the fetus appears to be producing endogenous glucose, and because fetal utilization of placentally produced lactate appears to be a significant component of fetal carbohydrate metabolism, some investigators examined the quantitative contribution of fetal gluconeogenesis. The key gluconeogenesis enzymes, pyruvate carboxylase (PC), phospho*enol*pyruvate carboxykinase (PEPCK), and fructose diphosphatase, are present from early fetal life onward in humans (discussed in the following section).[70,71] Hepatic cytosolic PEPCK appears for the first time after birth in the rat.[72] In humans, isolated liver tissue (explants) early in gestation can incorporate [^{14}C]alanine and [^{14}C]pyruvate into glucose.[73,74] The recycling of the glucose carbon in isolated human fetal liver preparation, however, is negligible.[75] These observations in humans are similar to those seen in guinea pigs and sheep.[76-80] Gleason and associates[81,82] and Levitsky and colleagues[83] quantified gluconeogenesis from [^{14}C] lactate in chronically catheterized sheep and baboon fetus. In sheep, although a significant amount of [^{14}C]lactate was taken up by the liver, only a negligible quantity of the tracer was incorporated into glucose, and the lactate to O_2 quotient was 2:1. These studies suggest that lactate in sheep fetal liver is probably used for nonoxidative metabolic processes, such as a source for glycogen.[81,82] In contrast to sheep, the baboon fetus had slightly greater incorporation of tracer lactate into glucose.[83] Finally, Palacin and colleagues[84] demonstrated that, even after 48 hours of maternal food deprivation, no gluconeogenesis was demonstrable from radioactively labeled glycerol or alanine in the rat fetus at 21.5 days gestation. Thus, in the mammalian species studied, even though the potential for hepatic gluconeogenesis exists, the gluconeogenic capacity is not expressed in utero under unperturbed circumstances, and the contribution of gluconeogenesis from lactate, pyruvate, or alanine to glucose is quantitatively negligible.

Expression and Regulation of Gluconeogenic Enzymes

The initiation of gluconeogenesis after birth is the result of a number of orchestrated events such as expression of key regulatory enzymes as a result of changes in hormonal and cytokine concentration and changes in hepatic redox state. The gluconeogenic pathway involves both the mitochondrial and cytosolic enzymes. Among these, four enzymes, PC, PEPCK, fructose-1,6-diphosphatase, and glucose-6-phosphatase (G-6-pase) catalyze irreversible reactions in the pathway. The developmental expression and regulation of these enzymes are discussed in the following section.

Pyruvate carboxylase: PC, a biotin-dependent mitochondrial enzyme, catalyzes the initial step in the gluconeogenic pathway, the adenosine triphosphate–dependent carboxylation of pyruvate to form oxaloacetate. The developmental expression of PC during the perinatal period in the rat liver and kidney have been quantitated by using enzymatic reverse transcriptase polymerase chain reaction and by immunologic methods. The data show that the activity of PC in the fetal rat liver is undetectable on day 20 of gestation (gestation in rat approximately 21 days), but increases to 8.23 ± 0.40 mU • mg^{-1} protein a day before birth. A further increase occurs in the activity in the immediate postnatal period, reaching a peak at 12 hours after birth (59.95 ± 9.17 mU • mg^{-1} protein). Thereafter a decline in the activity takes place followed by a further increase over the next few days, reaching a maximal activity on the sixth postnatal day (254 ± 16 mU • mg^{-1} protein). The activity of PC decreases to the adult values (86 ± 2.9 mU • mg^{-1} protein) after weaning. The in vitro activity of PC correlates with the amount of protein detected by the Western blot method. The transcription of PC gene parallels its activity during the perinatal period. In contrast to the developmental pattern in the liver, the PC activity in the fetal kidney is undetectable until the sixth postnatal day, suggesting a minimal or no contribution of kidney to gluconeogenesis in the perinatal period. PC activity in the kidney remains at low levels until after weaning, when it reaches the adult values. The rise in glucagon and catecholamines and decline in the levels of insulin in the immediate period after birth further increase the expression of the PC gene after birth.[85] Biotinylation of carboxylases is important for their action. Biotin deficiency during pregnancy in the mice has been shown to reduce the activity of PC in both the maternal and the fetal liver.[86]

Phosphoenolpyruvate carboxykinase catalyzes the guanosine triphosphate–dependent decarboxylation of oxaloacetate to phospho*enol*pyruvate. Two forms of PEPCK have been identified in mammalian species: the mitochondrial form (PECPK-M) and the cytosolic form (PEPCK-C). The mitochondrial form, present in larger proportion in certain species, (e.g., guinea pigs and humans) may be important in gluconeogenesis and lipogenesis particularly in the fetus in utero. The cytosolic PEPCK is the predominant form in the rat. The perinatal development of PEPCK-C in the rat shows that the messenger RNA (mRNA) levels are undetectable in the fetal liver. The gene is induced for the first time at birth, and the activity of PEPCK-C increases to 2.61 units • g^{-1} of liver within 2 to 3 hours after birth. A number of transcription factors, glucoregulatory hormones, and hepatic redox state in concert regulate the transcription of the gene for PEPCK-C.

An extensive analysis of the PEPCK-C gene promoter showed a number of binding sites for the transcription factors. Among these binding sites, CRE-1 region (cyclic adenosine monophosphate [cAMP] response element) is crucial for the expression of PEPCK-C gene during the perinatal period. The transcription factors–CAAT/enhancer binding proteins (C/EBP) bind to the CRE region of the PEPCK-C promoter. The developmental expression of C/EBPs (C/EBPα and C/EBPβ) follows closely with the expression of PEPCK-C gene. The important role of C/EBPα in the cAMP induction of PEPCK-C gene expression in the liver was demonstrated by undetectable PEPCK-C mRNA levels in the mice homozygous for a deletion in the C/EBPα gene. These data suggest that C/EBPα is required for the induction of the dormant PEPCK-C gene at birth. In addition to the transcription factors, hormonal changes associated with birth, that is, an increase in glucagon and catecholamines and decline in insulin levels, and increase in the intracellular concentration of cAMP induce PEPCK-C gene transcription. The inhibitory effect of insulin on PEPCK-C has been shown to be mediated by the transcription factor, SREBP-1c (sterol regulatory element binding protein 1c). Finally, the expression of PEPCK-C gene in the oxygen-rich periportal area of the liver, and its induction at birth associated with a change in the hepatic redox state, also suggests an oxygen-dependent modulation of PEPCK-C gene transcription. Putative sites in the CRE region of the PEPCK-C promoter were identified, indicating the role of oxygen in the PEPCK-C gene transcription.[87,88]

Fructose-1,6-diphosphatase catalyzes the hydrolysis of fructose-1,6-diphosphate to fructose-6-phosphate and inorganic phosphate. It is expressed in the liver, kidney, and pancreatic

beta cells. In the liver, fructose-1,6-diphosphatase is involved in both glycolysis and gluconeogenesis, and it has been suggested to participate as a component of metabolic sensing mechanism in the pancreas.The activity of fructose-1,6-diphosphatase is very low in the fetal period (less than 1 unit), increases after birth, peaks between 8 and 10 days after birth (10 units), and declines to reach the adult values at 25 to 30 days.[89]

Southern blotting of the rat genomic DNA showed a single copy of the fructose-1,6-diphosphatase gene. Three consensus cAMP regulatory elements have been identified in the 5′ flanking region of the gene. Studies in primary adult hepatocyte culture system demonstrated that cAMP increases the abundance of fructose-1,6-diphosphatase mRNA and insulin reduces the mRNA levels. The response to insulin occurs independent of the changes in cAMP levels.[90] Recent in vitro data show that insulin inhibits fructose-1,6-diphosphatase gene expression via activation of the c-Jun amino terminus kinase pathway.[91] Administration of glucagon intravenously has been shown to increase the activity of hepatic fructose-1,6-diphosphatase activity in humans.[92] The regulation of fructose-1,6-diphosphatase gene has not been examined in the perinatal period. However, the preceding in vitro and in vivo data support the role of glucagon and insulin in the expression of fructose-1,6-diphosphatase gene at birth.

Glucose-6-phosphatase is a membrane-bound, endoplasmic reticulum–associated enzyme found mainly in the liver and kidney cortex, the two important gluconeogenic tissues. In addition, G-6-pase is expressed in pancreatic beta cells and jejunum. G-6-pase catalyzes the terminal step in gluconeogenesis and glycogenolysis. Glucose-6-phosphate is transported into the microsomes by the glucose-6-phosphate transporter, where G-6-pase converts glucose-6-phosphate to glucose and inorganic phosphate. The products are then exported out of the vesicles by specific glucose and pyrophosphate transporters. The developmental expression of G-6-pase is tissue specific. Hepatic G-6-pase mRNA is present in significant amount in the fetal liver. Highest levels of hepatic G-6-pase mRNA are observed at birth and at weaning. In the human fetal liver, the pyrophosphate protein and transport capacity appears 2 to 3 weeks after the expression of G-6-pase mRNA. Its level increases by 20-fold in the first 3 days after birth in conjunction with an increase in the G-6-pase mRNA. In contrast to the developmental pattern in the liver, the intestinal G-6-pase mRNA levels are low in the fetal period and increase steadily during the first 2 weeks after birth.[93]

A number of regulatory elements have been identified in the G-6-pase promoter.The data show that higher levels of cAMP and long-chain fatty acids up-regulate the transcription of G-6-pase gene, whereas insulin represses its expression. Long-chain fatty acids have been proposed to enhance the expression of G-6-pase gene transcription via peroxisome proliferator activated receptor (PPAR) dependent pathway.[87] Little is known about the regulation of microsomal phosphate–pyrophosphate transport. In contrast to findings in term infants, persistence of low activity of G-6-pase beyond the immediate neonatal period is common in preterm infants.[94] As a consequence, preterm infants are more prone to have hypoglycemia during periods of starvation.

Fetal Glucose Utilization

The quantitative aspects of fetal glucose utilization have been studied extensively in large animals, such as sheep, by several investigators.[66-68,87,95,96] These data suggest that, because the fetus of a fed normoglycemic sheep, late in gestation, takes up approximately 5 mg/kg per minute of glucose and uses a similar amount, fetal hepatic glucose production approaches zero.[58,67,95] The only exception has been the study by Hodgson and colleagues,[68] which showed a higher utilization rate compared with umbilical uptake, suggesting fetal production of glucose.This difference may be related to the isotopic tracer method used in which the loss of tracer from the fetal to maternal compartment was not taken into account.[90] In humans, no such estimation of fetal glucose utilization has been possible. The only approximation has been based on the increase in total maternal glucose production, measured in late gestation, and it suggests a fetoplacental glucose utilization rate of 5 to 6 mg/kg per minute.[50] Whether such a magnitude of glucose uptake can account for the oxidative metabolism of the fetus has been examined by calculating glucose/O_2 quotient.[97,98] The glucose/O_2 quotient represents the fraction of the total fetal O_2 consumption required to metabolize aerobically and completely the glucose acquired by the fetus across the placental circulation and is calculated as follows: Glucose/O_2 quotient = 6 × [Umbilical vein − Umbilical artery difference of glucose (millimoles)]/[Umbilical vein − Umbilical artery difference of O_2 (millimoles)]. The fetal glucose/O_2 quotient in the fed sheep[98] has been estimated to be approximately 0.5 and in humans approximately 0.8.[97] These data suggest that the maternally acquired glucose could not provide for the entire oxidative metabolism of the fetus and that other substrates, such as lactate and amino acids, may also be used by the fetus in utero for its energy needs.

The role of insulin in fetal glucose use and fetal glucose production was further evaluated by the following means: (1) infusion of somatostatin, resulting in insulin and glucagon withdrawal from the fetus; (2) infusion of insulin directly to the fetus; and (3) injection of streptozocin to the fetus, to produce chronic fetal hypoinsulinemia. Acute infusion of somatostatin to the sheep fetus caused a decrease in plasma insulin and glucose concentration without any change in glucagon concentration.[99] Under these circumstances, fetal glucose utilization and umbilical glucose uptake did not change. Exogenous infusion of glucose did not elicit an insulin response; however, glucose utilization increased even though insulin secretion had been suppressed by somatostatin infusion. These data suggest that acute withdrawal of insulin does not affect fetal glucose utilization and that fetal glucose utilization can be increased even in the absence of a significant insulin stimulation.[99] Chronic withdrawal of insulin by streptozocin injection to the fetus resulted in fetal hypoinsulinism, fetal hyperglycemia, and a decrease in fetal glucose utilization and glucose carbon oxidation.[100-102] An associated decrease also occurred in umbilical uptake of glucose, probably as a result of fetal hyperglycemia.[101] Finally, direct infusion of insulin to the fetus increased fetal glucose utilization in both sheep and rat fetus.[103-106] In addition, acute insulin infusion also increased the glucose/O_2 quotient across the hind limb of fetal lamb.[107] These data are important in the context of human infants of mothers with diabetes in whom intrauterine hyperinsulinemia results in increased fetal glucose utilization, increased accretion of carbon in insulin-sensitive tissue, and increased growth and fetal macrosomia.[108] Such a change in fetal body composition, that is, increased fat deposition and increased hepatic lipogenesis in response to fetal insulin infusion, has been demonstrated in the sheep, monkey, and rat.[109-115] The role of other glucose-regulatory hormones, such as glucagon, cortisol, and catecholamine, in fetal glucose production, utilization, and umbilical glucose uptake needs to be evaluated.

Utilization of Other Substrates by the Fetus

Because glucose could not account for the total O_2 uptake by the fetus, attention has been focused on the utilization of other substrates such as lactate and amino acids by the fetus. Studies of quantitative aspects of lactic acid metabolism by the fetus are confounded by the rapid changes in the concentration of lactic acid in response to small perturbations in the fetus. Therefore, particularly in humans in whom previous blood samples had been obtained only at the time of vaginal or elective cesarean delivery, the umbilical arterial concentration of lactate has

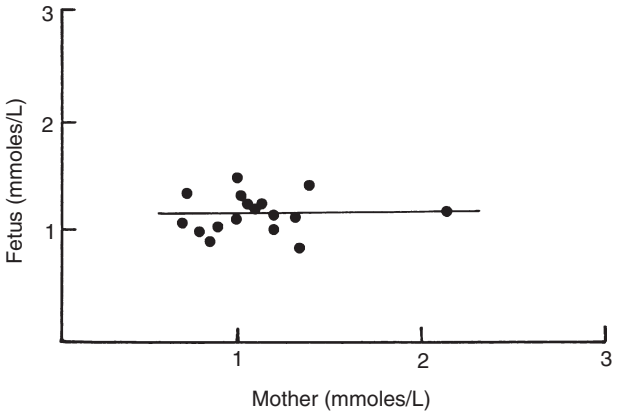


Figure 48-6 Lack of relationship between maternal and fetal blood lactate concentrations. The fetal samples were obtained by percutaneous umbilical blood sampling late in gestation. *(Data from Ashmead GG, Kalhan SC, Lazebnik N, et al: Maternal-fetal substrate relationships in the third trimester in human pregnancy,* Gynecol Obstet Invest *35:18, 1993).*

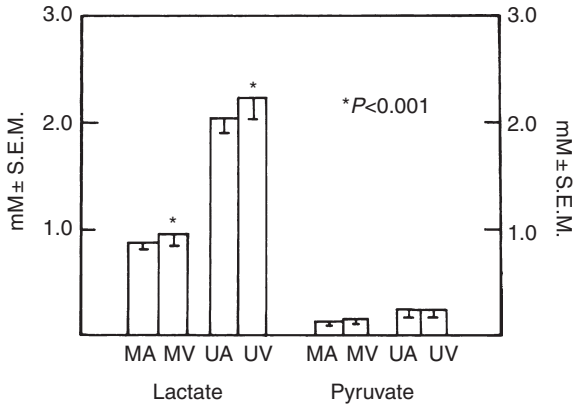


Figure 48-7 Maternal-fetal lactate and pyruvate gradients in the sheep, late in gestation. *MA,* Maternal artery; *MV,* uterine vein; *UA,* umbilical artery; *UV,* umbilical vein. *(Reprinted by permission from* Nature*, vol 254, p 710. Copyright 1975, Macmillan Magazines Ltd.)*

been observed to be higher than the simultaneously obtained umbilical venous and maternal venous blood concentrations.[116] These data would suggest fetal production and placental clearance of lactate but are subject to criticism because of a lack of steady state and the effect of labor on fetal metabolism. Even in a relatively unperturbed state in which percutaneous umbilical blood samples have been obtained from the fetus in utero, the fetal blood lactate concentrations were found to be similar to the simultaneously obtained maternal venous blood concentrations.[45] However, in a more recent study in which fetal blood samples were obtained by cordocentesis late in gestation, no correlation between maternal and fetal blood lactate concentrations was observed (Figure 48-6).[51]

In contrast to the data in humans, stable chronic preparations in the sheep fetus provide near-optimal conditions to study the maternal-fetal lactate relationship. As shown in Figure 48-7, the umbilical venous and uterine venous concentrations of lactate have been shown to be higher than the simultaneously measured umbilical arterial and uterine arterial lactate concentrations.[58] These data, which have been confirmed by Char and Creasy,[59] indicate uteroplacental production and maternal and fetal utilization of lactate. The metabolic fate of the lactate produced by the uteroplacenta and used by the fetus was not examined in these studies. However, estimation of the lactate/O_2 quotient suggested that complete oxidation of lactate could account for 25% of fetal O_2 consumption.[58] These studies are of interest in that the lactate derived from the placenta could potentially be used by the fetus for both oxidative and nonoxidative purposes such as glycogen synthesis.

In addition to glucose and lactate, amino acids actively transported from the mother to the fetus are an important potential source of energy as well as substrates for glycogen synthesis. Amino acids are actively transported across the human placenta from the mother to the fetus.[117] Lemons and colleagues[118,119] examined the fetal uptake of amino acids in the unstressed sheep and observed that the fetal uptake of amino acids was far in excess of that found in the carcass. These investigators suggested that a significant amount of amino acids taken up by the fetus was oxidized. Gresham and colleagues[120] estimated that up to 25% of the total fetal O_2 consumption in the sheep fetus could be accounted for by the oxidation of amino acids. In the human, on the basis of urea concentration gradients across the umbilical circulation, it can be assumed that the human fetus in utero catabolizes amino acids and synthesizes urea.[121] Using stable isotopic tracer methods, Gilfillan and colleagues[122] demonstrated synthesis of alanine by the human fetus at term gestation. They estimated that endogenous alanine synthesis could account for approximately 44% of alanine flux in the fetus. Because alanine is a major glucogenic precursor and because alanine nitrogen is a major precursor of urea nitrogen, the major oxidation product of amino acids, these data are consistent with utilization of amino acids by the human fetus for energy metabolism.

Regulation of Umbilical Glucose Uptake and Fetal Glucose Utilization

The fetal uptake of glucose appears to be closely regulated by the maternal plasma glucose concentration. As cited earlier, in human studies, the fetal glucose concentration closely follows maternal glucose concentration. In studies of fetal sheep, when the maternal glucose concentration was decreased by fasting[123] or by acute insulin infusion,[66] the umbilical uptake of glucose also decreased. During acute hypoglycemia produced by insulin infusion, the glucose-specific activity in the maternal and fetal compartment was identical, a finding suggesting no compensatory glucose production by the fetus and hence an associated decrease in fetal glucose utilization.[66] In contrast, in the experiments on fasted sheep, the fetal glucose utilization measured by isotopic tracer did not decline as much as the decrease in umbilical uptake, a finding suggesting a compensatory fetal glucose production.[123] The reason for the difference in these two studies may be that, in the former, hypoglycemia was produced acutely by insulin infusion, whereas, in the latter, the hypoglycemia was produced slowly after 5 to 7 days of fasting, which probably permitted the fetus to mount a compensatory response. DiGiacomo and Hay[124] showed that, during chronic maternal hypoglycemia produced by up to 3 weeks of insulin infusion, the sheep fetus increases glucose production and thus maintains glucose utilization. Whether such compensation also occurs in humans is not known. Nevertheless, isolated perfused human liver early in gestation is capable of release and autoregulation of uptake of glucose.[75] In this context, during acute umbilical cord compression in the sheep fetus that resulted in decreased umbilical glucose delivery to the fetus, Rudolph and associates[125] also showed an increase in net glucose production by the fetal liver. However, under these circumstances, a decrease in O_2 delivery to the fetus and a decrease in fetal arterial O_2 content also occurred, even

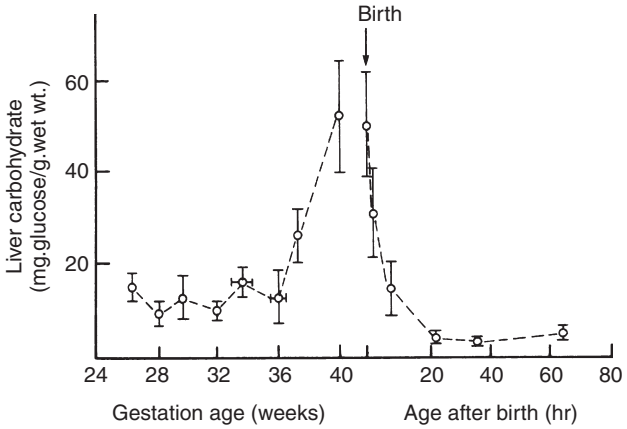


Figure 48-8 Changes in liver glycogen concentration in the human during fetal life and after birth. *(Modified from Shelley HJ, Nelligan GA: Neonatal hypoglycaemia,* Br Med Bull *22:34, 1966.)*

though umbilical O_2 uptake was maintained. Therefore the mechanism of compensatory glucose production during these conditions may be different from that seen during sustained hypoglycemia.

Glycogen Metabolism and Sources of Glycogen in the Fetus

The hepatic glycogen content in the human fetus is low early in gestation. As measured by Capkova and Jirasek,[126] the glycogen content of the fetal liver between 51 and 60 days (average, 8 weeks) gestation was 3.4 mg/g. A slow continuous increase in hepatic glycogen took place, so that between 121 and 130 days gestation it had reached 24.6 mg/g. In preparation for birth, a rapid steep increase appears to occur at around 36 weeks gestation. As shown by Shelley and Neligan,[127] in the human fetus at 40 weeks gestation, the hepatic glycogen content approaches 50 mg/g net weight (Figure 48-8). Soon after birth, hepatic glycogen decreases rapidly, so that by 24 hours of age it has reached a very low concentration. The human data have been obtained from abortions, fresh stillbirths, or infants who died soon after birth. Therefore the consequences of the events responsible for death should be considered when interpreting these data. It is likely that these estimates are lower than the actual levels in the liver. Similar changes in hepatic glycogen in relation to development have been observed in other animal species.

The fetal liver contains the full complement of enzymes required for the synthesis and breakdown of glycogen.[128] The potential for the synthesis of glucose from three carbon substrates has been demonstrated from early on in gestation in most mammalian species, except the rat. Evidence has been presented in mature animals and in humans suggesting that glucose may not be the immediate precursor of hepatic glycogen and that three carbon substrates—lactate, pyruvate, and alanine—derived from glucose in the periphery are likely to be the precursors for hepatic glycogen.[129-131] Whether a similar "indirect" pathway is also operative in the fetus was examined by Levitsky and associates[132] in chronically catheterized sheep fetuses late in gestation. By simultaneously infusing [^{14}C]lactate and [3-^{3}H]glucose in the fetal circulation and measuring ^{14}C- and ^{3}H-specific activity of glycogen, these investigators showed that glycogenesis from glucose, in the ovine fetus, is partly through the "indirect" gluconeogenic route, and lactate may be an important glycogenic precursor. These data are consistent with high lactate turnover rates observed in the sheep fetus.[133,134]

GLUCOSE METABOLISM IN THE NEWBORN

As discussed in the preceding section, the fetus in utero under normal circumstances is entirely dependent on the mother for glucose, and negligible amounts, if any, of fetal glucose production have been observed in most mammalian species studied. At birth, with the discontinuation of the maternal supply of substrates and nutrients as a result of cutting the umbilical cord, the newborn has to mobilize glucose and other substrates to meet energy needs. Therefore the process of birth involves certain well-orchestrated endocrine and metabolic events that result in adaptive responses for independent existence. Studies in newborn humans and in newborn animals have shown major changes in circulating glucoregulatory hormones, in association with birth and cutting of the umbilical cord. The plasma concentration of epinephrine, norepinephrine, and glucagon has been shown to increase rapidly, whereas the concentration of insulin decreases.[134-139] The net effect of these changes is mobilization of glycogen[140] and fatty acids.[135,136] After birth in the human newborn, the plasma glucose concentration declines, reaching a nadir by 1 hour of age and then rising spontaneously so that plateau levels are reached between 2 and 4 hours.[141-144] The lowest level of glucose observed and the speed with which stable levels are reached are in part dependent on whether the mother received glucose during labor and delivery.[145] With the current obstetric management of the mother and the nutritional practices for the neonate, low blood glucose levels are not seen as often as in the past. In addition, the average glucose levels observed in the nursery are higher than those in the past.[142-144] At present, a term healthy neonate in the first 6 hours of life maintains plasma glucose between 40 and 80 mg/dL. The glucose levels increase slightly with the initiation of feeding; therefore, after 24 hours of birth, the normal infant's plasma glucose levels range between 45 and 90 mg/dL. Of course, these data vary with the individual institution's clinical practice regarding nutritional management of the newborn. The data in animal studies are variable depending on the individual species differences. Suffice it to say that most newborn animals go through a brief period of transient hypoglycemia with spontaneous recovery.

Glucose Kinetics in the Newborn

By using an isotopic tracer dilution method, radioactive tracers in animals, and nonradioactive (stable) tracers in humans, the rates of glucose production and utilization have been quantified in several studies.[146-150] Although these measurements fit within a certain range, these quantitations are influenced by the following factors:

1. The type of tracer used, that is, recycling versus nonrecycling. In general terms, labeled carbon tracers are recycling tracers and result in underestimation of glucose production rate, the only exception being the use of uniformly labeled [^{13}C]glucose and quantitation of only the masses representing the whole molecule of glucose that is uniformly labeled.[149,151] In contrast to labeled carbon tracers, labeled hydrogen tracers are nonrecycling.[147] However, in some of the later tracers, an excessive loss or exchange of label may occur, resulting in an overestimation of the rate of turnover.
2. The clinical condition of the subject. Although it has been described that the subjects were "well," in many of these studies, the infants were receiving intravenous fluids or were completing a course in antibiotics. In one study, the infants were receiving intravenous glucose, which was discontinued before initiation of isotopic tracers.[147] These infants should not be considered "well" in the strict sense, and their underlying problems may have contributed to

the variance in measurements. Similarly, the earlier animal studies were performed by either tying the animals down or administering anesthesia to them.[150] Anesthesia has been documented to have significant effect on glucose metabolism.[152]

3. The blood-sampling technique, such as with repeated venipuncture or heelsticks versus indwelling needles, may influence glucose measurements. It has been well recognized and confirmed that the newborn infant can respond to pain by mounting a "stress"-hormone response, which can result in changes in glucose kinetics.[153]

Glucose Kinetics During Fasting

The rates of glucose production and utilization in the human newborn during fasting, that is, 3 to 4 hours after birth, before feeding, or after 8 to 9 hours after the last feeding, have been estimated to be between 4 and 6 mg/kg per minute.[146-149] These rates are higher than those measured by recycling tracers.[148] The rate of glucose production in the newborn human is somewhat lower than that observed in newborn dog, lamb, or pig,[150,154] and this situation probably is caused by species differences or by experimental conditions.

The rates of glucose production estimated in the newborn are significantly higher than those observed in adult humans and animals and reflect the higher metabolic rate and the higher brain–body weight ratio of the newborn, the brain being the major glucose-using organ. As the newborn grows, the rate of glucose production per unit body weight decreases; therefore, by adolescence, the rates of glucose production approximate the rates in adults, which are one half to one third those seen in the newborn.[147]

During steady state, in the presence of a constant blood glucose concentration, the rates of glucose production and utilization are equal. The fate of used glucose and its contribution to oxidative metabolism was quantified in human newborns by Denne and Kalhan.[149] By infusing glucose labeled uniformly with ^{13}C in combination with indirect respiratory calorimetry, these investigators showed that only 53% (range, 40.9% to 68.1%) of glucose produced was oxidized to CO_2, and oxidation of plasma glucose contributed up to 38% of the total calorie expenditure. Another interesting finding of this study was the small discrepancy (approximately 20%) between the estimation of total carbohydrate oxidation by respiratory calorimetry and that measured by tracer isotope studies. A similar discrepancy was shown by Sauer and colleagues[155] in their study in glucose-infused newborn infants. This difference has been postulated to result from the inclusion of local glycogen mobilization and oxidation, not included in the tracer isotope estimations.[149] However, this does not explain the observed discrepancy during prolonged fasting conditions.[19]

The study of Denne and Kalhan[149] raises an important question regarding the energy metabolism of the brain in newborn humans during fasting. As demonstrated by studies in adult humans, the brain derives almost all its energy from glucose.[156,157] The calculated amount of glucose necessary to supply the energy for the brain correlates well with the measured oxidation rates of glucose.[158,159] The O_2 consumption rate of the brain in the newborn human has been determined to be 104 μmol/100 g brain tissue per minute.[160] If the average weight of brain in newborn infants is estimated to be 360 g,[161] approximately 3.7 mg/kg per minute of glucose would be required to meet the metabolic needs of the brain. Thus, as shown by Denne and Kalhan,[149] the measured rates of glucose oxidation in the human newborn will supply only approximately 70% of energy needs of the brain. These data suggest that in the human newborn during fasting, the energy requirements of the brain are supported in part by fuels other than glucose.

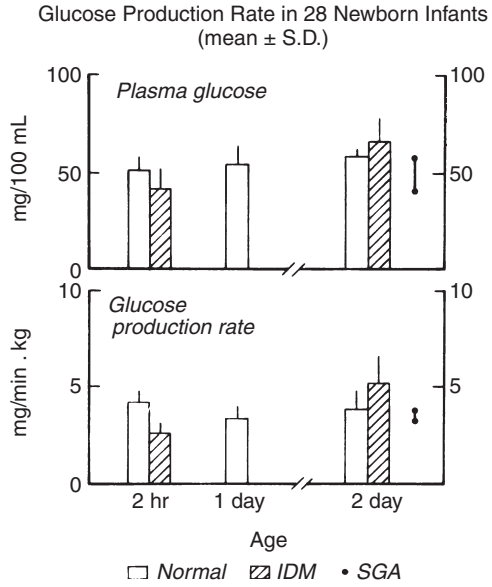


Figure 48-9 Changes in the rate of glucose production and blood glucose concentration in the first 2 days in physiologically normal infants, infants of mothers with diabetes *(IDM),* and small-for-gestational age infants *(SGA).*

Effect of Age

The data presented earlier were obtained in the first 48 hours after birth. Over the next few days, with the introduction of feeding, the normal infant maintains the plasma glucose level between 40 and 70 mg/dL during fasting.[162] The endogenous rate of glucose production and utilization, as measured by [1-^{13}C]glucose tracer dilution, also either remains unchanged or decreases slightly (Figure 48-9). Thus the glucose production rate in seven infants older than 2 days was 4.1 mg/kg per minute. As shown, in contrast to the finding in physiologically healthy infants, in the infant of a mother with diabetes the plasma glucose concentration and endogenous glucose production rate increased and reached those observed in the normal infants. In the infant with intrauterine growth restriction, the endogenous glucose production rate expressed per kilogram of body weight had been noted to be either the same as in healthy infants or slightly increased, a finding reflecting the higher brain weight–body weight ratio in these infants.

Regulation of Glucose Production in the Newborn

Interest in the regulation of hepatic glucose production in the newborn stems from the observation of marked fluctuation in glucose homeostasis during this period. Both in human and in animal models, hypoglycemia and hyperglycemia have been observed and constitute a significant problem in the clinical management of the infant. The problem is further compounded in the premature newborn and in the presence of intrauterine growth restriction. These responses in the newborn led some investigators to suggest that glucose homeostasis in the newborn is "transitional" as a result of immaturity of the regulatory mechanisms.[154] Evaluation of regulation of glucose homeostasis is rather difficult because of the small size of the newborn infant and animal, the prolonged nature of the experiments, and, finally, the rapid changes in the physiologic state as the newborn adapts to the extrauterine environment.

The role of glucose and the pancreatic hormones insulin and glucagon in the regulation of glucose production has been examined in both adults and newborns. During the basal state

in normal adult humans and animals, the hepatic glucose output is regulated by the interaction of insulin and glucagon. An unopposed action of insulin causes a decrease in hepatic glucose output, whereas glucose output increases in the presence of unopposed action of glucagon.[163,164] When glucose is infused in normal adults, the endogenous glucose production is suppressed as a result of the autoregulatory effect of glucose and the action of insulin on the liver.[165,166] At low rates of glucose infusion, when the insulin concentrations are similar to those in the basal state, 70% to 90% inhibition of endogenous glucose production occurs.[164,167,168] However, at higher rates of glucose infusion, with higher levels of circulating insulin, endogenous glucose production is completely suppressed.[155,159] Thus endogenous glucose production in adult animals and humans appears to be regulated by both glucose and insulin. Glucose alone appears to have its effect in suppressing endogenous glucose production on an insulinized liver via its effect on glycogen metabolism.[169-172]

Whether such mechanism of glucose regulation is functional in the newborn infant and animals has been a subject of study for some time. Numerous studies have examined the newborn human's and animal's response to exogenous glucose infusion. Varma and associates[173] observed a continuous increase in plasma glucose concentration during a constant-rate glucose infusion in newborn dogs, while at the same time the rate of endogenous glucose production decreased to a lesser degree than in adult dogs. In this study, the animals were anesthetized with sodium pentobarbital (Nembutal), which may have affected their glucose metabolism; these investigators used [2-^{3}H]glucose tracer, which is lost in excess as compared with other tracers because of futile cycling; and finally, because the newborn dogs had fasted for a very short time, as compared with adults, they were not in a true fasted state and may have been absorbing glucose from the gut. Sherwood and associates,[174] in a study of rhesus monkey neonates, showed a marked reduction of endogenous glucose output in response to glucose. However, in a group of "sick" newborns, possibly as a result of counterregulatory hormonal responses, marked hyperglycemia developed with no suppression of endogenous glucose output. Finally, in a study of newborn lambs, Cowett and colleagues[154] observed persisting endogenous glucose production until glucose was infused at high rates. However, in this study, the animals had consistently high glucose concentrations, a finding suggesting some influence of counterregulatory hormones, possibly as a result of experiment-related "stress."

In human studies, data have been variable. King and associates[175] observed 80% suppression of endogenous glucose production in term newborn infants. In contrast, Cowett and colleagues[176,177] observed a variable response—complete suppression in some and incomplete in others. The latter was seen more often in preterm infants. In another study, Kalhan and colleagues[178] examined the role of plasma glucose concentration in the regulation of endogenous glucose production by infusing glucose at 2.6 to 4.6 mg/kg per minute as a continuous infusion to term, preterm, and small-for-gestational age infants. Because of the different rates of exogenous glucose infusion, the measured endogenous glucose production rate was variable. However, the peak glucose concentration was related to the rate of glucose infusion, and a negative correlation between peak glucose concentration and the rate of endogenous glucose production was seen (Figure 48-10). All infants in this study mounted a significant insulin response, yet the hepatic response in terms of glucose production rate was variable, a finding suggesting that insulin alone does not appear to be the dominant factor in the regulation of hepatic glucose production. The inverse relationship between plateau glucose concentration and the endogenous glucose production (Re) supports the hypothesis that, in the presence of insulin, plasma glucose concentration appears to be the major regulator of hepatic glucose output in the human neonate. Such a regulatory effect of glucose level on hepatic release and uptake

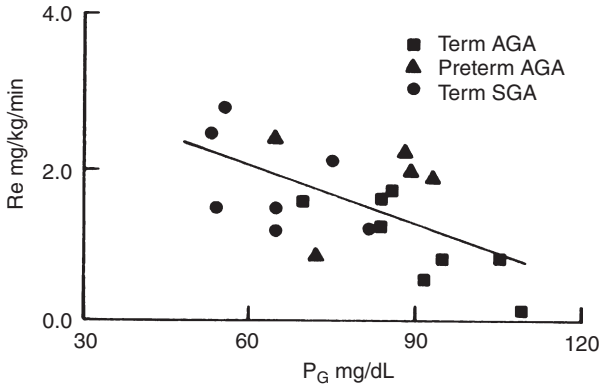


Figure 48-10 Relationship between peak glucose concentration *(P_G)* and endogenous glucose production rate *(Re)* in newborn infants during intravenous glucose infusion at varying rates. The rate of glucose production was measured by [6,6-$^2H_2$]glucose tracer as a prime–constant-rate infusion. A negative correlation between P_G and Re is shown. *AGA,* Appropriate for gestational age; *SGA,* small for gestational age. $Y = 3.56 - 0.03x$; $r = 0.59$; $p = .006$. *(From Kalhan SC, Oliven A, King KC, et al: Role of glucose in the regulation of endogenous glucose production in the human newborn,* Pediatr Res *20:49, 1986.)*

of glucose has been demonstrated in the isolated liver from the midterm human fetus.[75] In that study, insulin in physiologic concentration had no detectable effect on hepatic glucose output.

The role of other regulatory hormones, glucagon and catecholamines, in neonatal glucose homeostasis was evaluated in dogs and lambs. As discussed previously, an increase in glucagon concentration and a decrease in insulin are important in the establishment of hepatic glucose production after birth. However, when the role of glucagon was further evaluated in the neonatal dog and lamb by infusing somatostatin (an inhibitor of insulin and glucagon secretion), a variable response was observed.[179-181] This variability was in part the result of "resistance" to somatostatin action on the pancreas; therefore neonatal pancreas does not show a magnitude of inhibition of insulin and glucagon secretion similar to that seen in adults.[179,181] Thus these data remain subject to ambiguity in interpretation. Finally, studies by Cowett[182,183] suggest that, under normal physiologic conditions, the neonatal liver is more responsive to β-adrenergic stimulation than to α-adrenergic stimulation. The role of hepatic adrenergic receptors in glucose production in vivo in the newborn needs further examination.

Gluconeogenesis in the Newborn

Although the capacity for gluconeogenesis is present in the fetal liver with the exception of the rat, and even though the fetus in utero as well as the isolated fetal liver preparation can synthesize glucose via gluconeogenesis under certain circumstances, gluconeogenesis, that is, synthesis of new glucose and release in circulation, is a postnatal event in most mammalian species. In the case of rat liver, the key gluconeogenesis enzyme PEPCK appears only after birth, and hence the rat fetus is incapable of gluconeogenesis in utero.[87]

In the human newborn, a steady plasma glucose concentration maintained by systemic glucose production is evident soon after birth. Although glucose production most likely is initiated at birth, because of technical problems such as infusion of isotopic tracers, the earliest time of measurement has been 2 to 3 hours after birth. The contributors to systemic glucose production are glycogenolysis and various glucogenic substrates—glycerol,

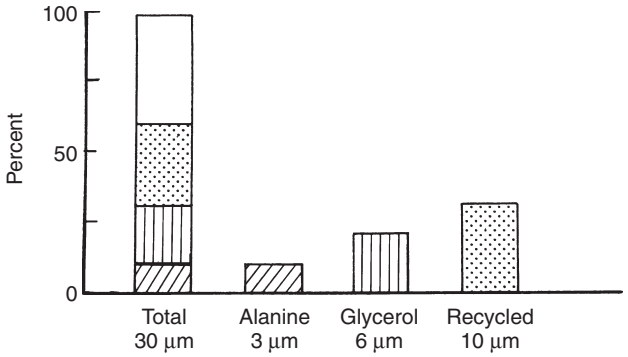


Figure 48-11 Sources of glucose in the human newborn. As measured by tracer isotopes, the major sources of glucose during fasting in the human newborn are glycogen *(unshaded area in the bar),* alanine, glycerol, and recycled glucose carbon.

lactate, and alanine via gluconeogenesis (Figure 48-11). Using various isotopic tracer methods, investigators have quantified the kinetics of these gluconeogenic substrates and their contribution to systemic glucose production in healthy newborn infants.

Gluconeogenesis from glycerol has been quantified in healthy term infants by simultaneous infusion of carbon-labeled glycerol along with labeled glucose. The appearance of tracer carbon in glucose is used to quantify the contribution of glycerol to glucose.[184-186] Data from two such studies in healthy newborn infants are displayed in Table 48-1. As shown, the rate of appearance of glycerol in healthy term infants during the first 48 hours after birth was approximately 9 μmol/kg per minute. Glycerol's contribution to glucose accounted for 10% to 20% of glucose produced. The wide range in estimates of glycerol's contribution to glucose may simply reflect the characteristics of the study population, their time since birth, and relation to the last feeding. Nonetheless, these data show that gluconeogenesis from glycerol is active in the healthy term infant immediately after birth, and approximately 10% to 20% of plasma glucose is derived from glycerol. Finally, these data show that lipolysis in adipose tissue is also active at a high rate in the neonate, because adipose tissue triglyceride depots are the primary source of plasma glycerol.

Because all other gluconeogenic substrates enter the gluconeogenesis pathway via pyruvate, some investigators have examined the contribution of alanine and lactate to glucose production,[87] by using carbon-labeled tracers. As discussed previously, all these methods result in an underestimation of the contribution of gluconeogenesis to glucose production because of the loss of tracer carbon via exchange in the TCA cycle. The data on lactate kinetics from one such study[187] are displayed in Table 48-2. The rate of appearance of glucose and lactate were quantified by using [6,6-$^2H_2$]glucose and [$^{13}C_3$]lactate, respectively. As shown, newborn infants have a relatively high rate of lactate turnover when compared with adults, and approximately 18% of glucose was derived from lactate. However, this estimate of gluconeogenesis from lactate did not correct for the loss of tracer. When corrected for the loss of tracer carbon (correction factor, approximately 1.5),[188] the lactate's contribution to glucose would be approximately 27%.

Newer techniques have been developed to avoid the errors associated with the loss of tracer carbon in the TCA cycle. These include the deuterium labeling of the body H_2O and mass isotopomer distribution method. These techniques have been applied to quantify gluconeogenesis in term and preterm infants.[187,189,190] Using the deuterium labeling of body H_2O method, Kalhan and colleagues[187] showed that the contribution of pyruvate to glucose production in healthy term infants amounted to 31% or 9.30 ± 1.96 μmol/kg per minute (mean ± SD). These estimates of gluconeogenesis via pyruvate are similar to the estimate of glucose carbon recycling, approximately 35%,[149] and the measurements of lactate contribution to glucose (see earlier). If the contribution of glycerol (10% to 20%) is also included, then the total gluconeogenesis in a healthy newborn infant will amount to approximately 50% or approximately 15 μmol/kg per minute of the total glucose Ra of 30 μmol/kg per minute. This is significant and implies an important role of gluconeogenesis, even after a brief fast. These data show that gluconeogenesis from pyruvate, and possibly from glycerol, is occurring at all times, although its contribution to glucose varies with feeding and fasting.

TABLE 48-1

Glycerol Metabolism in Healthy Newborn Infants

No.	Glycerol Ra (μmol/kg/min)	Glucose Ra (μmol/kg/min)	Fraction, glucose from glycerol	Quantity, glucose from glycerol (μmol/kg/min)	Reference
6	9.47 ± 2.11	21.09 ± 1.71	0.20 ± 0.06	4.34 ± 1.30	Patel and Kalhan[185]
8	8.7 ± 1.2	25.0 ± 3.5	0.11 ± 0.02	2.75 ± 0.59	Sunehag et al.[186]

Values are expressed as mean ± SD.
Ra, Rate of appearance.

These data also suggest that gluconeogenesis is one of the several substrate cycles in the body, like the triglyceride fatty acid cycle and protein turnover, which are active at all times and can be rapidly accelerated at the time of acute demand.[187] The regulation of gluconeogenesis in the human newborn has not been examined, and the reader is referred to a review by Girard for a discussion of regulation in the rat.[72]

Very-Low-Birth-Weight Infant

Low-birth-weight infants have become the largest clinical population in the neonatal intensive care unit as a result of improved clinical care and enhanced survival.[191] Studies in this population using isotopic tracer methods have shown that low-birth-weight infants can produce glucose at an appropriate weight-specific rate to meet their metabolic needs, and they respond appropriately to exogenous glucose and amino acid infusions.[178,192,193] Because several of the studies reported in these infants were performed while the babies were receiving intensive support, the hepatic response to glucose and insulin has been reported to be variable, that is, complete suppression of glucose production in some infants and incomplete in others.[178,193-197] However, such a response may be related to the stress response in these infants, as documented by persistent increase in circulating catecholamine levels.[198] In relation to glucose production, provision of exogenous lipids (i.e., a source of energy for gluconeogenesis) caused an increase in glucose levels but did not cause an increase in hepatic glucose production.[199,200] The utilization of glucose by low-birth-weight infants has been examined both during fasting and in response to intravenous glucose. These data suggest that healthy low-birth-weight infants can dispose of infused glucose appropriately without the development of glycosuria and osmotic diuresis, and glucose disposal is related to both plasma insulin levels and plasma glucose concentrations.[201,202] Finally, very-low-birth-weight infants less than 28 weeks gestation show that even this population can regulate endogenous glucose production in response to an infusion of glucose.[203,204]

Quantification of gluconeogenesis in very-low-birth-weight infants has also been done with use of more newly developed tracer techniques.[187-189] Because the data on low-birth-weight infants cannot be obtained during fasting, these studies have

TABLE 48-2

Glucose and Lactate Kinetics in Newborn Infants

	Glucose (mmol/L)	Lactate (mmol/L)	Glucose Ra (μmol/kg/min)	Lactate Ra (μmol/kg/min)	Glucose from lactate (%)
Healthy infants (n = 10)	2.96 ± 0.51	1.83 ± 0.29	20.66 ± 3.28	38.3 ± 11.90	17.9 ± 5.2
SGA infants (n = 4)	3.46 ± 0.43	2.22 ± 0.36	17.71 ± 3.34	32.5 ± 5.74	18.0 ± 9.4
Infants of IDDM (n = 6)	3.42 ± 1.12	2.14 ± 0.43	25.74 ± 8.92	48.6 ± 10.40	18.4 ± 4.1

Values are expresssed as mean ± SD.

Glucose Ra, Rate of appearance of glucose quantified by [6,6-$^2H_2$]glucose tracer dilution; *IDDM,* mothers with insulin-dependent diabetes; *Lactate Ra,* rate of appearance of lactate measured by [$^{13}C_3$]lactate tracer dilution; *SGA,* small for gestational age.

been performed while the infants were receiving parenteral glucose, glucose plus amino acids with or without lipids. Therefore these data should be interpreted in view of the specific metabolic and physiologic state. In addition, low-birth-weight infants are also receiving other clinical support that may confound the data obtained. These studies in low-birth-weight infants show that gluconeogenesis is active in these infants, even when they are receiving parenteral nutrients, and it contributes as much as 30% to 70% to endogenous glucose production. Because of the study conditions, that is, parenteral glucose infusion, endogenous glucose production is significantly suppressed. Thus, even under these circumstances with a low rate of appearance of glucose, gluconeogenesis makes a significant contribution to endogenous glucose production.

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Edward S. Ogata

Carbohydrate Metabolism During Pregnancy

The effects of pregnancy on maternal glucose metabolism should be considered as two epochs. The first half of gestation is a period of sustained maternal anabolism. During this time, the increased calories ingested by the mother not only sustain fetal growth but also are stored as maternal fat. Normally expected maternal insulin secretion drives this period of anabolism.[1,2]

The increase in maternal metabolic energy stores is important for the second half of gestation, a period of logarithmic fetal growth. The substrate and energy demands of the rapidly growing fetus require not only a greater fraction of maternal food intake but also utilization of the maternal energy stores laid down during the first half of pregnancy. Alterations in maternal sensitivity to insulin are central to this process.[3] The development of insulin resistance can unmask a tendency toward diabetes mellitus (gestational diabetes mellitus).[4,5] The conceptus is responsible for these adaptations. Indeed, these changes parallel the growth of the conceptus.[1] These alterations become increasingly apparent as the conceptus rapidly increases in mass from 20 to 24 weeks of pregnancy onward, and they are promptly reversed after delivery.[1] In the immediate postpartum period, normal glucose tolerance returns, even in those women with gestational diabetes mellitus.

EFFECT OF THE CONCEPTUS ON MATERNAL INSULIN METABOLISM

The fetus and placenta exert multiple metabolic effects. With respect to insulin, maternal insulin does not cross the placenta,[6] although some insulin may be bound there. The placenta clears some maternal insulin by directly degrading it.[7]

Changes in maternal insulin secretion and action are dramatic during the second half of pregnancy.[8] During the first half, basal insulin secretion is normal, and insulin secretion mediates the laying down of metabolic fuels.[9] In the second half of gestation, insulin secretion is greatly exaggerated.[9-11] Intravenous glucose tolerance testing indicates that, by the third trimester, physiologically normal pregnant women secrete three times the insulin that they do in the nonpregnant state.[1]

The development of insulin resistance during the second half of pregnancy is responsible for this exaggerated insulin secretion. Insulin sensitivity is reduced 40% to 60% during the second half of gestation.[9,12] This insulin resistance is caused in part by the generation of human placental lactogen, progesterone, and estrogen, which become available in ever-increasing amounts as gestation progresses.[13-15] These hormones directly antagonize the effect of maternal insulin. Insulin resistance increases in liver, adipose tissue, and skeletal muscle.[16] Insulin binding appears to be normal during late gestation, a finding indicating that insulin resistance is mediated by events distal to receptor and insulin binding. It is possible that tyrosine kinase inhibition may also contribute to insulin resistance.[17,18] Increased insulin clearance may also add to the impact of insulin resistance.[19] This possibility remains to be confirmed.[20]

EFFECT OF THE CONCEPTUS ON MATERNAL GLUCOSE AVAILABILITY

These changes in maternal endocrine controls ensure the availability of glucose, amino acids, and lipids to meet the ever-increasing needs of the fetus during the second half of gestation.

This is not trivial, because the energy and substrate needs of the fetus are considerable. Glucose utilization by the human fetus is estimated to be three times that of the adult. Growth of the human fetus during the third trimester requires the net transplacental transfer of 54 mmol of nitrogen per day.[21] The blunted response to insulin ensures that, during the postprandial state, glucose and amino acids are made available to the fetus at the mother's expense.

During maternal fasting, the ongoing insulin resistance allows a rapid and profound mobilization of maternal fat stores and stimulates ketogenesis. This also causes a decrease in maternal blood glucose and amino acid concentrations despite increased hepatic gluconeogenesis.[22] The reduction in maternal blood glucose may result in hypoglycemia. This fasting hypoglycemia of pregnancy is a result of a failure of amino acid mobilization to keep up with rates of glucose removal. This "accelerated starvation" of the mother ensures fuel availability to the fetus at the mother's expense during pregnancy.[23,24]

CONSEQUENCES OF PROLONGED MATERNAL FASTING

If maternal fasting is prolonged, maternal ketogenesis as a result of these endocrine changes will be prolonged and exaggerated. Ketone bodies readily cross the placenta, and the fetal brain can use ketones as early as 10 to 12 weeks gestation.[23] Although the capability to use an alternative fuel to glucose may be beneficial for the fetus, limited data suggest that the children of mothers who had ketosis during pregnancy are at risk for cognitive and psychomotor delays during childhood. The mechanisms responsible for this potential association between ketone body oxidation and impaired brain function are unclear.[25]

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Anthony F. Philipps

Oxygen Consumption and General Carbohydrate Metabolism of the Fetus

This chapter covers those factors related to general fetal metabolism, with specific reference to fetal energy balance and substrate uptake. Although some direct information regarding human fetal energy metabolism and balance is available, much of the information presented here relies heavily on data obtained from animal experiments and theoretic considerations. Because human fetuses of less than 30 weeks gestation are common in newborn intensive care units, an understanding of those factors involved in determining fetal metabolic needs may be of use in such settings. The word "metabolism" has been used here to describe a variety of biochemical reactions that either require or produce energy. This term is used here in a general sense to illustrate chemical processes in the fetus that alter carbon-containing substrates in order to synthesize tissue (accretion/growth), or provide the organism with energy needed for basic homeostasis (fuel/energy).

FETAL ENERGY REQUIREMENTS

For many years after the discovery of oxygen by Lavoisier,[1,2] it was assumed that the fetus had no metabolism of its own. John Hunter had, however, demonstrated in 1786 that fetal and maternal circulations within the placenta were distinct from each other[3] and Erasmus Darwin, Charles Darwin's grandfather, surmised correctly in his text *Zoonomia* (1794) that at least some of the function of the placenta was to supply oxygen for fetal demands. However, it was not until the late 1800s that fetal blood was demonstrated to contain oxygen[4,5] derived from mother's blood and in the 1930s that fetal blood oxygen content and hemoglobin saturation could be accurately measured.[5] Even so, mammalian fetuses were thought to live in hypoxic environments[5,6] because human and animal umbilical venous blood had a lower partial pressure than adult blood. The normal fetal umbilical venous pO_2 at term was found to be 25 mm Hg or less. This finding led to the "Mount Everest in utero" concept attributed to Sir Joseph Barcroft,[5] and attempts were then made to understand how the fetus adapted to such conditions. Because of the leftward shift in the oxyhemoglobin dissociation curve and elevated levels of fetal (versus adult) hemoglobin, normal umbilical venous oxygen content, measured by the standard of mL or mmol of oxygen per mL of blood, was found to be the rule.

Interestingly, more recently it has also been demonstrated that acquiring hemoglobin concentrations of 16 to 18 g/dL, similar to those found in the fetus, also appears to confer significant survival advantage for some adults living at high altitude (i.e., 3800 m),[7] another condition placing constraints on arterial blood oxygen tension. This is also likely to spare cardiac work (i.e., obviating the need to increase cardiac output, which would be limited in the fetus). Oxygen delivery to tissues in the fetus due to fetal hemoglobin, when studied in 5 mammalian species[8] (for a variety of reasons), was found to be more efficient than that of adult hemoglobin, as was the ability of hemoglobin to load oxygen at the placental level, due to the Bohr pH effect (Figure 50-1). Overall, therefore, in both human cord blood and umbilical venous samples from fetuses of a number of species, fetal oxygen delivery as measured by blood oxygen content (not pO_2) is adequate for aerobic metabolic needs.[9]

It follows that fetal mammals, under steady-state, or unstressed, conditions, ought to achieve adequate oxygen delivery (the product of umbilical venous blood oxygen content and umbilical venous blood flow) and should not exhibit evidence of hypoxia (low tissue oxygen availability), unless specific organ or total blood flow falls dramatically or fetal blood oxygen content declines below a critical level. Studies of fetal acid-base or lactate balance indirectly test this hypothesis. The consensus is that no evidence exists to support a hypoxic fetal milieu because (1) umbilical cord blood pH values of unstressed fetal humans, pigs, monkeys, and sheep are all similar to each other and to the normal adult range of 7.35 to 7.45; (2) venoarterial $[H^+]$ concentration differences in cord blood of these species are only modest, suggesting relatively small fetomaternal $[H^+]$ transfer, inconsistent with hypoxia-induced lactic acid production; (3) both in vitro and in vivo, the placenta has been shown to deliver lactate into the fetal circulation, where it is taken up, particularly by the fetal liver, for purposes of accretion and oxidation[10,11]; and (4) experimental production of maternal and therefore relative fetal hyperoxia in the sheep is not associated with an increase in fetal oxygen consumption.

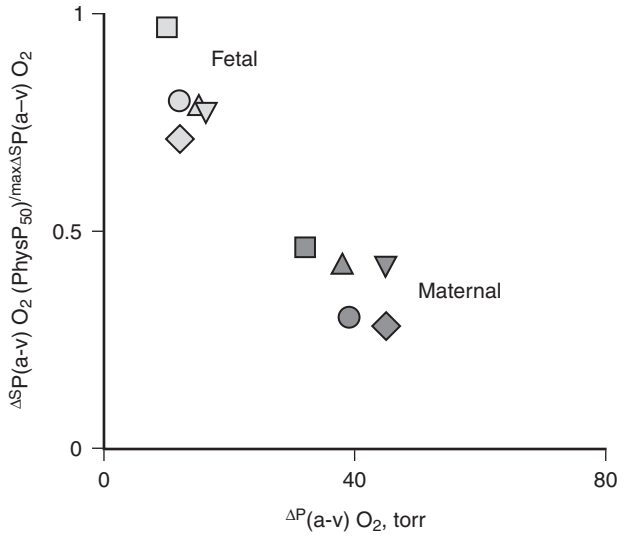


Figure 50-1 Enhanced efficiency of oxygen delivery of fetal vs maternal blood in five mammalian species. Efficiency calculated as $\Delta S_{(P(a\text{-}v)\ O2)PhysP50}/Max\Delta S_{(P(a\text{-}v)\ O_2}$ and plotted as a function of the arteriovenous (a-v) O_2 partial pressure difference of oxygen $(P(a\text{-}v)\ O_2)$. The term $\Delta S_{(P(a\text{-}v)\ O2)PhysP50}/Max\Delta S_{(P(a\text{-}v)\ O_2}$ represents the change in hemoglobin (Hb) saturation at any given a-v po_2 value at physiologic P50 (partial pressure at which Hb is 50% saturated) relative to a theoretically derived "optimal" Hb saturation at the same a-v pO_2 difference. (Open symbols: fetal; closed symbols: maternal; human ◇; cow ○; sheep △; pig □; horse ▽) *(From Zhang Y, et al:* Zool Sci *23:49, 2006)*

Thus in the unstressed fetus, energy processes appear to operate for the most part through oxidative pathways, and estimation of fetal oxygen consumption may be used as a direct indicator of the fetal metabolic rate.[9,12] Although rates of substrate uptake and carbon dioxide production provide alternate measurements of fetal metabolic activity, the calculation of each requires a significant number of assumptions. In the case of substrate utilization, the relative partitioning of carbon uptake by the fetus for use as fuel (oxidative needs) or growth (accretion needs) must be considered. In the case of carbon dioxide production measurements, the overall respiratory quotient (RQ) must be estimated (i.e., the relative contributions of carbon from the oxidative metabolism of carbohydrates, amino acids, and fats toward carbon dioxide production must be taken into account).[9,13]

FETAL OXYGEN CONSUMPTION

Measurement

Fetal oxygen consumption (Vo_2) has been estimated using a variety of techniques. Two methods have been used to gauge fetal oxygen consumption in vivo. Because the fetus has little stored oxygen, the vast majority of fetal requirement for oxidative metabolism of substrate is met by transfer of oxygen to the fetus via the placenta and umbilical venous circulation. Little, if any, transfer is thought to occur across the fetal membranes. The earliest known method for measuring fetal VO_2 accurately in the intact animal was that derived by Christian Bohr (circa 1900). By measuring VO_2 before and after intermittent umbilical occlusion in pregnant guinea pigs (or in subsequent studies by others before and after delivery of the fetus), fetal VO_2 could be calculated as follows:

$$\text{Fetal } VO_2 = \text{Predelivery maternal } VO_2 - \text{Postdelivery maternal } VO_2 \quad (1)$$

Fetal VO_2 is usually expressed as either mL or mmol o_2/kg or g/minute. As shown in Table 50-1, estimates for fetuses of several species, including humans, are available.

The major assumption in such studies is that the act of delivery (or cord occlusion) does not measurably alter maternal, uterine, or placental VO_2. Such assumptions may not be entirely true because of parturition-induced changes in fetal, placental, and maternal rates of metabolism, but the results of these studies provide a framework for estimates obtained using other methods.

The second technique for measuring fetal VO_2 relies on the principle derived by Adolf Fick (circa 1870), whereby uptake of substrates or oxygen for the whole organism can be calculated as the product of cardiac output and the arteriovenous difference in substrate or oxygen content. In the case of specific fetal organ uptake (or in this case, the whole fetus), the product then can be transposed as that of the organ or whole fetal blood flow multiplied by the arteriovenous or venoarterial concentration difference of the substance in question across the organ or umbilical circulation, respectively. Thus for oxygen consumption of the mammalian fetus, the principle may be restated according to Equation 2:

$$\text{Fetal } VO_2 = \text{Fumb} \times (V - A)\, O_2 \quad (2)$$

where Fumb = umbilical blood flow (mL/minute or mL/kg/minute) and (V – A) O_2 = the venoarterial difference in O_2 content (mL or mmol of O_2/100 mL blood) across the umbilical circulation.

Noninvasive methods for determination of human fetal VO_2 are being developed, such as magnetic resonance imaging (MRI)[14,15,15a] and near infrared spectroscopy (NIRS)[16] (Figure 50-2). Both techniques make use of the differences observed in either image density or spectroscopic patterns between saturated and desaturated hemoglobin and may be particularly useful for assessing specific organ (i.e., liver, brain) oxygen requirements or abnormalities of metabolic function due to maternal or fetal disease states.[17]

TABLE 50-1

Fetal Oxygen Consumption in Various Species

Species	Measurement	VO_2 (mL/kg/min)
Human at term[19,19a]	B	6.0-6.8
Human <28 wk[135]	F	5.4
Rhesus monkey[136]	F	7.0
Sheep[115]	F	8.0
Cow[137]	F	6.7
Horse[138]	F	7.0
Guinea pig[139]	B	8.8

B, Bohr principle measurement; *F,* Fick principle measurement.

Experimental Data

The difficulties involved in obtaining Fick principle estimates of fetal VO_2 include the necessity of obtaining samples of umbilical venous and distal aortic blood for oxygen content analysis, which requires use of a relatively large fetus (i.e., sheep, cow, pig) for catheter placement, and the requirement of a reliable estimate of umbilical blood flow. The former problem has been overcome either by sampling at these sites acutely in anesthetized animals or by the implantation of fetal catheters[9] for serial measurements under nonstressed conditions. The latter problem has been overcome by the development of several methods for the accurate measurement of umbilical blood flow, which include the use of laser-Doppler, ultrasonic, or electromagnetic flow transducers; radiolabeled microspheres; or steady-state diffusion of substances such as antipyrine, ethanol, or tritiated water across the placenta. The advantages of the Fick method over the original Bohr technique include (1) the ability to perform serial studies in the same animal during late gestation, (2) the ability to measure uptake and excretion of potential fetal metabolites simultaneously with the uptake of oxygen, and (3) the ability to observe changes in metabolic rate before and after experimental manipulation. As noted, other noninvasive methods are being developed that are hoped to allow for diagnostic as well as research use in humans, such as blood oxygenation–level dependent (BOLD) MRI[14-15a] and optical methods such as NIRS and a modification of NIRS, intensity-modulated optical spectroscopy (IMOS).[16] These latter two techniques estimate with significant accuracy differences in hemoglobin saturation in intra-organ circulations. Other noninvasive methods, such as the doubly labeled water technique,[18] have been used to estimate long-term oxygen consumption or oxygenation in humans, but significant obstacles in estimating human fetal VO_2 preclude their use at this time.

As can be seen in Table 50-1, when corrected for fetal weight, fetal VO_2 as measured in a variety of species is remarkably consistent, with a range of 6.5 to 8.5 mL $O_2 \times kg^{-1}$/ minute. In studies of fetal VO_2 in term human fetuses using the Bohr or modified Fick methods,[19,19a] values of 6.0 to 6.8 mL/kg/minute obtained fit well within the range observed for other species.

Determining Factors

As stated, fetal requirements for energy may be estimated from measured fetal VO_2 or by measuring uptake of a variety of fetal substrates to gauge both fuel and accretion requirements. The factors that control fetal energy expenditure are not fully understood but one main tenet is that a relatively constant relationship exists between mass and metabolic rate across species in the adult. Kleiber[20] originally formulated the allometric scaling relationship:

$$\text{Metabolic rate} = (\text{Mass})^{3/4} \quad (3)$$

In this relationship, however, it is important to point out that metabolic rate is expressed in kcal/day and so the total daily

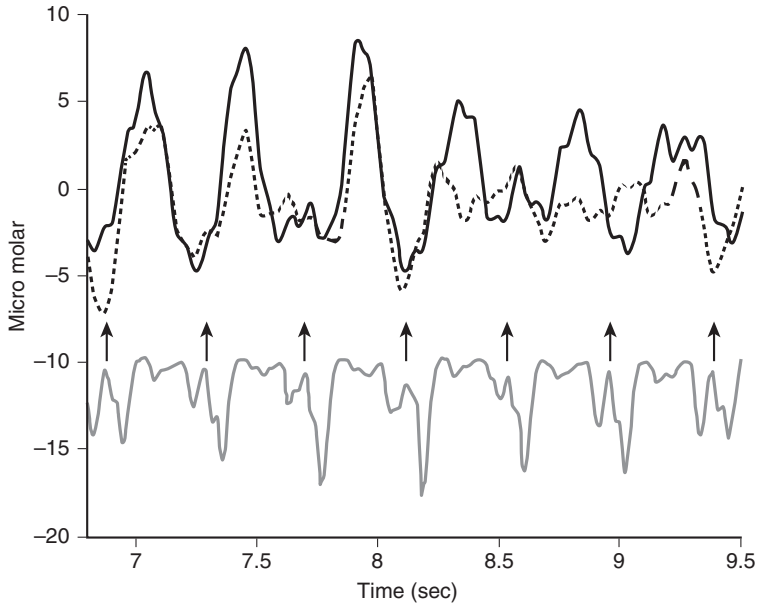


Figure 50-2 Near infrared spectroscopic (NIRS) analysis of blood volume *(solid line)* and saturated oxyhemoglobin *(dashed line)* changes in a human fetus measured transabdominally along with the fetal heart rate *(bottom dotted line). Arrows* represent fetal heart rate signals. *(From Vinzileos A, et al:* Am J Obstet Gynecol*192:129, 2005.)*

energy expenditure (not mass specific) rises in proportion to $(mass)^{3/4}$. As shown by Singer and others,[21] if mass-specific (non-allometric) basal metabolic rate (kcal/kg/time unit) is used instead, the relationship becomes an inverse one, with smaller animals having relatively larger mass-specific metabolic rates. It has been suggested that as mammalian species evolved, there was a general tendency for metabolic rate to increase directly in proportion to body mass (i.e., to $mass^{1.0}$). However, given that, geometrically speaking, surface area changes (which also must be taken into account relative to such factors as heat dissipation) are only proportional to $mass^{2/3}$,[22,23] an incongruity existed and the three-fourths power relationship may represent a basic biologic compromise. Alternatively it has also been recently argued[24,24a] that in living systems a variety of scaling relationships relative to mass are all based on multiples of 1/4 (life span, 1/4; heart rate, 1/4; mitochondrial density, 1/4; whole body RNA and DNA content, 1/4; cardiac output, 3/4). Over the 70 years since Equation 3 was first formulated, this basic principle has been extended and is reasonably predictive of the aerobic metabolic rates not only for a variety of mammals and other vertebrates, but also for the metabolic rates of single cells, mitochondria, and even for the turnover rate for substrates in the respiratory enzyme chain within mitochondria[25] (Figure 50-3).

The general relationship above may be predicted theoretically due to the basic limitations of nutrient and oxygen transport placed on respiring cells that relates to the geometry of branching networks, such as blood vessels or mitochondrial cristae. Subsequently this scaling relationship has been corroborated by a number of observers.[12,22,23] The theory requires that three basic factors be present for the scaling rule to be accurate: (1) a space-occupying branching network for transport purposes that extends throughout, (2) a "final" size-invariate end branch (such as a capillary), and (3) a requirement for minimization of the energy needed for substrate or oxygen transport purposes.[24,25] The caveats in such a relationship are that the metabolic rate is measured (classically as VO_2) (1) in the resting state as opposed to a "field" metabolic rate (i.e., during normal physical activity and changing milieu), and (2) in non-growing adults (i.e., does not include subjects that are actively growing, such as the fetus).

Although the basic three quarters rule would otherwise probably also apply in fetal and early neonatal life, the additional metabolic needs for growth and development clearly increase the demand for energy. At this point it may be more instructive to examine the nonallometric relationship between mass and energy as noted previously for the fetus and newborn. As it turns out, mass-specific metabolic rate is relatively constant from fertilization through the morula stage,[23] and increases (three- to four-fold) thereafter for the fetus. However, probably because of the diminished needs for such metabolic functions as heat control, VO_2 in late fetal life more clearly matches the weight-specific metabolic rate for the adult (Figure 50-4). According to recent work,[21] metabolic rate then rises above that of the adult postnatally, with a slow decline thereafter.[21,23] In fact, studies in human infants by a variety of investigators concluded that the relationship between body mass and VO_2 falls back to "adult" levels by about 18 months of age, or at roughly 12 kg.[26] Some of the differences noted relate to growth and changes in mass-specific surface area and some also to a poorly understood increment in cellular sodium pump activity in the newborn.[27] At the cellular level, the control of metabolic rate has not been clearly elucidated but involves complex interactions between a number of factors, including (1) the concentration of adenosine triphosphate (ATP), the product of mitochondrial oxidative phosphorylation; (2) the ratio of intracellular adenosine diphosphate (ADP) to ATP; (3) rates of intracellular protein synthesis; and (4) the generation of proton ($[H^+]$) gradients by respiratory enzymes across the mitochondrial and cell membranes.[12,28] For example, it has been estimated that approximately 15% of VO_2 of the term fetus might be spent on total active transport processes. One emerging factor related to regulation of metabolic rate (and potential changes during development)[29] is that polyunsaturated fatty acid (PUFA) composition of cellular phospholipid bilayers is directly related to the activity of the Na^+,K^+-ATPase pump, whereby increasing PUFA concentration is associated with increased metabolic activity. Whether this has significant applicability to the human fetus and newborn is unknown.

It has been assumed that at the organ or tissue level, metabolic rate is not influenced by oxygen availability unless the diffusion of oxygen is severely limited. Justification for this view stems from

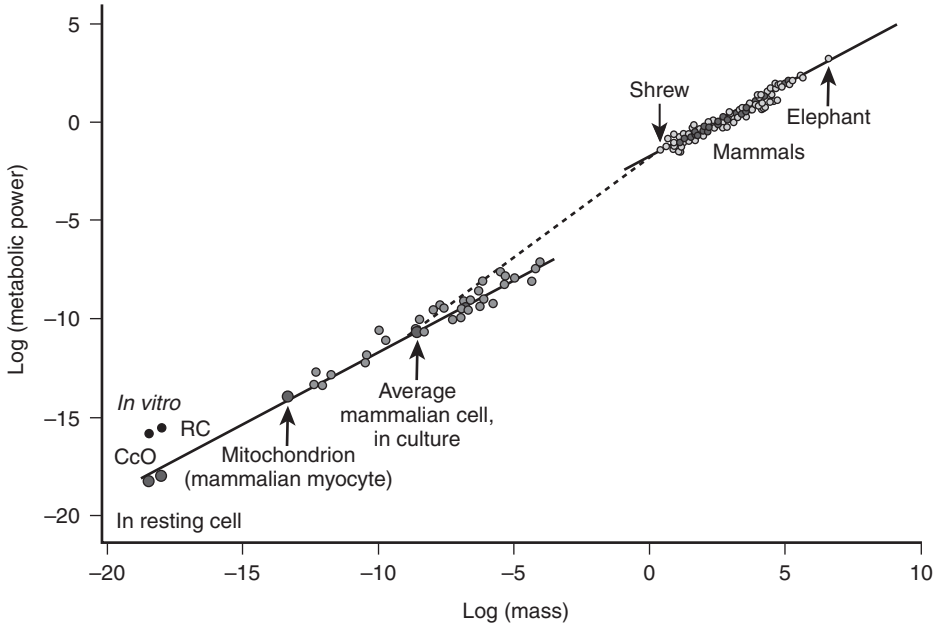


Figure 50-3 Basal metabolic rate as a function of mass using a logarithmic scale. The solid line is representative of the mass$^{3/4}$ relationship in mammals over 27 orders of magnitude from individuals, and extrapolated to unicellular organisms and uncoupled mammalian cells, mitochondria, cytochrome oxidase molecules (CoC) and the respiratory chain (RC) complex. *(From West GB, et al:* PNAS *99(Suppl 1):2473, 2002;West GB, et al:* J Exp Biol *208:1575, 2005.)*

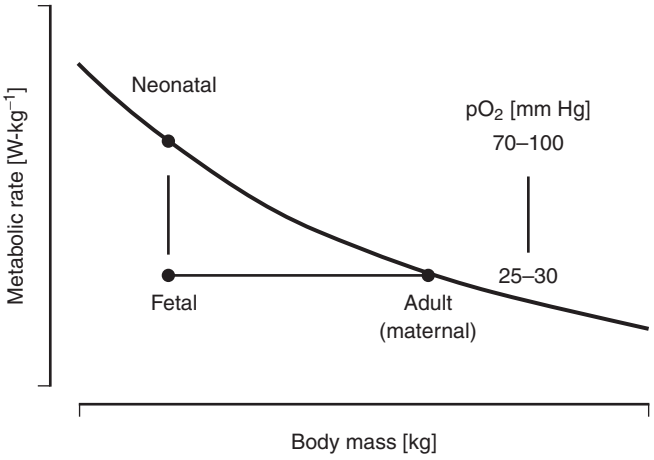


Figure 50-4 Change in relationship between body mass and metabolic rate (mass-specific, or nonallometric) from fetus to newborn. *(From Singer D, Mühlfeld C: Perinatal adaptation in mammals: The impact of metabolic rate.* Comp Biochem Physiol *Part A 148:780, 2007.)*

the observation that several mammalian tissues (liver, kidney) have similar in vivo and in vitro rates of VO_2, suggesting an intrinsic regulatory mechanism, perhaps related to genetically defined tissue-specific respiratory enzyme activities. However, some contradictory in vivo studies in mammals genetically adapted to living at high altitude (the South American Andean llama, family Camilidae)[30-31a] and in sheep recently acclimatized to high altitude[32] have shown that chronic exposure to relative fetal hypoxemia can, in addition to increasing hemoglobin concentration, induce significant depression of cerebral Na^+, K^+-ATPase pump activity, reduced expression of at least two Na channel proteins and decreased cerebral heat production, as well as decreased cerebral VO_2 without apparent biochemical or histologic evidence of brain damage.[30] At least some of these changes appear to be mediated by endothelin-1 and intrinsic nitric oxide, (NO) both potent mediators of vascular tone.

At sea level, however, classic studies have shown the characteristic fetal blood flow (and consequent oxygen) redistribution during graded maternal hypoxia, in which blood flow and oxygen delivery are preferentially shunted to brain and away from carcass.[33] Interestingly, in vitro studies have shown that tissues such as fetal muscle[34] do decrease VO_2 in response to change in media pO_2. Similar findings have been observed in other fetal organs such as liver and kidney.[35] A series of studies have shown that several oxygen-sensitive factors, most notably, hypoxia-inducible factor-1 (HIF-1),[36] a potent gene transcriptional factor, and calcium channel receptors[37] such as RyR1 may act to stimulate local organ intravascular synthesis of the intrinsic vasodilator NO, thereby increasing local blood flow and therefore oxygen delivery during times of relative hypoxemia. Expression of HIF-1, as well as several other hypoxia-inducible factors, appears to be developmentally and evolutionarily regulated.[38,38a] Studies of two distinct human populations living at high altitude for many centuries (Andeans and Tibetans) suggest divergent HIF-1 responses have evolved to deal with the problem of oxygen delivery.[19a,38a] Such studies have important implications for understanding the manner in which the fetus might be protected against sudden changes in oxygen delivery

INTEGRATION OF FETAL METABOLIC RATE

In terms of integrated metabolic rate of the whole fetus, most data available are from animals studied under steady-state conditions. Under a wide variety of circumstances, changes in O_2 delivery to the fetus are met by fetal changes in O_2 extraction to meet metabolic demands.[39] In the fetal sheep, O_2 extraction may be measured as the ratio between O_2 consumption (as in Equation 2) and O_2 delivery (the product of umbilical blood flow and umbilical venous O_2 content). In the steady state, this ratio

Box 50-1

Factors Influencing Fetal Oxygen Consumption

Specific organ metabolism*
Activity
Fetal breathing
Fetal limb movement
Fetal cardiac activity
Fetal sleep state
Fetal growth
Substrate uptake
Maternal exercise
Fetomaternal temperature gradient
Fetal hormonal status
Thyroid hormones
Catecholamines
Caffeine
Insulin

*Including ion pump activity and other processes necessary for cellular homeostasis.

TABLE 50-2

Tissue-Specific Oxygen Needs in the Fetus

	VO_2 per 100 g tissue Tissue μmol/100 g tissue/min	VO_2 per kg body weight μmol/kg body weight/min (mL/kg/min)	% Total VO_2
Whole fetus	—	360 (8.0)	100.0
Brain[120]	190	30 (0.7)	8.8
Heart[111]	400	25 (0.6)	7.5
Liver[140]	174	67 (1.5)	18.0
Intestine[33,46]	100	40 (0.9)	11.3
Kidneys[9]	100	8 (0.2)	2.5
Adrenal	68	2	0.05
Muscle[9,141*]	20	50 (1.1)	14.0
Other growth[4,9]	—	20 (0.45)	5.6
Activity[49*]	—	54 (1.2)*	15.0
Total	—	296 (6.6)	83

*Data for muscle (in vitro) and activity (in vivo) must reflect both sedentary and active states of striated muscle respiration.

is usually roughly 0.4 in the fetal sheep, meaning that only 40% of the oxygen delivered to the fetus via the placental circulation is actually extracted. The ratio during experimental hypoxemia may rise to some extent (to 0.5 to 0.6). Studies in healthy human fetuses during delivery found extraction ratios of 0.62 to 0.64.[11,40] Interestingly, even in fetuses with intrauterine growth restriction that were excreting significant amounts of lactate (and presumably had relative fetal hypoxia), no increase in extraction was observed, suggesting a relative maximum in extraction efficiency had been reached.[9,11] Of interest is the relative constancy of fetal VO_2 across species lines when corrected for fetal weight. In species whose fetal weights differ by as much as a factor of 300, weight-specific fetal VO_2 estimates (ml/kg/minute) differ by only 15% with a mean value of 7.4 mL/kg/minute (see Table 50-1).[9] Corroboration of these observations is apparent when resting fetal heart rates of a number of species are compared. Fetal and neonatal heart rates (and therefore cardiac output) have been used as reliable indicators of the metabolic rate.[41] Meier and co-workers[42] have observed that fetal heart rate differs little across species lines, in contrast to the adult, in whom heart rate is universally related to body weight. The reasons for such constancy of metabolic rate between various species are unclear but may relate to the previously mentioned allometric scaling principles,[25] the rapid rate of growth in late perinatal life, and changes in membrane lipid composition.[29] In addition, it has been hypothesized that the fetus is relatively free from the "adult" oxidative demands of temperature homeostasis and, to a lesser extent, gravity and muscular activity, all of which are somewhat dependent on body mass and surface area.

Of those factors influencing fetal VO_2, several are particularly prominent (Box 50-1). "Specific organ metabolism" refers to those various cellular processes common to most tissues. Under basal conditions a certain minimal level of VO_2 is necessary to maintain cellular homeostasis. Virtually all mitochondrial and plasma membrane ion pumps, vital for maintenance of intracellular and intraorganelle homeostatic reactions, require energy in the form of ATP for their activity and are thus heavily dependent on cellular respiratory activity. Such pumps consist of F-type H^+ pumps (mitochondria), V-type H^+ pumps (lysosomes, storage granules), or P-type Na^+,K^+, or H^+ pumps (plasma membranes).[43] The degree of mitochondrial proton leak is also allometrically related to body mass among different species.[23] In the adult, overall pump activity has been estimated at approximately 20% of the basal metabolic rate.[44] Although it has thus far been difficult to estimate the contribution of such processes to the overall demand on the fetus for oxygen, it is likely that for many tissues, pump activity is important in determining tissue VO_2. For example, in one estimate,[45] ouabain (via inhibition of Na^+,K^+-ATPase pump activity) caused a decline in fetal sheep liver and placental VO_2, which accounted for 20% and 34% of whole organ VO_2, respectively. Similarly in the newborn rat, approximately 50% of liver metabolism was due to activity of the sodium pump, with a significant decline in infancy.[27]

Other metabolic processes, such as protein synthesis and attendant energy-dependent chemical reactions such as transamination and decarboxylation have metabolic costs, but the contributions of these to fetal VO_2 have not been determined. Such processes that are common to all cells must make up a significant fraction of what is estimated when whole-organ or cellular VO_2 is measured. It is clear that fetal work (i.e., muscle oxidative requirements to perform such activities as fetal breathing movements, limb movements, etc) also accounts for a significant fraction of the total fetal VO_2. For example, striated muscle activity (excluding cardiac work), as assessed in the in vivo fetal sheep, has been estimated to account for as much as 15% of the total fetal VO_2, which agrees with an in vitro measurement of approximately 20 μmol/100 g/minute, assuming muscle mass in the term fetal lamb of 25% fetal wet weight. If specific cardiac VO_2, as also measured in the lamb, is added, slightly greater than 20% of the fetal requirement for oxygen is necessary for normal striated muscle activity.

Unfortunately further information of tissue-specific oxygen needs in the fetus is limited to several accessible organs in the sheep fetus although some in vitro tissue data from premature humans is available for comparison.[46] From estimates in Table 50-2, however, it can be seen that approximately 50% of the fetal VO_2 is attributable to fetal brain, liver, kidney, and intestine. Lastly, the energy cost of growth (i.e., the energy necessary to synthesize new tissue as opposed to the energy stored in new tissue) has been estimated to be approximately 10% of the caloric value of new tissue, which is equivalent to about 6% of the fetal VO_2. Thus in the sheep fetus, approximately 83% of the fetal VO_2 can be accounted for in the resting state. It is assumed that measurement of VO_2 in other tissues (e.g., bone marrow, lung, adrenal gland, cartilage, skin, etc.) will provide for the remaining 17% of whole fetus oxygen needs.

Several factors may influence the fetal metabolic rate (see Box 50-1). Interestingly, other than active fetal cooling, such adverse stimuli such as mild hypoxemia, manipulations such as mild hypoxemia, maternal exercise,[47] or severe maternal starvation[48] do not appear to depress overall fetal metabolic activity. Although not well studied, those maternally administered drugs that cross

the placenta and have a depressive effect on fetal activity would be expected to decrease fetal VO_2 as well. For example, skeletal muscle paralysis in the fetal lamb causes a 10% to 15% decrease in fetal VO_2.[49] Significant depression of fetal respiratory or body movement has been noted after maternal exposure to ethanol, antidepressants, cigarette smoking, hypoxemia, or steroids.[50,51] The fetal metabolic rate can be stimulated by such factors as change in fetal sleep state,[50,52] excessive fetal muscular activity, maternal fever, and possibly external stimuli such as sound.[53] Studies regarding changes in fetal behavior are possible using fetal ultrasound to gauge fetal movement and breathing. Using these techniques, it has been shown that fetal breathing is more prominent after maternal food intake[50] or glucose infusion[54] and appears to be stimulated by maternal drug abuse (cocaine, methadone).[51]

The hormonal milieu also plays a significant role in setting the background for the fetal metabolic rate at rest and when changes occur in the fetal environment. The interplay between the hypothalamic-pituitary-adrenal axis, catecholamines, and other fetal hormones influence cellular respiration and metabolic rate. In the adult, changes in both sympathetic and parasympathetic nervous activity during meals may be partially responsible for the well-known postprandial increase in metabolic rate. In the steady state, fetal insulin deficiency has not been shown to cause a change in resting umbilical VO_2,[55] although maternal and therefore fetal hypoglycemia has been shown to lower VO_2 by as much as 30%.[56] As noted in the following text, both fetal hyperinsulinemia and hyperglycemia appear to have independent effects upon accelerating fetal VO_2.[56] Fetal thyroid ablation has been shown to cause a 20% to 30% reduction in fetal metabolic rate[57] and exogenous thyroxine can be shown to reverse these metabolic changes. These observations may have important implications for such disease states as fetal thyrotoxicosis or maternal diabetes.

FETAL ENERGY SUBSTRATES

As noted in the previous sections, fetal VO_2 in most species, including humans, has been measured to be approximately 7 to 8 mL/kg/minute or roughly 11.5 L of oxygen per kg body weight per day. Assuming a "fetal diet" (see later) relatively low in fat intake for most species and a conversion factor of approximately 4.8 to 4.9 kcal energy liberated per liter oxygen consumed, the energy requirement (caloric value of substrate used for oxidation) by the fetus would be equivalent to approximately 55 kcal × kg^{-1} × day^{-1}. Because fetal energy requirements dictate a significant source of carbon uptake, it has been of interest to determine the relative uptakes of potential substrates across the fetal circulation. This has been accomplished both by classic Fick principle physiologic methods in ruminant species and by isotope infusion studies (e.g., radioactive carbon [^{14}C]-labeled glucose, lactate). Although the latter method has been more completely defined in fetal sheep, transfer studies are also available in small mammals such as the guinea pig, rabbit, and rat,[58,59] as well as in human fetuses to a limited extent.[60]

METHODS AND TERMINOLOGY

Uptake

Uptake of a substrate such as glucose may be calculated using the equation derived for fetal VO_2:

$$U_{substrate} = Fumb \times (v - a)_{substrate} \quad (4)$$

where $U_{substrate}$ = the umbilical uptake of a particular substrate in mmol or mg/kg/minute and $(v - a)_{substrate}$ = the umbilical vein–distal aortic concentration difference of substrate in mmol or mg/mL. Note that U does not necessarily measure fetal uptake because, theoretically, endogenous production of a particular substrate (such as glucose or lactate) from other substrates must be taken into account. U, therefore, measures net umbilical uptake and provides only a minimum estimate of fetal uptake. Another limitation of this method for estimating fetal substrate utilization is the sensitivity of the method used to assay substrate concentration (usually colorimetric or spectrophotometric methods) relative to the percent extracted from the circulation. For example, in the fetal sheep, the umbilical venous concentration of glucose is approximately 30 mg/dL (1.7 mmol/L), and the extraction coefficient ([uptake/delivery] × 100) ranges from 10% to 15%. The coefficient of variation of the glucose assay is approximately 3%, and thus it is possible to measure umbilical glucose uptake with accuracy. By contrast, the umbilical venous concentration for the amino acid serine is 700 μmol/L with a coefficient of variation of the assay of 1%.[61] In this case, however, the extraction coefficient of serine is only 0.6% of the delivery rate. It is therefore obvious that for substances with small extraction coefficients, accuracy in measuring umbilical uptake using the Fick principle is problematic.

Oxygen Quotient

Previously it was difficult to determine directly whether specific fetal substrates were taken up by the umbilical circulation and oxidized completely. The concept of substrate-oxygen quotient was adapted for the fetus,[9] predominantly using sheep to address this question. To estimate the maximum fraction of fetal oxygen consumption accounted for by the oxidation of any given substrate, the following equation is applied:

$$\text{Substrate}/O_2 \text{ quotient} = \frac{F_{umb} \times (v - a)_{substrate}}{F_{umb} \times (v - a)\ O_2} \times n \quad (5)$$

where n = the number of moles of oxygen required for the complete oxidation of substrate:

Substrate	n
Glucose	6.0
Lactate	3.0
Glycerol	3.5
Palmitic acid	23.0
β–Hydroxybutyrate	4.5
Acetoacetate	4.0

Using Equation 5, the blood flow terms in numerator and denominator (F_{umb}) cancel out, leaving the ratio of the venoarterial concentration differences of substrate versus oxygen. Thus, as adapted for fetal metabolic studies under steady-state conditions, this method of estimating substrate requirements for energy has the advantage of not requiring measurement of umbilical blood flow or fetal mass. The demonstration of a substrate-oxygen quotient near or equal to 1.0 suggests that if the particular substrate measured were completely oxidized, it could provide 100% of the carbon necessary for the measured fetal VO_2. A substrate-oxygen quotient significantly less than 1.0 would indicate the requirement for other carbon sources to be used to sustain metabolic rate even if the measured substrate were completely oxidized. It then follows that substrate-oxygen quotients greater than 1.0 indicate use of a significant portion of measured substrate for purposes other than energy production (i.e., for synthesis or transformation into other substrates). In fetal sheep, substrate-oxygen quotients measured under steady-state conditions[9] include glucose, 0.5; total amino acids, 0.25; and lactate, 0.25 with negligible fatty acid uptake. In other species including the human, estimates vary from 0.5 to 0.8 (Table 50-3) for glucose-oxygen quotients.

Although useful conceptually and in situations in which steady-state umbilical blood flow is difficult or impossible to determine, substrate-oxygen quotients that have been measured have had limited usefulness. Major limitations to the interpretation of this method include (1) the inability to trace metabolic pathways of

TABLE 50-3

Umbilical Glucose Uptake and Glucose/Oxygen Quotient

Species	Umbilical Glucose Uptake ($mg/kg^{-1}/min^{-1}$)	Umbilical Glucose/O_2
Sheep[69,114]	6.4	0.45
Cow[137]	5.2	0.57
Horse[138]	6.8	0.69
Human[19a,69,81]	4.2-5.0	0.80-1.2

degradation or transformation and (2) the necessity for conditions of steady state (i.e., no change in metabolic rate or blood flow) to be fulfilled to determine V-A differences accurately.

Substrate Utilization

The application of radioactive and nonradioactive isotope ("tracer") methodology[62] has provided interesting new insights into fetal metabolic processes and added substantially to the body of knowledge obtained from interpreting substrate-oxygen quotients and substrate umbilical uptakes via the Fick method. Fetal infusion of substrates such as glucose or lactate,[63] labeled with such tracers as ^{13}C or ^{14}C, can be performed and the fluxes of both radioactive and nonradioactive substrates then measured across the umbilical circulation. In addition, the transformation into other substrates or carbon dioxide can be quantified. Using this method calculation of fetal glucose utilization (i.e., glucose used for oxidation and storage from exogenous and endogenous sources) can be accomplished. As discussed later, under nonstressed conditions in ruminants uptake studies are either in agreement with (glucose) or underestimate (lactate, amino acids) actual fetal uptake and utilization. Limited data have also become available[64-65] in humans using stable isotopes, particularly related to fetal gluconeogenesis (see later). Additional techniques are being perfected for the study of fetal organ metabolism, particularly the brain. For example, positron-emission tomography (PET) has been used in fetal sheep to explore cerebral glucose uptake via estimation of intracellular glucose phosphorylation.[66] Additionally, a recent study by Kok and colleagues[67] has demonstrated that cerebral metabolic information may be obtained noninvasively in human fetuses using proton magnetic resonance spectroscopy.

Respiratory Quotient

In the nongrowing adult mammal, the ratio between generated carbon dioxide and consumed oxygen, the RQ, can provide some indirect information regarding the relative oxidation rates of carbohydrates, fats, and proteins. For example, the RQs of adult subjects oxidizing either carbohydrates or fat are 1.0 or 0.7, respectively, with those on a mixed diet having an RQ of 0.85. In the resting state, the RQ of the fetal lamb is approximately 0.94, suggesting little fetal oxidation of fat. Because of the rapid rate of fetal growth, however, the inequity between carbon uptake and excretion across the umbilical circulation invalidates this method as providing a sensitive measure of differential substrate utilization by the fetus.

Substrate Uptake: Carbohydrate

In most species studied a significant relationship is present between maternal and fetal levels of glucose, the carbohydrate present in highest concentration in fetal blood.[68,69] Other carbohydrates, such as fructose (ruminants) or galactose, are present in fetal blood but appear to play relatively minor roles in oxidative metabolism during fetal life. Fetomaternal transfer of glucose has been best studied (although not exclusively so) in ruminant species, in which determinants of transfer include (1) the maternal glucose concentration, (2) the fetomaternal glucose gradient, (3) the fetal endocrine milieu (specifically fetal insulin and glucagon secretion), and (4) the degree of placental metabolism of glucose. In several other species, including humans, these general determinants of glucose transfer also appear to be applicable. In humans, since the advent of periumbilical blood sampling (PUBS), fetomaternal substrate relationships have been examined more closely,[68] and these have documented that in both midgestation and late-gestation human pregnancies, a high degree of correlation between maternal and fetal blood glucose concentrations exists under relatively steady-state conditions. Marconi and associates[68] have also demonstrated in humans that the fetomaternal glucose gradient increases with advancing gestational age in normal pregnancies (Figure 50-5) and that this difference is more pronounced in growth-restricted fetuses. Using ^{13}C-labeled glucose injected before cesarean section or PUBS, fetal glucose utilization has been estimated in pregnant humans.[70] Although the measured glucose disposal rate of the human conceptus included placenta as well as fetus, glucose disposal rate related directly to the maternal plasma glucose concentration and increased with increasing conceptus mass. Furthermore, estimates of fetal glucose disposal were in agreement with studies in neonatal humans as well as fetuses of several other species.

Understanding of mechanisms of placental glucose transport has been expanded with the discovery of two families of specific membrane associated carrier proteins: the glucose transporter proteins, now labeled GLUT-1-14, and sodium-dependent glucose transporters 1-2 (SGLT 1-2).[71,72] Specifically the GLUT-1 isoform, known also as the brain erythrocyte glucose transporter, is expressed on both the maternal and the fetal facing sides[73] of human placentas. Although the density of transporters on the maternal side (microvillous membranes) remains constant after the first trimester, the concentration of transporters on the fetal facing (basal) side increases at least two-fold after the first trimester.[74] Furthermore, measured rates of placental glucose uptake in vitro match the density of placental GLUT-1 transcripts. In addition, GLUT-1 expression is inhibited by glucose but is not regulated by insulin.[56] In both humans and sheep, it has been estimated that glucose transport capacity is considerably higher than necessary given the normal range for maternal serum glucose concentrations. This suggests that changes in fetal and maternal glucose concentration or metabolism are key factors in determining fetal glucose transport. Within certain tissues, GLUT expression is important for determining the overall contribution of glucose as a major substrate for the fetus. In addition, various factors including insulin and glucose concentrations may modify synthesis of these proteins. For example, acute hyperinsulinemia or glycemia in the sheep fetus leads to an increase in muscle GLUT-4 expression, with a commensurate increase in hindlimb glucose uptake. However, chronic hyperglycemia in the sheep fetus promotes a state of relative insulin resistance, presumably due to a depression of muscle GLUT-4.[56,75] The complex roles and interactions between these tissue-specific GLUTs in regulation of glucose utilization and metabolic rate are not yet fully understood.

The placenta metabolizes glucose derived from both mother and fetus.[73] The rate of placental glucose consumption is not static and appears to be related to the fetal glucose concentration as studied in the fetal sheep.[69] The placental contribution to total uterine glucose uptake is significant and approximates 50% of uterine glucose uptake when measured in the sheep. In addition to concentration gradient, a number of studies have documented that the fetus is capable of exerting some hormonal control over glucose uptake, at least in the last trimester. Several investigators[76,77] have shown that fine control of glucose uptake under steady-state conditions is also mediated by changes in the endogenous fetal insulin concentration. In addition, surgical or chemical ablation of fetal insulin release induces relative

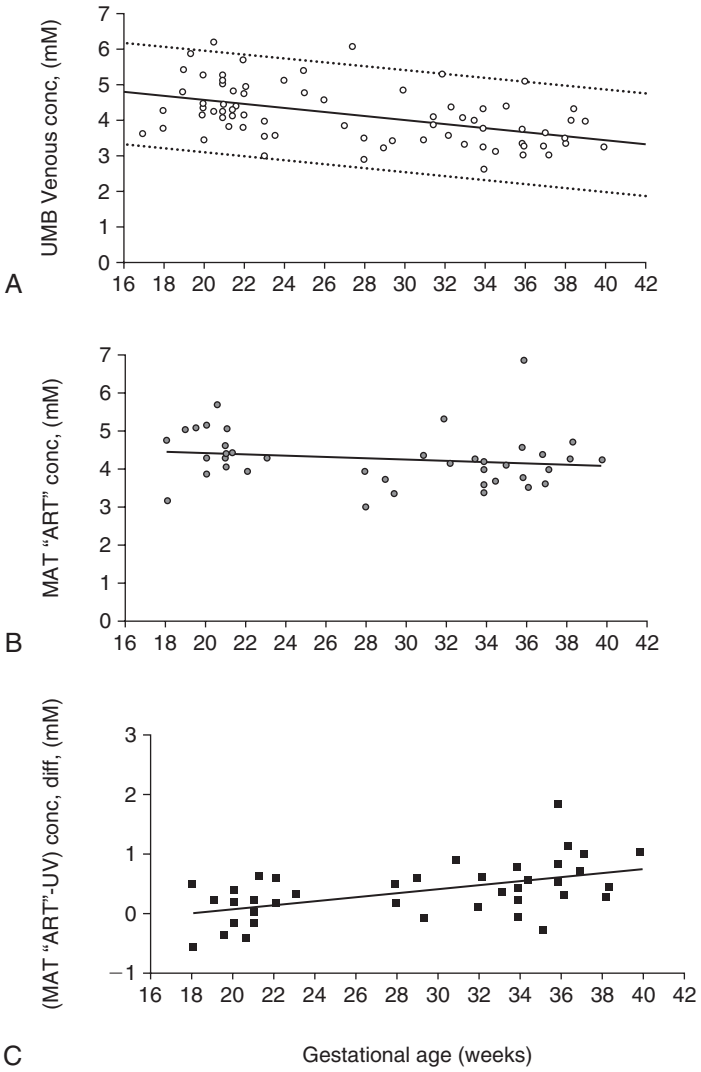


Figure 50-5 Human maternal versus fetal blood glucose concentration changes during gestation. **A,** Umbilical venous glucose versus gestational age in normal fetuses (UV = 5.66-0.56 GA); **B,** maternal arterial glucose versus gestational age; **C,** maternal-fetal glucose difference versus gestational age *(Marconi AM, et al:* Obstet Gynecol *87:937, 1996.)*

hyperglycemia and a decline in umbilical glucose uptake and fetal glucose utilization.[55,78,79] As noted earlier, Hay and colleagues[56] have shown conclusively that changes in circulating insulin concentration act as the principal regulator of glucose oxidation (versus other potential substrates in the fetus), but that regulatory control is more related to the combined factors of glucose and insulin concentrations than either taken separately. Other potential glucose regulatory hormones, such as glucagon, cortisol, and thyroid hormones, have been studied to a lesser extent in the fetus. In the absence of maternal or fetal disease, changes in physiologic concentrations of these hormones in fetal blood do not seem to be major factors in regulating steady state fetal glucose uptake until late gestation. It is highly likely that they are important coregulators of glucose metabolism during parturition and may also be very important in providing glucose homeostasis during times of stress, such as during maternal fasting or malnutrition.[57,57a,80]

Glucose Uptake and Utilization

Because of estimates of the fetal RQ approaching 1.0 as well as the relatively high concentrations of glucose in fetal blood, it was previously thought that a significant fetal uptake of glucose occurred. To test this hypothesis, a number of investigators measured glucose uptake in the fetus using classic Fick principle techniques (net umbilical uptake, Equation 2) as well as radiolabeled glucose transfer (fetal uptake and utilization). In one comparison of the two methods, Hay and colleagues[69] reported essentially identical results—measured net umbilical uptake of glucose was similar to the rate of fetal glucose utilization in the sheep, and both were related to the maternal glucose concentration. These results in the sheep fetus imply negligible endogenous fetal glucose production in the resting nonstressed state (see later discussion).

Studies in a variety of species, including human, show that net umbilical glucose uptake near term is between 4 and 7 mg (22 to 39 μmol)/kg/minute. This equates to approximately 6 to 10 g glucose/kg/day or roughly 32 kcal/kg/day in potential energy production. It is, however, significantly less than the energy estimated to be required by the measured fetal VO_2, which is approximately 55 kcal/kg/day. In fact, in estimates of umbilical glucose-oxygen quotients obtained in several ruminant species, the complete oxidation of glucose accounted for only roughly 50% of the fetal VO_2 (i.e., umbilical $G/O_2 = 0.5$; see Equation 5). These estimates, however, represent only the maximum percent

of the fetal VO_2 accounted for by complete oxidation of glucose and therefore overestimate the actual fraction if any significant glucose conversion or storage (such as to glycogen, fatty acids, or triglycerides) occurs. Only a few similar studies in humans are available for comparison[19a,70,81] (see Table 50-3) but are remarkably similar to data obtained in animals. Thus, although glucose represents a quantitatively significant source of fetal energy (fuel), it probably does not supply enough carbon to support the total oxidative demands of fetal life. Studies suggest that at least 80% of measured fetal glucose uptake can be accounted for by brain and striated (skeletal plus cardiac) muscle. Although the fetal liver consumes and oxidizes glucose, net consumption as measured in the sheep is small,[82,83] with significant concomitant hepatic outputs of pyruvate and glutamate. Use of other methods may provide more information. For example, Thorngren-Jerneck and associates[66] using PET scans, have determined that the global cerebral glucose metabolic rate (rate of intracellular cellular glucose phosphorylation) in near-term fetal lambs was 37.8 μmol × min^{-1} × 100 g^{-1}. Calculation of the cerebral glucose/O_2 quotient from Table 50-2 and Equation (5) yields a value of 1.1, similar to previous estimates measured more directly.

Unfortunately similar measurements of glucose uptake and oxidation are not available in other species. Less direct work in species such as the rat, pig, or guinea pig,[59,84,85] however, is in agreement with these estimates. In the human, demonstration of glucose uptake and utilization by fetal brain, liver, and placenta has also been documented. Estimates of glucose production (not utilization) in normal term and preterm newborns[65] using stable isotopes have suggested values of 3 to 5 mg/kg/minute. Although cord blood venoarterial differences of glucose in human studies[68,81] are positive, that is, suggesting net umbilical uptake, direct measurements of uptake or oxidation are limited. As noted previously, however, measurement of glucose disposal rate in human fetuses appears in the range of 5 mg (28 μmol)/kg/minute.[70]

GLUCOSE STORAGE

Glycogen

In the fetus as in the adult, the major storage form of glucose is glycogen, a glucose polymer stored intracellularly as a precipitate, with an average molecular weight of 500,000 daltons or greater. In fetal life, glycogen is stored in significant concentration in liver, skeletal and cardiac muscle, kidney, intestine, brain, and placenta. From the work of Shelley,[86] it is apparent that glycogen storage in most fetal species reaches a maximum concentration at term gestation in organs such as liver and skeletal muscle. In most other storage sites (i.e., lung, myocardium, kidney, and placenta), however, peak values are attained somewhat earlier (50% to 70% gestation), with a gradual decline near term to adult levels. The factors responsible for glycogen synthesis and induction of enzymes necessary for glycogen synthesis are complex (Figure 50-6) and reviewed elsewhere.[87]

Of importance are the following generalizations: (1) Activity of the enzyme necessary for glycogen synthesis (glycogen synthase) correlates well with the relative presence or absence of glycogen in fetal tissues. (2) Fetal liver glycogen synthesis appears to be regulated by both endogenous fetal insulin production and intactness of the fetal pituitary-hypothalamic-adrenal axis. Studies show lack of hepatic glycogen accumulation in decapitated fetal rats or rabbits with little effect on glycogen synthesis in other organs.[88,89] Fetal thyroid function may also be an important contributor to hepatic glycogen storage.[57,57a] (3) Significant fetal glycogen turnover with active degradation probably occurs in the last trimester but with relative net accumulation in tissues such as liver. (4) Certain organs, such as the placenta, accumulate glycogen in response to maternal stimuli such as human placental lactogen or insulin with little response to changes in fetal hormonal levels.[90] Maternal nutritional sufficiency plays a major role in late fetal glycogen deposition, but some discrepancies in the literature currently exist, probably as a result of species differences as well as differences in experimental design. In general, when studied in the rat or sheep,[91,92] significant maternal malnutrition causes a decrease in maternal hepatic glycogen stores but inconsistent changes in fetal hepatic glycogen.[93] Interestingly, in late gestation fetal leptin infusion in fetal sheep was shown to inhibit glycogen storage as well as enzymes of gluconeogenesis, suggesting an important role for the less well-characterized hormone in control of glucose homeostasis.[94] (5) Glycogen synthesis is highly energy dependent: both glycogen stores and glycogen synthase activity are depressed in experimentally induced intrauterine growth–restricted rats (IUGRs).[95] The mechanism appears to be related to impaired oxidative phosphorylation in liver and muscle, leading to depressed levels of ATP. Synthesis of glycogen classically derives from glucose. It has been demonstrated, however, that a significant portion of glycogen in the postabsorptive adult actually is synthesized from precursors of gluconeogenesis such as lactate and pyruvate (see Figure 50-6). This also appears to be the case in the sheep fetus.[96]

Another potential pathway for glycogen production (transformation of glycolytic intermediates through serine for the production of glycogen) has been documented in fetal hepatocytes.[97] This pathway allows for bypass of the gluconeogenic step oxaloacetate to phosphoenolpyruvate (which is rate limiting because of the developmental lag in fetal gluconeogenic enzyme synthesis (see later). It has been estimated that the alternative serine pathway could account for up to 25% of the glycogen produced.[97] In addition, serine and glycine are cycled between fetal liver and placenta,[60,61] providing another convenient source of precursor for fetal glycogen deposition.

Glycogen breakdown (glycogenolysis) with resultant availability of glucose for use as fuel substrate is an obvious important factor contributing to glucose homeostasis during the perinatal period and has significant survival value for the fetus in times of stress. The regulation of the activity of the enzyme involved in glycogenolysis (glycogen phosphorylase) is reciprocal to that of synthase. Thus when one is stimulated (as when phosphorylase expression is stimulated by catecholamines or glucagon), the other (synthase) is inhibited. In fetal tissues such as liver and kidney, glycogen breakdown can be induced by such hormones as catecholamines or glucagon or by stimuli such as cold stress or hypoxia. In these tissues the presence of glucose-6-phosphatase allows for glucose dephosphorylation and net glucose exit from intracellular stores. Thus free glucose can be released into the peripheral circulation for use at sites (e.g., brain) distant from the original storage tissue. In other organs such as lung, placenta, and myocardium, phosphorylase, but not glucose-6-phosphatase, is present, and stored glycogen is available only for intracellular consumption within the storage site. For some tissues such as myocardium and placenta, which have large obligate glucose requirements, this offers a distinct degree of protection against the sudden onset of hypoglycemia. For whatever reason, central nervous system glycogen storage in fetal life is minimal. In the late-gestation fetus it is possible to demonstrate glycogenolysis in response to pharmacologic doses of such secretagogues as glucagon.[98] In malnourished sheep, however, as in humans with intrauterine growth restriction, there is inconsistent evidence as to whether significant glucose production may occur from stored glycogen.[64,96] However, growth-restricted rat fetuses[99] have been shown to have markedly diminished glycogen synthesis, due to deficiencies in pyruvate oxidation and mitochondrial ATP generation.

GLUCONEOGENESIS

Another major pathway for the endogenous production of glucose is via synthesis from nonglucose precursors, such as certain amino acids, lactate, or pyruvate (see Figure 50-6)

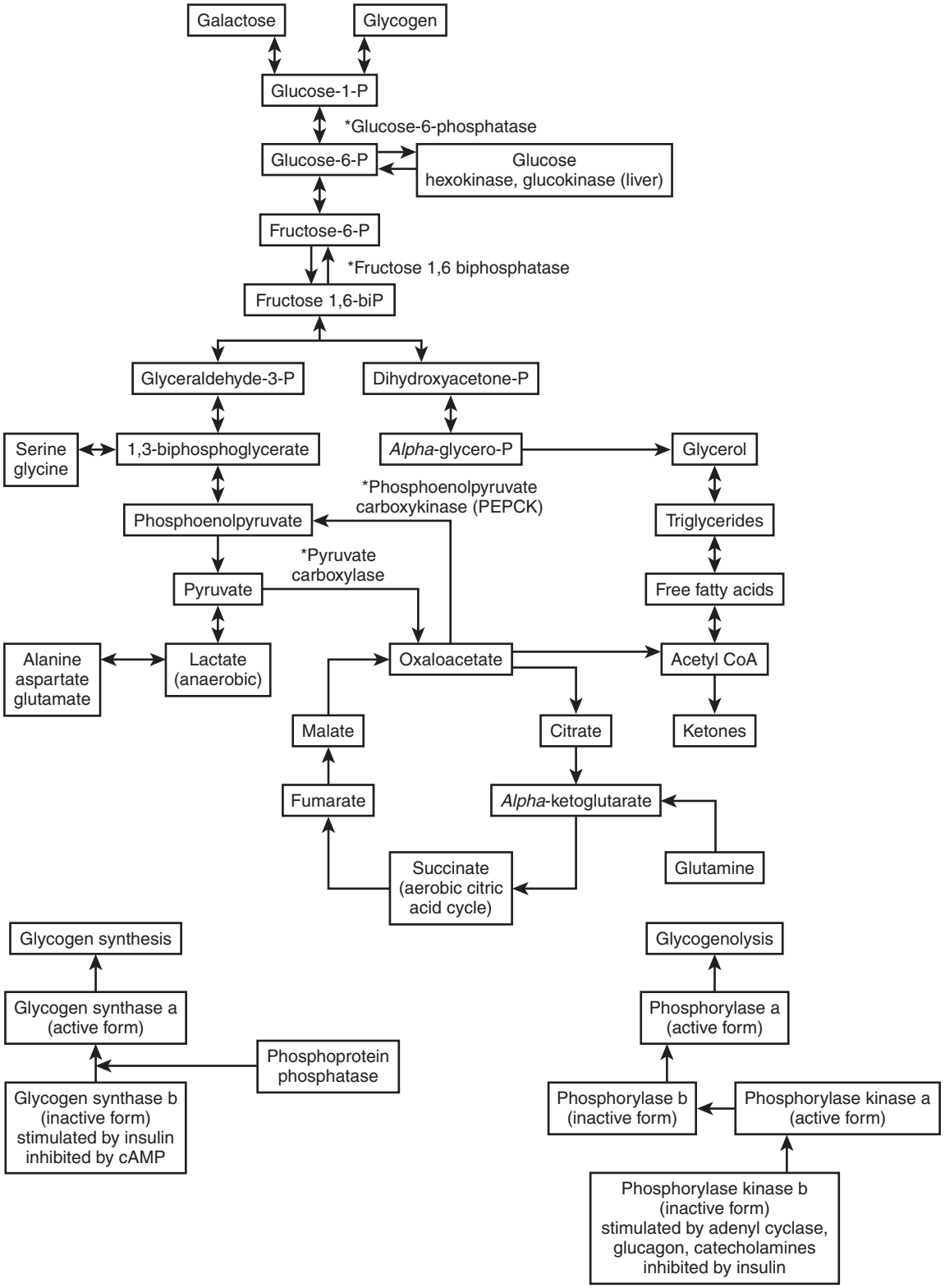


Figure 50-6 Metabolic pathways involving fetal metabolic substrates. Key gluconeogenic enzymes (*) include (1) glucose-6-phosphatase, (2) fructose-1,6-biphosphatase, (3) phosphoenolpyruvate carboxykinase, and (4) pyruvate carboxylase, and metabolic pathways involved in glycogen synthesis and degradation. *(Redrawn after Pagliara AS, et al:* J Pediatr *82:365, 1973; Milner RDG: In Beard RW, Nathanielsz PW [eds]:* Fetal Physiology and Medicine. *New York, Marcel Dekker, 1984. Reprinted courtesy Marcel Dekker, Inc.); Harris RA: Carbohydrate metabolism I: Major metabolic pathways and their control. In Devlin TM [ed]:* Textbook of Biochemistry, *6th ed: with clinical correlations. Hoboken, NJ, Wiley-Liss, 2006.*

(i.e., gluconeogenesis). Although the regulation of gluconeogenesis has been extensively studied in adult humans, significant capacity for gluconeogenesis in the human fetus has not been documented. For glucose to be synthesized from substrates such as glutamate or lactate, key enzymes located largely in liver and kidney must be present. Activities of phosphoenolpyruvate carboxykinase (PEPCK), pyruvate carboxylase (PC), fructose-1, 6-biphosphatase, and glucose-6-phosphatase have been measured in fetal liver from several species, including the human and are summarized elsewhere.[65] Although glucose-6-phosphatase activity is present from the twelfth week of gestation in the human, PEPCK activity is considerably less than in postnatal life and appears to be rate limiting in terms of glucose production. Activities of the other two gluconeogenic enzymes appear to be intermediate. In animal studies of gluconeogenic enzyme capacity, such as in the rat, similar patterns of activity are present. When the rate of gluconeogenesis has been measured via either in vivo or in vitro studies, variable results have been obtained, but the consensus is that significant gluconeogenesis does not occur under nonstressed conditions in the fetus.[65] For example, even though the liver extracts significant amounts of lactate from the umbilical circulation,[82] gluconeogenesis in fetal sheep or rat liver in vitro is nil unless stimulated by glucagon in the incubation medium. Small but significant rates of gluconeogenesis have been documented using either fetal rat kidney or liver from pig, guinea pig, and monkey. Even in these species, however, there appears to be a developmental increase in gluconeogenic activity only after approximately 90% of gestation. In vivo radiolabeled tracer studies in sheep and rats before term fail to detect significant gluconeogenesis in the resting state,[96,100] although glucagon has recently been shown to stimulate gluconeogenesis in the near-term fetal sheep.[101]

The reasons for these inconsistencies have recently been elucidated by Fowden and colleagues,[96] who have shown in the fetal sheep that the capacity for gluconeogenesis is restricted until the last few days of gestation, when it rises to account for roughly 50% of the glucose utilization rate and parallels the rate of rise in fetal blood concentrations of cortisol and catecholamines. In these studies maternal fasting induced endogenous glucose production only in late gestation fetuses, but 5 to 6 days earlier than would normally have been observed in nonfasted animals. As noted earlier, thyroid hormone, cortisol and catecholamines are all likely to mediate gene regulation and stimulation of synthesis of enzymes involved in hepatic gluconeogenesis. As stress hormones, this may explain the recently proven link between gluconeogenic enzyme synthesis and hypoxic stress (see later).

It is probable (but as yet unproved) that significant endogenous glucose production occurs in the stressed human fetus, such as during maternal hypoglycemia or starvation or in some states of intrauterine growth restriction. In human growth-retarded fetuses exposed to a maternal overnight fast, Marconi and colleagues[64] were unable to detect endogenous fetal glucose production when a maternal infusion of ^{13}C glucose was used. Whether the near-term human fetus has the capacity for significant glucose production under stress conditions such as intrauterine growth restriction is as yet unknown.[11]

MATERNAL DISORDERS: FETAL EFFECTS

In common clinical situations that affect the maternal milieu, fetal metabolic changes may occur as well. These may include (1) fetal hypoxemia induced by maternal hemorrhage or by maternal hypoxemia, (2) fetal hyperglycemia as a consequence of maternal diabetes, and (3) fetal hypoglycemia due to maternal malnutrition or insulin therapy. The majority of information available was derived from studies performed in animals under steady-state conditions. Such studies, in addition to clinical observations, have allowed for some insight into adaptive mechanisms by which the fetus may respond to potentially damaging insults.

Fetal Hypoxemia

Deterioration of umbilical venous oxygen content may occur due to a number of different adverse events. The predominant factors responsible for oxygen transport across the placenta include uterine and umbilical blood flow, the fetomaternal oxygen tension difference, and the hemoglobin concentration in fetal blood.[9] Thus clinically relevant abnormalities that could affect fetal oxygenation include (1) abnormal uterine or placental blood flow as may occur in hypertensive diseases of pregnancy, abruptio placentae, cord occlusion, or following nicotine or cocaine use; (2) maternal hypoxemia due to maternal pulmonary or cardiac disease, seizures secondary to eclampsia, or living at high altitude; and (3) fetal disorders such as sepsis, hemorrhage, anemia, or heart block and certain other tachy- or bradyarrhythmias.

Acute fetal hypoxemia is known to produce a variety of circulatory adaptations that may improve the chances for fetal survival[33,39,102-104] (Box 50-2). These fetal responses include the development of reflex bradycardia, hypertension, redistribution of blood flow (toward central nervous system, myocardium, and adrenals and away from intestine and muscle), depression of fetal breathing and skeletal muscle activity, and increase in fetal oxygen extraction from umbilical blood. Some of these processes are mediated by endothelial NO[105] and may be even noted in relatively immature fetuses.[106] In addition, Gardner and co-workers[104] have demonstrated that hemoglobin concentration rises rapidly by as much as 10% to 15% during controlled cord occlusion. This cannot be explained on the basis of newly generated red cells or splenic sequestration. Such an increase could work to ameliorate the concomitant reduction in transplacental oxygen transport.

Fetal hypoxemia also induces changes in fetal metabolic processes, particularly if it is of acute onset. Theoretically depression of oxygen delivery to respiring tissues increases the ADP-ATP ratio. This acts to stimulate glycolysis (the Pasteur effect), with the effect of subsequently increasing ATP production anaerobically.[87] Thus acute hypoxia would be expected to produce relatively high rates of glucose uptake and lactic acid production in most fetal tissues. If hypoxia is severe, both hypoglycemia and metabolic acidosis with ultimate tissue necrosis result. Several factors, however, appear to lessen the effect of hypoxemia on fetal metabolic processes. When oxygen transport is limited experimentally in the sheep fetus, by partial occlusion of uterine blood flow[107] or by mild maternal hypoxemia,[108] redistribution of blood flow occurs, but little change in fetal VO_2 is noted. In sheep fetuses exposed to severe hypoxemia, whether by occlusion of uterine blood flow or by maternal hypoxemia, fetal oxygen consumption

Box 50-2

Fetal Response to Acute Hypoxemia or Ischemia

Redistribution of blood flow
To: Brain
Myocardium
Adrenal
From: Gastrointestinal tract
Skin
Muscle
Increased umbilical O_2 extraction
Decreased fetal movement
Selective decrease in VO_2 (muscle)
Bradycardia
Selective shift toward increased anaerobic glycolysis

declines by 25% to 40%.[33,104,107] Because, as mentioned earlier, the metabolic rate of fetal muscle is related to the ambient oxygen tension, it is not surprising that carcass VO_2 declines more than that of the whole fetus. Although not yet determined, it is likely that the decline in overall fetal oxygen consumption is due to depressed metabolism in other nonessential organs as well, such as fetal intestine, liver, and kidney, and that decreased metabolic work of skeletal muscle (decreased activity) and myocardium (bradycardia)[33,104] also may play a role.

Long-term fetal hypoxemia, as demonstrated in both simulated and true high-altitude animal experiments induces an acclimatization, with increases in fetal erythropoiesis, cerebral blood flow, and oxygen extraction, at the expense of a significant decrease in blood flow to carcass and other organs and a modest fall in cerebral metabolic rate at very low oxygen tensions.[31,32,108] The acclimatization of fetal cerebral arteriolar structure and changes in sensitivity to adrenergic agents both allow for stable cerebral oxygen delivery (product of cerebral arteriolar blood oxygen content and cerebral blood flow). Interestingly, this adaptation, particularly of structure, has been suggested as having long-term consequences on cerebrovascular contractility later in life. The above responses of the fetus to a superimposed potentially hypoxic environment are in contrast to those of animals apparently genetically adapted to living at high altitude,[30] where hypoxic stimuli superimposed on high altitude do produce more significant changes in fetal cerebral VO_2.

In contrast, at sea level, fetal cerebrum and myocardium appear relatively protected from generalized acute fetal hypoxemia because of the increase in blood flow to these organs, probably induced by catecholamine secretion as outlined earlier. In studies of hypoxemia in fetal guinea pigs[109] and sheep,[110] relatively small changes in cerebral VO_2 occur (at the expense of other organs). An increased rate of anaerobic glycolysis can also be demonstrated, allowing for the ongoing production of high-energy phosphates such as ATP. The fetal heart also has a greater capacity for anaerobic glycolysis than that of the adult.[84] It has also been suggested that elevated levels of adenosine 5-monophosphate (AMP) (the precursor for adenosine) constitute an important regulator of blood flow in brain and heart (i.e., that increased levels of AMP and ADP result in increased local concentrations of adenosine, a potent vasodilator). Several investigators have documented a greater dependence of fetal compared with adult myocardium on energy supplied via glycolysis.[13,111] As proof of this concept, inhibitors of glycolysis affect fetal myocardial function to a much greater degree than adult function is altered, but inhibitors of oxidative phosphorylation depress both equally.

Lastly, in states of chronic maternal hypoxemia, such as may occur in a variety of clinical situations, fetal growth restriction is not uncommon.[19a,112,113] Depression of potent fetal growth factors such as insulin-like growth factor I during hypoxic states may have an important protective effect by conserving fetal substrate for energy as opposed to accretion needs.[112]

Another factor that provides protection against the effects of hypoxia is an increased fetal availability of glucose, particularly during late gestation. Hypoxia, and concomitant secretion of catecholamines and cortisol, are major stimuli for glycogenolysis in the adult and probably in fetal life as well.[103] In fetal lambs and monkeys at term, hypoxia causes a fall in hepatic glycogen content and concomitant rise in blood glucose concentration.[86] In fetal lambs exposed to severe hypoxemia, the net fetal glucose uptake doubles within 30 minutes, as hepatic glucose output rises to equal umbilical glucose uptake, and suggests active fetal glycogenolysis. However, in more chronic states of hypoxemia, replenishment of glycogen stores appears unlikely. For example, in the rat fetus exposed to maternal hypoxia, fetal hepatic glycogen synthase mRNA levels fall to approximately 60% of control with a concomitant 50% fall in hepatic glycogen content.[95] Using adult rat hepatocytes raised in tissue culture, recent studies have demonstrated a link between gene expression of key glucogenic enzymes, such as PEPCK, and specific nuclear modifying transcription factors, such as hypoxia and normoxia regulatory elements (HREs and NREs).[19a,36,114] For example, the gene regulating PEPCK has been shown to contain an oxygen-sensing element that modulates action of glucagon in stimulation of gluconeogenesis. Although, not yet proven, it is likely that HREs and other transcription factors play significant roles in modulating fetal responses to stress.[19a,38a] In summary, the simultaneous actions of increased cerebral blood flow (to preserve fetal cerebral oxygen delivery), decreased overall fetal oxygen consumption, and rapid fetal glycemic response ensures adequate substrate and oxygen delivery to vital tissues such as brain and myocardium and provides at least some measure of fetal tolerance to an interruption of placental oxygen transport.

Maternal Hyperglycemia

The diabetic state in pregnancy is associated with a number of specific fetal and neonatal abnormalities, some structural and some metabolic. The development of fetal hyperglycemia as a result of excessive fetomaternal glucose transfer has been well documented in a number of species, including the human, and appears to be the major but not sole factor in the development of such neonatal complications as macrosomia, hyperinsulinemia, and postnatal hypoglycemia. Evidence in sheep[56,115] and monkeys[116] suggests that fetal hyperglycemia or hyperinsulinemia may accelerate fetal metabolism. As noted above in the fetal sheep, changes in the circulating concentrations of glucose and insulin appear to have independent stimulatory effects on fetal VO_2 and glucose oxidation.[56] In relatively short-term studies, direct fetal glucose infusion over 3 to 4 days caused increases in fetal insulin concentrations, fetal glucose and lactate uptake and placental lactate production as well as 20% to 30% increase in fetal VO_2, all suggestive of a significant increase in fetal metabolic rate.[115] More severe fetal hyperglycemia induced a pronounced fetal metabolic change, including fetal acidosis and ultimate death. These latter findings are all consistent with the effects of glucose-induced hypoxia. However, in other work by Hay and co-workers,[56,117] prolonged maternal glucose infusions (2 to 5 weeks) were performed in sheep that induced similar levels of fetal glycemia to those of the more acute studies. These investigators observed considerably lower fetal plasma insulin concentrations during the study and no significant changes in fetal VO_2 despite large increases in glucose uptake and uteroplacental lactate production.

The differences in fetal metabolic responses between the acute and more chronic studies are not clearly understood, but may have to do with the blunting of fetal insulin response to more prolonged glycemia or to the difference between glucose infusions given directly to the mother or fetus. One possible conclusion would be that intermittent, repetitive glycemic insults to the late gestation fetus might be more injurious than chronic sustained hyperglycemia, but this remains only speculation at present. In humans, fetuses and infants of diabetic mothers have been thought to exhibit signs of in utero oxygen deprivation, such as late fetal demise, increased hematopoiesis occasionally leading to polycythemia, and neonatal hyperbilirubinemia. However, conclusive proof of a link between maternal and fetal hyperglycemia and in utero oxygen deficiency remains speculative. Glucose infusion in human pregnancy, however, does result in cord blood metabolic changes that mirror those in sheep studied over the long term.[118] It is presumed that the increase in fetal fuel needs for sustaining the increased VO_2 are met by increased uptake of glucose, placentally derived lactate, and perhaps amino acids and ketones. Interestingly, although human fetuses of diabetic mothers who exhibit excessive growth in utero remain at risk for late fetal demise, macrosomic fetuses of nondiabetic women appear not to be at risk.[119]

The factors responsible for the increase in fetal metabolic rate noted in some studies are unclear, although as mentioned, both glucose and insulin appear to be codependent factors.[117] Fetal hyperglycemia is known to increase the rate of fetal breathing movements in sheep and humans,[54] which might serve to increase muscular work and thus increase the Vo_2. In addition, specific organ responses to accelerated substrate influx or hyperinsulinemia, particularly in liver and brain, may play important roles. For example, fetal glucose infusion in the sheep may accelerate cerebral VO_2 by as much as 70%, with a concomitant increase in cerebral blood flow and glucose entry as well as changes in fetal electrocortical activity.[120] Catecholamine secretion[121] is likely to be responsible for at least some of these changes. The demonstration in prematurely delivered neonates of stimulation of VO_2 by intravenously administered nutrients[122] suggests that metabolic rate may be altered by increased delivery of substrates other than glucose.

Maternal Hypoglycemia

Because the major factor responsible for placental glucose transport to the fetus is the fetomaternal glucose concentration gradient,[73] it follows that induction of maternal hypoglycemia should ultimately depress fetal glucose uptake. In the sheep, maternal hypoglycemia induced by fasting is associated with a fall in fetal plasma glucose concentration and in depression of both umbilical glucose uptake and fetal glucose utilization.[63] Because fetal VO_2 does not change during hypoglycemia induced by maternal fasting[123] or maternal insulin infusion,[124] it is likely that other fuel sources, particularly amino acids and endogenously produced glucose and lactate, are used instead. For example, myocardial lactate uptake alone in the fetus may account for the majority of oxygen consumption in this organ,[111] even in steady state. Fetal muscle and brain also have the capacity to use ketones.[125] In addition, endogenous glucose production has been demonstrated in fetal sheep,[96,117] probably secondary to hepatic glycogenolysis and gluconeogenesis[93] under such circumstances. Chronic fetal hypoglycemia induced by infusing pregnant sheep with insulin was associated with fetal hypoinsulinemia, endogenous glucose production, fetal stunting and marked increases in fetal expression of key enzymes of gluconeogenesis.[63,93] During hypoglycemia of short duration, intracellular stores of glycogen, particularly in myocardium, liver, and kidney, clearly offer an available source of glucose for cellular metabolic processes. More prolonged chronic hypoglycemia caused by malnutrition, however, depletes cellular stores of glycogen in a variety of organs, particularly in brain, liver, and heart.[91,92] Lastly, expression of glucose transporter proteins[71,126,127] (see Substrate Uptake: Carbohydrate) such as GLUT-1 in placenta and GLUT-4 in muscle may also up-regulate in response to changes in circulating glucose (and insulin) concentration and act to increase the cellular uptake of glucose during relative glucose deprivation.

During induction of maternal hypoglycemia, evidence also suggests that other fetal counterregulatory mechanisms can blunt the fall in fetal blood glucose. For example, in sheep fetuses whose mothers were made hypoglycemic either by fasting[76] or by insulin infusion,[128] endogenous fetal insulin levels fell significantly. In the latter case, chronic fetal hypoglycemia appears also to render the fetus less sensitive to later challenges of glucose or arginine, suggesting a state of insulin resistance. In the fetal rat exposed to insulin-induced maternal hypoglycemia, catecholamines rise rapidly[129] and may be elevated in some newborn humans with hypoglycemia.[130] These effects act to stimulate both fetal glycogenolysis and gluconeogenesis. The role that other potential glucose regulatory hormones, such as glucagon, growth hormone or cortisol, play in defense against fetal hypoglycemia is not known, but seems more limited in scope.[131]

The effect of fetal hypoglycemia on fetal metabolism, particularly fetal cerebral metabolism, has not been studied in great depth. As the principal cerebral substrate, glucose is of major importance in maintaining neuronal and white matter integrity. Postnatal hypoglycemic brain damage and resultant neuronal and white matter necrosis have been well documented. Theoretically similar damage should result from sustained fetal hypoglycemia as well. In sheep, however, modest hypoglycemia of 2 to 4 hours' duration produces no changes in fetal cerebral metabolic rate or in cerebral glucose uptake.[132] Richardson and co-workers[133] have proposed an enhancement of cerebral glucose transport in the fetus during hypoglycemia that may have protective value. In addition, use of fetal brain lactate, fatty acids, and ketone bodies as alternate substrates for cerebral oxidative needs have been demonstrated in the fetal and neonatal rat under steady-state conditions.[134]

SUMMARY

The integration between substrate uptake and metabolic rate, as well as the interactions between maternal and fetal environments, makes for a very complex subject. The topics reviewed in this chapter included (a) basic concepts of how metabolic studies may be performed (and interpreted) in the fetus, with examples and a summary of information derived from organ and whole fetus information; (b) fetal carbohydrate metabolism, including the topics of glycogen deposition and breakdown and gluconeogenesis; and (c) several clinically relevant disorders and the fetal responses to them. In all, it is apparent that the fetal mammal in late gestation is able to some extent to partition and control energy needs under most circumstances through a variety of interconnected means to maintain homeostasis when the maternal milieu is altered adversely.

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Richard M. Cowett

Role of Glucoregulatory Hormones in Hepatic Glucose Metabolism During the Perinatal Period

In terms of metabolism in general and carbohydrate (glucose) metabolism in particular, the neonate is considered to be in transition between the complete dependence of the fetus and the complete independence of the adult.[1-3] In this transitional state, the neonate must become capable of balancing between glucose deficiency and excess, to maintain euglycemia. The dependence of the conceptus on the mother for continuous substrate delivery in utero contrasts with the variable and intermittent exogenous oral intake that is the hallmark of the neonatal period and beyond. Development of carbohydrate homeostasis results from a balance between the morbidities to which the neonate is subject and the factors involved in the development of regulatory control. The maintenance of euglycemia, especially in the sick or low-birth-weight neonate, is difficult. This difficulty reinforces the concept that the neonate is vulnerable to dysequilibrium. This issue recently has become a topic of hightened interest. Hyperglycemia has been identified as an associated pathophysiologic factor in the compromised neonate, infant, and older child in an ever-increasing number of published studies.[4-10] The etiopathogenesis of hyperglycemia has been evaluated relative to whether it relates to primarily hepatic (glucose production) versus pancreatic (insulin secretion) mechanisms, and whether a peripheral mechanism is in play as well in those tissues responsive to the effects of insulin such as muscle and fat. Many of these issues are highlighted in the following discussion. What is not evaluated in this physiologically oriented discussion is the increasing number of clinical studies focusing on the infusion of insulin as a treatment modality—a topic that is of considerable importance as the mechanisms of the dysequilibrium become better understood.[11-18] Whereas Chapter 50 focuses on carbohydrate metabolism in the fetus, this chapter concentrates on the control of glucose homeostasis in the neonatal period, specifically by regulatory hormones.

INSULIN AND GLUCAGON INFLUENCES ON GLUCOSE HOMEOSTASIS

Numerous investigations have focused on the measurement of insulin and glucagon singly and in combination with each other relative to development of glucose homeostasis in the perinatal-neonatal period. Ktorza and associates[19] evaluated insulin and glucagon secretion during the perinatal period. They noted that insulin and glucagon are detected in most species early in gestation. The insulin-glucagon molar ratio is high in the fetus at term, but then it decreases dramatically after birth and remains low during the first hours after birth. This change favors glycogenolysis and gluconeogenesis after birth.

King and colleagues[20] studied postnatal development of insulin secretion in the premature neonate (born at 26 to 30 weeks of gestation) for 110 days after birth and in the full-term neonate for up to 47 days after birth. Insulin levels were measured before and 30 minutes after the beginning of a glucose infusion given parenterally or enterally. The premature neonate exhibited a small response to glucose on day 1 that gradually increased over the course of the study; the full-term neonate was more responsive. The investigators concluded that the premature neonate may take up to 18 weeks to respond fully to an increase in plasma glucose concentration.

Ghiglione and associates[21] measured immunoreactive glucagon (IRG) levels in dogs between 12 hours and 60 days of age relative to four peaks—designated IRG >20,000, IRG 9000, IRG 3500, and IRG 2000—obtained by gel filtration. Changes with age were confined to IRG 9000 and IRG 3500. IRG 9000 was nine times higher in 12- to 36-hour-old dogs than in adults (108 ± 24 pg/mL versus 12 ± 3 pg/mL), decreasing to two times higher (27 ± 5 pg/mL) at 31 to 60 hours. IRG 3500 was higher in the adult only during the first 36 hours after birth (36 ± 5 pg/mL versus 15 ± 3 pg/mL). Insulin infusion (0.2 units/kg, administered intravenously) produced hypoglycemia, but no change was noted in any IRG component in the neonate. In response to an arginine infusion (0.5 g/kg over 15 minutes), an increase in plasma concentrations of IRG 9000 and IRG 3500 was observed in the neonate; however, only IRG 3500 increased in adults. An impaired secretory response to hypoglycemia was noted in the neonate—a conclusion revisited later in the chapter.

Grasso and colleagues[22] infused either glucose (1 g/kg) or saline in 37 term and 35 preterm neonates and measured plasma glucagon, serum insulin, and blood glucose concentrations on the first day of life before feedings were initiated or during the first week after birth. Glucose infusion diminished plasma glucagon secretion by 61% ± 6% in the term neonate and 38% ± 4% in the preterm neonate. Serum insulin response to glucose infusion was variable—attesting to the heterogeneity that exists in the neonatal period.

Mehta and associates[23] reported data for four neonates with severe hypoglycemia in whom the glucose production rate and the plasma concentrations of insulin and glucagon were measured. The hepatic glucose production rate was less than 20% of normal, and plasma insulin concentration was never greater than 12 μU/mL. Two of the four neonates had low plasma glucagon concentration as well (i.e., less than 60 pg/mL); however, a bolus infusion of glucagon restored the glucose production rate toward normal. In one neonate, use of diazoxide further depressed an already low plasma insulin concentration from 4.2 to 1.6 μU/mL. These investigators speculated that the insulin-glucagon ratio may be more important than the absolute concentration of insulin in controlling glucose metabolism.

Glucagon responsiveness of the preterm neonate to glucagon was studied by Jackson and co-workers in a two-phase study comprising 78 premature neonates born at 25 to 36 weeks of gestational age.[24] In the first phase, the neonates were fasted until they became hypoglycemic or were fasted maximally for 8 hours. In the second study, a bolus of glucagon (100 mcg/kg) replaced one omitted feeding. Specific differences identified in those neonates who exhibited an inadequate response to glucagon (i.e., a glucose concentration less than 1 mmol/L) included the following: relative fasting hyperglycemia and hyperinsulinemia; an increased insulin-glucagon ratio; and a lower insulin

sensitivity index. These workers concluded that some premature neonates, without a distinctive phenotype, mount an inadequate response to glucagon and demonstrate features of insulin resistance.

OTHER COUNTERREGULATORY HORMONE INFLUENCES ON GLUCOSE HOMEOSTASIS

Glucocorticoids and insulin mediate the rate of glycogen accumulation in fetal life. In the presuckling period, muscle glycogenolysis supplies lactate moieties that are subsequently oxidized by neonatal tissue and act as alternative substrate until glucose and ketones are available. The subsequent increase in plasma catecholamines and the decrease in the insulin-glucagon ratio result in liver glycogenolysis and gluconeogenesis to maintain euglycemia postnatally. During suckling, oxidation of free fatty acids, ketone body utilization, and gluconeogenesis supply energy for anabolism. Subsequently, the increase in the insulin-glucagon ratio (which occurs during feeding) promotes lipogenesis.

Padbury and colleagues[25] evaluated the catecholamine surge at birth in the preterm and term lamb using an exteriorized fetal lamb preparation in which the preterm animals were treated with surfactant before the first breath. Similar baseline concentrations of catecholamines and a marked rise in circulating epinephrine and norepinephrine were found in both groups after cord cutting. The preterm lamb, however, exhibited a delayed but exaggerated elevation of both catecholamine concentrations compared with those in the term lamb. Changes in heart rate were less profound and more gradual. Likewise, a blunted elevation in blood glucose concentration was noted. These data suggest that the catecholamine surge at birth represents an important adaptive physiologic response.

Hägnevik and associates[26] evaluated the immediate postnatal adaptation and sympathoadrenal activation in neonates delivered vaginally versus in those delivered by elective cesarean section. As expected, the vaginally delivered neonates exhibited higher catecholamine concentrations at birth than those measured in neonates delivered by cesarean section using either epidural or general anesthesia. Likewise, glucose concentration in the umbilical artery was higher in the former group of infants than in those delivered by cesarean section. The investigators speculated that in view of the marked differences in catecholamine concentrations, the differences in metabolic adaptation were unexpectedly small. This finding implied an attenuated metabolic response to sympathoadrenal stimulation in the neonate.

Gripois and colleagues[27] evaluated the interrelationships between thyroid secretion and adrenal medullary secretion in the neonatal rat. The adrenal medulla of normal, hypothyroid, and hyperthyroid rats were stimulated by insulin-induced hypoglycemia. In the euthyroid animal, insulin-induced epinephrine secretion increased during the first 10 days of postnatal life. Hypothyroidism retarded the development of that response, and hyperthyroidism accelerated the response. During adrenal medullary depletion after insulin-induced hypoglycemia, recovery was slower for the hyperthyroid animal than for the hypothyroid or euthyroid animal.

Finally, Hume and associates[28] found that up to 20% of preterm neonates about to be discharged home are at risk for hypoglycemia if a feeding is delayed. Cortisol, corticotropin, and epinephrine concentrations were higher in those neonates who were hypoglycemic, whereas insulin, glucagon, and human growth hormone concentrations in the hypoglycemic neonates were not different from those in the normoglycemic neonates. These workers concluded that hormonal dysfunction in the pretem neonate may contribute to the failure in postnatal development of glucose homeostasis, specifically, the postnatal expression of hepatic enzymes.

KINETIC ANALYSES OF HORMONAL CONTROL OF GLUCOSE HOMEOSTASIS

Kinetic analyses have been used to evaluate hormonal control of neonatal glucose metabolism. Originally, an indirect technique of stepwise incremental glucose infusion was used to infer the rate of basal glucose output or glucose turnover in the neonate compared with the adult.[29] This inference assumed that the neonate was as sensitive as the adult to minimal changes in glucose concentration. Subsequently, studies in the puppy by Varma and associates[30] indicated that fine control is not developed during the neonatal period. Kornhauser and colleagues[31] first used the Steele steady-state infusion technique to show that basal glucose production in the newborn puppy was two to three times the adult rate when expressed per unit of body weight. The data of Varma and colleagues substantiated this observation.

Cowett and associates[32] hypothesized that the insensitivity of the hepatocyte to insulin appeared to have a dominant effect in controlling the turnover (i.e., production) rate of glucose. In the initial series of experiments, it was expected that the newborn lamb, unlike the adult sheep, would exhibit a developmentally blunted hepatic response with a persistent output of glucose in response to a glucose infusion. After a 7-hour fast, basal plasma glucose, insulin, and glucagon concentrations were determined in term (newborn) lambs and in adult sheep, after which the lambs received 0, 5, 6, 11.7, or 21.7 mg glucose/kg/minute over a period of 6 hours. Older sheep received either saline or 5.7 mg glucose/kg/minute. Glucose turnover was determined by the prime constant infusion technique of Steele using D-[6-3H] glucose. Both newborn and adult animals maintained a constant plasma glucose concentration and glucose-specific activity during the turnover period. Glucose production rates persisted in the term newborn lambs until an infusion rate of 21.7 mg/kg/minute was reached. By contrast, in the adult lambs, the glucose production rate was reduced with a glucose infusion rate of 5.7 mg/kg/minute. At the point when the glucose production rate was significantly reduced, the plasma insulin concentration in the newborn lamb was five times greater than in the adult (270 μU/mL versus 56 μU/mL). Blunted hepatic responsiveness to insulin appeared to be a major factor explaining the inefficiency in glucose homeostasis in the neonatal lamb.

In these studies, hyperglycemia and hyperinsulinemia were produced simultaneously. Therefore the effect of peripheral hyperinsulinemia could not be differentiated from that of hyperglycemia. Subsequently, varying concentrations of glucose and insulin were infused in six groups of newborn lambs for sufficient time to produce steady-state equilibrium conditions of euglycemia and hyperinsulinemia.[33] Glucose production rates and gluconeogenesis from lactate were measured. The latter measurement was accomplished by determining the ratio of U-[^{14}C]lactate to D-[6-3H]glucose (*r* in Figure 51-1).

Increasing the rate of glucose infusion without administering insulin (groups II and III in the figure) produced a stepwise increase in plasma glucose and insulin concentrations when compared with controls (group I). Elevation of the plasma insulin concentration induced by hyperglycemia was associated with a significant reduction in the glucose production rate, but it was seen only when marked hyperglycemia and hyperinsulinemia were achieved (group III). With insulin administration, a significant and stepwise increase in plasma insulin concentration was observed, depending on the dose of insulin administered. By simultaneous glucose infusion, a state of euglycemia or hyperglycemia was produced with concomitant hyperinsulinemia. With a slight increase in plasma insulin (61 μU/mL) (group IV), a significant reduction in gluconeogenesis was noted, together with a slight but insignificant reduction in the rate of glucose production. When hyperinsulinemia was moderate to marked (236 and 481 μU/mL) (groups V and VI), there was a significant reduction

of gluconeogenesis and the rate of glucose production. Insulin is known to inhibit glycogenolysis and gluconeogenesis (while enhancing glycogenesis), and it suppresses glucose production in the adult.[34-36] These data suggested that a moderate elevation of plasma insulin concentration effectively reduced gluconeogenesis (groups II and IV) but did not influence the endogenous glucose production. The latter was reduced only when a much higher insulin concentration was achieved (groups II, V, VI). The investigators concluded that insulin, rather than glucose, controls the rate of glucose production in newborn lambs.

In neither of the studies cited was the pancreatic beta cell secretory activity evaluated. It was subsequently hypothesized that the pancreatic beta cell response to glucose concentration was comparable in the term neonate and the older adult (i.e., secretory activity by the neonatal beta cell is normal). To confirm this hypothesis, a steady-state insulin secretion study was performed using [^{131}I]insulin as the tracer.[37] Plasma glucose and insulin concentrations and insulin-specific activity were determined. Endogenous posthepatic insulin secretion and the metabolic clearance rate were derived in the spontaneously delivered term lamb, the betamethasone-treated preterm lamb, and the 4- to 5-month-old adult sheep. After a 7-hour fast, animals received 0.45% saline or glucose (5.7 mg/kg/minute) for 6 hours, followed by the tracer insulin infusion for 11 minutes. Posthepatic insulin secretion rates after infusion of 0.45% saline did not differ among any of the three groups—term, premature, or adult. Correspondingly, similar posthepatic insulin secretion rates also were noted in the three groups after infusion of 5.7 mg/kg/minute of glucose. The metabolic clearance rate did not differ among the groups. The investigators concluded that the posthepatic insulin secretion rate and the metabolic clearance rate were similar in the term gestation lamb, the prematurely delivered lamb, and the 4- to 5-month-old adult sheep. It had been previously suggested that precise control of the rate of glucose production is characteristic of the mature (adult) animal, whereas the neonate evidences a decreased ability to suppress the rate of glucose production when glucose is infused. The investigators suggested that this immaturity may be explained by hepatic unresponsiveness to insulin in the neonate and probably is not related to secretory capacity of the pancreatic beta cell.

The neonatal lamb is metabolically comparable to the human, and it frequently is used to evaluate perinatal glucose homeostasis. Kinetic studies in the human neonate, however, are more physiologically relevant. After the development of stable isotope methodology, it became possible to study the human neonate.[38] Figure 51-2 depicts the glucose production rate for preterm and term neonates and adults who were studied using stable isotope techniques. Five of 13 preterm and 2 of 7 term neonates had persistent glucose production rates (above 1 mg/kg/minute) during glucose infusion. By contrast, the glucose production rate in adults was not measurable. No correlation was found between plasma glucose concentration and the glucose production rate in the neonate or the adult (Figure 51-3). In both the neonate and the adult, however, a correlation was found between the plasma insulin concentration and the glucose production rate, but with considerable variability in the neonate (Figure 51-4). These data point to significant developmental differences in neonatal glucose homeostasis and suggest that insulin is important in neonatal hormonal control of glucose production.

The studies described earlier raise the question of when maturation (i.e., adultlike control) of glucose homeostasis is achieved in the newborn. As noted previously, suppression of the endogenous glucose production rate (Ra) is the adult response to glucose infusion. Persistent endogenous glucose production (i.e., 1 mg/kg/minute or greater, or less than an 80% decrease in Ra) in response to glucose infusion is evidence of a transitional homeostatic state in the neonate during the first days after birth. To determine when an adult-like response developed, Ra was measured in 11 prematurely born neonates (33 ± 0.3 weeks) at 2 to 5 weeks after birth. In these paired studies, 4 μg/kg/minute D-[U-^{13}C]glucose tracer was infused by prime constant infusion to determine Ra, during saline or

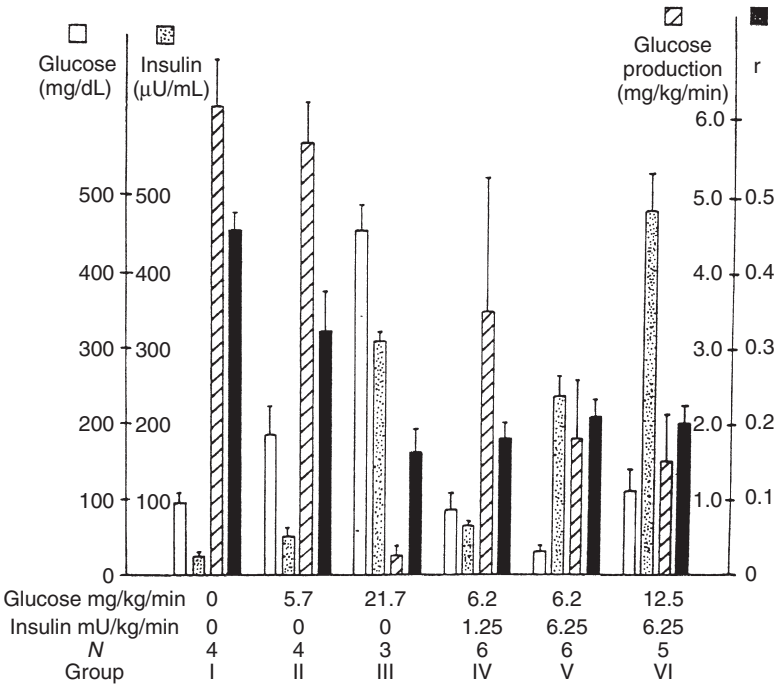


Figure 51-1 Plasma glucose and plasma insulin concentrations, glucose production rates, and ratio of U-[^{14}C]lactate to D-[$^{6\text{-}3}$H]glucose for all groups. *(From Susa JB, Cowett RM, Oh W, Schwartz R: Suppression of gluconeogenesis and endogenous glucose production by exogenous insulin administration in the newborn lamb,* Pediatr Res *13:594, 1979.)*

glucose infusion, the latter at a rate of 5.3 ± 0.2 mg/kg/minute. In comparison with that during the saline infusion turnover period, the plasma glucose concentration increased significantly during the glucose infusion turnover period, from 88 ± 3 to 101 ± 4 mg/dL. Plasma insulin concentration remained unchanged (12 ± 5 μU/mL versus 8 ± 3 μU/mL). Ra was heterogeneous during glucose infusion, and persistent Ra was present in 6 of 11 neonates (Figure 51-5). Of the 5 infants who showed decreased Ra during glucose infusion, 3 received glucose at a rate exceeding basal Ra. Of the remaining 6 infants who exhibited persistent Ra during glucose infusion, 3 received glucose at a rate equal to or in excess of basal Ra. These data suggest that glucose homeostasis in low-birth-weight infants is transitional throughout the neonatal period.[39]

A decreased response to epinephrine in the neonate (a decreased rate of production and decreased plasma glucose concentration) has been reported.[40,41] This lack of response to epinephrine has been correlated with the lack of response to insulin discussed earlier. It is possible, as noted by Hetenyi and colleagues,[42] that the decreased neonatal response to insulin and other hormones (e.g., glucagon or epinephrine) may protect the neonate from rapid fluctuations in substrate supply and may ensure ready availability of glucose for the developing brain. Diminished responsiveness to both insulin and counterregulatory hormones should assist in maintaining glucose homeostasis in the neonate. Other investigators, in more recent studies, examined the hypothesis that the imprecise control of glucose production by insulin is mirrored by a corresponding lack of response to the various counterregluatory hormones. In 30 spontaneously delivered term lambs, glucose kinetics were measured after administration of radiolabeled isotope using the prime constant infusion technique. Insulin infusion at 2.0 mU/kg/minute produced hyperinsulinemic hypoglycemia. Endogenous insulin, glucagon, and growth hormone release were blocked by administration of somatostatin. The addition of metyrapone blocked cortisol release. The control animals received only the isotope. The results suggested that insulin exerted a greater effect on glucose uptake than on glucose production. Glucagon, growth hormone, and cortisol did not affect Ra during hyperinsulinemic hypoglycemia. The imprecise effect of these counterregulatory hormones on neonatal glucose production mirrors the previously documented imprecise control by insulin.[43]

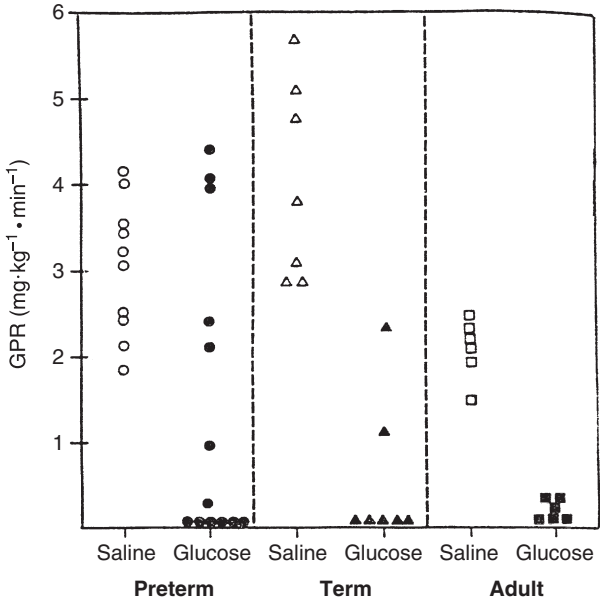


Figure 51-2 Glucose production rate (GPR) during saline or glucose infusion in preterm and term infants and adults. Plotted values are for individual patients. *(From Cowett RM, Oh W, Schwartz R: Persistent glucose production during glucose infusion in the human neonate,* J Clin Invest *71:467, 1983.)*

INSULIN RESISTANCE AND SENSITIVITY IN THE NEONATE

Lack of the precise control of glucose homeostasis in the human neonate has been postulated as secondary to either a decreased sensitivity or a decreased resistance to insulin compared with that in the adult.[38,44] Although methods for assessing insulin resistance have varied considerably, most studies evaluated only the insulin effect on plasma glucose concentration in the hyperglycemic, stressed neonate.[11-18] Fewer studies have evaluated the effects of insulin (or glucose) on glucose production in the healthy term or preterm neonate.[38,45,46]

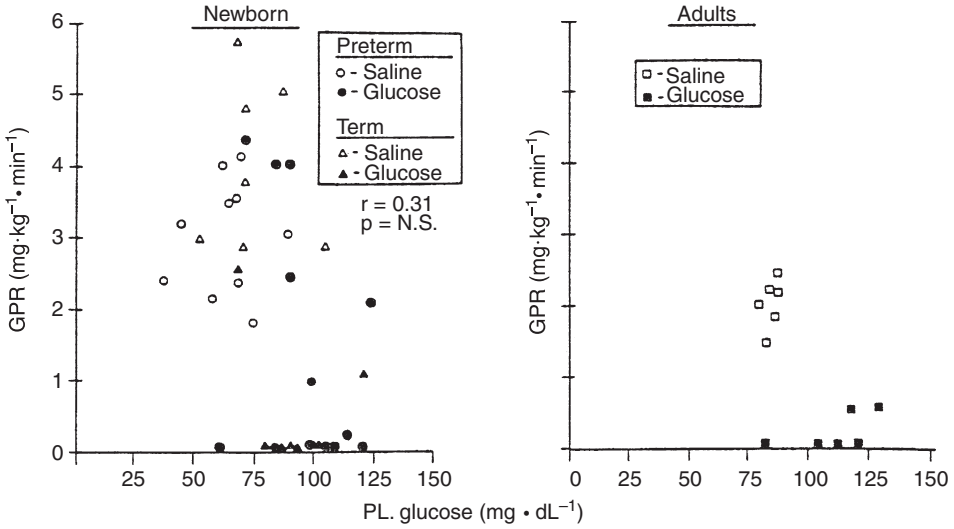


Figure 51-3 Correlation between plasma (PL.) glucose concentration during the turnover period and glucose production rate (GPR) in neonates and adults. *N.S.,* Not significant. *(From Cowett RM, Oh W, Schwartz R: Persistent glucose production during glucose infusion in the human neonate,* J Clin Invest *71:467, 1983.)*

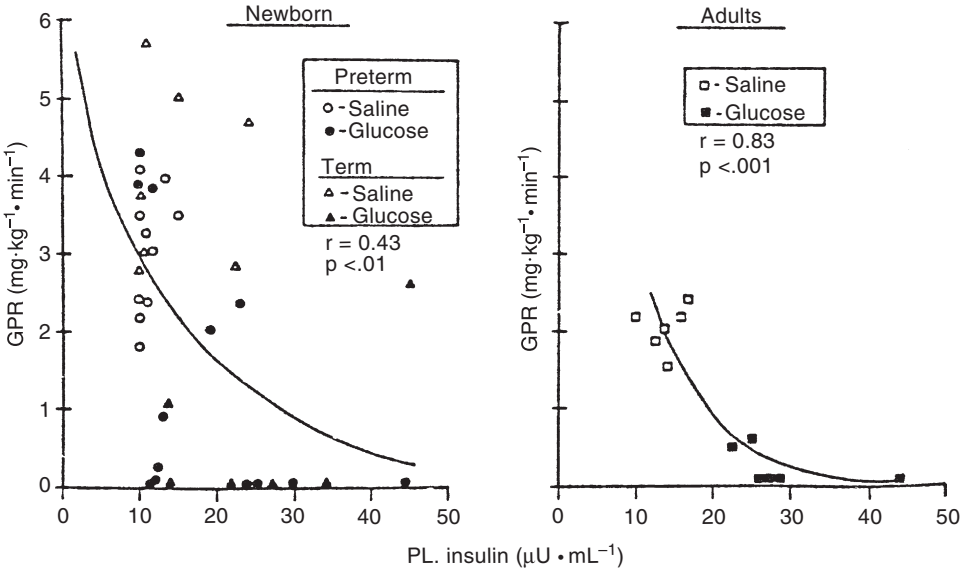


Figure 51-4 Correlation between peripheral plasma (PL.) insulin concentration during the turnover period and glucose production rate (GPR) in neonates and adults. *(From Cowett RM, Oh W, Schwartz R: Persistent glucose production during glucose infusion in the human neonate,* J Clin Invest *71:467, 1983.)*

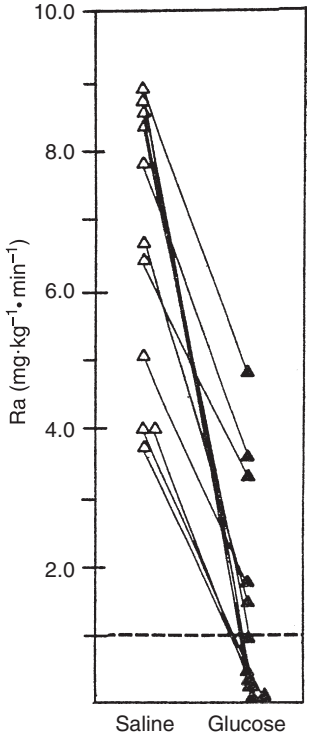


Figure 51-5 Endogenous glucose production rate (Ra) during saline and glucose infusion. *(From Cowett RM, Andersen GE, Maguire CA, Oh W: Ontogeny of glucose homeostasis in low birth weight infants,* J Pediatr *112:462, 1988.)*

Hulman and Kliegman[47] and Kliegman and colleagues[48] are credited with the initial use of the euglycemic hyperinsulinemic clamp in the neonatal period in a beagle puppy model, as first published by DeFronzo and associates.[49] Graded insulin infusions ranging from 3.75 to 100 mU/kg/minute were used to generate a dose-response curve. Complete suppression of glucose production was observed in the adult group, whereas an 80% reduction was achieved in the newborn puppy group. These workers attributed a lack of complete suppression of glucose production in the neonate to hepatic resistance to insulin and persistent gluconeogenesis.

Kahn[50] defined insulin resistance and provided a distinction between insulin insensitivity and insulin unresponsiveness: *Insulin insensitivity* is characterized by a shift to the right on the insulin dose-response curve such that a higher concentration of insulin is necessary to produce a half-maximal effect, with a maximal effect achieved eventually. This shift usually is the result of decreased affinity or a decreased concentration of insulin receptors. With *insulin unresponsiveness,* all responses to insulin are reduced (including the maximal response), but the dose-response relationship remains normal (i.e., the insulin concentration required to produce a half-maximal response is normal). This condition usually is the result of a postreceptor defect. A combination of both forms of insulin resistance may exist simultaneously, so that a higher insulin concentration would be required to produce a half-maximal effect, and the maximal effect would be reduced compared with that of a normal response.

In fact, the euglycemic hyperinsulinemic clamp technique to study in vivo insulin sensitivity and the hyperglycemic clamp technique to assess insulin secretion are being used to investigate a number of conditions featuring evidence of insulin resistance.[51]

Farrag and colleagues[52] performed the euglycemic hyperinsulinemic clamp in the human preterm neonate to evaluate insulin sensitivity in the neonatal period. As noted in Figure 51-6, hepatic glucose production was reduced in the preterm neonate at a relatively low insulin concentration. This effect did not significantly change despite the use of higher insulin infusion rates that resulted in as much as 10-fold higher plasma insulin concentrations. The percent reduction in endogenous glucose production ranged from 41% to 58% of basal rates, and persistent glucose production (i.e., at a rate of 1 mg/kg/minute or greater) during steady-state insulin infusion was noted throughout the study. If complete suppression of glucose production is considered to be the maximal effect of insulin on the liver, the data demonstrate that this maximal effect could not be reached in the human preterm neonate. This pattern of response to insulin is most consistent with a postreceptor defect.[53] This mechanism

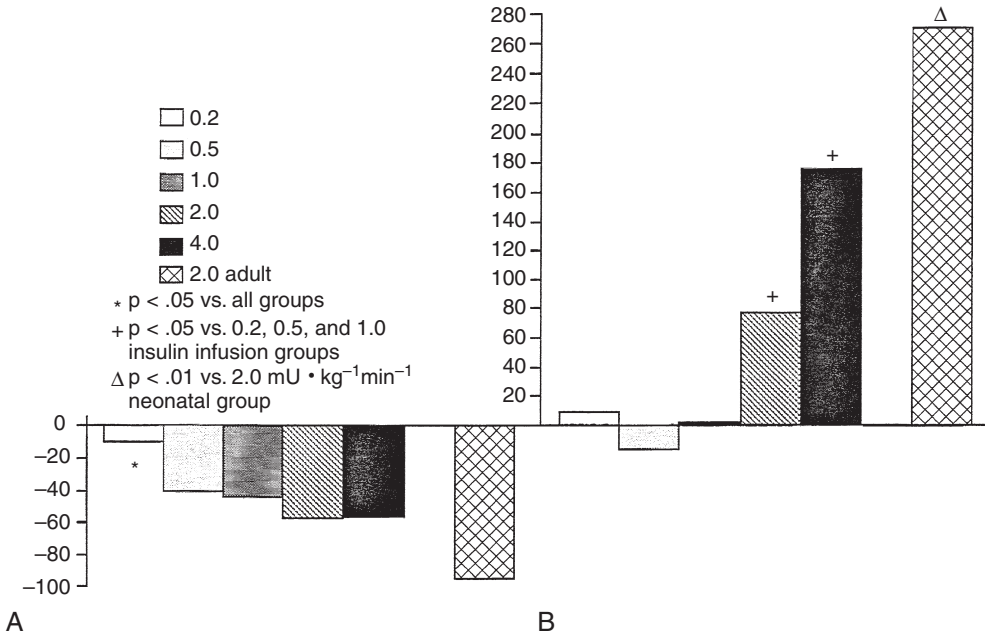


Figure 51-6 The percent decrease in endogenous glucose production and the percent increase in glucose utilization for the various insulin infusion rates of administration in the neonate as well as the insulin rate of administration in the adult (2 mU/kg/minute). *(From Farrag HM, Nawrath LM, Healey JE, et al: Persistent glucose production and greater peripheral sensitivity to insulin in the neonate vs. the adult,* Am J Physiol *272:E86, 1997.)*

may include factors at the membrane level (aside from receptor concentration and affinity) such as (1) the state of aggregation of the receptor, (2) its ability to interact with other membrane proteins required for signal generation, or (3) a variety of intracellular factors including all of the events that occur at site distal to the membrane, including the stimulatory or inhibitory effect of insulin on glucose transporters or key enzymes in the glycolytic, glycogenolytic, or gluconeogenic pathways. Decreased receptor concentration or affinity is an unlikely explanation, because at least in the human adult, plenty of "spare" insulin receptors are available after the number of receptor sites required to achieve a maximal response are occupied.[47,54] Second, an increase in both receptor concentration and affinity is seen in the human neonate compared with those in the adult.[55] Finally, a reduction in receptor concentration to less than that required for a maximal response will result not only in decreased responsiveness but also in decreased sensitivity, which was not apparent in Kahn's investigation.[50]

In the study by Farrag and colleagues just described, a significant increase in peripheral glucose utilization from basal rates occurred at insulin infusion rates of 2 and 4 mU/kg/minute.[52] It was not possible to determine whether a maximal effect on glucose utilization was achieved, because a plateau was not reached. The data denote a significant increase in glucose utilization at a higher insulin concentration than that required to reduce the rate of glucose production significantly (Figure 51-7). Even though that plateau was not ultimately reached at these insulin infusion rates, the neonatal glucose utilization response to insulin, even at a lower plasma insulin concentration, far exceeded that reported in the literature as the maximal response in the adult.[54]

The indirect effect of insulin on endogenous glucose production has been evaluated.[56] Inhibition of lipolysis (in adipose tissue) and reduction of free fatty acid concentrations were shown to act as signals to the liver to suppress endogenous glucose production. This particular issue is of current interest, but the limited amount of adipose tissue in the preterm neonate may potentially interfere with the indirect effect of insulin on endogenous glucose production.

As noted previously, Goldman and Hirata[13] suggested that an attenuated response to insulin in the very-low-birth-weight neonate is the reason for the occurrence of hyperglycemia. They attributed this response to insulin resistance, rather than lack of the beta cell response. In other studies, by contrast, abrupt and sustained improvement in glucose tolerance has been reported to occur in response to short-term and long-term insulin infusions.[11-18] Insulin infusion rates and plasma insulin concentrations used were higher than in the study by Goldman and Hirata, and no evaluation of glucose production or glucose utilization was documented.

An approximate 50% reduction in glucose production has been reported in response to an exogenous glucose infusion rate of approximately 4 to 6 mg/kg/minute (i.e., similar to the basal glucose production rate in the neonate), which resulted in a plasma insulin concentration of approximately 19 mU/mL.[39,46] The literature is unclear whether this reduction in glucose production is primarily a response to a higher plasma insulin concentration or plasma glucose concentration.[32,46] Kalhan and associates[46] suggested that the decline in the glucose production is secondary to a higher plasma glucose concentration, in contrast with the conclusions of Cowett and associates, as noted earlier.[38]

The data of Farrag and colleagues[52] are in accord with those reported by Hertz and associates,[57] supporting a strong positive correlation between plasma insulin concentration and glucose utilization. Hertz and associates noted a complete suppression of glucose production in stable, extremely premature infants when a high glucose infusion rate (i.e., 9.5 mg/kg/minute) was used. The glucose load resulted in a significant increase in both plasma glucose and insulin concentrations. At a similar plasma insulin concentration, a 41% reduction in glucose production was noted, so the combined effect of glucose and insulin seems necessary for complete suppression of glucose production. By contrast, a subsequent investigation from that laboratory suggested that infusion of glucose at a rate of 5.5 mg/kg/minute resulted in a 90% suppression of glucose production during intravenous infusion administered with or without lipid.[58] Suppression was achieved at a glucose

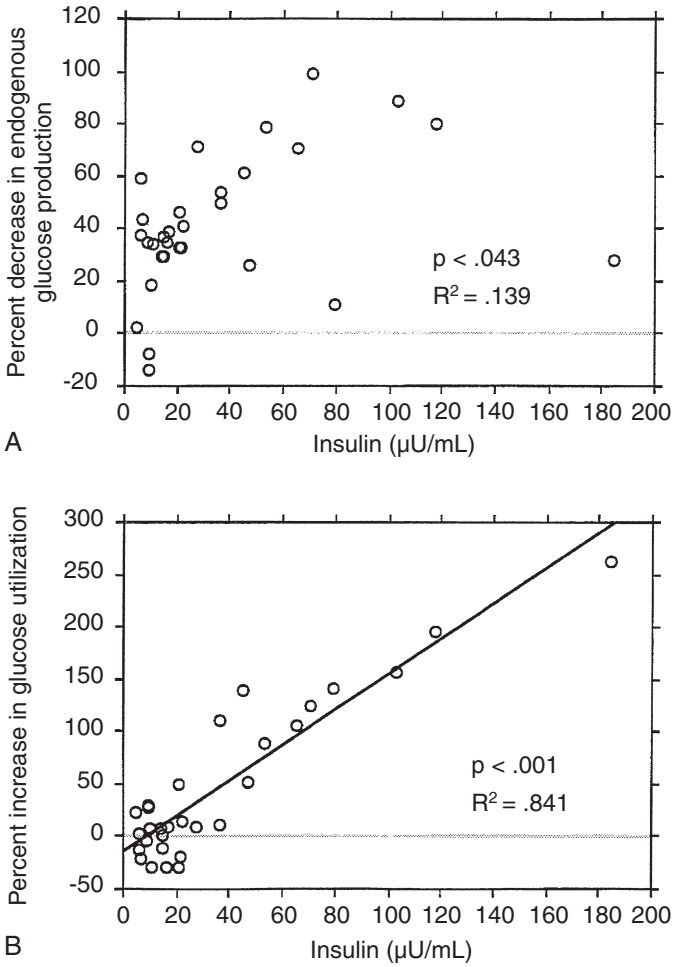


Figure 51-7 **A,** Regression correlating the percent decrease in endogenous glucose production relative to plasma insulin concentration in the neonate. **B,** Regression correlating the percent increase in glucose utilization relative to plasma insulin concentration in the neonate. *(From Farrag HM, Nawrath LM, Healey JE, et al: Persistent glucose production and greater peripheral sensitivity to insulin in the neonate vs. the adult,* Am J Physiol *272:E86, 1997.)*

concentration of approximately 90 mg/dL and an extremely low insulin concentration of approximately 6 μU/mL. The mechanisms explaining the dichotomy that exists in the two datasets are unclear.

It is difficult to compare neonatal with adult data, especially when basal glucose production rates, glucose utilization rates, and plasma insulin concentrations are different. As in the adult, a strong positive linear correlation has been found between plasma insulin concentration and the glucose utilization rate. The insulin effect on the glucose production rate begins at a lower insulin concentration than that required for the glucose utilization rate. If insulin sensitivity is calculated at euglycemia (that is, not confounded by non–insulin-mediated glucose utilization),[59] it is apparent that the neonate has a greater peripheral sensitivity to insulin than that in the adult. This increased sensitivity may result from a higher receptor concentration and affinity, provided that the postreceptor cascade is intact peripherally. Conversely, unlike in the adult, complete suppression of glucose production could not be achieved in the human neonate by Farrag and colleagues.[52]

Gelardi and associates,[60] using a lamb-sheep model, performed the euglycemic hyperinsulinemic clamp studies in two specific age-groups: 3- to 6-day-old lambs and 31- to 35-day-old sheep. Initially, the younger animals needed a higher rate of glucose infusion (15.87 ± 3.47 mg/kg/minute; p <.05) to maintain euglycemia compared with the older animal group (4.30 ± 1.11 mg/kg/minute) receiving the same amount of insulin—100 mU/kg/minute. In fact, endogenous glucose production persisted in both groups; however, the percent decrease compared with that in aged-matched controls receiving no insulin was greater in the younger group (53%; p <.001) than in the older group (34%; p <.01). The younger animals exhibited greater glucose utilization than that seen in the older animals (215% versus 90%, respectively; p <.01) (Figure 51-8). As in the human neonate, endogenous glucose production was not completely suppressed in the lamb despite very high plasma insulin concentrations. The younger animal group appeared to be more responsive to insulin, resulting in a significantly greater percent decrease in endogenous glucose production than in the older animals. The older animals also required significantly lower glucose infusion rates to maintain euglycemia during insulin infusion compared with the 3- to 6-day-old lambs. These data are consistent with findings in human preterm neonates, who exhibited persistent glucose production and greater peripheral sensitivity to insulin.

The appropriate distribution of whole-body glucose is regulated, at least in part, by the tissue-specific expression and regulation of several glucose transporter isoforms with distinct kinetic properties.[61] GLUT2 is the major glucose transporter isoform expressed in hepatocytes, beta cells, and the kidney. The distinguishing feature of this isoform is that it is a low-affinity, high-turnover transport system. GLUT2 forms part of a glucose-sensing apparatus that responds to subtle changes in blood glucose uptake with alterations in the rate of glucose uptake into the cell. GLUT4 glucose transporter is expressed in adipocytes and muscle cells. These are the "insulin-sensitive" cell types, so called because they respond to insulin with a rapid and reversible increase in glucose transport. Glucose transport in insulin-sensitive tissues has received attention because of the importance of this process in the maintenance of whole-body glucose homeostasis.[61]

In the study by Gelardi and associates, an apparent developmental increase in GLUT2 was noted in the older animal groups versus the neonatal groups (p <.05). This increase may signal the onset of an insulin-resistant state in the ruminant.[62] After an initial decrease, GLUT2 expression increased with time. The reduction in the expression of GLUT2 with euglycemic hyperinsulinemia is in agreement with findings in clamp studies in the diabetic rat. However, acute euglycemic hyperinsulinemia caused no change in the expression of GLUT4. This finding is consistent with previous studies showing that acute hyperinsulinemia does not regulate GLUT4 expression.[63] The investigators speculated that changes in GLUT4 expression are not directly responsible for the changes in insulin sensitivity (Figure 51-9). Increased GLUT2 expression with age, as well as decreased expression with hyperinsulinemia, is consistent with the development of an insulin-resistant state in the adult.

SUBSTRATE AVAILABILITY IN THE NEONATE

Several investigations have focused on the differential effects of substrate availability versus physiologic hormonal control in the human.[64-66] Jahoor and associates[64] suggested that in the adult, reduction in glucose turnover after an 86-hour fast was not secondary to a lack of gluconeogenic substrates. Conversely, dependence of gluconeogenesis on an adequate supply of precursors was demonstrated. When dichloroacetate was used to reduce lactate and alanine concentrations, a decrease in the rate of glucose production was observed. Bennish and associates[65] suggested that defective gluconeogenesis was the origin of the hypoglycemia in older children with diarrhea. Haymond and colleagues[66] studied differences in circulating gluconeogenic substrates in

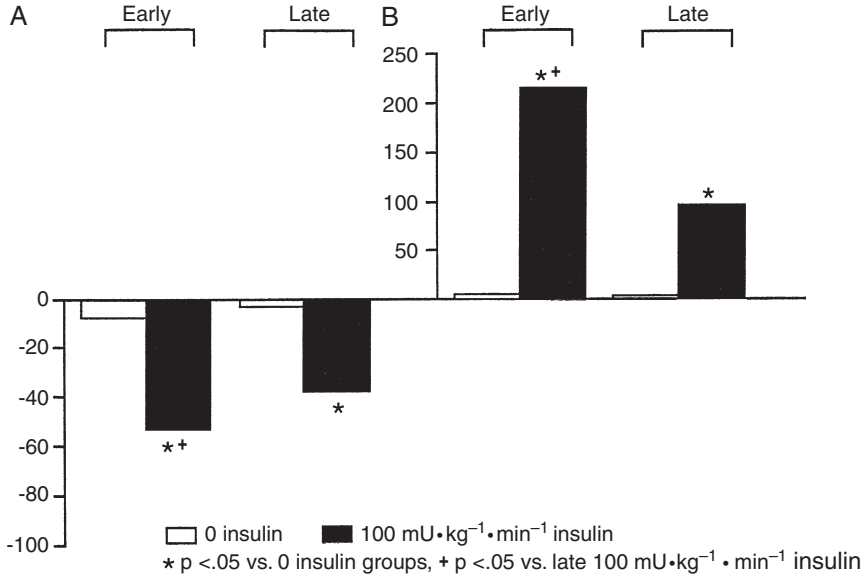


Figure 51-8 The percent decrease in endogenous glucose production **(A)** and the percent increase in glucose utilization **(B)** during the clamp period in the early (3-6 days old) and late (31-35 days old) groups. *(From Gelardi NL Rapoza RE, Renzulli JF, Cowett RM: Insulin resistance and glucose transporter expression during the euglycemic hyperinsulinemic clamp in the lamb,* Am J Physiol *277:E1142, 1999.)*

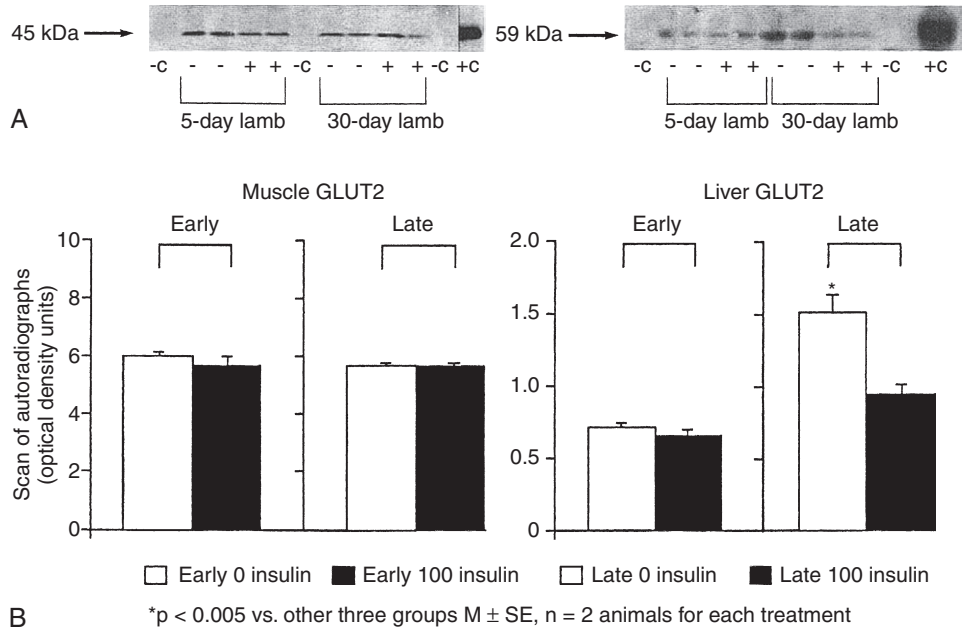


Figure 51-9 A, Representative Western blots for muscle *(left)* and liver *(right)* of 5- and 30-day-old 0 (–) and 100 (+) insulin-infused groups, showing also positive (+C) rat membrane and negative (–C) protein controls. **B**, Results of scans of autoradiograms from Western blots for muscle *(left)* and liver *(right)* for early (3-6 days old) and late (31-35 days old) groups. *(From Gelardi NL, Rapoza RE, Renzulli JF, Cowett RM: Insulin resistance and glucose transporter expression during the euglycemic hyperinsulinemic clamp in the lamb,* Am J Physiol *277:E1142, 1999.)*

men, women, and children subjected to short-term fasting and suggested that differences in glucose requirements among the three groups could be responsible for the differences noted in plasma substrate responses to fasting. The men and women evidenced nearly identical plasma lactate and pyruvate concentrations; the initial venous lactate and pyruvate concentrations were highest in children and increased during the 6 hours of fasting. The investigators speculated that lactate production may be accelerated in the pediatric patient in whom hepatic substrate uptake is normal. Neonates were not studied in that investigation. Cowett and Wolfe evaluated the potential for gluconeogenesis from lactate and found it to be accelerated in the preterm neonate compared with the adult.[67] These workers speculated that substrate acquisition may not be the primary problem in the neonate.

One of the issues of substrate availability related to glucose homeostasis involves the effect of glucose (with or without other substrates) on glucose production. In one series of investigations,

the Ra was negligible under conditions in which glucose was infused as part of the hyperalimentation mixture before administration of an intravenous fat emulsion.[68] However, a clear dichotomy is seen for data evaluating the ability of the neonate to diminish endogenous glucose production, which occurs regularly in the adult.[44] In studies in the term neonate,[38] the infant of the diabetic mother,[69] and the premature neonate,[38] persistent endogenous glucose production greater than 1.0 mg/kg/minute was reported in response to administration of exogenous glucose infusion alone. Similar results were observed in the newborn canine model using the euglycemic hyperinsulinemic clamp technique.[47] In other investigations using different techniques such as the glucose clamp[70] or glucose and amino acid infusion,[71] no persistent endogenous glucose production was reported in the neonate. For the second technique, both an amino acid mixture and glucose at a relatively moderate rate were infused (approximately 8 mg/kg/minute).As pointed out by Lafeber and colleagues,[71] suppression may be incomplete under conditions in which insufficient glucose is administered or glucose is the sole constituent of the infusate.The data reported in another study supports this conclusion in the latter condition. No endogenous glucose production was noted when 6.8 mg/kg/minute of glucose with amino acids was administered during the basal state or after administration of glucose, amino acids, and an intravenous fat emulsion combined.[68] Further work is necessary to determine whether the quantity of glucose administered or the addition of amino acids or lipid emulsion is the primary cause of the observed decline in endogenous glucose production in the neonate. In addition, the relative role of each of these substrates as a secretagogue for insulin is unknown. Such studies should assist in differentiating hormonal control of neonatal glucose metabolism from availability of substrate as a limiting factor in homeostatic maturation.

In another study, Cowett and associates investigated whether glucose alone tightly controls neonatal glucose homeostasis.[72] Fifteen spontaneously delivered term lambs were studied after receiving radiolabeled glucose for isotope-based measurement of glucose production. To isolate the contribution of glucose, after a baseline period five lambs received glucose at a rate of 8.5 mg/kg/minute in combination with the following: somatostatin to block insulin, glucagon, and growth hormone release; metyrapone to block cortisol release; phentolamine to block α-adrenergic release; and propranolol to block β-adrenergic release. Under conditions of glucose infusion at a rate 49% greater than the basal rate, the endogenous glucose production persisted; only an evanescent decrease from levels in the control group, which was not statistically different over time, was observed.As a substrate, glucose did not tightly control neonatal glucose homeostasis.

In conclusion, it is apparent that hormonal control by glucoregulatory hormones (including insulin and the various counterregulatory hormones) and possibly glucose plays a significant role in the developing regulation of glucose homeostasis in the neonatal period. Further research will be required to determine the relative contribution of the various factors important in the development of carbohydrate (glucose) homeostasis in the neonatal period.

The multiplicity of factors that influence both the treatment of neonatal hyperglycemia and its prevention resulted in two rigorous evaluations using the highly regarded Cochrane Collaborative methodology to evaluate randomized or quasirandomized clinical trials.[73,74] Because of the paucity of studies, the reviewers concluded that it remained uncertain whether the hyperglycemia per se was a cause of adverse clinical outcomes or how the hyperglycemia should be treated.[73] Relative to the issue of prevention, a parallel conclusion was adopted because of a similar paucity of studies—namely, that the evidence reviewed did not support the routine use of insulin infusion to prevent hyperglycemia in very-low-birth-weight neonates.[74] In both reviews, additional randomized clinical trials were advocated to answer the questions at hand.[74,75] Finally, a major issue in this ongoing area of research is the interrelationship of hormonal control of glucose homeostasis and the presence or development of insulin sensitivity or insensitivity.These studies are germaine to the study of an increasingly important area of metabolic investigation—namely, the fetal origins of adult disease.[73] This topic is evaluated in Chapter 18 by the investigator credited with originating the hypothesis.

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52 Cell Glucose Transport and Glucose Handling During Fetal and Neonatal Development

Rebecca Anne Simmons

Glucose is a vital substrate for the growing and developing fetus. It is required by most cells for oxidative and nonoxidative adenosine triphosphate (ATP) production and serves as a precursor for other carbon-containing compounds. It is the primary fuel used for several specialized cells and is the major fuel used by the brain. Its storage in the liver as glycogen provides a means by which glucose homeostasis can be maintained, particularly during the neonatal period. Glycogen stores also represent the primary source of energy for muscle tissue during exercise in postnatal life. Because of the diverse metabolic roles played by glucose, defects in its uptake or metabolism can alter cellular functions and can lead to significant morbidity and even death. This chapter focuses on the molecular biology and regulation of glucose transporters in the fetus and newborn.

The plasma membranes of most mammalian cells, except those of the proximal kidney and small intestine, have a passively mediated transport system for glucose. Facilitative entry of glucose into the cell is controlled by glucose transporters (GLUTs), structurally related proteins that are encoded by a *SLC2A* gene family[17] and are expressed in a tissue-specific manner. A different family of proteins, sodium-coupled glucose transporters (SGLTs), actively transport glucose across the apical membranes of polarized intestinal and renal epithelial cells.[8-13] The driving force for active glucose absorption is the electrochemical sodium gradient across the membrane.

Most cells contain at least one glucose transporter isoform, and many contain more than one. In most cell types, glucose transporters mediate a net uptake of glucose. Under some circumstances, glucose is transported out of the cell. For example, the sodium-coupled transporter actively transports glucose into epithelial cells of the small intestine, and a facilitative transporter mediates the efflux of glucose from the cell into the interstitium. In hepatocytes, facilitative glucose transporters are responsible for the uptake of glucose from the portal circulation and for the release of glucose generated by glycogenolysis or gluconeogenesis. Thus glucose transporters ensure efficient tissue uptake and distribution of glucose.

SODIUM-DEPENDENT GLUCOSE TRANSPORTERS

It has long been known that dietary sugars are actively absorbed from the small intestine; however, only recently has the molecular mechanism been elucidated. Active absorption of glucose across epithelial cells of the small intestine and the kidney proximal tubule is accomplished by sodium-glucose cotransporters located in the brush border membranes. Transport of each glucose molecule is coupled to the cotransport of two Na^+ ions (SGLT1) or of one Na^+ ion (SGLT2). This transport system uses the energy from an extracellular to intracellular Na^+ ion electrochemical gradient, generated by Na^+,K^+-ATPases, to drive the accumulation of glucose into the cell. These transporters belong to a major class of membrane proteins called co-transporters (or symporters). The sodium-dependent glucose transporter (SGLT) family, with the gene name *SLC5A,* comprises sodium-dependent glucose cotransporters (SGLT1 and SGLT2), the glucose sensor SGLT3, the widely distributed inositol,[14] the multivitamin transporters SGLT4 and SGLT6,[15] and the thyroid iodide transporter SGLT5.[16] These exist in bacteria, plants, and animal membranes, and they actively transport sugars, amino acids, carboxylic acids, and some ions (chloride, phosphate, sulfate, iodide) into cells.

SGLT1 is a hydrophobic integral membrane protein with approximately 12 membrane-spanning domains. The gene encoding the human intestinal SGLT has been localized to the q11.2–qter region of chromosome 22.[17] SGLT1 is abundantly expressed in the brush border of the small intestine and at lower levels in kidney, lung, and liver. The transporter has the same affinity for both glucose and galactose. An excellent review of the molecular regulation of SGLT1 is available.[18]

Clinical interest in the intestinal brush border sodium-glucose cotransporter has focused on diarrhea and malabsorption. Glucose-galactose malabsorption is a rare autosomal recessive disorder characterized by onset of severe, watery diarrhea in the newborn period. Unless glucose and galactose are eliminated from the diet, death rapidly ensues. Wright and colleagues demonstrated that a single missense mutation in the gene encoding the intestinal sodium-glucose cotransporter is sufficient to cause a syndrome of life-threatening diarrhea.[19]

SGLT2 complementary DNA (cDNA) originally was isolated by Hediger and colleagues from a human cDNA library.[8] The SGLT amino acid sequences are approximately 60% identical with those of SGLT1, and the proteins have the same predicted secondary structure. The expression of this cotransporter is restricted to the renal cortex, and it is located in epithelial cells of proximal tubule S1 segments. The bulk of the filtered glucose is believed to be reabsorbed in the proximal convoluted tubule by a low-affinity, high-capacity SGLT2, with reabsorption of the remainder by the high-affinity cotransporter SGLT1.

SGLT3 is expressed mainly in cholinergic neurons of the small intestine and in skeletal muscle at the neuromuscular junctions.[20] SGLT3 may act as a glucosensor by conveying information to the cell about external glucose concentration directly through the membrane potential or indirectly coupled through another molecule such as a G-protein.

Disorders of sodium coupled glucose transport are rare. Familial renal glycosuria is an autosomal dominant disorder (an autosomal recessive mode of inheritance has not been excluded in all cases) affecting 0.2% to 0.6% of the general population and is characterized by the excretion of large amounts of glucose into the urine in the presence of normal blood glucose concentrations. The molecular basis of benign renal glycosuria has not been determined. It is possible that mutations in the low-affinity sodium-glucose cotransporter, SGLT2, may be responsible for the defect in renal absorption of filtered glucose.

Sodium-glucose cotransporters appear to be active prenatally, and as a consequence, the intestine is ready to absorb the first ingested glucose.[21] The cloned cDNAs and specific antibodies for the different sodium-glucose cotransporters can be expected to be valuable tools for identifying the specific cells in the intestine and kidney that express these proteins and for studying the

regulation of their expression during development and in altered metabolic states such as diabetes mellitus or pregnancy.

FACILITATED GLUCOSE TRANSPORTERS

The energy-independent process of transporting glucose across the cell membrane occurs by facilitative diffusion. Transport of glucose is saturable, stereoselective, and bidirectional. The kinetics of glucose transport inward and outward are not necessarily identical,[22] and in fact, in the erythrocyte, the rate of exchange flux for glucose is faster than net flux rate.The primary function of the facilitative glucose transporters is to mediate the exchange of glucose between blood and the cytoplasm of the cell.This mechanism may involve a net uptake or output of glucose from the cell, depending on the type of cell, its metabolic state, and the metabolic state of the organism. In most cells, cytoplasmic glucose is rapidly phosphorylated by hexokinase or glucokinase, and levels of glucose-6-phosphatase are low, so little intracellular free glucose is available.These cells are involved only in net uptake and metabolism of blood glucose. The hepatocyte also is a net producer of glucose in the postabsorptive state. Glycogenolysis and gluconeogenesis increase intracellular free glucose to levels greater than its concentration in the blood, resulting in net efflux of glucose from the cell. In the postprandial state, glucose is transported into the hepatocyte to replenish glycogen stores.

The facilitative glucose transporters constitute a family of structurally related proteins. Several facilitated glucose transporter isoforms have been identified and are designated GLUT (with the gene symbol SLC2A, for *sol*ute *c*arrier family *2*).[2,3,23-32] The GLUT family of proteins has been subdivided into three classes: Class I consists of GLUT1 to GLUT4; class II contains GLUT5, GLUT7, GLUT9, and GLUT11; and class III consists of GLUT6, GLUT8, GLUT10, and GLUT12. These proteins vary in size, ranging from 492 to 524 amino acids long.They exhibit 39% to 68% sequence identity and 50% to 76% sequence similarity in pairwise comparisons.[1,3,24,25,33-38] A topology map of the GLUTs has been proposed based on analysis of the primary amino acid sequence of GLUT1.[25] Each isoform consists of 12 membrane-spanning domains, an intracytoplasmic hydrophilic loop, and an exofacial loop bearing a single *N*-glycosylation site. Both the amino-terminal (N-terminal) and carboxyl-terminal (C-terminal) ends are exposed intracellularly (Figure 52-1). Comparisons among the different isoforms have revealed that the sequences of the transmembrane segments and the short cytoplasmic loops connecting these transmembrane regions are highly conserved. Most likely, these regions are responsible for the transport of glucose.The N terminus and C terminus are unique for each of the different isoforms and may contribute to isoform-specific properties, such as kinetics, hormone sensitivity, and subcellular localization.[1,3,23-25,27,34-37]

STRUCTURE AND PROPERTIES OF FACILITATIVE GLUCOSE TRANSPORTERS

GLUT1

GLUT1 was the first glucose transporter to be cloned. Antibodies were raised against the purified erythrocyte glucose transporter to screen antigen expression cDNA libraries from RNA from the human hepatoblastoma cell line (HepG2). The amino acid sequence of GLUT1 is highly conserved, with 98% identity between the sequences of human and rat GLUT1 and 97% identity between the sequences of human and mouse, rabbit, or pig GLUT1. This high degree of sequence conservation implies that all domains of this 492-residue protein are functionally important.

GLUT1 is the most ubiquitously distributed of the transporter isoforms. It is found in virtually all tissues in the fetus and in many tissues and cell types in the adult.[39-46] GLUT1 has a very high affinity for glucose. These properties make it likely that GLUT1 is responsible for constitutive glucose uptake. In many organs, GLUT1 is concentrated in endothelial cells of blood-tissue barriers. Thus one of the specialized roles of GLUT1 is to shuttle glucose between blood and organs that have limited access to small solutes through passive diffusion.

GLUT1 is the predominant isoform in the fetus.This transporter also is expressed in fetal tissues that fail to express it significantly in the adult. Most fetal cells exhibit rapid growth and differentiation, necessitating an increased supply of energy-producing substrates. This energy need may be the reason for the prevalence of GLUT1 in fetal tissues. After birth, GLUT1 levels decrease, and levels of other isoforms such as GLUT2 in the liver and GLUT4 in the muscle increase.[39,42,45,46] The signals responsible for the decline in GLUT1 expression during the neonatal period are not known. It is hypothesized that the switch from a carbohydrate to a fat source of fuel may induce this change in some organs.[45]

Most of the studies concerning the regulation of GLUT1 have been carried out in cultured cells and cell lines from humans and rodents. Growth factors, insulin, insulin-like growth factor-I (IGF-I), growth hormone, glucose, estrogen, transforming growth factor-β, thyroid hormone, cyclic adenosine monophosphate (cAMP), fibroblast growth factor, and oncogenes increase GLUT1 expression in many different cell types.[47-56] Cellular ATP decreases GLUT1-mediated sugar import capacity.[57] GLUT1-mediated sugar transport displays acute and adaptive regulation. *Acute* responses occur within seconds to minutes and involve stimulation of existing cell surface glucose transporters.[58] *Adaptive* responses occur over several hours in response to hypoxia and hypoglycemia and involve changes in glucose transporter expression.[59]

GLUT1 deficiency syndrome was first described by De Vivo in 1991.[60] To date, more than 100 cases have been reported. It is caused by a defect of glucose transport into the brain, mediated by the facilitative glucose transporter GLUT1. A seizure disorder is the most common clinical manifestation. Other potentially associated clinical features include developmental delay, ataxia, and spasticity. The diagnosis is suggested by hypoglycorrhachia, defined as a cerebrospinal fluid (CSF) glucose value less than 2.2, with a CSF–plasma glucose ratio generally less than 0.4. The disease is treated effectively with a ketogenic diet, because ketone bodies easily penetrate the blood-brain barrier and serve as an alternative fuel for the brain.

GLUT2

GLUT2 is the major transporter isoform expressed in adult liver, pancreatic beta cells, and epithelial cells of the intestinal mucosa and kidney.[34-36] Levels of this isoform are quite low in the fetus. GLUT2 has 55% amino acid identity with sequences of GLUT1, and it has a similar structure and orientation in the plasma membrane. In contrast with GLUT1, whose sequence is highly conserved, only 81% identity between the sequences of human and rat GLUT2 has been documented.The most characteristic feature of this isoform is its low affinity for glucose. GLUT2 and glucokinase form a glucose-sensing apparatus in hepatocytes and beta cells that responds to subtle changes in blood glucose concentrations by altering the rate of glucose transport into the cell.The transport capacity of GLUT2 is in excess of the glucokinase trapping reaction, thus making phosphorylation of glucose the rate-limiting step for glucose uptake in hepatocytes and beta cells. In the intestine and kidney, the high-capacity, low-affinity system is necessary to transport glucose under conditions of large transepithelial substrate fluxes that occur after meals.

Mutations in the gene encoding GLUT2 in the human give rise to Fanconi-Bickel syndrome, caused by a rare autosomal-recessive inborn error of metabolism, which resembles type I glycogen storage disease.[61,62] More than 100 patients with this syndrome

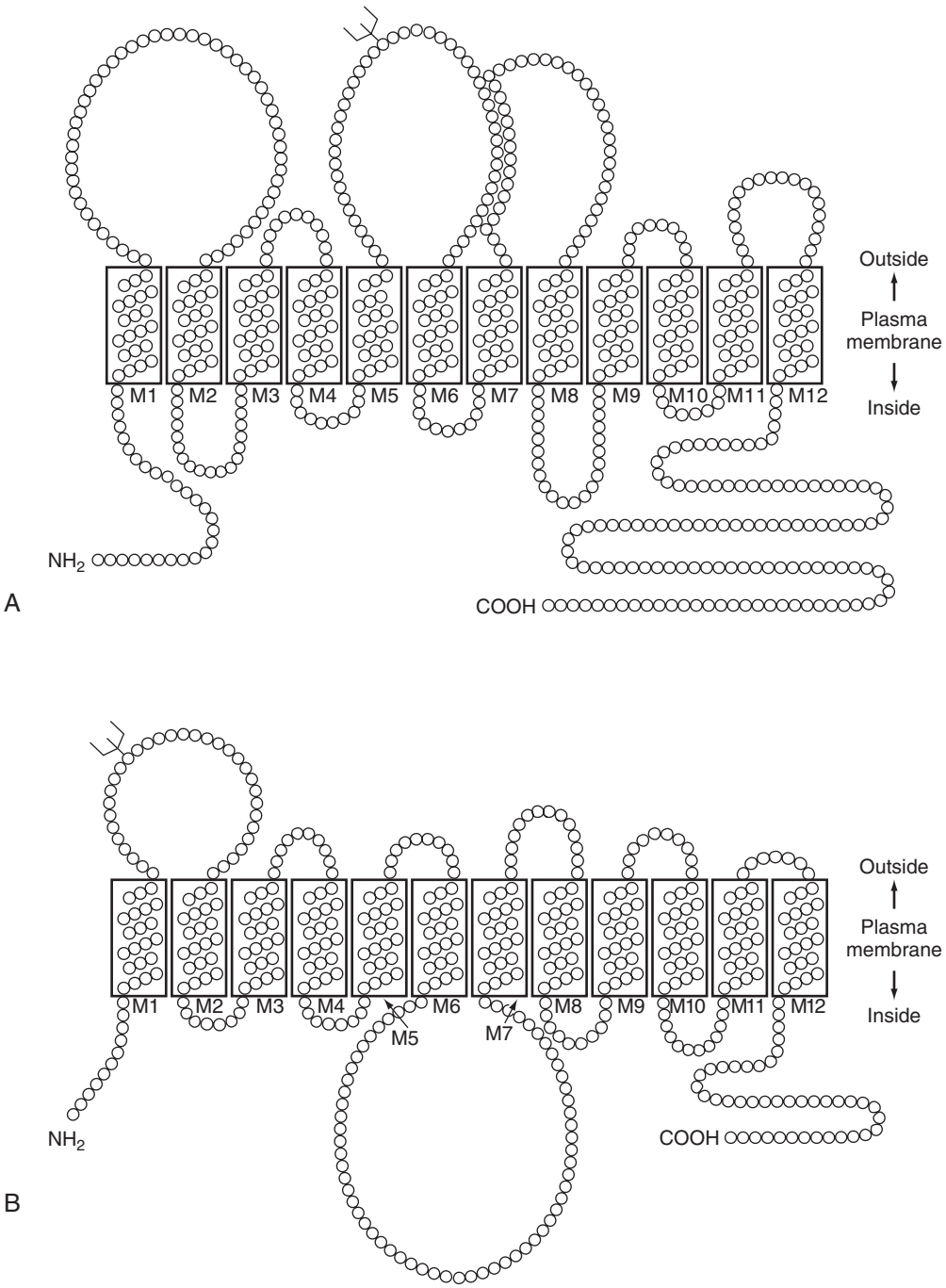


Figure 52-1 Models for the orientation of the human sodium-glucose cotransporter SGLT1 **(A)** and the facilitative glucose transporters GLUT1 to GLUT5 in the plasma membrane **(B).** The 12 potential membrane-spanning α-helices are shown as *boxes,* numbered M1 to M12.

and 34 different GLUT2 mutations have been reported in the recent literature. Because GLUT2 is the predominant glucose transporter in hepatocytes, pancreatic beta cells, enterocytes, and renal tubular cells, a loss of GLUT2 leads to a typical clinical syndrome of hepatorenal glycogen accumulation, glucose and galactose intolerance, fasting hypoglycemia, a characteristic tubular nephropathy, and severely stunted growth.[61]

Expression of GLUT2 appears to be developmentally regulated. Beta cell content of GLUT2 protein in the fetus is approximately half that in the adult rat.[63] Despite the reduction in GLUT2 content, the blunted insulin secretory response seen in fetal islet cells is not the result of a limitation of glucose transport. At least a 10-fold decrease in transport activity would be required to reduce metabolism of glucose sufficient to perturb glucose-induced insulin secretion.[64,65] Other factors appear to be responsible for the blunted insulin secretory response observed in the fetus.

Studies done in fetal rats have demonstrated that GLUT2 levels are markedly diminished in fetal hepatocytes compared with the adult cells.[66-69] Shortly after birth, GLUT2 protein content dramatically rises and increases again, coinciding with weaning of the newborn pup from high-fat maternal milk.[68] Although an altered hormonal or substrate milieu often is implicated etiologically in

the metabolic maturation associated with birth, the mechanism of this change is still unknown.

Glut3

GLUT3 was first isolated from human fetal skeletal muscle.[70] Human GLUT3 has 64% and 52% identity with human GLUT1 and GLUT2, respectively, with 83% amino acid sequence identity between the sequences of human and mouse GLUT3. Thus, as with GLUT2, the sequence of GLUT3 is not as highly conserved among species as that of GLUT1. GLUT3 messenger RNA (mRNA) is present at variable levels in all human tissues and is most abundant in brain, kidney, and placenta. The ubiquitous distribution of GLUT3 in human tissues suggests that it, together with GLUT1, may be responsible for basal glucose transport. In other animals such as rats, monkeys, and mice, the pattern of expression of GLUT3 is much different from that observed in humans.[3] In these animals, GLUT3 is abundant only in brain. The expression of GLUT3 in brain indicates that two facilitative glucose transporters are involved in the uptake of glucose. GLUT1 is primarily responsible for transport of glucose across the blood-brain barrier, and GLUT3 controls the uptake of glucose into the neuron.

Relatively little information is available about the regulation of GLUT3 in the fetus. Studies done in fetal rat brain suggest that glucose concentrations do not regulate expression of this transporter isoform.[71] This finding is in contrast with those for GLUT1, in which high levels of glucose down-regulate GLUT1 protein and mRNA abundance.[49] In contrast with the findings for glucose, chronic hypoxia increases GLUT3 mRNA expression in embryonic (day 14) rodent brain.[72]

Glut4

GLUT4 is expressed primarily in adult tissues that exhibit insulin-stimulated glucose transport, such as adipose tissue and skeletal and cardiac muscle.[49] It also is expressed at low levels in fetal rat brain.[72] In comparison with adult levels, little GLUT4 is expressed in fetal muscle[47] and brown fat,[73] and levels do not increase until well after birth.[43,45,73]

The sequence of GLUT4 is highly conserved, with 95% and 96% identity between the sequences of human and rat or mouse GLUT4.[74] GLUT4 contains unique sequences in its N-terminal and C-terminal cytoplasmic domains that direct its characteristic membrane trafficking capability. GLUT4, unlike other facilitave glucose transporters, is localized primarily intracellularly in the unstimulated state and is acutely redistributed to the plasma membrane in response to insulin and other stimuli.[74,75]

Glucose transport in insulin-sensitive tissues has received considerable attention because of the importance of this process in the maintenance of whole-body glucose disposal in the adult. The transport step is rate-limiting for glucose uptake into fat and muscle under most conditions.[76,77] A central role for GLUT4 in whole-body metabolism is strongly supported by a variety of genetically engineered mouse models. Heterozygous GLUT4$^{+/-}$ mice, which display decreased GLUT4 protein in muscle and adipose tissue, are insulin-resistant and develop diabetes later in life.[78,79]

Glut5

GLUT5 is the most divergent member of the glucose transporter family.[27] Human GLUT5 shares 42%, 40%, 39%, and 42% identity with human GLUT1, GLUT2, GLUT3, and GLUT4, respectively.[80] GLUT5 is expressed at high levels in the apical membrane of intestinal enterocytes and mature spermatocytes in adults.[81] Fructose is transported across intestinal epithelial cells by passive transport. A high rate of fructose utilization by testes also has been documented. In view of these findings, it seems likely that GLUT5 is the major mammalian fructose transporter.

GLUT5 also is found in smaller quantities in adult human kidney, brain, muscle, and adipose tissue.[81,82] The physiologic significance of GLUT5 in these tissues is unknown.

Novel Glucose Transporters

The facilitative glucose transporters GLUT1 to GLUT4 have considerable sequence similarity and different tissue distributions. Although the diverse tissue distribution and the specific functions of GLUT1 to GLUT5 appear to indicate that these transporters are sufficient to control glucose uptake in all mammalian tissues, it is likely that additional sugar transport facilitators exist. By searching the expressed sequence tag (EST) databases and taking advantage of the conserved sugar transporter signatures, several novel GLUT-like genes have been identified.

GLUT7, GLUT9, and GLUT11 are considered to be class II facilitative glucose transporters. GLUT7 is a high-affinity transporter for glucose and fructose and can be detected in small intestine, colon, testis, and prostate; within the small intestine, GLUT7 is expressed predominantly in enterocyte brush border membrane.[83]

GLUT9 exhibits highest expression levels in kidney and liver; lower mRNA levels were detected in small intestine, placenta, lung, and leukocytes.[30] GLUT9 has been detected in the mouse at a preimplantation stage of development and may be critical for early preimplantation development.[29] Three different isoforms have been identified that seem to be differentially expressed during development.[29] Two of the mouse GLUT9 isoforms demonstrate glucose transport activity when expressed in *X. laevis* oocytes.[28]

GLUT11 exhibits a low affinity for glucose and is thought to be a fructose transporter with low affinity for glucose. GLUT11 is expressed predominantly in pancreas, kidney, and placenta and also exhibits moderate expression in heart and skeletal muscle.[84]

GLUT6, GLUT8, GLUT10, and GLUT 12 are the family of class III transporters. The low-affinity glucose transporter GLUT6 is expressed predominantly in brain, spleen, and peripheral leukocytes.[27,85] GLUT8 is a high-affinity glucose transporter but also may transport fructose and galactose.[28,86] This transporter exhibits significant sequence similarity with the GLUT1 (29.4%).[28] In human tissues, it is found predominantly in testis, and lower amounts are detected in most other tissues including skeletal muscle, heart, small intestine, and brain.[23] GLUT8 is expressed in testis only from adult but not prepubertal, rats, and expression is markedly inhibited by estrogen treatment.[28,87] Thus GLUT8 may be involved in the provision of glucose required for DNA synthesis in male germ cells. GLUT8 also has been found to be the glucose transporter responsible for insulin-stimulated glucose uptake in the blastocyst.[88] In addition, GLUT8 may play a role in glucose uptake of adipocytes.[89]

GLUT10 (35% sequence identity with human GLUT1 to GLUT8) is expressed in human heart, lung, brain, liver, skeletal muscle, kidney, and placenta.[32] It also is detected in fetal brain and liver.[32] GLUT12 is expressed predominantly in heart and prostate and exhibits glucose transport activity when expressed in *X. laevis* oocytes.[90] This activity is competitively inhibited by D-galactose and to a lesser extent by D-fructose.[91]

LOCALIZATION AND REGULATION OF FACILITATIVE GLUCOSE TRANSPORTERS

Embryo

An excellent review of the role of facilitative glucose transporters in preimplantation development has recently been published.[92] GLUT1 is expressed in all stages of embryonic development of the mouse, rat, rabbit, cow, and human, including the oocyte and the blastocyst. It is readily detectable in trophectoderm and inner cell mass cells of the mouse blastocyst, and it is associated with intracellular membranes as well as plasma membranes of all cell

types.[93-96] During organogenesis in the rat embryo, GLUT1 can be found in the neural tube, as well as in the heart tube, gut, and optic vesicle.[97]

GLUT2 also appears to be an important mediator of glucose uptake in the early embryo, although this role is somewhat controversial.[98,99] GLUT2 is expressed as early as during the eight-cell blastocyst stage. It is reportedly expressed on trophoectoderm membranes facing the blastocyst cavity and on the plasma membrane of the inner cell mass.[93,94] The function of GLUT2 at these locations in the blastocysts has yet to be elucidated. It has been speculated, however, that GLUT2 may be responsible for glucose transport into the blastocoele cavity.[100] Other studies also conducted in mice did not demonstrate GLUT2 expression.[98,99] In addition, studies conducted in other species—namely, rabbit and cow—did not find GLUT2 to be expressed during the preimplantation period.[101,102]

Expression of GLUT3 appears by day 4 of gestation (in rat), and it is found on the apical membranes of trophoectoderm cells and seems to be responsible for the uptake of maternal glucose.[97,100] Both knock-down and knock-out of GLUT3 significantly impair preimplantation development, suggesting that at least in the mouse, GLUT3 is essential for preimplantation development.[92,103]

GLUT8 is expressed primarily within vesicles in the cells lining the blastocoele.[29] Insulin and IGF-1 stimulate translocation of GLUT8 to the plasma membrane of these cells through binding to the IGF-1 receptor.[29] Studies suggest that GLUT8 expression and translocation in response to insulin are critical for blastocyst survival.[104] However, the physiologic role that insulin-regulated glucose transporters play in vivo in the human blastocyst remains to be determined.

Recently, two additional facilitative glucose transporters have been identified in the preimplantation embryo: GLUT9 and GLUT12.[29,105] Down-regulation of a GLUT9 isoform does not decrease glucose uptake in blastocysts. When antisense-treated embryos are transferred into pseudopregnant female mice, however, an increase in pregnancy loss is noted.[29]

GLUT12 is abundantly expressed at the two-cell stage and decreases to low levels in the morula and blastocyst stages.[91] Because the promoter region of murine GLUT12 contains sequences that are homologous to known insulin response elements,[105] this transporter may be an insulin-responsive glucose transporter.

One of the characteristics of preimplantation embryos is a metabolic shift from a dependence on the tricarboxylic acid cycle during the precompactation stages to a metabolism based on glycolysis after compactation.[106] This change in substrate utilization is coincident with the rapid proliferation that occurs during this developmental stage. Similar changes in substrate preference occur in numerous other cells as they undergo proliferation. Therefore high-affinity glucose transporters such as GLUT1 and GLUT3 can be assumed to be required for glucose uptake.

Pregnant women with diabetes are at increased risk for both first-trimester spontaneous abortions and major fetal malformations. Data suggest that hyperglycemia-induced apoptosis in the embryo may contribute to early pregnancy loss.[107,108] Preimplantation studies have shown that maternal hyperglycemia down-regulates GLUT1, GLUT2, and GLUT3 at the blastocyst stage of development,[109] which is associated with increased apoptosis.[107] Only 40% of the cells showed evidence of apoptosis, which has been shown to result in neural tube defects, limb abnormalities, and abdominal wall malformations similar to those seen among infants of diabetic women.[110]

Placenta

In the human placenta, placental villi are in direct contact with maternal blood. The surface of the placental villus is covered with a single syncytiotrophoblast layer, formed by the fusion of the underlying cytotrophoblast elements. Fetal capillaries lie directly beneath the syncytiotrophoblast. Transfer of glucose from maternal to fetal blood occurs by way of the placental villi and probably is mediated by GLUT1. GLUT1 is abundantly expressed in the plasma membranes of both the basal and apical sides of the syncytiotrophoblast. GLUT1 may facilitate the entry of glucose into the cytoplasm of the syncytiotrophoblast from maternal blood, whereas GLUT1 at the basal plasma membrane may aid in the exit of glucose from the cytoplasm of the syncytiotrophoblast to the pericapillary space of the fetus. GLUT1 on the endothelial cell of the capillary then transfers glucose into the fetal circulation.[111-114] Protein and mRNA levels of GLUT1 increase in the placenta as the fetus matures, underscoring the importance of this transporter in fetal development.[112]

GLUT3 is distributed throughout placental villous tissue and decreases during gestation.[112,114-116] Although GLUT3 mRNA is abundantly expressed in villous tissue, GLUT3 protein is localized primarily to the vascular endothelium. GLUT3 may play a role in transporting glucose from mother to fetus after transsyncytial transport. This hypothesis is supported by the finding that decreased GLUT3 expression (from knockdown of a single allele) in murine placenta causes fetal growth restriction.[103]

A body of data regarding the regulation of glucose transporter expression in human placenta is beginning to accumulate. An excellent comprehensive review of placental nutrient transport by Jones and co-workers is available.[117] A number of studies have been carried out in placentas from pregnancies complicated by intrauterine growth restriction and diabetes. Growth-restricted fetuses often are hypoglycemic, and impaired placental glucose transport has been implicated as a pathophysiologic mechanism. Growth-restricted fetuses have a reduced umbilical venoarterial concentration difference in glucose and lower fetal weight–specific umbilical volume flow.[118,119] However, placentas from preterm and term infants with intrauterine growth restriction have not shown a difference in levels of GLUT1 protein.[120]

Gestational diabetes is associated with placenta overgrowth and an increase in transplacental glucose transfer to the fetus. Levels of GLUT1 protein are increased in the basal membranes and lead to an increase in glucose transport activity of syncytial basal membranes.[121,122] Microvillous expression and activity are unaffected by hyperglycemia.[123,124] Likewise, levels of GLUT3 and GLUT4 are not altered in placentas of women with diabetes during pregnancy.[123,124] Thus it appears from these studies that increased glucose transport to the fetus of the diabetic mother is facilitated by increased levels of GLUT1. Illsley proposed that this process leads to fetal hyperglycemia, which in turn stimulates the production of IGF-1, leading to excess fetoplacental growth.[125]

Brain

Brain glucose utilization accounts for approximately 80% of whole-body glucose disposal in humans.[126] Furthermore, heterogeneity in glucose utilization has been observed among different regions of the brain. Circulating glucose crosses the blood-brain barrier and enters brain parenchyma cells by means of facilitative glucose transporters. Most studies of GLUT expression in the nervous system of the developing animal have been performed in the rat. Before the formation of the blood-brain barrier, GLUT1 is abundant in the germinal neuroepithelium, which gives rise to both neurons and neuroglia cells.[127] Just before birth, GLUT1 is abundant in the brain vasculature, meninges, ependyma, and choroid plexus. After birth, GLUT1 also is found in glial cells.[127] GLUT1 is developmentally regulated in rat and rabbit brain.[39-41] Its expression is highest in adult brain, followed by fetal and neonatal brain, respectively.

Few localization studies have been done in the human fetus. One report demonstrated that the localization of GLUT1 in the mid- to late-gestation human fetus is similar to the rat—that is,

it is located primarily in the microvascular endothelial cells that constitute the blood-brain barrier.[82] A more recent study, however, suggested a much wider distribution of GLUT1 in the developing brain.[128] From 10 to 21 weeks of gestation, GLUT1 is expressed in all regions of the fetal brain and is present primarily in the endothelial cells of the brain capillaries, in the epithelial cells of the choroid plexus, and in neurons.[128] GLUT 2 is not expressed until midgestation (21 weeks), and at that time it is highly expressed in the granular layer of the cerebellum.[128] No study to date has been able to detect GLUT3 or GLUT4 in human fetal brain.

In the rodent, after birth, GLUT3 is found in the cerebellum in neurofilament-positive cellular transverse fibers, cell bodies of Purkinje cells, and other neuronal elements in close proximity to the Purkinje layer.[82] This region-specific pattern of GLUT3 expression may reflect the differing glucose needs of anatomically distinct regions of the brain. Localization of GLUT3 is similar to the distribution of glucose utilization, which during early infancy is mainly infratentorial, and later in development occurs in supratentorial structures as well.[129,130]

Regulation of glucose transport in the fetal brain is uniquely different from that in the adult. Before birth, low levels of glucose (in vivo or in vitro) fail to up-regulate glucose transport in whole fetal rat brain[71] or isolated glial cells. After birth, however, hypoglycemia induces a marked increase in GLUT1 expression[131] in whole-rat-brain isolated glial cells. Furthermore, glucose transport in the fetal brain does not respond to insulin or IGF-I,[47] two hormones that increase GLUT1 expression in glial cells of older animals.[132,133] The mechanisms underlying these differences in regulation of glucose transport that occur with maturation are unknown.

Hypoxia during gestation induces a marked increase in both GLUT1 and GLUT3 levels in fetal rat brain.[72] Hypothyroidism decreases protein levels of GLUT1 but not GLUT3 in postnatal rat brain.[134]

Lung

Glucose is an important metabolic substrate for the lung and provides carbon moieties for energy production and synthesis of surfactant. In adult lung, transport of glucose across the apical membrane of the type II pneumocyte occurs by sodium-coupled transport,[135-137] and it occurs across the basolateral membrane by facilitative glucose transport. To date, GLUT1 is the only isoform found to be expressed in type II pneumocytes of fetal rats and humans.[138] It is hypothesized that SGLT1 also is expressed in type II pneumocytes; however, no study has thus far been able to localize this transporter in fetal lung.

GLUT1 is abundantly expressed in the fetal lung when compared with that of the juvenile and adult rat.[46] Glucose utilization and levels of GLUT1 mRNA and protein dramatically decline as the animal matures.[46,139] By day 14 of life, GLUT1 is undetectable in rat pups. The factors responsible for this significant decrease in the synthesis of GLUT1 are unknown.

Insulin and IGF-I are important modulators of glucose transport in type II pneumocytes of fetal rats. Physiologic levels of insulin and IGF-I stimulate glucose transport,[47] whereas higher levels of insulin inhibit glucose uptake.[140] Several animal studies suggest that hyperinsulinemia, through its inhibitory effects on glucose transport, contributes to the decrease in surfactant synthesis observed in infants of diabetic mothers. In a model that somewhat mimics human gestational diabetes, diabetes is induced in pregnant rats by streptozotocin. Fetal rats are hyperglycemic, hyperinsulinemic, and large for gestational age. Type II pneumocytes from these animals exhibit markedly diminished glucose uptake and GLUT1 expression.[141] It is possible that the decrease in glucose uptake diminishes the supply of glucose available for surfactant synthesis. This limited synthesis could be one factor that increases the risk for respiratory distress syndrome in infants of diabetic mothers.

Male fetuses exhibit delayed lung maturation and surfactant production in comparison with female fetuses. This delay may be related to sex hormone effects: Estrogen enhances and androgens delay lung development. The uptake of glucose, an important precursor for surfactant synthesis, appears to be differently affected by estrogen and androgens. In vitro studies performed in fetal rat have shown that estradiol and dehydrotestosterone differentially regulate glucose uptake in fetal rat lung tissue. This regulation of substrate supply (glucose) by estradiol and dehydrotestosterone may be another mechanism underlying the sexual dimorphism observed in lung development and surfactant synthesis.[142]

Liver

Transport of glucose across the hepatocyte does not appear to be rate-limiting for glucose metabolism. However, glucose transport is developmentally regulated in the human and rat, and glucose transport contributes to the changes in glucose metabolic capacity from fetal to extrauterine life. The major glucose transporter in the adult hepatocyte is GLUT2. GLUT1 is expressed only in perivenous hepatocytes.

By contrast, in the fetus, GLUT1 and GLUT2 are abundantly expressed in hepatocytes.[66-68] In this context, the fetal to neonatal transition is characterized by a shift from abundant GLUT1 in the hepatocyte to an adult pattern of little GLUT1 expression.[39,68] Many metabolic and hormonal factors dramatically change during the perinatal period. The factors responsible for the switch in GLUT1 expression remain to be delineated.

Acute hyperglycemia and hyperinsulinemia are associated with increased expression of GLUT1 in fetal ovine liver,[143] whereas chronic hyperglycemia is accompanied by decreased GLUT1 levels.[144] Thus chronically elevated circulating glucose concentrations appear to induce an adaptive mechanism to prevent entry of excess glucose, which is toxic to the cell.

Muscle

Most of the studies regarding glucose transport in muscle have been carried out in adults. As described earlier, GLUT4 is the predominant isoform expressed in adult muscle. In response to insulin, this transporter isoform significantly increases the transport of glucose into the myocyte. In contrast with the marked insulin responsiveness observed in the adult, fetal muscle responds only modestly to insulin.[145] Both acute and chronic adminstration of glucocorticoids to fetal sheep increase GLUT4 expression, which is associated with increased insulin sensitivity.[146] Insulin and IGF-I increase GLUT1 expression 1.5-fold in normal fetal rat muscle explants,[47] compared with the 20-fold increase observed in adult muscle.[147,148] Insulin does not stimulate GLUT1 expression in isolated myoblasts from fetal rats,[45] suggesting that stimulation of glucose transport by insulin requires tissue-specific additional factors.

GLUT1 is localized to the myoblast, and levels are quite high in the fetal and newborn rat pup. GLUT1 decreases significantly during weaning.[45,149] It appears that the switch from GLUT1 to GLUT4 expression during this period is secondary to dietary factors. If rats are weaned to a diet rich in fat, the normal increase in GLUT4 is prevented.[45,149,150] The molecular mechanisms responsible for this effect are unknown.

Kidney

The kidney, small intestine, and liver all can release glucose during periods of decreased glucose availability. Although the liver is the principal supplier of glucose during short fasts, the kidney also produces glucose during prolonged starvation. The

sodium-glucose cotransporter, SGLT1, transports glucose into the brush border cell of the proximal tubule of the kidney. GLUT2, localized on the basolateral membrane of epithelial cells lining renal tubules, is involved in the net release of glucose into the blood during absorption of renal glucose.

No data are available concerning the regulation of glucose transport in the fetal kidney, and only a few reports have described the ontogeny of renal glucose transport. SGLT1 is expressed in lower quantities in fetal than in adult kidney. GLUT2 also is present in the fetal kidney, and its expression increases with maturation.

SUMMARY AND FUTURE DIRECTIONS

Glucose transporters have acquired distinct physiologic and biochemical properties that allow them to serve specific functions in the tissues in which they are expressed. An understanding of the mechanisms underlying tissue-specific expression of these transporters will facilitate an understanding of in vivo glucose utilization and clearance processes that occur normally and in disease states. Although studies in adults provide insight into the regulation of glucose transport, similar studies are required in the fetus and newborn for full elucidation of the role of the glucose transporter in fetal and neonatal development.

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Charles A. Stanley and Olga T. Hardy

Pathophysiology of Hypoglycemia

Hypoglycemia is more common in the newborn than at any other age, particularly in the first 12 to 24 hours of extrauterine life. After the first 24 hours of life, true neonatal hypoglycemia is uncommon; once detected, it must be investigated and treated appropriately. The importance of detecting and treating neonatal hypoglycemia is two-fold: First, hypoglycemia deprives the brain of its primary source of metabolic fuel and can lead to seizures and brain damage; and second, hypoglycemia is a presenting feature of numerous endocrine disorders and inborn errors of metabolism that require specific diagnosis and treatment.

The finding of hypoglycemia in a neonate mandates urgent investigation to define the cause, together with rapid restoration of physiologically optimal blood glucose levels. Neonatal hypoglycemia invariably reflects a failure of fasting adaptation; therefore, this chapter focuses on the metabolic and hormonal systems involved in fasting homeostasis and abnormalities of the fasting systems that result in hypoglycemia in the newborn period.

CHARACTERISTICS OF HYPOGLYCEMIA

Despite the frequency with which low blood glucose is encountered, considerable controversy surrounds the definition of hypoglycemia in the newborn. On the basis of the finding that low blood glucose levels are common in the first 12 to 24 hours of life, it has been argued that low blood glucose in the neonate represents a physiologic phenomenon[1-4] and that the newborn has unique physiologic adaptation to low blood glucose levels.[5,6] No evidence for this hypothesis, however, has been offered. Although transient hypoglycemia may occur during the first 12 to 24 hours in otherwise normal neonates[7] (particularly with a delay in feeding), it is essential to ensure that the condition does not persist. Hypoglycemia that does not respond to feeding, or that emerges after the first 24 hours of life, needs to be investigated promptly, to avoid overlooking endocrine disorders or inborn errors of metabolism with consequent exposure of the infant to the risk of cerebral damage.

Plasma glucose is determined by a balance between glucose supply and glucose utilization. In the newborn, a large proportion of glucose utilization is accounted for by cerebral metabolism.[8] Glucose transport into the brain occurs by a carrier-mediated facilitated diffusion process dependent on circulating arterial glucose concentration[9-11]; therefore a fall in the arterial glucose concentration places the infant at risk for neuroglycopenia. By depriving the brain of an important metabolic fuel, hypoglycemia can result in acute symptomatic cerebral dysfunction with manifestations that include lethargy, poor feeding, and seizures, among others, as listed in Box 53-1. These signs and symptoms are particularly common in circumstances in which alternative metabolic fuels such as lactate and ketone bodies are not available. However, many infants with low blood glucose may not exhibit obvious clinical signs. If the hypoglycemia is prolonged, permanent damage to the brain can occur. No universally accepted level or duration of hypoglycemia has been recognized to be associated with the risk of cerebral damage.[12,13]

PHYSIOLOGY OF FASTING GLUCOSE HOMEOSTASIS

Studies using radioisotope tracer techniques in fetal sheep[14] and in humans[15] have demonstrated that the fetal glucose supply is derived from the mother, with no endogenous glucose production in the fetus. The time of birth is marked by an abrupt change from a high-carbohydrate, low-fat diet to a high-fat, low-carbohydrate diet[16] The neonate must make the transition from an environment of continuous glucose supply from the placenta to one of intermittent periods of feeding and fasting.

The healthy neonate who is able to absorb nutrients from oral feedings uses glucose for metabolic processes, with storage of part of the surplus glucose in the form of glycogen in the liver and muscle and conversion of the remainder to fat, which is stored in adipose tissue. Hypoglycemia in the newborn occurs in the fasted state. Fasting hypoglycemia can result from developmental delays in the maturation of fasting metabolic systems, or it may be caused by endocrine disorders or inborn errors of intermediary metabolism. Five systems are required for successful fasting adaptation:

- Hepatic glycogenolysis
- Hepatic gluconeogenesis
- Adipose tissue lipolysis
- Fatty acid oxidation and hepatic ketogenesis
- Hormonal regulation of these systems

BOX 53-1

Signs and Symptoms of Hypoglycemia in Neonates

- Cyanotic episodes
- Apnea
- Respiratory distress
- Refusal to feed
- Myoclonic jerks
- Seizures
- Somnolence
- Staring spells
- Sweating

Glycogenolysis

Breakdown of glycogen stored in the liver occurs as soon as intestinal digestion of carbohydrate is exhausted, at 2 to 3 hours postprandially. Glycogenolysis results in the production of glucose-6-phosphate, which can be released as free glucose by the action of glucose-6-phosphatase. Muscle glycogen cannot be released into the circulation, because muscle cells lack glucose-6-phosphatase. Conditions that promote glycogenolysis are those associated with suppressed insulin levels or elevated glucagon and epinephrine levels. Liver glycogen accumulates in utero and is readily available to maintain plasma glucose in the immediate postnatal period. The stored glycogen is, however, depleted within 12 hours in a healthy term neonate, and more rapidly in a preterm or stressed neonate,[17] if there is no other source of glucose. Congenital defects in the enzymes of glycogenolysis are referred to as glycogen storage diseases (GSDs), and the one most likely to be associated with hypoglycemia is GSD type III.

Gluconeogenesis

During periods of fasting, the neonate is dependent on active gluconeogenesis to maintain plasma glucose levels. *Gluconeogenesis* is the production of glucose from precursors such as lactate, amino acids, and glycerol. This process requires four enzymes to bypass the unidirectional steps in glycolysis: glucose-6-phosphatase, fructose-1,6-bisphosphatase, phosphoenolpyruvate carboxykinase (PEP-CK), and pyruvate carboxylase. Most gluconeogenesis occurs in the liver, with a minor contribution from the kidney. Gluconeogenesis requires the provision of gluconeogenic substrates from peripheral tissues. The principal substrates for gluconeogenesis are lactate, alanine, and glutamine; during prolonged fasting, associated with increased adipose tissue lipolysis, glycerol becomes a major substrate for gluconeogenesis.

The normal newborn is not able to carry out gluconeogenesis in the first few hours of life.[18] Gluconeogenesis increases rapidly after birth in parallel with the activation of PEP-CK transcription, the rate-limiting enzyme of this pathway. The rise in plasma glucagon and the fall in plasma insulin that occur immediately after birth are the main determinants of liver PEP-CK induction. Levels of this enzyme in liver reach adult values approximately 24 hours after birth.

Adipose Tissue Lipolysis

The release of free fatty acids from adipose tissue occurs in physiologic conditions similar to those favoring gluconeogenesis (e.g., low plasma insulin, elevated plasma epinephrine and growth hormone). Plasma free fatty acid concentrations rise rapidly soon after birth. Although free fatty acids cannot be converted to glucose, they can be oxidized in place of glucose in heart and skeletal muscle. They are oxidized by the liver to produce ketones—acetoacetate and β-hydroxybutyrate (see later). By supplying an alternative source of fuel, fatty acid oxidation has a glucose-sparing effect.

Hepatic Ketogenesis

The liver produces the ketones β-hydroxybutyrate and acetoacetate as its major end products of fatty acid oxidation. These ketones are exported and metabolized by the brain,

TABLE 53-1

Hormonal Regulation of Fasting Metabolic Systems

Hormone	Hepatic Glycogenolysis	Hepatic Gluconeogenesis	Adipose Tissue Lipolysis	Hepatic Ketogenesis
Insulin	Inhibits	Inhibits	Inhibits	Inhibits
Glucagon	Stimulates	—	—	Stimulates
Cortisol	—	Stimulates	—	—
Growth hormone	—	—	Stimulates	—
Epinephrine	Stimulates	Stimulates	Stimulates	Stimulates

which cannot use fatty acids as a source of fuel. Infants born at a weight appropriate for gestational age (AGA) and those born small for gestational age (SGA) fail to show the increase in plasma ketone bodies during hypoglycemia that is observed in older children. This delay in development of hepatic ketogenesis probably is the result of delayed expression of two enzymes that catalyze the first and last steps in hepatic ketogenesis: carnitine palmitoyl-transferase-1 and β-hydroxy-β-methylglutaryl–coenzyme A synthase. Delayed expression of these enzymes has been demonstrated in animal models.[17] Fatty acids provided by the first feeding appear to play a critical role in activating transcription of key fatty acid oxidation and ketogenesis enzymes.[19-22]

Hormonal Regulation

A fall in blood glucose is the stimulus for a series of hormonal changes that act to restore blood glucose toward normal and to increase the supply of precursors for gluconeogenesis and alternative fuels.[23-25] The most important mechanism is the suppression of insulin secretion by hypoglycemia. The counterregulatory hormones become important if blood glucose levels continue to fall: Glucagon release is stimulated, followed by the release of epinephrine, cortisol,[26] and growth hormone.[27] The combined action of these hormone suppresses glucose utilization, stimulates hepatic glycogenolysis, activates gluconeogenesis, promotes adipose tissue lipolysis, and stimulates ketogenesis. These effects on carbohydrate metabolism serve to increase plasma glucose levels, and the effects on lipolysis and ketogenesis provide an alternative source of fuel for tissue metabolism. The overlapping effects of the counterregulatory hormones means that the loss of one can be partly compensated by other hormones that have a similar counterinsulin action.

At the time of birth, major changes occur in the concentrations of the hormones that regulate plasma glucose concentrations. An abrupt increase in glucagon concentration is observed within minutes to hours after birth.[8,28] This increase is accompanied by an increase in catecholamine concentrations and a fall in plasma insulin concentration. Together, these hormonal changes promote hepatic glycogenolysis and gluconeogenesis and mobilization of free fatty acids from adipose tissue. The hormonal regulation of fasting systems is summarized in Table 53-1.

TRANSIENT HYPOGLYCEMIA IN NEONATES

Delayed Development of Fasting Systems in Appropriate-for-Gestational-Age Term and Preterm Neonates

Physiologically normal AGA infants are highly susceptible to the development of hypoglycemia on the first day of life if feedings are delayed. For example, if the first feeding is not given until 6 to 8 hours after birth, 30% of term AGA neonates cannot maintain plasma glucose levels higher than 50 mg/dL, and in 10% to 15% of these infants, glucose values may drop to below 30 mg/dL. In normal newborn infants, the risk of hypoglycemia declines quickly after 12 to 24 hours, and by the second day of life, the frequency of blood glucose levels lower than 50 mg/dL drops to less than 0.5%.[7,29]

This susceptibility to transient hypoglycemia is associated with developmental lags in the capacity for both hepatic ketogenesis and hepatic gluconeogenesis in the newborn, as described earlier, and is aggravated in infants who have limited stores of glycogen.[30] (A classification of the various pathophysiologic mechanisms in neonatal hypoglycemia, both transient and persistent forms, is presented in Box 53-2.) Studies performed in the guinea pig and rat have shown delays for up to 12 hours after birth in expression of the gluconeogenic enzyme PEP-CK, as well as those enzymes mediating the first and last steps in the pathway of hepatic ketogenesis, carnitine palmitoyl-transferase-1 and β-hydroxy-β-methylglutaryl–coenzyme A synthase.[19,20,31,32]

Premature infants have an increased risk for hypoglycemia as a result of markedly reduced glycogen stores, in addition to the usual low activity of key gluconeogenic and glycolytic enzymes.[33] It has been shown that up to 20% of preterm infants who were about to be discharged home were at risk for hypoglycemia if a feeding was delayed.[33] SGA infants also have demonstrated similar mechanisms for the development of hypoglycemia (low glycogen stores in addition to usual delayed activation of fatty acid oxidation and induction PEP-CK).[34,35]

BOX 53-2

Classification of Hypoglycemia in the Newborn

Neonatal Transient Hypoglycemia
- Developmental lags in gluconeogenesis and ketogenesis
- Transient hyperinsulinism
  - Infant of diabetic mother
- Perinatal stress-induced hyperinsulinism
  - Small for gestational age
  - Discordant twin
  - Birth asphyxia
  - Infant of toxemic mother

Neonatal Persistent Hypoglycemia
- Hyperinsulinism
  - Potassium-ATP (K_{ATP}) channel hyperinsulinism (recessive, dominant, focal)
  - Glucokinase hyperinsulinism (dominant)
  - Glutamate dehydrogenase hyperinsulinism (dominant)
  - Short-chain L-3 hydroxyacyl–coenzyme A dehydrogenase deficiency (recessive)
  - Beckwith-Wiedemann syndrome
- Counterregulatory hormone deficiency
  - Panhypopituitarism
  - Isolated growth hormone deficiency
  - Cortisol deficiency
- Glycogenolysis disorders
  - Debrancher enzyme deficiency (small for gestational age)
- Gluconeogenesis disorders
  - Glucose-6-phosphatase deficiency (GSD type IA)
  - Glucose-6-phosphatase translocase deficiency (GSD type IB)
  - Fructose-1,6-diphosphatase deficiency
  - Pyruvate carboxylase deficiency
- Fatty acid oxidation disorders

ATP, Adenosine triphosphate; *GSD,* glycogen storage disease.

Hyperinsulinism in Birth Asphyxia and in Small-for-Gestational-Age Infants

Among SGA newborns and infants born in circumstances of perinatal stress, a high incidence of prolonged neonatal hypoglycemia has been reported. Up to 10% of SGA infants in one series continued to have hypoglycemia beyond the first week of life.[36] For reasons that are not clear, the incidence of hyperinsulinism, which may persist for weeks to months, is increased in these infants.[37-40] This perinatal stress-induced hyperinsulinism is responsive to diazoxide and is not related to genetic forms of hyperinsulinism such as mutations in the adenosine triphosphate (ATP)-dependent potassium (K_{ATP}) channel or glutamate dehydrogenase (GDH) enzyme.The specific pathomechanism for hyperinsulinism in SGA infants and in those with birth asphyxiaor erythroblastosis fetalis has yet to be elucidated.

Infants born to diabetic mothers have an increased incidence of hypoglycemia, which results from hyperinsulinism.[41] Fetal insulin secretion increases in response to maternal hyperglycemia; accordingly, the degree of fetal hyperinsulinemia correlates with maternal diabetic control during pregnancy.[40] The condition tends to be short-lived and usually resolves over the first few days of life. Deficiencies of the counterregulatory hormones, epinephrine and glucagon, also have been demonstrated in infants born to diabetic mothers, particularly women with poor control of their disease during pregnancy.[42,43]

CONGENITAL HYPOGLYCEMIC DISORDERS MANIFESTING IN THE NEONATE

Congenital Hyperinsulinism

The term *hyperinsulinism* is applied when hypoglycemia results from inadequate suppression of the plasma insulin. Hyperinsulinism in infancy usually is a congenital defect in the regulated release of insulin from pancreatic beta cells associated with inappropriate secretion of insulin. Both sporadic and familial genetic forms of the disease are recognized, with an incidence ranging from 1 in 50,000 births in Western Europe to 1 in 2500 births in societies with high rates of consanguinity.[44]

Congenital hyperinsulinism is both clinically and genetically heterogeneous. The clinical presentation ranges from extremely severe, life-threatening disease to very mild clinical symptoms, which may even be difficult to identify. Furthermore, clinical responsiveness to medical and surgical management is extremely variable and genotype-specific.[45] Early diagnosis and therapy are essential to prevent brain damage.[46]

The diagnosis of hyperinsulinism cannot rely solely on the measurement of plasma insulin levels at the time of hypoglycemia, because the insulin assays are not sufficiently sensitive in the low range to reliably distinguish a normal from an inadequately suppressed insulin level. Normally insulin concentrations are suppressed to values that are undetectable when plasma glucose levels fall to less than 65 mg/dL (less than 3 μU/mL for conventional assays). Thus an insulin level in the "normal" range when the blood glucose is 50 mg/dL or less is consistent with hyperinsulinism.

Therefore, in addition to plasma insulin measurements at the time of hypoglycemia, other markers of excessive insulin action should be sought, including demonstration of inappropriately low plasma ketones and free fatty acids and an inappropriately large glycemic response to glucagon.[47,48] At a plasma glucose level less than 50 mg/dL, evidence of hyperinsulinism includes the following: plasma insulin level greater than 3 μU/mL, plasma β-hydroxybutyrate less than 2 mmol/L, plasma free fatty acids less than 1.5 mmol/L, and occurrence of glycemic response to 1 mg of glucagon measured as a change in serum glucose greater than 30 mg/dL within 15 to 20 minutes.[49] These values reflect insulin suppression of adipose tissue lipolysis and ketone body production and preservation of glycogen stores despite falling plasma glucose levels.

The genetic basis of hyperinsulinism continues to be elucidated; mutations in five different genes have been identified. Most cases are caused by mutations in either of the two subunits of the beta cell adenosine triphosphate (ATP)–sensitive potassium channel (K_{ATP}), SUR1 (sulfonylurea receptor-1) and Kir 6.2 (inward-rectifying potassium channel protein 6.2).[50] Others are caused by mutations in the beta cell enzymes GDH and glucokinase and in short-chain L-3-hydroxyacyl–CoA dehydrogenase (SCHAD).[51-54] In as many as 50% of the cases, no genetic defect has been determined.[55]

Insulin Secretion

The regulation of insulin secretion is under the control of at least four mechanisms: (1) substrate-mediated mechanisms involving glucose, amino acids, and fatty acids; (2) neural control by the autonomic nervous system; (3) paracrine control by glucagon and somatostatin secreted within the pancreatic islets; and (4) endocrine control by intestinal incretins such as glucagon-like peptide (GLP-1). All of the currently known genetic forms of hyperinsulinism involve abnormalities in substrate-mediated pathways of insulin release (Figure 53-1).

The mechanism of insulin release that is best characterized is substrate-mediated insulin release by way of the K_{ATP} channel and the voltage-gated calcium channel. Glucose stimulates insulin release by metabolism to increase the ratio of ATP to adenosine diphosphate (ADP), leading to inhibition of the plasma membrane K_{ATP} channel, depolarization of the plasma membrane, activation of voltage-gated calcium channels, and influx of calcium to activate insulin release from storage granules.[56] An increase in the ATP-ADP ratio also can be generated by an increase in the oxidation of glutamate, a reaction catalyzed by the enzyme GDH. GDH activity is controlled allosterically by guanosine triphosphate (GTP) inhibition and is stimulated by ADP or leucine.[57,58] Activating mutations in the GDH gene causes the hyperinsulinism and hyperammonemia syndrome.

K_{ATP} Channel Hyperinsulinism

Recessive K_{ATP} Hyperinsulinism

In recessive K_{ATP} hyperinsulinism, previously referred to as nesidioblastosis, affected infants typically are large for gestational age (LGA), with severe hypoglycemia manifesting soon after birth. The genetic defect impairs either the SUR1 or Kir6.2 component of the K_{ATP} channel; most mutations discovered to date affect the SUR1 gene.[55,59,60] The genes for SUR1 *(ABCC8)* and Kir6.2 *(KCNJ11)* are located on the short arm of chromosome 11 (11p), immediately adjacent to one another.[61] Therapeutic interventions that act by way of the K_{ATP} channel, such as diazoxide, usually are ineffective. Thus many of these infants require near-total pancreatectomy.

Focal K_{ATP} Hyperinsulinism

In the focal form of hyperinsulinism, the area of abnormality is in a specific region of the pancreas; the characteristic histopathologic entity is described as focal adenomatous hyperplasia. The clinical presentation is indistinguishable from that in the recessive form of hyperinsulinism, described earlier, that involves the entire pancreas. The focal lesions are an area of beta cell adenomatosis caused by loss of heterozygosity for the maternal 11p15 region and expression of a paternally derived K_{ATP} channel mutation.[62-67] Approximately half of infants with hyperinsulinism requiring surgery may have focal lesions and are therefore potentially curable. Positron emission tomography (PET) scans using ^{18}F-fluoro-L-dihydroxyphenylalanine ([^{18}F]-dopa) have proved to be an accurate noninvasive technique to diagnose the condition and localize the focal lesion.[68-70]

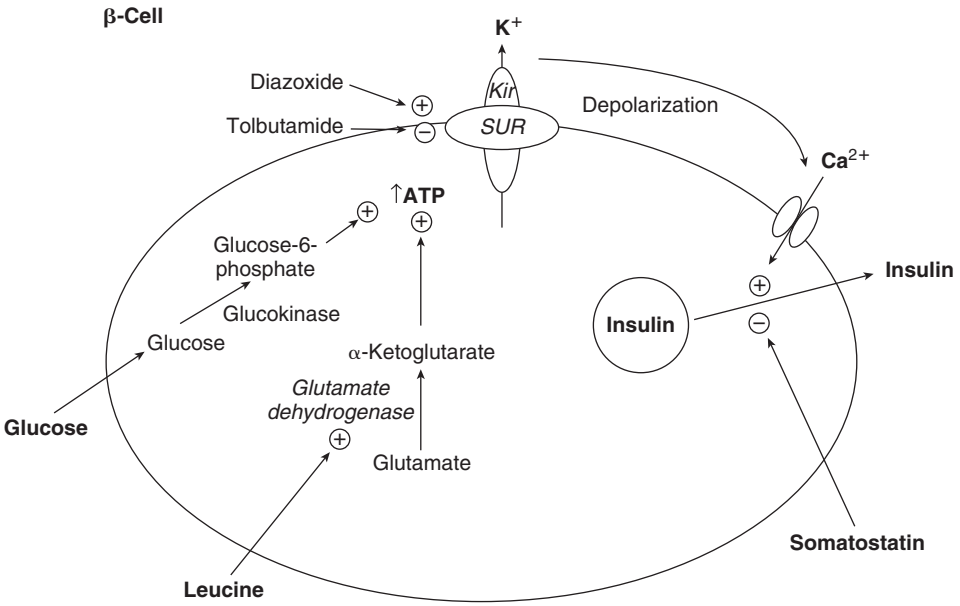


Figure 53-1 Pancreatic beta cell insulin secretion. Glucose stimulates insulin secretion through its oxidation, which increases levels of intracellular adenosine triphosphate (ATP), which in turn inhibits the potassium efflux through the potassium (K_{ATP}) channel (the sulfonylurea receptor [SUR]–inward-rectifying potassium channel [Kir] protein complex). Closure of the K_{ATP} channel depolarizes the plasma membrane, which activates the voltage-gated calcium channel (Ca^{2+}) to increase intracellular calcium and to trigger exocytosis of insulin granules. Leucine is an allosteric activator of glutamate dehydrogenase (GDH); increased activity of GDH increases glutamate oxidation, which also increases intracellular ATP. Diazoxide inhibits insulin release by preventing closure of the K_{ATP} channel. Tolbutamide stimulates insulin secretion by closing the K_{ATP} channel. Somatostatin decreases insulin release by downstream mechanisms.

Patients with the focal form have the best chance of successful treatment when managed at a center that can provide a multidisciplinary approach with a team including an endocrinologist, radiologist, pathologist, and surgeon.[71]

Dominant K_{ATP} Hyperinsulinism

Several families have been described with dominantly inherited congenital hyperinsulinism, associated with SUR1 and Kir 6.2 mutations.[72-74] Many of these patients have a less severe presentation than that typical for other forms of K_{ATP} hyperinsulinism, and all cases have been responsive to diazoxide.

Glutamate Dehydrogenase Hyperinsulinism

The hyperinsulinism and hyperammonemia syndrome has been reported as a cause of moderately severe hyperinsulinism with diffuse involvement of the pancreas.[75] GDH is a mitochondrial matrix enzyme that functions in the beta cell to mediate leucine-stimulated insulin secretion (see Figure 15-1). The mutations causing hyperinsulinism impair allosteric inhibition of GDH by GTP and result in uninhibited insulin release.[76-78] Clinical manifestations have included normal birth weight, late onset of hypoglycemia, diazoxide responsiveness, and protein-sensitive hypoglycemia.[77,79]

Glucokinase Hyperinsulinism

A less frequent form of hyperinsulinism, glucokinase hyperinsulinism (GK-HI), is caused by activating mutations in *GCK,* which encodes glucokinase.[80] GK-HI is inherited in an autosomal dominant manner, and five mutations have been identified to date.[81] In beta cells, glucokinase controls the rate-limiting step of glucose metabolism and is responsible for glucose-stimulated insulin secretion.[82] In GK-HI, activating mutations result in increased affinity of glucokinase for glucose, closure of K_{ATP} channels and inappropriate insulin secretion. The beta cell glucose threshold for glucose-stimulated insulin secretion in children with GK-HI can be as low as 27 mg/dL, much lower than the normal glucose threshold (90 mg/dL). The age at onset and severity of symptoms, as well as response to diazoxide treatment, vary markedly.[80,83-85]

SCHAD Hyperinsulinism

A mutation in *HADHSC,* the gene encoding the mitochondrial enzyme SCHAD, has been reported to be associated with hyperinsulinism.[51-54] SCHAD catalyzes the conversion of 3-hydroxyacyl-CoA to 3-ketoacyl-CoA, the third of four steps in the mitochondrial fatty acid oxidation spiral. SCHAD hyperinsulinism is inherited as an autosomal recessive trait, and patients have hypoketotic hypoglycemia with normal free fatty acids, 3-hydroxyglutaric acid in the urine, and raised plasma levels of 3-hydroxybutyryl carnitine and exhibit responsiveness to diazoxide. The clinical presentation of SCHAD hyperinsulinism is heterogeneous, ranging from late onset of mild hypoglycemia to severe onset of early hypoglycemia in the neonatal period. Patients do not have evidence of hepatic dysfunction, cardiomyopathy, or skeletal muscle involvement typically seen with fatty acid oxidation disorders.[54] The mechanism for increased insulin secretion in patients with SCHAD deficiency remains unknown but is thought to involve a K_{ATP} channel–independent pathway.[86]

Other Conditions with Hypoglycemia Resulting From Hyperinsulinism

Beckwith-Wiedemann syndrome is a congenital overgrowth syndrome associated with isodisomy for 11p that includes fetal overgrowth, hypoglycemia, and increased risk for some cancers. The incidence of hypoglycemia in affected children is approximately 50%.[87] The hypoglycemia can be transient but in approximately 5% of the cases can extend beyond the neonatal period, necessitating either continuous feeding, medical therapy, or in some

cases, partial pancreatectomy.[88,89] The cause of this hypoglycemia is unclear but may involve genes in the 11p region: either deficiency of K_{ATP} channels or excessive insulin-like growth factor-2 (IGF-2) activity.

Congenital disorders of glycosylation (CDGs) comprise a group of inherited multisystem disorders that are due to defects in the synthesis of the carbohydrate moiety of glycoproteins. In CDG type Ib, caused by phosphomannose isomerase (PMI) deficiency, clinical features include protein-losing enteropathy, liver disease, and hyperinsulinemic hypoglycemia. Mannose treatment bypasses the PMI deficiency and can be successful in correcting the clinical and biochemical abnormalities seen in affected patients.[90,91] Additional variants of CDG also have been found to be associated with hypoglycemia. These disorders can be detected by identifying the transferrin isoelectric focusing pattern.

Counterregulatory Hormone Deficiencies

Neonatal hypoglycemia occurring secondary to counterregulatory deficiency usually results from pituitary deficiency with various combinations of cortisol and growth hormone deficiency. The clinical presentation of neonates with panhypopituitarism can be identical to that seen in hyperinsulinism, requiring a high glucose infusion rate to maintain euglycemia, inappropriate hypoketonemia, and a positive glycemic response to glucagon at the time of hypoglycemia. Physical features such as micropenis or midline facial malformations such as cleft palate should suggest pituitary dysfunction as the cause of hypoglycemia.[92] Neonatal hypopituitarism sometimes is associated with cholestatic liver jaundice.[93] A low cortisiol or growth hormone value obtained at the time of hypoglycemia does not prove hormone deficiency. Therefore stimulation testing often is required to exclude pituitary insufficiency, because the critical sample may miss the peak hormonal response, thereby providing misleading results.

Defects in Gluconeogenesis

GSD IA, due to glucose-6-phosphatase deficiency, and GSD IB, secondary to glucose-6-phosphate translocase deficiency, result in a complete inability of the liver to release glucose into the circulation, because the formation of glucose from glucose-6-phosphate is the final step in both glycogenolysis and gluconeogenesis. Affected infants present with hypoglycemia, hepatomegaly, lactic acidemia, hyperuricemia, hypertriglyceridemia, and growth failure.

Deficiency of the enzyme fructose-1,6-diphosphatase blocks gluconeogenesis from precursors below the level of fructose-1,6-diphosphate (fructose, lactate, glycerol, alanine). The disease, which is inherited as an autosomal recessive trait,[94] can manifest in the neonatal period and can be fatal. Affected infants present with hypoglycemia, lactic acidemia, and hepatomegaly.[95] Hypoglycemia occurs later in this disorder and in pyruvate carboxylase deficiency (described next) than in G6Pase deficiency, because glycogenolysis is unimpaired.

Fasting hypoglycemia can occur in infants and children with deficiency of pyruvate carboxylase, the enzyme mediating the first step in gluconeogenesis.[96] Depending on the severity of the deficiency, the clinical presentation can range from devastating neurologic damage, lactic acidemia, hyperammonemia, and ketosis in early infancy to intermittent attacks of fasting-induced hypoglycemia accompanied by lactic acidemia in childhood.[97]

Deficiency of the last of the four gluconeogenic enzymes, PEP-CK, has not been convincingly demonstrated, despite several reported "cases."[98] Part of the reason for confusion in this area is that both mitochondrial and cytosolic enzymes with PEP-CK activity, encoded by two separate genes, are known to exist. True PEP-CK deficiency has yet to be described, but the clinical presentation is likely to include features similar to those of fructose-1,6-diphosphatase and pyruvate carboxylase deficiencies, such as lactic acidemia and hypoglycemia.

Glycogen Storage Diseases

GSDs are a group of disorders characterized by abnormal glycogen synthesis or degradation. Hypoglycemia is a feature of several but not all of these disorders. The two disorders in this group most likely to manifest in the neonatal period with hypoglycemia are GSD type I (glucose-6-phosphatase deficiency) and GSD type III (debrancher enzyme deficiency). GSD I is better classified as a disorder of gluconeogenesis (see earlier), because the enzyme catalyzes the final common step in glycogenolysis and gluconeogenesis.

Debrancher enzyme deficiency results in an inability to degrade stored glycogen. Inheritance is autosomal recessive, with more than 30 mutations reported. Clinical manifestations include hepatomegaly and hypoglycemia; skeletal myopathy and cardiomyopathy may develop in later childhood and adolescence in one third to two thirds of cases.

Fatty Acid Oxidation Defect

Fatty acid oxidation defect is the term applied to an error in the pathway of fatty acid mitochondrial oxidation. Such errors disturb oxidative phosphorylation and ATP generation from fat stores and result in loss of hepatic ketogenesis.[99] Episodes of illnesses are provoked by fasting and result in hypoglycemia associated with inappropriate hypoketonemia. The manifestations may be multisystemic, involving liver, heart, and skeletal muscle, leading to a Reye-like syndrome eventuating in coma and death.[100] The diagnosis of some but not all disorders of fatty acid oxidation can be made by mass spectrometry determination of the plasma acyl-carnitine profile.

Medium-chain acyl coenzyme A dehydrogenase (MCAD) deficiency is the most common fatty acid oxidation defect, with a frequency of 1 in 8000.[101,102] This disorder is now included in newborn screening protocols in many states. The gene for MCAD is located on chromosome 1 (1p31). An A985G point mutation causing a change from lysine to glutamate at position 304 in the mature MCAD protein has been found in 90% of patients with MCAD who were identified retrospectively,[103] although the frequency of this mutation may be lower among patients identified by neonatal screening.[104,105] The disorder is readily managed by avoidance of prolonged fasting.

Miscellaneous

Reactive hypoglycemia associated with late dumping syndrome is a frequent sequela of Nissen fundoplication. Treatment with acarbose to delay carbohydrate digestion often is helpful.[106] Finally, an atypical clinical presentation should raise the possibility of factitious hypoglycemia (Munchausen syndrome by proxy) secondary to surreptitious administration of insulin or insulin secretagogues. In such cases the infant will have elevated insulin levels with suppressed C-peptide values. However, some new insulin analogues do not demonstrate reactivity on the insulin assay.

SUMMARY

The occurrence of hypoglycemia in the neonate reflects failure of fasting adaptation. The endocrine and metabolic systems required during fasting and their normal developmental sequence are emphasized in this chapter. Neonatal hypoglycemia secondary to maturational delay in two of these fasting systems, gluconeogenesis and ketogenesis, is likely to occur if feeding is delayed after birth. Beyond 12 to 24 hours, neonatal

hypoglycemia requires specific diagnosis, to permit institution of appropriate treatment and to avoid adverse outcomes. Persistent neonatal hypoglycemia most commonly is the result of transient hyperinsulinism in infants with birth asphyxia, in SGA infants, and in infants of diabetic mothers. Genetic causes of persistent neonatal hypoglycemia such as congenital hyperinsulinism, hypopituitarism, and gluconeogenesis disorders also need to be considered. Inborn errors of metabolism often do not manifest as hypoglycemia in the newborn period; of those that do, fatty acid oxidation defects are the most common.

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SECTION VIII

Protein Metabolism

54

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General Concepts of Protein Metabolism

This chapter describes the methods used to evaluate dynamic changes in protein metabolism in the newborn infant. In a consideration of the relevant kinetics, the fundamental point of interest is that amino acids—the building blocks of protein—differ from carbohydrate and fat *only* by the inclusion of nitrogen. Alanine without its lone nitrogen atom is pyruvate, which is half a glucose molecule. It is the nitrogen atom or atoms in amino acids that set protein apart from carbohydrate and fat, and this distinction is the basis for investigation of amino acid and protein metabolism.

NITROGEN BALANCE

The oldest (and most widely used) method to follow changes in body protein is the nitrogen balance method, which has been the standard modality for defining minimum levels of dietary protein and essential amino acid intake in humans of all ages.[1-3] Of note, however, nitrogen balance defines only the difference between that going in and that coming out of the body. This determination is done by carefully recording all food consumed and collecting all material excreted: urine, feces, sputum, and so on. The nitrogen from aliquots of each food, urine, and fecal collection is tediously converted to ammonia by boiling the specimens in concentrated acid. The ammonia content is determined, and nitrogen intake and excretion are calculated. This simple nitrogen balance technique is fraught with technical difficulties: Nitrogen losses are routinely *underestimated* because of incomplete collections of urine and feces and insensible losses through skin, sweat, and so on, and nitrogen intake is routinely *overestimated* because of variable content or availability of protein in foods consumed, inaccurate recording of ingested amounts, and so on.[4,5]

Alternatively, the anthropometric measure of growth may be used to determine efficacy of the level and quality of protein in the diet of infants. Measurement of growth is an indication of well-being but gives no information specific to protein metabolism. Although measures of nitrogen balance and growth often have been used to study infant nutrition,[2,6] both methods require days or weeks to measure effects. Neither method provides any information concerning the turnover of nitrogen *within* the body. For example, an infant may be receiving adequate protein but insufficient energy intake for growth. Owing to this restricted intake, the child is not growing and has a nitrogen balance of zero. When more calories are provided, the infant starts growing and shows a positive nitrogen balance. How does the child's body respond to produce this effect? Nitrogen balance and growth measurements do not provide this information.

Figure 54-1 shows four possible responses to the original clinical situation. Case 0 is the starting zero nitrogen balance. Positive nitrogen balance could be obtained by increasing protein synthesis (case A in the figure), decreasing protein breakdown (case C), increasing or decreasing (cases B and D). The effect is a positive nitrogen balance for all four scenarios, but the energy implications are considerably different. Because protein synthesis costs energy, cases A and B are more "expensive," whereas cases C and D require less energy than in the starting scenario (case 0). Investigation of these possible mechanisms requires direct assessment of rates of protein breakdown, protein synthesis, and amino acid turnover within the body. Such assessment can be accomplished with the use of stable, isotopically labeled tracers.

MODELS FOR WHOLE-BODY AMINO ACID AND PROTEIN METABOLISM

The simplest approach to the study of amino acid and protein kinetics is to hypothesize a single, free pool of amino acid nitrogen, with *two inflows*—amino acid from dietary protein and amino acid released from protein breakdown—and *two outflows*—amino acid oxidation to end products (carbon dioxide [CO_2], urea, and ammonia) and amino acid uptake for protein synthesis (Figure 54-2). This model can be considered either for whole-body protein turnover, in which the pool is the total free amino acid pool (see Figure 54-2), or for the kinetics of a single amino acid, in which the pools are for a particular amino acid (illustrated for a ^{13}C tracer in Figure 54-3). The difference between the two approaches is related to how the system is viewed: looking at protein turnover, on the one hand, versus

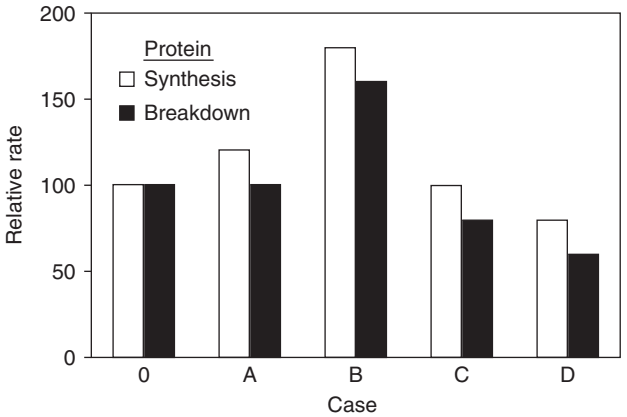


Figure 54-1 Four different hypothetical responses to a change from a zero balance (case *0*) to a positive nitrogen balance (cases *A to D*). A positive nitrogen balance can be obtained by increasing protein synthesis, as in case *A*; by increasing synthesis more than breakdown, as in case *B*; by decreasing breakdown, as in case *C*; or by decreasing breakdown more than synthesis, as in case *D*. The nitrogen balance method does not distinguish among any of the four possibilities.

looking at the kinetics of a specific amino acid from which inferences to whole-body protein turnover are drawn, on the other hand.

All protein in the body (structural, enzymes, and so on) is constantly being made and broken down. For most of the protein in the body (e.g., muscle protein), this turnover process occurs slowly, and can be considered to consist of a large amorphous inflow of amino acid from protein breakdown and a large amorphous uptake of amino acid from the fast-turnover free amino acid nitrogen pool. This system defines the traditional single-pool model of protein metabolism. The single pool is the free amino acid pool. Because of the size and slow turnover of the body protein pool, it is considered to be both a *sink* via extraction of amino acids for protein synthesis and a *source* for amino acid entry via protein breakdown. Whatever tracer enters this protein pool during the time course of the study is not likely to come out. Therefore, even though a box or circle may be used to depict this hypothetical compartment in diagrams of models for whole-body protein turnover, mathematically it is not considered to constitute a "pool" at all.

Lumping all body protein into a single entity is a gross oversimplification. Each of the many different tissues of the body is made up of a wide range of proteins with different turnover rates. Obviously, following the individual rates of hundreds of proteins would be impracticable. Because most of the important stores of nitrogen in the body turn over at similarly slow rates, it is possible to simplify the system into a conceptual model of clinical and experimental utility.

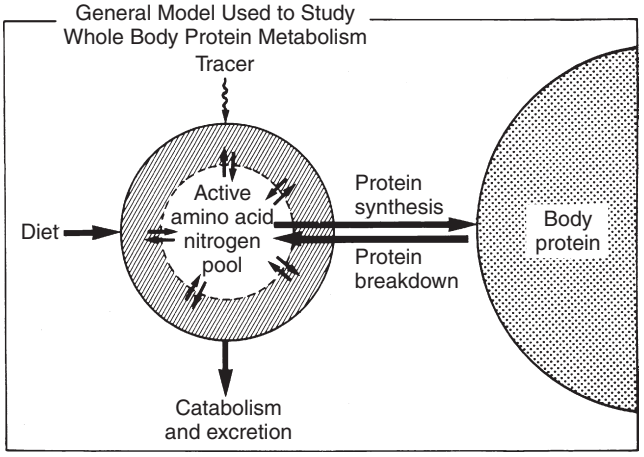


Figure 54-2 Single-pool amino acid model for whole-body protein metabolism. The model is applied without requiring definition of individual pools. All free amino acid nitrogen is lumped together. The *shaded outer circle* indicates interchange of free amino acids with various intracellular free amino acid pools and with incorporation into and release from faster-turnover proteins. Slower-turnover protein, such as muscle protein, appears as an exit from the system for free amino acid by way of protein synthesis and as a source of free amino acid entry by way of protein breakdown. Amino acids also leave the system by oxidation to the end products (carbon dioxide, urea, and ammonia) and can enter through absorption of dietary protein or amino acids. *(From Matthews DE: Stable isotope applications in human metabolism research. In Duncan WP, Susan AB, editors:* Synthesis and Applications of Isotopically Labeled Compounds, *Amsterdam, 1983, Elsevier, pp 279-284.)*

METHODS FOR MEASURING PROTEIN METABOLISM IN HUMANS

Box 54-1 lists the methods used to measure protein and nitrogen metabolism in humans. As already discussed, measurements of growth and nitrogen balance do not provide information on how protein balance is regulated. Changes in amino acid metabolism for a specific tissue can be inferred by measuring the difference between the amino acids delivered to the tissue (arterial blood levels) and the amino acids released from the tissue (venous blood levels). In adults, this technique has been applied to studies of forearm, leg, liver, kidney, and brain metabolism.[7-9] In animals and more recently in pregnant women, this model has been important in dissecting fetal and placental metabolism.[10,11] Measuring an arterial-venous difference, however, is similar to the nitrogen balance technique—it provides information about the balance across the tissue bed but tells nothing about the mechanism within the tissue affecting the balance. Most exciting is the combination of tracer infusion with the measurement of amino acid balance across the tissue bed. This technique allows for a complete solution of the various pathways operating in the tissue for each amino acid tracer used and can measure tissue-specific rates of protein synthesis and balance.[12-14] However, the use of arterial and venous catheters restricts the applicability of this method primarily to animal models.[10,15-21]

Only a limited number of investigations have addressed human fetal amino acid metabolism by infusing amino acid tracers into the mother before delivery and obtaining cord blood at delivery to determine fetal metabolism.[22] The only way to get to specific tissue amino acid metabolism in human neonates is with the simultaneous use of enteral and intravenous tracers. First-pass amino acid metabolism of the small intestine and liver is measured by comparison of the tracer enrichments of

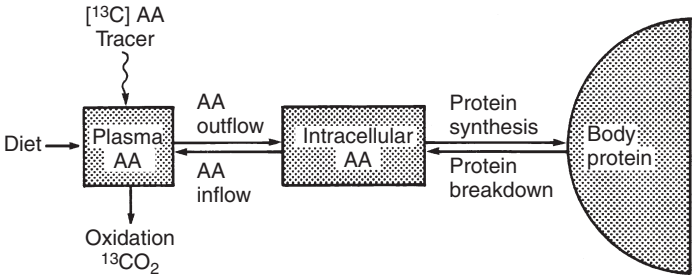


Figure 54-3 Simplified model for following the kinetics of an individual essential amino acid (AA) using a 13C label. The ^{13}C-labeled amino acid is infused intravenously. The tracer equilibrates with the free amino acid in plasma and in intracellular compartments. Amino acid is taken up from and released into the intracellular compartment through protein synthesis and breakdown. Amino acid oxidized intracellularly to form carbon dioxide (CO_2) is released into plasma as bicarbonate, and then into exhaled breath as CO_2. *(From Bier DM, Matthews DE, Young VR: Interpretation of amino acid kinetic studies in the context of whole-body protein metabolism. In Garrow JS, Halliday D, editors:* Substrate and Energy Metabolism in Man, *London, 1985, John Libbey, pp 27-36.)*

BOX 54-1

Methods for Measuring Protein and Nitrogen Metabolism in Humans

- Growth assessment
- Nitrogen balance method
- End product method
- Turnover of individual components
- Essential amino acids turnover (index of protein breakdown)
- Nonessential amino acids turnover (de novo synthesis and gluconeogenesis)
- Urea production (protein oxidation)
- Arteriovenous measurement of amino acids across a tissue bed
- Protein synthesis of a specific protein by following tracer incorporation
- Protein degradation of a specific protein by following disappearance of tracer from the protein

both tracers in plasma. The plasma enrichment of the enteral administered tracer will be lower than that of the intravenously infused tracer by the amount of material sequestered by the gut and liver on the first pass during enteral absorption of the tracer. The ratio between the enrichment of the intravenous administered tracer and that of the enteral administered tracer is used to calculate the first-pass uptake.[23]

End Product Approach to Measurement of Protein Metabolism

Because glycine is the only amino acid without an optically active α-carbon center, [^{15}N]glycine is the easiest labeled amino acid to synthesize and was the earliest tracer used for measuring protein turnover in the body. Using this tracer, Sprinson and Rittenberg[24] performed pioneering studies of protein metabolism based on a compartmental analysis of a single pool. As indicated in Figure 54-2, the free amino acid nitrogen pool is far too complex to be forced into a single kinetic pool when the metabolism of multiple amino acids is considered. The problem lies not in conceptualizing a single amino acid pool but in applying mathematical equations specific to a single pool. Therefore almost no progress was made in studying protein metabolism in humans from the time of the initial description by Sprinson and Rittenberg until 1969, when Picou and Taylor-Roberts[25] proposed a significant and simple alternative. Conceptually, the same single-pool approach was used, but no specific single-pool kinetic restriction was applied—that is, the system was viewed in stochastic fashion.[26] The [^{15}N]glycine tracer is given, and the ^{15}N-glycine mixes (scatters) among the free amino acids. The ^{15}N in the free amino acid pool is diluted with unlabeled amino acid entering from protein breakdown and from dietary intake. Using the rate of ^{15}N infusion and after measuring the dilution of ^{15}N in the free amino acid pool, the rate of unlabeled nitrogen appearance is readily calculated:

$$Q = 100 \bullet i/E$$

where Q is the free amino acid nitrogen pool turnover rate (typically expressed as mg nitrogen/kg/day), i is the rate of [^{15}N]glycine infusion (mg ^{15}N/kg/day), and E is the ^{15}N enrichment (dilution of ^{15}N by unlabeled nitrogen, expressed as *atom* or *mole % excess*). The 100 factor converts mole % to mole fraction. The dilution of the ^{15}N-glycine tracer (i.e., enrichment in the free amino acid nitrogen pool) is sampled indirectly via either the urea or ammonia end products, which are produced from the free amino acids. According to the standard precursor-product relationship,[26] a product (i.e., urinary urea or ammonia) formed from a single precursor will have an enrichment equal to the precursor (i.e., the free amino acid nitrogen pool).

The rate of whole body protein breakdown *(B)* is determined from the turnover *(Q)* by subtracting the known rate of dietary nitrogen intake *(I)*:

$$B = Q - I$$

In these calculations, the standard value of 6.25 g protein = 1 g nitrogen is used to interconvert protein and N. Attention to the units (g of *protein* versus g of *nitrogen*) is important, because both units often are used concurrently in the literature.

Like most of the kinetic methods, the end product method assumes steady-state conditions with respect to the free pool (i.e., the free nitrogen pool is neither expanding nor contracting). Obviously, the free pool does both, but over the period of most measurements, increases cancel decreases so that the steady-state assumption is reasonable. When *inflows* equal *outflows*,

$$Q = I + B = C + S$$

where the outflows are amino acid nitrogen oxidation *(C)* to end products urea and ammonia, and amino acid nitrogen uptake for protein synthesis *(S)*. Amino acid nitrogen oxidation is simply the sum of the ammonia and urea production rates, which are determined by urine collection. Therefore the rate of whole-body synthesis is

$$S = Q - C$$

Occasionally encountered in the literature[27] is the term *net protein balance* or *net protein gain,* which refers to the difference between the measured synthesis and breakdown rates ($S - B$). Rearranging the balance equation for Q shows that

$$S - B = I - C$$

and $I - C$ is simply the difference between intake and excretion, that is, *nitrogen balance*. Therefore the $S - B$ term is a misnomer in that it is based solely on the nitrogen balance measurement and not on the administration of the ^{15}N tracer.

The overwhelming advantage of the end product method of Picou and Taylor-Roberts[25] is that it can be completely noninvasive; accordingly, it became the cornerstone method for protein metabolic research in pediatrics. This method, however, is not without its problems.

As described by Picou and Taylor-Roberts,[25] the [^{15}N]glycine tracer is given orally at short intervals (e.g., every 3 hours) until a plateau for ^{15}N is reached in urinary urea. The time required to reach this plateau is approximately 60 hours, regardless of whether adults,[28] children, or infants[29-31] are studied. The delay in attaining a plateau results from the time required for the ^{15}N tracer to equilibrate within the free glycine, serine, and urea pools.[28,32] An additional problem is plateau definition. Often the urinary urea ^{15}N time course does not show, either on visual inspection or with curve-fitting regression, the anticipated single exponential rise to plateau.

Although [^{15}N]glycine tracer is the cheapest amino acid to purchase, any ^{15}N-labeled amino acid can be used in the end product method, but widely divergent results have been obtained when different tracers have been used.[33-35] Table 54-1 shows the values of protein turnover measured for a single adult subject receiving different ^{15}N-labeled amino acid and protein preparations on different days and highlights the differences in the metabolism of the different amino acid tracers and distribution of the ^{15}N in the free amino acid pool. Glycine, for example, rapidly transfers its ^{15}N to serine, glutamate, and glutamine.[28] That distribution is not changed with changing dietary protein intake.[32] Although it may be fortuitous that [^{15}N]glycine is the best label for use with the end

TABLE 54-1

Rates of Protein Turnover Determined on Different Occasions for the Same Patient Using the End Product Method but Different ^{15}N-Labeled Tracers*

	PROTEIN TURNOVER (g/kg/day)	
^{15}N Tracer	*From Urea*	*From Ammonia*
Glutamine	2.6	3.1
Alanine	2.8	2.6
Wheat protein	3.5	5.8
Glutamate	3.5	8.0
Glycine	4.5	4.8
Leucine	9.6	4.7
Lysine	11.8	21.7
Aspartate	17.8	7.5

Data from Fern EB, Garlick PJ, McNurlan MA, Waterlow JC: The excretion of isotope in urea and ammonia for estimating protein turnover in man with [^{15}N]glycine, *Clin Sci* 61:217-228, 1981.

*The whole-body protein turnover rate was calculated using urinary urea and urinary ammonia ^{15}N enrichments.

product method,[36] an important point is that a change in end product ^{15}N enrichment may be attributable either to a real change in protein turnover or to an artifactual change in the distribution of ^{15}N resulting from changes in glycine metabolism, which can be *independent* of changes in protein metabolism. Another clue to difficulties with the end product method is that the urinary ammonia ^{15}N enrichment usually is different from the urinary urea ^{15}N enrichment,[31,36,37] because the amino acid ^{15}N precursor for ammonia synthesis is of renal origin, whereas the amino acid ^{15}N precursor for urea synthesis is of hepatic origin. Of the two types of enrichment, probably the urea ^{15}N should be used, but no direct evidence for either one is available.

In two studies,[31,38] an interesting observation was reported: ^{15}N could not be measured in urinary urea of some preterm infants receiving [^{15}N]glycine, but ^{15}N was observed in urinary ammonia. These results suggest that glycine may be inadequate in the diet of the preterm infant to meet growth requirements.[38] This event could happen only if glycine synthesis was limiting, that is, if glycine was conditionally indispensable.[1,39] Alternatively, the amount of ^{15}N-glycine given could have been too low to produce detectable levels of ^{15}N greater than the natural abundance in urea. Other groups of investigators,[40,41] however, have had no difficulty measuring ^{15}N enrichment in urinary urea when [^{15}N]glycine was administered to infants or synthesis of glycine,[42] suggesting that glycine can be considered an essential amino acid for preterm and possibly SGA infants.

As with all methods, limitations are recognized. The best approach with the end product method is to design the experiment to minimize the obvious problems of changes in ^{15}N tracer distribution within the pool or changes in ammonia metabolism. The utility of the method is readily apparent from its application.[43] For example, Nissim and co-workers have used the end product method to study protein turnover as a function of conceptual age[44] (Figure 54-4). Energy is required for turnover (i.e., synthesis and breakdown) of protein in the body, and energy is required for accretion of new protein in the growing infant. Therefore growth is intimately tied to both energy and nitrogen intakes, and both must be considered.[6,27]

In view of all of the foregoing evidence, it seems reasonable to conclude that the potential pitfalls with using [^{15}N]glycine outweigh the benefits of using the end product method in infants to study protein metabolism.

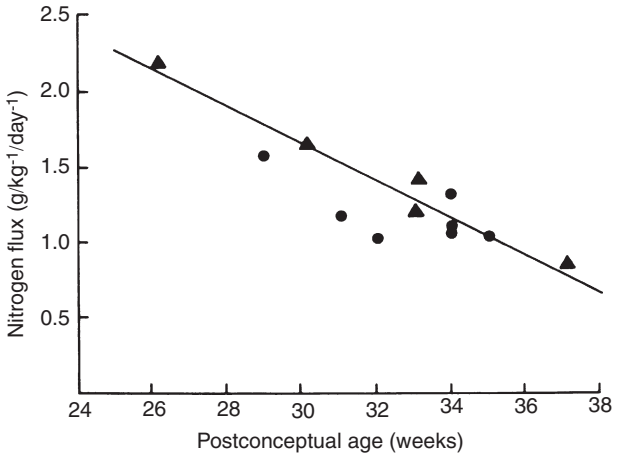


Figure 54-4 Whole-body nitrogen flux versus conceptual age measured using a single dose of [^{15}N]glycine given intravenously to eight infants, some studied twice. Triangles indicate five infants who received a diet of the same energy and protein content based upon infant body weight when studied. The significant correlation of whole body nitrogen flux in these infants further emphasizes that the correlation is indicative of postconceptual age, not level of dietary energy or protein intake. *(From Nissim I, Yudkoff M, Pereira G, Segal S: Effects of conceptual age and dietary intake on protein metabolism in premature infants,* J Pediatr Gastroenterol Nutr *2:507-516, 1983.)*

TURNOVER OF INDIVIDUAL COMPONENTS OF NITROGEN METABOLISM

Discussion up to now has considered the turnover of whole-body protein metabolism per se. Alternatively, the kinetics of individual components can be considered (see Figure 54-3). As outlined in Box 54-1, the interesting components are (1) the essential (or indispensable) amino acids, (2) the nonessential (or dispensable) amino acids, and (3) end products such as urea. Essential amino acid kinetics can be extrapolated to rates of protein metabolism. An essential amino acid enters the free pool from dietary intake (I_{aa}) and protein breakdown (B_{aa}); it disappears from the free pool by oxidation (C_{aa}) and uptake for protein synthesis (S_{aa}). These are the same components (*I, B, C,* and *S*) discussed previously, but cast in terms of the turnover, or *flux,* of a specific individual amino acid:

$$Q_{aa} = I_{aa} + B_{aa} = C_{aa} + S_{aa}$$

The turnover rate (or flux, Q_{aa}) of a metabolite is measured by the tracer dilution measured directly in the free pool. Typically, a tracer of an essential amino acid is infused until isotopic steady state (constant dilution) is reached in the blood free amino acid pool. Knowing the tracer enrichment and infusion rate and measuring the tracer dilution in blood from samples taken at plateau allow determination of the rate of unlabeled metabolite appearance (Q_{aa}):

$$Q_{aa} = i_{aa} \bullet [E_i / E_p - 1]$$

where i_{aa} is the infusion rate of tracer with enrichment E_i (mole % excess), and E_p is the plasma or blood amino acid enrichment.[45-47]

For a ^{13}C-labeled tracer, the amino acid oxidation rate can be measured from the rate of $^{13}CO_2$ excretion.[45,46] This approach assumes that the label is *quantitatively* released with the oxidation of the amino acid. For example, the ^{13}C of an L-[1-^{13}C] leucine tracer is removed at the first irreversible step of leucine catabolism[47] (Figure 54-5), and leucine oxidation is determined directly from $^{13}CO_2$ excretion. Even phenylalanine, for which the pathway to oxidation is not direct, has been used to define phenylalanine-tyrosine oxidation.[48-50]

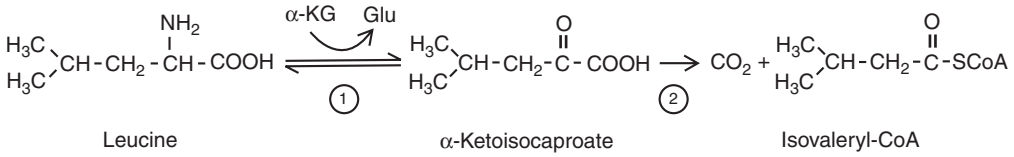


Figure 54-5 Leucine metabolism. *1,* Leucine is first transaminated to α-ketoisocaproate. This reaction is rapid and reversible. *2,* The ketoacid is then decarboxylated, releasing a carbon dioxide (CO_2) and an organic acid, which is further oxidized. The other two branched-chain amino acids, valine and isoleucine, also are metabolized by the same enzymes in these first two metabolic steps. *CoA,* Coenzyme A; *Glu,* glutamate; *KG,* ketoglutarate.

Determining an amino acid flux does not require a ^{13}C label per se. Any label (e.g., ^{15}N, ^{2}H, ^{18}O) can be used, but the caveat is that the label must follow the metabolic pathway expected. For example, a deuterium label in the tail of leucine or any ^{13}C label will trace leucine metabolism through the critical, irreversible second step of catabolism (see Figure 54-5), but an [^{15}N]leucine tracer is removed with transamination.[51] Because more than 70% of the leucine transaminated to α-ketoisocaproate (KIC) is immediately reaminated to leucine,[51] the ^{15}N label measures a leucine nitrogen turnover rate that is considerably faster than the leucine carbon flux. Therefore the ^{15}N-leucine tracer cannot be used to follow leucine carbon metabolism. For all metabolites to be studied, the biochemistry of the tracer must be carefully considered when choosing an isotopic label.

The rates of amino acid release from protein breakdown (B_{aa}) and uptake for protein synthesis (S_{aa}) are calculated by subtracting dietary intake and oxidation from the flux, respectively, just as is done with the end product method. The primary distinction is that the measurements are for a specific amino acid, not protein. Flux components typically are determined in units of μmol/kg/hour and then extrapolated to whole-body protein kinetics by dividing the amino acid rates by the assumed concentration of the amino acid in body protein (μmol of amino acid/g of protein).

The principal *advantages* of following individual metabolite kinetics are that (1) the results are specific, improving confidence in the measurement, and (2) the turnover time of the free pool usually is fast. With use of a priming dose to reduce the time required to come to isotopic steady state, the tracer infusion study can be completed in less than 4 hours.

The principal *disadvantages* are that (1) the method is invasive, (2) the true intracellular precursor pool usually is not sampled, and (3) dietary intake often is through a route different from that of the tracer. The amount of blood drawn is not an issue; blood loss can be kept below 1 mL total. However, placing catheters for sampling of blood amino acids or for tracer administration is almost unavoidable. Catheters may already be in place in the seriously ill infant, but invasive procedures are not warranted for studying healthy infants. Yet it is in the healthy infant that the tracer techniques offer the greatest tool for defining normal metabolism and nutritional requirements.

The bioavailability of dietary urea nitrogen has been determined in infants noninvasively by adding ^{15}N-urea tracer to the feeding solution and collecting and measuring the tracer in the urine.[52-54] The purpose of the studies using this technique was to determine what fraction of the urea nitrogen was retained in the body, rather than passing into the urine. Retention of urea nitrogen would reflect gut microorganism hydrolysis of the urea and incorporation of the resulting ammonia (presumably by the liver) into other nitrogen-containing compounds, including essential amino acids. This result has been demonstrated in both adults[55] and infants.[56] Because a significant portion of human milk nitrogen is urea nitrogen, urea nitrogen represents a potentially important nitrogen source. Because that the major portion of the urea ^{15}N consumed by infants is recovered in the urine, not much of the urea nitrogen will be bioavailable.

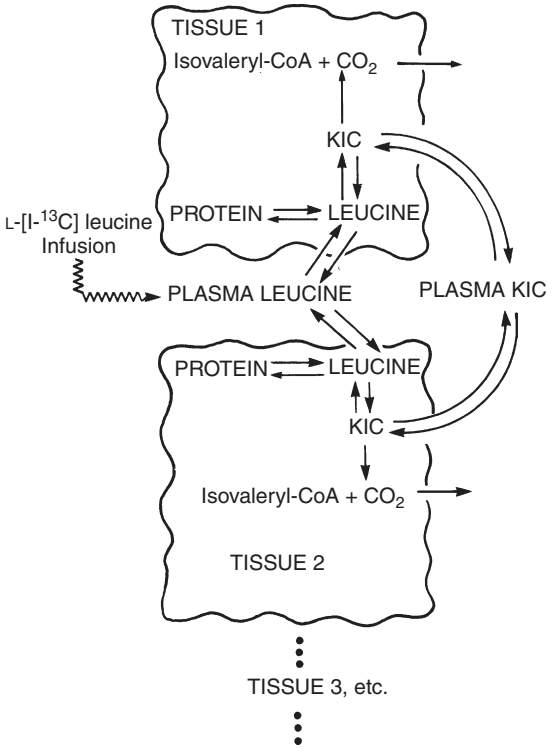


Figure 54-6 Multitissue model of whole-body leucine metabolism. Leucine is transported into and out of cells of various tissues in the body (e.g., muscle, kidney, liver, adipose tissue). α-Ketoisocaproate (KIC), formed within cells from leucine by transamination, is released into plasma. During an infusion of [1-^{13}C]leucine, the plasma KIC ^{13}C enrichment is the weighted average of the intracellular leucine ^{13}C enrichment of those tissues releasing KIC. *CO_2,* Carbon dioxide; *CoA,* coenzyme A. *(From Matthews DE, Schwarz HP, Yang RD, et al: Relationship of plasma leucine and alpha-ketoisocaproate during a L-[1-13C]leucine infusion in man: A method for measuring human intracellular leucine tracer enrichment,* Metabolism *31:1105-1112, 1982.)*

To measure amino acid kinetics, the amino acid tracer is infused into the blood and sampled from the blood, but the metabolic action takes place within cells. Amino acids do not freely pass through cells as does urea; amino acids are transported.[57,58] For the neutral amino acids, which are leucine, isoleucine, valine, phenylalanine, and tyrosine, transport into and out of cells is rapid, with only a small concentration gradient between plasma and intracellular milieus.[46] Still, the intracellular enrichment of leucine, as measured by plasma KIC, which is formed from intracellular leucine and then released into plasma (Figure 54-6), is approximately 20% lower than plasma leucine enrichment.[59] Using the plasma leucine enrichment to calculate leucine turnover underestimates whole-body flux by approximately 20%.

The plasma KIC enrichment can be used during the leucine tracer infusion to resolve the question of intracellular leucine enrichment for calculating leucine kinetics. For amino acids such as glutamate and glutamine, which have extremely large intracellular to extracellular gradients, very little intracellular glutamate or glutamine exchanges with plasma, and the sampled plasma amino acid enrichment reflects primarily interorgan transport of amino acid, rather than whole-body flux.[60] The partitioning of amino acid between intracellular and extracellular milieus must therefore be considered carefully for every individual amino acid studied.

Adults are commonly studied in the postabsorptive state after an overnight fast. The purpose usually has been to study the effect of mediators such as hormones or of specific interventions on protein metabolism. In the past several years, a few reports of amino acid metabolism using tracers in infants have appeared.[40,61-75] In terms of nutrition, many of the questions to be addressed in infants are related to oral or enteral feeding. However, the fed state complicates calculating kinetics more than by just adding a dietary intake term to the flux equation. Any dietary amino acid oxidized or taken up for protein synthesis by either gut or liver during absorption of food will not have mixed with the systemic circulation in which the tracer is administered. The flux of amino acid released from protein breakdown will therefore be underestimated by an amount equal to the amount of amino acid sequestered on the first pass through the splanchnic bed. It is possible under some circumstances to have a *negative* protein breakdown after dietary amino acid inflow has been subtracted from the flux. This problem is negated if the tracer is administered with the food, that is, orally or by intragastric tube feeding.[76] Oral feeding will not provide a constant flow of tracer into the system, however, unless tube feeding is used.[76]

Addition of the tracer to the enteral feeding solution solves the problem of following the metabolism of the enteral amino acids. An enteral tracer does not necessarily help, however, when the goal is to compare the effects of parenteral and enteral feeding regimens. For those kinds of studies, the tracer may be administered intravenously with the parenteral formula or enterally with the enteral formula. The use of two different administration routes for delivery of the amino acid tracer yields data that do not allow direct comparison—the proverbial apples and oranges.[40] A more elaborate design is required for comparing enteral and parenteral feeding regimens. For example, if two tracers of the same amino acid are available that give identical measures of metabolism (e.g., [1-^{13}C]leucine and [5,5,5-$^{2}H_3$]leucine), they can be administered simultaneously by both enteral and intravenous routes.[23,46] This approach gives direct measurement of tracers by both routes simultaneously during both parenteral and enteral feeding regimens.[15,69,77] More recent studies have shown that turnover rates may vary up to 80%, depending on the route of tracer.[78,79] Enterally administered tracer will yield a much higher turnover rate, reflecting first-pass removal by gastrointestinal tract and liver.

An alternative to invasive blood sampling is sampling amino acids from urine.[76] Small amounts of amino acids are continually filtered through kidney and normally appear in the urine. Because urinary amino acids are derived from blood, they should be a reasonable substitute for blood amino acids measured by means of tracer enrichment under isotopic steady-state conditions. De Benoist and colleagues[76] demonstrated in 1984 in that urinary leucine enrichments in infants were nearly identical to plasma leucine enrichments during infusion of [1-^{13}C] leucine, indicating that the urinary leucine came directly from plasma and was not contaminated significantly with unlabeled renal-derived leucine. Since then, several applications using this technique have been described in the literature.[40,72,80] In some commercial preparations of stable isotopically labeled L-amino acid tracers, however, D-amino acid tracer also will be present in variable amount. Because D-amino acids are not metabolized by the same pathways as for L-amino acids, but usually are removed by kidney and excreted in the urine, D-amino acid tracer may accumulate in urine, resulting in distortion of L-amino acid tracer measurements in urine. This problem can be avoided by using a chiral column to separate the D and L forms before measurement of amino acid tracer enrichment using the mass spectrometer.[81]

REQUIREMENTS FOR SPECIFIC AMINO ACIDS

Current dietary recommendations for essential amino acids are based on nitrogen balance studies and short-term growth studies. More recently, the indicator amino acid oxidation (IAAO) method has been developed to establish specific amino acid requirements.[82-84] It has been validated extensively in animal models of infancy.[85-87] This technique is based on the partitioning of essential amino acid outflow under steady-state conditions between oxidation and protein synthesis ($Q_{aa} = C_{aa} + S_{aa}$, as defined earlier). When a single essential amino acid is present in limited quantity in the diet, the amount of protein that can be synthesized likewise is curtailed. With an essential amino acid in limited supply, the use of all other dietary amino acids for protein synthesis also will be limited, and the body will have no choice but to oxidize the excess amounts of these amino acids. If the dietary amount of the limiting amino acid is increased, protein synthesis will increase, and so will the utilization of the other dietary amino acids—which in turn reduces their oxidation. Once the requirement for the limiting amino acid is reached, further increases in its dietary intake will cause no further increase in protein synthesis, nor decrease in the oxidation of the other essential amino acids.

The IAAO method works on this principle.[84,88] Subjects are given a series of diets containing various amounts of the amino acid for which the requirement is to be determined. The amounts vary below and above requirement. All other amino acids are furnished at constant amounts *above* requirement. At the end of each diet period, a dose of another essential amino acid with a ^{13}C or ^{14}C label (the indicator amino acid) is given, and its oxidation is measured. The oxidation of the labeled indicator amino acid will decrease as the amount of test amino acid increases, until requirement is reached, and then the oxidation will plateau. As shown in Figure 54-7, plotting the oxidation of the labeled indicator amino acid against test amino acid intake should show a breakpoint at the requirement level for the test amino acid. More recent studies have used the IAAO technique to determine sulfur (methionine and cysteine) amino acid requirements for neonates both in a piglet model[89,90] and in human infants.[91] Providing preterm infants with different intakes of cysteine while methionine (the sulfur donor of cysteine) and serine (the carbon donor of cysteine) were supplied in adequate amounts did not influence the oxidation rate of the indicator amino acid, suggesting that cysteine can be adequately synthesized from serine and methionine and is not conditionally essential.[91]

A different indirect technique yielded similar results, demonstrating that glucose carbon (labeled with ^{13}C) is incorporated into cysteine. This finding indicates that cysteine synthesis is not limited by serine availibility.[92]

FREE, EXTRINSICALLY OR INTRINSICALLY LABELED AMINO ACIDS

Almost all tracer studies have been performed using labeled amino acids as *free* amino acids, rather than amino acid tracers incorporated into proteins. When amino acids and proteins are given enterally, differences in the time course of hydrolysis and absorption of the amino acids and small peptides have been documented.[93] Although it takes time to hydrolyze enterally delivered proteins to peptides, peptides are absorbed more rapidly than free amino acids.[94] The net effect is that free amino

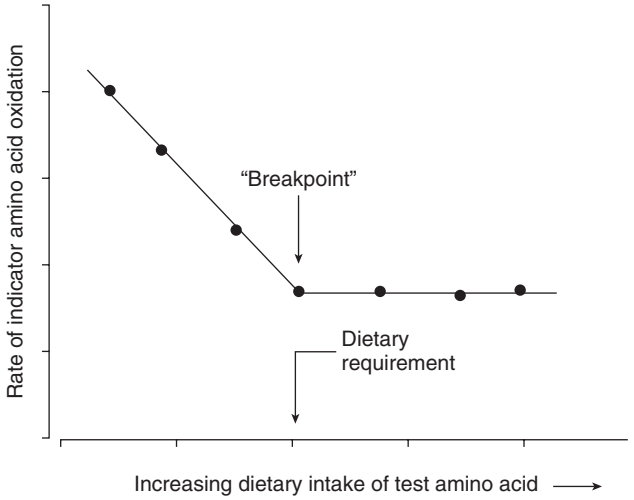


Figure 54-7 The rate of oxidation of an indicator amino acid in response to various dietary intakes of a test amino acid. The inflection or breakpoint in the rate of indicator oxidation represents the physiologic requirement of the test amino acid for the individual patient. *(From Brunton JA, Ball RO, Pencharz PB: Determination of amino acid requirements by indicator amino acid oxidation: Applications in health and disease,* Curr Opin Clin Nutr Metab Care *1:449-453, 1998.)*

acids still appear in systemic blood more quickly than amino acids arising from intact proteins, but that different proteins are hydrolyzed at different rates. Thus an enterally delivered labeled free amino acid will have different absorption kinetics from those for the corresponding protein-bound amino acids it is meant to trace.

Metges and co-workers[95] measured the prandial metabolic fate of ^{13}C-labeled dietary leucine when it was ingested as an intrinsically labeled component of mixed meals either incorporated into casein protein or as a free amino acid (extrinsically labeled) in a mixture of crystalline free amino acids defined to simulate the casein amino acid pattern. To serve as control data, leucine kinetics also were measured when free labeled leucine was given together with the intact casein. Leucine oxidation was higher for the free ^{13}C-leucine tracer combined with a free amino acid mixture than for an intrinsically ^{13}C-leucine–labeled casein. This result, together with the finding of a higher uptake of leucine for protein synthesis with the intrinsically labeled casein, suggests that protein-bound leucine was better utilized for whole-body protein synthesis than as a free amino acid. Such studies have not yet been performed in neonates but may prove to be important in defining infant formulas in terms of amino acid and protein composition.

SYNTHESIS OF SPECIFIC PROTEINS

Some proteins are readily sampled. Lipoproteins, albumin, and other plasma proteins are obtained from blood; muscle can be obtained by biopsy. If a protein or group of proteins can be sampled, the synthesis rate can be determined directly from the rate of tracer incorporation into the protein. For proteins with slow turnover (e.g., muscle protein or albumin), incorporation of tracer is linear with time during the first several hours of tracer infusion.[30,96-98] If the tracer infusion was continued for several half-lives of turnover, the tracer concentration in the protein would rise exponentially to match that of its precursor, the intracellular amino acid enrichment.[99,100] For proteins with slower turnover, then, it is more convenient to measure the synthesis rate from the initial rate of tracer incorporation into the protein.

To convert the initial rate of tracer incorporation in protein into a protein synthesis rate requires knowledge of the intracellular amino acid precursor enrichment for synthesis. For muscle protein synthesis, L-[1-^{13}C]leucine often is used as the tracer,[101] because plasma KIC ^{13}C enrichment approximates the intracellular muscle leucine enrichment. Various other schemes have been used to estimate intracellular liver amino acid tracer enrichment.[102-104]

For proteins such as very-low-density lipoprotein apolipoprotein B for which turnover occurs within the period of tracer infusion (typically 4 to 8 hours), tracer incorporation into the protein rises exponentially to reach a plateau. If the tracer enrichment in the protein does not reach plateau during the time course of the tracer infusion, curve fitting usually can predict the plateau. If the enrichment plateau can be defined, then the precursor enrichment (the enrichment of the intracellular free amino acid from which the protein was made) will now have been measured directly.[102] Several groups of investigators have used plateau enrichment of tracers in apolipoprotein B as a surrogate measure of intracellular hepatic tracer enrichment in both a piglet model[17,105,106] and human infants.[92]

Garlick and colleagues have proposed an alternative method to measure protein synthesis of slow turnover proteins.[107] Their method is to administer a "flooding dose" of tracer amino acid, producing a large momentary concentration gradient between the extracellular and intracellular spaces. The gradient pushes the amino acid rapidly into cells, "flooding" them with the tracer. This scheme is meant to force the intracellular tracer enrichment (which cannot be readily measured) to equalize with the extracellular enrichment (which can be measured), thereby removing the uncertainty of what is the precursor enrichment for protein synthesis.[108-112] An obvious drawback is that the administered dose is a *pharmacologic* dose of material that may alter metabolism and induce secretion of various hormones (e.g., insulin) and factors that are known to regulate protein metabolism. Nevertheless, the flooding dose approach is a convenient method for determining protein synthesis of slow-turnover proteins, which can be sampled readily in a short period of time.

DEGRADATION OF SPECIFIC PROTEINS

Measurement of protein degradation is much more difficult. It usually requires prelabeling a protein and following its disappearance over time. The first problem arises with prelabeling the protein. Prelabeling can be done for plasma proteins using radioactive iodine. When the protein degrades, the radioactive iodine is no longer present in the system. If the protein is labeled by synthesizing it with one or more labeled amino acids, a second problem emerges in relation to recycling of amino acid tracer. As the protein degrades, the newly released amino acids can again be taken up for new protein synthesis; that is, some of the amino acids are *reutilized* or recycled back into protein. This recycling greatly complicates interpretation of the labeled protein data.

A few amino acids cannot be reutilized for protein synthesis. Hydroxyproline, methyl lysine, and 3-methylhistidine are examples. No transfer ribonucleic acid exists for these amino acids. Proline is hydroxylated and histidine and lysine are methylated after the protein has been synthesized. This posttranslational process occurs in very specific proteins—for example, collagen for hydroxyproline and actin and myosin for 3-methylhistidine. When the proteins degrade, these modified amino acids are not reutilized. Hydroxyproline has been used to follow collagen kinetics,[113] and 3-methylhistidine has been used extensively to follow myofibrillar protein breakdown.[114,115] Because most of the myofibrillar protein is in muscle, muscle should be the principal source of urinary 3-methylhistidine. However,

3-methylhistidine also is present in gut actin. Although the gut actin pool is very small relative to muscle protein, protein turnover within the gut is rapid and therefore can be a significant contributor to urinary 3-methylhistidine output. Assay of urinary 3-methylhistidine to reflect muscle protein breakdown requires cautious interpretation because of the gut 3-methylhistidine source.[116]

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Fetal Requirements and Placental Transfer of Nitrogenous Compounds

INTRAUTERINE GROWTH AND PROTEIN (NITROGEN) ACCRETION

Nutritional requirements for fetal protein accretion depend directly on the rate of fetal growth. Because direct measurements of fetal growth are not possible, fetal growth rates have been estimated by inference from anthropometric measurements of infants born at different gestational ages and chemical composition studies of infants who died close to birth but appeared normally grown at autopsy. The better of these studies have excluded obviously abnormal infants and have involved defined, relatively homogeneous populations. Even with such precautions, however, there has been no assurance that infants born preterm had grown normally, making suspect whether their weights represent those of normally growing fetuses. Furthermore, anthropometric measurements have their own inherent range of accuracy, and cross-sectional, static size-for-gestational-age groupings of neonates may not accurately reflect the dynamics of fetal growth. In addition, population means of fetal growth rate do not necessarily reflect growth of a given fetus or the changes in body composition with growth that occurs with advancing gestational age.[1]

Despite such concerns about the reliability of data, most composite evaluations of human fetal growth based on neonatal anthropometric measurements at birth are relatively similar. Most mean percentiles among the many different growth curves differ by ± 5% or less and can be accounted for primarily by factors such as suboptimal pregnancy dating, adverse maternal and/or fetal medical and obstetric complications of pregnancy, and variations in maternal race, ethnicity, socioeconomic status, altitude, and diet.[2] Such neonatal-derived fetal weight curves appear as sigmoidal functions of weight versus gestational age. In some studies that accounted for many of the factors that can affect fetal growth and fetal size, fetal weight gain appears to be a linear function of gestational age through to term (12 to 13 g per day for white race, middle-class pregnancies), with only slight increases for male versus female gender and for maternal obesity.[3] In contrast, weight change (the rate of weight gain per kg body weight per day) is relatively linear at 1.5% per day from 24 to 37 weeks but tapers off to a plateau between 37 and 42 weeks, and a decrease may occur after 43 to 44 weeks.

Chemical composition studies of allegedly normal human infants have been accomplished in relatively few cases. Sparks[1] reviewed data from 15 studies with a combined 207 infants. Based on the data from these studies, nonfat dry weight and nitrogen content (both reasonably good predictors of protein content) show a linear relationship with fetal weight (Figure 55-1) and an exponential relationship with gestational age (Figures 55-1 and 55-2).[1] However, at each gestational age, a variety of fetal weights is observed. Thus nonfat dry weight and nitrogen content for a given fetus can also be compared with the "average" fetus. When these comparisons are made, larger fetuses grow faster than smaller fetuses at the same gestational age; protein accretion and therefore protein nutritional requirements follow accordingly.

Tables 55-1 and 55-2 present nitrogen, protein, and selected amino acid composition and accretion rates, respectively, in reportedly normal human fetuses.[4] According to data from sheep and guinea pigs, however, 80% of the nitrogen content of the fetus in these species is found in protein; the remainder largely occurs in urea, ammonia, and free amino acids. The data for human fetal protein content and accretion in these tables thus may be high because they are based solely on nitrogen content. Additional nitrogen requirements for urea excretion (and for other possible nitrogen excretion products) are not known for human fetuses. An analysis of complete nitrogen balance of this sort has been accomplished only in fetal sheep and is discussed later. Several items of comparative chemical and physical growth in six species are summarized

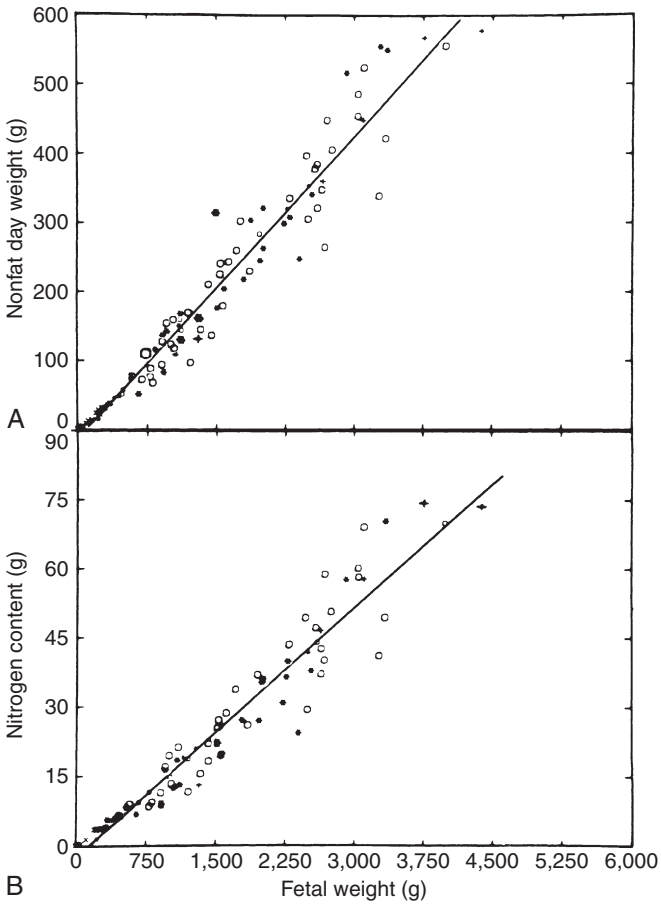


Figure 55-1 **A,** Relationship of nonfat dry weight (NFDW) to fetal weight in 169 human fetuses. *, Small-for-gestational-age (SGA) infants; +, large for gestational age (LGA) infants; ○, appropriate for gestational age (AFA) infants. NFDW = 0.0589 g. **B,** Relationship of nitrogen (N) content to fetal weight in the same infants. N = 0.00665 g. *(From Sparks JW: Human intrauterine growth and nutrient accretion,* Semin Perinatol *8:74, 1984.)*

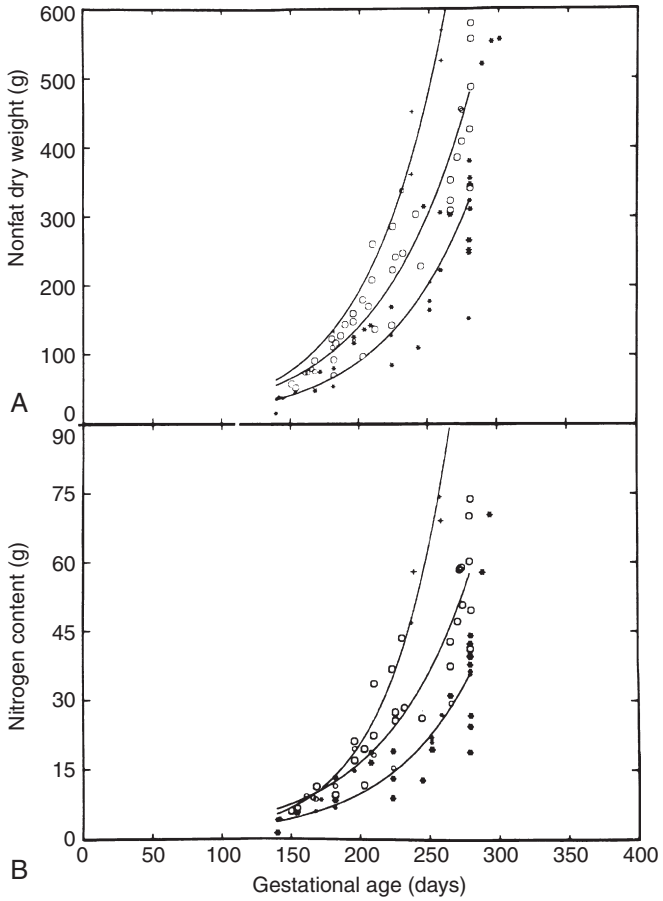


Figure 55-2 ***A,*** Relationship of nonfat dry weight (NFDW) to gestational age in 97 human fetuses (see Figure 55-1 for explanation of symbols). Increase in NFDW:LGA-NFDW (g) = $4.78 \cdot e^{(0.0184 \cdot GA)}$, r = 0.9869; AGA-NFDW (g) = $6.38 \cdot e^{(0.01548 \cdot GA)}$, r = 0.9511; SGA-NFDW (g) = $3.60 \cdot e^{(0.0161 \cdot GA)}$, r = 0.9380. ***B,*** Relationship of nitrogen content to gestational age in the same infants. Increase in total body nitrogen (N); LGA-N (g) = $0.227 \cdot e^{(0.0225 \cdot GA)}$, r = 0.9870; AGA-N (g) = $0.703 \cdot e^{(0.0157 \cdot GA)}$, r = 0.9385; SGA-N (g) = $0.377 \cdot e^{(0.0163 \cdot GA)}$, r = 0.9351. *(From Sparks JW: Human intrauterine growth and nutrient accretion,* Semin Perinatol *8:74, 1984.)*

in Table 55-3.[5] The variation among certain parameters is considerable; for example, growth rate variation is 20-fold and weight-specific content of fat at term varies 16-fold. In contrast nonfat dry weight and protein weight specific contents (as percentage of total weight) at term are constant. Limited chemical analyses at different gestational ages indicate that in other animals, as in the human, fetal protein content is linearly related to fetal weight and, therefore, fetal protein requirements are linearly related to fetal weight gain. Species-related differences in fetal weight gain are remarkable; for example, protein accretion in the fetal rat occurs approximately 23 times as fast as it does in humans. The rat must possess a placental amino acid transport system with enormous capacity to support this high protein accretion rate, as well as mechanisms to process and support the high fetal protein synthetic rate.

PLACENTAL AMINO ACID SUPPLY: PLACENTAL TRANSPORT SYSTEMS

Placental amino acid supply involves active, energy-dependent transport of amino acids through molecular transporter protein systems across the placental membranes. The transport of

TABLE 55-1

Body Composition of the Human Fetus

Fetal Age (wk)	12	16	20	24	28	32	36	40
Weight (kg)	**0.02**	**0.1**	**0.3**	**0.75**	**1.35**	**2.0**	**2.7**	**3.4**
Total Nitrogen (N) and Protein in Fetal Body								
Total N (g)	0.18	1.0	3.6	10.4	19.6	30.2	44.3	64.3
Protein (g) (N × 6.25)	1.1	6.3	22.5	65	123	189	227	402
Content of Individual Amino Acids in Fetal Body (g)								
ILE	0.04	0.22	0.77	2.3	4.2	6.5	9.6	13.7
LEU	0.08	0.46	1.7	4.8	9.0	13.9	20.3	29.6
LYS	0.08	0.44	1.6	4.6	8.6	13.3	19.5	28.3
MET	0.02	0.12	0.42	1.2	2.3	3.5	5.2	7.8
PHE	0.05	0.25	0.91	2.6	4.9	7.6	11.1	16.3
TYR	0.04	0.18	0.65	1.9	3.5	5.4	8.0	11.5
THR	0.05	0.25	0.91	2.6	4.9	7.6	11.1	16.3
VAL	0.05	0.28	1.0	3.0	5.6	8.7	12.8	18.6
ARG	0.08	0.47	1.7	4.9	9.2	14.1	20.7	30.3
HIS	0.03	0.16	0.59	1.7	3.2	4.9	7.2	10.4
ALA	0.08	0.44	1.6	4.6	8.6	13.3	19.5	28.5
ASP	0.10	0.56	2.0	5.8	10.9	16.8	24.7	35.7
GLU	0.14	0.80	2.9	8.3	15.7	24.2	35.5	51.4
GLY	0.14	0.73	2.6	7.6	14.3	22.0	32.3	46.6
PRO	0.09	0.51	1.8	5.3	10.1	15.5	22.7	33.2
SER	0.05	0.27	0.97	2.8	5.3	8.2	12.0	17.3

From Widdowson EM: In Aebi H, Whitehead R (eds): *Maternal Nutrition During Pregnancy and Lactation*. Beme, Switzerland, Hans Huber, 1980, pp 39-48.

TABLE 55-2

Increments per Day of Nutrients in the Fetal Body at Selected Intervals During Gestation

Fetal Age Range (wk)	12-16	16-20	20-24	24-28	28-32	32-36	36-40
Weight Range (kg)	**0.02-0.1**	**0.1-0.3**	**0.3-0.75**	**0.75-1.35**	**1.35-2.0**	**2.0-2.7**	**2.7-3.4**
Increments of Nitrogen (N) and Protein in Fetal Body/24 hr							
Total N	29	93	243	326	386	504	714
Protein (g) (Nx6.25)	0.18	0.58	1.52	2.04	2.41	3.15	4.46
Increments of Individual Acids in Fetal Body (mg/day)							
ILE	6	26	53	71	82	109	148
LEU	13	43	111	151	174	231	330
LYS	13	41	107	145	167	222	313
MET	4	11	28	39	44	59	92
PHE	7	23	61	83	95	127	184
TYR	5	17	44	59	68	91	127
THR	7	23	61	83	95	127	184
ARG	14	43	114	154	177	236	340
HIS	5	15	39	53	61	81	112
ALA	13	41	107	145	167	222	319
ASP	17	52	136	183	211	281	392
GLU	23	74	195	263	303	403	568
GLY	21	68	177	240	276	367	513
PRO	15	48	125	168	194	258	300
SER	5	25	66	89	102	136	191

From Widdowson EM: Chemical composition and nutritional needs of the fetus at different stages of gestation. In Aebi H, Whitehead R (ed): *Maternal Nutrition During Pregnancy and Lactation*. Beme, Switzerland, 1980, Hans Huber, pp 39-48.

TABLE 55-3

Growth Characteristics and Chemical Composition at Term of Selected Mammals and a Representative Human Fetus

Human	Monkey	Sheep	Guinea	Pig	Rabbit	Rat
Gestation (days)	280	163	147	67	30	21.5
Number of fetuses	1	1	1	3-5	4-6	10-12
Growth rate (g/kg/day)	15	44	60	70	300	350
Fetal weight (g)	3500	500	4000	100	60	5
Dry weight (g/%)	1050/30	125/25	760/19	25/25	9/15	0.2/4
Nonfat dry weight (g/%)	490/14	—	640/16	14/14	—	—
Protein (g/%)	490/12	—	480/12	12/12	7.2/12	0.6/12

From McCrance RA, Widdowson EM: Glimpses of comparative growth and development. In Falkner F, Tanner JM (eds): *Human Growth*, 2nd ed. New York, Plenum Press, 1985, p 139.

amino acids is altered qualitatively and quantitatively by transport system location and activity, competition among amino acids, placental metabolism of amino acids, and relative concentrations of the amino acids in maternal and fetal plasma, as well as by changes in the overall size of the placenta, architecture of the placental tissues, and developmental and pathologic changes in placental transport capacity. In addition, various aspects of placental-fetal amino acid exchange can be affected by specific metabolism within certain fetal organs and tissues, demonstrating a unity of function of the conceptus.

For most (but not all) of the amino acids, transport from maternal to fetal plasma occurs against a concentration gradient among all mammalian species studied and involves energy-dependent transport mechanisms.[6-17] However, quantitative differences exist among species. For example, the fetal/maternal ratio in humans for the basic amino acids lysine and histidine is greater than 1.0, whereas in sheep the ratio is less than 1.0. Even for amino acids with a fetal/maternal concentration ratio of less than 1.0, overall transport may be energy dependent because the concentration of a specific amino acid (e.g., citrulline) within the trophoblast tissue is greater than in the maternal plasma.[12,13,18] This is especially true for the very high intracellular trophoblast concentrations of taurine, glutamate, and aspartate, as well as for moderately increased intracellular concentrations of alanine, glycine, serine, glutamine, and threonine in the trophoblast.[16] For most amino acids, placental transport is sufficiently rapid that despite high concentrations of the amino acids in the trophoblast and the requirement for energy to promote amino acid uptake and transport, maternal concentrations of at least some amino acids can affect transport. This has been shown primarily for certain essential amino acids (e.g., leucine[6,19] and lysine[9]), providing reasonable evidence for how maternal diet and metabolic condition that determine maternal plasma amino acid concentrations directly affect fetal amino acid supply.

The syncytial epithelium of the human placenta, the *syncytiotrophoblast,* is a polarized multinucleate epithelium. The maternal-facing, apical surface of this epithelium has a microvillous plasma membrane; the basal surface facing the fetus is a relatively smooth membrane. The transport of amino acids across the syncytiotrophoblast involves three steps: (1) uptake from the maternal circulation across the microvillous membrane; (2) movement through the trophoblast cytoplasm; (3) transport out of the trophoblast, across the basal membrane into the umbilical circulation. Transport systems are required at

TABLE 55-4

Placental Amino Acid Transport Systems, Substrates, Conditions and Location

System	Substrates	Conditions	Membrane
Sodium Dependent			
A	Neutral amino acids: alanine, glycine, serine, proline, threonine, glutamine, MeAIB	Na^+ dependent, slowed by extracellular H^+ Excludes anionic and cationic amino acids, BCH, and leucine	M, F
ASC	Alanine, serine, cysteine, anionic (acidic) amino acids	Na^+ dependent, slowed by extracellular H^+ Excludes MeAIB, cationic aminoi acids, and proline	F
N	Glutamine, histidine, asparagine	Na^+ dependent Excludes cysteine, MeAIB, transstimulated	M
X-AG	Anionic (acidic) amino acids, glutamate, aspartate	Na^+ dependent Uptake from fetal blood; noncompetitive with other amino acids Excludes nonanionic amino acids	M, F
β	Taurine	Na^+, CI^- dependent Highest trophoblast intracellular concentration of all amino acids Excludes α-amino acids	M
B^o	Neutral amino acids	Excludes cationic and anionic amino acids	M, F
Gly	Glycine	Na^+ dependent	M
Sodium Independent			
L(l)	Branched chain amino acids: (leucine, isoleucine, valine), tryptophan, BCH, phenylalanine, tyrosine, alanine, serine, threonine, glutamine	Na^+ independent, enhanced by extracellular H^+ Excludes anionic amino acids, proline, MeAIB	M, F
y^+	Lysine, arginine	Major cationic amino acid transporter excludes anionic amino acids inhibited by neutral amino acids (fetal side > maternal side)	M, (F?)
$b^{o,+}$	Lysine, arginine	Excludes anionic amino acids	(F?)
y^+L	Lysine, arginine	Excludes anionic amino acids	(M?), F
Asc	Small neutral amino acids	Excludes cationic and anionic amino acids	ND

Compiled from references 10,14,20,23.

BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; *F,* fetal; *M,* maternal; *MeAIB,* α-(methylamino)isobutyric acid; *ND,* not determined.

the two steps that involve transport across plasma membranes (i.e., the maternal and fetal surfaces). Molecular transporter systems for amino acid uptake by placental tissues have been identified (Table 55-4).[10,14,20-22] Specific amino acids transported by each system, conditions favoring or inhibiting or affected by each system, and location (maternal or fetal trophoblast membrane) for each system are presented in Table 55-4.

PLACENTAL AMINO ACID TRANSPORTER PROTEINS

Historically placental amino acid transport has been described in terms of systems (see Table 55-4). More recently, the ability to clone, sequence and study the expression of individual transporter proteins has led to an explosion of data concerning

the molecular basis of amino acid transporter systems and their function.[10,20,23,24] Transport systems operate in one of two ways, either in a monomeric manner, or in a heterodimeric manner.[24-26] Monomeric systems transport amino acids via single membrane proteins, similar to amino acid uptake mechanisms associated with permeases in microorganisms.[27,28] Heterodimeric systems on the other hand involve the grouping together of two proteins through the formation of disulfide bridges[25,29] to facilitate amino acid transport.[23,24,30] Presently monomeric transport systems in placental tissues include System A y+31 and most likely the X_{AG}^- system, whereas heterodimeric transport systems include System L, y^+L,[32] and the Asc system.[33,34] Those systems that operate in a heterodimeric manner are generally made up of a common heavy chain, a type II membrane glycoprotein, such as 4F2hc (comparable with the CD98 surface antigen, CD98hc[35,36]), NBAT (equivalent to rBAT or D,[26,37-40]), or as yet undefined heavy chains,[41] and associate with one of a variety of light chain transport proteins to yield a range of differing amino acid transport systems.[25,30,32,34] A summary of the traditional transport systems and their heavy and light chain associations that have been reported in placental tissues and membranes is presented in Table 55-5, and a brief description of them follows.

TABLE 55-5

The Placental Transport Systems and Associated Light Side Chains

Transporter system	Amino Acid Transporter Type	System interaction	Heavy Chain Protein	Light chain Protein	References
A	Neutral Sodium dependent			ATA-1	46
				ATA-2	47
ASC	Neutral Sodium dependent			ASCT-1	69
B^o	Neutral Sodium dependent			ATB^o (ASCT-2)	257,258
β	Neutral Sodium dependent			TAUT	66
N	Neutral Sodium dependent			SN1	
Asc	Neutral	Heteromeric	4F2hc	Asc-1	34
	Sodium independent		Unknown	Asc-2	42
L	Neutral	Heterodimeric	4F2hc	LAT-1	84,85
	Sodium independent			LAT-2	35,88,89
y^+L	Cationic	Heterodimeric	4F2hc	y^+LAT-1	97
	Sodium independent			y^+LAT-2	
y^+	Cationic Sodium independent	Monomeric		CAT-1, CAT-2B, and CAT-4	36,131,259
X_{AG}^-	Anionic			EAAT-1, EAAT-2, and EAAT-3	99,101,260

Refer to text for expanded abbreviations of light chain proteins.

Neutral Amino Acid Transporter Light Chain Proteins

The placental system A amino acid transporter is encoded by three members of the SLC38 gene family giving rise to three subtypes: SNAT1 (SLC38A1), SNAT2 (SLC38A2), and SNAT4 (SLC38A4). System A activity has been demonstrated in microvillous and basal membranes.[42-44] The SNAT1 isoform is expressed predominantly in the placenta and heart and is responsible for the transport of small short-chain neutral amino acids, such as alanine, serine, methionine, asparagine, and glutamine.[45] The functional characteristics of SNAT2 are similar to those of SNAT1, although it is found in a wider range of tissues, including the placenta.[45,46] SNAT4 appears to have a unique tissue distribution predominantly in hepatic and skeletal muscle tissues,[47] although recent reports also place it in the placenta, in both microvillous and basal membranes, and being expressed in the human placenta from early pregnancy onwards, similar to SNAT1 and SNAT2.[48]

Regulation of this system has been demonstrated through amino acid deprivation and interaction with the well characterized phosphatidylinositol 3-kinase (PI3K), mammalian target of rapamycin mTOR, and S6Kinase-dependent pathways, hypoxia, cortisol exposure,[49-52] and recently insulin-like growth factor (IGF) interactions through both mitogen-activated protein MAP kinase-dependent and independent pathways.[53] Additionally, angiotensin II has been demonstrated to decrease System A activity in human placental villous fragments through activated AT1 receptor.[54] Additionally of particular interest is that imprinted genes control the supply of nutrients to the fetus, and in the mouse, the *Slc38a4* gene has been shown to be imprinted. In studies utilizing the placental-specific *Igf2* transcript (P0) knockout, placental growth is compromised, although fetal demands are likely met through an up-regulation of transporter genes *Slc2a3* (glucose transporter) and the imprinted *Slc38a4*.[55,56] Interestingly when fetal demands for nutrients are removed through removal of the fetal *Igf2*, the up-regulation does not occur, providing evidence of regulation of placental transport activity via fetal growth demand genes. Both the *Igf2* and *Slc38a4* are paternally expressed, and this interaction is seen as a display of paternally inherited genes maximizing extraction of maternal nutrients.[56]

A related system, System N, shares many characteristics of System A, although it differs in its preference for amino acids containing nitrogen-bearing side chains and transports only glutamine, asparagine, and histidine.[57] System N activity has been localized in the human placenta,[58] and the gene SNAT3 (SLC38A3) has been identified as being associated with this activity.[23] In terms of amino acid movement in and out of trophoblast cells, it is interesting to note that SNAT1 and SNAT2 are sodium coupled and function under physiological conditions as influx transporters. On the other hand, SNAT3 and its other system N isoform, SNAT5 (SLC38A5) are coupled to a sodium gradient as well as a proton gradient and mediate a transport process in which sodium and glutamine move in one direction and H+ moves in the opposite direction; this suggests the importance of considering and studying transport systems as a collective, rather than as individual systems in isolation. Furthermore, the direction of glutamine flux through these transporters can be altered, even under normal physiological conditions.[59,60]

The β transporter, which transports taurine, has been found in placental microvillous and basal membrane vesicles.[61-63] Basal membrane activity is only approximately 6% of that in the microvillous membrane[64] and the cDNA associated with this activity, TAUT (SLC6A6), has been reported in placental cell lines.[64,65] Classical ASC transport of neutral amino acids—alanine, serine, and cystine—is reported in the microvillous membrane and in the basal membrane.[42,66] The cDNA, ASCT-1 (SLC1A4), sequences this classical sodium dependent ASC transport system and has been localized to human placental tissue[67] with other related activity also being weakly associated.[68] Another member of this family of sodium dependent neutral amino acid transport systems, the B° system, is responsible for

neutral amino acid transport, including the branch chain amino acids.[69-71] The cDNA, ASCT-2 (SLC1A5), represents this system in human placental choriocarcinoma cell lines,[70,72] and hypoxia has also been reported to play a role in its expression.[73] The sodium independent Asc system is responsible for the transport of small neutral amino acids and is comprised of Asc-1 and Asc-2 components, both of which have been isolated in placental mRNA.[33,74] Although Asc-1 forms a heterodimeric complex with 4F2hc,[33] Asc-2 appears to interact with a presently unknown heavy chain.[41]

Functional System L activity occurs through specific light chain proteins associating with 4F2hc,[75,76] catalyzing the uptake of a wide range of neutral amino acids in a sodium-independent manner, and System L activity is reported on both microvillous and basal membrane.[43,77,78] Regulation of placental System L activity does not seem to be under the control of hypoxia,[52] although evidence exists that it may not be the case in other cell preparations.[79,80] Although evidence is lacking in placental preparations, IGF appears to regulate System L activity where chronic infusions of IGF-I have corrected impaired transport of leucine in the brush-border of the rat intestine.[81]

Four associated light chains have been reported in placental tissues: System L-amino acid transporter-1 LAT-1 (SLC7A5); LAT-2 (SLC7A8); LAT-3 (SLC43A1); and LAT-4 (SLC43A2).[31,34,76,82-88] Immunological and functional studies suggest that at term, the light chain LAT-1 is located predominantly in the microvillous membrane and the syncytiotrophoblast layer of the villi.[84,86] Furthermore, the L transport system phenotype associated with the microvillous membrane occurs when the LAT-1 catalytic subunit is co-expressed with the 4F2hc, not LAT-2.[66] Human LAT-2 mRNA also has been found in the human choriocarcinoma BeWo cell line and placental villous tissues.[34,87-89] Placental basal membrane studies concerning the inhibition of specific amino acid and/or synthetic amino acid uptake have determined that the basal membrane L transport system phenotype is that which is associated with the co-expression of LAT-2 and 4F2hc, and not LAT-1, as observed to occur in the microvillous membrane.[76,89,90] LAT-1 and LAT-2 function as classic obligatory exchangers, moving substrates in exchange for other amino acids.[91] The two additional system L light chains, LAT-3 and LAT-4, have recently been reported in placental tissues. These appear to transport a restricted group of system L substrates (leucine, isoleucine, and phenylalanine) and also differ from the traditional system L transport in that they do not appear to require the co-expression of 4F2hc and therefore likely function as facilitative diffusion transporters.[82,92]

Cationic Transport System Light Chain Proteins

The transport of cationic amino acids occurs though the y^+ and the y^+LAT systems. System y^+ activity has been extensively studied in the human placenta.[93] This system operates in a sodium-independent manner and accumulates cationic amino acids within the placenta aided through the negative trophoblast potential. Activity is primarily localized to the microvillous membrane,[7,35] as opposed to the basal membrane, though there is conflicting data as to which CAT isoform is responsible for system y^+ activity in either trophoblast membrane.[35,94] The system y^+ activity is dependent only on the expression of these monomeric proteins (or CAT gene products),[95] unlike systems L and y^+L, which require expression of both a common heavy chain and a related light chain.[31]

System y^+L activity is localized to both the basal and microvillous membranes.[7,31,35] This system appears to provide an essential role in supplying cationic amino acids to the fetal circulation by exchanging cationic amino acids from within the trophoblast, for neutral amino acids such as glutamine and leucine from fetal circulation. This mode of operation provides a route of efflux, overcoming the trophoblast potential.[23] Similar to system L, placental y^+L system activity is also determined by which light chain, system y^+L-amino acid transporter-1 (y^+LAT-1, SLC7A7) or system y^+L-amino acid transporter-2 (y^+LAT-2, SLCA76), forms a heterodimer with the 4F2hc.[30,32,96] The y^+LAT-1 mRNA is detected in human placental Poly(A)$^+$ RNA and when co-expressed with 4F2hc in oocytes, displays characteristics similar to y^+L system.[96] Discrepancies concerning the expression of y^+LAT-2 in placenta have been reported.[31,94]

Anionic Transport System Light Chain Proteins

Five cDNAs encoding for proteins capable of mediating high-affinity sodium-coupled transport of anionic amino acids have been reported,[97] and three of these have been cloned from rat and human placenta and are present on both the microvillous and basal membranes of the placenta.[97,98] The rat basal membrane preparation is reported to have a higher maximal velocity,[98] an observation that is consistent with the predominant flux of glutamate from fetus to the placenta as has been demonstrated in the sheep.[99] These proteins—excitatory amino acid transport EAAT-1 (SLC1A3, GLAST1), EAAT-2 (SLC1A2, GLT1), and EAAT-3 (SLC1A1, EAAC1)—mediate placental sodium dependent D-aspartate-inhibitable anionic amino acid transport or system X_{AG}^- activity.[100] EAAT-1, EAAT-2, and EAAT-3 have been detected in human placental tissue.[101] The regulation of these proteins appears to be under the control of growth hormone (GH) and IGF family members,[102] and up-regulation of the placental system has been demonstrated through nutrient deprivation studies.[98] These later studies demonstrated that EAAT-1 and EAAT-3 play a key role in the basal anionic amino acid transfer, whereas EAAT-2 may be involved in conditions of amino acid depletion.[98] Of interest is that in rat cardiac myocytes, X_{AG}^- system activity is up-regulated after hypoxia,[79] although similar experiments are yet to be conducted in placental tissues or lines.

With a greater number of studies now using molecular biology to determine the specific transporter proteins in the plasma membranes, it is likely that the identification of individual transporter proteins in the trophoblast will be expanded considerably as sequence data for specific transporters permit rapid identification of other members of the same family of transporters. Thus the information provided in Table 55-5 should be regarded as an interim report in a rapidly changing field.

DEVELOPMENTAL AND ADAPTIVE CHANGES OF PLACENTAL AMINO ACID TRANSPORT

Development and Growth of Amino Acid Placental Transport Capacity

As pregnancy advances, the increasing nutrient demands of the developing conceptus must be met through an appropriate increase in placental nutrient transport capacity. This enhanced performance, as it relates to amino acid transport, is facilitated through alterations in placental perfusion, including increased uteroplacental blood flow and changes in membrane exchange area together with changes in amino acid transporter concentrations and activity in the plasma membrane (see Box 55-1).

In conjunction with the dramatic increases in uteroplacental blood flow during pregnancy (six-fold in sheep[103]) the developing surface area of the trophoblast is an important factor regulating total placental nutrient transport. Between week 16 and term of pregnancy, human fetal weight increases approximately 20-fold, whereas the peripheral villous surface area increases only nine-fold.[104-106] It should be emphasized, however, that only a few careful ontogenetic studies are available on how total surface area, including microvillous surface, changes

Box 55-1

Factors Affecting Placental Amino Acid Transfer

1. Transporter proteins in the trophoblast membranes: ontogeny, location (maternal-facing microvillus, fetal-facing basal, or both), regulation by local or circulating factors
2. Ion channel activity, ion gradients: Na^+, K^+-ATPase; Na^+, H^+, Cl^- gradients: (e.g., inward Na^+ gradient with system A co-transport)
3. Transport capacity, measured by V_{max} (maximum transport rate, affected by number of active transporters, the number of transporters per unit membrane area, and total membrane surface area)
4. Transporter-substrate binding affinity, measured by K_m (plasma amino acid concentration at half V_{max})
5. Placental disease, for example, fetal growth restriction and preeclampsia
6. Intracellular concentrations of amino acid, maternal amino acid concentrations, fetal amino acid concentrations, maternal-fetal amino acid concentration gradients leading to competition for transporters among amino acids
7. Turnover of transporters (rates of synthesis, degradation, or both)
8. Metabolism of amino acids by the trophoblast (e.g., oxidation of glutamate, conversion to other amino acids or substrates such as serine to glycine and leucine to KIC, protein synthesis, deamination-producing NH_3)
9. Uterine/umbilical blood flows: absolute rates (substrate delivery), the ratio of their flows (substrate delivery versus uptake and transport capacity), and the role of vasoactive substances (local or circulating) that alter uterine and/or umbilical blood flow (e.g., reduction in flow with epinephrine, enhancement of flow with nitric oxide)
10. Circulating hormone concentrations, placental receptors, and second messengers (e.g., hormone stimulation of cAMP-responsive increase in calcium channel and intracellular calcium release, activating amino acid metabolism and transporter activity)
11. Diffusional leaks into or out of cells or via paracellular pathways
12. Inhibitory effects of drugs (e.g., alcohol, nicotine, cocaine)

over gestation.[105-107] Although some more recent surface area studies have been more detailed,[106-108] they have not yet been conducted in conjunction with in vivo amino acid flux studies. The demonstrated increases in total surface area of the placenta, however, alone cannot account for the exponential fetal growth occurring over this time period. These data suggest that fetal growth is supported not by changes in villous surface area alone, rather the total transport capacity of the trophoblast is likely to be determined by changes in surface area and the concentration and activities of specific amino acid carrier proteins on the cell membrane surface.

As amino acids require active transport, they fit into the category of substances transported by the placenta according to diffusion-limited clearance. As such, placental transport rates of amino acids most likely are not affected much by moderate fluctuations in uterine or placental blood flow. More severe reductions in flow may affect their transport (but this has not been observed or measured) and, if it does occur, it may be mediated through flow-reduction effects on placental energetics and ion gradients, rather than by a direct reduction of delivery. Furthermore, changes in flow may alter transport of certain amino acids more than others, potentially leading to changes in the relative proportions of amino acids delivered to the fetus.[109] Such highlighted energetics and ion gradient deficiencies may be as, or more, important than amino acid delivery as the means by which amino acid uptake and transport are altered by deficient placental blood flow.

In addition to changes and the placental exchange surface area, capacity for amino acid transport is also affected by the differing expression and transport parameters of transport systems that appear to change through gestation.[35,44,110-112] For example, in the first trimester and term human microvillous membrane preparation, L-arginine transport is through both y^+ and y^+L systems, whereas in the basal membrane, transport may be restricted to only the y^+L system at term.[35] In addition, the kinetic properties of transport systems may also change as gestation advances. First-trimester microvillous membrane has increased y^+L transport activity compared with term placenta vesicles,[111] and y^+ activity appears to be related to gestational age, increasing with gestational age.[93] Additionally, 4F2hc protein levels differ between early pregnancy and term placenta.[35] Such studies highlight the complex interactions that occur between developing microvillous and basal membranes, within the trophoblast, and between the two circulations to facilitate an increase in nutrient delivery to the growing fetus as gestation advances.

Changes in Amino Acid Transport in Intrauterine Growth Restriction

Both in vitro and in vivo studies indicate that reduced fetal amino acid concentrations lead to enhanced amino acid transport (increased V_{max}, reduced K_m).[113-115] Furthermore this adaptive increase in transport can be blocked by selective inhibition of protein synthesis, indicating that the synthesis of new transporter proteins is potentially responsible for this altered amino acid transport pattern. The in vivo studies have been conducted in pregnant rats made hypoaminoacidemic by glucagon infusion. In these studies, fetal weight was not compromised until the mother became hypoglycemic while fasting.[116] These results were interpreted as indicating placental compensation for the hypoaminoacidemia to maintain fetal amino acid supply and concentrations. In contrast, no placental compensation for hypoglycemia (which reduced the fetal energy [glucose] supply) that was unaccompanied by hypoaminoacidemia was apparent.

These results take on added importance relative to the observations by Cetin and colleagues,[117] who showed that plasma concentrations of α-amino nitrogen were lower in growth restricted infants and their mothers, whether the measurements were made at the time of term delivery or many weeks before term. Furthermore, these investigators have shown that amino acid concentrations were lower in pregnancies with intrauterine growth restriction (IUGR), even if the fetuses had normal fetal heart rate and velocimetry monitoring, suggesting that the decrease in amino acid concentrations preceded other clinical pathologic changes in these pregnancies and in the fetuses.[118] Data collected from babies with IUGR at delivery show reduced total amino acid concentrations,[119,120] also suggesting alterations in amino acid transporter systems.

Although changes in amino acid concentrations have been used to interpret reduced supply and decreased utilization rates for amino acids, these values actually represent a balance among the rates of amino acid supply, synthesis into protein, release by protein catabolism, and oxidation. As such, reliance on concentration data alone in assessing the transport capacity of a placenta may not be entirely accurate. For example, it is interesting to note that more recent studies of fetal plasma amino acid concentrations in well defined groups of human fetuses with IUGR, where samples were derived from fetal blood sampling, have not repeated earlier observations of reduced values for fetal amino acid concentrations.[121] Similarly, recent studies in models of sheep with IUGR have not shown reductions in all of the fetal

plasma amino acid concentrations, especially the branch chain essential amino acids noted by the earlier studies.[122,123] These more recent placental amino acid transport studies in human and sheep IUGR, using radioactive and stable tracer techniques, do support the earlier conclusions of reduced placental transport, but together they highlight that tissue uptake and fetal metabolism of amino acids might also be impaired in IUGR, as there appear to be situations of IUGR in which fetal concentrations are maintained despite decreased placental transport.

In vitro observations, in which microvillous membrane vesicles from babies who were appropriate-for-gestational-age (AGA) and small-for-gestational-age (SGA) were prepared, demonstrated markedly lower activity (by 63%) of the A-system transporters in the infants who were SGA.[124] A number of investigators have described that, in cases of human IUGR, System A membrane activity per milligram of microvillous membrane is reduced, suggesting a positive association between fetal growth and System A activity.[110,125,126] Also, in placentas in the context IUGR, the sodium-dependent and -independent transport of taurine from maternal to fetal circulation also is reduced, thereby reducing fetal taurine concentration.[64]

Regarding the essential amino acids, the uptake of leucine in both microvillous and basal membranes of the placenta with IUGR is reduced, suggesting alterations in the placental L-transport system.[127] In these studies, basal membrane uptake was also reduced, suggesting that the changes in the basal membrane could be an important adaptive response by the trophoblast, limiting the backflux from the fetal circulation to the placenta. In vivo studies of ovine IUGR placental transport have indeed shown a reduced backflux of leucine and additionally threonine from the fetal circulation to the placenta, indicating that changes in basal membrane function may be the case.[122,123] In addition, under steady state in studies of stable isotopic tracer leucine infusion into the mother, the fetal/maternal enrichment ratio of leucine in normal human pregnancies is approximately 0.8, a much higher ratio than that 0.4 in sheep.[123,128] However, in both species, this ratio is significantly lower in pregnancies with IUGR, suggesting similarly reduced placental transport and metabolism characteristics. Clinical studies have shown that the fetal/maternal leucine enrichment ratio is significantly lower in human IUGR compared with normal pregnancies.[128] Furthermore, the magnitude of its reduction correlates with a clinical classification of IUGR severity based on a completely different set of clinical data, namely fetal arterial velocimetry and fetal heart rate data.[118] Interestingly the uptake of another essential amino acid—lysine—is not altered in IUGR placental preparations.[127] This amino acid uses the y^+ system and further studies confirm that the y^+ and also y^+L system activities are not altered in IUGR placental vesicles.[93] Finally, by way of additional species comparison studies in rats, in which IUGR was induced through maternal protein deprivation, have demonstrated a down-regulation of placental amino acid transport through reductions in system A, X_{AG}^-, and, interestingly, y^+ transport.[129,130]

One final component of change in IUGR that relates to placental amino acid transport is that of surface area,[107,108,131] indicating that morphometric changes also are likely to contribute to the overall reduction in placental amino acid transport capacity. Thus it currently appears that both reductions in surface area for exchange and reductions in specific transporter activity and/or number contribute to the reduction in amino acid transport in pregnancies with IUGR.

UTERINE UPTAKE AND PLACENTAL CONSUMPTION OF AMINO ACIDS

The greater bulk of amino acid in vivo studies have occurred in the sheep model. Net uptake rates of amino acids by the uterus have been difficult to measure because of small arteriovenous concentration differences and relative inaccuracies in the determination of the high rate of uterine blood flow (600 to 1600 mL/minute in late gestation pregnant sheep). In the pregnant sheep, the uterine arteriovenous concentration differences are positive, indicating net uptake by the uterus for all amino acids tested except glutamate; furthermore, the ratio of umbilical/uterine arteriovenous differences (1.9:1) is approximately equal to the uterine/umbilical blood flow ratio.[132] This evidence indicates that most of uterine amino acid uptake is transported to the fetus and that uterine requirements for amino acids are small relative to those of the fetus. In contrast, the placenta is highly active in amino acid utilization and modification; examples including leucine, threonine, glycine, alanine, and glutamate[99,122,123,133,134] are discussed in more detail in Placental-Fetal Amino Acid Cycling section, later. Quantitative aspects of placental amino acid requirements at any gestational age have yet to be determined. Early in gestation, the placental/fetal weight ratio is several-fold higher than at term, and placental growth (by increase in surface area relative to weight or by absolute increase in mass, depending on species and gestational age) is greatest at this period, indicating that placental amino acid metabolism might be quantitatively significant relative to that of the fetus. As examples of the high metabolic rate of the placenta for amino acids, based on placental nitrogen content at term in the human placenta, Lemons[135] estimated that placental growth over gestation would require 10.6 g of nitrogen (or 66 g of protein). Also, infusions of certain radioactively labeled amino acids (lysine, leucine, glycine, aspartate, and MeAIB) into the in situ perfused guinea pig placenta showed 12% to 16% of the label incorporated into placental proteins. This process was inhibited (81% to 96%) by cycloheximide, suggesting that an active process of placental synthesis of proteins was, in fact, occurring.[136]

The placenta near term contains a large variety of enzymes that are capable of metabolizing amino acids through pathways such as gluconeogenesis, glycogen synthesis, protein synthesis, amino acid oxidation, ammoniagenesis, and so forth.[135] Amino acid flux through these pathways has been demonstrated in vitro. So far, amino acid oxidation by human placental mitochondria has been demonstrated for alanine, aspartate, glutamate, and glycine.[137] Ammoniagenesis occurs in vivo in the placentas of sheep,[138,139] rabbit,[140] and guinea pig.[141] Other amino acid requirements include at least a small amount for oxidation and an undetermined amount for synthesis of secreted protein products (e.g., hormones such as human chorionic gonadotropin, luteinizing hormone, and placental lactogen).

UMBILICAL (FETAL) AMINO ACID UPTAKE

The net uptake of amino acids by the umbilical circulation represents the dietary or nutritional supply of amino acids for fetal growth and protein metabolism. Although net protein uptake by the fetus has been observed, this additional amount of protein supply to the fetus probably provides little nutritional value in that total amino nitrogen uptake, at least in the fetal lamb, is not different from the total amino nitrogen uptake in the form of amino acids. Protein molecules as small as albumin and as large as γ-globulin pass from maternal to fetal plasma with increasing efficiency as gestational age progresses.[142,143]

The uptake of amino acids by the fetus via the umbilical circulation has been studied in only one experimental animal (fetal sheep) because only this well-characterized large animal model has allowed accurate quantification of plasma amino acid concentrations in both the umbilical artery and vein. Specifically, the coefficients of extraction for several amino acids (umbilical venous-umbilical artery concentration/umbilical artery concentration) across the umbilical circulation are near zero. Therefore a high degree of accuracy is needed to measure the venous, arterial, and umbilical venous-umbilical artery concentration

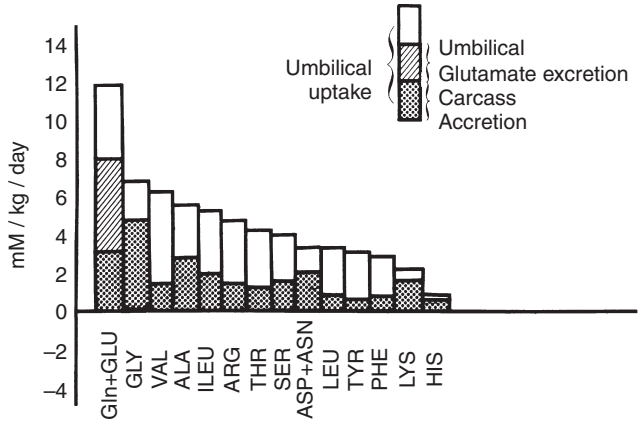


Figure 55-3 Umbilical uptake of individual amino acids by the late gestation fetal sheep, partitioned into amino acid utilization for carcass accretion *(lower, dotted portion of bar)* and that presumably used for oxidation *(upper, open portion of bar).* There was a net excretion to the placenta from the fetus for glutamate in this study. *ALA,* Alanine; *ARG,* arginine; *ASP,* aspartate; *ASN,* asparagines; *Gln,* glutamine; *GLU,* glutamate; *GLY,* glycine; *HIS,* histidine; *ILEU,* isoleucine; *LEU,* leucine; *LYS,* lysine; *PHE,* phenylalanine; *SER,* serine; *THR,* threonine; *TYR,* tyrosine; *VAL,* valine. *(From Lemons JA, Adcock EW 3rd, Jones MD Jr: Umbilical uptake of amino acids in the unstressed fetal lamb,* J Clin Invest *58:1428, 1976.)*

differences to estimate net uptakes of these amino acids by application of the Fick principle,[132,144,145] whereby the product of umbilical blood flow times the umbilical venous-arterial blood concentration difference for each amino acid yields the *net* uptake of each amino acid by the fetus.[12] Figure 55-3 illustrates the net uptake for each amino acid in fetal sheep studied during the last 20% of gestation (net uptake equals the total height of each bar). In a recent study in fetal sheep at term,[13] total fetal umbilical nitrogen uptake was 0.91 g nitrogen/kg/day (± 2 SD; range 0.56 to 1.27 g nitrogen/kg/day). This value is not very different from the calculated total fetal nitrogen requirement of approximately 1 g nitrogen/kg/day) based on nitrogen accretion data and estimated fetal urea production rates in near-term fetal sheep.[13] Figure 55-3 also shows, in the solid portion of the bars, the net accretion of each amino acid in the fetal carcass. The net uptake of most of the basic amino acids exceeds their net accretion by considerable amounts. In contrast, the net uptakes of the two basic amino acids, lysine and histidine, and the neutral amino acid glycine barely exceed net accretion. Similarly, the combined accretion of asparagine and its product aspartate and the combined accretion of glutamine and its product glutamate are very close to the net uptakes of asparagine and glutamine, respectively. For these amino acid pairs, no net uptake of the acidic forms, glutamate and aspartate, occurs. This is true not only in sheep[146,147] but also in primates and in humans.[148] These forms are derived in the fetus from deamination. These observations demonstrate that for these amino acids fetal requirements must be met by production within fetal tissues. Limitation of supply of these five amino acids (lysine, histidine, glycine, glutamine, and asparagine) very likely would lead to reduced protein accretion and growth.

PLACENTAL-FETAL AMINO ACID CYCLING

The placenta and fetus interact in a variety of ways to ensure amino acid supply to vital developmental, metabolic, and signaling processes that are unique to fetal growth and development[149] (Figure 55-4). For example, in addition to net transport of amino acids to the fetus, the placenta contributes to fetal amino acid and nitrogen balance by contributing to selective interorgan cycling,[9] an important concept in fetal physiology. For example, the placenta actively produces ammonia, which is delivered into both the uterine and umbilical circulation.[139,150] This process appears to be normal and occurs over a large part of gestation; for example, in sheep, the absolute rate of uteroplacental ammonia production (~25 μmol/min) at 73 to 97 days gestation is as high as that estimated from data collected in sheep near term,[151] which is consistent with in vitro studies demonstrating negligible urea cycle activity in sheep placental tissue over the entire length of gestation.[150,152] This relatively high placental ammonia production rate in midgestation probably also accounts for the higher concentration of ammonia (approximately two-fold) in fetal blood at midgestation. At that stage of development, fetal weight and the capacity of the fetus to clear ammonia are markedly reduced relative to term,[150,153] despite an in vitro fetal hepatic capacity for urea synthesis at midgestation that is equal to that near term.[154] A fraction of the net umbilical ammonia uptake is extracted by fetal tissues in sheep, perhaps contributing to hepatic urea formation and to other specific metabolic pathways. However, the ammonia taken up by the fetal sheep liver near term (~6.5 μmol/min) is 1.5 to 1.8 times that taken up by the umbilical circulation, demonstrating considerable fetal endogenous ammonia production, consistent with observations of net ammonia efflux across the fetal hind limb.[13]

Measurements of umbilical and fetal hepatic concentrations of amino acids in fetal sheep have demonstrated reciprocal relationships among three sets of amino acids.[13] Glutamine and glycine are taken up in net by the fetus from the placenta and by the liver from the umbilical vein, whereas their metabolic products glutamate and serine, respectively, are produced in net by the fetal liver and are taken up in net by the placenta (although net fetal-to-placental transfer of serine in the fetal sheep is more characteristic of midgestation than near-term gestation). Similar but less marked relationships have been found for net hepatic uptake of asparagine and release of aspartate with a reciprocal change across the umbilical circulation. Thus, for these three amino acids, it is clear that fetal requirements must be met by net production within fetal tissues during all or at least a major portion of gestation.

For serine, tracer studies in near-term fetal sheep indicate that 30% of fetal hepatic serine production is derived from glycine and that approximately 30% of the glycine uptake by the fetal liver is directed to serine production.[155] However, only 5% of fetal plasma serine is derived from plasma glycine, indicating that most of fetal serine is derived from fetal production in peripheral tissues. The bulk of fetal plasma serine oxidation occurs in extrahepatic tissues.[156] These data confirm the interorgan cycling of glycine and serine between the placenta and at least one fetal organ, the liver. Fetal serine production and uptake by the placenta from the umbilical circulation are much larger at 50% of gestation than at term; however, the source of such a relatively higher serine production rate early in gestation (greater synthesis or decreased incorporation into protein) is not known.

Glycine entry into the fetal pool is principally derived from umbilical uptake (30%) and from protein breakdown (56%). Glycine production in the placenta utilizes serine derived from both maternal and fetal circulations.[157] An additional source of glycine (14%) is derived from fetal glycine synthesis. The production of this nonessential amino acid in late fetal life[158] confirms the importance of the fetal glycine synthetic pathway found in liver and other organs. The hepatic amino acid–oxygen quotient is significantly higher than the umbilical amino acid–oxygen quotient (0.63 ± 0.87 VS. 0.13 ± 0.03), which indicates a high rate of amino acid utilization by the fetal liver.[13] The

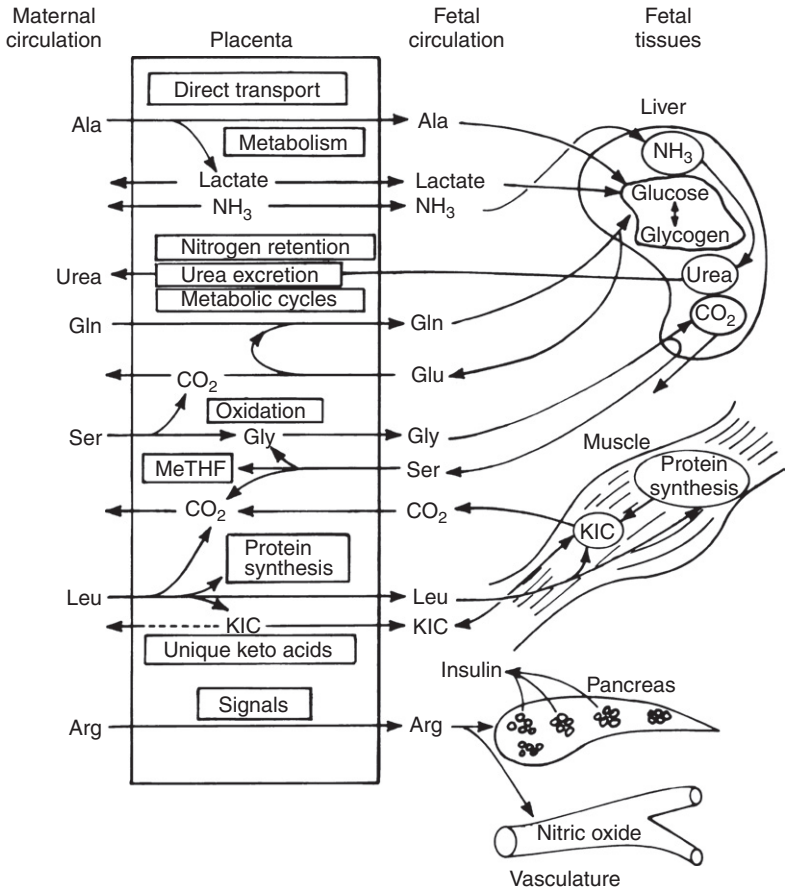


Figure 55-4 Schematic representation of a variety of placental-fetal metabolic interactions with respect to amino acid uptake by the placenta, metabolism in the trophoblast cells, direct transfer to the fetus, signaling of fetal vascular and metabolic processes, and utilization in fetal tissues. *Ala,* Alanine; *Arg,* arginine; *Gln,* glutamine; *Glu,* glutamate; *Gly,* glycine; *KIC,* α-ketoisocaproic acid; *Leu,* leucine; *MeTHF,* methyltetrahydrofolate; *NH_3,* ammonia; *Ser,* serine. *(From Hay WW Jr: Metabolic interrelationships of placenta and fetus,* Placenta *16:19, 1995.)*

oxidation rate for glycine is high in the fetus, and occurs almost exclusively in the fetal liver. Fetal hepatic glycine oxidation and serine production from glycine are linked by the enzyme systems, glycine oxidase and serine hydroxymethyl transferase.[159] A byproduct of placental glycine production via serine hydroxymethyl transferase is methylene tetrahydrofolate, which can be involved in a variety of methylation reactions within the placenta.[157,159]

The actual release of glutamate and serine from the fetal liver could be a direct reflection of the uncoupling of gluconeogenesis from amino acid oxidation.[99,157] Ordinarily, glutamine produces glycogen as well as glutamate in the fetal liver.[160] Almost 100% of fetal glutamate production is accounted for by fetal hepatic production from glutamine.[160] Placental oxidation of glutamate serves as its principal pathway of disposal. At birth, this pattern shifts so that with the loss of the placenta and the development of net hepatic production of glucose, glutamine carbon can be shunted directly to gluconeogenesis. Placental glutamate oxidation also provides nicotinamide adenine dinucleotide phosphate, which is required for placental lipid metabolism and steroidogenesis, particularly that of progesterone.[160] It is noteworthy that the term human trophoblast lacks an active pentose phosphate pathway, which is another pathway for nicotinamide adenine dinucleotide phosphate production.[161]

The ovine placenta takes up glutamine from maternal plasma. Most is transported directly to the fetus, and some is deaminated to glutamate in the trophoblast.[162] The ovine placenta also contains glutamine synthetase, and a small fraction of glutamate that is taken up by the placenta from the fetal circulation is returned to the fetus as glutamine. No proof is available, however, of a direct placental-fetal cycle for glutamine and glutamate. The amount of glutamine produced from glutamate in the placenta accounts for less than 10% of the total glutamate uptake by the placenta and represents less than 10% of placental glutamate output.[163] It is the oxidation of glutamate in the placenta that appears to be the predominant pathway of glutamate disposal in the fetal-placental system (70% to 80% of that taken up from the umbilical circulation is oxidized).[99] The ovine placenta receives two major influxes of glutamate: the placental uptake of fetal plasma glutamate at approximately 4.8 μmol/kg/min, and the placental production of glutamate from deamination of branched-chain amino acids at 3.2 μmol/kg/min.[164,165] In the sheep, practically no uptake of glutamate by the placenta from the maternal circulation occurs and thus no contribution of maternal plasma glutamate to placental-fetal glutamate metabolism. Conditions may be different in the human placenta, which has glutamate transporters on both maternal and fetal surfaces.[166,167]

Another example of placental-fetal amino acid cycling involves the relatively high concentrations and activities of branched-chain amino acid aminotransferases found in the placenta (sheep, human).[168,169] The transamination of a branched-chain amino acid provides the placenta with nitrogen which, in addition to other placental metabolic functions, is used to

sustain a high rate of glutamate production rather than the direct placental utilization of branched-chain amino acids as energy substrates.[127,170] Studies in sheep suggest that net placental uptake and transamination to the corresponding α-ketoacid can occur for leucine,[171] with 10% to 15% of leucine carbon transferred to the fetus as ketoisocaproic acid (KIC). The existence of these pathways implies a complex level of regulation in fetal-placental amino acid metabolism and clearly indicates that the placental-free amino acid pool, whether derived from protein breakdown in the placenta or from the maternal circulation, cannot be treated as a single homogeneous pool.[172]

Alanine also is consumed by the ovine placenta.[133,173] Placental metabolism of alanine may contribute significantly to placental lactate production[174] and also to placental carbon oxidation, accounting for 20% of alanine oxidation by the entire conceptus. The placental oxidation of alanine is much higher than the decarboxylation rate of leucine in the placenta.[175] Furthermore, alanine entering the placenta is metabolized and exchanged for placental alanine. In fact most of the alanine delivered to the fetus is of placental origin.[133] As the fetus matures, fetal hepatic urea cycle enzyme activity and production rates increase, approaching 0.4 g nitrogen excretion/kg/day.[139] In addition, the sheep fetus,[176] like the preterm human infant,[177] responds to an increased intake of ammonia and nitrogen with increased urea production. These observations indicate that the fetus responds effectively to a varied rate of amino acid supply (uptake or degradation) by appropriate forms of nitrogen excretion (e.g., serine and glutamate uptake by the placenta with placental ammonia and hepatic urea production). The higher ratio of ammonia production to urea excretion rate ratio that occurs earlier in gestation may be an advantage to the smaller fetus, providing nitrogen for reincorporation into amino acids and amines essential for rapid fetal growth.[178] The lower urea cycle activity in earlier gestation may also favor conversion of ornithine to polyamines that are essential in development.[179] All five enzymes of the urea cycle are present in the human fetus by 10 to 12 weeks postconceptional age; however, the levels of arginase are lower than those of the other urea cycle enzymes and may be rate-limiting for fetal urea production.[180] Arginase may provide a mechanism in the placenta for urea production from arginine in spite of undetectable levels in the human placenta of ornithine carbamyltransferase.[181] Quantitative aspects of ammonia and urea production in the human fetus are as yet unknown. This knowledge will become important as efforts persist to increase nitrogen intake as a therapy for fetal growth restriction and to help determine the developmental capacity of the preterm infant to metabolize amino acids and protein and excrete nitrogen in response to allegedly growth-promoting, high-protein diets.

NUTRITIONAL ASPECTS OF FETAL AMINO ACID UPTAKE FROM THE PLACENTA

The nutritional requirement for amino acids is high in the mid gestation to early trimester fetus and very preterm infant (24 to 30 weeks gestation). This requirement for amino acids is necessary to provide for the exceptionally high fractional protein synthetic and growth rates at this early developmental age.[182] Estimates of amino acid requirements at this period of development in fetal sheep range from 3.5 to 4.5 g/kg/day when scaled to human fetal growth rates, values comparable with that of 4 g/kg/day determined by the factorial method.[183] Amino acid and protein insufficiency has produced growth failure of the whole fetus and preterm infant.[123,184] Protein deficits in the growth restricted fetus (primarily based on animal studies) also adversely affect the growth and development of the individual organism. For example, in rats, IUGR produced by low protein diet in the mother during pregnancy decreases the growth of the fetal kidney.[185] Such changes appear to last into adulthood, leading to hypertension and diabetes, respectively; mechanisms have not been determined, although reduced glomerular development and associated reductions of the rennin-angiotensin system have been noted in adult rats that were born after IUGR.[185] IUGR from placental insufficiency in fetal sheep reduces the growth of the pancreas and the secretion of insulin in response to normal secretagogues.[186] This model of IUGR produces fewer islets with fewer insulin-producing β-cells in the pancreas due to cell cycle arrest and thus reduced rates if mitosis.[187]

FETAL AMINO ACID OXIDATION

Studies in the fetal sheep have demonstrated that the net uptake of amino acids by the fetus in excess of accretion requirements for growth. This observation implies that this excess portion of amino acid uptake is used for oxidation. More direct evidence for the fetal oxidation of amino acids comes from two observations: the high fetal urea production rate[137,138,143,176,188] and the direct measurement of labeled carbon dioxide production and excretion during fetal infusions of carbon-labeled amino acids.[147] Estimates of fetal urea production rate ranging from 0.4 mg/minute/kg in humans[143] and rhesus monkeys[138] to 0.7 mg/minute/kg in sheep.[176] The urea production rate in sheep could account for approximately 0.36 g/kg/day of nitrogen excretion (or 25% of fetal nitrogen uptake in amino acids) and 0.2 g/kg/day of carbon (or 2% of total fetal carbon uptake and 6% of fetal carbon uptake in amino acids). Such fetal urea production rates are large, exceeding neonatal and adult body weight–specific rates, indicating relatively rapid protein turnover and oxidation in the fetus.

Direct measurement of fetal amino acid oxidation with carbon-labeled isotopic tracers of selected amino acids, quantifies oxidation as the net excretion of labeled carbon dioxide from the fetus via the umbilical circulation relative to the plasma labeled amino acid–specific activity.[145] An important refinement of this methodology is to use the plasma amino acid reciprocal pool–specific activity; for example, for leucine, the plasma KIC pool should be used because it equilibrates with the intracellular specific activity that represents the dilution of intracellular leucine by cellular protein degradation and the final pathway to oxidation.[189]

Central to this methodology has been the documentation that, at least in the fetal lamb, almost 100% of fetal carbon dioxide production (e.g., that produced by fetal infusion of $NaH^{14}CO_3$) is excreted via the umbilical circulation.[145] One limitation of this methodology is its overestimation of fetal oxidation of a specific substrate to the extent that carbon-labeled nonoxidative products derived from placental and maternal metabolism of the substrate reenter the fetus and are oxidized.[190] For example, the carbon dioxide production rate from tracer leucine may include carbon dioxide derived from the decarboxylation of KIC molecules reentering fetal plasma after the placental deamination of fetal leucine.[171] Although estimates of this additional labeled carbon dioxide suggest that it is a fraction of an amino acid's direct oxidation, experimental verification has not been accomplished.

Several ^{14}C-labeled amino acids have been infused into fetuses in vivo, documenting $^{14}CO_2$ production (leucine, lysine, alanine, tyrosine, glycine, serine, threonine, glutamate).[99,122,123] Oxidation rates have been calculated for leucine (25% of utilization), lysine (10% of utilization), and glycine (13% of utilization), demonstrating that the oxidation/disposal ratio is directly related to the excess of umbilical uptake that is greater than accretion and to the plasma concentration of the amino acid.[12,13] Leucine oxidation occurs largely in muscle, whereas glycine oxidation occurs in liver as well. The net umbilical uptake of alanine in

the sheep fetus is approximately 5 μmol/kg/minute, which is less than the amount of plasma alanine that is decarboxylated by the fetus (9 μmol/kg/minute), emphasizing the need for a relatively high rate of fetal alanine production.[173]

AMINO ACID METABOLISM IN MIDGESTATION

Studies of the fetal lamb in midgestation have emphasized several important developmental characteristics of fetal amino acid metabolism. First, at midgestation the fetal uptake of amino acid and ammonia nitrogen via the umbilical circulation is comparable with fetal nitrogen requirements estimated from nitrogen accretion and urea excretion data.[153] Second, the contribution of amino acid carbon to energy needs and carbon accretion is comparable with that of glucose.[191] Similar observations have been made near term. However, in relation to fetal dry weight (a comparison made necessary by the large decrease in fetal water content over gestation), the umbilical uptake of amino acid nitrogen at midgestation is approximately four-fold greater than it is near term. This much higher dry weight–specific nitrogen uptake at midgestation agrees with measurements of protein synthetic rate and oxygen consumption. Third, leucine oxidation at midgestation is at least as great as it is at term, indicating that amino acids provide carbon for fetal oxidative metabolism over a large part of gestation.[191]

FETAL PROTEIN SYNTHESIS AND TURNOVER

In the growing fetus, net protein synthesis exceeds net protein degradation, although both processes continue simultaneously. Protein synthetic rate equals the protein accretion rate together with the amount of protein degradation, where protein accretion has been measured in fetuses by whole carcass protein analysis at different gestational ages. The protein synthetic rate has been determined using isotopic tracers of various amino acids. The rate of incorporation of tracer amino acid into proteins is the difference between infusion rate and the combined loss of tracer to catabolic pathways (oxidation) and diffusional loss to the placenta.[145,175,192] Such tracer methodology is limited in vivo by several factors. First, it calculates a whole body protein synthetic rate, which does not address the markedly different rates of growth and protein synthesis that occur among organs. Also, the protein synthetic rate is calculated using the plasma-specific activity (unless a reciprocal pool product is available, such as KIC for leucine), which may be greater than the intracellular-specific activity because of intracellular reutilization of amino acids released by protein degradation.[193] Thus the protein synthetic rate is frequently underestimated.

Fractional protein synthetic rate (K_S, or the fraction of body proteins that are synthesized/unit time) can also be calculated and compared with fractional growth rate (K_G). K_S is calculated by infusing an amino acid tracer until a plateau-specific activity (SA) in proteins and in the plasma is reached,[143,194] thus $K_S = SA_{proteins}/(SA_{plasma} \times \text{time of infusion})$. This calculation can be made more accurate by calculating the rate at which steady-state SA is obtained and by accounting for the recycling of tracer from degradation of newly synthesized (thus tracer-containing) proteins. In the fetal lamb, K_S and K_G have been compared using two tracers, ^{14}C-leucine and ^{14}C-lysine, at different gestational ages.[182,191] Figure 55-5 is a composite of data from these studies, demonstrating that both K_S and K_G decrease with gestational age, but the decline in K_S is greater. The higher protein synthetic rate in the midgestation fetus is proportional to the higher metabolic rate and the greater glucose uptake and utilization rates at that stage of gestation. Thus protein synthesis per mmol of oxygen consumed is essentially constant from midgestation until term.[191] The same appears to be true for the relationship between protein synthesis and glucose consumption.[1] Because other studies in pregnant sheep have shown increasing placental nutrient transport capacity with advancing gestational age, these data suggest that the reduced rate of fetal growth toward term is an intrinsic quality of fetal development and not the result of placental limitation of nutrient supply. The mechanisms underlying the reduction in protein synthetic rate over gestation remain to be determined. At least a partial explanation can be offered according to the changing proportion of body mass contributed by the major organs (Table 55-6). Based on the relatively increased mass of skeletal muscle (which has a relatively lower fractional synthetic rate in late gestation), for example, it is logical that the whole body fractional synthetic rate should decrease.[148] It is clear, however, that a direct relationship with anabolic endocrine-paracrine factors, such as insulin, pituitary and placental growth hormone, placental

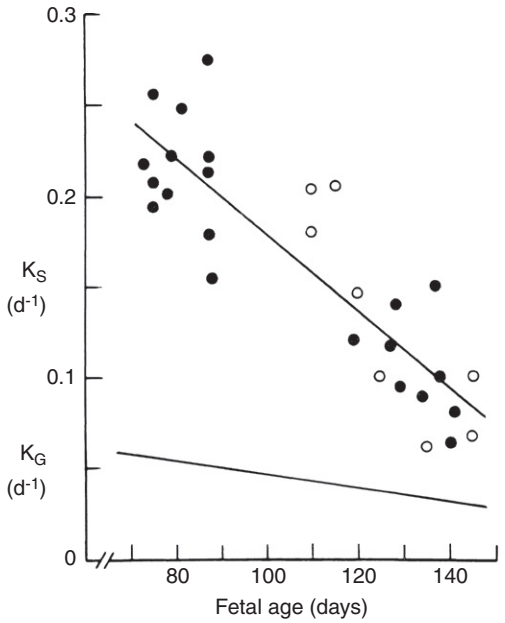


Figure 55-5 Fractional rate of protein synthesis *(K_S)* over gestation in fetal sheep studied with leucine *(●)* and lysine *(○)* radioactive tracers compared with fractional rate of growth *(K_G)* in the lower portion of the figure *(—). (From Battaglia FC, Meschia G: Fetal and placental metabolism: Part II. Amino acids and lipids. In Battaglia FC, Meschia G:* An Introduction to Fetal Physiology. *Orlando, Academic Press, 1986, p 120; Kennaugh JM, Bell AW, Teng C, et al: Ontogenetic changes in the rates of protein synthesis and leucine oxidation during fetal life,* Pediatr Res *22:688, 1987; Meier PR, Peterson RG, Bonds DR, et al: Rates of protein synthesis and turnover in fetal life,* Am J Physiol *240:E320, 1981.)*

TABLE 55-6

Fractional Synthetic Rates Among Organs in Early Postnatal Life

	Organ Percent Synthesis/Day (K_s)	
Liver	57.0	± 12.0
Kidney	50.0	± 10.0
Brain	17.0	± 3.0
Heart	18.5	± 4.0
Skeletal muscle	15.2	± 2.8

From Waterlow JL: *Protein Turnover in Mammalian Tissues and in the Whole Body*. Amsterdam, Elsevier/North-Holland Biomedical Press, 1978.

lactogen, insulin-like growth factors (somatomedins), epidermal growth factors, and so forth, cannot be proposed because most studies suggest an increasing concentration or secretion of these substances over gestation.[1]

Role of Insulin in Fetal Amino Acid Metabolism

Insulin is an important growth hormone for the fetus. All studies have shown that a reduction in fetal insulin concentration—from genetic defects in human infants that produce pancreatic agenesis or from experimental surgical removal of the pancreas or chemical ablation of pancreatic islets or β cells—decreases the rate of fetal growth. Growth still occurs in all of these conditions, just at a slower rate. Insulin action in the fetus and newborn infant has the same general properties as it does later in life. With respect to growth, the primary action of insulin is to increase amino acid uptake by the cells, promoting their direct synthesis into protein and, when energy is deficient, into oxidative metabolism and energy production. Insulin also acts to decrease protein breakdown. This is observed consistently in adults,[1,195,196] but less so in the fetus and the preterm newborn who is receiving only intravenous glucose for energy requirements.[197] For example, stable isotopes of glucose, leucine, and phenylalanine/tyrosine have been used to study glucose and protein metabolism during a euglycemic, hyperinsulinemic clamp in preterm infants receiving only parenteral glucose.[198] These infants demonstrated a doubling of glucose utilization with high doses of insulin. Protein breakdown was decreased by 20%, but protein synthesis was also diminished by 20%, resulting in no net protein gain. In contrast insulin may be anabolic and induce a net gain in protein balance in the fetus and preterm infant when given with amino acids as well as glucose.[198,199] A potentially confounding factor in assessing the specific effect of insulin in such studies, however, is that insulin also increases glucose utilization and oxidation at the same time it enhances amino acid utilization. This occurs by insulin's effect to translocate GLUT4 to cell membranes, increasing the capacity for cellular glucose uptake. A recent study in fetal sheep, however, showed that when glucose uptake was reduced to normal rates in the presence of continued amino acid infusion during a hyperinsulinemic clamp, leucine oxidation did not increase significantly, indicating that the acute effect of insulin on amino acid utilization for protein synthesis, net protein accretion, and oxidation is largely independent of simultaneous glucose utilization.[198,200,201]

Several studies have addressed the interactive roles of insulin and glucose, the principal energy substrate in the fetal metabolism, in fetal skeletal muscle metabolism of amino acids.[202-207] Under hyperinsulinemic conditions in which glucose and amino acids were also infused to maintain their concentrations relatively in the normal range, an increased net uptake of most amino acids by the hind limb occurred, reflecting reduced rates of proteolysis more than increased rates of protein synthesis. Protein synthesis was more strongly regulated by the plasma concentration of amino acids than by insulin alone. Glucose uptake also plays a role, indicating that a positive energy balance, as well as the provision of amino acids, allows insulin to promote nitrogen accretion most effectively.[12,202,204]

Hormonal/Nutrient Regulation of Protein Translation

More recent studies of fetal amino acid metabolism have shown that insulin can enhance the MAPKinase (mitogen activated pathway) in fetal skeletal muscle[208] and that both insulin and IGF-I regulate protein synthesis through well-recognized intermediates in their signal transduction pathways, including mTOR (mammalian target of rapamycin) and the eukaryotic initiation factors[209] (Figure 55-6).

Translation initiation and protein synthesis in mammalian cells are regulated through mTOR. In mammals, this evolutionary-conserved protein functions as a sensor for growth factors,

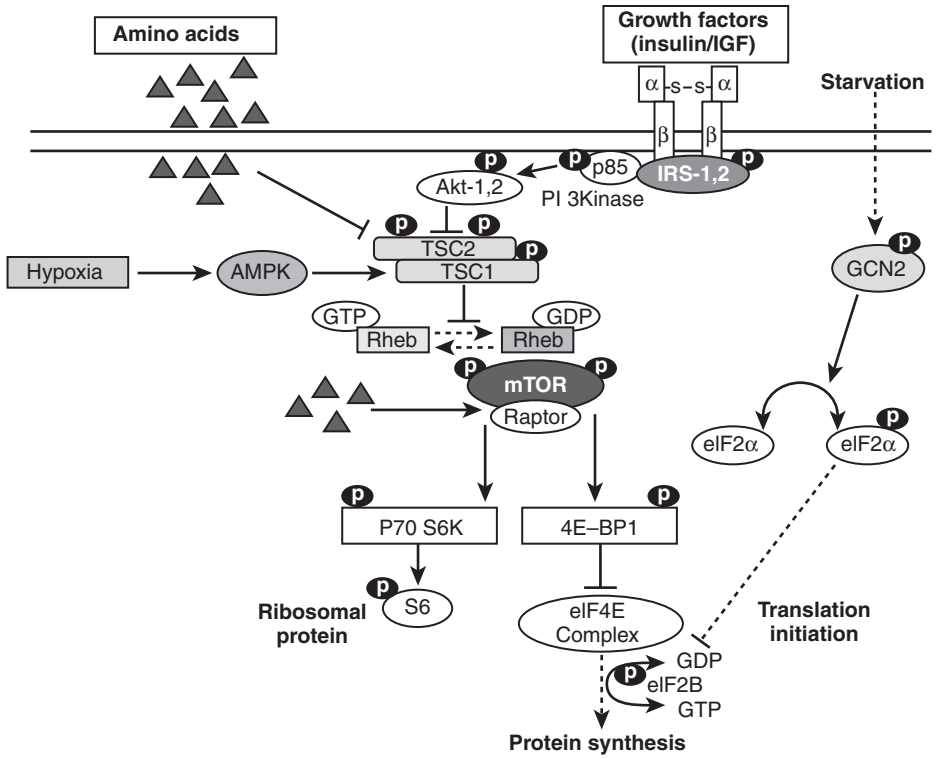


Figure 55-6 Schematic representation of the cellular signal transduction pathways of regulatory proteins leading from stimulation by amino acids, growth factors such as insulin and IGF, or energy substrate supply to protein synthesis.

nutrients, energy and stress, and coordinates these signals to regulate cell growth and proliferation.[210]

Insulin, via a well-described cascade of serine/threonine phosphorylation events, activates mTOR.[211] Amino acids also function as direct-acting nutrient signals that activate mRNA translation initiation via mTOR through what appears to be a separate pathway not as well defined.[212] Activated mTOR is able to phosphorylate eukaryotic translation initiation factor 4-binding protein (4E-BP1), releasing it from one of the eukaryotic initiation factors eukaryotic translation initiation factor 4E (eIF4E). This enables eIF4E to form the eIF4E translation initiation complex, which binds mRNA and the ribosome to initiate protein synthesis. mTOR also phosphorylates ribosomal protein p70S6 kinase, which in its phosphorylated (activated) form will increase the translation of 5'-TOP mRNAs that encode for ribosomal proteins and other translation factors. In addition, hypoxic regulation of mTOR, independent of energy metabolism, also is emerging as an important signal for modulating cellular translation.

Several other signaling pathways contribute to the regulation of mammalian growth. Phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) by cellular stress is recognized as a suppressor of global protein synthesis and is regulated by general control nonrepressed 2 (GCN2), a kinase activated specifically by amino acid starvation.[213] Detailed analysis in drosophila and mammalian cells has revealed that tuberous sclerosis protein 1/2 (TSC1/2) complex negatively regulates mTOR signaling.[210] Several environmental factors act either positively or negatively on TSC2, thereby inhibiting or stimulating mTOR. TSC2 is phosphorylated and functionally inactivated by Akt in response to insulin.[214] Insulin also activates the Ras/extracellular signal-regulated kinases (ERK) pathway, leading to both inhibition of TSC1/2 and activation of p70S6 kinase.[215] Low cellular energy of any stress that depletes cellular ATP, such as hypoxia, oxidative stress or nutrient deprivation will activate 5'AMP-activated protein kinase (AMPK) and decrease mTOR activity. Finally, mTOR signaling is inhibited by hypoxia (through hypoxia-inducible factor [HIF1] and REDD), and protein synthesis is downregulated.[216]

In vitro studies have demonstrated that insulin and amino acids interact to regulate translation initiation downstream of mTOR. Insulin, independent of external amino acid supply, decreases the amount of 4E-BP1 associated with eIF4E and increases formation of the translation initiation complex.[217] Leucine deprivation of L6 myoblasts causes dephosphorylation of p70 S6 kinase, and leucine replenishment restores the phosphorylation state to control patterns.[218] These studies show insulin and amino acids to be positive and/or permissive regulators for mTOR signaling.

In vivo studies also have shown positive effects of insulin and amino acids on the stimulation of translation initiation and muscle protein synthesis. Several studies in the neonatal piglet have demonstrated that insulin and amino acid infusion independently promote muscle protein synthesis with additive effects. Amino acids will signal the phosphorylation of 4E-BP1, increase eIF4E translation initiation complex information, phosphorylate p70S6 kinase and increase muscle protein synthesis, but only in the presence of fetal serum insulin at postprandial concentrations.[219,220] Leucine infusion alone will stimulate the phosphorylation of p70S6 kinase and 4E-BP1.[221] A series of studies in the sheep fetus have shown that insulin, IGF-1, and amino acid infusions have significant effects on whole fetal protein synthesis and accretion. Acute fetal infusions of insulin and IGF-1 increase whole body protein accretion and stimulate muscle protein synthesis[222] and insulin infusion leads to phosphorylation of 4E-BP1 and p70S6 kinase.[209] Acute amino acid infusion, independent of insulin, increases amino acid disposal by means of stimulating both protein synthesis and catabolism.[223]

Additional studies in late gestation fetal sheep have shown that insulin and amino acids act independently to modulate key signal transduction proteins in the mTOR pathway. Recent studies observed a tendency for a greater proportion of mTOR protein to be in a phosphorylated, activated state, and phosphorylation states of ERK1/2 and p70S6 kinase were increased three-fold and four-fold when both amino acids and insulin were present. Amino acids alone did not activate the phosphorylation of any of these proteins or the phosphorylation of 4E-BP1. Thus insulin appears to be a more effective regulator of the mTOR signaling pathway, at least acutely.[224]

Independent Regulation of Signal Transduction by Amino Acids in the Fetus With Intrauterine Growth Restriction

Studies similar to those in normal fetuses described above also have been applied to the fetus with IUGR. In such studies, total 4E-BP1 protein did not change, but significantly less 4E-BP1 in its phosphorylated, activated form relative to the total protein amount in the animals with IUGR compared with the control animals was seen.[225] Giving amino acids to these animals with IUGR failed to restore the ratio of phosphorylated to total 4E-BP1 back to control animal proportions. Because 4E-BP1 is downstream from mTOR, these data indicate that translation initiation in IUGR may be inhibited through selective inactivation of 4E-BP1. These results also indicate that persistent fetal growth deficits that are programmed in utero may not be easily up-regulated or corrected simply by giving more amino acids, particularly when the growth restriction has been chronic and the rate of fetal growth and its regulatory mechanisms have adapted to the long-standing conditions in IUGR of reduced amino acids supply and low plasma concentrations of anabolic hormones (insulin, IGF), glucose, and perhaps oxygen. It also is possible that the adaptation in IUGR might simply be, in part, due to a decrease of amino acid–stimulated insulin secretion; other studies have documented reduced endocrine pancreas development and insulin secretion in IUGR. Thus a simultaneous increase in fetal insulin concentrations with amino acid stimulation or an independent insulin infusion might be required to reactivate the intracellular protein synthesis initiation pathways in fetuses with chronic IUGR (and, presumably, neonates).

FETAL PROTEIN METABOLISM IN RESPONSE TO MATERNAL FASTING

Because protein synthetic and growth rates are high in fetal life, it is appropriate that a number of investigators have studied changes in fetal protein and amino acid metabolism in response to maternal conditions that restrict amino acid, energy supply, or both to the fetus. As described earlier, for example, sustained fetal amino acid concentrations and fetal growth have been observed during selective maternal hypoaminoacidemia in pregnant rats; fetal growth was reduced only when maternal fasting reduced the fetal glucose (energy) supply. Similarly, normal to increased fetal amino acid concentrations have been observed during maternal fasting in pregnant sheep.[226] Fetal uptake of amino acids did not change with fasting, indicating either a reduced fetal protein synthetic rate or an enhanced rate of proteolysis that was directly related to fetal glucose energy supply. Other studies have demonstrated enhanced hind limb release of alanine and glutamine[227] (major potential sources of gluconeogenesis) in fasted sheep. Evidence for fetal gluconeogenesis during prolonged maternal fasting–induced hypoglycemia and reduced glucose supply have been demonstrated.[228]

The balance among energy supply, amino acid supply, protein synthesis, and protein catabolism has been further investigated in two separate models. In fasted, pregnant sheep, no change was seen in leucine disposal rate, but a doubling of the leucine oxidation/disposal rate ratio that occurs after a week of maternal fasting was observed (Figure 55-7).[147] In acutely fasted pregnant rats, fetal proteolysis is enhanced but rates of protein synthesis are normal.[229] These animals had relatively

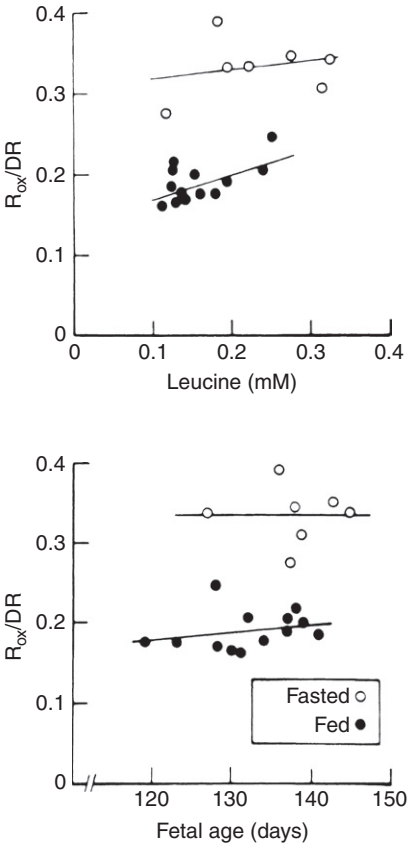


Figure 55-7 Oxidation/disposal rate ration for leucine in fetal sheep near term, studied during fed and fasted conditions compared with fetal arterial plasma leucine concentration *(top)* and fetal age *(bottom). (From van Veen LC, Teng C, Hay WW Jr, et al: Leucine disposal and oxidation rates in the fetal lamb,* Metabolism *36:48, 1987.)*

normal concentrations of amino acids but markedly reduced glucose concentrations.[230] In a subsequent study, prolonged maternal malnutrition with both energy (e.g., glucose) and protein restriction resulted in decreased protein synthesis and proteolysis, but in this case, protein synthesis was reduced to a greater extent.[231] Together these data support the concept that fetal protein synthesis is both amino acid and energy dependent, whereas fetal protein catabolism may be more specifically regulated by nonprotein energy substrate supply. Furthermore, certain amino acids released by proteolysis can be oxidized to maintain fetal energy balance at the expense of growth, whereas others can be directed toward gluconeogenesis to maintain glucose energy requirements.

AMINO ACID, PROTEIN, NITROGEN, AND CARBON BALANCE

Amino acid balance has been determined for several amino acids, including leucine, glutamine (and glutamate), alanine, glycine, and serine, but thus far in only one species, the fetal sheep. Details of these studies for these amino acids were discussed earlier and are presented schematically in Figure 55-4. More complete results for leucine from fetal sheep studied near term and at 50% to 60% of gestation have been determined.[232] Protein turnover/wet weight is higher in the early-gestation fetus, accounted for primarily by relatively increased rates of umbilical leucine uptake (exogenous entry) and protein synthesis. This results in a 50% higher rate of net protein accretion.

TABLE 55-7

Metabolic Balance for the Late Gestation Fetal Sheep

	Carbon Calories	
	Carbon-Caloric Balance (g/kg/day) (kcal/kg/day)	
Requirement		
Accumulation in carcass	3.2	32
Excretion as CO_2	4.4	0
Excretion as urea	0.2	2
Excretion as glutamate	0.3	2
Heat (oxygen consumption)	0.0	50
TOTAL	8.1	86
Uptake		
Amino acids	3.9	45
Glucose	2.4	17
Lactate	1.4	14
Fructose	1.0	7
Acetate	0.2	3
TOTAL	8.9	86
Nitrogen Balance Requirement		
Urea nitrogen excretion	0.4	
Nitrogen accretion	0.6	
TOTAL	1.0	
Uptake (in amino acids)	1.0	
TOTAL	1.0	

From Battaglia FC, Meschia G: Fetal nutrition. *Annu Rev Nutr* 8:43, 1988; Meier P,Teng C, Battaglia FC, Meschia G:The rate of amino acid nitrogen and total nitrogen accumulation in the fetal lamb. *Proc Soc Exp Biol Med* 167:463, 1981; Battaglia FC, Meschia G: Fetal and placental metabolism: Part I. Oxygen and carbohydrates. *An Introduction to Fetal Physiology.* Orlando, Academic Press, 1986, pp 49-99.

Table 55-7 summarizes the contributions of amino acids to fetal carbon, calories, and nitrogen balance compared with other known substrates in the late-gestation fetal sheep.[143,232,233] Differences among species can be expected based primarily on the capacity of the placenta to transfer fat to the fetus and on the contribution of fat to fetal body composition. Even in those species that take up fat from the placenta and deposit fat in fetal tissues, fatty acid oxidation is presumed low. This presumption has not been tested adequately and may provide a partial explanation for the difference between carbon and caloric requirements and carbon and caloric uptakes.

CONCLUSIONS

Important lessons for amino acid nutrition of the preterm infant are to be learned from evaluation of fetal amino acid uptake and metabolism. As emphasized here, amino acid supply to the fetus by the placenta is quantitatively large and qualitatively unique. Individual amino acids are actively transported into the fetal circulation at relatively high rates to meet the rapid growth and energy needs of the fetus. The active transport also produces amino acid concentrations in the fetal circulation that are generally higher than those in the maternal circulation. These concentrations also are higher than most preterm infants have in their plasma after birth. By way of comparison, the early third trimester fetal sheep uses approximately 6 g/kg/day of amino acids. When scaled to the fractional growth rate of lean body mass characteristic of the normally growing human fetus at the same stage of development,[234] this rate of amino acid utilization is 3.6 to 4.8 g/kg/day. This rate is similar to the required amino acid intake (4.0 g/kg/day) estimated for very preterm infants by the factorial method, but noticeably greater than the rate of amino acid or protein intake that these infants are usually fed.[184] Only

when amino acid intake rates and plasma concentrations are achieved in preterm infants that meet the fetal values do nitrogen and protein balance as well as oxidation rates of the preterm infant match those of the normally growing fetus.[235] The relative concentrations of amino acids achieved in the fetal circulation also are unique. This occurs as a combined result of unique transport characteristics for each amino acid and for groups of amino acids in the placenta, unique rates of placental metabolism of amino acids and unique amino acid derivatives produced by placental amino acid metabolism, and unique placental-fetal hepatic amino acid metabolic cycles. Such information from animal models needs to be expanded by human studies to help determine the optimal supply of each of the amino acids and the unique concentrations that should be achieved in the circulation of the preterm infant in order to optimize preterm neonatal amino acid metabolism and nitrogen and protein anabolism.

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56 Protein and Amino Acid Metabolism and Requirements

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Proteins are the major structural and functional components of all cells of the body. Enzymes, membrane carriers, blood transport molecules, intracellular matrix, and even hair and fingernails are proteins, as are many hormones. Proteins also constitute a major portion of all membranes, and the constituent amino acids of protein act as precursors of many coenzymes, hormones, nucleic acids, and other essential molecules. An adequate supply of dietary protein is therefore necessary to ensure cellular integrity and function.

PROTEIN AND AMINO ACID CHEMISTRY AND METABOLISM

Proteins are among the most complex molecular compounds of the body and of the diet. They consist of chains of amino acid subunits joined together by peptide bonds between the carboxyl group of one amino acid and the amino group of the adjacent amino acid. The chains range from two (dipeptide) to thousands of amino acids, with molecular weights ranging from hundreds to hundreds of thousands daltons.

Proteins are not simply long straight chains of amino acids. Rather the chains fold intospecific three-dimensional structures depending on the function of the protein and its intended interaction with other molecules. Many proteins have several separate peptide chains held together by ionic or covalent links. From a nutritional perspective the most important aspect of a protein is its amino acid composition. However, its structure may influence its nutritional availability. Some proteins are insoluble and hence are resistant to digestion, and others are resistant to the hydrolytic enzymes of the intestine.

The amino acid components of mammalian proteins are α-amino acids. They have a carboxyl group, an amino nitrogen group, and a side chain attached to a central α-carbon. The structure of the side chains determines the functional differences among amino acids. The side chains also have different charges at physiologic pH, and they may be either hydrophobic or hydrophilic. The side chains therefore are important determinants of how the protein structure is stabilized and hence how the protein functions. Attractions between positive and negative charges, for example, pull different parts of the molecule together, with hydrophobic groups tending to cluster in the center of globular proteins and hydrophilic groups tending to remain at the periphery. The formation of disulfide bonds between the thiol groups of cysteine is another important factor in stabilization of the folded structure of a polypeptide and is crucial for formation of interpolypeptide bonds. Some proteins contain complex oligosaccharide side chains attached to the hydroxyl and amide groups of amino acids. Ion-binding proteins (e.g., calcium binding proteins, iron-binding proteins) contain large amounts of histidine and the dicarboxylic acids.

Some amino acids are classified as "indispensable" or "essential," and others are classified as "dispensable" or "nonessential." The carbon skeletons of the former cannot be synthesized by humans from simpler molecules and hence must be provided in the diet, whereas the latter can be synthesized from other amino acids or from other precursors. Some amino acids also are classified as "conditionally indispensable" or "conditionally essential." These can be synthesized from other amino acids, but their synthesis is limited under some conditions.[1-3] These may be particularly important for the preterm LBW infant in whom developmental delays in several enzymes involved in amino acid synthesis have been demonstrated.[4-8] Dietary amino acids classified as essential, nonessential, and conditionally essential are shown in Table 56-1.

Protein Digestion and Absorption

Ingested proteins are denatured by gastric acid and are cleaved into smaller peptides by the enzyme pepsin, which is activated by the feeding-induced increase in gastric acidity. In the small intestine, the peptide bonds of the proteins and peptides are hydrolyzed by various bond-specific proteolytic enzymes of pancreatic origin. This process results in a mixture of free amino acids and small peptides that are transported into the enterocytes by substrate-specific carrier systems. The free amino acids are secreted into the portal blood and pass into the liver, where some are removed and used. The remaining amino acids reach the systemic circulation and are transported to specific sites throughout the body.

The efficiency of digestion of most common dietary proteins (e.g., casein, whey, grain, and legume proteins), measured as removal of labeled amino acids from the small intestinal lumen, is thought to be at least 90%. Nonetheless, nutritionally significant quantities of indispensable amino acids appear to be metabolized by the splanchnic tissues, including the enterocytes.[9] Thus all amino acids removed from the intestine do not reach the peripheral circulation, and the amounts that do not vary from amino acid to amino acid, with intestinal threonine metabolism being particularly high. Absorbed amino acids that reach the systemic circulation are transported into tissues throughout the body, where they are either metabolized to other amino acids, glycogenic or lipogenic precursors, or are used for protein synthesis.

Protein Synthesis

Amino acids destined for protein synthesis bind with transfer ribonucleic acid (tRNA). Information concerning the amino acid sequence of each protein to be synthesized is contained in the sequence of nucleotides in messenger RNA (mRNA) molecules that are synthesized in the nucleus from regions of the deoxyribonucleic acid (DNA). The mRNA molecules then interact with different cytoplasmic tRNA molecules to synthesize protein by linking the individual amino acids. The specific proteins expressed in any cell and the rates at which they are synthesized are determined by the relative abundances of the different mRNAs (i.e., the rates of transcription) and the stability of the messages. Changes in the rate of translation alter the number of tRNA molecules or the efficiency of the tRNA and, hence, alter the rate of synthesis of total cellular protein without altering the composition of the mixture of proteins produced.

TABLE 56-1

Indispensable, Dispensable, and Conditionally Indispensable Dietary Amino Acids

Indispensable	Dispensable	Conditionally Indispensable
Histidine	Alanine	Arginine
Isoleucine	Aspartic acid	Cysteine
Leucine	Asparagine	Glutamine
Lysine	Glutamic acid	Glycine
Methionine	Serine	Proline
Phenylalanine		Tyrosine
Threonine		
Tryptophan		
Valine		

Protein synthesis is a continuing process that occurs in most cells of the body. In the absence of either growth or protein loss, it is balanced by an equal amount of protein degradation.

Protein Degradation

The mechanism by which intracellular protein is hydrolyzed to free amino acids is more complex than that of protein synthesis and has not been as well characterized. Although various enzymes that can hydrolyze peptide bonds are present in cells, the bulk of hydrolysis is thought to be shared by two multienzyme systems: the lysosomal and proteasomal systems. The lysosome, a membrane-enclosed intracellular vesicle, contains proteolytic enzymes called *cathepsins*. This system appears to be relatively unselective, hydrolyzing all engulfed proteins. It is thought to operate when entire areas of the cell or complete organelles need to be degraded (e.g., to provide a rapid supply of free amino acids as substrates for gluconeogenesis). The system is also highly regulated by hormones and amino acids.[10]

The second system, the adenosine triphosphate–dependent ubiquitin-proteasome system, is present in the cytoplasm. The first step is to combine molecules of the very basic 76-amino acid peptide, ubiquitin, with lysine residues of the target protein. This process, which selectively targets proteins for degradation by a second component, the proteasome, involves several enzymes. The proteasome is a very large complex of proteins with numerous different proteolytic activities. The system is very selective and hence can account for the wide range of degradation rates (e.g., half-lives ranging from minutes to days) among proteins. It is thought to be responsible for degrading abnormal or damaged proteins as well as regulatory proteins, which typically are synthesized and degraded very rapidly.[11,12]

Protein Turnover

The process of continuous degradation and resynthesis of protein is known as *protein turnover*. It has been estimated that the human adult synthesizes and degrades approximately 250 g of protein daily despite a mean daily protein intake of only 60 to 100 g. The rate of protein turnover on a body weight basis is considerably greater in infants, particularly preterm LBW infants, and is lower in older adults.[13] The liver and intestine, despite their small contribution to the protein content of the body, are thought to account for approximately 50% of whole body protein turnover.[9,14] In contrast, skeletal muscle, which comprises about 50% of body protein mass, accounts for only about 25% of total body protein turnover.[13]

Total body protein turnover of adults as well as both term and preterm infants has been studied using stable isotope tracers. This method assumes a simple two-pool model of protein metabolism in which amino acids are either free or protein bound. Amino acids enter the free pool from the diet, I, from protein breakdown, B, or from endogenous synthesis (e.g., dispensable amino acids). They leave the pool to be incorporated into newly synthesized protein, S, or to be catabolized, E. Because I and E can be measured, S and B can be calculated from the flux of a single amino acid, which also can be measured: Q = I + B = S + E. Isotopic tracers of both essential ([1-^{13}C] leucine; [^{13}C] or [$^{2}H_5$] phenylalanine) and nonessential amino acids ([^{15}N] glycine) have been used to label the amino acid pool, which is usually assumed to be represented by plasma. However, use of a single amino acid tracer to calculate body protein kinetics may not adequately represent whole body protein metabolism.[15,16]

PROTEIN REQUIREMENTS

Methods for studying the protein requirements of any population (including preterm infants) include nitrogen balance, stable isotope tracer kinetics, and the factorial method. All these methods have advantages as well as inherent shortcomings and limitations.

Net gain or loss of protein, that is, the difference between nitrogen intake and nitrogen output, can be determined by the nitrogen balance technique. Although nitrogen intake can be determined precisely, accurate determination of all nitrogen losses, particularly losses from the skin and breath,[17,18] is difficult. In infants nitrogen output is usually measured as the sum of nitrogen lost in the urine and stool. Losses from the skin and breath are quantitatively small and either are not taken into account or are estimated.

The most commonly used stable isotope tracer method allows assessment of the dynamic aspects of protein metabolism and estimation of protein accretion as the difference between rates of synthesis and breakdown. Protein accretion calculated in this way usually is very close to retention calculated by a carefully conducted, conventional nitrogen balance.

The factorial method requires determining obligatory nitrogen losses (i.e., those that occur on a diet that meets energy needs but lacks protein) and adding to these the protein needs for growth, including a correction for incomplete use of dietary protein. This latter factor is usually assumed to be approximately 70% (as has been suggested by several sets of data).[19-21]

Alternatively, because human milk is considered the ideal food for term infants less than 6 months of age, the protein requirement of infants can be assumed to be the amount in the average intake of human milk by normally growing infants. This is the method used by the Food and Nutrition Board of the Institute of Medicine to establish an adequate intake of protein for term infants less than 6 months of age.

Numerous studies provide considerable insight into the growth and nutrient accretion rates incident to different protein intakes as well as the effects of these protein intakes on other indices of protein adequacy or excess. Thus it is possible to define the protein intake required to achieve various goals. In this regard it is quite clear that the protein intake of preterm infants fed a reasonable volume (i.e., 180 mL/kg/day) of either term (~2 g/kg/day) or preterm human milk (~2.5 g/kg/day) is insufficient to support the intrauterine rate of nitrogen accretion as recommended by the American Academy of Pediatrics Committee on Nutrition,[22] the Canadian Pediatric Society,[23] and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition.[24] These intakes also are insufficient to maintain plasma concentrations of albumin and transthyretin at more than 3.25 g/dL and 10 mg/dL, respectively.[25]

Increasing nitrogen retention has been reported with increasing protein intake.[26,27] It appears that a protein intake of 2.8 g/kg/day is close to the minimal intake needed to ensure rates of

TABLE 56-2

Composition of Available Preterm Infant Formulas

Component*	Similac Special Care Advance	Enfamil Premature Lipil
Protein (g/120 kcal)†	3.6	3.6
Fat (g/120 kcal)‡	6.5	6.1
Carbohydrate (g/120 kcal)§	12.4	13.2
Calcium (mg)	216	198
Phosphorus (mg)	120	100
Magnesium (mg)	14.4	10.8
Iron (mg)	2.2	2.2
Zinc (mg)	1.8	1.8
Manganese (μg)	14.4	7.6
Copper (μg)	300	144
Iodine (μg)	7.2	30
Sodium (mg)	51.6	69.6
Potassium (mg)	155	118
Chloride (mg)	97	108
Vitamin A (IU)	1500	1500
Vitamin D (IU)	180	288
Vitamin E (IU)	4.8	7.6
Vitamin K (μg)	14.4	9.6
Thiamin B_1 (μg)	300	240
Riboflavin B_2 (μg)	744	360
Vitamin B_6 (μg)	300	180
Vitamin B_{12} (μg)	0.66	0.3
Niacin (μg)	6000	4800
Folic acid (μg)	44.4	48
Pantothenic acid (μg)	2280	1440
Vitamin C (mg)	44.4	24

*Amount/120 kcal.

†Protein content of the formulas is composed of bovine milk and whey proteins with a 60:40 ratio of whey proteins–caseins.

‡Similac Special Care Advance contains medium-chain triglycerides and a mixture of soy and coconut oils, and Enfamil Premature Lipil contains medium-chain triglycerides and a mixture of soy and high-oleic vegetable oil; both formulas have less than 0.5% of *Crypthecodinium cohnii* oil and *Mortierella alpina* oil as source of docosahexaenoic acid (DHA) and arachidonic acid (ARA). The DHA and ARA content (mg/120 kcal) of Similac Special Care Advance is 16.3 and 26.1 and of Enfamil Premature Lipil is 20.4 and 40.8, respectively.

§Carbohydrate content of the formulas is lactose and corn syrup solids.

weight gain and nitrogen retention at least equal to intrauterine rates and also to maintain acceptable plasma albumin and transthyretin concentrations.[28,29] Moreover, the blood urea nitrogen concentration and the plasma concentration of most amino acids in infants receiving this intake are only minimally different from concentrations observed in infants fed preterm human milk.[25]

A protein intake of 3.5 to 4 g/kg/day results in rates of both weight gain and nitrogen accretion well in excess of intrauterine rates and also maintains acceptable plasma albumin and transthyretin concentrations.[28,29] Although blood urea nitrogen and plasma amino acid concentrations of infants fed these intakes are higher than those of infants fed lower intakes, blood urea nitrogen concentrations greater than 10 mg/dL are uncommon and the plasma concentration of only a few amino acids exceeds the concentration of cord plasma[30] or plasma obtained at fetoscopy.[31]

Today most LBW infants in the United States are fed either preterm human milk fortified with protein and other nutrients, one of several formulas designed specifically for LBW infants, or a combination of human milk and one of the preterm formulas. When volumes sufficient to provide an energy intake of 120 kcal/kg/day are fed, the LBW infant formulas available in the United States provide a protein intake of 3.6 g/kg/day (Table 56-2). Infants fed these formulas gain weight and retain nitrogen at rates equal to or slightly in excess of intrauterine rates,[32-34] without appreciable "metabolic stress." However, data indicate that most conventionally managed infants who weigh less than 1500 g at birth, although of appropriate size for gestational age at birth, weigh less than the 10th percentile of intrauterine standards at hospital discharge.[35] This finding suggests that most of these infants fail to compensate for the lack of growth or actual weight loss during the first 2 to 4 weeks of life.

The Life Sciences Research Office Expert Panel on Assessment of Nutrient Requirements for Preterm Infant Formulas[36] recommends minimum and maximum protein contents of 2.5 and 3.6 g/100 kcal, respectively. At an energy intake of 120 kcal/kg/day, these recommendations equate to protein intakes of 3 to 4.3 g/kg/day.

SPECIFIC AMINO ACID REQUIREMENTS

The intake of each indispensable amino acid required to maintain nitrogen equilibrium in adults[37,38] and, to a lesser extent, normal growth and nitrogen retention in term infants[39] has been defined as the amounts necessary to restore growth, nitrogen balance, and plasma concentration values observed before elimination of each amino acid from an otherwise adequate diet.

More recently a number of oxidation techniques using ^{13}C-amino acids (i.e., direct amino acid oxidation; 24-hour amino acid balance; indicator amino acid oxidation) have been used to determine indispensable amino acid requirements of adults,[40] children,[41] and parenterally fed term infants.[42] The theoretic basis of all these methods is that the nutritional indispensability of an amino acid is a function of its carbon skeleton; thus administration of a test amino acid labeled with ^{13}C results in production of breath $^{13}CO_2$, which is a measure of the irreversible oxidative loss. For the direct amino acid oxidation method, a break point in the relationship between carbon catabolism of the amino acid, that is to say, breath $^{13}CO_2$, and its intake is determined. When intake is less than the requirement, the amino acid is conserved and when the requirement is reached, oxidation increases.

The 24-hour amino acid balance method is similar but involves measurements over an entire 24-hour period rather than a shorter period thus encompassing both fed and fasting periods. It is used less frequently because it is labor and time intensive. Moreover, to measure balance, turnover must also be measured. The indicator amino acid oxidation method also is based on measurement of amino acid oxidation but measurements of the catabolism of a nonlimiting amino acid, the indicator amino acid, is used as the carbon analogue of nitrogen balance. If a single indispensable amino acid is provided at intakes of less than its requirement, the ability to retain other nonlimiting amino acids in body protein is limited. Thus the other amino acid, including the indicator amino acid, are present in excess and are oxidized. When intake of the test amino acid is zero, protein retention is minimal and oxidation of the indicator amino acid is maximal. As the intake of the test amino acid increases, oxidation of the test amino acid decreases until the requirement is reached, after which oxidation of the test amino acid is lower and essentially constant. Data are analyzed to obtain estimates of the intersection of the constant and linear portions of the relationship.

To date these methods have not been used to determine specific amino acid requirements of LBW infants. Rather, the requirements of this population have been estimated by a variety of other methods. Snyderman defined the amino acid requirements of the LBW infant as the amount of each provided by an intake of protein that results in a normal plasma amino acid pattern.[43] Based on observations of "normal" plasma concentrations of most amino acids in LBW infants fed 2 g/kg/day of bovine milk protein, the intake of each amino acid provided by this intake was proposed by Snyderman as the minimum requirement for that amino acid.

TABLE 56-3

Recommendations for Minimum and Maximum Amino Acid Requirements for Low-Birth-Weight Infants

Amino Acid Requirement*	Minimum (mg/kg/day)	Maximum (mg/kg/day)
Isoleucine	245	171
Leucine	434	303
Lysine	297	207
Methionine	69	48
Cyst(e)ine	95	66
Phenylalanine	172	120
Tyrosine	202	141
Threonine	202	141
Tryptophan	77	54
Valine	241	168
Histidine	103	72
Arginine	155	108
Alanine	163	114
Aspartate and asparagine	387	270
Glutamate and glutamine	791	552
Glycine	103	72
Proline	370	258
Serine	219	153

*Amino acid content of 3 g and 4.3 g of human milk proteins, the minimum and maximum protein contents (g/120 kcal) recommended for preterm infant formulas. Human milk amino acid composition is derived from references 47 to 50.

TABLE 56-4

Accretion of Amino Acids by the Human Fetus

Age (days)	160-180	180-220	200-220	220-240	240-260	260-280
Weight (kg)	0.5-0.9	0.9-1.4	1.4-1.9	1.9-2.4	2.4-2.9	2.9-3.4
	Amino Acid Accretion (mg/kg/day)					
Isoleucine	84	62	52	47	45	44
Leucine	181	133	112	102	97	96
Lysine	174	128	107	98	27	92
Methionine	49	35	29	27	25	25
Cyst(e)ine	—	—	—	—	—	—
Phenylalanine	101	74	62	56	53	53
Tyrosine	70	52	44	40	38	37
Threonine	101	74	62	57	54	53
Tryptophan	—	—	—	—	—	—
Valine	116	84	70	64	61	60
Histidine	64	46	39	36	34	33
Arginine	186	137	114	105	99	98
Alanine	176	129	107	99	93	92
Aspartate	220	161	135	123	117	116
Glutamate	317	231	194	178	168	166
Glycine	289	210	176	161	152	151
Proline	206	150	126	115	108	108
Serine	107	78	66	60	57	56
Total nitrogen	393	283	242	221	208	206

Data from Widdowson EM, et al: In Visser HKA (ed): *Fifth Nutricia Symposium: Nutrition and Metabolism of the Fetus and Infant.* The Hague, Martinus Nijhoff, 1979, pp 169-177.

Infants receiving this intake, however, have a low plasma concentration of lysine and a somewhat elevated plasma concentration of glycine, findings that are often associated with deficient protein intakes.[44] Thus considering these plasma amino acid data and the more recent evidence that a protein intake of approximately 2.8 g/kg/day is required to ensure intrauterine rates of nitrogen retention and weight gain as well as "normal" plasma protein concentrations (see earlier),[28] the amount of each amino acid provided by a protein intake of 2.8 g/kg/day seems more reasonable.

This essentially is the approach proposed by Fomon and Filer,[45] who defined the range of requirements of each amino acid for term infants as the range of intake of each amino acid provided by the amount of commonly used proteins sufficient to support normal growth. For the preterm infant, the range of specific amino acid requirements (per kilogram per day) may reasonably be assumed to encompass the amounts of each amino acid present in 2.8 g of human milk, bovine milk, or modified bovine milk protein. This approach relies heavily on the assumption that a protein intake of 2.8 g/kg/day is adequate, but this has been demonstrated only for modified bovine milk.[28]

The Life Sciences Research Office Expert Panel on Assessment of the Nutrient Requirements of Term Infant Formulas[46] defines the minimum and maximum contents of each amino acid for these formulas as the amount of each present in the amounts of human milk protein corresponding to the recommended minimum and maximum protein contents—1.7 and 3.4 g/100 kcal, respectively. The Expert Panel on Assessment of the Nutrient Requirements of Preterm Infant Formulas adopted the same approach, defining the minimum and maximum amino acid contents for preterm infant formulas (mg/100 kcal) as the amounts of each present in 2.5 and 3.6 g/100 kcal of human milk protein, the minimum and maximum protein contents (g/100 kcal) recommended for preterm infant formulas. Because the panel envisioned the energy intake of the "average" preterm infant to be 120 kcal/kg/day, these recommendations for the amino acid contents of preterm formulas can be translated to a range of requirements (mg/kg/day) as shown in Table 56-3.[47-50]

Widdowson and associates[51] suggested that the amino acid requirements of LBW infants could be estimated as the amount of each that accumulates during normal intrauterine development. These investigators also provided data concerning the accretion of almost all amino acids during intrauterine development, thus making this approach feasible. As illustrated in Table 56-4, the rates of amino acid accretion during different stages of intrauterine development vary somewhat. However, this variation results from variation in rates of protein accretion at different stages of development, rather than from variation in the pattern of amino acid deposited. Thus the amount of each amino acid deposited during any interval can be calculated by assuming a constant rate of growth over the interval of interest. The amount of each amino acid that accumulates as the fetus grows at a constant rate (~15 g/kg/day) from 900 to 2400 g and the estimated intake of each necessary to ensure deposition rates equal to those of the fetus, assuming retention of 70% of total protein intake, can be calculated. However, the latter estimate does not include an allowance for "maintenance" requirements or obligatory losses (i.e., the amounts necessary to maintain nitrogen equilibrium in the absence of nitrogen intake). According to a recent study, maintenance protein needs of preterm infants are 0.55 to 0.75 g/kg/day.[27] Assuming that the amino acid pattern of the maintenance protein needs is the same as that accumulating during development, the intake of each amino acid required to ensure intrauterine rates of growth and nitrogen retention can be calculated (shown in Table 56-5).

A major assumption inherent in this approach is that the various amino acids contribute proportionally to maintenance needs for protein and the roughly 30% of total nitrogen intake lost in the stool and urine. Another is that the increments of amino acids in the body during development reflect the sole need for amino acids. Both are unlikely to be completely valid. For example, as discussed in more detail later, the content of aromatic amino acids (phenylalanine, tyrosine, tryptophan) in most acute phase proteins is roughly double the content of these amino acids in endogenous protein stores. Hence the stressed infant may require greater intakes of these amino acids to support an adequate acute phase protein response to stress without increasing breakdown of endogenous protein stores.

TABLE 56-5

Amino Acid Requirements (mg/kg/day) of the Low-Birth-Weight Infant as Predicted from Fetal Rates of Amino Acid Accretion and Obligatory Losses

Amino Acid Requirement*	Accretion Rate (0.9-2.4 kg†)	Obligatory Losses	Estimated Requirement
Isoleucine	52	19	101
Leucine	113	41	220
Lysine	108	40	211
Methionine	30	11	59
Cyst(e)ine	—	—	—
Phenylalanine	62	23	121
Tyrosine	44	13	81
Threonine	62	23	121
Tryptophan	—	—	—
Valine	71	26	139
Histidine	39	14	76
Arginine	115	42	224
Alanine	109	40	213
Aspartate	136	50	266
Glutamate	196	72	383
Glycine	178	65	347
Proline	127	46	247
Serine	66	23	127
Total nitrogen	242	86	469‡

*Accretion rate plus obligatory losses/0.7, based on assumption that 70% of intake is retained (see text).

†Data concerning fetal accretion rate of cyst(e)ine, tryptophan, asparagine, and glutamine are not available.

‡Equivalent to a protein intake of 2.99 g/kg/day (i.e., 469 mg × 6.38).

TABLE 56-6

Comparison of Amino Acid Requirements (mg/kg/day) of Low-Birth-Weight Infants as Estimated from Intakes of Various Proteins (2.8 g/kg/day) and from Fetal Accretion Rates of Individual Amino Acids

	ESTIMATES BASED ON INTAKES*			Estimates Based on Accretion† and Obligatory Losses
	Human Milk	Bovine Milk	Modified Bovine Milk	
Isoleucine	160	134	151	101
Leucine	266	274	280	220
Lysine	196	213	235	211
Methionine	59	73	64	59
Cyst(e)ine	53	22	48	—
Phenylalanine	123	120	106	121
Tyrosine	132	123	101	81
Threonine	132	120	162	121
Tryptophan	48	25	48	—
Valine	171	165	157	139
Histidine	67	70	56	76
Arginine	118	98	84	224
Alanine	104	92	118	213
Aspartate	258	218	258	266
Glutamate	504	571	546	383
Glycine	73	53	53	347
Proline	221	277	227	247
Serine	140	154	154	127

*Amino acid content of various proteins provided by Ross Laboratories.

†Data concerning fetal accretion rate of cyst(e)ine, tryptophan, asparagine, and glutamine are not available.

Because of metabolic immaturity, preterm/LBW infants require some amino acids (e.g., cysteine and tyrosine) that are not considered indispensable for the older infant and adult. The basis for the preterm infant's and, perhaps, the young term infant's requirement for cysteine/cystine is thought to be low hepatic activity of cystathionase,[4-6] a key enzyme in conversion of methionine to cysteine. In addition, removal of cysteine/cystine from an otherwise complete synthetic diet has been shown to decrease rates of weight gain and nitrogen retention as well as plasma cysteine/cystine concentration, all of which return to control values when the cysteine/cystine intake of the control diet (85 mg/kg/day) is restored.[43] In this study, lower cysteine/cystine intakes (44 and 66 mg/kg/day) were not effective; hence it appears that the cysteine/cystine requirement of LBW infants is near 85 mg/kg/day.

The intake of tyrosine necessary to maintain control rates of weight gain and nitrogen retention as well as normal plasma tyrosine concentrations was shown in a similar fashion to be 50 mg/kg/day,[43] slightly less than the estimate based on the intrauterine accretion rate. The reason for the infant's requirement for tyrosine is not as clear as that for cysteine/cystine. Hepatic activity of phenylalanine hydroxylase, the key enzyme in conversion of phenylalanine to tyrosine, is substantial throughout the last two trimesters of gestation.[52] Moreover, studies using stable isotopes show that even very immature infants can convert phenylalanine to tyrosine.[53,54]

The probable requirement for each amino acid, estimated both as the amount provided by an intake of 2.8 g/kg/day of human milk, bovine milk, or modified bovine milk protein and as the amount theoretically necessary to ensure deposition of each at the intrauterine rate, is shown in Table 56-6. It is obvious that estimates of requirements derived by the various approaches are quite different. In general, the requirements for indispensable amino acids as estimated from the intake of 2.8 g/kg/day of human milk, bovine milk, or modified bovine milk protein are greater than those estimated from fetal accretion rates, whereas the requirements for many dispensable amino acids (arginine, alanine, and, particularly, glycine) estimated from this intake are lower. This finding raises the intriguing possibility that the dispensable amino acid content of proteins commonly used for feeding LBW infants may be limiting, particularly if the infant is unable to synthesize adequate amounts of one or more indispensable amino acid(s). Jackson and co-workers[55] reported that some preterm infants fed banked human milk do not form [^{15}N]urea from administered [^{15}N]glycine, suggesting that the availability of glycine is limited, although perhaps not sufficiently to interfere with growth. Miller and colleagues[56] showed that LBW infants also have a limited capacity for synthesizing proline from glucose.

Studies in animals and in human adults suggest that glutamine becomes conditionally essential during catabolic illness.[57] However, addition of glutamine to enteral feedings of very LBW infants results in no difference in growth, mean hospital stay, or prealbumin concentrations. On the other hand, feeding tolerance appears to be better, and the risk for infection lower in glutamine-supplemented infants.[58]

Further research concerning the LBW infant's requirement for individual amino acids is needed. Moreover, the requirements are likely to depend on the goal for which they are calculated. For example, if the goal is to produce rates of nitrogen retention and weight gain in excess of the intrauterine rate, the additional requirement for each amino acid over that required to support intrauterine rates of nitrogen retention and weight gain will be greater in proportion to the increase in protein intake necessary to achieve the goal.

AMINO ACID REQUIREMENTS FOR PARENTERAL NUTRITION

Because at least 10% of the nitrogen content of most enterally ingested intakes is lost in stool whereas fecal loss of parenteral nitrogen intake is minimal, it follows that the minimal parenteral nitrogen intake required to support the intrauterine rate

of nitrogen retention should be less than the minimal enteral intake. However, this does not appear to be the case. Zlotkin and co-workers[59] found that the minimal parenteral amino acid intake required to achieve the intrauterine rate of nitrogen retention was 3 g/kg/day, approximately 10% more than the required minimal enteral intake (2.8 g/kg/day).[28] The reason appears to be greater urinary nitrogen losses with parenteral versus enteral nutrition. Although 70% or more of most enteral intake is retained despite fecal losses of at least 10%,[19-21] no more than 60% to 65% of most parenteral nitrogen intakes is retained (see later) despite minimal fecal losses. This finding suggests that the quality of available parenteral amino acid mixtures is lower than that of human milk and the proteins commonly used in infant formulas.

In contrast to the relative lack of differences in the overall amino acid pattern of the proteins commonly used in enteral feeding of LBW infants (Table 56-7), the overall quality of available parenteral amino acid mixtures varies considerably, as shown in Table 56-8. In large part, the plasma amino acid pattern of infants receiving parenteral nutrition infusates reflects the pattern of the amino acid mixture used to formulate the infusate.[60,61] Most of the older mixtures, now referred to as general purpose mixtures, result in low plasma concentrations of several indispensable amino acids (e.g., the branched-chain amino acids, tyrosine, and cysteine/cystine) and high concentrations of others (e.g., phenylalanine, methionine), as well as elevated concentrations of some dispensable amino acids (e.g., glycine).[60] Based on conventional theory, these mixtures provide insufficient amounts of the amino acids that are present in low concentrations in plasma and excessive amounts of those that are present in high concentrations.

The indispensable amino acid pattern of most early parenteral amino acid mixtures mimicked the pattern of a high-quality dietary protein (e.g., egg yolk protein) and little attention was given to the dispensable amino acid pattern. Glycine comprised the bulk of the dispensable amino acid content of many mixtures (see Table 56-8). Parenteral amino acid mixtures designed for use in pediatric patients are available. The amino acid pattern of one of these (Neopham, Kabi-Vitrum, Sweden) is similar to that of human milk protein. The amino acid pattern of another mixture (TrophAmine, B. Braun Laboratories, Irvine, CA) is that predicted by a mathematic model to result in plasma concentrations of all amino acids similar to those of normally growing, 30-day-old, breast-fed, term infants. The pattern of yet another pediatric parenteral amino acid mixture (Aminosyn-PF, Abbott Laboratories, Chicago, IL) is a modification of the pattern of the older, general purpose mixture, Aminosyn (Abbott Laboratories).

These pediatric parenteral amino acid mixtures, like human milk protein and the combinations of bovine milk proteins used in most LBW infant formulas (see earlier discussion), provide more indispensable and less dispensable amino acids than the protein deposited by the developing fetus (Table 56-9). Whether a parenteral amino acid mixture with a pattern identical or similar to that of the protein deposited by the fetus may be used more efficiently is not known. The amino acid pattern of TrophAmine is that predicted mathematically to result in normal plasma

TABLE 56-7

Amino Acid Patterns (mg/g) of Proteins Commonly Used to Feed Low-Birth-Weight Infants and of the Developing Fetus

Amino Acid	Human Milk	Bovine Milk	Modified Bovine Milk	Fetal Tissue
Isoleucine	57	48	54	35
Leucine	95	98	100	75
Lysine	70	76	84	72
Methionine	21	26	23	20
Cyst(e)ine	19	8	17	—
Phenylalanine	44	43	38	41
Tyrosine	47	44	36	29
Threonine	47	43	58	41
Tryptophan	17	9	17	—
Valine	61	59	56	47
Histidine	24	25	20	26
Arginine	42	35	30	77
Alanine	37	33	42	72
Aspartate	92	78	92	90
Glutamate	180	204	195	130
Glycine	26	19	19	118
Proline	79	99	81	84
Serine	50	55	55	41

TABLE 56-8

Amino Acid Content (mg/g) of Commercially Available Amino Acid Mixtures

Amino Acid	Aminosyn (Abbott)	Aminosyn-PF (Abbott)	Travasol(B) (Baxter)	FREAmine III (B. Braun)	Trophamine (B. Braun)	Neopham (Kabi-VITRUM)
Isoleucine	72	76	48	70	82	49
Leucine	94	119	62	91	140	110
Lysine	72	68	58	73	82	83
Methionine	40	18	58	53	33	20
Phenylalanine	44	43	62	56	48	42
Threonine	52	52	42	40	42	56
Tryptophan	16	18	18	15	20	22
Valine	80	64	46	66	78	56
Histidine	30	32	44	30	48	33
Cystine	0	0	0	0	<3	0
Tyrosine	9	6	4	0	23*	8
Taurine	0	7	0	0	2	5
Alanine	128	70	207	70	53	99
Aspartate	0	53	0	0	32	64
Glutamate	0	82	0	0	50	111
Glycine	128	38	207	140	37	33
Proline	86	82	42	112	68	88
Serine	42	50	0	59	38	59
Arginine	98	123	103	95	122	64
Total	991	1001	1001	970	995	1002

*As 17 mg tyrosine and 50 mg *N*-acetyl-L-tyrosine.

concentrations of all amino acids. Hence, by this criterion, the infant appears to be able to convert the indispensable amino acids to the needed dispensable amino acids. Exceptions include cysteine/cystine and tyrosine.

Because tyrosine and cystine are insoluble and cysteine is unstable in aqueous solution, none of the mixtures contains more than trace amounts of these two amino acids. TrophAmine contains *N*-acetyl-L-tyrosine, a soluble tyrosine derivative, but approximately 50% of this derivative is excreted unchanged in urine and is not available to meet the apparent tyrosine needs.[62,63] Because both tyrosine and cysteine/cystine are thought to be indispensable for the infant, their absence in parenteral amino acid mixtures may be cause for concern.

Cysteine/cystine, in addition to being a component of protein, also is a component of glutathione, a tripeptide that is an important biologic antioxidant. The plasma concentration of this antioxidant is low in LBW infants,[64] and although cysteine/cystine supplementation does not appear to increase plasma glutathione concentration,[65] it does increase whole blood glutathione concentration.[66] Because oxidant stress has been implicated in several neonatal disorders, clarification of the role of cysteine/cystine in maintaining plasma and tissue levels of glutathione is important. The lack of adequate tyrosine in parenteral amino acid mixtures may also be important. As discussed, because of the high content of tyrosine in most acute phase proteins, inadequate tyrosine intake may necessitate endogenous protein breakdown to supply adequate tyrosine for ongoing synthesis of these important proteins.

Few studies have examined either the relative efficacy of the available pediatric parenteral amino acid mixtures or the efficacy of any of these mixtures versus that of the general purpose parenteral amino acid mixtures. One exception is the study of Helms and co-workers,[67] showing that LBW infants who received a regimen containing TrophAmine gained weight more rapidly and retained a greater percentage of administered nitrogen than did similar infants who received an isocaloric, isonitrogenous regimen containing a general purpose parenteral amino acid mixture (i.e., 78% versus 66%). The nutritional benefits of TrophAmine were attributed to a greater intake of tyrosine (as *N*-acetyl-L-tyrosine) and cysteine/cystine (as cysteine hydrochloride, added when the infusate was prepared). However, other explanations, including the more normal plasma amino acid pattern of the group that received the TrophAmine regimen, are equally feasible. This latter explanation was proposed by Duffy and co-workers[68] to explain the better utilization of a crystalline amino acid mixture versus a protein hydrolysate.

TABLE 56-9

Amino Acid Pattern (mg/g) of Pediatric Parenteral Amino Acid Mixtures and the Developing Fetus

Amino Acid	Aminosyn PF	Trophamine	Neopham	Fetal Protein
Isoleucine	76	82	49	35
Leucine	119	140	110	75
Lysine	68	82	83	72
Methionine	18	33	20	20
Cyst(e)ine	0	3	0	—
Phenylalanine	43	48	42	41
Tyrosine	6	23	8	29
Threonine	52	42	56	41
Tryptophan	18	20	22	—
Valine	64	78	56	47
Histidine	32	48	33	26
Arginine	123	122	64	77
Alanine	70	53	99	72
Aspartate	53	32	64	90
Glutamate	82	50	111	130
Glycine	38	37	33	118
Proline	82	68	88	84
Serine	50	38	60	41

FACTORS AFFECTING PROTEIN UTILIZATION

Many factors can affect protein and amino acid metabolism and, hence, protein and amino acid requirements. These include clinical status, quality of protein intake, concomitant energy intake (quantity and quality), concomitant intake of other nutrients, and some hormones. Each of these factors is discussed in the sections that follow.

Clinical Status

Clinical status affects protein metabolism and requirements for protein as well as requirements for all other nutrients. Although data from infants are limited, it is likely, as has been established for adults, that any type of stress increases protein requirements. In adults, trauma and sepsis result in increased protein degradation (proteolysis) and, as reflected by higher rates of urinary nitrogen excretion and higher rates of oxidation of essential amino acids, irreversible catabolism of free amino acids.[69,70] The severity of trauma also is directly associated with nitrogen excretion.[71,72] The increased nitrogen excretion incident to stress appears to be mediated, in part, by catecholamines and glucocorticoids. Concentrations of both are elevated in stressed subjects, and increased urinary nitrogen excretion has been reported after infusion of these hormones in healthy volunteers.[73] However, although the infusion of glucocorticoids alone results in increased protein degradation,[74] infusion of catecholamines alone does not have a significant effect on proteolysis.[75]

One mechanism by which stress increases urinary nitrogen losses may be related, in part, to synthesis of acute phase proteins. Waterlow[76] estimated that the mixed acute phase protein response of an infected adult is approximately 1.2 g/kg/day. Although similar data for neonates are not available, it is likely that the response of term as well as preterm infants is similar qualitatively, if not quantitatively. Because maintenance of an appropriate acute phase protein response obviously is important for survival, the amino acids needed to support this response, if not provided exogenously, must be derived from breakdown of endogenous protein stores. Reeds and colleagues[77] estimated that roughly 2 g of endogenous protein must be mobilized to support an acute phase protein response of 1 g. Primarily this is because the aromatic amino acid content of endogenous protein stores is only about half that of most acute phase proteins. Because the other amino acids released from endogenous protein stores cannot be used for endogenous protein synthesis, they are catabolized, thereby increasing urinary nitrogen excretion. The specific cellular and molecular mechanisms involved have not been completely elucidated.

The extensive studies of Knutrud[78] in the 1960s demonstrated that infants subjected to operative stress respond qualitatively in the same manner as adults but to a lesser degree. Although the subjects of these studies were primarily term infants (<1 year of age), there is little reason to believe that the LBW infant does not behave similarly. More recent studies[79] indicated that rates of whole body amino nitrogen flux, synthesis, and breakdown in postsurgical LBW infants are approximately 25% higher than expected. Boehm and colleagues,[80] comparing the metabolic responses of LBW infants (birth weight <1500 g) who experienced bacterial sepsis during the first week of life with those of healthy control infants and infants with respiratory distress syndrome, found that the urinary nitrogen excretion of infants with septicemia was double that of the other groups. In addition, decreasing nitrogen balance with increasing degree of illness has been reported in infants, with the lowest balances observed in infants with sepsis.[81]

Although many clinicians maintain that it is impossible, regardless of nitrogen intake, to achieve positive nitrogen balance during periods of stress, it is clear that this is not the case. Positive nitrogen balance is achievable in stressed patients, even in adults with severe burns; however, the nitrogen intakes required to achieve positive balance are considerable. The LBW infant with sepsis, for example, would require a nitrogen intake of at least 400 mg/kg/day (equivalent to a protein or amino acid intake of approximately 2.5 g/kg/day) to achieve nitrogen equilibrium and an even greater intake to promote nitrogen accretion. Whether provision of smaller amounts of specific amino acids (e.g., aromatic amino acids as discussed earlier) would be beneficial remains to be determined.

Reasonably stable LBW infants who receive no exogenous nitrogen intake during the first week of life excrete, on average, 150 to 200 mg/kg/day of nitrogen,[26,82-85] equivalent to a daily protein loss of 0.94 to 1.25 g/kg, or about 1% of endogenous protein stores. However, these data must be interpreted cautiously because it is likely that the study populations included some nonstressed as well as some moderately (but not severely) stressed infants. Thus the daily nitrogen losses of some infants may be considerably greater and those of others considerably smaller. However, in the studies cited, a parenteral amino acid intake of 1.5 to 2.5 g/kg/day effectively reversed negative nitrogen balance and, in fact, resulted in achievement of positive balance, although not of the magnitude achieved in older, growing, enterally fed infants. Because conditions associated with greater endogenous excretion of nitrogen are usually short-lived (e.g., infection), such intakes over the short term may be adequate.

Based on the usual interpretation of nitrogen balance, a parenteral amino acid intake of 1.5 g/kg/day should preserve lean body mass and a higher intake should support at least modest rates of protein accretion. Further, because the catecholamines, glucocorticoids and cytokines released in response to stress also interfere with utilization of energy substrates, particularly carbohydrate, it is unlikely that stressed infants can tolerate the energy intake required for normal growth. If the period of stress is not long, the effects of less than optimal anabolism for a short time usually can be corrected once the infant has recovered from the precipitating clinical insult. Conversely, if the infant can tolerate a nonprotein energy intake of 80 to 90 kcal/kg/day, a greater amino acid intake very likely will result in the intrauterine rate of nitrogen retention.[59]

Although it is clear that the rate of weight gain of infants with chronic conditions such as congenital heart disease and chronic pulmonary disease is less than optimal, it is not clear that the nutrient requirements of these infants are much greater than those of healthy infants. A thorough evaluation of the cause of the less than optimal rate of weight gain often reveals that the infant's intake is less than that of the normally growing infant with no underlying disease. Thus although the protein requirement of infants with these conditions, as well as their requirements for other nutrients, may be somewhat greater than the requirements of the healthy infant, it is unlikely that they are considerably greater. However, the data on which this statement is based are scarce.

Quality of Protein Intake

An important factor in defining the protein requirement of any population is the overall quality of the protein intake. In large part this is a function of the amino acid pattern of the protein. For example, the initial use of soy protein formulas resulted in lower rates of weight gain and nitrogen retention than did the use of formulas with a similar content of bovine milk protein.[86] Hence the intake of soy protein required to produce a specific rate of nitrogen retention was considerably greater than the required intake of bovine milk protein. The reason for this was related to the lower methionine content of soy versus bovine milk protein. Modern soy protein preparations are supplemented with methionine, and their intake results in rates of weight gain and nitrogen retention equivalent to those resulting from a similar intake of bovine milk protein.

LBW infants fed similar intakes of human milk protein, unmodified bovine milk protein, and modified bovine milk protein (whey predominant) experience similar rates of both weight gain and nitrogen retention. Thus the overall quality of these three proteins appears to be similar. Although the amino acid patterns of the three proteins differ somewhat (see Table 56-7), all contain a greater percentage of indispensable amino acids than does the protein synthesized by the fetus during development; further, all contain more of each indispensable amino acid as a percentage of total indispensable amino acid content than does newly synthesized fetal protein. An intake of only approximately 2 g/kg/day of any of the three proteins provides the requirements of all indispensable amino acids, as estimated from fetal accretion rates of individual amino acids (see Table 56-5). This intake does not provide sufficient amounts of some dispensable amino acids (e.g., glycine, alanine, and arginine) to ensure intrauterine accretion rates. This finding raises the intriguing possibility that these proteins may be limiting with respect to dispensable amino acids, particularly if energy intake is sufficient to favor maximal protein deposition. Indeed, as discussed, Jackson and co-workers,[55] studying [^{15}N]glycine turnover in infants fed human milk, found that glycine intake was inadequate (see previous discussion).

Despite the apparent lack of difference in the overall quality of modified and unmodified bovine milk proteins, most available LBW infant formulas contain modified bovine milk protein. One reason for this is that the plasma amino acid pattern of infants fed modified versus unmodified bovine milk protein is somewhat more desirable. For example, because plasma tyrosine concentrations during infancy appear to be related inversely with neurodevelopmental outcome,[87] the lower plasma tyrosine concentration of infants fed modified (i.e., whey predominant) versus unmodified bovine milk protein[88] may be desirable. In addition, because cysteine/cystine is thought to be an indispensable amino acid for the LBW infant and, perhaps, the term infant, the greater cysteine/cystine content of modified bovine milk protein and the associated higher plasma cysteine/cystine concentrations of infants fed this protein[88] may be another advantage of modified bovine milk protein. Additional data suggest that metabolic acidosis may be less common in infants fed modified versus unmodified bovine milk protein.[89] Modified bovine milk protein, conversely, results in higher plasma threonine concentrations than either human milk or unmodified bovine milk protein.[88]

The quality of protein intake needed in all situations may not be the same. For example, if more aromatic amino acids are needed to support the acute phase protein response to stress (see earlier discussion), a protein with an amino acid pattern resembling the endogenous protein stores of the developing fetus is not likely to be the best quality to provide during periods of stress. However, until the possibility that increasing the intake of aromatic amino acids during stress reduces endogenous nitrogen losses is tested directly, recommendations cannot be made.

Concomitant Energy Intake

The effect of concomitant energy intake on protein, or nitrogen, utilization has been studied extensively in animals and to some extent in human adults,[90,91] but few data from human infants are available. The usual concept is that any nitrogen intake is used better if it is accompanied by a higher versus a

lower concomitant energy intake. For any specific nitrogen intake, however, there is a concomitant energy intake beyond which further increases will not improve nitrogen retention. Although the few reported studies of the effect of energy intake on nitrogen utilization of LBW infants fall short of defining the precise quantitative aspects of this interaction, all suggest that the same principle outlined earlier applies.

Zlotkin and associates,[59] studying LBW infants receiving parenterally delivered nutrients, found that utilization of amino acid intakes of both 3 and 4 g/kg/day was greater with a concomitant energy intake of 80 versus 50 kcal/kg/day. Duffy and co-workers[68] and Pineault and associates[92] also observed a beneficial effect of intakes of 80 to 90 versus 60 to 70 kcal/kg/day on utilization of parenterally delivered amino acid intakes of 2.5 to 2.9 g/kg/day.

Kashyap and co-workers[29] found no statistically significant difference in nitrogen retention of LBW infants fed an enteral protein intake of 3.5 to 3.6 g/kg/day with a concomitant energy intake of 150 vs 120 kcal/kg/day. However, the blood urea nitrogen concentration and the plasma concentration of several amino acids were somewhat lower in the group that received the higher energy intake, suggesting that this energy intake was at least minimally beneficial in enhancing nitrogen utilization. In subsequent studies these investigators found that nitrogen retention of infants who received enteral protein intakes of either 3.8 to 3.9 g/kg/day or 4.2 to 4.3 g/kg/day with a concomitant energy intake of 140 to 150 kcal/kg/day was significantly higher than that of infants who received the same protein intakes with a concomitant energy intake of 120 kcal/kg/day.[28,93]

These data from LBW infants, summarized in Table 56-10, are insufficient to permit definitive conclusions concerning the precise energy intake required to ensure maximal utilization of a specific protein or amino acid intake. Nonetheless, it appears that a concomitant energy intake of 80 kcal/kg/day is sufficient to ensure near-maximal utilization of a parenterally administered amino acid intake of 2.7 g/kg/day and that a concomitant energy intake of 60 kcal/kg/day is sufficient to ensure reasonable utilization. Similarly it appears that a concomitant energy intake of 120 kcal/kg/day is sufficient to ensure at least near-maximal utilization of an enterally administered protein intake of 3.6 g/kg/day.

TABLE 56-10

Nitrogen Retention and Weight Gain of Low-Birth-Weight Infants Receiving Various Protein (Amino Acid) and Energy Intakes

Protein (Amino Acid) Intake (g/kg/day)	Energy Intake (kcal/kg/day)	Nitrogen Retention (mg/kg/day)	Weight Gain (g/kg/day)
Zlotkin et al[59]			
3	50	256 ± 20	2 ± 4
3	80	320 ± 8	16.2 ± 2.4
4	55	274 ± 11	1.5 ± 3.2
4	80	432 ± 21	15.6 ± 1.9
Duffy et al[68]			
2.6	70	214	—
2.9	96	284	—
Pineault et al[92]*			
2.7	60	220	11.5 ±3.2
2.7	80	248	14.6 ± 2
Kashyap et al[28, 29]			
3.6	120	422 ± 22	18.3 ± 2.8
3.5	150	425 ± 12	22 ± 3.1
3.8	120	420 ± 31	19.1 ± 3.2
3.9	142	473 ± 20	21.5 ± 2.2

*Data from high-fat (3 g/kg/day) groups at each energy intake are combined; there were no effects of quality of energy intake on either nitrogen retention or weight gain.

Extrapolating from these data, it seems safe to conclude that utilization of the amino acid content of a parenterally administered regimen will be efficient, although perhaps not maximally efficient, if the energy–amino acid ratio of the regimen is at least 20 to 25 kcal/g and, similarly, that utilization of the protein content of an enterally administered regimen will be efficient if the energy-protein ratio of the regimen is at least 30 kcal/g. These energy-protein ratios, however, may not be optimal for maximal utilization of protein and amino acid intakes outside the range for which they were calculated. In addition, the ratio may be different if the distribution of energy intake between carbohydrate and fat is different from the distribution of these nutrients in the regimens for which the ratios were calculated. In this regard, there is considerable evidence that carbohydrate is more effective than fat in promoting nitrogen retention.[91,94,95] There is no apparent benefit of an energy intake much in excess of that necessary to ensure utilization of the concomitant protein intake. Such intakes result simply in excessive fat deposition relative to protein deposition.[21,28,96-99]

Concomitant Intake of Other Nutrients

In theory, an inadequate intake of any nutrient required for production of new tissue also limits the extent to which protein can be deposited as new tissue. The best example of this phenomenon is a study in adults receiving only parenterally administered nutrients showing that withdrawal of sodium, potassium, or phosphorus from an otherwise complete parenteral nutrition regimen resulted in retention of less nitrogen and a lower rate of weight gain.[100] There is no reason to believe that this result is not applicable to all subjects. If so, an inadequate intake of any nutrient required for production of new tissue is likely to interfere with utilization of protein and amino acid intake.

Kashyap and colleagues[29] suggested this possibility to explain apparent differences between the results of their studies and those of Räihä and associates.[89] The latter investigators found no difference in rates of weight gain of LBW infants fed protein intakes of 2.25 versus 4.5 g/kg/day (with an energy intake of 120 kcal/kg/day), whereas Kashyap and colleagues[29] found a marked difference in rates of weight gain as well as nitrogen retention between infants fed protein intakes of 2.25 g/kg/day versus 3.6 g/kg/day, both with an energy intake of 120 kcal/kg/day. The only apparent difference between the two studies concerned sodium and phosphorus intakes, which were considerably lower in infants studied by Räihä and associates.[89] Based on the intrauterine relationships between sodium and phosphorus accretion and the accretion of nitrogen,[101] the sodium and phosphorus intakes of the infants studied by Räihä and associates, even if 100% absorbed, were insufficient to permit deposition of the higher protein intake. In contrast, the sodium and phosphorus intakes of the infants studied by Kashyap and colleagues were generous, and retention of sodium and phosphorus relative to the retention of nitrogen was roughly the same as the intrauterine relationships.

Unfortunately, few data concerning this potentially important relationship are available. Nonetheless, it seems reasonable to conclude that inadequate intake of any nutrient required for synthesis of new tissue is likely to inhibit utilization of nitrogen for production of lean body mass. If so, excessive losses of nutrients required for synthesis of new tissue (e.g., electrolyte losses with chronic diuretic usage) also are likely to inhibit utilization of protein and amino acids for production of new tissue.

Hormones

Insulin and insulin-like growth factor I (IGF-I) are important regulators of fetal and postnatal growth.[102] Secretion of insulin is dependent on the plasma concentration of glucose as well

as that of certain amino acids, namely arginine and leucine. In adults, reduction in proteolysis has been reported with plasma insulin concentrations within the physiologic as well as the pharmacologic range.[103,104] In newborn infants, pharmacologic concentrations of insulin (79 ±13 μU/mL) result in reduction of proteolysis, but concentrations within the physiologic range do not.[105] Further, the anabolic effect of insulin on protein synthesis has not been demonstrated consistently in infants, possibly because the effect of insulin on protein synthesis may be underestimated if its effect on protein degradation significantly reduces amino acid availability.

IGF-I also has been shown to enhance protein anabolism.[106] Although IGF-I infusion under conditions of adequate substrate supply enhances protein synthesis, it does not affect proteolysis,[107] whereas insulin infusion appears to decrease proteolysis without affecting protein synthesis. When amino acid concentrations are maintained, infusions of both insulin and IGF-I increase protein accretion of the ovine fetus, but the greatest increase is observed with combined infusion.[108]

Growth hormone (GH) increases lean body mass and promotes growth of GH-deficient individuals.[109,110] It also stimulates muscle and whole body protein synthesis of healthy adults.[111] At least some of these effects of GH are thought to be mediated through IGF; however, the exact mechanism for GH-stimulated protein synthesis remains unclear. Although GH has been shown to prevent glucocorticoid-induced protein wasting in adults,[112] administration of recombinant human GH does not appear to prevent the catabolic side effects of dexamethasone in extremely low birth weight infants with bronchopulmonary dysplasia.[113]

CONCLUSION

Given the sparsity of available data and the fact that much of the data available are confusing and often in conflict with other data, it is difficult to make concrete statements concerning the LBW infant's requirement for protein and amino acids. However, the available data appear to support the following conclusions:

1. The "average" LBW infant who receives no nitrogen intake loses approximately 1% of endogenous protein stores daily, or approximately 1.25 g/kg.
2. A parenteral amino acid intake of approximately 3 g/kg/day or an enteral protein intake of about 2.8 g/kg/day is required to achieve the intrauterine rate of nitrogen accretion.
3. Enteral protein intakes as high as 4.2 to 4.3 g/kg/day are well tolerated by LBW infants and result in rates of weight gain and nitrogen accretion that exceed intrauterine rates.
4. An enteral protein intake in excess of 3.5 to 3.6 g/kg/day is not used maximally unless accompanied by an energy intake greater than 120 kcal/kg/day.
5. There are no major differences in the overall quality of the proteins used in modern preterm infant formulas. In addition, the biologic quality of these proteins differs minimally, if at all, from that of human milk protein.
6. The overall quality of most available parenteral amino acid mixtures appears to be less than optimal.

If these conclusions are warranted it seems reasonable to conclude further that the LBW infant who can tolerate enteral feedings immediately after birth requires a protein intake of at least 2.8 g/kg/day to ensure continued accretion of nitrogen at the intrauterine rate. If the infant cannot be fed enterally, the parenteral amino acid intake required to achieve the same goal is about 3 g/kg/day (this requirement may be somewhat less if a parenteral amino acid mixture designed specifically for infants is used; however, further data are required to confirm the superiority of the pediatric mixtures).

For various reasons some LBW infants receive little or no protein or amino acids for the first few days of life. Because these infants lose at least 1% of their endogenous protein stores daily (or >1 g/kg/day), their subsequent protein and amino acid needs are greater than required simply to support the intrauterine rate of protein accretion. Otherwise this early loss will not be replaced until well after discharge. In other words if the infant receives no protein or amino acid intake for the first several days of life, the minimum requirement is no longer simply the amount required to ensure the intrauterine rate of nitrogen accretion; rather, it is this amount *plus* that required to replace the earlier endogenous losses.

Viewed in this way, the protein (amino acid) requirement of individual infants is variable, depending on the extent of endogenous losses prior to feeding. For example, the infant who receives no amino acid or protein intake for the first week of life sustains endogenous protein losses during this time of at least 7 to 8 g/kg. Assuming that only 70% of intake is retained, these losses increase the infant's subsequent enteral protein requirement by at least 10 g/kg. If this loss is replaced over a period of 40 days, the daily increment in protein requirement is about 0.25 g/kg. If it is replaced over a period of 20 days, the daily increment, of course, is 0.5 g/kg. Thus once the "average" LBW infant is able to tolerate full enteral feeding, it is likely that the protein requirement will be at least 3 g/kg/day. The protein content of available LBW infant formulas is sufficient to provide the minimal protein needs (assuming, of course, that a sufficient volume to provide 120 kcal/kg/day is ingested). A greater protein intake is likely to be tolerated by most LBW infants. Moreover, a greater protein intake also will result in a greater rate of weight gain. Such an intake therefore may be particularly desirable for the infant who has sustained extensive endogenous protein losses.

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SECTION IX

Thermoregulation

57

Gordon G. Power and Arlin B. Blood

Perinatal Thermal Physiology

The temperature of any living organism at equilibrium with its surroundings is a balance between heat produced as an inevitable product of metabolism within the organism and the net movement of heat from the organism into the environment. Regulation of body temperature is accomplished by a combination of control over rates of heat flow to the environment and heat production by the body. Newborns and adults use a number of sensory and control mechanisms to balance heat loss with production in order to hold core body temperature relatively constant over a wide range of environmental temperatures. For example, in response to a cold environment, blood flow patterns are altered in order to minimize heat loss, and metabolic processes are increased for the sole purpose of generating additional warmth. Indeed, thermoregulatory responses of endothermic animals are extremely sensitive, constituting one of the most tightly controlled feedback systems known to biology.

At birth the thermoregulatory responses of the mammalian newborn are rapidly activated in order to defend core body temperature against dramatic heat loss due to evaporation of amniotic fluid from the skin and decrease in ambient temperature. Although thermogenic mechanisms are developed in the fetus weeks before birth, they are held for the most part inactive in utero. As a result, the fetus can be considered ectothermic, its temperature fluctuating passively with that of the uterine environment. Indeed, as is discussed in this chapter, heat transfer from the fetus to the mother is so efficient and tightly linked to gas exchange in the placenta that any attempts by the fetus to alter the fetal-maternal temperature gradient would be futile. Much of what is known about fetal and neonatal thermoregulation has been learned using the chronically instrumented sheep as an experimental model, due in part to its large size and tolerance to surgical instrumentation. Comparative studies in other mammals, including humans, suggest that most observations made in sheep can be extrapolated qualitatively to the human. This chapter discusses (1) fetal metabolic heat production and the pathways of heat exchange between the fetus and mother in utero; (2) the metabolic rate of the fetus and placenta and how they pertain to fetal heat production and oxygen consumption; (3) the development and control of thermoregulatory mechanisms in the fetus; and (4) factors controlling thermogenesis in utero and during parturition and in the immediate newborn period.

BASAL FETAL HEAT PRODUCTION AND DISSIPATION

Because the mother provides a warm environment for the fetus in utero, it is tempting to think of heat as flowing from mother to fetus. In fact, the net flow of heat between the two is from fetus to mother due to heat production by the fetus (Figure 57-1). For purposes of this chapter, it is helpful to note that "heat production" is a generic term referring to the generation of heat as a by-product of any biochemical reaction, whereas "thermogenesis" is a more specific term referring to biochemical reactions whose primary purpose is to generate heat to maintain core body temperature. As is discussed later, fetal thermogenesis is suppressed in utero such that fetal heat production stems solely from biochemical processes vital to the growth and function of the fetus. During the last third of gestation, the fetus produces heat at a rate of 3 to 4 W • kg-1, roughly twice that of the adult. At this rate, balanced with the resistance to flow of heat from fetus to mother, fetal body temperature is maintained at approximately 0.5° C greater than that of the mother.

Basal Fetal Heat Production

Fetal heat production has been measured experimentally by two different experimental methods, *indirect* calorimetry and *differential* calorimetry. More than 200 years ago it was observed that fertile chicken eggs produced more heat and consumed more oxygen than did spoiled ones.[1] It has since become widely accepted that these two variables are directly related to each another in a proportion that remains constant across most aerobic organisms, such that 4.9 calories (20.5 joules) of heat are produced for every milliliter of oxygen consumed. Using this assumption, the method of indirect calorimetry calculates fetal heat production based on measurements of oxygen consumption. Measured oxygen consumption of the fetal sheep is approximately 8 mL • min^{-1} • kg^{-1} during the last third of gestation.[2] Thus by indirect calorimetry, fetal heat production can be estimated at 39.2 calories • min^{-1} • kg^{-1}, or 2.7 W • kg^{-1}.

The other means of measuring fetal heat production, differential calorimetry, relies on measurement of changes in the fetal-maternal temperature gradient following administration of known quantities of heat to the fetus.[3] The method is based on the idea that when maternal temperature and resistances to heat flow between the fetus and mother are stable, only changes in fetal heat production can alter the fetal-maternal temperature gradient. Administration of heat into the core of the fetus widens the fetal-maternal temperature gradient just as normal metabolic heat production by the fetus does. As a result, the ratio of a known quantity of heat administered centrally to the fetus and the resulting change in fetal temperature is equal to the ratio of overall fetal metabolism and the fetal-maternal temperature gradient as follows:

$$H_{heater} / \Delta T_{heater} = H_{fetus} / \Delta T_{fetus}$$

where H_{heater} is the known amount of heat produced by a heater implanted within the fetal body, ΔT_{heater} is how much the implanted heater raises fetal temperature above baseline, H_{fetus} is the amount of heat produced by the fetus, and ΔT_{fetus} is the fetal-maternal temperature gradient. The heat production of the fetus is derived by solving the equation for H_{fetus}. In using this equation, experiments in sheep have resulted in measurements of 3.1 W • kg^{-1}, which is within 15% of measurements by indirect calorimetry.[3]

Pathways of Fetal Heat Dissipation

Heat travels within the body by either conduction (the diffusive transmission of heat through matter) or convection (the transfer of heat within moving fluids, i.e., blood). Therefore heat

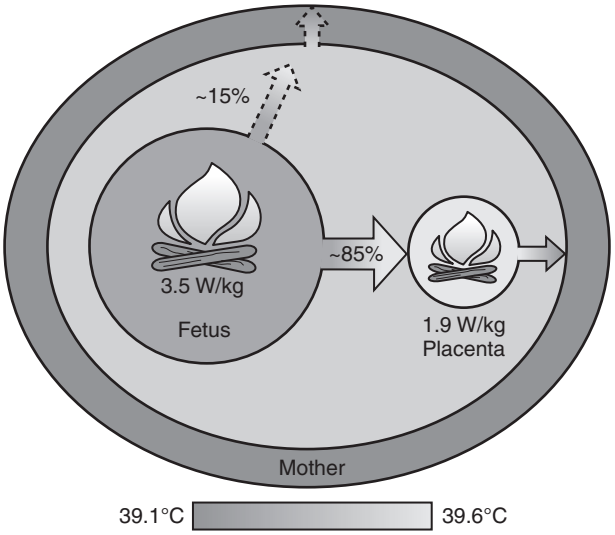


Figure 57-1 A stylized illustration of the net movement of heat from the fetal sheep to the mother. About 85% of total heat produced by the fetus passes to the mother through the placenta, with the remaining 15% passing through the fetal skin, amniotic fluid, and uterine wall. At equilibrium, the temperature of the fetus is approximately 0.5° C more than that of the mother. Temperatures average 2° C lower in the human.

produced by metabolically active tissues leaves the organ where it is produced via conduction to the surface or by entry into the bloodstream, where it is carried away by convection in the venous blood. Heat flows from the fetus to the mother by either conduction through the fetal skin and into the amniotic fluid and uterus, or by convection in the blood to the placenta, where it is conductively transferred to the placental tissues and maternal blood.[4]

The fraction of overall fetal heat dissipation accounted for by each of these pathways has been determined experimentally in fetal sheep by measuring changes in fetal, maternal, and amniotic fluid temperatures following injection of known quantities of ice-cold saline into the amniotic fluid. The rate of heat exchange among these three compartments was measured with the fetus either alive or dead, and the difference between the two taken as the contribution of convective heat exchange due to umbilical blood flow. The results of these experiments suggest that approximately 85% of fetal heat production is dissipated through the placenta and 15% is dissipated through the fetal skin (see Figure 57-1).[4]

Placental Heat Production and Exchange

As mentioned, the placenta handles approximately 85% of the overall heat flux from the fetus to the mother. The overall permeability of the placenta to heat (0.8×10^6 W • C^{-1} • kg^{-1}) is very high compared with the amount of heat produced by the fetus and the permeability of the placenta to the molecules it exchanges.[5,6] Paradoxically, however, the placenta appears to be much more efficient at transferring matter than heat. For example, the capacity of the isolated perfused guinea pig placenta to transfer water from fetus to mother is much greater than its capacity for heat transfer despite the fact that heat diffuses through tissue much faster than water.[5,7] This counterintuitive finding can be explained by assuming that heat exchange is not limited to the same utero-umbilical interface area as that of matter exchanged in the placenta. Instead, due to its extremely diffusive nature, heat likely also shunts from the umbilical arterial

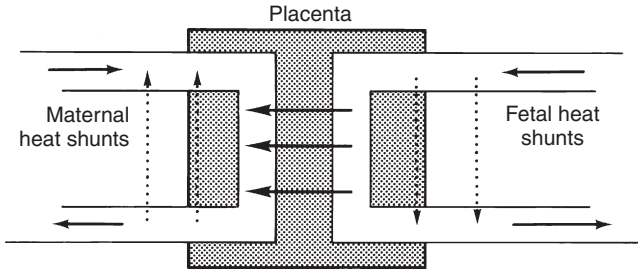


Figure 57-2 An illustration of heat exchange and shunting in the placental circulation. Because heat is highly diffusible, shunts may develop between vessels supplying and returning from the placenta. Such shunts diminish the efficiency of the placenta as a heat exchanger.

circulation across to the umbilical vein, where it is carried back into the fetus. It may also enter the amniotic fluid from vessels in the umbilical cord and on the surface of the placenta. These factors result in decreased efficiency of the placenta to transfer heat to the mother (Figure 57-2).

The transfer of heat across the placenta is also affected by the heat production of the placenta itself. The placenta is very metabolically active, consuming as much or more oxygen per kilogram of tissue weight as the fetus itself. Therefore, assuming normal fetal and placental weights of 3.5 and 0.7 kg, respectively, a fetal and placental heat production of 3.5 W • kg^{-1}, and that 85% of fetal heat dissipation passes through the placenta, it can be seen that 25% of the heat passed from the placenta to the mother is derived from the placenta itself. By lowering the temperature gradient between umbilical and uterine blood, this locally produced placental heat diminishes the efficiency of the placenta as a heat exchanger.

Simulating Heat Movement in the Fetus

As a result of the predictable physical properties of heat, the movement of heat from the fetus to the mother can be expressed in rather simple equations defining the flow of heat by either conduction or convection. For example, the rate of heat conduction across the fetal skin and the placenta can be defined by

$$H_s = (T_f - T_a) \bullet G_s$$

and

$$H_p = (T_f - T_m) \bullet G_p$$

respectively, where H_x is the heat flux across the skin (H_s) and placenta (H_p) in watts, T_f is the fetal core temperature, T_a is the mean temperature of the amniotic fluid surrounding the fetus, and G_x is the experimentally measured conductance of the skin (G_s) or placenta (G_p) in W • kg^{-1} of tissue weight per degree C temperature difference. Equations describing heat convection must include the rate of flowing matter and its specific heat, or capacity of the matter to hold heat. Using the umbilical cord as an example, heat carried in blood flowing from the fetus to the placenta can be defined by the equation

$$H_{umb} = Q\,(T)\,(SH_{blood})$$

where H_{umb} is the heat flux, in watts, of the flowing blood, Q is the blood flow rate (mL/min), T is the temperature of the blood (C), and SH_{blood} is the specific heat capacity of the blood (3.6 joules • mL^{-1} • C^{-1}).

By using equations such those described to define the transfer of heat in the fetal-maternal unit, it is possible to create computer models capable of taking data from multiple experiments into account and to accurately predict experimental measurements.

In this manner, the use of computer modeling of fetal-maternal thermal homeostasis has improved the understanding of pathophysiologic disturbances in fetal heat production or dissipation.[8] For example, by varying simulated umbilical blood flow, heat production, and conductances individually while holding maternal temperature constant, it can be demonstrated that changing umbilical flow and fetal heat production are the only two means by which the fetus could alter its temperature significantly from that of the mother. As a result, any attempt by the fetus to raise its temperature in response to cold is met with the impossibility of simultaneously *increasing* heat production (hence oxygen consumption) and *decreasing* umbilical blood flow (hence oxygen supply). These models thus emphasize the futility of the fetus to regulate its own temperature in utero.[7]

Changes in Maternal Temperature

The fetus is largely ectothermic, its temperature at equilibrium approximately 0.5° C more than that of the mother despite changes in maternal temperature. This is demonstrated by a number of studies examining the effects of maternal hyperthermia or hypothermia on the fetal-maternal temperature gradient. Because it lacks the ability to regulate its own body temperature independently, the fetus is vulnerable in situations in which maternal temperature varies significantly from normal body temperature.

Maternal Hyperthermia

Following an unusually severe heat wave in 1965 that caused a number of the pregnant guinea pigs in his laboratory to abort prematurely, Marshall Edwards observed an increase in birth defects in guinea pigs who continued on to parturition.[9,10] Subsequent studies established that hyperthermic insult during the first or second trimester of pregnancy is teratogenic, leading to a long list of birth defects including anencephaly, microcephaly, neural tube defects, microphthalmia, talipes, arthrogryposis, heart defects, renal anomalies, abdominal wall defects, and limb reduction defects.[11,12] The teratogenic effects of maternal hyperthermia have been observed in a number of species including guinea pigs, rats, mice, pigs, sheep, and monkeys. Hyperthermia as a result of maternal fever or hot tub or sauna exposure has also been linked to human birth defects in a number of studies,[13-19] making hyperthermia the first human teratogen discovered through animal studies.[11]

In humans the precise thresholds at which hyperthermia results in birth defects are not well defined. However, animal experiments in a number of different species indicate that temperature elevations of more than 2° to 2.5° C for an hour or more are necessary. Exposures to hyperthermia of less than 2° C do not appear to be teratogenic regardless of duration. Evidence that avoiding spas and hot tubs during the first trimester of pregnancy results in decreased birth defects together with the overwhelming amount of animal data has resulted in recommendations that spa temperatures 39° C for more than 15 minutes and 40° C for more than 10 minutes should be avoided by women during the first and second trimesters of pregnancy.[20]

The mechanisms underlying the teratogenic effects of hyperthermia are likely related to the effects of heat on protein conformation and function. Mitotic cells appear to be the most susceptible to heat stress, dying in a pseudo-apoptotic manner 6 to 8 hours post insult. Cells in S-phase are also especially sensitive, dying apoptotically 12 to 15 hours after exposure.[12] Interestingly, there also appear to be cellular protective response mechanisms to heat stress. Exposure of rat embryos to subteratogenic levels of hyperthermia results in significant protection against subsequent heat stress that would be teratogenic in nonpreconditioned embryos. These protective effects persist for up to 8 hours following insult and are likely related to a family of proteins transcribed in response to heat, aptly termed heat shock proteins. These proteins serve to protect nascent proteins against aggregation as they are being synthesized, as well as aiding in the folding of new or denatured proteins to their functional configurations. In this manner, heat shock proteins serve to rescue and protect proteins from heat denaturation.[21]

Although the teratogenic effects of hyperthermia appear to be limited mainly to the first half of gestation when cell differentiation is most prevalent, heat stress in the latter half of gestation can also be detrimental to the fetus. During strenuous exercise or exposure to elevated ambient temperatures, maternal temperatures may rise by as much as 2° C above normal. Fetal temperatures, being inexorably linked to those of the mother by the heat-clamp function of the placenta discussed earlier, also increase under these circumstances but tend to lag behind maternal changes. As a result, the difference between fetal and maternal temperature is decreased or even reversed during the first 30 to 40 minutes of heat exposure.[22,23] This thermal buffering may be partly due to a phenomenon known as thermal inertia, which refers to the time required for additional heat to build up in or escape from tissues as a result of alterations in heat production or dissipation. The transient decrease in fetal-maternal temperature gradient occurring soon after maternal heat accumulation might also be explained by increases in placental blood flows, but this does not seem likely because both uterine and umbilical blood flows of sheep decrease in response to transient hyperthermia.[22,24,25]

The effects of chronic mild hyperthermia are known to be deleterious in the fetal sheep. In fact, exposure to chronic 0.6° to 0.8° C elevations in maternal body temperature for the final 0.75 of gestation is a well-validated animal model of placental insufficiency and intrauterine growth restriction.[26] These conditions result in decreased placental and fetal weight, mild to severe fetal hypoxemia, and decreased placental amino acid, oxygen, and glucose transport. Uterine and umbilical blood flows are also decreased, although the decrease is proportional to fetal body weight and it is not clear whether this is the primary cause of fetal growth restriction and hypoxia.[27]

Maternal Hypothermia

Compared with the harmful effects of even 1° or 2° C of hyperthermia, hypothermia in utero is better tolerated by the fetus and occurs less frequently. Hypothermia applied selectively to the fetus has been well studied and is discussed in later portions of this chapter devoted to thermogenesis. Maternal hypothermia finds clinical relevance in cases of cardiopulmonary bypass surgery or medical complications such as hypoglycemia or septic shock.

Cardiopulmonary bypass surgery usually involves administration of hypothermia to the patient to temperatures as low as 25° C or less. When the procedure is required in pregnant women, reports of fetal survival rates range from 71% to 91%.[28,29] Clinical studies that include measurements of fetal heart rate report profound fetal bradycardia during bypass. However, it is not clear whether the primary cause of the bradycardia is hypothermia or perhaps fetal hypoxia secondary to changes in placental oxygen exchange. In sheep, mild hypothermia results in increased sympathetic activity and heart rate, but this response is overridden by superimposed hypoxia, which causes bradycardia that is mediated vagally by central chemoreceptors (Figure 57-3).[30] Likewise, in at least one clinical case in which maternal temperatures were held normothermic during cardiopulmonary bypass surgery, fetal bradycardia occurred abruptly on ligation of the maternal aorta, suggesting bradycardia may not be caused by hypothermia per se.[31] In a study of cardiopulmonary bypass performed on pregnant sheep, fetal heart rate increased initially on initiation of maternal cooling, but then fell as body temperatures were lowered to more than 5° C below normal.[32] However, arterial hypoxia was also observed in lambs held at 20° to 24° C, making

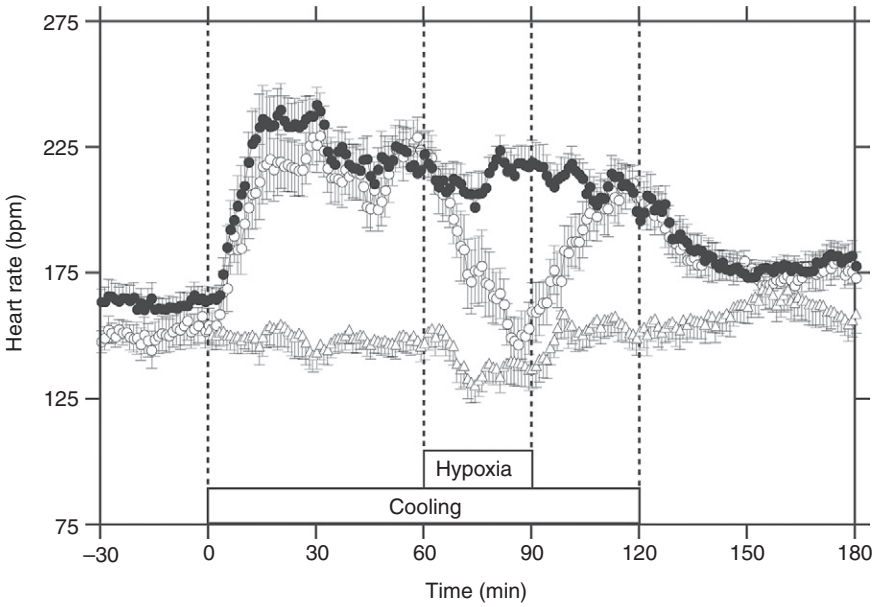


Figure 57-3 The effects of mild cooling (decrease in core body temperature of 1.5° C) hypoxia (maternal Fio_2 of ~0.12) or hypoxia superimposed during cooling on heart rate of the fetal sheep in utero. Cooling alone *(closed circles)* results in rapid and profound tachycardia. In contrast, hypoxia alone *(open triangles)* results in bradycardia, mediated vagally by central chemoreceptors. When superimposed on cooling *(open circles),* hypoxic bradycardia is overriding.

it difficult to distinguish between low arterial Po_2 or cooling of the sinus node as the primary cause of bradycardia. The distinction may be important because the latter indicates fetal distress, whereas the former tends to be protective. It is worth noting that the lambs were not acidotic and maintained normal Pco_2 levels throughout the procedure, suggesting that stress as a result of hypoxia or poor placental exchange was minimal. At any rate, although further studies are needed on the fetal effects of maternal hypothermic cardiopulmonary bypass, available reports suggest that deep cooling of the mother (20° to 24° C) is safer than very deep cooling (<20° C). In addition, cooling as low as 25° C may not be any more harmful or is perhaps even less harmful to both the fetus the mother than normothermia during bypass by virtue of the lowered metabolic demands during the procedure.

PERINATAL THERMOREGULATION

Homeotherms are often categorized based on the developmental stage of thermoregulatory systems at the time of birth as immature, altricial, or precocial. Immature newborns, such as marsupials and hamsters, are largely poikilothermic during early postnatal development. Body temperature thus varies as a function of ambient temperatures during at least the first 2 weeks of life, at which time thermogenic responses develop only in those animals exposed to hypothermic stress. Altricial (which means "nesting") newborns, such as rodents, dogs, and rabbits, are born with little or no thermogenic capacity during the first 1 to 6 days of life, after which responses develop only if exposed to hypothermic stress. Precocial newborns, such as humans, guinea pigs, and lambs, develop the capacity for thermogenic responses in utero capable of being activated immediately at birth and which are vital to the survival of species born in cold environments.

Fetal Thermosensory Development

Control of thermoregulatory mechanisms begins with sensory input from either sensory neurons located in the preoptic chiasma/anterior hypothalamic nuclei (POAH), or from peripheral cutaneous receptors that send information to the POAH. Thermosensitive cells within the POAH fire at frequencies that vary with changes in temperature. Signals from the POAH are subsequently processed through a number of central inhibitory and stimulatory steps en route to activation of nonshivering thermogenesis by sympathetic norepinephrine-secreting nerve fibers that innervate brown adipose tissue (BAT), and to activation of shivering thermogenesis via the posterior hypothalamic nucleus.[33,34]

The in utero development of central thermoregulatory controls has not been fully explored, although these mechanisms do appear to be in place long before the time of parturition in some species. By cooling fetal sheep in utero, researchers have observed thermoregulatory responses such as shivering[35] and increased sympathetic activity as demonstrated by tachycardia, hypertension, and increased circulating concentrations of epinephrine and norepinephrine.[36] As is discussed later, sympathetic output is the key stimulus of thermogenesis by BAT. Interestingly, cutaneous cooling but not central cooling of the premature fetal sheep also results in coordinated breathing movements, suggesting cutaneous cold sensors also play a role in the initiation of ventilation at birth.[35] Although less studied than the sheep, premature human infants as young as 25 weeks gestation also demonstrate thermoregulatory responses to cold stress at birth, although the responses are clearly not as vigorous as those of the term infant.[37]

Behavioral Thermoregulation

Although initial studies of thermoregulation in animals focused primarily on physiologic changes in blood flow and metabolism to control heat loss and production, behavioral mechanisms of thermoregulation are a key factor for many nesting animals. For example, although rat pups in isolation appear relatively poikilothermic, in a litter they move closer and farther apart in response to changes in ambient temperature, a behavior known as thermotaxis. By dynamically changing the overall surface-to-volume ratio of the group in this manner, the litter as a whole is able to maintain euthermic body temperatures and conserve metabolic energy of the individual pups within.[38] These thermoregulatory behavioral responses are developed at the time of

birth. Interestingly, isolated 1-day-old rats in a cool environment are even capable of learning head-turn movements that result in brief rewards of heat.[39]

The full extent to which behavioral mechanisms of thermoregulation interact with physiologic responses remains to be determined, but it is clear that in many cases they are closely linked. For example, endogenous thermogenic mechanisms such as BAT activation are required for huddling behavior to be fully effective in newborn rats.[40] Likewise, exposure of infants to mild cold stress results in increased sympathetic nervous activity that not only activates thermogenesis but also increases crying, which in turn attracts the attention of caregivers.[41]

Mechanisms of Thermogenesis

Thermogenic responses fall naturally into three broad categories: shivering of skeletal muscle, nonshivering thermogenesis in BAT, and futile cycling of ions in specialized heat-producing tissues. Each has a distinctive mechanism of action. During shivering, for example, small random muscle contractions hydrolyze adenosine triphosphate (ATP), stimulate respiration, and dissipate the free energy as heat, without doing useful mechanical work.[42] During nonshivering thermogenesis, energy from mitochondrial respiration is not conserved as ATP but rather is released as heat. During futile ion cycling, respiration remains coupled, but significant amounts of heat are dissipated from specialized tissue by cycling of Ca^{2+} in the sarcoplasmic reticulum with associated use of ATP.[43] It has been speculated that similar mechanisms may be associated with malignant hyperthermia in humans, but whether aberrant Ca^{2+} cycling contributes to cold-induced thermogenesis in newborn mammals remains unknown. Other nonshivering thermogenic mechanisms include protein synthesis–degradation cycling of proteins and K^+-ATPase cycling, but again their importance in the neonatal period is unknown.[44]

The proportion of thermogenesis provided by shivering or nonshivering means varies widely across species. In the newborn lamb, nonshivering thermogenesis provides the first line of defense against cold exposure, with shivering commencing only after body temperature falls significantly. However, although shivering is a second line of defense, it accounts for nearly 50% of maximal heat production during cold exposure, with nonshivering thermogenesis accounting for more than 30%.[45] In the human newborn, the relative importance of the different mechanisms is not well established, but is thought to vary with gestational age and severity of the temperature stress. Nonshivering thermogenesis is clearly not essential for survival in all species, demonstrated by the fact that it is lacking altogether in the newborn pig and ox.

Carbohydrate metabolism is also stimulated by cold exposure. Evidence for augmented carbohydrate metabolism includes a fall in hepatic glycogen stores and increased glucose turnover. During summit metabolism, when cold-induced increases in metabolic rates are at a maximum, glucose, glycogen and lipid energy stores are adequate for 8 to 12 hours in the newborn lamb.

Shivering Thermogenesis

Shivering is mediated by information processed in the primary motor center for shivering located in the posterior hypothalamus. This center receives inhibitory signals from the POAH when euthermic or hyperthermic and excitatory signals from cutaneous nerve endings during hypothermia. The POAH stimulates general increases in skeletal muscle tone via motor neurons. Although shivering is a primary thermogenic response to cold in the adult and can increase body heat production by four- or five-fold, in most newborns (human included) it is a second line of defense.[45,46]

The shivering response of the fetal sheep to hypothermia is present in utero as early as 106 days gestation (~0.75 of 147 days full term).[35] Shivering is inhibited by hypoxia in the fetal sheep and, as in the newborn and adults of many species,[47] during rapid eye movement (REM)–like sleep electrocortical activity. This inhibition emerges at approximately 120 days in the sheep.[35]

Nonshivering Thermogenesis

The importance of nonshivering thermogenesis in thermoregulation was not widely acknowledged until it was observed that animals treated with curare paralytics to block shivering retained an ability to produce significant amounts of heat in response to cold.[48] The findings were supported in human infants when cold-induced increases in metabolic rate were still observed in pharmacologically sedated infants displaying no signs of shivering.[46] These observations were made in the context of growing acceptance that BAT was, in fact, a thermogenic organ responsible for a predominant portion of nonshivering thermogenesis, thus creating an entirely new field of study. As a result, over the past 30 years much progress has been made toward understanding the regulation and contribution of nonshivering thermogenesis to thermal homeostasis.

In utero, BAT first appears in the fetal sheep less than halfway through gestation,[49] and for the last trimester of gestation constitutes 0.5% to 1.5% of total human infant body weight.[50] BAT is found near highly perfused organs or along the course of large vessels, with a distribution that has been described to resemble a "high-collared vest."[50] In the human infant the most predominant deposits are around the kidneys and in the intrascapular area of the back, extending up the dorsal neck.

The oxygen consumption of BAT cells in vitro is increased by 10-fold on exposure to norepinephrine, which mediates its effects primarily via β_3 but also via α_1 receptors on the cell surface.[33,51] The thermogenic properties of BAT are a result of the action of uncoupling protein 1 (UCP1), which is found only in BAT. Activation of β_3 receptors results in increased G_s subunit activity, leading to adenylate cyclase activation and increased cyclic adenosine monophosphate (cAMP) production, which ultimately results in the simultaneous release of free fatty acids from cytosolic stores and activation of UCP1 (Figure 57-4). UCP1, which is located in the mitochondrial membrane, facilitates the free movement of protons into the mitochondrial matrix, allowing them to bypass ATP synthase, and hence uncoupling oxidative respiration from the production of ATP. The result is unhindered oxidative metabolism with the resulting energy released as heat rather than being captured in the form of ATP.[33]

In vivo, functional innervation of BAT by sympathetic neurons occurs as early as gestational day 120 in the fetal sheep and increases gradually until birth.[52] Thus BAT is not only readily activated immediately on birth at term, but can also play an important role in thermoregulation of premature infants, as is discussed later.

In addition to sympathetic input are a number of hormonal regulators of BAT growth and activation. BAT is rich in 5′-monodiodinase, the enzyme that converts thyroxine (T_4) to (T_3). Plasma T_4 concentrations increase gradually during the last half of gestation, and then double abruptly at birth, with corresponding increases in both plasma and BAT-localized T_3. Increases in circulating concentrations of T_4 result in increased UCP1 content in BAT, an effect likely mediated by local conversion of T_4 to T_3 within the BAT rather than by circulating T_3.[53,54]

Leptin, a 16 kDa protein member of the cytokine family, is released primarily from white adipose tissues, but also by BAT and the placenta.[55] Leptin concentrations in the fetal sheep rise abruptly within days of birth and then fall just as abruptly following parturition, when UCP1 levels are at a peak.[56] In the adult rat, plasma leptin concentrations stimulate increases in UCP1 levels.[57] In contrast, leptin administration in the newborn lamb results in decreased UCP1 and UCP1 mRNA concentrations

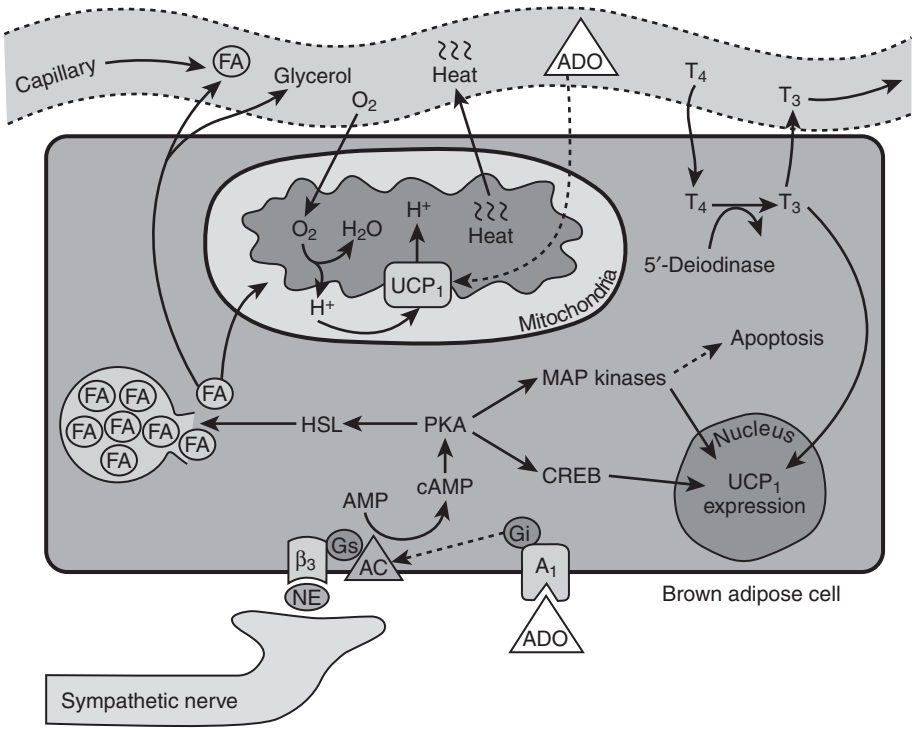


Figure 57-4 Some metabolic and hormonal controls of brown adipose tissue (BAT). *Solid arrows* indicate stimulatory influences, *dashed arrows* indicate inhibitory ones. The principal means of BAT activation is through norepinephrine (NE) released from sympathetic nerve endings. NE binds to β_3 receptors on the cell surface, activating G_s and adenylate cyclase (AC), resulting in increased concentrations of cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA). PKA phosphorylates hormone-sensitive lipase (HSL), which in turn liberates free fatty acid (FA) from triglyceride droplets. FA stimulates the activation of uncoupling protein 1 (UCP1), resulting in increased oxygen consumption and heat production. PKA also activates myosin-activated protein (MAP) kinases responsible for inhibiting apoptosis, and stimulates expression of UCP1 by activation of MAP kinases and the transcription factor cAMP responsive element binding protein (CREB), thyroxine (T_4) from the circulation is converted to triiodothyronine (T_3) by 5′-monodeiodinase. Activation of BAT results in increased concentrations of circulating FA, glycerol, and T_3, and increased consumption of oxygen (O_2). Adenosine (ADO) is thought to inhibit BAT activation by binding to A_1 receptors on the cell membrane, resulting in G_i activation and decreased AC activity. ADO may also have a direct inhibitory effect by binding to UCP1.

with little effect on thermoregulatory capability,[55] and although leptin concentrations appear to rise and fall in advance of those of UCP1 in the fetal and newborn lamb, whether leptin actually effects BAT remains uncertain.

Plasma concentrations of cortisol, a corticosteroid released by the adrenal gland, surge during the 72 hours prior to birth of the fetal sheep. Cortisol appears to have a direct effect on BAT, by stimulating increased production of UCP1, and indirect effects by stimulating the release of T_4 from the thyroid.[58]

Prolactin is a single 24 kDa chain of 199 amino acids secreted from the anterior pituitary. Its effects are mediated by cell surface receptors located on a number of tissues, including BAT. Plasma prolactin concentrations and BAT prolactin receptor numbers increase shortly before birth, peaking at 6 hours after birth in the newborn lamb. Recent evidence suggests that prolactin may have potent BAT-activating effects because its intravenous infusion into newborn lambs results in rapid increases in core body temperature and circulating concentrations of fatty acids and glycerol.[59] These metabolites are reliable indices of BAT activity as is discussed in the following text.

INDICATORS OF THERMOGENIC RESPONSE

After birth the temperature of the mammalian newborn typically falls, and the newborn responds by increasing O_2 consumption and thus heat production. These responses begin within a matter of minutes and can persist for many hours.[45] They are particularly strong in the lamb, calf, and rabbit,[60] species in which heat production increases three- to five-fold, and are less powerful in the human, in whom heat production approximately doubles. When at their maximum level, these responses are called *summit metabolism*.

Much of what is known about the control of nonshivering thermogenesis in utero and at parturition has been determined from the fetal sheep model represented in Figure 57-5. In these experiments fetal sheep are surgically instrumented with tracheal intubation for mechanical ventilation, cooling coils wrapped around the thorax to simulate cutaneous cooling, and an umbilical cord snare to eliminate placental blood flow. After allowing the fetus to recover from the surgery for 2 to 3 days, the stimuli of birth can be administered in utero in a stepwise manner and in any combination, while responses of oxygen consumption, temperatures, and circulating concentrations of thermogenic mediators and metabolites are sampled.[61,62]

Glycerol and fatty acids are the two major biochemical products of lipolysis, and their plasma concentrations provide useful indicators of nonshivering thermogenesis (Figure 57-6). With glycerol, only negligible quantities of glycerol kinase are present in brown adipose tissue.[33] Therefore, glycerol released within the adipocyte spills into the circulation and is metabolized elsewhere, principally in the liver. Once released into the circulation, glycerol is cleared slowly from the blood and does not pass freely across the ovine placenta. For these reasons circulating concentrations of glycerol have proved to be a useful index of lipolysis, and serve as an index of nonshivering thermogenesis in the fetus and newborn.[63]

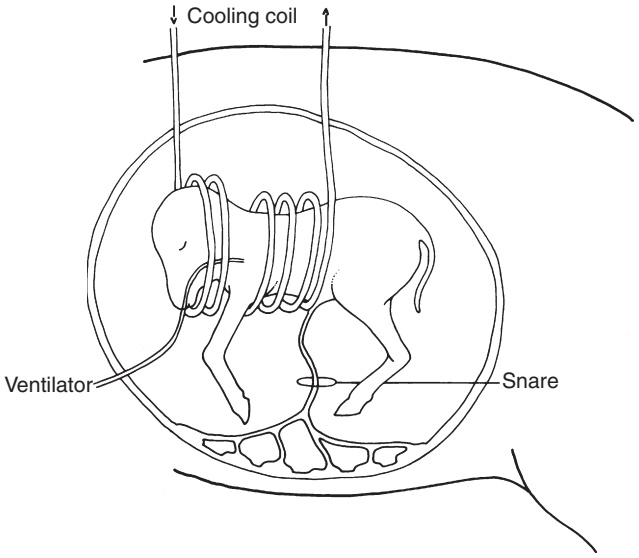


Figure 57-5 Method to study thermogenesis in fetal sheep. Responses to cooling ventilation, and oxygenation, cord occlusion, and the infusion of hormones can be tested separately. *(From Power GG, Gunn TR, Johnston BW, Gluckman PD: Oxygen supply and the placenta limit thermogenic responses in fetal sheep,* J Appl Physiol *63(5):1896, 1987.)*

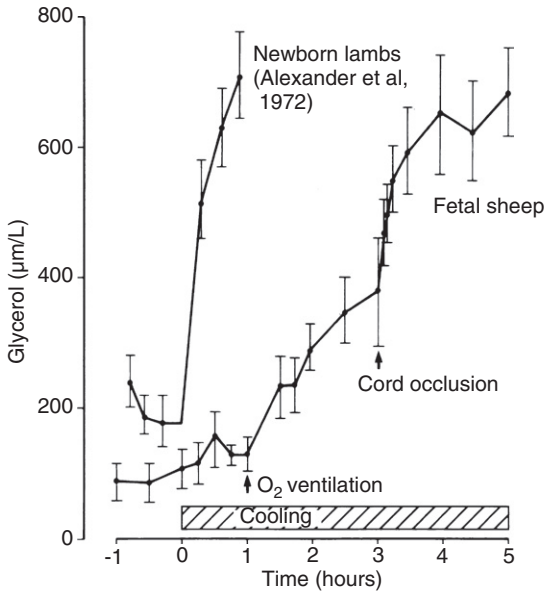


Figure 57-6 Plasma glycerol, an indicator of nonshivering thermogenesis, rises rapidly and markedly in newborn lambs exposed to cold. It rises only negligibly, however, in cooled fetal sheep and only moderately after supplemental oxygen is given. Only after cord occlusion do responses approach newborn levels. This result suggests an intrauterine inhibitor of fetal thermogenesis, possibly of placental origin.

Fatty acids differ from glycerol in several important respects. Most are oxidized or re-esterified locally within the fat itself. Some, however, enter the circulation and are transported to the heart, brain, diaphragm, and skeletal muscle in which they provide metabolic fuel and serve as the predominant substrate for shivering. This dispersal of lipid energy reserves permits a more generalized and prolonged thermogenic response than would otherwise be

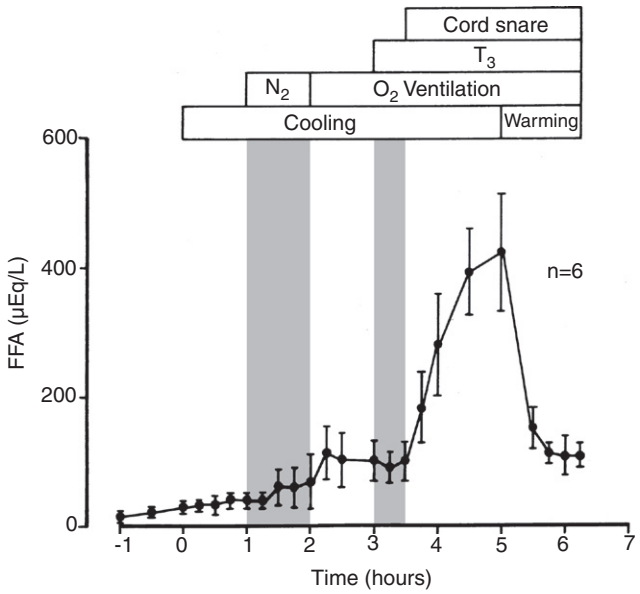


Figure 57-7 Plasma free fatty acids, an index of nonshivering thermogenesis, do not rise appreciably in response to cooling and nitrogen (N_2) ventilation in fetal sheep. Supplemental oxygen and infusion of large quantities of triiodothyronine induce only modest responses. Only after cord occlusion do responses approach a significant fraction of newborn levels. These results suggest that a metabolic inhibitor of placental origin circulates before birth. *(From Power GG, Gunn TR, Johnston BM, Nichols G, Gluckman PD: Umbilical cord occlusion but not increased plasma T_3 or norepinephrine stimulate brown adipose tissue thermogenesis in the fetal sheep,* J Dev Physiol *11(3):171, 1989).*

possible and links shivering in skeletal muscle with nonshivering thermogenesis in BAT. As is the case with glycerol, plasma concentrations of fatty acids are closely related to nonshivering thermogenic activity in the newborn period (Figure 57-7).[64]

Thermogenesis In Utero and at Birth

Fetal thermogenesis is normally inactive despite the ability of fetal BAT to be activated when studied in vitro[65] and despite the fact that even the prematurely delivered lamb can achieve thermoregulation. A number of studies indicate that both shivering[35] and nonshivering[63,66] thermogenic responses to cold are minimal in utero. This is shown in Figure 57-6, in which cold-induced responses in plasma glycerol in newborn lambs are compared with those in fetal sheep. Despite intense cooling by artificial means, and despite reductions in fetal core temperature of more than 2° C, the fetal response can be seen to be relatively weak. Responses in plasma free fatty acids and other indicators of thermogenesis (Table 57-1) are also attenuated and are not stimulated by ventilation with either nitrogen or oxygen nor by exogenous triiodothyronine (see Figure 57-7). Infusions of norepinephrine to the fetus in utero also result in only minimal BAT activation.[67,68] All these observations suggest a functionally capable but unused heat-producing capacity in the fetus.

Inhibition of Thermogenesis In Utero

The inability to activate fetal thermogenesis despite intense stimulation and supplemental oxygenation raises the possibility that nonshivering thermogenesis is suppressed in utero. It can be seen from Figures 57-6 and 57-7 that maximal responses are not observed until the umbilical cord is clamped. In fact, in the

presence of cooling and oxygen ventilation in utero, BAT can be activated and deactivated repeatedly by occluding the umbilical cord with a reversible occluder.[69] Likewise, newborn lambs exposed to cold are unable to initiate lipolysis if patency of the umbilical cord is maintained.[70] These studies suggest the presence of one or more circulating inhibitors of thermogenesis produced by the placenta that suppress BAT.

There is evidence that adenosine and certain prostaglandins (such as prostaglandin [PG] E_2) are circulating inhibitors of fetal thermogenesis. Both adenosine[71] and PGE_2[72] are produced by the placenta and have short plasma half-lives. The short effective biologic half-life of these compounds is consistent with the onset of thermogenesis within minutes after placental separation at birth. Pharmacologic blockade of these compounds in utero during oxygenation and cooling stimulates BAT activation without umbilical cord occlusion.[72,73] Conversely, administration of either of these compounds or their analogues following cord occlusion inhibits the activity of BAT.[61,72] These findings provide strong evidence of thermogenic inhibitors that circulate before birth. An alternative mechanism is that cord occlusion may stimulate BAT by diverting blood flow from the placenta to the BAT, though this possibility has not been studied thoroughly. Current knowledge about potential stimuli and suppressors of nonshivering thermogenesis at the time of birth is summarized in Table 57-2.

CAESAREAN SECTION AND THERMOGENESIS OF THE NEWBORN

The route of delivery influences the strength of thermoregulatory responses immediately following birth. Full-term infants delivered by cesarean section have lower body temperatures that average approximately 0.3° C less than temperatures of infants delivered vaginally.[74] BAT activation and UCP1 levels are depressed in newborn lambs delivered by cesarean section compared with those delivered vaginally.[75] Sympathetic activity, plasma levels of catecholamines,[75,76] and plasma levels of thyroid hormones[77] are reduced in infants and lambs delivered by cesarean section. The ability of the newborn lamb to regulate its temperature is improved by intravenous administration of norepinephrine, T_3, or thyrotropin-releasing hormone at the time of cesarean section delivery.[78] The potential benefit of similar treatments in the human newborn appears not to have been tested in a controlled study.

TABLE 57-1

Indicators of Thermogenic Activity in the Mammalian Fetus and Newborn

Sign	Usefulness and Comment
Plasma free fatty acid concentration	Fatty acids are products of lipolysis; many re-esterified without being oxidized; rapid turnover and quick response
Plasma glycerol concentration	Glycerol is also a product of lipolysis; slow turnover and damped response
Body temperature	Nonspecific; thermal inertia causes delays; insensitive
Difference between brown adipose tissue and body core temperature	Highly specific; difficulty arises in verifying location of brown adipose tissue temperature sensor
Oxygen consumption	Relatively nonspecific because measurement includes basal oxygen use as well as shivering and nonshivering components
Direct calorimetry	Research laboratory procedure requiring specialized equipment

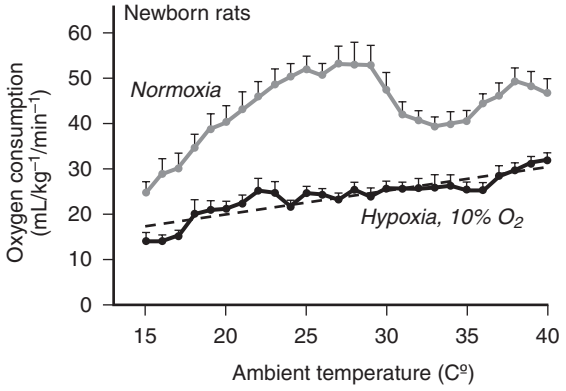


Figure 57-8 An illustration of the relationship between ambient temperature and oxygen consumption of the normoxic *(open circles)* and hypoxic *(closed circles)* 2-day-old rats. Normoxic rats respond to cooling with a marked increase in oxygen consumption due to thermogenic efforts, resulting in a summit metabolic rate at approximately 28° C. These thermogenic increases in oxygen consumption are abolished by hypoxia. *(From Mortola JP: Implications of hypoxic hypometabolism during mammalian ontogenesis,* Respir Physiol Neurobiol *141(3): 345–356, 2004.)*

TABLE 57-2

Sources and Effects of Major Mediators of Nonshivering Thermogenesis in the Mammalian Newborn

Compound	Concentration Changes During Gestation and Parturition	Major Fetal/Newborn Source	Effect on Brown Adipose Tissue
Norepinephrine	Increases after placental separation and in response to cooling	Sympathetic nerve endings within BAT	Stimulates activation of BAT through the β_3 and α_1 receptors
Triiodothyronine	Rises gradually during gestation, then rises sharply at birth	Conversion of thyroxine within BAT by 5′-monodeiodinase	Stimulates production and increases activity of UCP1 within BAT
UCP1	Rises gradually during gestation, peaking at birth, declines thereafter	BAT	Mediates the uncoupling of oxidative phosphorylation in BAT
Prostacyclin	Low during gestation, then increases with onset of ventilation	Lungs	Stimulates lipolysis
Cortisol	Rises near the end of gestation	Adrenal gland	Stimulates thyroid activity and UCP1 production in BAT
Adenosine	High during gestation then falls sharply at birth	Placenta and other organs	Inhibits BAT activation
Prolactin	Increases during gestation, peaking within hours after birth	Anterior pituitary	Activates BAT, increases UCP1
Prostaglandin E_2	High during gestation, then falls sharply at birth	Placenta and other organs	Inhibits BAT activation

BAT, Brown adipose tissue, brown fat; *UCP1,* uncoupling protein 1.

HYPOXIA AND THERMOGENESIS OF THE NEWBORN

Thermogenic responses are costly in terms of oxygen and substrate use. In the newborn pig, dog, rat, and sheep, exposure to cold can increase oxygen use by 100% or more. This increase in metabolic rate is attenuated if hypoxia accompanies exposure to cold (Figure 57-8).[79] The inhibition of thermogenic activity by hypoxia appears to be an adaptive response in that it conserves oxygen that would otherwise be used to generate heat.[80,81] Normally thermogenic activity occurs when temperature in the hypothalamus drops below the normal setpoint. Hypoxia may decrease thermogenic metabolism by reducing or overriding the activation of BAT by sympathetic outflow in response to cold.[82] By allowing body temperature to remain at lower than normal levels, the organism also rations the limited oxygen available by depressing the rate of enzymatic processes due to the Q_{10} effect. This phenomenon of metabolic hibernation in response to hypoxia is particularly prominent in smaller, younger, and more metabolically active newborns of many species[83] and has been demonstrated in the fetal sheep.[84] Careful clinical testing is needed to evaluate its presence and possible use in medical practice.

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58

Rakesh Sahni and Karl Schulze

Temperature Control in Newborn Infants

This chapter is an updated revision of Dr. Kurt Brück's comprehensive treatise on neonatal thermal regulation presented in earlier editions; it is not an original synthesis of the current authors. Brück remains the senior contributor despite his death in 1995. We have immense admiration for his many contributions to our understanding of the physiology of human neonates. His classic study of temperature regulation[1] provided fundamental knowledge, which has been validated and refined in subsequent years. Using continuous measurements of heat production and cutaneous blood flow, before, during, and after discrete and timed environmental cold stress, Brück defined the fundamental features of the neonatal response to chilling and its dependence on gestational and postnatal ages. These important observations concerning the basic responses of infants to cold—increased heat production and decreased heat loss, as well as the timing of these events and their development pattern—remain central to our understanding of how best to care for human neonates.

CURRENT MODELS AND TERMINOLOGY

It is useful to model the temperature control system of newborn infants using the basic biocybernetic concept of a passive (i.e., controlled) system, the temperature of which depends on metabolic heat production and heat transfer to the environment. The controller consists of sensor, integrator, and effector components (Figure 58-1). Current understanding is that information about heat storage and heat gradients within the body is monitored continuously by various central and peripheral thermal sensors. These multiple inputs are transmitted to an integrating neural control network, located in the hypothalamus and limbic systems, which, in turn, is linked by means of efferent neuronal pathways to effector mechanisms that are capable of controlling heat storage. The earlier concept that the controlled variable is a single temperature at a single site within the body has been refined to take into account evidence that temperatures at multiple sites provide important input to the controller.[2-5]

For clarity, the following discussion is structured according to this controller model, reviewing information about the sensors, integrator, and effectors as separate components followed by a consideration of how the integrated control system functions in response to environmental thermal transients, how the system changes during development, and how it operates under special circumstances, such as fever, heart failure, and hypoxia.

PRINCIPLES OF PHYSIOLOGIC TEMPERATURE REGULATION

The maintenance of a stable body temperature that is much warmer than the environmental temperature is a property of the two higher classes of the animal kingdom: birds and mammals.

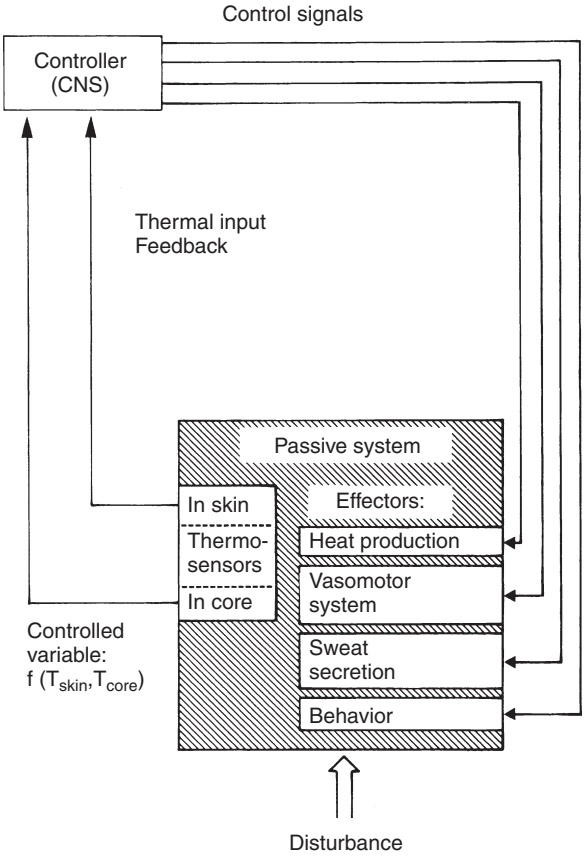


Figure 58-1 Diagram representing the biocybernetic concept of temperature regulation in humans. Temperature is sensed at various sites of the body, and the temperature signals are fed into the central controller *(multiple-input system)*.

These classes form the group of *homeothermic* beings, all others designated as *poikilothermic*. A prerequisite of *homeothermy* is a basal rate of metabolism several times higher than that in poikilothermic animals. The former are thus referred to as *tachymetabolic* and the latter as *bradymetabolic* organisms. Furthermore, homeothermy requires a balance among heat production, skin blood flow, sweating, and respiration in such a way that changes in heat loss or gain from the environment are precisely compensated.

METABOLISM AND HEAT PRODUCTION

Metabolic processes that provide energy for maintenance of homeostasis and physical exercise are closely linked with heat production. The overall efficiency of energy transformation in homeotherms is only on the order of 10% to 25%, meaning that most of the energy transformed during metabolic activities is liberated as heat and must either be eliminated or stored depending on the needs of the organism. For organisms that are tachymetabolic (including human neonates), the at rest metabolic rate alone is sufficient to increase body temperature by several degrees Celsius greater than the ambient temperature. The at rest metabolic rate is of great importance for the state of the controlled system (see Figure 58-1).

Standard Metabolic Rate

The at rest metabolic rate depends on several factors, including physical activity, environmental temperature, feeding, thermic effect of food, diet-induced thermogenesis (formerly called *specific dynamic action*), time of day (diurnal rhythmicity), age, and growth rate. By convention, the conditions used for the measurement of at rest metabolic rate have been standardized: (1) subjects must be awake and have fasted at least 12 hours; (2) they must be fully relaxed; and (3) thermoneutral conditions should be maintained.

Metabolic rate measured under these standard conditions is termed the *basal metabolic rate* (BMR). Neonates are unlikely to fulfill the first and second standard conditions at the same time; thus special conditions for measurement must be defined. The following conditions have been suggested[6]:

1. The infant should remain on a normal feeding schedule (this suggestion is not unreasonable because the maximum increase in energy metabolism in infants is only 4% to 10% after an ordinary feeding).
2. The measurements must be made in a period of 5 to 10 minutes, during which the infant is fully relaxed (in contrast with the BMR standard conditions, the infant does not have to be awake). The metabolic rate determination may even be made during postprandial sleep.[7]
3. The measurements must be made under thermoneutral conditions.

The minimum metabolic rate measured in this way is called the standard metabolic rate (SMR) or minimum observed metabolic rate.[8] The designation BMR should be used only for determinations performed under standard conditions as employed in adults. Depending on why the metabolic rate is being measured, longer periods of observation (i.e., 3 to 6 hours) are required.[9,10]

Standard Metabolic Rate in Relation to Body Mass and Age

Total heat production is related to body size; for example, the overall metabolic rate of sheep is greater than that of rabbits. Because body temperatures of homeothermic species are close to one another at similar preferred environmental temperatures, it can also be anticipated[11] that the metabolic rate per kilogram of body mass will be larger in a rabbit than in a sheep. In fact, the correlation of the logarithm of body weight and the logarithm of at rest metabolic rate (oxygen consumption) is close and has a slope of 0.75 for adult animals of various sizes. This means that the metabolic rate per kilogram$^{3/4}$ of at rest animals of different sizes, including adult humans, is independent of body size. This relationship has been termed the *law of metabolic reduction* by Kleiber[11] and has been found useful by some observers for cross-species comparisons of the physiology of metabolism.

Although the Kleiber equation can be useful for predicting metabolic rates when comparing different species, attempts to predict metabolic rates for a group of individual organisms within the same species but of different body sizes reveals its limited applicability. Several deviations from the law of metabolic reduction must be introduced. Age, sex, body shape, and body composition affect metabolic rate. The age dependency of metabolic rate makes it impossible to predict the metabolic rates of neonates and adults from an individual species with a single exponential function of weight, even if a higher exponent than Kleiber's is used.[1]

As illustrated in Figure 58-2, newborn infants to 1 week of age have a SMR that is lower than that predicted by Kleiber's equation; that is, the SMR is lower in a 3-kg human newborn than in a 3-kg rabbit. Conversely, in the weight range of 5 to 20 kg, the SMR is higher in human infants than in adult animals of the same weight. During this period of growth, the SMR demonstrates a nearly linear relationship to body weight. Mathematically, this relationship can be expressed by an exponent of $b = 1$ in the equation $SMR = a \times m^b$ where a is age and m is mass. During the

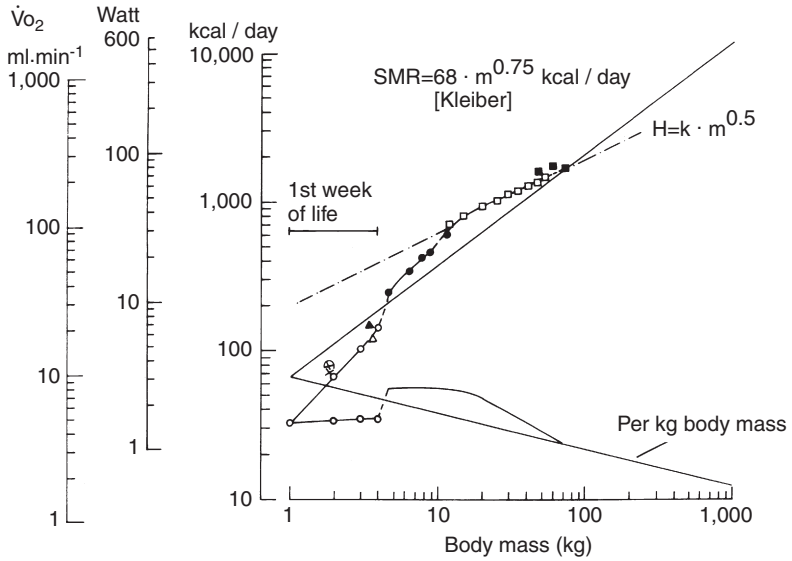


Figure 58-2 Relationship of standard metabolic rate (SMR) to body mass and age. *Top curve,* SMR and the corresponding $\dot{V}o_2$ in relationship to body mass. *Bottom curve,* The same data but expressed in relation to unit of body mass. — = SMR according to Kleiber's equation; ·—·—·—, heat production that would yield equal temperature differences between body core and environment. (See text for further discussion.) ○, first week of life[1]; △, 0-6 hr[123]; ▲, 18-30 hr[123]; ×, 4-12 hr[109]; ⊗, 1-2 days[109]; ●, 1-36 mo[124]; □, 3-15 yr[125]; ■, 14-30 yr.[126] *(Modified from Brück K: Heat production and temperature regulation. In Stave U [ed]:* Perinatal Physiology. *New York, Plenum Publishing, 1978, pp 455-498.)*

period that corresponds to the weight range of 20 to 70 kg, the SMR approaches data in Kleiber's curve.

An exponent close to 1, or somewhat larger than 1, should also be used to calculate the SMR for infants weighing between 1 and 4 kg. This means that during the neonatal period, the SMR/body mass unit is almost independent of weight, whereas a decrease would be expected with increasing weight according to Kleiber's equation (see Figure 58-2). These SMR values demonstrate a relatively large amount of scatter during the first day of life. A survey of these data and a discussion of the possible reasons for the scattering may be found in the references.[7,12] Given these limitations, attempts to refer observations of the metabolism of human neonates to standards derived for other mammals should be avoided.

Certain quantitative changes in the SMR during early postnatal development are worth keeping in mind:

1. Immediately after birth, the SMR/kg body mass is lower in human infants than in adult animals of the same weight (see Figure 58-2).
2. After birth, the SMR/kg body weight increases and eventually attains a value that is up to 50% higher in infants than in adult animals of the same body mass. The time course of this process appears to be variable. It may take 2 days to a few weeks (particularly in premature infants) to attain values that are characteristic of the postneonatal period.[7,12]

The seeming violation of the law of metabolic reduction (see Figure 58-2) during the early neonatal period may be only a fictitious problem if one takes into account the considerable change in extracellular water content (extracellular fluid [ECF]) that occurs from the early fetal to the adult stage. Sinclair et al[13] have suggested using body weight minus ECF as the reference value in metabolic reference standards. According to their calculations, ECF composes as much as 44% of body weight in a 4000-g newborn infant and 58% in a 1000-g premature neonate. The corresponding average value for ECF in the adult is only 20%. Because the ECF does not participate in oxidative metabolism, the expression "body weight minus ECF" may be considered representative of the active tissue mass. Thus it seems theoretically justified to relate metabolic rate to this active tissue mass rather than to total body weight. In contrast, the lean body mass (i.e., total body mass minus fat mass) has not been found to be a suitable reference value.

One can also relate metabolic rate in a neonate to the expression "active tissue mass plus adult ECF," that is, to a body mass that corresponds in composition to that of the adult organism. Metabolic rates related to this calculated value closely approximate Kleiber's curve, whereas the metabolic rate related to the actual body weight remains less than the predicted values.[7]

Metabolic Rate in Relationship to Body Temperature

All chemical reactions in an organism are temperature dependent and obey van't Hoff's law. According to this law, oxygen uptake and body temperature of a poikilothermic animal are expected to decrease with decreasing environmental temperature. This decrease has, in fact, been demonstrated in frogs, reptiles, and other poikilothermic animals without exception. The ratio of the reaction rates at temperatures differing by 10° C is called the Q_{10}. In general, the Q_{10} for the metabolic rate in poikilothermics is approximately 2 to 3. van't Hoff's law applies to homeotherms in the same way but is masked by regulatory processes.

Thus, in the intact homeothermic organism, the metabolic rate increases initially with decreasing body temperature (Figure 58-3). This *regulatory metabolic rise* can be prevented by pharmacologic intervention, in particular by deep general anesthesia. As shown in Figure 58-3 (which illustrates the relationship between metabolic rate and rectal temperature in anesthetized dogs), metabolic rate decreases with decreasing body temperature in both cold-induced and basal conditions (bottom curve in figure). In other words, van't Hoff's law is obeyed. The slope of the curve corresponds to a Q_{10} of 2 to 3. At body temperatures less than 30° C, the metabolic rate may drop steeply to the basal curve (*dashed line* in Figure 58-3) because the thermoregulatory drive generated in the thermointegrative areas of the central nervous system vanishes with increasing hypothermia. The body temperature range at which this reduction occurs may be species dependent.

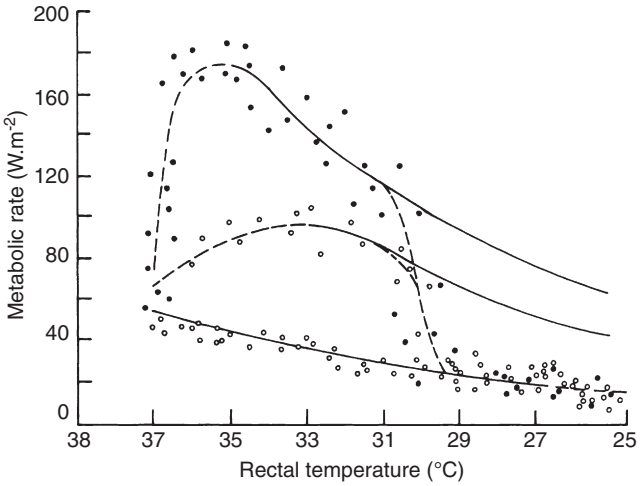


Figure 58-3 Relationship between metabolic rate and rectal temperature in anesthetized dogs, the body temperatures of which were manipulated by intravascular heat exchangers. *Top curve,* Light anesthesia; thus a marked cold-induced increase in heat production with decreasing body temperature was observed. *Middle curve,* Moderate anesthesia; the response of heat production to cooling is reduced. *Lower curve,* Deep anesthesia; no cold defense reaction. After having reached a maximum, thermoregulatory heat production follows van't Hoff's law. Note that the upper and middle curves tend to approach the lower curve as soon as the rectal temperature drops below a critical temperature (about 30° C). (*From Behmann FW, Bontke E: [Regulation of heat formation in artificial hypothermia. I. Experimental studies on the effects of depth of anesthesia.]* Pflügers Arch *266:408, 1958.*)

Before describing the response of the integrated system to changes in environmental temperature, we review what is known about the individual components of the biocybernetic model.

THERMAL SENSORS: THERMORECEPTIVE STRUCTURES AND THE THERMOAFFERENT SYSTEM

Location and Properties of Cutaneous Thermal Receptors

The cutaneous thermal receptors are a group of structures that function as temperature sensors in the temperature control system (the term *sensor* is gaining preference to *receptor* to avoid confusion with chemical structures reacting with specific substances. Both terms are used interchangeably within this section). It is generally agreed that the cutaneous thermosensors in the thermoregulatory system are identical with those mediating thermal sensation. The distribution of the cutaneous thermoreceptors can thus be studied in adult humans by stimulation of so-called warm and cold spots using fine temperature probes. There is scarcely any area of the skin that does not respond to cold stimulation, although the number of cold spots/cm^2 may vary between one and five on the palm of the hand and more than 15 on the face. The number of warm spots/cm^2 is much less in all skin areas (0.3 to 1.7). This agrees with the better spatial resolution of cold stimuli, but it does not mean that cutaneous warm sensitivity is any less developed than is cold sensitivity. In fact, with larger stimulus areas (using thermodes of 10- to 100-cm^2 contact areas), warm sensitivity can be demonstrated on all skin areas with few exceptions (cornea, glans penis).[14] Employing electrophysiologic methods (recording single-receptor activity from nerves), warm and cold receptors have been demonstrated on the faces of cats and other species and on the lower arms and legs of monkeys.[14] By inserting fine metal electrodes through the intact skin into a branch of the radial nerve, it has even been possible to record the activity of single warm and cold receptors in humans in response to thermal stimulation on the skin of the back of the hand.[15]

The morphologic correlate of cold sensors is the fine unmyelinated nerve endings penetrating into the basal layer of the epidermis.[16] These endings contain numerous mitochondria, providing energy for a temperature-sensitive Na^+, K^+ pump, which seems to be part of the transduction of the cold stimulus into an electrical signal.[17]

Figure 58-4 illustrates the average response characteristics of cold and warm receptors from different species (including humans) under static conditions (i.e., after the temperature of the skin had been constant for several minutes). Minimum activity occurs at a skin temperature of 35° C. The maximum discharge frequency of cold receptors is found between 35° and 20° C and that of warm receptors takes place between 40° and 45° C. At temperatures greater than 45° C, warm-receptor activity decreases. At temperatures lower than approximately 25° C, the activity of cold receptors may also be reduced.

Sensor activity during temperature changes is noteworthy in that receptor discharges may reach frequencies that are two- to threefold greater than under static conditions. Irrespective of the initial temperature, a warm receptor will always show an overshoot of its discharge on sudden warming and a transient inhibition on cooling, whereas a cold receptor will respond in the opposite way (with an inhibition on warming and an overshoot on cooling). Correspondingly, sensitivity to temperature changes is amplified and increases with the size of the exposed area. With exposure of larger areas (e.g., the whole hand), a temperature change of less than 0.01° C per second may evoke a thermal sensation[18] and probably a regulatory reaction.

In summary, thermoregulatory responses elicited through cutaneous thermoreceptors are determined by the average skin temperature (T_{skin}), the rate and direction of temperature change ($\Delta T_{skin}/\Delta t$), and the size of the stimulated area.[14,18] Because of the temperature discharge characteristics (see Figure 58-4), warm receptors may contribute to the stimulation of heat dissipation actions only when mean skin temperature is considerably increased higher than normal levels.

Location and Properties of Internal Thermosensitive Structures

Evidence for the existence of deep body thermosensors has been obtained in three ways: (1) by demonstrating that there is poor correlation between body temperature and the action of the final control elements if only skin temperature is taken into consideration,[19] (2) by observing thermoregulatory actions after heating and cooling of circumscribed areas within the body using implanted thermodes and vascular heat exchangers,[20] and (3) by recording the activity of single units of the central nervous system and relating these responses to their own local temperatures.[21]

Hypothalamus

According to thermal stimulation studies (using thermodes implanted on a long-term basis), deep body thermosensors are located in the preoptic area and the anterior hypothalamus. Local warming of this region stimulates heat-dissipating mechanisms (i.e., vasodilation, panting, sweating), whereas it inhibits heat production (i.e., metabolic reactions [Figure 58-5] and vasoconstriction). Inversely, cooling evokes heat production and vasoconstriction, whereas heat dissipation mechanisms are inhibited.

Thermal stimulation does not permit discrimination between internal cold and warm receptors (e.g., the effects of local

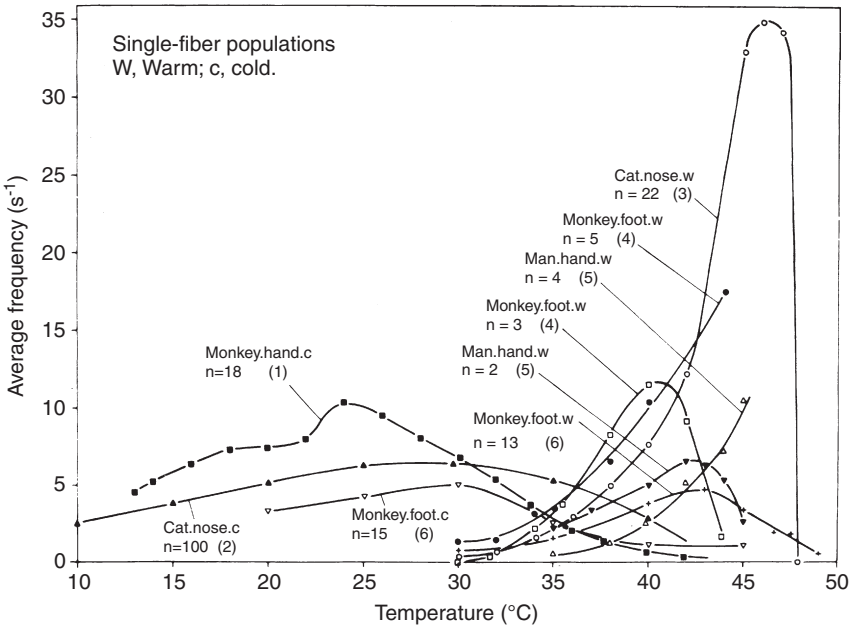


Figure 58-4 Average static discharge frequency of populations of cutaneous cold and warm receptors as a function of skin temperature obtained from various species and body sites by several authors. (*From Hensel H:* Thermoreception and Temperature Regulation. *New York, Academic Press, 1981.*)

cooling might be ascribed to actuation of central cold receptors and to the inhibition of central warm receptors). Here further clarification is obtained from single-unit studies.[21] As shown in Figure 58-6, warming of the preoptic area results in markedly increased activity of one single unit and leads to an increase in respiratory frequency (panting). Units like this are considered to be warm sensors.

In vitro studies of hypothalamic slices[22] and of cell cultures[23] have addressed the question of whether hypothalamic thermosensitivity is tied to individual thermosensitive ganglion cells or is based on the temperature dependence of synaptic transmission between afferent and efferent neurons (Figure 58-7). After synaptic transmission is blocked by electrolyte solutions with a low Ca^{2+} content and a high Mg^{2+} content, the structures in question remain sensitive to thermal stimuli. This implies the existence of thermosensitive cells within the preoptic region and anterior hypothalamus. Both areas contain not only warm-sensitive cells but also thermosensitive and cold-sensitive cells, the latter two being less numerous than the warm-sensitive cells.

Lower Brain Stem and Spinal Cord

Thermosensitive structures have also been demonstrated in the lower brain stem (midbrain and medulla oblongata), and thermoregulatory reactions can be initiated by local warming of these areas.[24] However, the thermosensitivity of this region is distinctly less than in the preoptic region and anterior hypothalamus.[20] In contrast, the spinal cord is extremely thermosensitive. When the temperature of the spinal cord is raised only a few tenths of a degree along its entire length in dogs and other animals, the results include panting, vasodilation, and inhibition of thermogenesis.[20,25] Cooling of the spinal cord elicits shivering, but in this case a greater temperature change is required. In newborn and young guinea pigs, a local temperature change in the region of the cervical cord suffices to trigger thermoregulatory reactions.[3]

Other Thermosensors

Quantitative considerations suggest the existence of thermoreceptive structures outside the central nervous system and the skin.[20] Experimental evidence for the presence of thermosensors in the region of the dorsal wall of the abdominal cavity and in the musculature exists. Evidence for the existence of subcutaneous thermosensors has also been confirmed.[26]

Afferent Thermosensitive Pathways

The cutaneous thermoreceptors are served by thin myelinated and unmyelinated axons belonging to the slowly conducting group III and group IV nerves. Warm fibers are mostly unmyelinated (group IV). The axons run within the afferent cutaneous nerve bundles, and they enter the spinal cord through the segmental dorsal root ganglia. Those axons cross over to the contralateral side and ascend within the spinothalamic tract in the anterolateral section of the spinal cord. On their way to the thalamus, the ascending thermal fibers join the medial lemniscus and are accompanied by the afferents coming from the trigeminal region. From the medial lemniscus, collaterals diverge and project to the hypothalamus through a pathway not definitively described. Evidence has been obtained that part of the cutaneous thermal input is conveyed through the spinoreticular pathway to the reticular formation; from there, it is projected to the hypothalamus through the raphe nuclei and the ventral noradrenergic system, which passes the subcerulean area.[24,27-29]

The spinal cord thermal sensors are connected to the posterior hypothalamus through axons running in an anterolateral pathway of the spinal cord, as has been shown in young guinea pigs[30] and in cats.[31,32] The thermosensors of the preoptic area also end in the posterior hypothalamus; however, these short pathways have not yet been identified.

INTEGRATION OF THERMAL INPUTS

Integration of Multiple Thermal Inputs

The theoretic concepts of thermoregulation require the demonstration of some elements that "process" the thermal information originating at the receptors in various sites of the body (multiple-input system) and that transform these inputs from the sensors into effector outputs.

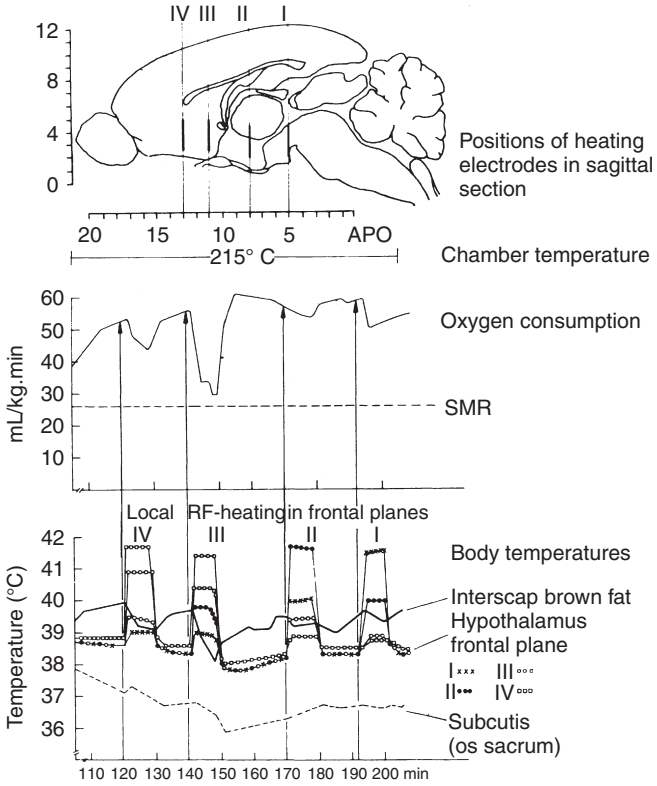


Figure 58-5 Effect of local radio frequency (RF) heating in different frontal planes of the hypothalamus on nonshivering thermogenesis (NST) induced by external cooling in a newborn guinea pig (for these small animals, an ambient temperature of 21.5° C is below neutral temperature). The upper part of the figure shows the projection of the four implanted electrodes on the sagittal section of the brain. The different frontal planes were locally heated in succession. Only heating of plane III, corresponding to the preoptic area and anterior hypothalamus, resulted in an almost complete suppression of the cold-induced NST (note reduction of the temperature within the interscapular brown fat). *SMR,* Oxygen uptake corresponding to standard metabolic rate. (*From Zeisberger E, et al: In Jansky L (ed):* Depressed Metabolism and Cold Thermogenesis. *Prague, Charles University, 1977, pp 182-187.*)

There are many experimental studies implicating the hypothalamus (especially the posterior hypothalamic area, which has no appreciable thermosensitivity) as an integration center for thermoregulation. This premise is further supported by electrophysiologic findings. For example, neurons exist in the posterior hypothalamic area, the activity of which (discharge rate) is influenced by local thermal stimulation in either the preoptic region or the cervicothoracic part of the spinal cord.[33] At the boundary between the anterior and posterior hypothalamus, neurons have been found that respond to changes in the skin temperature on the limbs and trunk.[24] The posterior hypothalamus, therefore, is characterized by the presence of thermoresponsive cells (i.e., cells that respond to changes in the temperature of distant structures but are not sensitive to changes in their own temperature). However, there is no absolute spatial separation of receptive and integrative functions. In the preoptic region and anterior hypothalamus, cells shown to be thermosensitive have also been found to be affected by skin temperature changes and thus are simultaneously thermoresponsive.[4,34]

A special feature of biologic thermoregulation (compared with the familiar simple technical system) is that two kinds of sensors in different locations (the cold and warm receptors) interact antagonistically. The cutaneous cold receptors are activated when the temperature decreases to less than the lower limit of the thermoneutral zone. This reaction is counteracted by heat-activated internal thermosensors when the body temperature increases as a result of overshooting cold-protective mechanisms or after bodily activity. This circuitry enables the protective mechanisms to be set in motion rapidly in case of external cooling, long before the core temperature has begun to decrease and internal thermoreceptors can be influenced.

Conversely, heat dissipation processes (vasodilation, sweating) may be activated by internal warm receptors, which are primarily stimulated when body core temperature increases. This effect is counteracted by cold activation of the cutaneous cold receptors. When the body is heated externally, sweat secretion is stimulated by the combined action of cutaneous and internal warm receptors.

Figure 58-8 shows a simplified model of the neuronal circuitry underlying the central nervous system integrative processes. Three kinds of neuronal elements are distinguished: (1) efferent neurons located in the hypothalamus, the axons of which activate the peripheral controlling elements (Figure 58-9) either directly or, more probably, by way of a chain of interneurons; (2) facilitative and inhibitory interneurons within the hypothalamus; and (3) thermal afferents, arising in part from the cutaneous thermoreceptors and in part from internal receptors (e.g., those of the preoptic region).

Cold receptors directly activate the effectors for thermogenesis. The inhibitory action on the efferents to heat loss effectors is exerted through interneurons. Activation of warm receptors excites the efferents to the heat loss effectors, simultaneously inhibiting (through interneurons) the efferents to the effectors for heat production. The various effector neurons may receive different combinations of thermal afferents. Thus, as shown in studies of newborn guinea pigs, nonshivering thermogenesis is driven by cutaneous cold receptors and is inhibited by hypothalamic warm receptors, whereas the inhibitory influence of shivering is exerted mainly by spinal cord warm-receptive structures (Figure 58-10). The cervical spinal cord is the region that preferentially receives (through vascular connections) the heat that is generated in the interscapular brown adipose tissue.[3,7,35] As a result of this "meshed control" of the two heat-generation mechanisms, shivering remains suppressed in neonates so long as sufficient heat is supplied from the interscapular brown adipose tissue to the spinal cord warm-sensitive structures and to intrathoracic organs.

Principles Guiding the Actions of the Integrator

Given that the thermosensitive structures are distributed over the entire body, the thermoregulatory effector actions (see Figure 58-9) cannot be described as a function of a single local temperature (e.g., the rectal temperature). Thus the goal of thermophysiology is to describe thermoregulatory actions as a function of as many as possible of the temperatures associated with the various thermosensitive parts of the body. Systems of equations with several variables are required for such a description. The data must be collected by experiments on animals in which spatially circumscribed temperature changes are produced by means of thermodes and heat exchangers[20]; in humans, limited opportunity exists for experimental manipulation of local temperature while the temperature of the rest of the body is kept constant. Only an approximate description is possible; the thermoregulatory parameters are presented as a function of two temperatures, the temperature of the interior of the body (measured at a representative site) and the mean skin temperature.[2,28,36] Shivering and sweating threshold temperatures, as well as the temperature pairs

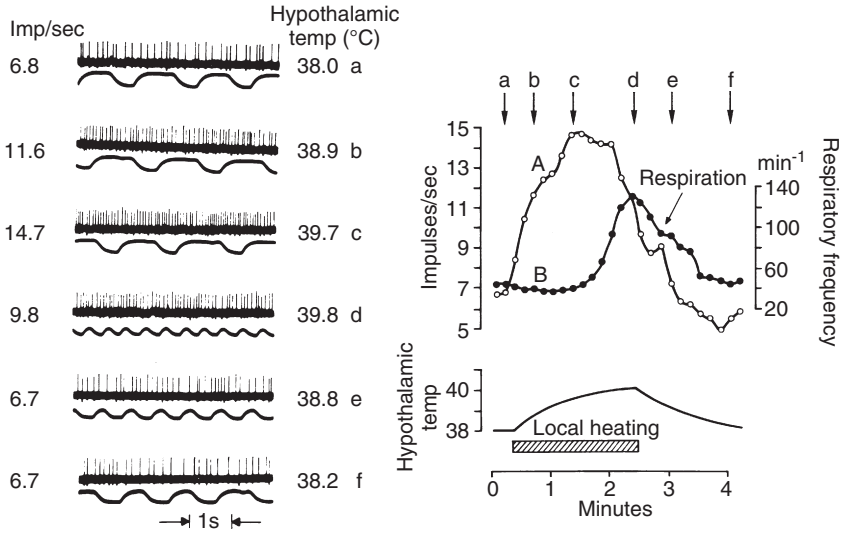


Figure 58-6 *Left panel,* Sections of original records of impulse frequency of a hypothalamic neuron and respiratory rate in relation to hypothalamic temperature. *Right panel,* Discharge of a warm-sensitive neuron in the preoptic region *(curve A)* and simultaneous record of respiratory rate during local heating of the hypothalamus. *Arrows* marked with lowercase letters designate time at which the original record *(left)* was taken. (*From Nakayama T, et al: Thermal stimulation of electrical activity of single units of the preoptic region,* Am J Physiol *204:1122, 1963.*)

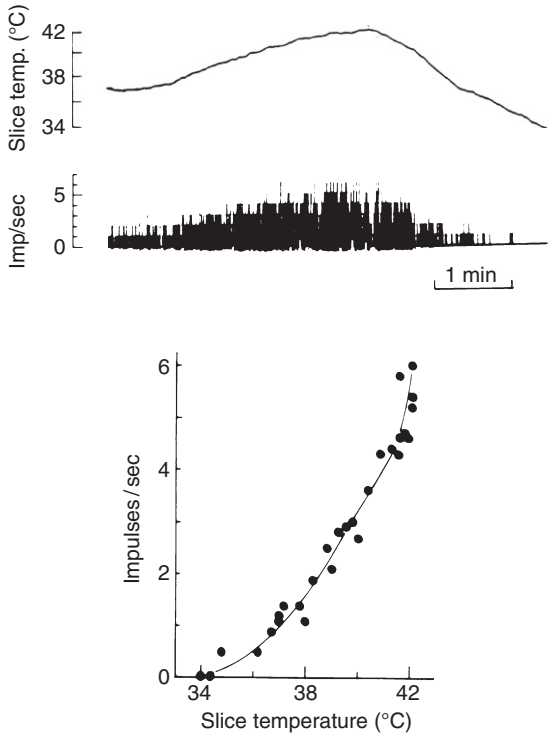


Figure 58-7 *Top,* Original record of a single warm-sensitive unit. *Bottom,* Thermal response curve of the same warm-sensitive unit located in the preoptic area of a slice preparation of the rat hypothalamus. (*From Hori T, Nakashima T, Hori N, Kiyohara T: Thermo-sensitive neurons in hypothalamic tissue slices in vitro,* Brain Res *186:203, 1980.*)

yielding equal magnitudes of shivering and sweating, are represented by contour lines in a coordinate system with mean skin temperature and core temperature as the coordinates (Figure 58-11). Blood flow through the skin follows similar contour lines located between shivering and sweating threshold lines.[37] The contour plots portrayed in Figures 58-11 and 58-12, although involving only two temperatures, are good semiquantitative illustrations of the performance of the multiple input control system. Note that the relative impact of the sensors increases as temperatures become increasingly "out of range," and that the whole contour plot depends on other attributes such as the warm/cold adapted state of the animal. Within a certain range of temperatures (i.e., near the set point), at which the contour lines can be approximated by straight lines, it is possible to express the effector responses and the threshold temperature conditions for the elicitation of effector responses in terms of a weighted mean body temperature, T_b, calculated with the following linear equation:

$$T_b = a \times T_{core} + b \times T_{skin}$$

Data from human adults can be best fitted if values of 0.9 and 0.1 are chosen for the coefficients a and b, respectively.[28] Figure 58-12 shows the shivering threshold contour lines of adult humans, rabbits, goats, and young (4-week-old) guinea pigs. In addition, the threshold contour line for nonshivering thermogenesis in newborn guinea pigs is given. The curvilinear contour lines in small animals do not allow the use of arithmetic mean body temperature except for small sections of the temperature ranges that may be approximated by straight lines.

SET POINT AND NORMAL BODY TEMPERATURE

In a multiple-input system, the control actions of the thermoregulatory system can be ineffective at various combinations of temperatures. For instance, shivering remains suppressed so long as the combination of body surface and body core temperatures stays at or greater than the threshold contour line (see Figures 58-11 and 58-12). Conversely, heat dissipation actions will be ineffective so long as the combination of surface and internal temperature values remains less than the heat dissipation threshold hyperbola (see Figure 58-11).

Under environmental conditions that allow the control actions to reach ineffective levels, the deep body temperature will stabilize at a value typical for the species (if enough time is allowed to attain steady-state heat flow). This temperature, whether measured within the tympanic canal, the rectum, the esophagus, or

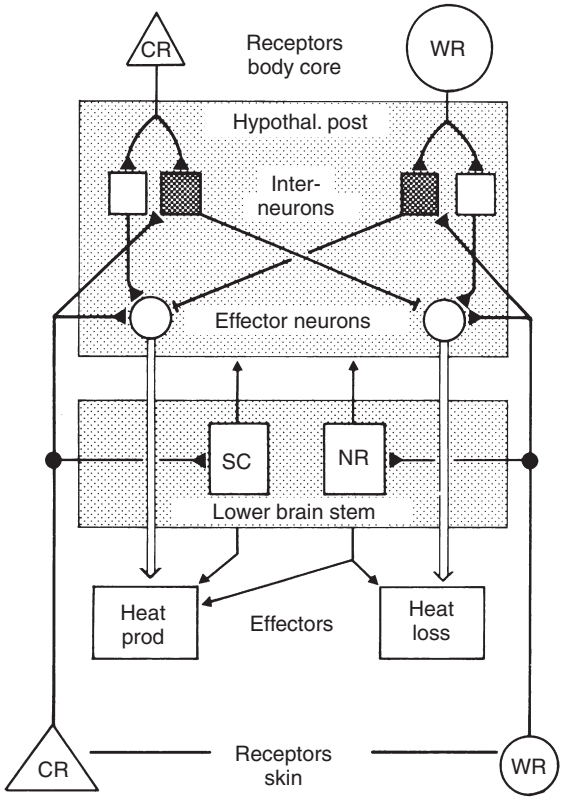


Figure 58-8 Highly simplified model of the connections between thermal afferents and the efferent neuronal networks that control the thermoregulatory effector elements. *Shaded areas* represent the thermointegrative regions of the hypothalamus (mainly the posterior hypothalamus) and the lower brain stem, which contain crucial structures for the processing of thermal information from the skin (*RN,* raphe nuclei; *SC,* subceruleus region). The inhibitory neurons shown *(cross-hatched squares)* mediate the reciprocal inhibition of the heat-losing and heat-generating processes. *CR,* Cold receptors; *WR,* warm receptors (size of the symbols indicates roughly the difference in numbers); ◀, activating; ⊣, inhibitory synaptic connections. The symbols for neurons represent not single neurons but neuron pools. Some details of the connections in the SC, RN, and hypothalamus are known but are not within the scope of this diagram. The *arrows* pointing down from the lower brain stem represent pathways to mononeurons and dorsal horn neurons in the spinal cord; the latter can suppress input from the warm afferents. (*Data from Brück K, Hinckel P: Thermoafferent systems and their adaptive modifications,* Pharmacol Ther *17:357, 1982.*)

elsewhere, may then be called the *normal body temperature*. Deviations of such a representative temperature from an empirically attained normal value may be described as a set-point displacement or, more specifically, as a deviation of the threshold temperatures for the respective control actions.

EFFECTOR RESPONSES OF THE THERMOREGULATORY SYSTEM

The effector outputs of the thermoregulatory controller restore the system to the desired set point by regulating both heat production and heat loss. After a brief review of the relationship between metabolic rate and environmental temperature, control of heat production and heat loss are discussed separately.

Metabolic Rate and Environmental Temperature

The cold-induced increase in metabolic rate (see Figure 58-3) is the most characteristic feature of homeothermic organisms. In the neonates of all homeothermic species studied thus far (except for the ground squirrel and golden hamster, which are hibernators), a cold-induced increase in metabolic rate has been demonstrated immediately after birth. Although this response may be small during the first hours of life, neonates should not be considered poikilothermic.

The influence of postnatal age on cold-induced thermogenesis is shown in Figure 58-13. As demonstrated by several investigators, oxygen uptake (directly proportional to thermogenesis) is distinctly greater at 28° C ambient temperature than at 32° to 35° C, even during the first few hours of life. At an ambient temperature of 23° C, oxygen consumption is even greater. Depending on the species, this cold-induced metabolic response increases to a greater or lesser extent during the first week of life. Preterm and small-for-date infants also increase their metabolic rate with cooling, and on average their response is not much less than that in full-term infants (see Figure 58-13). Oxygen uptake values of 15 mL/kg/minute measured at an ambient temperature of 23° C[1] and of 16.8 mL/kg per minute measured at 26° C[38] appear to represent the maximum (summit) metabolic response to cold in 1-week-old full-term infants. For comparison, in well-trained young adults, summit oxygen consumption may increase to five times the at rest level, that is, to 17 to 20 mL/kg per minute, when the environmental temperature is maintained at 0° to 5° C.

The maintenance of a constant body temperature requires that heat loss and heat production be equal in the steady state. Figure 58-14 illustrates the possible ways in which body temperature can be kept constant when the environmental temperature changes. According to Newton's law, dry (nonevaporative) heat loss is proportional to the temperature difference between the body core and the surroundings. Therefore, in humans, heat loss should be nonexistent at an ambient temperature of 37° C, and it should increase linearly with decreasing ambient temperature. Because heat loss also depends on heat conduction and convection within the body, and thus on peripheral blood flow, two heat loss curves may be generated (see Figure 58-14), one for peripheral vasodilation and another for vasoconstriction. Within the thermoneutral zone, heat production (corresponding to the at rest [basal] metabolic rate) is in equilibrium with heat loss only if skin blood flow is progressively reduced as the temperature decreases from the upper end of the thermoneutral zone *(T_3)* to its lower end *(T_2)*. At temperatures less than T_2, body temperature can be kept constant only if heat loss is exactly compensated for by increasing thermoregulatory heat production (cold-induced thermogenesis). The maximum thermogenesis (which amounts to three to five times the BMR in adults but only two to three times the SMR in neonates) determines the lower limit of the thermoregulatory range (T_1 in Figure 58-14). The value for T_1 is approximately 5° C in adults and 23° C in full-term neonates.[1] When this limit is exceeded, hypothermia ensues.

At temperatures greater than T_3, thermal equilibrium can be achieved only by an additional heat loss mechanism—the secretion and evaporation of sweat. It is not possible for the organism to down-regulate BMR. In Figure 58-14, point T_4 indicates the upper limit of the range of regulation, which is determined by the sweat rate capacity. Between points T_3 and T_4, body temperature inevitably increases because of a load error typical for a proportional control system, resulting in an increasing metabolic rate according to van't Hoff's rule.

The term *thermoneutral zone* was originally defined solely with respect to metabolic rate, and some problems arose concerning its use in perinatal physiology.[39] The thermoneutral zone is now defined as "the range of ambient temperature at which metabolic rate is at a minimum, and within which the

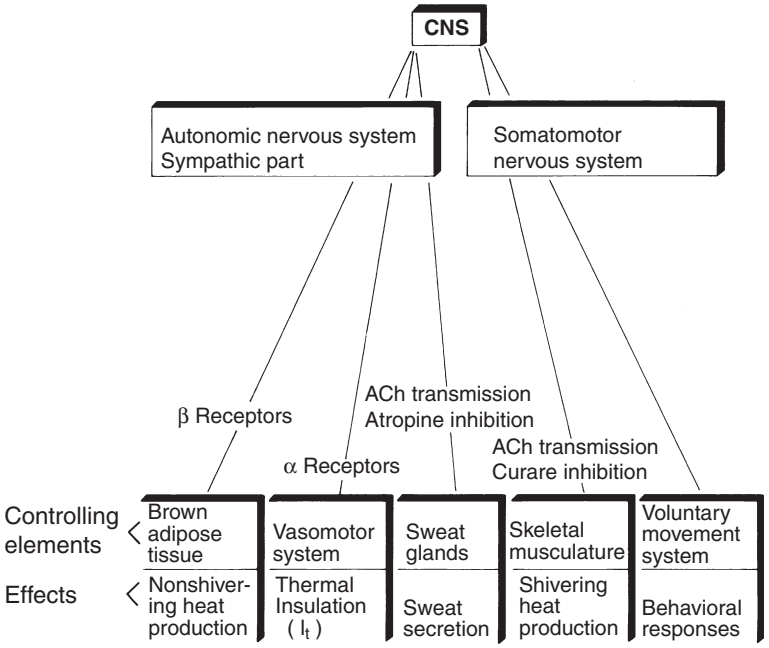


Figure 58-9 Schematic representation of the neural control of the thermoregulatory effector systems. (*From Brück K: In Schmidt RF, Thews G (eds):* Human Physiology. *Berlin, Springer-Verlag, 1983, pp 531-547.*)

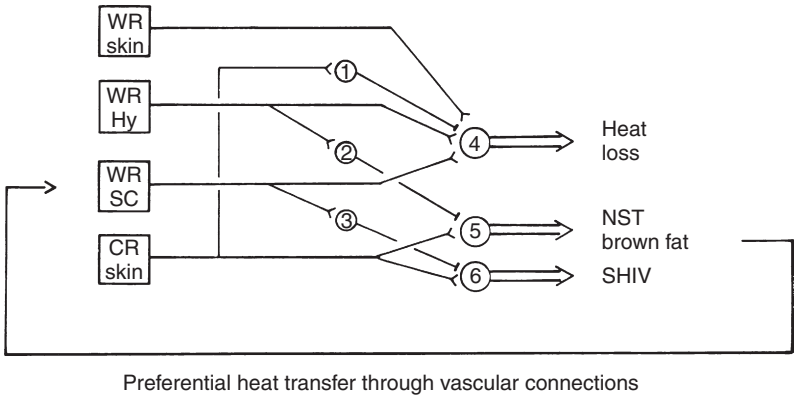


Figure 58-10 Schematic representation of the presumed neuronal circuitry underlying the control of heat loss and thermogenesis. Note that nonshivering thermogenesis *(NST)* and shivering *(SHIV)* are differentially controlled. *CR,* Cold receptors; *WR,* warm receptors; *SC,* spinal cord; *Hy,* hypothalamus; *1, 2, 3,* inhibitory interneurons; *4, 5, 6,* effector neurons. It must be inferred from experimental evidence that with increasing age, connections are being formed between WR_{Hy} and the no. 6 effector neurons.

temperature regulation is achieved only by control of sensible ("dry" or "nonevaporative") heat loss, that is, without regulatory changes in metabolic heat production or evaporative heat loss."[8] This range is delineated by the symbols T_2 and T_3 in Figure 58-14. In unclothed at rest adults, the lower range limit of the thermoneutral zone (T_2) is 26° to 28° C (50% relative humidity, still air); however, it is 32° to 35° C (operative temperature, 50% relative humidity, still air) in naked full-term newborn infants.[1,39] This difference is of importance because it shows that environmental temperature conditions that do not require any thermoregulatory effort in adults may seriously overtax the metabolic thermoregulatory system of neonates.

In small premature infants (1 kg), the lower limit of the thermoneutral zone may be as high as 35° C.[39] The lower end of the thermoneutral zone may change with postnatal development as body size increases[39] and as small changes in the sensitivity of the thermoregulatory system occur. It is thus extremely difficult to provide exact standard values for the thermoneutral zone in neonates.

CONTROL OF HEAT PRODUCTION

Modes of Extra Heat Production

The ability to produce extra heat in a cool environment is one of the characteristic features of homeothermy. There are three principal modes of heat production that are responsible for the increase of heat production with decreasing environmental temperature: (1) voluntary muscle activity, (2) involuntary tonic or rhythmic muscle activity (the latter may be manifest as shivering or may be invisible and detectable only by electromyography), and (3) nonshivering thermogenesis. The existence of nonshivering thermogenesis was originally evidenced by the demonstration of a cold-induced increase in oxygen uptake that persisted after neuromuscular blockade with curare.

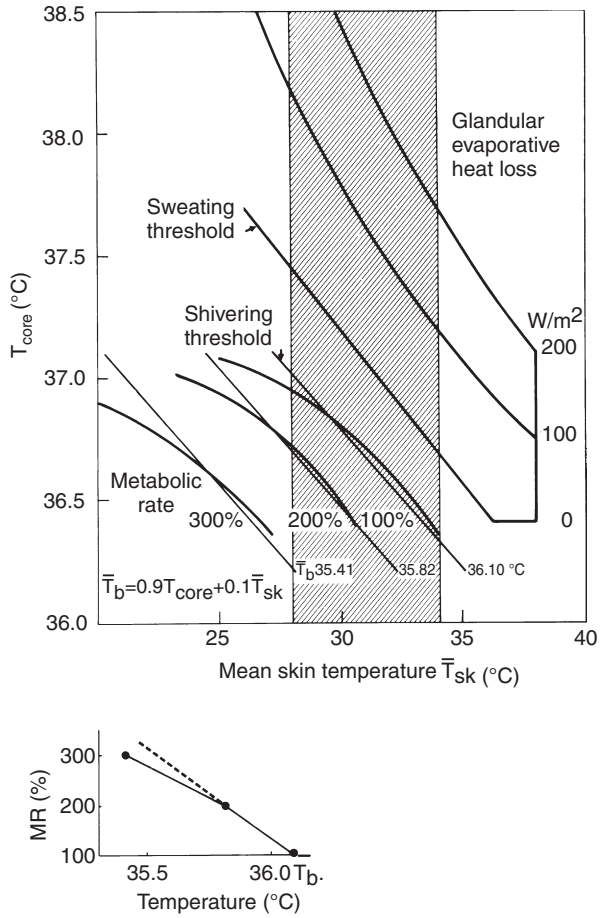


Figure 58-11 Contour plot of metabolic rate and sweat rate (glandular evaporative heat loss) as a function of core temperature and mean skin temperature. The *curved lines* for metabolic rate were approximated by *straight lines* (and so might be the 100 and 200 W curves for evaporative heat loss). Each *straight line* represents all pairs of core and skin temperature with equal $\overline{T}_b$ calculated from the inset equation. *Hatched area* indicates range of mean skin temperatures relevant for the comparison of shivering threshold values. Percentage metabolic rate (MR) in relation to $\overline{T}_b$ is shown at the bottom of the figure. (*Data from human adults in Benzinger TH: Heat regulation: homeostasis of central temperature in man.* Physiol Rev *49:671, 1969. and Nadel ER, Bullard RW, Stolwijk JA: Importance of skin temperature in the regulation of sweating,* J Appl Physiol *31:80, 1971.*)

Shivering and Nonshivering Thermogenesis

In adult humans and in larger adult mammals, shivering is quantitatively the most important involuntary mechanism of thermoregulatory heat production. In contrast, nonshivering thermogenesis is an important and effective mechanism of heat production in the neonates of many mammalian species, including human infants. In comparison with nonshivering thermogenesis, shivering is a less economical form of heat production in that it inevitably increases convective heat loss because of the body oscillations. Moreover, shivering interferes with body movement. This economical aspect becomes more important in smaller organisms (high surface-to-mass ratio) with poorer thermal insulation. Thus it is satisfying, from a teleologic point of view, to find that the maximum extent of nonshivering thermogenesis available in one species is inversely related to its order of body size (Figure 58-15). Extrapolating the regression line in Figure 58-15, one can conclude that subjects with body weights heavier than 10 kg lack the capacity for nonshivering thermogenesis.

Loss of Nonshivering Thermogenesis

In relatively mature newborns, such as guinea pigs, the extent of nonshivering thermogenesis is greatest at the time of birth and it vanishes within a few weeks (Figure 58-16). This involution of nonshivering thermogenesis can be retarded and partly inhibited by rearing the animals in a cold environment. After nonshivering thermogenesis has been extinguished through exposure to a warm environment, it can again be evoked by exposing older guinea pigs to a cool environment. In human neonates, no experimental data are available to document the process of the disappearance of nonshivering thermogenesis. It may be assumed that nonshivering thermogenesis is the prevailing mechanism of thermoregulatory heat production during the first 3 to 6 months of life.[12] By contrast, in rats, the extent of nonshivering thermogenesis increases during the postnatal period.[40,41] Such behavior may be expected to occur in other altricious neonates that, like rats, are born in a relatively immature stage. This would explain the gradual increase in the maximum cold-induced heat production that can be observed during the first few weeks of life in premature infants.[1]

Lack of Nonshivering Thermogenesis

At least one example exists of a species in which the neonates, although small, do not possess nonshivering thermogenesis—pigs. Piglets shiver vigorously when exposed to cold, even on the first day of life.[42] Miniature piglets have no demonstrable nonshivering thermogenesis even when they are reared for a few weeks in a cold environment.[43] In agreement with the data depicted in Figure 58-15, calves (a larger neonate) have no metabolic response to norepinephrine, indicating the lack of nonshivering thermogenesis.[44]

Sites of Nonshivering Thermogenesis

For many decades, the liver, other intestinal organs, white subcutaneous adipose tissue, and the skeletal musculature were suggested as sites of nonshivering thermogenesis. During the early 1960s it was suggested that brown adipose tissue was the primary site of nonshivering thermogenesis.[40,45-47]

Shivering

Newborn animals and newborn infants are rarely seen to shiver in response to cooling, although the metabolic rate is considerably increased. As shown in Figure 58-17, no muscle activity is demonstrable by electromyography on day 2 of life in the cold-stressed guinea pig. In contrast, on day 12 of life, exposure to the same ambient temperatures (16° and 8° C) evokes bursts of electrical muscle activity that are accompanied by visible muscle oscillations. This does not mean, however, that the shivering mechanism is not developed in the immediate postnatal period. To the contrary, it has been shown that shivering can be elicited in the guinea pig on the first day of life when nonshivering thermogenesis is blocked by a β-receptor-blocker (Figure 58-18) and body temperature is thereby allowed to fall. One may conclude, therefore, that the shivering mechanism is well developed at the time of birth but is normally suppressed by nonshivering thermogenesis. Shivering has also been observed in human neonates with severe hypothermia after birth. It has thus been concluded that the shivering threshold is displaced in neonates to a lower body temperature in comparison with the adult.

The control of the thermoregulatory effector responses (thermogenesis, skin blood flow changes, sweat secretion, and behavioral responses) is predominantly exerted by the nervous system (see Figure 58-9); hormonal transmission has a role only in long-term modifications of the thermoregulatory system.

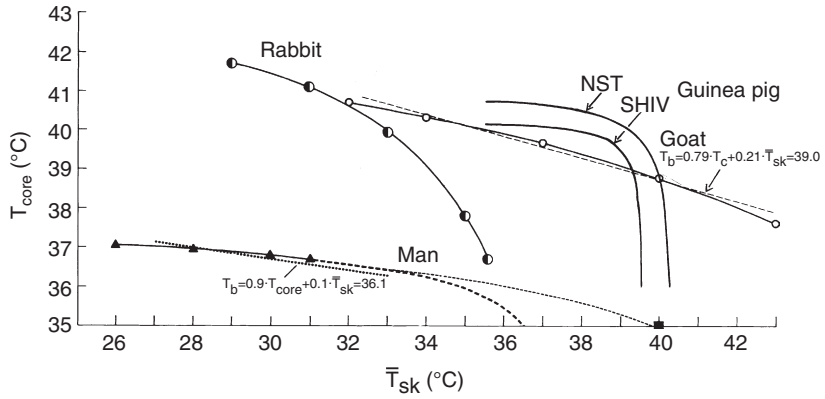


Figure 58-12 Thresholds for shivering *(SHIV)* and nonshivering thermogenesis *(NST)* as a function of core *(T_{core})* and mean skin temperature *($\bar{T}_{skin}$)*. Compared are the shivering threshold contour lines from adult humans, rabbits,[127] goats,[20] and 4-week-old guinea pigs[3] as well as the contour line representing threshold conditions for NST in newborn guinea pigs.[128] Note that core temperature is the local hypothalamus temperature in the case of rabbits and newborn guinea pigs and the spinal cord temperature in the case of 4-week-old guinea pigs.The *stippled* and *dashed straight lines* represent pairs of T_{core} and $\bar{T}_{skin}$ with equal $\bar{T}_b$, *36.1* and *39,* as calculated from the respective inset equations. (- - -, *Data from Benzinger TH: Heat regulation: homeostasis of central temperature in man*. Physiol Rev *49:671, 1969;* —, *data from Hayward JS, Eckerson JD, Collis ML: Thermoregulatory heat production in man: prediction equation based on skin and core temperatures,* J Appl Physiol *42:377, 1977.*)

Two neuronal systems participate in thermoregulation: (1) the somatomotor system and (2) the sympathetic system.

Metabolic Responses: Neural Control of Shivering and Nonshivering Thermogenesis

Shivering is controlled by means of the somatomotor system. The descending axons of the central shivering pathway project from the posterior hypothalamus to the reticular formation of the midbrain and pons.There they contact the supraspinal pathways and descend to the motor neurons in the anterior horns of the spinal cord. From there the musculature is rhythmically actuated through the motor nerves that leave through the anterior roots.

Nonshivering thermogenesis is controlled from the hypothalamic ventromedial nucleus[48] through the sympathetic nervous system.The transmitter in the target system (in particular, brown adipose tissue) is norepinephrine, which acts on adrenergic β-receptors located in the cell membranes. Nonshivering thermogenesis can thus be blocked by β-receptor-blocking agents (see Figure 58-18).The norepinephrine released at the nerve endings liberates free fatty acids from the lipid droplets and stimulates their subsequent oxidation.

CONTROL OF HEAT LOSS

Vasomotor Responses

Thermoregulatory control of blood flow varies regionally.[49] At least three functionally different regions can be distinguished: (1) acral areas (e.g., fingers, hands, ears, lips, nose), (2) trunk and proximal limbs, and (3) head and brow. Blood flow through the acral areas is controlled exclusively through noradrenergic sympathetic nerves.An increase in sympathetic tone causes vasoconstriction, and a decrease in tone results in vasodilation. In contrast to the distal extremities, heat-induced reflex vasodilation in the trunk and proximal limbs results in a much larger blood flow than that observed after sympathectomy.[50] It has been suggested that in these areas there are specific vasodilator nerves that inhibit the vascular smooth muscles (active vasodilation).[50] Conversely, additional vasodilation has been ascribed to an enzyme secreted in sweat that catalyzes the formation of a vasoactive mediator, possibly bradykinin. The latter concept is supported by the frequent occurrence of a second phase of vasodilation coinciding with the onset of sweat secretion in the forearm (Figure 58-19) and by the observed lack of substantial vasodilation in people congenitally lacking sweat glands.[45] Vasomotor nerves exert only a slight effect on the forehead, and practically no vasoconstriction occurs in response to cold stress; however, vasodilation does occur in these regions along with sweat secretion in response to heat stress.

Well-developed vasomotor responses to environmental temperature changes have been demonstrated in full-term and premature newborn infants.[7]

Sweat Secretion

Apocrine sweat glands, as found in various mammals such as the Bovidae and Equidae, are controlled by an adrenergic sympathetic mechanism. However, in humans, apocrine sweat glands are controlled by cholinergic sympathetic fibers.[51,52] Consequently, sweat secretion can be inhibited by atropine.Acetylcholine, pilocarpine, and other parasympathetic drugs evoke sweat secretion.

Behavioral Regulation

Behavioral measures must not be neglected in considering the constancy of body temperature. In adults of various species, particularly humans, behavioral measures may have a more important part in temperature constancy than do the autonomic reactions, and under some circumstances they may be the only way to maintain thermal comfort.A civilized human is rarely seen to shiver; he or she would rather increase the set temperature of the air conditioning system or decrease it before perspiration begins.

Behavioral regulation has been generally looked on as a highly developed type of thermoregulation in comparison with the autonomic regulatory measures described previously. More recent studies, however, have shown that behavioral regulation is, from a phylogenetic point of view, the more primitive state of thermoregulation. Behavioral thermoregulatory actions may even be manifested in a species that is not at all able to respond by autonomic effector actions.Thus numerous fish species have been shown to seek out a preferred water

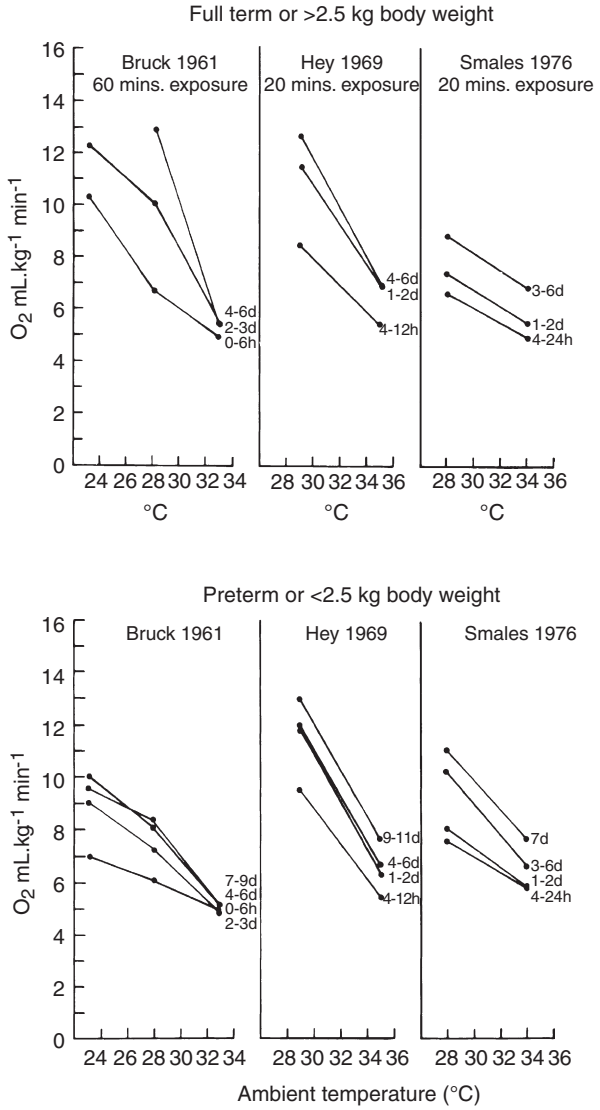


Figure 58-13 Average rates of oxygen consumption of mature *(top panels)* and premature infants *(bottom panels)* at different ages and different ambient temperatures as measured by Brück,[1] Hey,[38] and Smales.[129] (*From Hull D, Smales ORC: Heat production in the newborn. In Sinclair JC [ed]:* Temperature Regulation and Energy Metabolism in the Newborn. *New York, Grune & Stratton, 1978, pp 129-156.*)

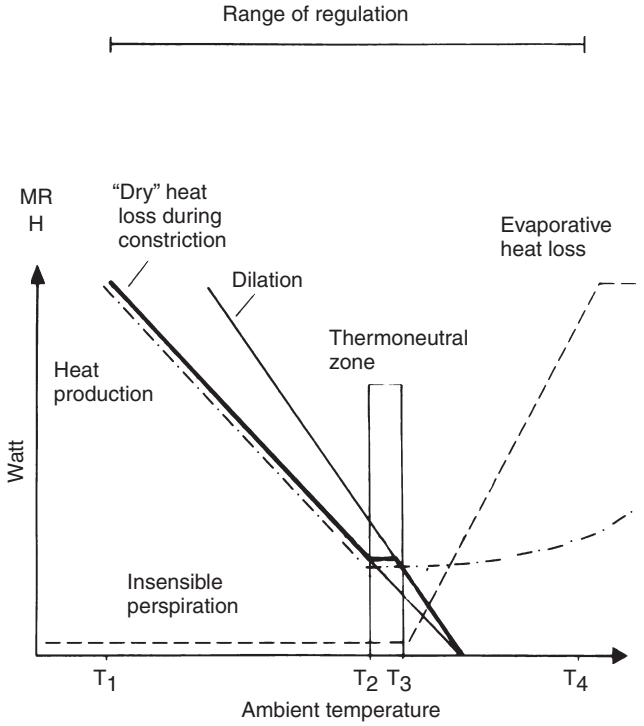


Figure 58-14 Schematic representation of thermal balance in a homeothermic organism. In the range between T_1 and T_3, heat loss is matched by heat production; between T_3 and T_4, evaporative heat loss matches heat production plus heat gain from the environment. In the range T_2 to T_3 (thermoneutral zone), heat loss can be matched to at rest heat production by vasomotor adjustments. Less than T_1, heat loss exceeds the thermogenetic capacity. Greater than T_4, the production and influx of heat exceed the capacity for evaporative heat loss. *MR,* Metabolic rate; *H,* heat loss. (For further discussion, see text.)

temperature when they are given a choice, thereby stabilizing body temperature at a certain level. The "sunbathing" of reptiles is another well-known behavioral thermoregulatory response by which the body temperature is increased to, and precisely maintained at, a level comparable with that of homeothermic animals.[53]

Neonates also make considerable use of behavioral measures. Both piglets and newborn rabbits[54] reduce heat loss by huddling together. Moreover, when newborn rabbits are put into a temperature-gradient environment (a tube with steadily increasing temperature from one end to the other), they will move to a section of the tube warm enough to cause their body temperatures to stabilize near the adult level.[55-57]

In human neonates, postural reactions against overheating have been described.[58] Moreover, babies may cry to signal "thermal discomfort." This would represent another type of behavioral thermoregulation, namely, one mediated by the parents.

ADAPTIVE AND DEVELOPMENTAL CHANGES IN THERMOREGULATION

Unlike engineered physical regulatory systems, thermoregulation by living organisms is not invariant; its properties are influenced by thermal loading itself—its duration, frequency, and intensity. Some of the so-called long-term thermoadaptive phenomena may be traced to changes in the thermoregulatory system. As for the "passive system" (see Figure 58-1), heat insulation may change with development because of an increase in the size of the subcutaneous fat layer or growth of a fur coat. The capacity of the effector systems (nonshivering and shivering thermogenesis) and some regulatory characteristics (namely, alterations in the threshold and gain of the relationship between effector response and body temperature)[28,59] can also undergo changes with development. As a consequence of these thermoadaptive modifications, the range of regulation (see Figure 58-14) may increase, and the thermal discomfort evoked by thermal stress may be relieved.

Acclimation Versus Maturation

So long as the neonatal temperature control system was considered merely as deficient, it was thought that thermal acclimation, in addition to maturational processes, improved this system postnatally. With regard to nonshivering thermogenesis, however, the capacity of this cold defense mechanism is already maximized at birth in some species (see Figure 58-16). Only a narrow margin exists for improving cold resistance by adaptive modifications in the metabolic system during the neonatal period.

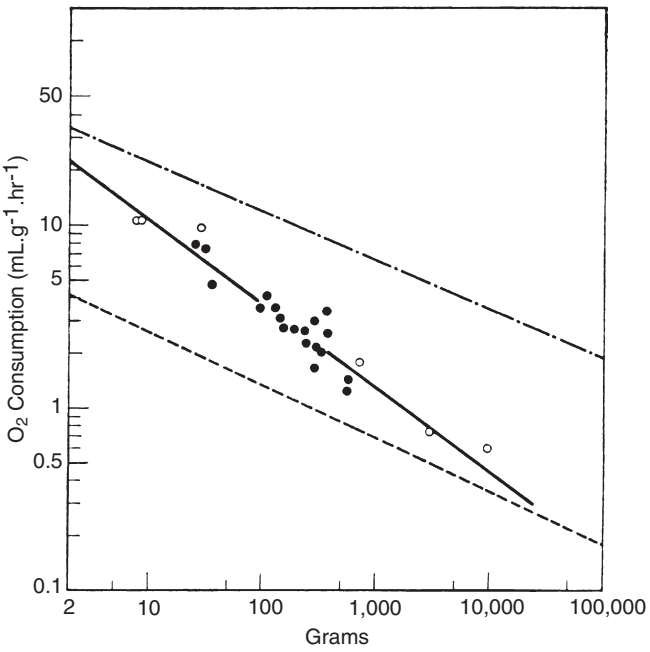


Figure 58-15 Maximum amount of nonshivering thermogenesis, measured as increase in O_2 uptake after norepinephrine injection, in relation to body mass (various species in the adult age). ----, Minimum O_2 uptake at neutral temperature; ——·——·——, calculated maximum O_2 uptake for exercise; ●, rodents; ○, other mammals. (*From Heldmaier G: Zitterfreie wärmbildung und Körper-gröBe bei Säugetieren.* Z Vergl Physiol *73:222, 1971.*)

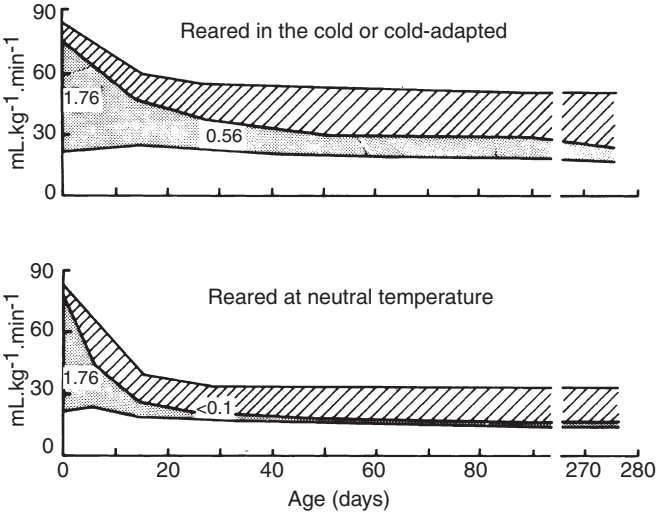


Figure 58-16 Reduction of the maximum extent of nonshivering thermogenesis (NST) with increasing age and the dependence of this process on the environmental temperature at which the guinea pigs were reared. *White area,* Minimal oxygen consumption; *shaded area,* NST; *striped area,* shivering. The inset figures indicate the percentage mass of interscapular brown adipose tissue, which is closely related to the maximum extent of NST. Based on data from Zeisberger et al.[130] (*From Brück K: Heat production and temperature regulation. In Stave U (ed):* Perinatal Physiology, *New York, Plenum Publishing, 1978, pp 455-498.*)

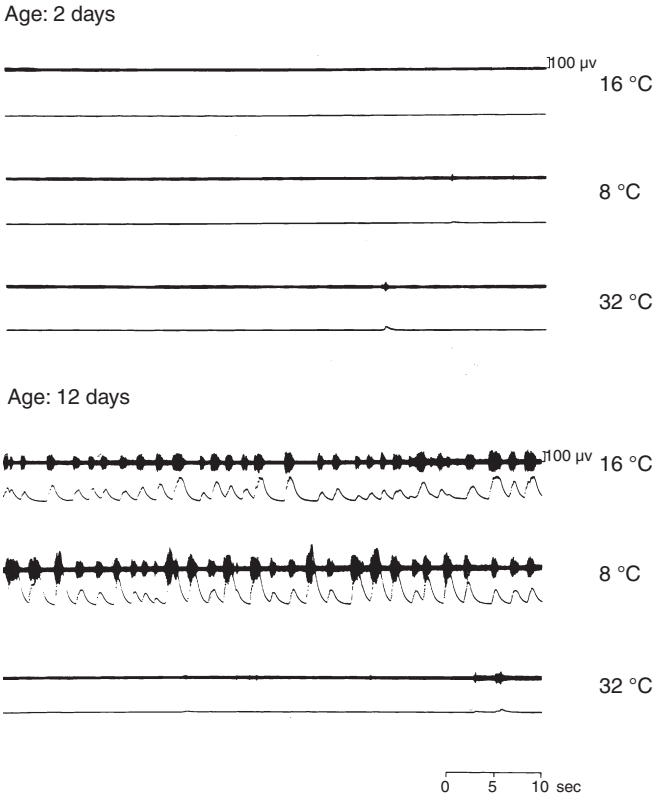


Figure 58-17 Electromyograms from a guinea pig examined on days 2 and 12 postnatally at three different environmental temperatures (indicated on the right side). The lower traces on each record represent the integrated electrical muscle activity. Note that no shivering occurred on day 2 (although oxygen uptake was increased to three times standard metabolic rate). (*From Brück K: In Linneweh F [ed]:* Fortschritte der Pädologie, *Berlin, Springer-Verlag, 1965, pp 96-108.*)

In neonates born in a less mature state, development of nonshivering thermogenesis and brown adipose tissue may proceed postnatally (even at thermoneutral conditions) and contribute to an improvement in temperature regulation. Thus the increasing summit metabolic rate in response to cold during the first few days of life in full-term and premature infants (see Figure 58-13) may be due to postnatal development of nonshivering thermogenesis and an increase in brown adipose tissue. In hamsters and rats, which are born in a rather immature state, guanosine diphosphate binding (as a measure of the thermogenic concentration) has also been shown to increase postnatally (altricious animals).[41,60]

Long-Term Threshold Temperature Displacement

Modifications in the threshold temperature for the elicitation of thermoregulatory effector actions have been demonstrated in animals other than humans. In guinea pigs reared in the cold, the shivering threshold is shifted to a temperature level about 1° C lower than in controls reared at neutral temperature (Figure 58-20).[28,59] This shift enables these animals to make full use of nonshivering thermogenesis before the less economical shivering mechanism is evoked. A similar shift in the shivering threshold can be produced in adult humans by repeated short-term cold exposure (Figure 58-21). Linked with the change in shivering threshold in humans is a shift in the threshold temperature for the experience of thermal discomfort, estimated by the subjects being studied on the basis of a subjective rating scale.[61]

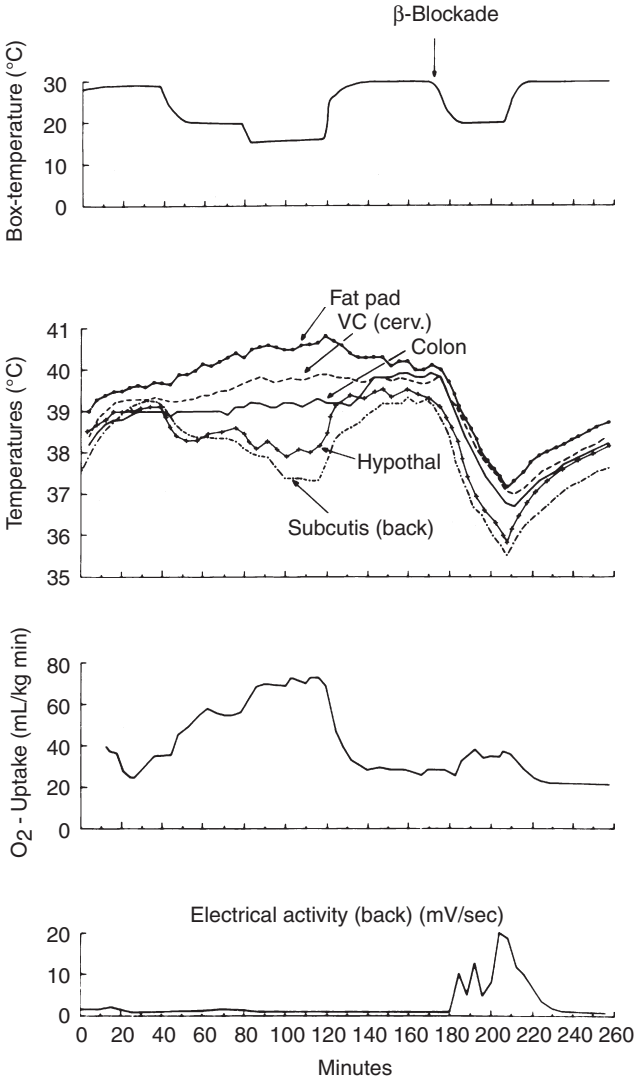


Figure 58-18 Demonstration of shivering and nonshivering thermogenesis (NST) in a newborn guinea pig, age 0 days, body mass 101 g. In the first part of the experiment, only NST occurs during cold exposure. After administration of a β-receptor-blocker and blockade of NST, shivering takes place and can be recognized by the increased electrical activity of the back muscles. NOTE: Increasing temperature in the area of the interscapular adipose tissue *(Fat Pad)* and in the cervical part of the vertebral canal *(VC [cerv])* occurs before, but parallel decrease of all temperatures occurs after blockade of NST. (For further explanation, see text.) (*Adapted from Brück K, Wünnenberg W: Beziehung zwischen Thermogenese im "brounen" Fettgewebe, Temperatur im cervicalen Anteil des VertebralKanals und Kgiltezittem,* Pflügers Arch *290:167, 1966.*)

This type of adaptation has been called *tolerance adaptation*, that is, larger deviations in body temperature are tolerated before actuation of the appropriate thermoregulatory effectors occurs. In other words, the precision in temperature regulation is reduced, but at the same time cold discomfort is diminished.

Repeated heat exposure has been known to decrease the sweating threshold, that is, a sensitization occurs in the course of repeated heat exposure rather than development of tolerance. The load error of the thermoregulatory system is thereby diminished, that is, a tendency occurs to keep body temperature at less than a critical level. The sweating threshold shift as it occurs during heat adaptation is accompanied by similar changes in the threshold temperatures for shivering and vasodilation (Figure 58-22). Such concurrent shift of the thresholds for all autonomic effector systems corresponds to a resetting of the set point of the thermoregulatory control system.[28,62] In some instances the threshold deviation may be accompanied by alterations in the gain of the temperature-response relationship.

Short-Term Threshold Temperature Displacement

In addition to the described threshold changes that occur during repeated or continuous cold-heat exposures for several days or weeks, threshold deviations developing on the order of minutes have been demonstrated in humans as well as in animals. Short-term shivering threshold displacement in men exposed twice to a low climatic chamber temperature with an interposed 20-minute rewarming period has been observed. During the second cooling phase, shivering occurred at a 0.4° C lower mean body temperature, whereas the slope of the temperature-response relationship was unchanged. In addition, the thresholds for skin blood flow and for sweating were shifted to lower values when the subjects were exposed to cold before they started an exercise test; this shift led to vasodilation and sweating. Thus a short cooling period causes a concurrent displacement of the threshold temperatures for all autonomic thermoregulatory responses in adult humans. In animals' adapted to normal room temperature (21° C), this phenomenon of short-term acclimation is even more pronounced.

Previous observations in premature infants who were maintained at ambient temperatures so low as to yield steady-state body core temperatures near 35° C without arousing any cold defense have been ascribed to such short-term acclimation. In a subsequent study on a group of premature infants (i.e., those weighing less than 1500 g), minimal oxygen uptake was compatible with rectal temperatures in the range of 35° to 36° C. As judged from the time spent in quiet sleep, these babies did not show any signs of thermal discomfort.[63] Thus the discomfort threshold can also shift to a lower level of body temperature. Moreover, the infants in that study exhibited a slight tendency toward vasodilation, indicating a decreased threshold for vasomotor responses. However, these effects may be related to postnatal maturation.

Maturation

Although the control system (passive system; see Figure 58-1) undergoes considerable alteration during ontogenesis, the thermoregulatory system tends to maintain deep body temperature from birth onward at a value typical for the species. This maintenance requires a number of adjustments by the thermoregulatory system to the smaller body size of neonates. These adjustments may be mediated by an increased thermogenic capacity, an additional heat production mechanism (nonshivering thermogenesis), and special changes in threshold conditions for eliciting effector responses. Conversely, modifications developing during thermal adaptation may consist of intrinsic mechanisms functioning in a neonatal organism that has not been stimulated by any environmental factor. These same mechanisms may serve to adjust the regulatory system either to body size or to special environmental conditions.

Stability of Deep Body (Core) Temperature During Ontogenesis

As shown in Figure 58-23, colon temperature in newborn guinea pigs or rabbits is maintained close to the adult level, even during severe cold exposure. Remarkably, thermostability seems to be even greater during the first few days of life than it is later on. In guinea pigs, this enhanced stability can be accounted for by

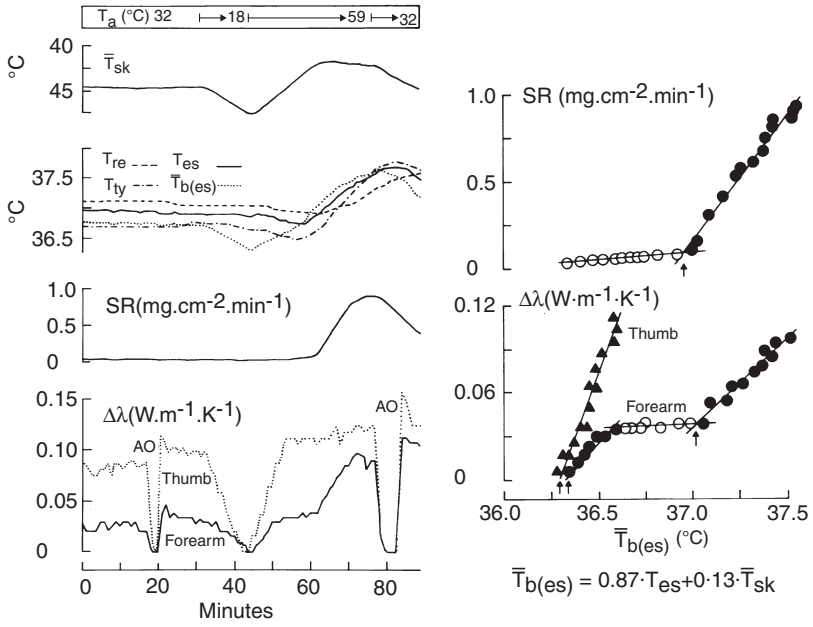


Figure 58-19 Temperature-induced changes in skin blood flow and local sweat secretion (chest) in an adult. *Left,* Before body heating, ambient temperature *(T_a)* was decreased from 32° to 18° C for establishing initial skin vasoconstriction (skin blood flow estimated from heat conductivity increment [Δλ]); thereafter T_a was raised to 59° C. *Right,* Skin blood flow and sweat secretion in relation to mean body temperature, $T_{b(es)}$ (calculated from esophageal temperature [T_{es}] and mean skin temperature[T_{sk}] as shown previously). Note second vasodilation on forearm *(arrow)* coinciding with onset of sweat secretion. *AO,* Arterial occlusion at upper arm; T_{re}, rectal temperature; T_{ty}, tympanic temperature; *SR,* local sweat rate (chest). (*Adapted from Hessemer V, Brück K: Influence of menstrual cycle on shivering, skin blood flow, and sweating responses measured at night,* J Appl Physiol *59:1902, 1985.*)

a greater metabolic response in the younger and smaller animal (Figure 58-24). As shown in Figure 58-16, newborn guinea pigs maintain body temperature mainly by nonshivering thermogenesis. In other species, for example, pigs or miniature pigs, nonshivering thermogenesis does not occur. In these species, shivering supplies the extra heat for stabilizing body temperature at ambient temperatures as low as 20° C in the neonates.[43] In human neonates exposed to ambient temperatures of 33° to 23° C, the thermoregulatory effort is much larger than in adults for a given ambient temperature (Figure 58-25). The metabolic increase is mainly due to nonshivering thermogenesis.

Changes in the Passive System

The neonatal passive system is characterized by a large body surface area-to-volume ratio and by decreased heat insulation resulting from the smaller absolute thickness of the body shell. With growth, the surface area-to-volume ratio decreases by a factor of 2.7. Comparison of data from neonates[64] and adults[65] indicates that overall insulation *(I_{a+cl})* (Figure 58-26) increases by a factor of 1.8 as body mass increases from 3.5 to 70 kg. In a cool environment, maximum tissue insulation *(I_t)* changes by a factor of 3, and air insulation *(I_a)* increases by a factor of 1.2 because of the change in the curvature radii of the trunk and extremities.

The heat production *(H)* required for equilibrium heat flow at any given overall temperature difference would therefore have to be nearly five times as large in neonates as in adults (per unit of body mass). From the preceding data, an equation is derived that predicts heat loss in relation to body mass *(m)* at a given temperature difference between body core and environment (see Figure 58-2):

$$H = k \times m^{0.5}$$

The SMR is actually lower in the neonates of various species than heat loss predicted from the preceding equation. It even falls short of Kleiber's equation in which metabolic rate is related to body mass to the 0.75th power (see Figure 58-2). In humans, beginning with the second year of life (corresponding to a body mass of more than 10 kg), the SMR is a function of the body mass to the 0.5th power. In the first year of life (especially during the first week of life), the SMR in relation to body mass is only 1.5 to 2 times larger than in adults. This means the overall temperature difference (ΔT) that can be maintained in full-term neonates under the condition of SMR is less than half that of adults (and even less in premature infants) (Figure 58-27). At an ambient temperature less than 33° C, deep body temperature will drop unless heat production is increased. In fact, metabolic rate is increased at such high ambient temperature that the lower limit of the neutral temperature range is shifted to a higher level of ambient temperature.

Adjustment of Effector Threshold Temperatures

Because of the small amount of tissue insulation, the difference between core and mean skin temperature is considerably smaller in neonates than in adults. Thus cold defense reactions should be elicited at higher mean skin temperatures than in the adult to maintain core temperature at the adult level (Figure 58-28). In other words, the threshold for increasing vasoconstrictor tone and for the metabolic reactions is shifted to a higher level in neonates, thereby adjusting the thermoregulatory system to the smaller body size.

The displacement of the neonatal threshold temperature can be explained in two ways. First, because of the increased body surface area to body mass quotient, the number of cutaneous cold receptors/unit body mass is increased (assuming that the density of the distribution of cold receptors is the same in adults and neonates). Second, the central processing of thermal input signals (see Figures 58-1 and 58-8) in neonates is different from that noted in adults. In any case, the increased cutaneous sensitivity is

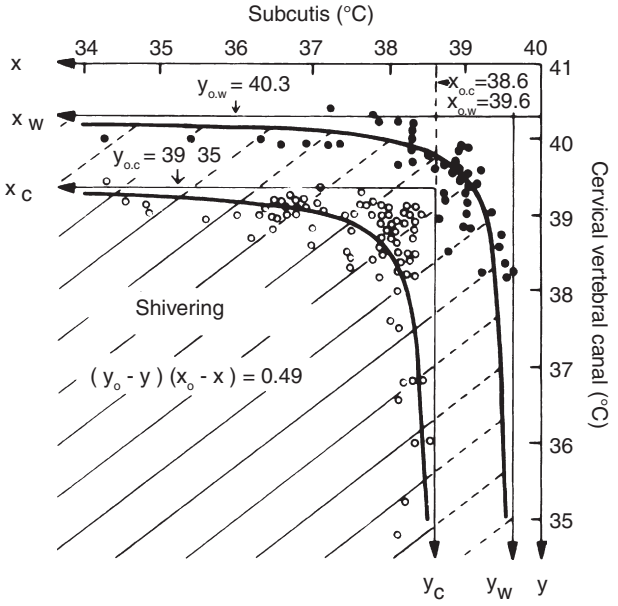


Figure 58-20 Shivering threshold curves for two groups of guinea pigs (aged 4 to 8 weeks) reared at different environmental temperatures. The values were obtained by independent changes of the body surface temperature and the temperature in the cervical vertebral canal. The diagram shows, for instance, that at a certain body surface temperature, which corresponds to a subcutaneous temperature of 37° C, shivering begins in warm-adapted animals *(•)* when the hypothalamic temperature drops below 40° C. In the cold-adapted animals *(small circle),* however, shivering does not occur until the hypothalamic temperature has reached a value slightly below 39° C. (*From Brück K, Wünnenberg W: Meshed control of two effector systems: nonshivering and shivering thermogenesis. In Hardy JD, Gagge AP, Stolwijk JAJ, et al [eds]:* Physiological and Behavioral Temperature Regulation, *Springfield, IL, Charles C Thomas, 1970, pp 562-580.*)

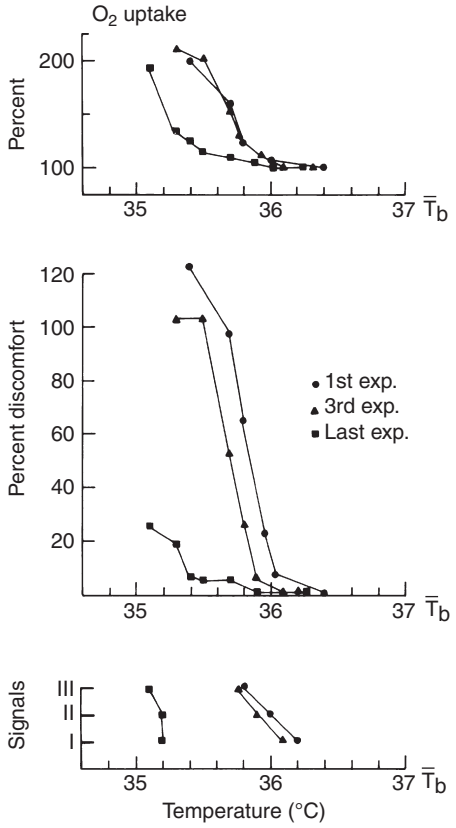


Figure 58-21 Responses of a young man wearing only shorts on exposure to cold (ambient temperature decreasing from 28° C to 5° C in 45 minutes) in the course of an acclimatization series. Note that shivering threshold (onset of increase in $\dot{V}o_2$) and discomfort threshold were shifted to lower mean body temperatures at the last (sixth day of the acclimatization period) exposure. Signals indicate subjective rating of cold sensation: *I,* Cool; *II,* cold; *III,* very cold. (*From Brück K, Baum E, Schwennicke HP: Cold-adaptive modifications in man induced by repeated short-term cold-exposures and during a 10-day and-night cold-exposure,* Pflügers Arch *363:125, 1976.*)

a prerequisite for enabling newborn infants to maintain core temperature (within a limited range of environmental temperature) at the same level and with the same accuracy as occurs in the adult.

Stage of Maturity of the Thermoregulatory System at Birth

Temperature regulation is frequently referred to as immature at the time of birth. However, one should be cautious in asserting immaturity of the thermoregulatory mechanisms even though neonates show greater fluctuations in body temperature than adults. Greater fluctuations of body temperature are to be expected in smaller organisms because of their large surface area-to-volume ratio, the relatively small insulating body shell, and the smaller body mass that acts as a heat buffer in large organisms. Because of these peculiarities in body size and shape, a reduced range of regulation (see Figure 58-14) should be expected in neonates.

The degree of maturity of the thermoregulatory system should be evaluated according to the following three criteria:

1. What is the capacity of the effector system (a) in comparison with adults and (b) in relation to body size?
2. What is the qualitative and quantitative (threshold, gain) responsiveness of the effector systems to thermal stimuli?
3. Are there qualitative or quantitative differences in the central thermointegrating system between adults and neonates?

Regarding the first criterion, the capacity of the metabolic system in full-sized human neonates is comparable with that of an adult system when the metabolic rate is related to body mass, but it is too small to compensate for heat loss in as wide a range of ambient temperatures as in adults. Therefore the tolerated ambient temperature range (range of regulation) (see Figure 58-14) is reduced. Full-term neonates are, at best, able to maintain a stable body temperature at an ambient temperature as low as 23° C (Figure 58-29), at which point maximum thermogenesis is required for thermal balance. In adults, by contrast, maximum thermogenesis (although smaller/unit body weight) is sufficient to balance heat loss at an ambient temperature as low as 0° to 5° C. It is notable that adults and neonates can maintain thermal balance under these extreme conditions only for restricted periods (30 to 60 minutes). More extended exposure results in exhaustion of the metabolic system and hypothermia. In neonates born at full-term gestation, the lower limit of the range of regulation (see Figure 58-14; T_1) comes closer to that of adults than it does in preterm neonates.

Regarding the second criterion, the threshold and gain for the metabolic reactions are adjusted to the smaller body size of neonates (see Figure 58-28). The sweating threshold is comparable with that in adults, except in premature and

small-for-gestational-age neonates[66] in whom the threshold is shifted upward. Even in the very small premature infant, metabolic and vasomotor control responses are developed at birth,[1,67] although the threshold for elicitation of these responses may not be appropriately adjusted to body size. In addition, the capacities of the effector systems may be smaller than in adults. In neonates, maximum evaporative heat loss is in the range of the SMR,[66,68] whereas in adults it is five times the SMR.

As for the third criterion, no substantial neuronal or hormonal differences have yet been described between the neonatal and adult thermoregulatory systems.

According to these criteria, one may distinguish three groups of mammals according to their functional stage of temperature regulation immediately after birth. The first group exhibits a thermoregulatory system more or less completely adjusted to a smaller body size. The body temperature is stable within a certain control range. This range is narrower, however, than in the adults. Members of this group include full-term human neonates, guinea pigs,[69] pigs,[42] miniature pigs,[43] lambs,[70] and larger mammals such as calves.[71]

The second group of mammals has the following characteristics: Thermoregulatory responses can be evoked at birth, but the capacity of the effector systems, the threshold temperatures, or both are not sufficiently adjusted to the smaller body size. Body temperatures drop on exposure to environmental temperatures slightly less than thermal neutrality. Mammals in this group include low-birth-weight human newborns,[1] kittens, rabbits,[72] puppies,[73] and rats.[74]

In the third group, thermoregulatory responses are not evocable, and oxygen uptake does not increase with decreasing environmental temperatures, but follows van't Hoff's law (see Figure 58-3). These animals (e.g., ground squirrels[73] and golden hamsters[60,75]) behave as poikilothermic animals do. This group is presumably restricted to the neonates of hibernators.

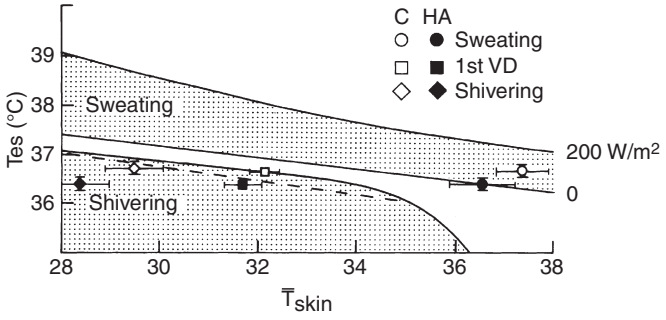


Figure 58-22 Concurrent shift of the threshold temperatures of all autonomic thermoregulatory effectors to lower body temperatures in the course of a 5-day heat adaptation series. Means of seven subjects. Each subject was exposed to heat for approximately 90 minutes daily. Shivering threshold was determined 1 day before and 1 day after the 5 heat-exposure days. (For explanation of lines, see Figure 58-12.) *C,* Control studies before acclimation; *HA,* after heat acclimation; *1st VD,* first vasodilation (see text). (*Data from Hessemer V, Zeb A, Brück K: Effects of passive heat adaptation and moderate sweatless conditioning on responses to cold and heat,* Eur J Appl Physiol Occup Physiol *55:281, 1986.*)

PATHOPHYSIOLOGY

Sudden Infant Death Syndrome

A possible role for hyperthermia in sudden infant death syndrome (SIDS) has been hypothesized because some victims are found in unusually warm environments, are warm and sweating when found dead, are wrapped tightly in clothing or bedding, have a history of febrile illness before death, or have high rectal temperatures at examination or autopsy.[76,77] Given the association between SIDS and another important risk factor, prone body positioning,[78] investigators have recently begun to examine the relationships in body position, body temperature, state of sleep, and cardiorespiratory activity. Infants sleeping prone have a higher peripheral skin temperature.[79,80] Heat loss is lower in infants sleeping prone than in those sleeping supine, perhaps because of less heat loss from the head. Thus prone infants take longer than supine infants to reach the lower rectal temperature that occurs after the onset of sleep and rectal temperature remains

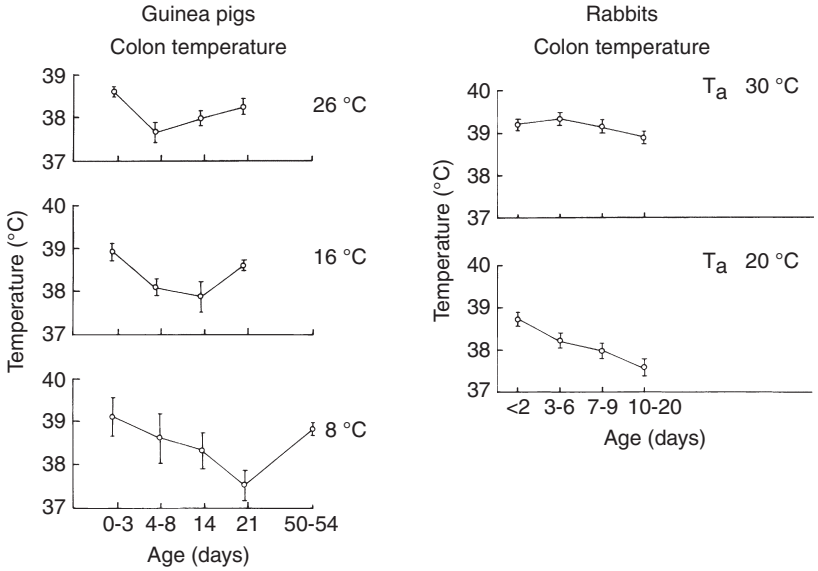


Figure 58-23 Colon temperatures in guinea pigs and rabbits in relationship to age and ambient temperature *(T_a).* (*Data from Brück K, Wünnenberg B: Über die Modi der Thermogenese beim neugeborenen Warmblüter. Untersuchungen am Meerschweinchen.* Pflügers Arch *282:362, 1965; and Várnai I, Farkas M, Donhoffer S, et al: Thermoregulatory heat production and the regulation of body temperature in the new-born rabbit,* Acta Physiol Acad Sci Hung *38:299, 1970.*)

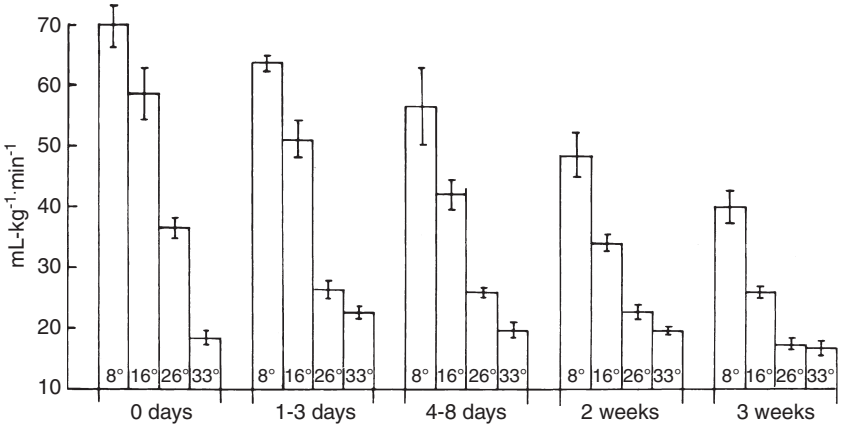


Figure 58-24 Heat production (expressed as oxygen uptake) in relationship to ambient temperature and age in guinea pigs. *(From Brück K, Wünnenberg B: Über die Modi der Thermogenese beim neugeborenen Warmblüter. Untersuchungen am Meerschweinchen,* Pflügers Arch *282:362, 1965.)*

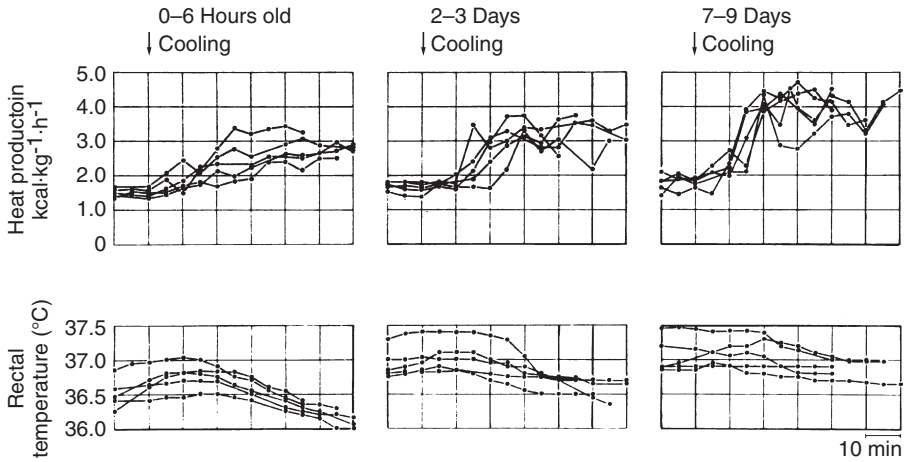


Figure 58-25 Course of rectal temperatures and heat production in full-term newborn infants. Ambient temperature to the left of downward-pointing arrow 33° C, then quick drop to 23° C. (*From Brück K: Temperature regulation of the newborn infant,* Biol Neonate *3:65, 1961.*)

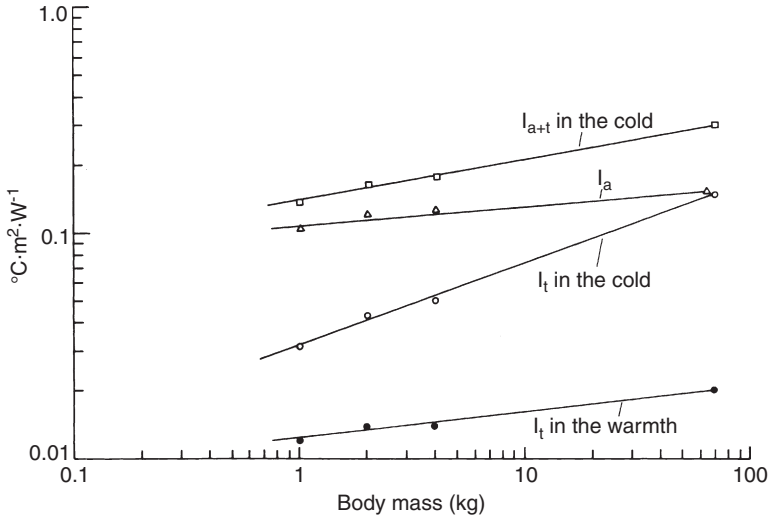


Figure 58-26 Tissue insulation (I_t) under cold and warm environmental conditions, ambient insulation (I_a), and total insulation (I_{a+t}) in relation to body mass in humans. (*From Brück K: Heat production and temperature regulation. In Stave U [ed]:* Perinatal Physiology, *New York, Plenum Publishing, 1978, pp 455-498.*)

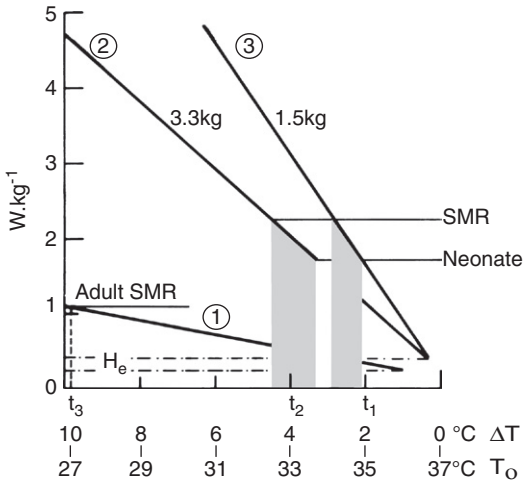


Figure 58-27 Schematic representation (based on data given in Figures 58-2 and 58-26) of heat production/unit body mass required to maintain the temperature differences (ΔT) in body core (37° C) and environment (operative temperature, T_O, Weighted mean of air and radiation temperature) in the adult (1) and in a 3.3-kg (2) and a 1.5-kg (3) infant (with maximum vasoconstriction). With the actual standard metabolic rate (SMR) given, the temperature differences T_1, T_2, and T_3 can be maintained under conditions of thermal neutrality; conversely, the neonates require an environmental temperature of about 33° C (2) or even 34° to 35° C (3) to maintain a deep body temperature of 37° C, whereas this level is much lower in the adult, 27° C (T3). H_e, Minimum evaporative heat loss. (*From Brück K: Heat production and temperature regulation. In Stave U [ed]:* Perinatal Physiology, *New York, Plenum Publishing, 1978, pp 455-498.*)

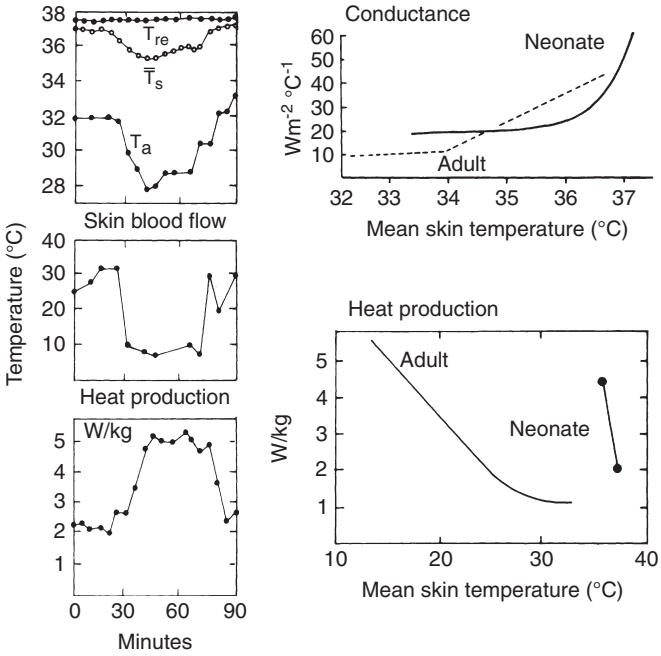


Figure 58-28 *Left,* Simultaneous response of skin blood flow (heel) and heat production to a slight drop in mean skin temperature ($\bar{T}_{skin}$) evoked by a decrease in ambient temperature (T_a) from 32° to 28° C. Study in a 7-day-old infant, 3290 g. *Right,* Relationship of $\bar{T}_{skin}$ and heat production in adults[131] and newborn infants[1] and thermal conductance (peripheral blood flow) in relationship to mean skin temperature in neonates[132] and adults.[133] Note onset of responses at higher body temperatures in the neonate. (*From Brück K: Heat production and temperature regulation. In Stave U [ed]:* Perinatal Physiology, *New York, Plenum Publishing, 1978, pp 455-498.*)

higher during sleep.[81,82] The weight of evidence obtained from term and low-birth-weight infants and infants a few months old also suggests that prone positioning is associated with reduced heat production. Even so, heart rate,[83,84] blood pressure,[85] and absolute skin temperatures[79,80] are higher and temperature gradients from central to peripheral skin are narrower.[86] Taken together, these data suggest that despite attempts to eliminate heat through adjustments in the peripheral circulation (narrowing central-peripheral gradients) infants are unable to maintain set-point temperature(s). Alternatively, the prone position may be linked to an elevation in the set point. Either way, the evidence points toward probable differences in autonomic control of metabolism and cardiorespiratory function in the prone versus the supine position.

Fever

It is well known that newborn infants may suffer severe infection without increased body temperature. In contrast, clinical reports show body temperature elevations higher than 38° to 39° C in newborn infants with septicemia, purulent meningitis, and pneumonia.[87] Experimental studies suggest that some peculiarities of fever mechanisms exist during the neonatal period. Newborn lambs fail to respond with fever after the administration of bacterial pyrogen or leukocyte pyrogen during the first few days of life.[88] Newborn guinea pigs have been shown to react to adult doses of pyrogen no sooner than a few days after birth, but they do respond with fever immediately after birth when this pyrogen is administered at much higher doses than in the adults.[87] If one takes certain precautions (e.g., providing an ambient temperature that does not overwhelm thermogenesis, sufficient nutrition, and so on), a pyrogen fever comparable to that of the adults may be evoked in the neonates of many species. However, many species-related differences in the pattern of the febrile response exist. For example, newborn guinea pigs demonstrate a biphasic response when endotoxin is administered intraperitoneally (Figure 58-30). In contrast, 0- to 3-day-old rabbits exhibit a short-lasting monophasic fever, and 3- to 6-day-old kittens show a more sustained monophasic fever. A hypothermic response is commonly observed in 8- to 10-day-old rats.[87] In most cases, pyrogen administration causes heat production to increase; as in thermoregulation, nonshivering thermogenesis is a prevailing mechanism of extra heat production during febrile response.

Mechanism of Fever

Set-point displacement. Fever develops as a result of an activation of cold defense reactions as they normally occur when an organism is exposed to cold. Generally the process begins with peripheral vasoconstriction and is followed by enhanced thermogenesis. In adults, the latter response is accompanied by shivering, whereas in neonates, nonshivering thermogenesis prevails. With increasing body temperature, these thermoregulatory effector actions, thermogenesis and vasoconstriction, are diminished just as if the organism aimed to reach a new target temperature. Recovery from the fever is induced by the activation of heat dissipation mechanisms—vasodilation and sweating. During the constant fever phase, thermal disturbances (see Figure 58-1) are compensated for by the appropriate control processes. It appears from this description that the thermoregulatory effector system remains completely functional but that the organism aims to

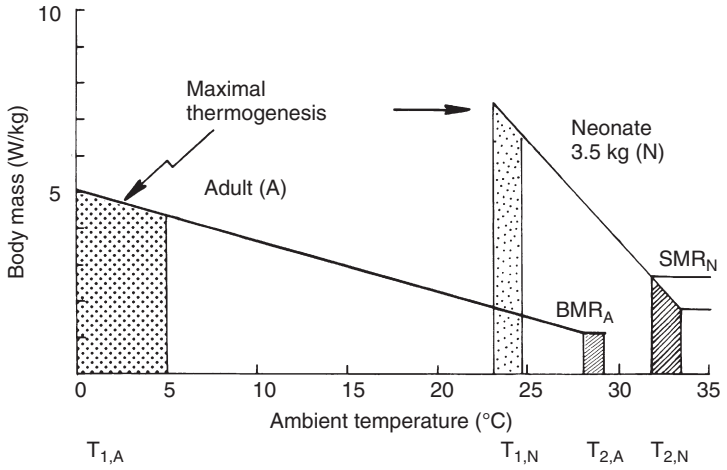


Figure 58-29 Minimal (basal or standard) and maximum metabolic rate in relation to ambient temperature in neonates *(N)* and adults *(A)*. The inflection point of each curve marks the lower limit of the thermoneutral zone *(T_2),* which is shifted to a higher temperature in neonates because of relatively low standard metabolic rate (*SMR*). As the SMR increases during the first few days of life, $T_{2,N}$ shifts to lower ambient temperature. The lower limit of the range of regulation, T_1 (see Figure 58-14), is determined by the maximal rate of heat production and is about 23° C in newborns and 0° to 5° C in adults. (*Data from Brück K: Heat production and temperature regulation. In Stave U [ed]:* Perinatal Physiology, *New York, Plenum Publishing, 1978, pp 455-498.*)

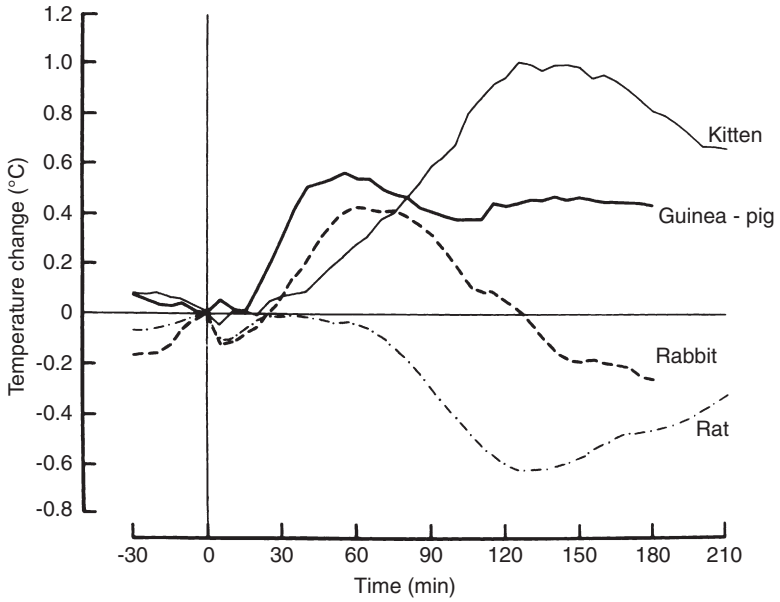


Figure 58-30 Average body temperature changes in the newborn of four species after two intraperitoneal injections of 20 mcg/kg *Escherichia coli* endotoxin at the individual thermoneutral ambient temperature. (*From Szeákely M, Szeleányi Z: The pathophysiology of fever in the neonate. In Milton AS [ed]:* Pyretics and Antipyretics. *Berlin, 1982, Springer-Verlag, pp 479-528.*)

assume and sustain a higher body temperature during fever. This phenomenon has been termed *set-point displacement*, thereby excluding other conceivable interpretations, for example, a toxic effect on one or more effector systems (see Figure 58-9). Current fever research is focusing on the question of how bacterial and viral toxins (exogenous pyrogens) act to finally affect the thermointegrative system (see Figure 58-8) in such a way that the body temperature is shifted to a higher level.

Pathogenesis of Fever

Fever is considered an immunoreactive process.[89,90] Certain fever-producing substances of external origin (e.g., exogenous pyrogens), such as the heat-stable lipopolysaccharides of bacterial membranes (endotoxins), stimulate granulocytes and macrophages of the reticuloendothelial system to produce a heat-labile peptide called *endogenous pyrogen*. Endogenous pyrogen is identical to the cytokine interleukin-1. Microinjection of endogenous pyrogen into small regions of the hypothalamus triggers typical fever reactions that are not observed when it is injected into other parts of the brain. Endogenous pyrogen initiates a cascade of processes by activating phospholipase A_2, which then converts phospholipids in cell membranes to arachidonic acid. This compound may then be converted to prostaglandins.

One of the prostaglandins, prostaglandin E_2 (PGE_2), has a pyrogenic action when injected into the hypothalamus in minute

amounts.[91] PGE_2 produces the set-point shift already described by interacting with thermosensitive, integrative structures (see interneurons; Figure 58-8), or both in the hypothalamus. Antipyretics (e.g., acetylsalicylic acid) inhibit cyclooxygenase activity and thus prostaglandin formation. However, PGE_2 is not the only fever mediator because specific PGE_2 antagonists do not prevent pyrogen-initiated fever.[92] Evidence suggests that endogenous pyrogen may penetrate, in minute amounts, into the hypothalamus through a special transport system located in the organon vasculosum of the upper brain stem,[91] which belongs to the so-called circumventricular system. Based on evidence that PGE_2 appears before the increase in plasma cytokine concentration and the increases in related enzymatic activity, it has been suggested that rapid neural pathways may be involved in the pyrogenic sensing and signaling.[93] A more complex model for the genesis of fever is warranted and a search is under way for other fever mediators, particularly circulating products of oxidative stress and their interactions with nitric oxide (NO) and the thermointegrative networks.[94] Interest in the role of NO in body temperature control is expanding. This ubiquitous molecule appears to mediate both peripheral (vasomotor tone and brown fat metabolism) and central (set-point) responses of the thermal control system to a variety of stimuli, including exogenous pyrogens, psychologic stress, and exercise.[95] Studies based on intradermal administration of nitric oxide synthase (NOS) inhibitors via microdialysis probes during hyperthermia have found that active vasodilation during heat stress was significantly attenuated by NOS inhibition.[96,97] These studies suggest that an active vasodilator requires functional NOS to achieve full expression. Alternatively, Farrell and Bishop[98] noted that nitroprusside could restore the vasodilation inhibited by previous NOS blockade during hyperthermia in rabbit ears, but the same dose of nitroprusside infused into the ear circulation in normothermia did not raise ear blood flow. This implies that active vasodilation in rabbit ears requires both NO production and activation of the vasodilator nerves, and that these two elements not arranged in series (vasodilator activity did not increase NO production), and suggests a "permissive" role of NO in active vasodilation. Human studies based on NO breakdown products during hyperthermia have shown that both skin blood flow and bioavailable NO concentrations begin to increase at the same core temperature. This indicates that NO could well be an active effector of cutaneous vasodilation during heat stress.[99,100] Also, Shibasaki et al[101] have shown that acetylcholine-mediated NO production via muscarinic receptor activation was only possible early in heat stress, and then only before the occurrence of substantial cutaneous vasodilation. Although, both neuronal NOS and endothelial NOS have been detected in human skin,[102,103] it is unclear which isoform produces the increased NO required for active vasodilation. Wong et al[104] reported evidence that H_1 histamine receptors may play a role in the generation of NO during cutaneous active vasodilation in hyperthermia. The source of histamine remains to be identified; however, work by Wilkins et al[105] suggests that (1) vasoactive intestinal peptide–induced release of histamine from mast cells could be involved, and (2) vasoactive intestinal peptide and histamine have NO dependent components to their vasodilatory effects. Thus several pathways may be involved in the generation of NO in the skin during hyperthermia.[101,104,105] The students of thermoregulation will want to follow developments in this area closely.

Behavioral Fever

In newborn rabbits, which are born in a very immature state, the thermogenic response to a standard pyrogen dose may be weak or even lacking. Nevertheless, these animals have been shown to experience fever when they are put into a temperature-gradient tube in which they are able to move to an area so hot as to cause their body temperatures to increase.[56] This response has been termed *behavioral fever* in contrast to the fever produced by the activity of the autonomous thermoregulatory effectors (see Figure 58-9).

Temperature Regulation in Heart Failure

Thermal instability is a common accompaniment of cardiac disease in newborns, often leading to unnecessary treatment for infection. It should be remembered that circulatory changes are central to control of body temperature and thus dependent on cardiac function. Cardiac dependent changes are noted within and outside the thermoneutral zone, and these alterations are likely to differ with the severity and etiology of heart failure. Unfortunately, there are limited studies addressing this issue, particularly during the neonatal period. The upper boundary of the zone of hypothermia is elevated to include environments that, at worst, are only mildly chilling for normal patients but induce shivering in patients with heart failure. The cardiac stress associated with the onset of shivering is likely to be compounded by increased thermal stress through enhanced rate of decline of core temperature and a further stimulus to shivering.[106] In the neutral zone, where core temperature is maintained by control of skin blood flow, pharmacologic interventions have the potential to deprive the individual of the ability to vasodilate appropriately and could lead to a 2° C decline in core temperature.[107] In the zone of hyperthermia, both skin and core temperatures are likely to increase. Thermal steady states are possible when reflex sweating and cutaneous vasodilation are adequate to achieve thermal balance. The vasodilator component of this response is undoubtedly influenced by the physiologic changes and pharmacologic interventions associated with heart failure and its treatment. Interestingly, a vasodilator response has been shown experimentally to result in improvement of cardiac function because of afterload reduction,[108] similar to that observed with pharmacologic vasodilator treatment. In this situation the increase in cardiac output is entirely diverted to the cutaneous vasculature. Although it has not been well studied, it is likely that monitoring central and peripheral temperatures would provide useful information about the about the adequacy of cardiac reserve of children with congenital heart disease.

Hypoxia

Acute and Chronic Hypoxia

Considerable impairment of temperature regulation should be anticipated if the oxygen supply is critically reduced to the tissues subserving thermoregulatory heat production (skeletal tissues, musculature, and brown adipose tissue). Anaerobic metabolic processes are quantitatively insufficient to provide enough heat for thermoregulation. In fact, a reduction of oxygen content in the inspired gas to 10% has been shown to completely block thermogenesis in newborn kittens exposed to an ambient temperature that is lower than neutral.[109,110] As a result, body core temperature drops. Remarkably, the at rest metabolic rate as measured at neutral temperature remains unaffected by this degree of hypoxia. Similar results have been obtained in newborn lambs,[111] rhesus monkeys,[112] guinea pigs,[109] and rabbits.[113] By contrast, in larger adult organisms that do not possess nonshivering thermogenesis (e.g., adult humans[114] and dogs[115]), cold-induced thermogenesis (shivering) is only transiently reduced by hypoxia. With sustained hypoxia, shivering thermogenesis appears to be almost unimpaired. Thus nonshivering thermogenesis appears to be more vulnerable to the effect of hypoxia.

This view is supported by a study in newborn rabbits.[113] As shown in Figure 58-31, oxygen uptake increases on cold exposure without shivering. Offering a 10% oxygen gas mixture immediately abolishes the cold-induced nonshivering thermogenic

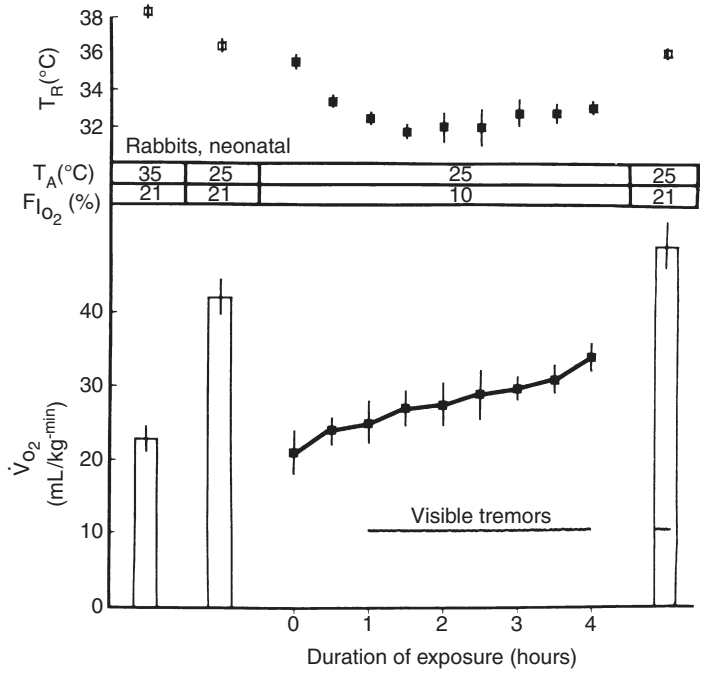


Figure 58-31 Effect of cold and hypoxia on the oxygen consumption and colonic temperature of unanesthetized newborn rabbits. Exposure to 25° C in air caused an increase in oxygen consumption, which was produced without shivering. This increase was abruptly abolished when the inspired oxygen content was decreased to 10%. During the next 4 hours, oxygen consumption gradually increased. Visible shivering developed after 1 hour and persisted into the posthypoxic period. (*From Blatteis CM: Shivering and nonshivering thermogenesis during hypoxia. In Smith RE [ed]:* Bioenergetics. Proceedings of the International Symposium on Environmental Physiology, *Bethesda, MD, 1988, pp 151-160.*)

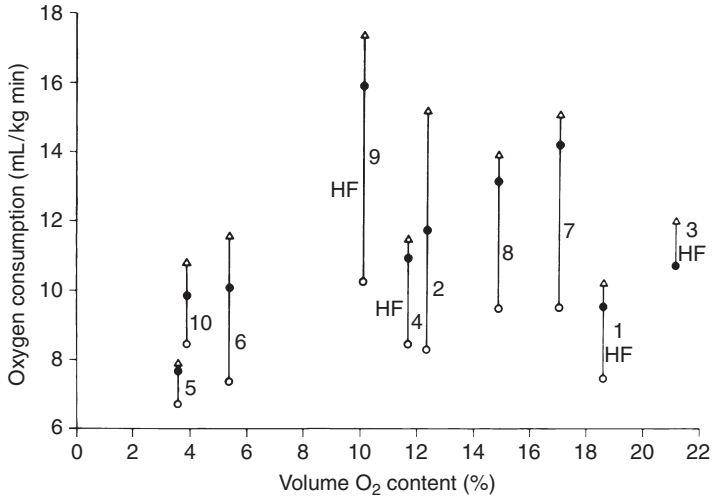


Figure 58-32 Responses of oxygen consumption on cold exposure (ambient temperature drop from 32° to 28° C) in relation to arterial oxygen content. ○, Minimal oxygen consumption *(MOC)* at neutral temperature; ●, average oxygen consumption during cooling period; △, peak oxygen consumption during cooling period. Three infants designated with heart failure (*HF*) had suffered severe heart failure. Note that oxygen consumption increased on cooling even in infants with arterial oxygen content as low as 5% vol. (*From Brück K, Adams M: Temperature regulation in infants with chronic hypoxemia,* Pediatrics *30:350, 1962.*)

response. In the subsequent hours, however, oxygen uptake steadily increases and is accompanied by shivering. Because the interscapular brown adipose tissue is a major site of thermogenesis and the heat produced in this organ inhibits shivering through cervicospinal warm-sensitive structures, the appearance of shivering during hypoxic blockade of nonshivering thermogenesis is understandable. With prolonged hypoxia, the capacity for nonshivering thermogenesis may more or less recover. Normal thermogenic responses without obvious shivering have been observed in some neonates and infants with cyanotic congenital heart disease in whom arterial oxygen contents range 10 to 20 mL/dL (Figure 58-32). Even with arterial oxygen content as low as 5 mL/dL, thermogenic responses can be evoked, although they are reduced in magnitude. These results suggest that in chronic hypoxia, adjustments take place that restore the capacity for nonshivering thermogenesis.

As for the mechanism of the hypoxic reduction of cold-inducible thermogenesis, two possibilities have been suggested: (1) reduced oxidative capacity from the lowered oxygen tension at the level of mitochondria in the organs involved in thermogenesis (brown adipose tissue, skeletal musculature) and (2) central inhibition of the effector systems. As shown by Blatteis,[113] the effect of norepinephrine infusion on nonshivering thermogenesis is abolished or grossly reduced during hypoxia. This would support the first hypothesis.

In hibernators, hypoxia and hypercapnia have been shown to displace the threshold for the elicitation of cold defense reactions to a lower body temperature level through a central action.[116,117] In a study in conscious adult cats,[118] hypoxia caused by inspiration of 11% oxygen reduced shivering. This suppression of shivering was partially reversed, however, when the end-tidal carbon dioxide concentration was returned to normal by addition of carbon dioxide to the inspired gas. These data would argue against the first possibility, at least with regard to moderate grades of hypoxia. Thus those authors concluded that "brain hypoxia lowers the regulated body temperature during cold stress." More recent observations suggest that, as in the case of fever, molecular mediators of the central effector response are likely to be important. Murine hypoxic hypometabolism and associated hypoventilation have been ameliorated by inhibitors of NOS.[119] Depending on the experimental design and species under study, various, often contradictory effects of NO have been reported. Overall, the evidence points toward a fundamental, but complex, role for NO in the control of metabolism, cardiorespiratory function, and temperature during hypoxia.

Effects of Fetal Hypoxia on Neonates

Fetal hypoxia has been shown to increase the plasma norepinephrine concentration. This, in turn, results in peripheral vasoconstriction and circulatory centralization of cardiac output.[120] Moreover, increased norepinephrine levels have been shown in guinea pigs to cause a downward displacement of the shivering threshold temperature.[121,122] Neonates who had fetal asphyxia may thus display a delayed metabolic response to cold exposure as well as a delayed cutaneous vasodilation on heat exposure. This has been demonstrated in neonates who have undergone severe asphyxiation during delivery.[1]

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Gunnar Sedin

Physics and Physiology of Human Neonatal Incubation

During fetal life, water and electrolytes are exchanged between the mother and the fetus through the placental circulation. The fetus contributes to the formation of amniotic fluid with urine[1] and with water transported through the respiratory epithelium and the airways.[2-5] During gestation, body water gradually decreases, and extracellular water and the contents of sodium and chloride decrease proportionally. The water content of a term newborn infant is approximately 77% of the body weight.[6] In preterm infants, the total body water at birth is higher, namely, 82% at 32 weeks gestation and 86% at 24 weeks.[6] Heat produced by the fetus results in a fetal temperature that is approximately 0.5° C higher than the maternal temperature and results in a transfer of heat from the fetus to the mother through the placental circulation and through the amniotic fluid and fetal membranes.[7,8]

Immediately after birth, the skin of the infant is covered with amniotic fluid and usually vernix caseosa. Evaporation from the body surface of amniotic fluid[9] and of water in amniotic fluid[10] causes a loss of heat. At the same time, the infant is exposed to a colder temperature than was experienced in utero. The body temperature will then be lowered, a change that is at least partially physiologic, because the body temperature at birth is higher than that in subsequent life.To make it possible for a newborn infant to maintain an almost constant body temperature, heat production and heat exchange with the environment have to balance each other. Exposure to cold may give rise to thermogenic responses that will increase basal heat production,[11-18] and the skin circulation may decrease to lower the heat losses.[19] Seriously ill and very preterm infants are usually nursed in an environment in which a normal body temperature can be maintained, either in an incubator at an ambient temperature within the thermoneutral zone[14,15,20] or under a radiant heater. Heat balance in newborn infants is, however, not dependent solely on the ambient temperature and the radiant energy but also on many other factors that determine the heat transfer between the infant and the environment.* One of these factors is water exchange between the infant and the environment.† With every gram of water evaporated from the body surface or the respiratory tract, the infant will lose heat because of the latent heat of evaporation.

ROUTES OF WATER EXCHANGE

The newborn infant loses water insensibly through the skin and respiratory tract and sensibly with the urine and stools.‡ Water is gained by the body through the intake of fluids and food and as a result of metabolism.

Water loss from the skin surface involves two processes: continuous diffusion of water vapor through the epidermis and losses from the sweat glands in term and near-term infants. In the absence of forced convection, that is, with airflow velocity lower than 0.2 m/second, the human body is surrounded by a boundary layer of water vapor,[67] which constitutes a transition zone for transportation of moisture and heat from the body to the ambient air. In this zone of diffusion, a linear relationship exists between the water vapor pressure and the distance from the evaporating surface.[68] Evaporation of water from the skin and from the respiratory tract implies transfer of mass and of energy.[16,24] Insensible water loss (IWL) has been studied by gravimetric methods§ and with the ventilated chamber technique.[15,51,63] Hey and Katz[15] found that the IWL from the skin (IWL_s) in term infants comprised about 75% of the total IWL.[15] It has also been shown that the IWL is influenced by the ambient humidity[15,31,51,63] and is

*References 9, 11, 12, 14-16, 20-32.
†References 9, 10, 15, 16, 19, 24-26, 29, 31, 33-57.
‡References 10, 15, 29, 31, 34, 36-41, 43-45, 48, 49, 54, 58-66.
§References 33, 46, 54, 57, 58, 59, 61, 62, 69, 70.

high in low-birth-weight infants.[62] Techniques for determining water loss from the skin[36,47,71] and from the respiratory tract[42,49] have made it possible to determine the relative contributions of these two routes of water loss to the total IWL in infants of different gestational ages* (under different conditions) and to calculate the different modes of heat exchange.[9,25,26,53,72]

Determination of Water Loss From the Skin

In the absence of forced convection, and if the effect of thermal diffusion is disregarded, the process of water exchange through a stationary water-permeable surface can be expressed in terms of the vapor-pressure gradient immediately adjacent to the surface, as

$$\text{Water exchange} = -D' \frac{\delta\rho}{\delta\chi}$$

where D' is the diffusion coefficient and $\delta r/\delta\chi$ is the gradient.

This expression includes both a diffusive mass flow caused by a vapor-concentration gradient and a convective mass flow caused by a temperature gradient.

D' varies with the temperature (T) and the atmospheric pressure (P_{atm}), as follows[73]:

$$D' = \frac{DM}{RT} \frac{T^{1.75}}{300} \frac{101 \times 10^3}{P_{atm}}$$

where M is the molecular weight and R the gas constant (8.314 J/mol-K).

Within the ranges of temperatures and atmospheric pressures in question, the maximal variation in D' is limited. D' can then be approximated by a constant given the value 0.670×10^{-3} g/mhPa, which is valid for the water vapor pressure at a temperature of 300 K and an atmospheric pressure of 101 kPa.

The evaporation rate (ER; g/m^{2}h) can thus be determined by a method based on calculation of the water vapor pressure in the layer of air immediately adjacent to the skin surface. In this zone, a linear relationship exists between the vapor pressure and the distance from the evaporating surface.[68] If the gradient in this layer is known, the amount of water evaporated per unit time can be calculated according to the following equation[47,71]:

$$\frac{I}{A}\frac{dm}{dt} = D' \frac{dp}{dx} \text{ (g/m}^2\text{h)}$$

where A is the area.

The gradient method (Evaporimeter; ServoMed AB, Kinna, Sweden) allows measurements of free evaporation and quick measurements without disturbing the infant.[36,47] Transepidermal water loss (TEWL; grams per square meter), which is a mean value of cutaneous water loss, can be calculated according to the following equation:

$$\text{TEWL} = 0.92 \bullet ER_{(a,b,c)} + 1.37$$

Measurements of Respiratory Water Loss

Respiratory water loss (RWL) is usually included in the measurements of total IWL when measurements are made with ventilated chambers, but in a few studies it has been estimated separately.[15,31,63] These authors all found that the RWL was higher at low ambient humidity than at high humidity.

In most of the more recent studies of RWL, an open flow-through system has been used with a mass spectrometer to measure the gas concentrations.[42] Ambient air is then sucked through a specially constructed Teflon funnel attached to the infant (or the lamb), so that all expired air is collected without inclusion of evaporation from the surrounding skin.[42,49] This system provides data on RWL, oxygen consumption, and carbon dioxide (CO_2) production.*

ROUTES OF HEAT EXCHANGE

Heat exchange between the infant and the environment occurs through the skin and to some extent through the respiratory tract. Heat exchange through the skin takes place through conduction (H_{cond}), evaporation (H_{evap}), radiation (H_{rad}), and convection (H_{conv}). Its magnitude depends on the body surface area of the infant and the proportions of the body surface in direct contact with the mattress or clothing and exposure to the air and surrounding surfaces. To determine the heat exchange between the infant and the environment, it is thus necessary to know the heat loss from the skin per unit surface area, the total body surface area, and the proportion of the surface area participating in the different modes of heat exchange.†

Calculation of Heat Exchange Between the Infant's Body Surface and the Environment

Heat exchange through conduction, evaporation, radiation, and convection can be calculated with knowledge of the TEWL, the temperature of the material on which the infant is placed (T_{bed}), the temperature of the infant's skin (T_{skin}), the temperature of the ambient air (T_{amb}), the temperature of the walls facing the infant (T_{wall}), and characteristics of the material in the infant's environment, by using the following equations[9,25]:

Heat exchange through conduction:

$$H_{cond} = K_0(T_{skin} - T_{bed})(\text{W/m}^2)$$

where K_0 is the conductive heat transfer coefficient. H_{cond} is dependent on the thermal characteristics of the skin, but even more on those of the mattress. T_{skin} is the temperature of the skin (K), and T_{bed} (K) is the temperature of the bed (mattress). With the thermal conductivity characteristics of most regular mattresses, the heat loss through conduction in incubators and under radiant heaters is very low.

Heat exchange through evaporation:

$$H_{evap} = K_1 \bullet \text{TEWL} \, (3.6 \times 10^3)^{-1} (\text{W/m}^2)$$

where K_1 is the latent heat of evaporation ($2.4 \cdot 10^3$ J/g), TEWL is the transepidermal water loss (g/m^{2}h), and $3.6 \cdot 10^3$ is the correction factor for time (seconds). TEWL is a mean value of evaporation of water from the skin surface measured with the gradient method.‡

Heat exchange through radiation:

$$H_{rad} = S_0 \bullet e_1 \bullet e_2 \bullet (T_1^4 - T_2^4)(\text{W/m}^2)$$

In this equation, S_0 is Stefan-Boltzmann's constant ($5.7 \cdot 10^{-8}$ W/m^{2}K^4), e_1 is the emissivity of the skin, e_2 is the emissivity of the surrounding walls (0.97), T_1 is the mean temperature of the skin (K), and T_2 is the mean temperature of the surrounding wall (K).

Heat exchange through convection can be calculated as follows:

$$H_{conv} = K_2 \, (T_1 - T_3)(\text{W/m}^2)$$

where K_2 is the convection coefficient (2.7 W/m^{2}K), T_1 is the mean temperature of the skin (K), and T_3 is the mean temperature of the ambient air (K). This calculation does not include fast convections, which in adults occur at air velocities greater than 0.27 m/second.[24] The coefficient for convection given earlier

*References 29, 36-41, 49, 50, 65.

*References 19, 29, 30, 42, 49, 50, 65, 74-78.
†References 9, 25-27, 32, 35, 48, 71.
‡References 30, 36-41, 52, 53, 77, 78.

and used in many studies referred to in this chapter has usually been determined in measurements on adult human skin. A convection coefficient suggested to be more valid for newborn infants[79] can be used; however, H_{conv} will then be 48% higher.

The extent of heat exchange between the body surface area and the environment depends on the type of heat exchange, on the position and geometry of the body, and on the magnitude and frequency of body movements. Because the different modes of heat exchange are unequally influenced by a change of body position, the relative contributions of the different modes of heat exchange may vary with time. When heat exchange is compared among different environmental conditions and among infants of different gestational and postnatal ages, it is often presented as heat exchange per unit area of body surface exposed to the ambient air or facing the walls of the incubator.

Heat Exchange Through the Respiratory Tract

The expired air is usually more humid (i.e., it has a higher water vapor pressure) than the inspired air. This implies an evaporative loss of water and of heat from the respiratory tract. A usually low degree of convective heat transfer also takes place in the respiratory tract. Often, these two processes are considered together.[16] In the newborn, heat may also be gained through the respiratory tract if the infant inspires warm air with a high humidity.

Because of the alternating displacement of air during the respiratory cycle, convective and evaporative heat transfer in the respiratory tract is complex. When ambient air, which is cooler than the body, passes along the mucosa during inspiration, it gains heat by convection and gains water vapor by evaporation from the mucosa. On reaching the alveoli, the air is at thermal equilibrium with the central body temperature and is saturated with water. During expiration, the expired air may become a little cooler than the body temperature before it leaves the infant.

Calculation of Heat Exchange From the Infant's Respiratory Tract and the Environment

The exchange of heat through convection in the respiratory tract, $H_{conv\text{-}r}$, is calculated from the air volume ventilated per unit time (V = ventilation volume) and from the temperature difference between expired and inspired air ($T_E - T_I$) according to the following relationship:

$$H_{conv\text{-}r} = V \bullet \rho \bullet c \bullet (T_E - T_I) m^{-1} (W/kg)$$

where V is the ventilation volume per unit time, ρ is the density of the air (1 g = 0.880 L), c is the specific heat (1 J/g/° C), m is the body weight (kilograms), and T_E and T_I are the temperatures of the expired and inspired air.[16]

Because of the alternating inspiratory warming and expiratory cooling of the air, the convective heat exchange in the respiratory tract depends mainly on the temperature of the inspired air. In human infants nursed in incubators, only a small difference exists between the temperatures of the inspired and expired air, and convective losses are therefore small.

Evaporative heat exchange from the airway ($H_{evap\text{-}r}$) depends on the difference in water content between expired and inspired air. This is the RWL.[29,42,49,50,65] Because the formation of water vapor in the respiratory tract requires thermal energy, the amount of heat exchange by evaporation per unit time will be as follows:

$$H_{evap\text{-}r} = K_1 \bullet \text{RWL} (3.6 \bullet 10^3)^{-1} (W/kg)$$

where K_1 is the latent heat of evaporation of water ($2.4 \bullet 10^3$ J/g), RWL is the respiratory water loss (milligrams per kilogram minute), and $(3.6 \bullet 10^3)^{-1}$ is the correction factor for time.

Heat Exchange in Incubators, Under Radiant Heaters, and in Heated Beds

Incubators

The first incubators were constructed when it was realized that a good thermal environment increases the chances of survival of newborn infants. Budin[80] found higher survival in infants whose temperature had never been lower than 32° C. Later studies by Silverman and co-workers,[81,82] Hey and Mount,[27] Hey,[14] Hey and Katz,[15,28] and Dahm and James[83] widened the knowledge concerning the influence of T_{amb} on the survival rate, oxygen consumption, and respiration of newborn infants.

In a convectively heated incubator, the warm air supplied is usually directed so that both the air temperature of the incubator and the incubator walls are kept warm. If the airflow velocity close to the infant is less than 0.2 m/second, a normal convective pattern will exist around the infant, and, at an airflow velocity lower than 0.1 m/second, the convective heat transfer will depend on the gradient between the skin and the air temperature. In this condition, the vapor-pressure gradient close to the skin surface will also be maintained, avoiding increased evaporative water loss resulting from airflow velocity.* In incubators available today, both the airflow velocities and the humidification capacity vary markedly.[85] The wall temperatures also vary among different incubators.[85] It is therefore necessary to determine airflow velocities, humidity, and wall temperatures carefully before calculations of heat exchange are performed.

Radiant Heaters

Infrared radiation energy was first used by Agate and Silverman[86] to control the body temperature of small newborn infants and has since become widely used in neonatal intensive care. The radiant warmer placed over an open bed platform provides a good accessibility and visibility for the care of the newborn infant. When nursed under a radiant heater, the infant gains heat but may also have extensive heat losses through evaporation and convection and, from some surfaces, through radiation.[14,46,54,57,70] It is thus difficult to estimate the relative contributions of different modes of heat exchange when the infant is nursed under a radiant heater. Because free air movements may occur above the body surface of the infant, both evaporative and convective heat loss may increase as a result of a high air velocity. Williams and Oh[57] and Bell and Oh[60] suggested that the vapor pressure of the ambient air may be lower under a radiant heater than in an incubator and that this could result in higher insensible loss of water from the skin or the respiratory tract. Baumgart[22] considered convective heat loss to be the major component of net heat loss under a radiant heater. It was also found that in critically ill preterm infants nursed under a radiant heater the IWL increased when the radiant power density was increased to the level required by these small infants.[33] In addition, Baumgart and co-workers pointed out that convective air currents under the radiant heater could cause an increase in evaporative water loss and heat exchange.[21,33,34] Determinations of heat exchange in infants nursed under a radiant heater are difficult to perform because of the variations in humidity and air velocities and in the proportion of the body surface facing the walls of the nursery.

Heated Beds

Sarman and co-workers showed that infants weighing 1000 to 2000 g can be kept warm by placing them on a heated water-filled mattress.[87] In infants with a low body temperature, heat can be gained by conduction. By covering most parts of the body except the head, direct heat exchange between the infant's skin and the environment through other modes will be almost eliminated for a large proportion of the body surface. The water and

*References 16, 24, 35, 48, 79, 84.

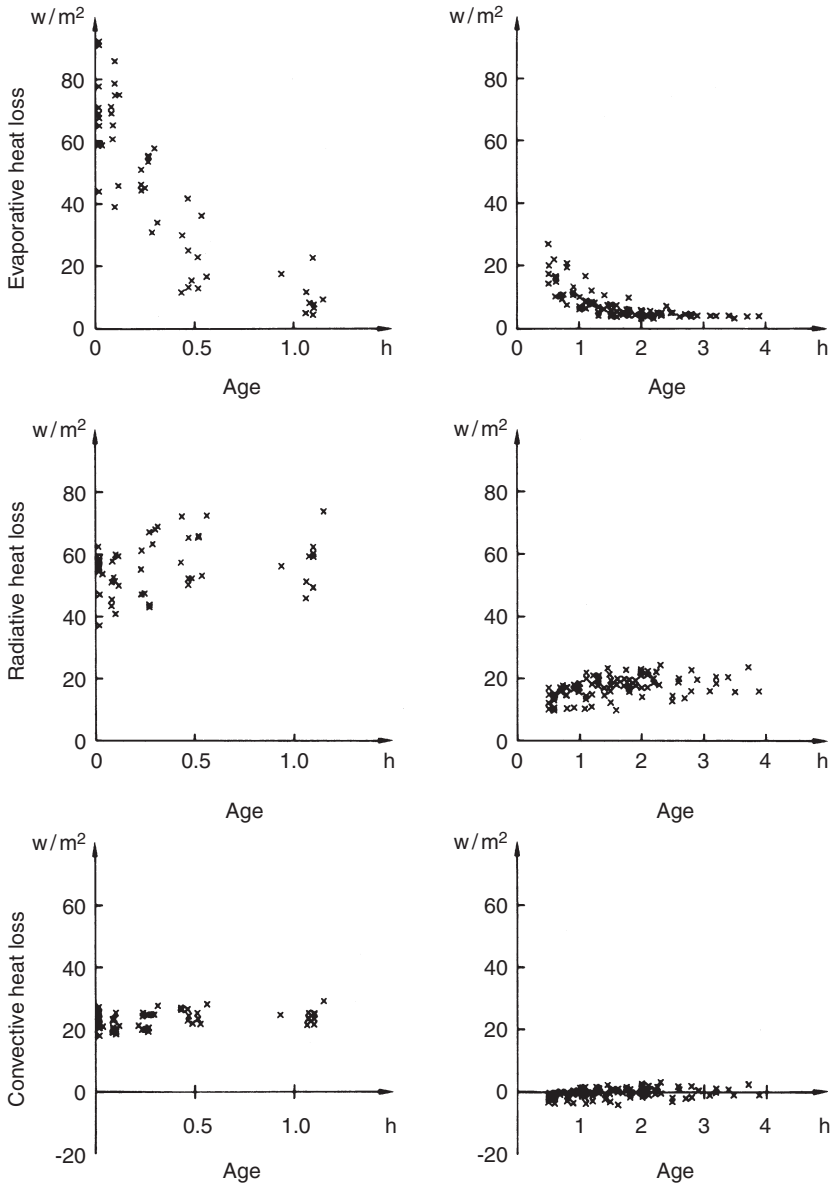


Figure 59-1 Heat exchange between the skin of term infants and the environment immediately after birth and during the first 1 to 4 postnatal hours. Data presented in the *left-hand scatter diagrams* are based on measurements in infants who were not washed or wiped. After the measurements at 1 and 5 minutes after birth, the infants were covered with a towel between the measurements. Data in the *right-hand diagrams* are based on measurements in infants who after birth were covered with a towel until they were placed in an incubator. *(From Hammarlund K, Nilsson GE, Oberg PA, Sedin G: Transepidermal water loss in newborn infants. V. Evaporation from the skin and heat exchange during the first hours of life,* Acta Paediatr Scand *69:385, 1980.)*

heat exchange through the respiratory tract will depend on the temperature and humidity of room air.

WATER AND HEAT EXCHANGE BETWEEN THE SKIN AND THE ENVIRONMENT

Water evaporation from the skin surface of infants nursed in incubators has been determined by use of the gradient method* (Evaporimeter; ServoMed AB, Stockholm, Sweden). TEWL[36-38,40,41,53] and heat exchange[9,25,26] have also been calculated. In these studies, the body temperature has been maintained at 36° to 37° C, except when the effect of warming has been investigated, and the ambient humidity has been kept at 50%, except when the effects of different humidities have been analyzed. Evaporative heat loss is usually insensible, but it may become sensible when term infants are nursed in a warm environment[59] and start to sweat.[37,55,56] In preterm infants, the sweat glands are nonfunctional, and no sweating occurs even when preterm infants are nursed in a warm environment.[15] Not all infants born at term and nursed in a warm environment start to sweat.[56] In infants who do, the sweating is preceded by an increase in skin blood flow, as measured with the laser Doppler technique,[55,56,88] and feeding cold glucose can then inhibit both sweating and vasodilatation.[56]

Heat Exchange During the First Hours After Birth

An enormous evaporative heat loss takes place early after birth, when the infant's skin is covered with amniotic fluid, and it gradually decreases during the first hours of postnatal life (Figure 59-1).[9] In the delivery room, heat loss to the environment

*References 10, 36-41, 43, 45, 52, 53, 55, 56.

through radiation is also considerable if the infant's skin surface is exposed to the room air and faces the walls of the delivery room. This early loss of heat through evaporation, radiation, and convection can usually be kept at a moderate level even when the infant is nursed in the mother's arms,[9] provided adequate precautions are taken to prevent excessive loss of heat, such as by gently wiping the infant's skin[10] and covering the infant with warm towels.[9]

Transepidermal Water Loss During the First Day After Birth and During the First 4 Postnatal Weeks

The TEWL from the skin surface of the newborn infant depends on certain factors, such as the gestational age at birth,[38] the postnatal age,[40,41] and being small[39] or appropriate for gestational age.[38,39,41,53] TEWL is also dependent on ambient factors such as the air temperature and humidity.[36,38-40,52,53] TEWL is 15 times higher in infants born at 25 weeks gestation than in term infants. An exponential relationship exists between TEWL and gestational age when measurements are made during the first day after birth.[38] This type of relationship prevails over the first 4 weeks after birth (Figure 59-2),[41] even though the difference in TEWL between the most preterm and term appropriate–for–gestational age infants gradually diminishes with age. However, at a postnatal age of 4 weeks, TEWL is still twice as high in the former as in the latter (see Figure 59-2). The loss of water from the skin surface also depends to a higher degree on the ambient relative humidity, with much higher losses at a low humidity than at a high humidity (Figure 59-3).[38] This relationship seems to be valid for all gestational ages and all postnatal ages studied.[36,38-40,52,53]

In very preterm infants, at an ambient humidity of 50%, water loss through the skin (Table 59-1) is the most important route early after birth. As seen in Table 59-1,[41] in infants born at 25 to 27 weeks gestation, the water loss amounted to 129 g/kg body weight per 24 hours during the first day after birth, after which a gradual decrease occurred, which was most marked during the first 7 days. These losses, however, depended on the ambient humidity, which meant that the IWL_s during the first day after birth in infants born at 25 to 27 weeks gestation was 205 g/kg per 24 hours at an ambient humidity of 20% and only 53 g/kg at an ambient humidity of 80% (Table 59-2). Therefore water balance in very preterm and extremely preterm infants is best maintained if excessive evaporative losses of water are prevented by nursing the infants in a humid environment.[66]

Heat Exchange During the First Day After Birth

Because the evaporative heat exchange between the infant's skin and the environment during the first day after birth is directly proportional to the amount of water evaporated from the infant's skin, evaporative heat loss shows the same kind of relationship with gestational age as TEWL (Figure 59-4). In the most preterm infants, the evaporative heat exchange may reach 50 W/m^2 of the body surface area,[25] whereas in term infants it is close to 5 W/m.^{2} Because of the high evaporative heat loss in very preterm infants, a high T_{amb} is needed to maintain a normal body temperature of between 36.0° and 37.0° C. To keep the infant's thermal balance at steady state, the servocontrol system of the incubator, which controls the skin temperature, increases the temperature of the air, which leads to an increase in the temperature of the incubator walls. Thus the loss of heat to the environment through convection and radiation decreases, and the most preterm infants can even gain heat through these modes of heat exchange (see Figure 59-4). Because these data were obtained while the infants were being nursed in an incubator with a relative humidity of 50%, the preterm infants who needed a higher T_{amb} were nursed at a higher ambient vapor pressure than more mature infants. The difference in evaporative heat exchange between very preterm and term infants would have been even greater if the comparison

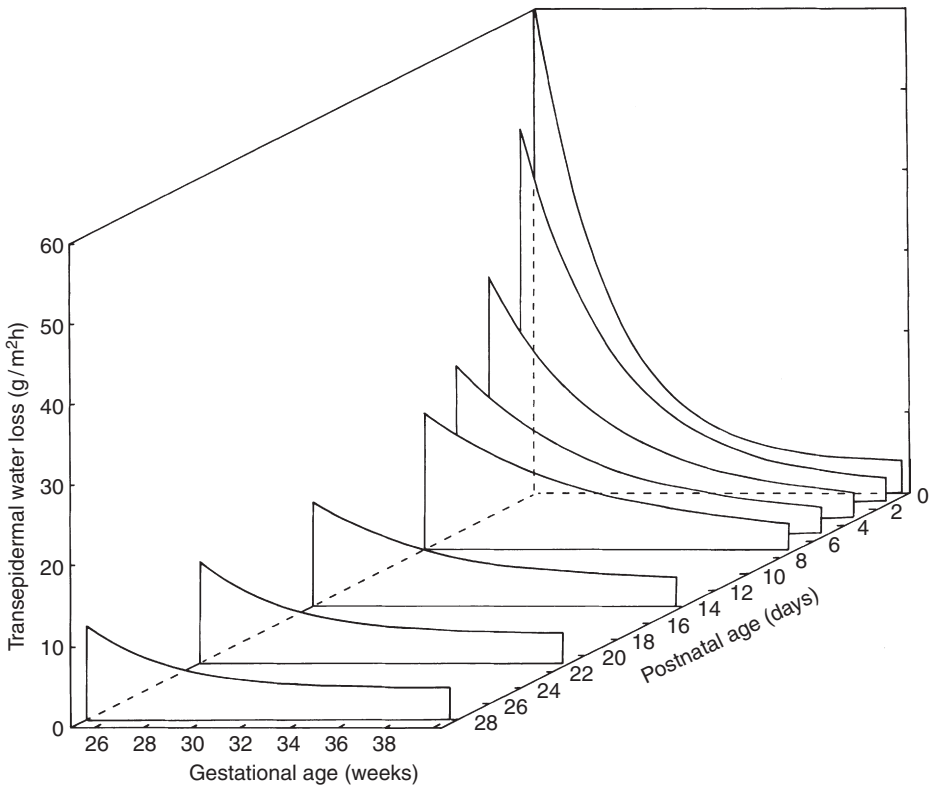


Figure 59-2 The regression of transepidermal water loss on gestational age at birth at different postnatal ages in appropriate–for–gestational age infants. *(From Hammarlund K, Sedin G, Strömberg B: Transepidermal water loss in newborn infants. VIII. Relation to gestational age and post-natal age in appropriate and small for gestational age infants,* Acta Paediatr Scand *72:721, 1983.)*

had been made at equal ambient vapor pressures instead of equal ambient humidities. Infants born at a gestational age of less than 28 weeks need a T_{amb} of approximately 40° C to maintain a normal body temperature at an ambient humidity as low as 20%, whereas term infants need a T_{amb} of approximately 34° C at this ambient humidity (Figure 59-5). At a higher ambient humidity, a somewhat lower T_{amb} is required to maintain a normal body temperature in the most immature infants.[25]

When calculations of heat exchange are made for three different ambient humidities, it is found that the evaporative heat exchange between the skin of very preterm infants and the environment is highest at a low humidity.[25] At a relative ambient humidity (RH) of 60%, the heat exchange through evaporation in these infants is only 50% of that at a RH of 20% (Figure 59-6). Other modes of heat exchange are also influenced by the ambient humidity (see Figure 59-6). Because the evaporative heat exchange is highest at a low humidity, the T_{amb} has to be kept high, and the infant will gain heat both through radiation and through convection. At higher ambient humidities, the evaporative heat exchange will be lower, especially in preterm infants, and, if the body temperature is kept constant, radiative heat loss will be higher and heat gain through convection will be somewhat lower.[25]

If the different modes of heat exchange from the skin surface are added together, the sum will be fairly constant throughout the gestational age groups. The total heat loss cannot, however, be calculated in this way, because the proportions of the body surface area exchanging heat in different ways are not exactly known.

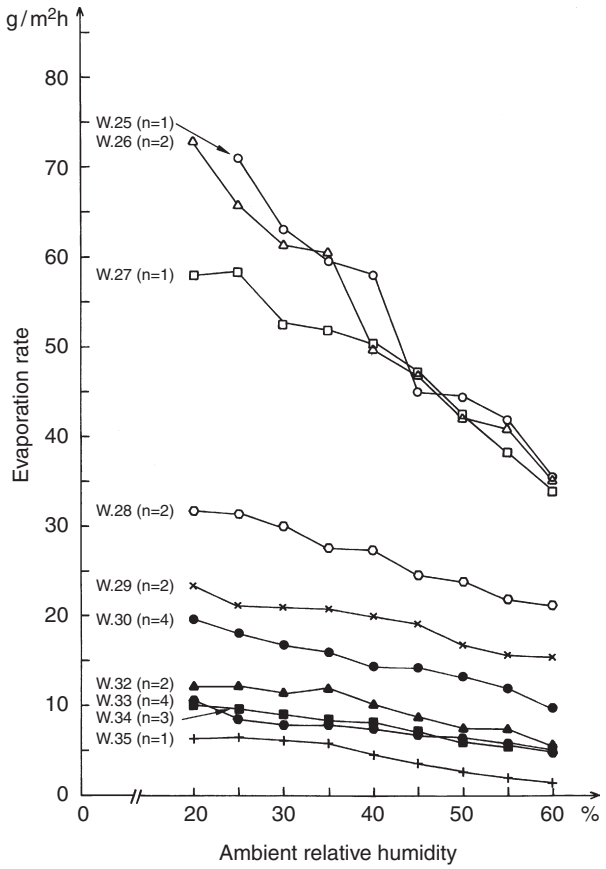


Figure 59-3 The relation between evaporation rate and ambient relative humidity in preterm appropriate–for–gestational age newborn infants in different gestational age groups on the first day after birth. *W,* Completed weeks gestation. *(From Hammarlund K, Sedin G: Transepidermal water loss in newborn infants. III. Relation to gestational age,* Acta Paediatr Scand *68:795, 1979.)*

Heat Exchange During the First Weeks After Birth

During the first weeks after birth, the temperature of the incubator air can gradually be lowered for both preterm and term infants.[26] The high evaporative losses of heat from the infant's skin on the first days after birth will gradually decrease with increasing postnatal age (Figure 59-7).[26] Heat loss through radiation, which is low early after birth in the most preterm infants born at 25 to 27 weeks gestation, will be the most important mode of heat exchange after the first postnatal week. In infants born at more than 28 weeks, radiative heat exchange is the most important mode of heat exchange from birth. The heat loss through radiation will gradually increase with age. The smallest preterm infants will gain heat through convection over the first 10 days, after which a low loss of heat will occur through convection.

During the first weeks after birth, the relative magnitude of the different modes of heat exchange will depend on the ambient humidity (Figure 59-8). In a dry environment, in infants born at 25 to 27 weeks gestation, evaporative heat exchange will be the most important mode of heat exchange for more than 10 days, whereas, at an ambient humidity of 60%, this mode of exchange will be much lower and will be exceeded by heat exchange through radiation from the fifth day after birth.[26]

Total Heat Exchange Between the Infant's Skin and the Environment

To calculate the total heat exchange between the infant's skin and the environment, the body surface area of the infant and the fractions of this area that participate in each mode of heat exchange must be calculated. With the available methods, it is difficult to determine how different fractions of heat exchange vary over a longer period.[26] The total heat exchange is basically dependent on the metabolic rate.

TABLE 59-1

Mean Insensible Water Loss From the Skin in 68 Newborn Appropriate–for–Gestational Age Infants, at an Ambient Humidity of 50%

			POSTNATAL AGE (d)					
Gestational age (wk)	No. of infants	Mean birth weight (g)	<1	3	7	14	21	28
25-27	9	860	129	71	43	32	28	24
28-30	13	1340	42	32	24	18	15	15
31-36	22	2110	12	12	12	9	8	7
37-41	24	3600	7	6	6	6	6	7

From Hammarlund K, Sedin G, Strömberg B: Transepidermal water loss in newborn infants. VIII. Relation to gestational age and post-natal age in appropriate and small for gestational age infants, *Acta Paediatr Scand* 72:721, 1983.

Values are expressed as grams per kilogram body weight per 24 hours.

TABLE 59-2

Mean Insensible Water Loss Through the Skin at Different Ambient Humidities in Appropriate–for–Gestational Age Infants Born at 25 to 27 Weeks Gestation

	POSTNATAL AGE (d)				
Ambient humidity (%)	<1	2	3	5	7
20	205	171	105	75	63
80	53	43	26	19	15

Data from Houdas Y, Ring EFJ: *Human Body Temperature: Its Measurement and Regulation.* New York, Plenum Publishing, 1982, and Kjartansson S, Hammarlund K, Riesenfeld T, Sedin G: Respiratory water loss and oxygen consumption in newborn infants during phototherapy. Acta Paediatr 81:769, 1992.

Based on data in Hammarlund K, Sedin G: Transepidermal water loss in newborn infants. III. Relation to gestational age. *Acta Paediatr Scand* 68:795, 1979, and Hammarlund K, Sedin G, Strömberg B: Transepidermal water loss in newborn infants. VIII. Relation to gestational age and post-natal age in appropriate and small for gestational age infants, *Acta Paediatr Scand* 72:721, 1983.

Values are expressed as grams per kilogram body weight per 24 hours.

Ambient Humidity and Skin Barrier Maturation

In our previously performed longitudinal studies on water and heat exchange between very preterm infants and their environment early after birth, we have during regular care always kept the RH at 85% during care in incubators. Before performing measurements of ER from the skin, the RH in the incubator is lowered to 50% and stabilized at this level. Then measurements of ER and calculation of transepidermal water loss are performed at 50% ambient humidity[89] with the aim to investigate whether the level of RH in which the infants are nursed influences the maturation of their postnatal skin development.

In 22 preterm infants born at a gestational age of 23 to 25 weeks, TEWL was determined at postnatal ages of 0, 3, 7, 14, and 28 days. The infants were nursed at a RH of 85% during the first week after birth. At a postnatal age of 7 days the infants were randomly assigned to continued care at either 50% or 75%. The results of the measurements were corrected to a RH of 50% ($TEWL_{50}$). $TEWL_{50}$ was very high on the first day after birth (62 ± 5 g • m^{-2} • h^{-1}) and decreased with increasing postnatal age. At postnatal days 3 and 7 no differences in $TEWL_{50}$ were seen between the two groups. At a postnatal age of 28 days $TEWL_{50}$ was twice as high in infants nursed at 75% RH (22 ± 2 g • m^{-2} • h^{-1}) as in those nursed at 50% RH (13 ± 1 g • m^{-2} • h^{-1}; $p < .001$). These results indicate that the level of RH influences the development of the skin barrier (Figure 59-9), presumably through its effect on transepidermal water flux. These findings are relevant for nursing strategies promoting barrier integrity in extremely preterm infants. The study has resulted in a shorter duration of nursing very preterm infants at a very high humidity. After 1 postnatal week, the RH can be lowered to 50%, promoting skin barrier development in extremely preterm infants.

Nonionizing Radiation and Evaporative Water and Heat Exchange Between the Infant's Skin and the Environment

Radiant Heaters

In studies of the ER in term, moderately preterm, and very preterm infants nursed in incubators with 50% ambient humidity and under a radiant heater, it was found that the ER from the skin surface of term infants was significantly higher under the radiant heater than in the incubator.[45] Preterm infants were observed both at 50% ambient humidity and at a lower ambient humidity of 30% to 40% in the incubator and then under a radiant heater. No significant difference was found between ER values obtained in the incubator at the lower ambient humidity and ER measured under the radiant heater. When using the measured values of ER and water vapor pressure in the incubator and the theoretical values of ER = 0 and the water vapor pressure in the incubator at a RH of 100% at incubator air temperature, the highest possible ER was calculated from the linear regression line for the value water vapor pressure = 0 (Figure 59-10). A calculated value for ER during care under a radiant heater could then be obtained on the line between the maximum ER value at water vapor pressure = 0 and ER = 0, that is, at the saturated vapor pressure for the air temperature under the radiant heater (see Figure 59-10).[45] The calculated values for ER determined in this fashion were always higher than the measured values, a finding that suggests that, even if the measured ER under the radiant heater is higher than the ER in the incubator on account of the higher humidity in the latter, no indication exists of an increased water loss resulting from a direct effect of nonionizing radiation on the barrier function of the infant's skin,[45] as suggested previously.[90]

In investigations of the effects of nonionizing radiation in an incubator equipped with a radiant hood warmer, a device that heats the incubator roof and ceiling independently of the incubator's main heat source, no change in TEWL in term and preterm infants was observed with use of this device.[30,78] This finding further supports the view that an isolated change in radiative heat exchange does not influence the evaporative water and heat exchange if the ambient air temperature and ambient humidity (i.e., the vapor pressure) are unaltered.

Phototherapy

An increase in the IWL during phototherapy was reported in several publications.[58,60,61,64,70] This could be caused either by increased water loss from the skin as a result of altered barrier properties or by increased RWL. In thermally stable term and preterm infants,[43] no increase in the ER from the skin surface was found during phototherapy. In that study, a significant increase was seen in the temperature of the ceiling of the incubator. T_{body}, T_{skin}, heart rate, and respiratory rate were unchanged in term and preterm infants. T_{amb} was unchanged during the studies in term infants, whereas T_{amb} sometimes had to be lowered in preterm infants to avoid heat stress.[43]

WATER AND HEAT EXCHANGE BETWEEN THE RESPIRATORY TRACT AND ENVIRONMENT

Water and Heat Loss From the Respiratory Tract

When an infant is nursed in room air, water is lost from the respiratory tract with each expiration. With use of indirect methods for the determination of RWL, it has been shown that RWL depends on the humidity of inspired air, with lower losses at a high humidity.[15,31,54,63] In a series of studies of RWL (milligrams per kilogram per minute) using a flow-through system with a mass spectrometer for measurements of gas concentrations,* we found that, in an environment with 50% ambient humidity, the evaporative heat loss from the airway will be of moderate magnitude.[49] Actually, in term appropriate–for–gestational age infants who are nursed in an environment with 50% ambient humidity, the IWL from the respiratory tract (IWL_r) and that from the skin (IWL_s) will be of equal magnitude.[49] Therefore the evaporative heat loss from the respiratory tract and the evaporative heat loss from the skin will also be of equal magnitude under these conditions.

The evaporative loss of water and heat from the airway has been found to depend on the ambient humidity in both lambs[42] and infants,[49] with lower losses at higher humidity. Whereas IWL_s decreased from 9 to 2 g/kg per 24 hours in term infants when the ambient humidity was increased from 20% to 80%, the corresponding change in IWL_r was much smaller, that is, from

*References 19, 29, 42, 49, 50, 65.

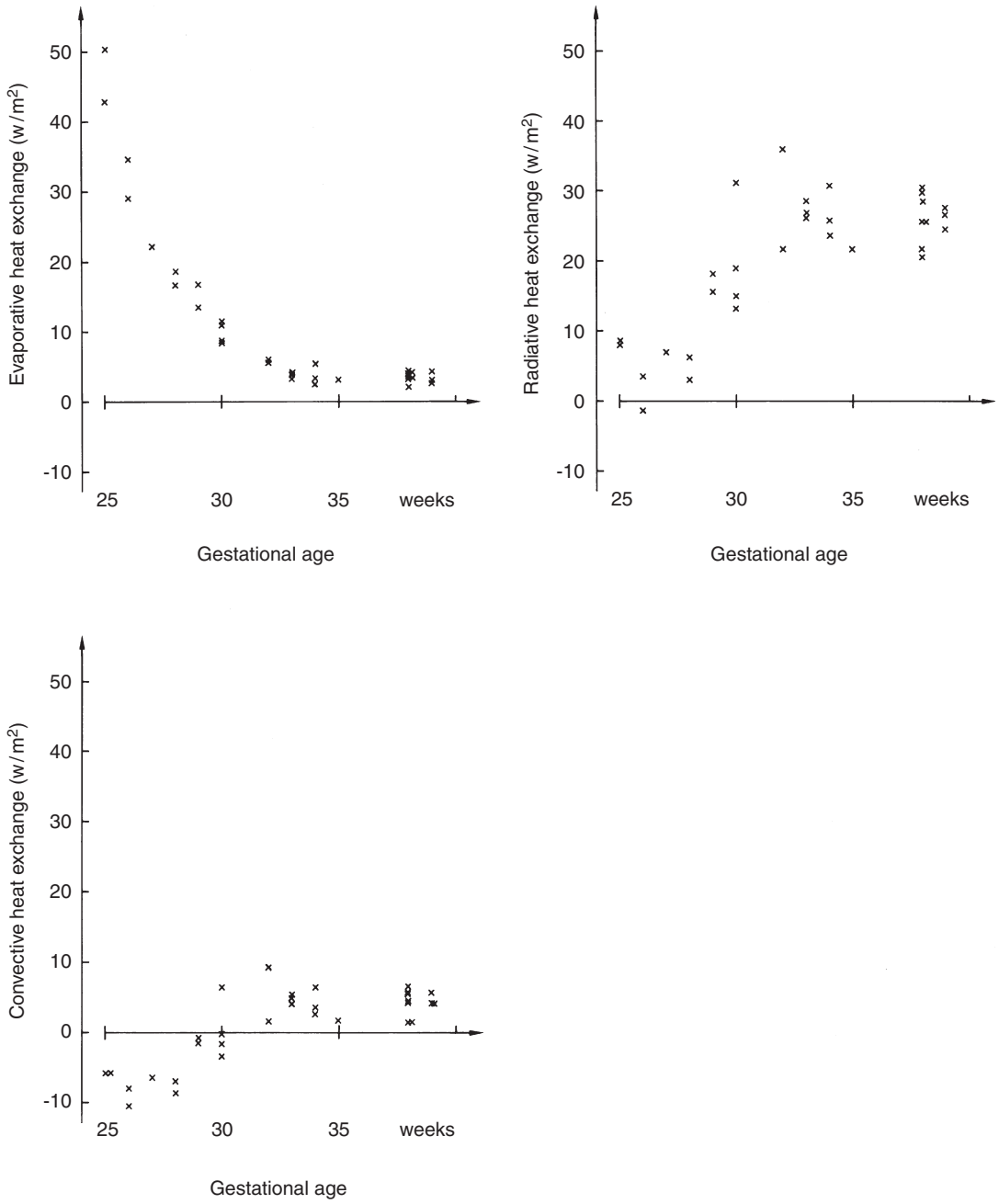


Figure 59-4 Heat exchange through evaporation, radiation, and convection at an ambient humidity of 50% in relation to gestational age. Measurements were made during the first 24 hours after birth in preterm infants and during the first 30 hours in term infants. *(From Hammarlund K, Sedin G: Transepidermal water loss in newborn infants. VI. Heat exchange with the environment in relation to gestational age,* Acta Paediatr Scand *71:191, 1982.)*

9 to 5 g/kg per 24 hours.[49] When measuring evaporative heat loss and respiratory heat loss in infants placed in a calorimeter, Sulyok and associates[31] found that both the total evaporative heat exchange and respiratory heat exchange were related to the ambient humidity; the respiratory heat loss ranged between 0.07 and 0.22 W/kg, or 3% to 10% of the total heat production from metabolism and approximately 40% of the insensible heat loss. In term infants, RWL and evaporative heat loss from the respiratory tract may increase during activity by up to 140% of the values at rest.[50] In Table 59-3, data on IWL_s and IWL_r and their sum, the total IWL, are given for the hypothetical situation in which the infant spends 24 hours at the same level of activity. The oxygen consumption will also increase with higher levels of activity.[50]

Infants are able to tolerate moderate heat stress without increasing their RWL.[29] In addition, both lambs and infants can increase their RWL and evaporative heat loss without increasing their oxygen consumption and CO_2 production.[29,75,76] An example is given in Figure 59-11, which shows how exposure to radiant heat can alter the RWL in a lamb while leaving the oxygen consumption and CO_2 production unchanged. The RWL is directly proportional to the rate of breathing,[29,75,76] and this means that lambs and infants will lose more water and heat when they have a high rate of breathing (Figure 59-12). Respiratory water and evaporative heat losses also decrease with postnatal age, at least in lambs.[42] In both lambs and infants, we observed irregular breathing during a period before a significant increase in RWL occurred.[91]

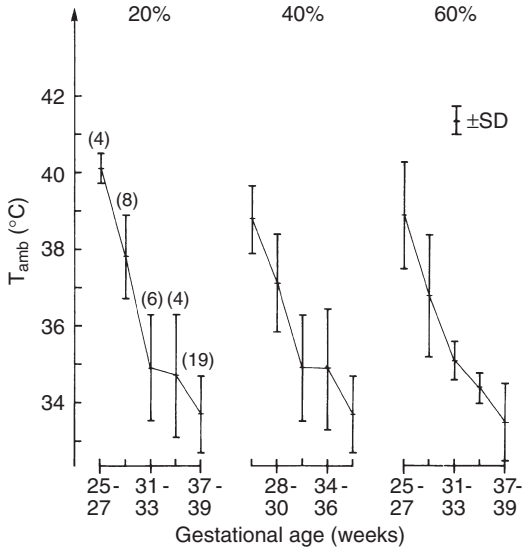


Figure 59-5 Ambient temperature *(T_{amb})* in relation to gestational age at ambient relative humidity of 20%, 40%, and 60%. *(From Hammarlund K, Sedin G: Transepidermal water loss in newborn infants. VI. Heat exchange with the environment in relation to gestational age,* Acta Paediatr Scand *71:191, 1982.)*

Respiratory Water and Evaporative Heat Exchange Before and After Intubation

Hammarlund and colleagues[74] studied young lambs exposed to heat stress before and after intubation. In nonintubated lambs exposed to a radiant heat source, the RWL increased from 10.5 to 33.4 mg/kg per minute, whereas the respiratory rate increased from 54 to 161 breaths/minute; oxygen consumption and CO_2 production were unaltered.[74] During exposure to the same heat source, intubated lambs increased their respiratory water loss from 8.1 to 18.7 mg/kg per minute and their rate of breathing from 46 to 125 breaths/minute. In intubated lambs, both oxygen consumption and CO_2 production increased significantly. RWL per breath did not change with intubation but increased significantly after extubation; the reason for this may be a larger tidal volume after intubation or to some extent a higher water content in the expired air resulting from a higher body temperature after heat stress.[74]

Respiratory Water and Evaporative Heat Loss in Relation to Gestational Age

RWL was found to be highest in the most preterm infants and lower in more mature infants observed in incubators with 50% RH and a T_{amb} that allowed the infant to maintain a normal and stable body temperature. The infants were usually asleep during the measurements. RWL per breath (milligrams per kilogram) was almost the same at all gestational ages. Therefore the higher RWL found in the most preterm infants as compared with the more mature infants was the result of the higher rate of breathing. As can be seen in Table 59-4, an increase in respiratory rate causes an increase in the loss of water and evaporative heat through the respiratory tract.[65]

Respiratory Water and Evaporative Heat Exchange During Phototherapy

When term and preterm infants[44] underwent phototherapy, no significant changes in RWL, oxygen consumption, CO_2 production, or rate of breathing were found (Table 59-5). In studies in term infants, the T_{amb}, T_{body}, and T_{skin} rose significantly. When preterm infants were studied, T_{amb} did not change; however, T_{roof} increased, and a small increase occurred in T_{body} and T_{skin}. Thus, in the absence of heat stress, no increase occurs in RWL, oxygen consumption, or CO_2 production during phototherapy in term or moderately preterm newborn infants.[44]

Respiratory Water and Heat Exchange During Mechanical Ventilation

In clinical neonatal care with a warm and humid environment or with warm and humidified gas supplied from a respirator, heat exchange through evaporation and convection between the respiratory tract and the environment will be low.[31,42,49] Infants who inspire cold air with a low water vapor pressure will have high evaporative loss from the respiratory tract and will also lose heat through convection. These losses may become clinically significant, for instance, during transport in a cold climate. Under these conditions, the total heat loss from the respiratory tract may increase to up to 20% of the total heat production of the infant.[72]

Skin-to-Skin Care and Heat Exchange

In skin-to-skin care, infants are placed lying naked, except for a diaper, on the mother's or father's chest, and they are covered with the parent's clothing or a blanket.[92-94] Only the head or parts of the head are exposed to the environmental air.[93-95] Several studies have shown that preterm infants can maintain a normal body temperature during skin-to-skin care even if no heat gain by conduction occurs. In an infant in a thermoneutral state, skin-to-skin care should cause loss of heat.[95] Covering the infant's trunk and extremities with a blanket will eliminate convective and radiative heat losses from these parts of the body. Little is known about the evaporative heat exchange between the infant and the environment under these conditions. The heat exchange between the infant's head and the environment can be reduced by using a cap or by partly covering the head, but from areas of the head that are exposed to the environmental air significant heat losses will occur through radiation, evaporation, and convection.

Studies of body and skin temperatures and oxygen consumption in infants have revealed that in preterm infants with a weight less than 1500 g and at an postnatal age less than 1 week and who are nursed in incubators the rectal and skin temperatures increase during 1 hour of skin-to-skin care with no increase in oxygen consumption.[96] In a later study, Bauer and colleagues[97] also found that infants born at 28 to 30 weeks gestation studied during the first and second week after birth have an increase in body temperature during 1 hour of skin-to-skin contact with no significant change in oxygen consumption. More immature infants (25 to 27 weeks gestation) showed no increase in oxygen consumption but a decrease in rectal temperature during the same duration of skin-to-skin contact during the first week after birth. During the second postnatal week, the body temperature did not change in infants born at 25 to 27 weeks gestation during skin-to-skin care.[97] The authors suggested that infants born at 25 to 27 weeks gestation may have a high evaporative heat loss during the first week after birth,[41] thus causing cold stress. Further studies on the different modes of heat exchange and oxygen consumption are needed to clarify the physiology of very preterm infants during skin-to-skin care.

SUMMARY AND CONCLUSION

The exchange of heat between the infant's skin and the environment is influenced by the insulation provided by the skin, the permeability of the skin, and environmental factors such as the T_{amb} and humidity, airflow velocity, and the temperature and characteristics of the incubator surfaces facing the infant. Evaporative heat loss from the skin is the major component of heat exchange

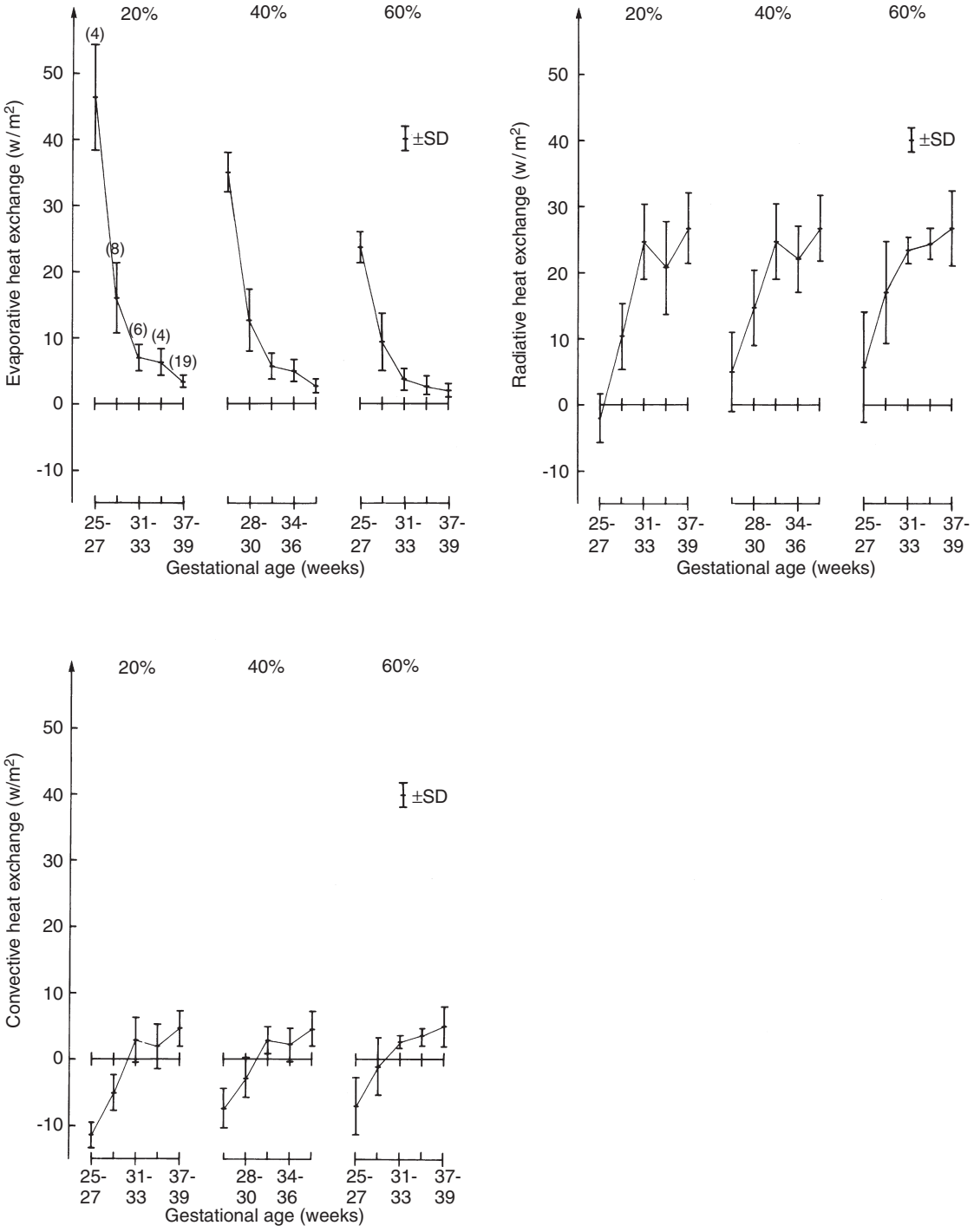


Figure 59-6 Heat exchange through evaporation, radiation, and convection in relation to gestational age at relative ambient humidities of 20%, 40%, and 60%. *(From Hammarlund K, Sedin G: Transepidermal water loss in newborn infants. VI. Heat exchange with the environment in relation to gestational age,* Acta Paediatr Scand *71:191, 1982.)*

in the most preterm infants early after birth. Infants gain heat through convection and, in a very dry environment, possibly also through radiation when they are nursed in incubators. As the water loss from the skin surface of these most preterm infants decreases with postnatal age, the heat loss through evaporation will also decrease. Concurrently, the need for a high T_{amb} diminishes, and, with the lower temperature of the incubator walls, the heat loss through radiation will increase and the heat gain by convection will change to a low loss of heat during the first weeks after birth. In infants nursed under a radiant heater, heat is gained through radiation, and, as a result of the low ambient humidity, evaporative loss of water and heat may be high in very preterm infants. The loss of heat through convection greatly depends on how the infant is protected from high air velocities by arrangements made under the radiant heater and also on the magnitude of air movements in the nursery.

In term infants nursed in incubators with an ambient humidity of 50%, the evaporative water and heat loss from the respiratory

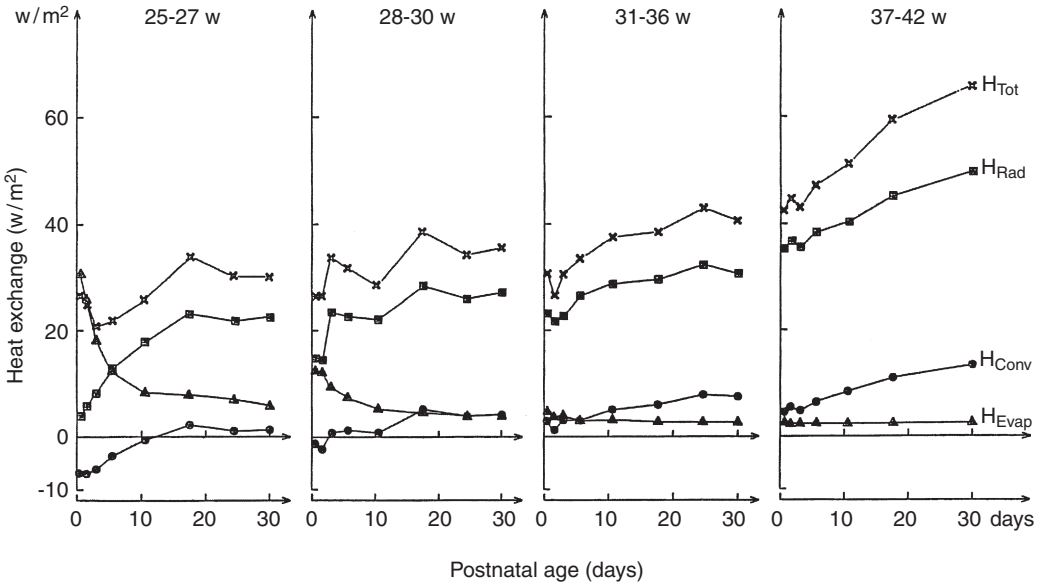


Figure 59-7 Heat exchange between the infant and the environment per square meter of body surface area in relation to postnatal age in different gestational age-groups at an ambient humidity of 50%. H_{Conv}, heat exchange through convection; H_{Evap}, Heat exchange through evaporation; H_{Rad}, heat exchange through radiation; H_{Tot}, total heat exchange; *w*, weeks. *(From Hammarlund K, Strömberg B, Sedin G: Heat loss from the skin of preterm and fullterm newborn infants during the first weeks after birth,* Biol Neonate *50:1, 1986.)*

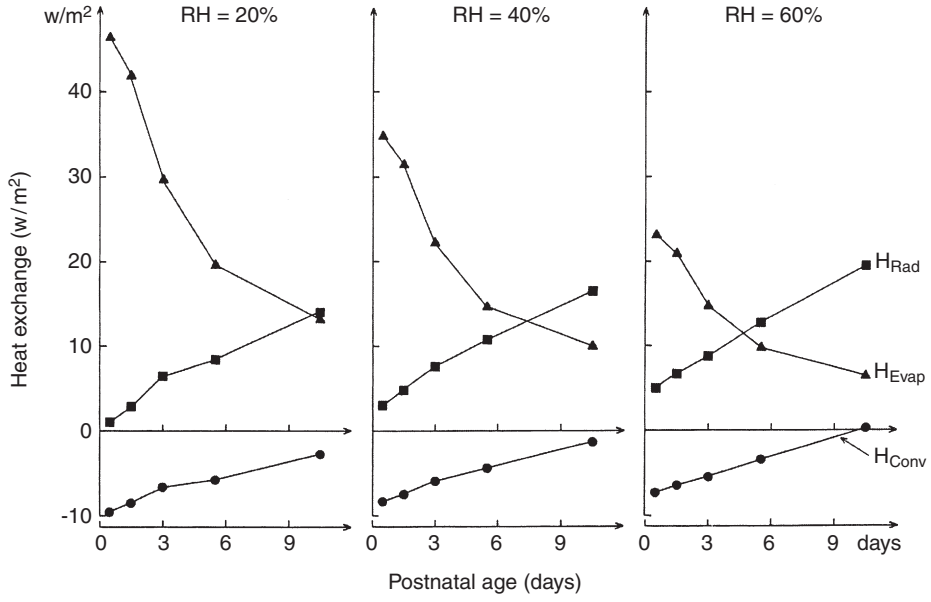


Figure 59-8 Heat exchange between the infant and the environment in relation to postnatal age at relative ambient humidity *(RH)* of 20%, 40%, and 60% in infants born at 25 to 27 weeks gestation. H_{Conv}, Heat exchange through convection; H_{Evap}, heat exchange through evaporation; H_{Rad}, heat exchange through radiation. *(From Hammarlund K, Strömberg B, Sedin G: Heat loss from the skin of preterm and fullterm newborn infants during the first weeks after birth,* Biol Neonate *50:1, 1986.)*

tract and that from the skin will be of about the same magnitude. Therefore respiratory water and evaporative heat losses are low in term infants. The respiratory evaporative water and heat loss per breath (milligrams per kilogram) is of approximately the same magnitude in calm preterm and term infants. In preterm infants, evaporative heat losses from the skin are much greater than such heat losses from the respiratory tract. The evaporative heat loss from the airway may be considered important only in infants with high motor activity or with tachypnea, especially if they are nursed in a very dry environment. Mechanical ventilation with dry and cold air will also result in a high loss of heat from the respiratory tract through evaporation and convection. Exposure to nonionizing radiation does not increase evaporative water or heat loss from the skin or the respiratory tract. Intubated lambs have a lower ability to increase respiratory water and evaporative heat loss during heat stress than nonintubated lambs.

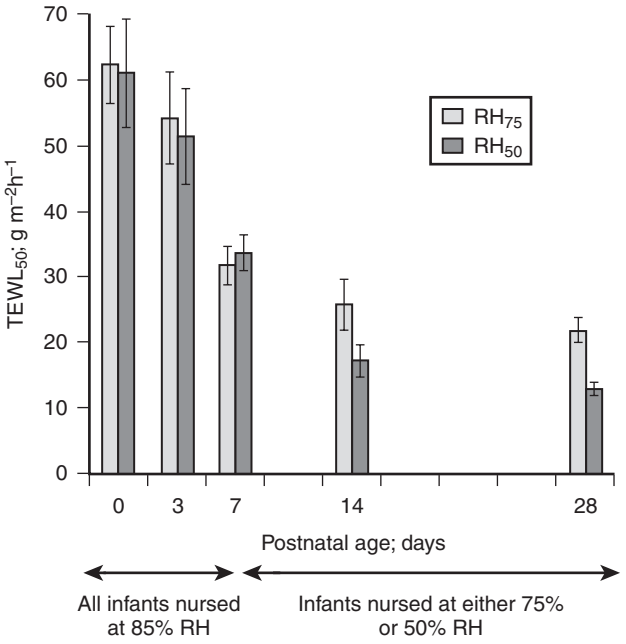


Figure 59-9 $TEWL_{50}$ in infants nursed at either RH_{75} or RH_{50} from postnatal ages of 7 to 28 days. During the first 7 days all infants were nursed at a relative ambient humidity (RH) of 85%, and no differences in TEWL were noted. After adjustment of RH at PNA of 7 days, skin barrier function improved more rapidly in infants nursed at a lower RH.

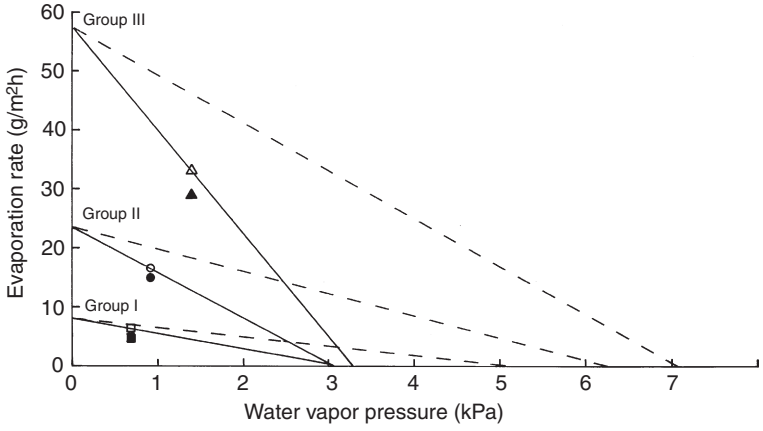


Figure 59-10 The calculated *(open symbols)* and measured *(filled symbols)* values for evaporation rate during care under a radiant heater together with corresponding regression lines for evaporation rate on water vapor pressure at the air temperatures of the incubator and the radiant heater in three infants, one from each of the studied groups: term infants (group I; n = 12), preterm infants born at 30 to 34 weeks (group II; n = 8), and very preterm infants born at 25 to 29 weeks (group III; n = 8). *(From Kjartansson S, Arsan S, Hammarlund K, et al: Water loss from the skin of term and preterm infants nursed under a radiant heater,* Pediatr Res *37:233, 1995.)*

TABLE 59-3

Insensible Water Loss From the Skin and From the Respiratory Tract and Their Sum and Oxygen Consumption, in Term Appropriate–for–Gestational Age Infants at Different Levels of Activity

Activity level	IWL_S	IWL_R	IWL_T	$\dot{V}O_2$
0	6	6	12	8
1	—	8	—	9
2	—	9	—	10
3	—	10	—	11
4	—	12	—	12
5	7	16	23	13

From Riesenfeld T, Hammarlund K, Sedin G: Respiratory water loss in relation to activity in fullterm infants on their first day after birth, *Acta Paediatr Scand* 76:889, 1987.

Water loss values expressed as grams per kilogram per 24 hours. Oxygen consumptions values expressed as liters per kilogram per 24 hours.

IWL_R, Insensible water loss from the respiratory tract; *IWL_S,* insensible water loss from the skin; *IWL_T,* total insensible water loss; *$\dot{V}O_2$,* oxygen consumption.

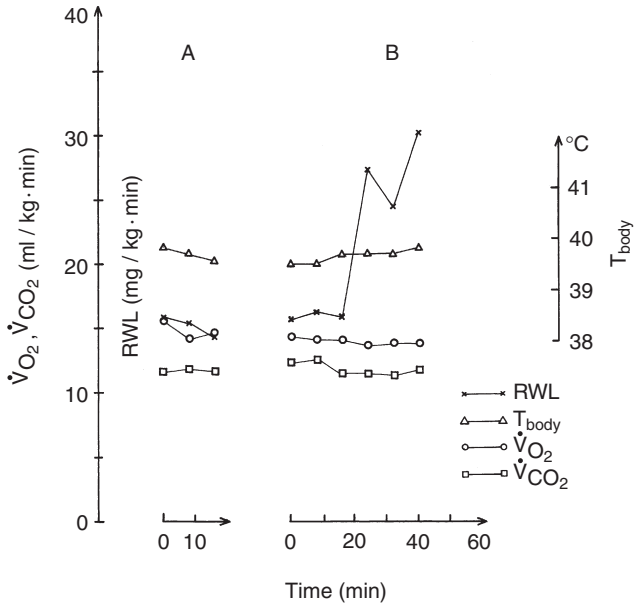


Figure 59-11 Respiratory water loss *(RWL),* body temperature *(T_{body}),* oxygen consumption *($\dot{V}O_2$),* and carbon monoxide production *($\dot{V}CO_2$)* in a 6-day-old lamb before **(A)** and during **(B)** heat stress. *(From Riesenfeld T, Hammarlund K, Jonzon A, Sedin G: Influence of radiant heat stress on respiratory water loss in new-born lambs,* Biol Neonat *53:290, 1988.)*

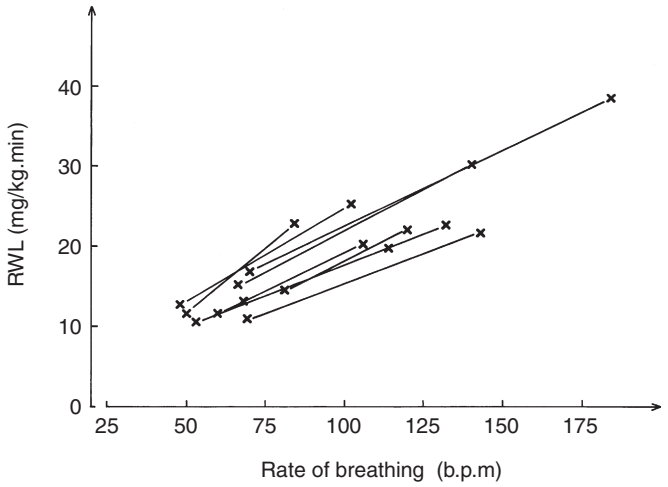


Figure 59-12 Respiratory water loss *(RWL)* in relation to rate of breathing before and during moderate radiant heat stress in lambs. For each lamb, the value before heat stress and the highest value during heat stress are joined. *(From Riesenfeld T, Hammarlund K, Jonzon A, Sedin G: Influence of radiant heat stress on respiratory water loss in new-born lambs,* Biol Neonat *53:290, 1988.)*

TABLE 59-4

Mean Respiratory Water Loss in Preterm and Term Infants on the First Day After Birth at an Ambient Relative Humidity of 50%

			RWL/min (mg/kg)		RWL/24 hr (g/kg)	
GA (wk)	RWL/breath (mg)	RWL/breath (mg/kg)	RR 40	RR 100	RR 40	RR 100
27-30	0.13 ± 0.05	0.12 ± 0.04	4.8	12	6.9	17
31-36	0.24 ± 0.12	0.11 ± 0.05	4.4	11	6.3	16
37-41	0.36 ± 0.06	0.10 ± 0.02	4.0	10	5.8	14

From Riesenfeld T, Hammarlund K, Sedin G: Respiratory water loss in relation to gestational age in infants on their first day after birth. *Acta Paediatr* 84:1056, 1995.
GA, Gestational age; *RR,* respiratory rate; *RWL,* respiratory water loss.
Values are expressed as mean ± SD.

TABLE 59-5

Respiratory Water Loss, Oxygen Consumption, and Carbon Dioxide Production in Term Infants Before, During, and After 60 Minutes of Phototherapy

	N	RWL (mg/kg/min)	$\dot{V}o_2$ (mL/kg/min)	$\dot{V}co_2$ (mL/kg/min)	RR (breaths/min)
Before	11	4.4 ± 0.7	5.9 ± 0.9	4.0 ± 0.7	48 ± 7
12 min	11	4.4 ± 0.6	5.9 ± 1.0	3.9 ± 0.6	
24 min	10	4.1 ± 0.8	5.5 ± 1.1	3.8 ± 0.7	
36 min	9	4.2 ± 1.0	5.4 ± 1.0	3.6 ± 0.6	
48 min	11	4.4 ± 0.7	5.9 ± 1.1	3.9 ± 0.7	51 ± 11
60 min	9	4.6 ± 0.9	5.9 ± 1.1	4.1 ± 0.8	
After	9	4.8 ± 0.8	6.1 ± 0.9	4.1 ± 0.4	

From Kjartansson S, Hammarlund K, Riesenfeld T, Sedin G: Respiratory water loss and oxygen consumption in newborn infants during phototherapy, Acta Paediatr 81:769, 1992.

Values are expressed as mean ± SD.

RR, Respiratory rate; *RWL,* respiratory water loss; $\dot{V}co_2$, carbon dioxide production; $\dot{V}o_2$, oxygen consumption.

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60

Alistair Jan Gunn and Laura Bennet

Responses of the Fetus and Neonate to Hypothermia

Moderate to severe hypoxic-ischemic encephalopathy continues to be a significant cause of acute neurologic injury at birth, occurring in approximately 1 to 2 cases per 1000 term live births.[1,2] The possibility that hypothermia may be able to prevent or lessen asphyxial brain injury is a "dream revisited."[3] Early experimental studies, mainly in precocial animals such as kittens, demonstrated that hypothermia greatly extended the "time to last gasp."[4] This finding led to a series of small, uncontrolled studies in the 1950s and 1960s in which infants who were not breathing spontaneously at 5 minutes after birth were immersed in cold water until respiration began and then allowed to rewarm spontaneously over many hours.[5-7] Although outcomes were said to be better than for historical controls, this experimental approach was overtaken by two major developments: the introduction of active ventilation and resuscitation of infants exposed to asphyxia and the recognition that even mild hypothermia is associated with a wide range of potential adverse effects,[8] including greater mortality in the premature newborn.[9] Thus resuscitation guidelines for the newborn exposed to asphyxia have, until recently, simply emphasized keeping the newly born warm: that is to say, avoiding hypothermia.[10]

The early experimental studies just noted focused entirely on the effects of cooling *during* severe hypoxia, which is well known to be associated with potent, dose-related, long-lasting neuroprotection.[11] The central clinical question is, of course, whether cooling *after* asphyxial or hypoxic-ischemic injury is beneficial. This chapter reviews recent developments that helped to delineate the experimental parameters that determine whether postresuscitation cooling is successful.

PATHOPHYSIOLOGIC PHASES OF CEREBRAL INJURY

The critical advance has been the clinical and experimental observation in term fetuses, newborns, and adults that injury to the brain is not a single "event" occurring at, or just after, an insult but rather an evolving process that leads to cell death well after the initial insult.[12,13] Using magnetic resonance spectroscopy, Azzopardi and co-workers[14] showed that infants with evidence of moderate to severe asphyxia often had normal cerebral oxidative metabolism shortly after birth, but many then went on to have delayed energy failure 6 to 15 hours later. Those infants who did not show transient recovery had a very high mortality, whereas in survivors the degree of secondary energy failure after 24 to 48 hours was closely associated with impaired neurodevelopmental outcome at 4 years of age.[15] An identical pattern of initial recovery of cerebral oxidative metabolism followed by secondary energy failure was seen after hypoxia-ischemia in the piglet[16] and closely correlated with the severity of neuronal loss.[17] This delay between insult and injury raised the tantalizing possibility that asphyxial cell death may be prevented even well after the insult.

Pathophysiologically, several phases have been identified, as illustrated in Figures 60-1 and 60-2. The actual period of hypoxia and ischemia is the *primary* phase of cell injury. During this phase, progressive hypoxic depolarization of cells occurs, leading to severe cytotoxic edema, with failure of reuptake, leading to extracellular accumulation of excitatory amino acids *(excitotoxins).*[18] Excessive levels of excitatory amino acids cause activation of their ion channels and promote further excessive entry of salt, water, and calcium into the cells. After reperfusion (see Figure 60-2) or return of cerebral circulation during resuscitation from an asphyxial insult, the initial hypoxia-induced cytotoxic edema may transiently resolve over approximately 30 to 60 minutes, with at least partial recovery of cerebral oxidative metabolism *(latent phase).* This is followed by a secondary phase of deterioration (approximately 6 to 15 hours later) that may extend over many days.[15,19] At term gestation, this so-called *secondary phase* is marked by delayed-onset seizures,[20,21] secondary cytotoxic edema (see Figure 60-1),[22] accumulation of excitotoxins,[18] failure of cerebral oxidative energy metabolism,[16,19] and ultimately neuronal death.

The studies discussed in this chapter strongly suggest that it is the early recovery, or latent phase, that represents the key window of opportunity for intervention.

FACTORS DETERMINING EFFECTIVE NEUROPROTECTION WITH HYPOTHERMIA

Experimentally, the efficacy of hypothermia is highly dependent on certain factors, including the timing of initiation of cooling, its duration, and its depth.

Cooling During Resuscitation and Reperfusion

Brief hypothermia, for 1 to 2 hours, appears to be modestly neuroprotective, provided it is initiated immediately after the insult. For example, after 15 minutes of reversible ischemia in the piglet, mild hypothermia (2° to 3° C) for 1 to 3 hours significantly improved recovery and reduced neuronal loss 3 days later.[23,24] Critically, protection appears to be lost if the brief interval of hypothermia is delayed by as little as 15 to 45 minutes after the primary insult.[25-28] This extreme sensitivity to delay is consistent with the hypothesis that resuscitative hypothermia can suppress damage resulting from oxygen free radical production during reperfusion.[29,30] Alternatively, however, this strategy may merely represent intervention at the end of the primary phase, when cerebrovascular perfusion is being reestablished, cell function is just starting to recover, and levels of excitatory amino acids are still high.[18]

Even if such immediate cooling during resuscitation were consistently effective, it would be almost impossible to test in practice. It is simply not possible at present to identify reliably the few infants requiring resuscitation who will go on to have significant encephalopathy until some hours after birth.

Prolonged Cooling

A more recent approach has been to try to suppress the secondary encephalopathic processes by maintaining hypothermia throughout the course of the secondary phase. Such extended periods of cooling of between 5 and 72 hours appear to be more

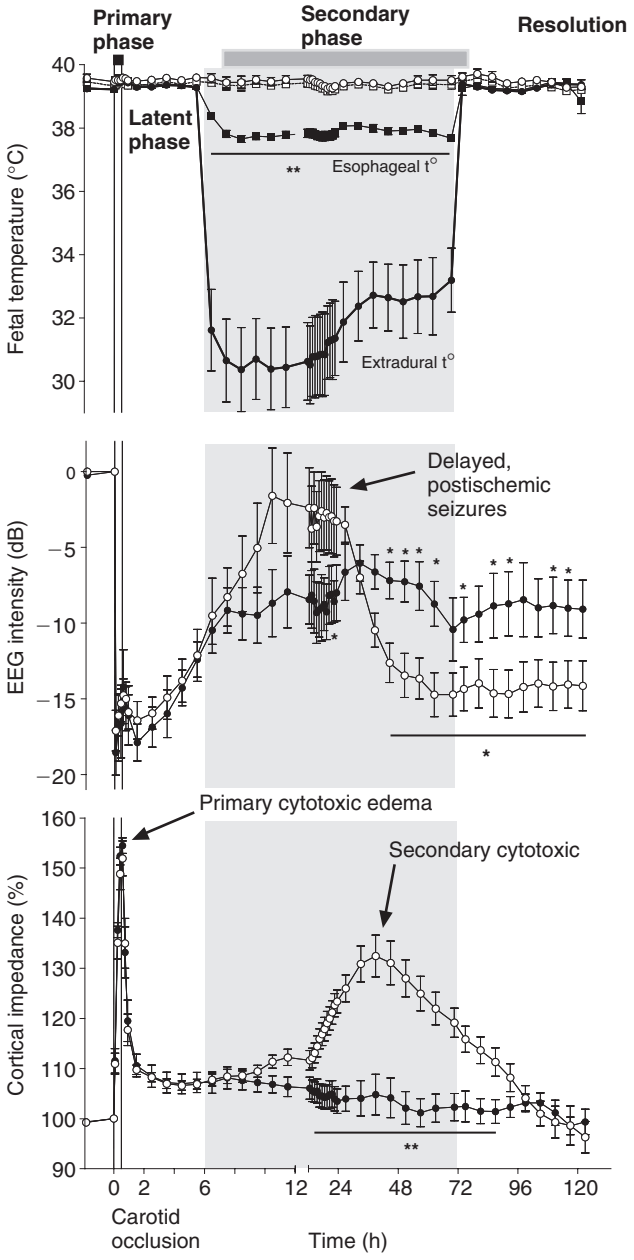


Figure 60-1 The effect of hypothermia started 5.5 hours after reperfusion from 30 minutes of cerebral ischemia in near-term fetal sheep. The period of ischemia is shown by *dotted lines*, whereas cooling is shown by the *bar*. The *top panel* shows changes in extradural *(solid circles)* and esophageal *(solid squares)* temperature in the hypothermia group and extradural *(open circles)* and esophageal *(open squares)* temperature in the sham-cooled group. The *lower two panels* show changes in electroencephalographic (EEG) intensity and cortical impedance (expressed as percentage of baseline) in the hypothermia *(solid circles)* and sham-cooled *(open circles)* groups. Impedance is a measure of cytotoxic edema (cell swelling). The hypothermia group shows greater recovery of EEG intensity after resolution of delayed seizures and complete suppression of the secondary rise in impedance. Mean ± SEM. $^{*}p < .05$, $^{**}p < .001$ hypothermia versus sham-cooled fetuses. *(Data from Gunn AJ, Gunn TR, Gunning MI, et al: Neuroprotection with prolonged head cooling started before postischemic seizures in fetal sheep,* Pediatrics *102:1098-1106, 1998.)*

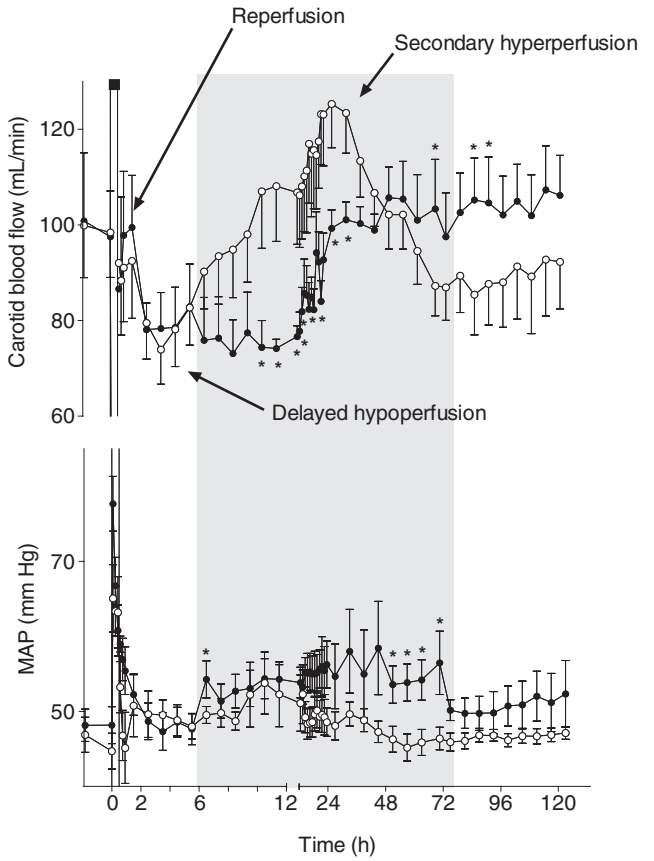


Figure 60-2 The effect of hypothermia started 5.5 hours after reperfusion from 30 minutes of cerebral ischemia in near-term fetal sheep on carotid blood flow and mean arterial blood pressure (MAP) in the hypothermia *(solid circles)* and sham-cooled *(open circles)* groups. Cerebral ischemia is shown by the *solid bar* at the top of the graph, whereas cooling is shown by the *highlighted region*. A significant increase in blood pressure occurred during cooling that rapidly resolved with rewarming. Both groups show a significant phase of secondary hypoperfusion that was beginning to resolve at 6 hours; hypothermia prolonged this phase and prevented the phase of secondary hyperperfusion such that carotid blood flow was significantly reduced in the hypothermia group between 10 and 36 hours. Mean ± SEM. $^{*}p < .05$ versus sham cooling. *(Data from Gunn AJ, Gunn TR, Gunning MI, et al: Neuroprotection with prolonged head cooling started before postischemic seizures in fetal sheep,* Pediatrics *102:1098-1106, 1998.)*

consistently effective.[31-33] For example, in a study of reversible middle cerebral artery occlusion in the adult rat, 21 hours, but not 1 hour, of mild hypothermia reduced the area of infarction after 48 hours recovery.[34] After severe global ischemia in the adult gerbil, 12 hours of mild hypothermia was ineffective, whereas extending the interval to 24 hours did afford protection.[32]

Newborn studies support these data. In unanesthetized infant rats subjected to moderate hypoxia-ischemia, mild hypothermia (2° to 3° C reduction in brain temperature) for 72 hours from the end of hypoxia prevented cortical infarction, whereas 6 hours of cooling had only intermediate, nonsignificant results.[35] In the same model greater reduction in body temperature (by 5° C) for 6 hours, starting immediately after the insult, gave significant neuroprotection after both 1 and 6 weeks survival, as well as neurobehavioral improvement.[36] Finally, in the anesthetized piglet

exposed either to hypoxia with bilateral carotid ligation or to hypoxia with hypotension, 12 to 48 hours of moderate whole-body hypothermia or head cooling with mild systemic hypothermia (started immediately after hypoxia) prevented delayed energy failure,[33,37] reduced neuronal loss,[38-40] and suppressed posthypoxic seizures.[39]

A Window of Opportunity for Treatment? Recent Evidence

At present, the window of opportunity for any particular therapy can be determined only empirically. However, some general principles can be discerned. For example, initiation of neuronal degeneration is accelerated by more severe insults. DNA fragmentation in the hippocampus can be detected as early as 10 hours after a 60-minute hypoxic-ischemic injury in the rat, whereas DNA fragmentation in the hippocampus is detectable only 3 to 5 days after a 15-minute hypoxic-ischemic injury.[41] However, the appearance of DNA fragmentation and classic ischemic cell change represent only the terminal events of this cascade and are thus not a good guide to determining when cell death may still be reversible. In vitro studies have distinguished *latent* and active or *execution* phases during the process of programmed or apoptotic cell death.[42] Whereas the active phase involves downstream factors that could induce DNA fragmentation and chromatin condensation within previously normal nuclei, the preceding latent phase is characterized by caspase activation (a large family of enzymes that mediate and amplify apoptosis)[43] confined to the cytoplasm, without downstream factors. These data suggest that activation of the downstream, intranuclear factors is the critical event that occurs at the transition from the latent to the execution phase of programmed neuronal death. Thus, in principle, it seems far more likely that intervention would be protective if applied during the initial, latent phase of programmed cell death rather than during the execution phase, even though the latter still precedes DNA fragmentation and cell death.[42]

Systematic in vivo studies support the central role of the latent phase. In the near-term fetal sheep, moderate hypothermia induced 90 minutes after reperfusion (i.e., in the early latent phase) and continued until 72 hours after ischemia prevented secondary cytotoxic edema and improved electroencephalographic recovery.[21] A concomitant, substantial reduction occurred in parasagittal cortical infarction, and neuronal loss scores improved in all regions. When the start of hypothermia was delayed in this paradigm until just before the onset of secondary seizures, 5.5 hours after reperfusion, only partial protection was seen (Figure 60-3).[44] With further delay (until after seizures were established 8.5 hours after reperfusion), no electrophysiologic or overall histologic protection occurred with cooling (see Figure 60-3).[45] Data from adult models are consistent with this concept. Thus 5 hours of moderate hypothermia (32.5° C) initiated under anesthesia in adult rats reduced selective neuronal loss when it was started up to 12 hours after brief global ischemia, although the degree and extent of neuroprotection markedly declined as the start of cooling was delayed beyond 2 hours.[46] In adult gerbils, when the delay before initiating a 24-hour period of cooling was increased from 1 to 4 hours after ischemia, neuroprotection in the CA1 field of the hippocampus after 6 months of recovery fell from 70% to 12%.[47] However, this deterioration could be largely overcome by extending the interval of moderate hypothermia (a reduction in body temperature of up to 5° C) to 48 hours or more, even when the start of cooling was delayed until 6 hours after reperfusion.[48,49]

Is Neuroprotection Maintained Long Term?

There have been reports that hypothermia may only delay, rather than prevent, neuronal degeneration after global ischemia in the adult rat[50-52] and after relatively mild hypoxia-ischemia in the 7-day-old rat.[53] The most likely explanation is that the duration or degree of hypothermia may have been inadequate, as suggested by the finding that 5° C of cooling for 6 hours[36] or 2° to 3° C for 72 hours in infant rats was associated with long-term improvement after carotid occlusion and hypoxia.[35] Subsequent studies both in the 7-day rat and in adult species have confirmed that a sufficiently prolonged phase of moderate cooling can be associated with persistent behavioral and histologic protection for many weeks and months.[36,48,49,54-57] Broadly, these studies tend to suggest that the later cooling is started, the more prolonged the treatment needs to be for neuroprotection.[58] An additional issue may have been rebound hyperthermia in the secondary phase. Even short periods of hyperthermia, 24 hours after either global or brief focal ischemia in the adult rat, exacerbated injury.[59,60] Similarly, in 10-day-old rat pups a 1.5° C increase in brain temperature for several hours during induced seizures after hypoxia-ischemia was associated with preventable exacerbation of neural injury up to 20 days later.[61] Further, when moderate hypothermia 2 to 9 hours after global ischemia in the rat was combined with prevention of spontaneous delayed pyrexia with antipyretics, histologic protection was seen after 2 months of recovery.[52] Each intervention alone had essentially short-term benefit only. Whether mild

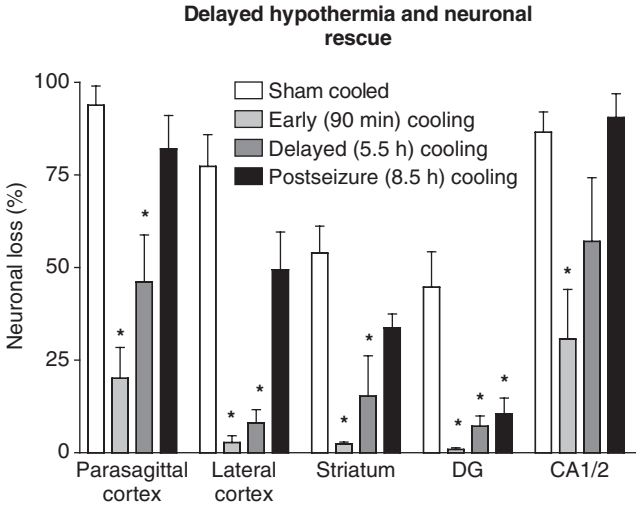


Figure 60-3 Comparison of the effect of cerebral cooling in the fetal sheep started at different times after reperfusion and continued until 72 hours on microscopically assessed neuronal loss in the neuronal regions of cortex after 5 days recovery from 30 minutes of cerebral ischemia. Compared with the sham-cooled group (n = 13), cooling that was started 90 minutes after reperfusion (n = 7) or just before the end of the latent phase (5.5 hours after reperfusion, n = 11) was protective, whereas cooling started shortly after the start of the secondary phase (8.5 hours after reperfusion, n = 5) was not. Only cooled fetuses in which the extradural temperature was successfully maintained at less than 34° C have been included. *DG,* Dentate gyrus. $^{*}p < .005$ compared with sham-cooled (control) fetuses, (Mann-Whitney *U* test; mean ± SEM). *(Data from Gunn AJ, Gunn TR, de Haan HH, et al: Dramatic neuronal rescue with prolonged selective head cooling after ischemia in fetal lambs,* J Clin Invest *99:248–256, 1997; Gunn AJ, Gunn TR, Gunning MI, et al: Neuroprotection with prolonged head cooling started before postischemic seizures in fetal sheep,* Pediatrics *102:1098–1106, 1998; Gunn AJ, Bennet L, Gunning MI, et al: Cerebral hypothermia is not neuroprotective when started after postischemic seizures in fetal sheep,* Pediatr Res *46:274–280, 1999.)*

hypothermia could have had additional benefit compared with normothermia in this late interval is, regrettably, unknown.

If Some Is Good, Is More Better?

A critical depth of cerebral hypothermia appears to exist between 32° and 34° C, that is required for effective neuronal rescue. In the fetal sheep cooled from 90 minutes after ischemia, substantial neuroprotection was seen only in fetuses that had a sustained fall in extradural temperature to less than 34° C (normal temperature in the fetal sheep is 39.5° C).[21] In the adult gerbil, cooling to a rectal temperature of 32° C was associated with greater behavioral and histologic neuroprotection than was 34° C.[56] Intriguingly, recent short-term recovery studies in the piglet suggest that the optimal depth of cooling may be greater for the cortex than for the basal ganglia.[40] Supporting this experimental observation, in a recent case series, head cooling but not whole-body cooling seemed to be associated with a reduction in the incidence of severe cortical lesions,[62] as examined by magnetic resonance imaging.

A trade-off is likely to occur between the adverse systemic effects of cooling, which increase below a core temperature of approximately 34° C,[8] and cerebral benefits. Although few specific perinatal data exist, in the adult dog, deep hypothermia (to a rectal temperature of 15° C) after cardiac arrest was detrimental, whereas mild hypothermia (34° to 36° C), from 10 minutes until 12 hours after cardiac arrest, was beneficial.[63]

Is It Possible to Cool the Head "Selectively"?

To provide adequate neuroprotection with minimal risk of systemic adverse effects in sick, unstable neonates, ideally only the brain would be cooled. Although this has been demonstrated experimentally by using cardiac bypass procedures,[64] it is clearly impractical in routine practice. More pragmatically, partially selective cerebral cooling can be obtained with use of a cooling cap applied to the scalp while the body is warmed by some method such as an overhead heater to limit the degree of systemic hypothermia.[65-67] In practice, mild systemic hypothermia is desirable during head cooling, first to limit the steepness of the intracerebral gradient that would otherwise be needed (avoiding excessively cold cap temperatures)[68] and second to provide greater cooling of the brain stem. This approach was demonstrated in studies in the piglet, with a substantial (median, 5.3° C) sustained decrease in deep intracerebral temperature at the level of the basal ganglia compared with the rectal temperature.[69,70] Similar results from studies of brief head cooling have been reported in the fetal sheep,[71] the young adult cat,[72] the newborn rat,[73] and the piglet.[74] Although direct temperature measurements are not feasible in asphyxiated newborns, head cooling has been shown to increase the gradient between nasopharyngeal and rectal temperature by nearly 1° C.[65]

In many ways, this approach is an extension of normal physiology. Even in the healthy neonate, no single cerebral temperature exists, but rather there is a gradient from the warmer deep regions to the cooler surface.[75] The brain is a major source of heat production, and it is cooled by a combination of surface radiation and blood flow convection.Thus the deep brain temperature is approximately 1° to 2° C higher than the scalp,[76] and it is 0.7° C higher than core body temperature.[75] Thus deep brain temperature is increased during reduced perfusion as occurs after severe asphyxia.[75]

MECHANISMS OF ACTION OF HYPOTHERMIA

The precise mechanisms of hypothermic neuroprotection are still unclear. Although this situation does not affect its pragmatic clinical use, it is critical to efforts to develop more effective strategies such as treatment in combination with other treatments.[77,78] Broadly, it is now well established that cooling suppresses many of the pathways leading to delayed cell death.

As well as reducing cellular metabolic demands,[79,80] hypothermia reduces excessive accumulation of cytotoxins such as glutamate and oxygen free radicals, suppresses the postischemic inflammatory reaction, and inhibits the intracellular pathways leading to programmed (i.e., apoptosis-like) cell death.

Cerebral Metabolism, Excitotoxins, and Free Radicals During the Primary and Reperfusion Phases

The combination of hypoxic depolarization and extracellular excitotoxin accumulation is a key factor in the initiation of neuronal injury in the primary phase. Hypothermia produces a graded reduction in cerebral metabolism of approximately 5% for every degree of temperature reduction[79] that delays the onset of anoxic cell depolarization.[81] However, the protective effects of hypothermia even in this phase are not simply the result of reduced metabolism, because cooling improves outcome even when the absolute duration of depolarization is controlled.[82] Cooling potently reduces postdepolarization release of numerous toxins including excitatory amino acids,[83] nitric oxide,[84,85] and other free radicals.[29,86] Similarly, cooling begun during reperfusion reduces levels of extracellular excitatory amino acids and nitric oxide production in the piglet.[30] However, these mechanisms are not active during the latent phase and thus cannot readily account for the protective effects of delayed cooling.

Intracellular Pathways in the Latent Phase

The effects of hypothermia on pathways distal to cell membrane ion channels are likely to be important. For example, intrainsult hypothermia did not prevent intracellular accumulation of calcium during cardiac arrest in vivo[87] or during glutamate exposure in vitro.[88] Indeed, evidence exists in the adult rat that the apparent neuroprotective effect of NBQX, a glutamate antagonist, administered from 1 hour after mild ischemia, was actually mediated by mild hypothermia.[51] In contrast, in vitro neuronal degeneration was prevented by cooling initiated after the excitotoxins had been washed out.[88] Thus the ability of hypothermia to reduce release of excitotoxins does not appear to be central to its postinsult neuroprotective effects; rather these data suggest that the critical effect of hypothermia is to block the intracellular consequences of excitotoxin exposure.

Suppression of Inflammatory Second Messengers

Brain injury leads to induction of the inflammatory cascade with increased release of cytokines and interleukins.[89] These compounds are believed to exacerbate delayed injury, either by direct neurotoxicity and induction of apoptosis or by promoting stimulation of capillary endothelial cell proinflammatory responses and leukocyte adhesion and infiltration into the ischemic brain.[90] Evidence is good that cooling can suppress this inflammatory reaction. In vitro, hypothermia potently inhibits proliferation, superoxide production, and nitric oxide production by microglia.[91] In the adult rat, hypothermia suppresses the posttraumatic release of interleukin-1β and accumulation of polymorphonuclear leukocytes.[92,93] Similarly, hypothermia reduces microglial activation after transient ischemia in the term-equivalent fetal sheep.[94] Thus these data suggest that the hypothermic protection against postischemic neuronal damage may be, in part, the result of suppression of microglial activation.

Does Hypothermia Specifically Prevent or Suppress Apoptosis (Programmed Cell Death)?

Some studies, particularly in adult species, show that hypothermia can prevent delayed necrotic cell death.[95] However, increasing data suggest that hypothermia has a particular role

in suppressing apoptotic processes, particularly in the developing brain. In neuronal culture, preconditioning using a brief period of hypoxia stimulated the expression of antiapoptotic gene products and regulatory components of the cell cycle; in contrast, induced hypothermia depressed cell activity and abolished hypoxia-associated protein synthesis.[96] Consistent with this, moderate hypothermia was associated with inhibition of apoptotic cell death and expression of apoptotic factors after cold-induced and ischemic brain injury in adult rats.[97,98] Similarly, in the piglet, hypothermia begun after severe hypoxia-ischemia reduced apoptotic cell death but not necrotic cell death,[38] and hypothermic neuroprotection in the near-term fetal sheep was closely linked with suppression of activated caspase-3.[94]

Summary

The experimental studies discussed earlier suggested that a prolonged duration of moderate cerebral hypothermia could improve long-term outcome if started as soon as possible, within approximately 6 hours of hypoxic-ischemic injury. On the basis of these extremely encouraging data, a number of clinical trials were undertaken.

CLINICAL TRIALS OF HYPOTHERMIA

Pilot Studies

A number of small controlled trials of head cooling with mild hypothermia[65,67,99,100] or whole-body cooling,[101] as well as case series using these techniques,[102-104] reported that cooling asphyxiated newborns was both feasible and apparently safe. Although none of these studies were powered to evaluate neurologic outcome, some suggestion was made of improved outcomes.[99,105,106] For example, in a controlled study of head cooling of infants with early stage II or III encephalopathy, mild systemic hypothermia was associated with a trend to reduced cerebral palsy in survivors (odds ratio 0.46 [0.08, 2.56] vs. normothermia).[99] A retrospective study of whole-body cooling to between 32° and 34° C in 10 infants found a significant reduction of major neurologic abnormalities and abnormal magnetic resonance imaging findings at follow-up compared with 11 historical controls.[105] A larger randomized pilot study of head cooling with mild hypothermia compared with normothermia found a significant reduction in neuron-specific enolase levels in cerebral spinal fluid with cooling, but only a marginal improvement in developmental outcome at 6 months of age.[106]

In the largest pilot study 65 infants were randomly assigned to normothermia or whole-body cooling to a rectal temperature of 33° C for 48 hours, initiated within 6 hours of birth. The relatively deep body cooling in this study was associated with some clinically manageable adverse effects, including longer dependence on inotropic agents, prolongation of prothrombin times, and lower platelet counts with more patients requiring plasma and platelet transfusions.[107] The combined outcome of death or severe motor scores was significantly lower in the hypothermia group (52%) than the normothermia group (84%) ($p < .02$).[108]

Large Randomized Controlled Trials

Two large multicenter randomized controlled trials of hypothermia for term infants with hypoxic-ischemic encephalopathy have been reported. In the CoolCap trial[109,110] infants with moderate to severe hypoxic-ischemic encephalopathy were randomly assigned to head cooling with mild systemic hypothermia, defined as a rectal temperature of 34° to 35° C (n = 116), or conventional care (n = 118). Death or disability at 18 months was reduced in infants with less severe electroencephalographic changes at trial entry (n = 172, odds ratio 0.42; 95% confidence interval 0.22-0.80, $p = .009$).[109] In contrast, however, no benefit was found in those with the combination of seizures and profound suppression of the amplitude integrated electroencephalogram (aEEG) recording before cooling was started. The improvement was primarily due to a reduction in motor disability with a more than 50% reduction in severe neuromotor disability in survivors and improved continuous Bayley Scale of Infant Development II scores, with no change in early neonatal mortality. The only consistent minor adverse effects were scalp edema under the cap, which resolved rapidly before or after removal of the cap; transient hyperglycemia from 4 to 24 hours compared with controls (mean ± SD, 7.6 ± 4.4 vs. 5.4 ± 3.1 mmol/L, at 4 hours, $p < .001$); and sinus bradycardia (which is a well-known, essentially normal response during hypothermia[111]) that did not require treatment. Conversely, an apparent reduction occurred in the incidence of elevated liver enzymes in the cooled group (38% of cooled infants vs. 53% of controls, $p = .02$).

In a further large multicenter trial of whole-body cooling from the National Institute of Child Health (NICHD), 208 were enrolled on the basis of clinical and laboratory criteria consistent with exposure to severe perinatal hypoxia plus moderate or severe hypoxic-ischemic encephalopathy.[112] Infants in the experimental group (n = 102) were cooled to a rectal temperature of 33.5° ± 0.5° C for 72 hours with use of a cooling blanket. The incidence of death and/or moderate-to-severe disability at 18 months of age was significantly reduced in the cooled infants (45%) compared with the normothermic group (62%; relative risk 0.72; 95% confidence interval, 0.55-0.93). No difference for death or disability alone was found.

Taken together, the remarkably similar effect sizes in two independent, well-controlled studies strongly suggest that induced hypothermia is beneficial. A recent meta-analysis of the CoolCap,[109] NICHD,[112] and Eicher[108] studies suggested that induced mild-to-moderate hypothermia is associated with a significant reduction (relative risk 0.76; 95% confidence interval, 0.65-0.89) in the incidence of death and disability after asphyxia.[113] However, as the authors emphasize, major differences exist between these studies including selection, method and duration of cooling, and outcome parameters.

Other Ongoing Trials

A number of trials are still in progress, including the Total Body Cooling trial (TOBY) in England, the NeoNetwork trial in central Europe, and the Infant Cooling Evaluation (ICE) trial in Western Australia. In a preliminary report Inder and colleagues[114] reported that whole-body–cooled infants had less cortical gray matter signal abnormality on magnetic resonance imaging (1 of 12 vs. 7 of 14 infants in the normothermic group; $p = .036$). Of interest, Rutherford and colleagues[62] have also reported a reduced incidence of severe cortical lesions in infants treated with head cooling. However, in that report, both head and whole-body cooling was associated with a decrease in basal ganglia and thalamic lesions that was significant in infants with moderate aEEG changes but not in those with severe aEEG findings.[62]

SYSTEMIC EFFECTS OF HYPOTHERMIA

Although the preceding studies have suggested that mild hypothermia is generally safe, they have also highlighted the clinical importance of understanding the physiologic changes associated with hypothermia.[8] In particular, a significant increase in blood pressure has been reported at initiation of cooling, both experimentally[44] and clinically.[102] This response is mediated by rapid peripheral vasoconstriction, that is, centralization of blood flow.[115] Further, hypothermia slows the atrial pacemaker and intracardiac conduction. Consequently, hypothermia to less than approximately 35.5° C is associated with mild but sustained sinus bradycardia; however, this has not required treatment.[67,101,109,112]

This relationship between heart rate and core temperature likely mainly reflects decreased metabolic demand with decreasing temperature. Some infants show markedly prolonged QT duration above the 98th percentile corrected for age and heart rate, without arrhythmia, during cooling, that resolves with rewarming.[116] Although such isolated prolonged QT does not seem to be associated with an increased risk of ventricular arrhythmias, other therapies that lengthen the QT interval (such as macrolide antibiotics) should be avoided.

One consistent metabolic effect associated with hypothermia was transient mild hyperglycemia, in both adult[117] and infant trials[109] but no increase in the rate of hypoglycemia. A similar transient rise in glucose concentrations has been observed in the piglet and near-term fetal sheep[21,118] and likely reflects hypothermia-induced catecholamine release.

It is also important to appreciate that although, for example, no increase occurred in the rate of complications such as infection in the newborn studies, this must in part reflect the routine screening and treatment for possible infection included in the trials.[109,112] Hypothermia has profound antiinflammatory effects and in older adults seems to increase the risk of infective complications such as pneumonia and bacteremia,[8,119] and thus this potential risk needs to continue to be carefully monitored in clinical use.

Although no increase in hemorrhagic complications was reported in either of the two large trials,[109,112] Eicher and colleagues[107] did find an increase. It is unclear at this time whether this difference was the result of deeper cooling or a rectal temperature of 33° C,[107] or was a chance finding in a small study. It is reassuring that in piglets where the cortex was cooled to less than 30° C no hemorrhagic changes were seen in the brain.[39,120] Finally, it is important to note that all the clinical studies have been in term infants. It is unknown whether, in the setting of modern intensive care, selected preterm infants with evidence of acute metabolic acidosis on cord blood and clinical encephalopathy would also benefit from hypothermia.[121] Studies in preterm fetal sheep, at an equivalent stage of neural maturation, now suggest that head cooling, started 90 minutes after profound asphyxia, can reduce neuronal loss in the striatum.[71]

CONCLUSION

Experimental evidence is now overwhelming that mild to moderate postasphyxial cerebral cooling can be associated with long-term neuroprotection. The key requirements are that hypothermia be initiated as soon as possible in the latent phase, within the first 6 hours, before secondary deterioration, and that it be continued for a sufficient period in relation to the evolution of delayed encephalopathic processes, typically 48 hours or more. These findings are now supported by large randomized trials of both head cooling combined with mild systemic hypothermia and whole-body cooling that found that cooling is safe, at least in the intensive care environment, and associated with a significant reduction in the incidence of death and severe disability after asphyxia. These studies show that although hypothermia is an important advance, as currently applied it is not a "magic bullet." Only approximately 15% have better outcome after cooling compared with standard care.

To further improve this outcome, it is vital to find ways of optimizing methods of applying hypothermia and of selecting infants who are most likely to benefit from treatment.[13] For example, increasing evidence exists that hypothermia can augment the effects of experimental neuroprotective therapies.[77,122] Further, the CoolCap trial suggested that EEG monitoring could identify a subgroup of infants with profound suppression of amplitude and onset of seizures at the time of randomization who did not respond.[109] It will be vital to refine these initial findings in focused experimental and clinical studies.

Thus, although many questions remain over how hypothermia should be best used and outcomes to school age or later are not yet known, we can now be cautiously optimistic that therapeutic hypothermia is here to stay and that greater understanding of the determinants of protection with ongoing studies will yield further improvements in outcome.

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SECTION X

Skin

61

David H. Chu and Cynthia A. Loomis

Structure and Development of the Skin and Cutaneous Appendages

OVERVIEW

Skin is a complex organ that comprises many different cells and cell types; it forms a critical physical barrier that protects the body and maintains fluid homeostasis, temperature regulation, and sensation. Skin cells derive from both embryonic mesoderm and ectoderm, and development from these precursors is tightly regulated. Perturbations in the developmental process, either from genetic anomalies or as a result of exogenous (e.g., teratogenic) agents, can result in severe abnormalities that have important consequences in the care of infants. Understanding the normal progression of molecular and cellular events that underlie the development and differentiation of skin allows for a more rational approach to infants who have defects in these processes. For clinicians, this knowledge will direct the diagnosis, therapy, and parental counseling necessary for care of the patients.

As with all organs, organogenesis of the skin proceeds through three distinct but overlapping stages, from early embryonic through fetal and neonatal development.[1,2] These stages are as follows: *specification,* in which portions of superficial ectoderm and lateral plate mesoderm become distinct from other portions of the body wall; *morphogenesis,* in which the specific structural and biochemical characteristics of skin begin to appear; and *differentiation,* in which the skin tissue further develops to its mature form.

For the sake of clarity, we have organized the discussion of the developmental progression of the skin to follow each component sequentially, first with a discussion of the epidermis, followed by the dermis and subcutaneous tissue, the dermal-epidermal junction (DEJ), and, finally, epidermal appendages. Each of these sections discusses the structural and biochemical changes that occur during the particular stage of development, followed by a discussion of related clinical syndromes and genetic disorders that are related to defects in this developmental progression.[3] However, all these tissues of the skin are, in fact, developing in parallel, and in some cases they require interaction with adjacent tissues for development. A timeline is included to illustrate the sequence of events that are occurring simultaneously (Figure 61-1).

EPIDERMIS

Overview

The mature adult *epidermis* is a stratified squamous epithelium that develops from the ectoderm. Keratinocytes form 80% of the cellular composition. The germinative keratinocytes reside in the deepest portion of the epidermis, known as the *basal layer*, and these cells are known as *basal cells.* As differentiation of the basal cells proceeds, these cells migrate to more superficial cell layers and become progressively flattened; they also begin to express large insoluble proteins that ultimately become crosslinked along the exterior of the cell to form an insoluble shell or brick, known as the *cornified cell envelope*.

Above the basal cell layer rests the spinous layer, or stratum spinosum. The "spines" seen in this layer result from the abundance of desmosomes, specialized regions of the keratinocyte cell surface that promote adhesion between these cells in a calcium-dependent manner. Desomosomal proteins include plakoglobin, desmoplakins I and II, keratocalmin, desmoyokin, band 6 protein, and the cadherins—desmogleins 1 and 3 and desmocollins 1 and 2.[4]

The cell layer superficial to the stratum spinosum is the stratum granulosum. Granular cells begin to express some of the components that will contribute to the cornified cell envelope, a protein-lipid polymer that is found on the outer boundary of terminally differentiated keratinocytes.[5] The cornified cell envelope serves a critical role in the barrier function of the epidermis. Keratohyaline granules, found within the granular cells, are principally composed of two proteins, loricrin and profilaggrin. Profilaggrin undergoes sequential proteolytic cleavage into filaggrin oligomers and finally monomers, as well as desphosphorylation, during its processing. This process is initiated at the time of formation of the granular layer and continues even after its eventual extrusion from the cornified cell. Loricrin, another major component of the cornified envelope, is also initially localized to the keratohyaline granules. Lamellar granules, which are also abundant in the granular cell layer, contain the lipid components that will be extruded from the cells and crosslinked to the cornified cell envelope. Other proteins that contribute to the cornified envelope include involucrin, small proline-rich proteins, annexin, elafin, desmoplakin, envoplakin, periplakin, repetin, and trichohyalin. Modifying enzymes such as transglutaminases are important in the final crosslinking of the cornified envelope components. As described in another section, mutations in either the structural proteins or the enzymes involved in protein crosslinking and lipid and steroid metabolism can have clinically significant outcomes in genetic skin disease.

The stratum corneum contains the terminally differentiated keratinocytes, flattened dead "squames" that lack nuclei and other organelles. These cells are composed primarily of keratin filaments, tightly packed within the crosslinked cornified envelopes. Some specialized components within the epidermis maintain its architecture. Keratins are some of the most important structural proteins within the epidermal cells. They assemble as filaments composed of "basic" and "acidic" keratin peptides, which form *obligate heterodimers*; that is, different keratins are found to associate with a limited number of partners.[6,7] Their expression is regulated in a tissue-specific and developmentally selective manner. Mutations in these genes often exhibit clinical

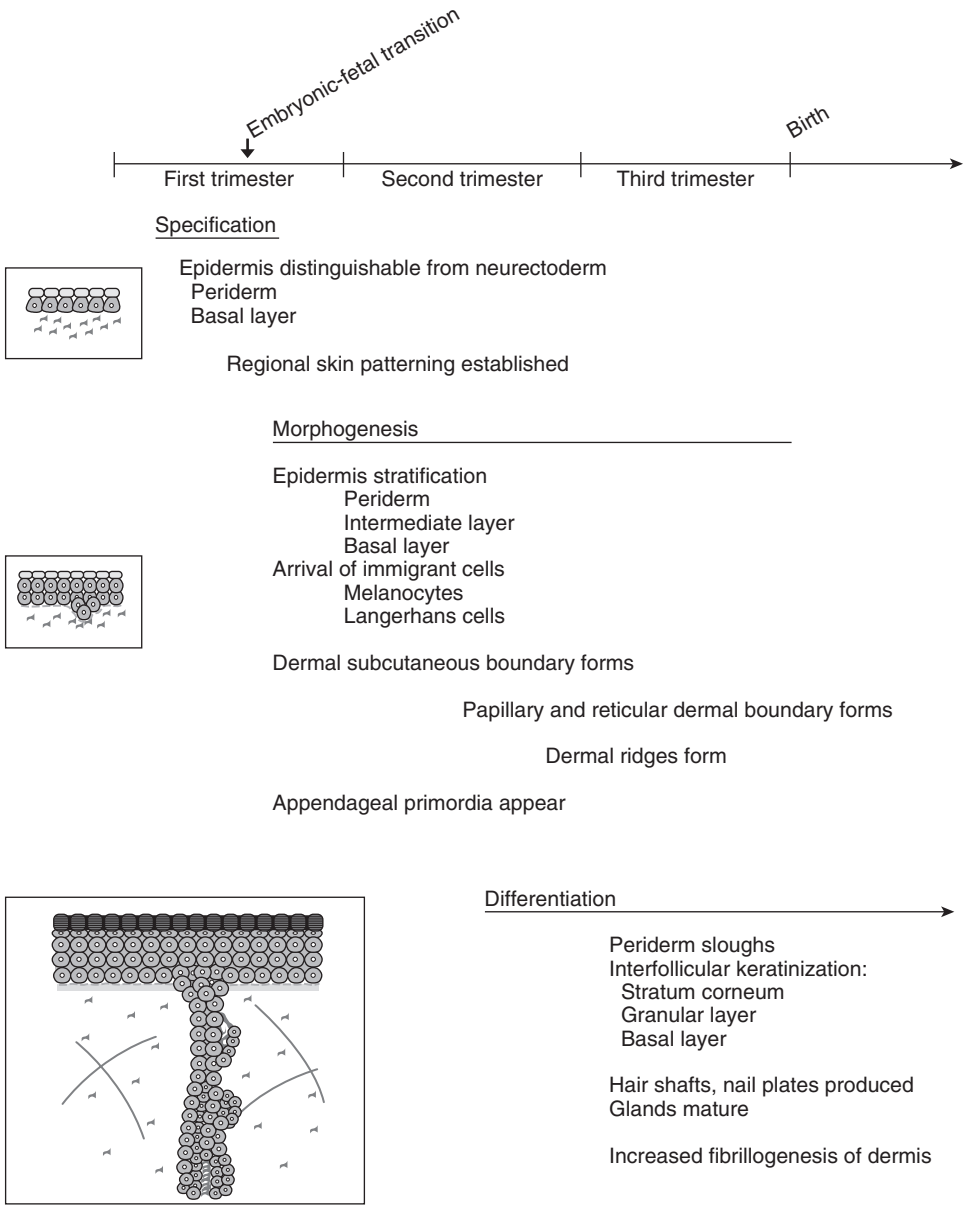


Figure 61-1 Timeline for the development of skin tissue. *(Adapted from Loomis CA, Koss T: Embryology. In Bolognia JL, Jorizzo JL, Rapini RP [eds]:* Dermatology. *Philadelphia, Elsevier, 2003.)*

phenotypes that reflect the tissue expression of the specific keratin gene. For example, whereas basal cells express K5 and K14, suprabasilar keratinocytes express the genes encoding K1 and K10 (K1 and K9 in palmoplantar epidermis).

Other critical structural elements of the epidermis are the cell-cell and cell-matrix adhesion systems. The major cell-cell adhesion junctions of the epidermis are desmosomes. The proteins in these complexes include specialized adhesion proteins (cadherins, calcium-dependent adhesion molecules) as well as intracellular plaque and adaptor proteins. An analogous adhesion structure, the hemidesmosome, attaches basal cells to the basement membrane at the DEJ. Although the proteins in the hemidesmosome are distinct from those of the desmosome, the plaque proteins have similar amino acid sequences, and both are tightly associated with the keratin filament network. Gap junctions form important intercellular bridges that allow small molecules to pass from one keratinocyte to another. These specialized junctions are formed by proteins known as connexins. Mutations in any of these molecules have effects on the normal formation of the epidermis that can result in certain genodermatoses, many of which have manifestations from birth.

Specification

Gastrulation of the embryo occurs during the third week after fertilization. This is a critical process that results in the generation of the three primary embryonic germ layers: endoderm, mesoderm, and ectoderm. The ectoderm then is further divided into the neuroectoderm and the presumptive epidermis. The epidermis is then subsequently specified into distinct regional domains, such as palmoplantar skin, scalp skin, and mammary skin.

The earliest presumptive epidermis consists of a basal cell layer, which covers the embryo.[8,9] By 6 weeks estimated gestational age (EGA), the surface ectoderm consists of two layers: basal cells and more superficial periderm cells. The periderm layer does not give rise to any portion of the definitive

epidermis, and as such it serves as a transient embryonic covering. This layer is ultimately sloughed during late gestation and contributes to the vernix caseosa, which covers the newborn.

Morphogenesis

The process of *morphogenesis* begins at approximately 8 weeks EGA, the classic transition between embryonic and fetal development, when hemopoietic production shifts to the bone marrow. At this point, the epidermis begins its process of stratification, by forming an intermediate layer between the basal cell and the periderm layers. This intermediate layer remains highly proliferative, and more layers are added as development proceeds over the next several weeks. In mice, it has been shown that this process of epidermal stratification requires the *p63* and *Dlx3* genes.[10-12] In humans, partial loss of function mutations in *p63* have been seen in various ectodermal dysplasias, as discussed in another section.

Differentiation

Maturation of the epidermal layers includes keratinization, which results in the *differentiation* of granular and stratum corneal layers and the formation of a water-impermeable barrier. Accompanying this stage of development is the sloughing of the periderm. Keratinization occurs first in the skin appendages between 11 and 15 weeks EGA, followed by the interfollicular epidermis between 22 and 24 weeks EGA.[13,14]

The process of keratinization involves the production of certain proteins, including filaggrin and loricrin. Posttranslational modification of the crosslinked proteins and production of specialized lipid and steroid components contribute to the water-impermeable matrix formed in the mature cornified layer. Structurally, the third trimester stratum corneum is similar to that of an adult, but functional studies have shown that it is much less effective at preventing water loss and is more permeable than the mature epidermis. It is actually not until the third week of life that the barrier function of a newborn's stratum corneum is comparable to that of an adult. Emollient use is of therapeutic benefit in many patients with defective epidermal barriers because the stratum corneum is insufficiently differentiated in very-low-birth-weight preterm infants.[15]

Clinical Relevance

Human mutations that inhibit the process of specification in the epidermis have not been reported, most likely because such mutations would be incompatible with further development of the embryo. However, experiments in animals have suggested that a group of proteins, the bone morphogenetic proteins (BMPs), as well as the Engrailed-1 transcription factor, may play an important early role in these processes.[16-18] Similarly, few mutations in humans have been found that affect epidermal morphogenesis although, as mentioned earlier, *p63* and *Dlx3* have been implicated in the process of epidermal stratification based on studies in mouse models.

In contrast to the relative lack of mutations that affect epidermal specification and morphogenesis, mutations that have an important impact on epidermal differentiation are quite numerous. In general, these genes are not required for development in utero, but they become critical for effective barrier function of the epidermis after birth. Thus defects in these genes can often cause significant postnatal morbidity.

Two groups of such diseases include the palmoplantar keratodermas and ichthyoses. The *palmoplantar keratodermas* are a diverse group of disorders that share a common presentation of hyperkeratosis of the palms and soles. These disorders commonly are also associated with other cutaneous as well as noncutaneous findings. The genetic bases of many of these disorders have been discovered, and they include defects in connexins, differentiation keratins, and desmosomal components.[19]

TABLE 61-1

Summary of Ichthyoses

Disease	Inheritance	Defective Protein(s)
Ichthyosis vulgaris	AD	Filaggrin
X-linked ichthyosis	XLR	Steroid sulfatase
Lamellar ichthyosis	AR	Transglutaminase 1
NBCIE	AR	Transglutaminase 1; ALOX12B;ALOXE3; CGI58
Bullous congenital ichthyosiform erythroderma	AD	Keratin 1; Keratin 10
Harlequin fetus	AR	ABCA12
Sjögren-Larsson syndrome	AR	Fatty aldehyde dehydrogenase
Refsum syndrome	AR	Phytanoyl-CoA Hydroxylase
Conradi-Hunermann syndrome	XLD	Emopamil binding protein
CHILD syndrome	XLD	NSDHL (NAD(P)H steroid dehydrogenase-like protein)
Netherton syndrome	AR	LEKTI (lymphoepithelial Kazal-type–related inhibitor)
Erythrokeratodermia variabilis	AD	Connexin 31; Connexin 30.3
KID syndrome	AD	Connexin 26

AD, Autosomal dominant; *AR,* autosomal recessive; *CHILD,* congenital hemidysplasia with ichthyosiform erythroderma and limb defects; *KID,* keratitis-ichthyosis-deafness; *NBCIE,* nonbullous congenital ichthyosiform erythroderma; *XLD,* X-linked dominant; *XLR,* X-linked recessive.

The *ichthyoses* are another diverse group of diseases that often present around the time of birth with diffuse scaly skin and defective epidermal barrier function.[20,21] These conditions differ in both pattern and morphology of scaling as well as extracutaneous features. Ichthyosis vulgaris, lamellar ichthyosis, and X-linked ichthyosis have phenotypes predominantly restricted to the skin (Table 61-1). Ichthyoses can also be a cause of erythroderma (e.g., in bullous and nonbullous congenital ichthyosiform erythroderma). Neurologic abnormalities can be associated with the skin condition, as in Sjögren-Larsson syndrome, Refsum syndrome, or Tay syndrome (IBIDS: ichthyosis, brittle hair, intellectual deficit, decreased fertility, and short stature). In X-linked dominant ichthyoses, female children inheriting the relevant mutation can develop asymmetry of limbs, as in congenital hemidysplasia with ichthyosiform erythroderma and limb defects (CHILD) syndrome or chondrodysplasia punctata (Conradi-Hünermann disease). Interestingly, certain mutations in the gene encoding filaggrin, a key component of the cornified envelope and the gene mutated in ichthyosis vulgaris,[22] also confer susceptibility to development of atopic dermatitis, a common inflammatory skin disease of childhood.[23]

One of the earliest phenotypic presentations of a group of these disorders is known as *collodion baby,* in which an infant is born encased in a parchment-like membrane. Over the next few days to weeks, the membrane is shed, eventuating in lamellar ichthyosis, nonbullous congenital ichthyosiform erythroderma, Netherton syndrome, Tay syndrome (IBIDS), Conradi-Hünermann disease, and occasionally, a normal baby.[3,20]

In contrast to the relatively benign outcome for collodion babies, the so-called *harlequin fetus* usually dies soon after birth. These babies are born encased in restrictive plates of thick, armor-like scale that have extremely poor barrier function, often resulting in fluid derangements, infections, sepsis, and death. Mutations in the gene ABCA12, which encodes an adenosine-5′-triphosphate (ATP) binding cassette transporter, have recently been discovered to be responsible for this condition.[24, 25]

Nonkeratinocytes in the Epidermis

Melanocytes

Although keratinocytes are the most abundant cell type that makes up the epidermis, other cell types migrate there early in development. In these are the melanocytes, neural crest-derived dendritic cells that synthesize and distribute pigment to the epidermis. Melanocyte precursors exit the dorsal region of the neural tube, migrate through the superficial mesenchyme, and ultimately come to reside in the epidermis by 7 weeks EGA.

Langerhans Cells

Langerhans cells are another migratory cell population that enters the epidermis before the embryonic-fetal transition. They are bone marrow-derived cells that, when mature, are important for antigen presentation within the skin. They remain relatively undifferentiated until the embryonic-fetal transition, at which time they begin to express CD1 on their cell surface and begin to produce their characteristic Birbeck granules, tennis racket-shaped intracellular vesicles that form during the processing of membrane-bound antigens. The density of Langerhans cells remains low until the third trimester, when it increases to those of a typical adult.

Merkel Cells

The third type of specialized cell within the epidermis is the Merkel cell, a highly innervated neuroendocrine cell type involved in mechanoreception. These cells are first detectable around 8 to 12 weeks in volar skin and slightly later in interfollicular skin. They are often associated with skin appendages. Merkel cells express both neural-specific markers and epithelial-specific proteins, such as keratins.

Clinical Relevance

Many mutations have been identified that are critical for melanoblast migration and survival and for melanocyte differentiation and function (summarized in Table 61-2). Defects in these genes result in disorders of pigmentation, which can be detected at an early age.

One of the most common abnormalities is that of albinism. There are variations in severity of depigmentation as well as associated abnormalities. These differences depend on the particular gene affected and its global function.

Defects in melanin synthesis result in oculocutaneous albinism (OCA), a set of disorders characterized by absent pigment in the skin, hair, and eyes at birth. Often associated with the skin findings are neurologic and ophthalmologic findings of nystagmus, photophobia, and visual disturbances, reflecting the neuroectodermal derivation of melanocytes. In OCA type 1, sequence alterations in the enzyme tyrosinase, critical for the conversion of tyrosine to melanin (either null or partial loss of function alleles), are responsible. In OCA type 2, tyrosinase functions normally (tyrosinase-positive albinism), but the P gene, a putative tyrosine transporter, is defective. This subtype can be distinguished from OCA type 1 because a child's skin and eyes begin to darken with age. The children usually have white, yellow, or red hair. OCA type 3 results from defects in tyrosinase-related protein-1. This condition is also known as *brown OCA*, featuring minimal to light brown skin pigmentation in some persons of African descent.[26]

Other genes result in a more patchy distribution of pigmentation defects, as in the white forelock (poliosis) seen in both piebaldism and Waardenburg syndrome. These diseases occur as a result of defective melanocyte specification, migration, or survival. The gene responsible for piebaldism encodes c-KIT, a growth factor receptor and protooncogene.[27] Waardenburg syndrome subtypes can be caused by defects in transcription factors Pax3, Sox10, MITF and in endothelin receptor B and endothelin 3 ligand. In Waardenburg syndrome, depending on the subtype, neural tube defects, heterochromia iridis, deafness, and Hirschsprung disease may be associated with depigmentation.[28]

Finally, defects in melanosome production result in the entities Hermansky-Pudlak, Chédiak-Higashi, and Griscelli syndromes. Patients with Hermansky-Pudlak syndrome have mutations in the HPS1 gene, which encodes a putative transmembrane component of a number of cytoplasmic organelles, or the AP3B1 gene, which is a protein subunit involved in the sorting of proteins in the exocytic/endocytic pathway. Patients present with creamy, light skin tones, dysfunctional platelets (prolonged bleeding time), pulmonary fibrosis, granulomatous colitis, and ceroid lipofuscinosis in phagocytic cells.[29] Chédiak-Higashi syndrome is characterized by nystagmus, decreased visual acuity, skin and respiratory infections, light skin tones, and silvery yellow to brown hair. Giant melanosomes are found in white blood cell smears and hair, and giant lysosomal inclusion granules are found in all leukocytes. The defect is in LYST, which encodes a protein proposed to be important in intercellular protein trafficking.[30] Griscelli syndrome also features hair shafts with large pigment granules and hypopigmentation with silver-gray hair. Patients have hemophagocytic syndrome and experience episodes of massive lymphocyte and leukocyte activation and organ infiltration. Polymorphonuclear leukocytes are morphologically normal, unlike in Chédiak-Higashi syndrome. The defect is in a guanosine triphosphate–binding protein involved in vesicular fusion and trafficking, RAB27A.[31]

TABLE 61-2

Disorders of Pigmentation

Disorder	Mutant Gene	Defective Protein/Function
Melanocyte specification, migration, or survival		
Piebaldism	KIT	Growth factor receptor, proto-oncogene
Waardenburg syndrome		
Types 1 and 3	PAX3	Pax3 transcription factor
Type 2	MITF	Transcription factor
Type 4	EDNRB	Endothelin receptor B
	EDN3	Endothelin 3
	SOX10	Sox-10 transcription factor
Melanin biosynthesis		
Oculocutaneous albinism		
Type 1	TYR, null alleles	Rate-limiting enzyme of pigment production
Type 1b	TYR, partially functional alleles	Rate-limiting enzyme of pigment production
Type 2	P gene	Putative tyrosine transporter
Type 3	TYRP1	Tyrosinase-related protein-1
Melanosome production		
Hermansky-Pudlak syndrome	*HPS1*	Putative membrane component of various organelles
	AP3B1	Putative component of endocytic protein trafficking pathway
Chédiak-Higashi syndrome	LYST	Putative involvement in protein trafficking
Griscelli syndrome	RAB27A	Guanosine triphosphate–binding protein involved in membrane fusion and protein trafficking

DERMIS AND SUBCUTIS

Overview

Unlike the epidermis, which is derived exclusively from ectoderm, the *dermis* originates from different tissues depending on the specific body site. Dermal mesenchyme of the face and anterior scalp comes from neural crest ectoderm, whereas that of the back is derived from the dermomyotome of the embryonic somite. Dermal mesenchyme of the limbs and ventral trunk, conversely, is likely derived from the lateral plate mesoderm.

Early fetal mesenchyme is highly cellular, containing few fibrillar elements. These fetal mesenchymal cells are thought to be pluripotent, able to give rise to adipose tissue, cartilage, and dermal fibroblasts. The superficial mesenchyme becomes visibly distinct from underlying skeletal elements by 60 days EGA. By 12 to 15 weeks, the different characteristics of the dermis can first be observed—the papillary dermis has a finer weave than the thicker reticular dermis. Collagen and elastic fibers begin to be assembled in the latter half of pregnancy, and a more rigid, fibrillar meshwork gradually forms.

Dermal repair shifts from nonscarring to scarring by the end of the second trimester. At birth, the dermis is thick and well organized but is still more cellular than in adults.

A distinct region identifiable as the subcutis can be seen by 50 to 60 days EGA. It is separated from the dermis by a plane of thin-walled vessels. By the end of the first trimester, the distinction between the fibrous dermis and the sparse matrix of the subcutis can be seen. Preadipocytes arising from the undifferentiated mesenchyme begin to mature and accumulate lipids by the second trimester. By the third trimester, the more differentiated adipocytes begin to aggregate into lobules separated by fibrous septa. Although the initial events leading to the commitment of mesenchymal cells to become adipocytes are not well understood, numerous regulators of later preadipocyte-adipocyte differentiation have been identified, including the hormone leptin.[32]

Blood Vessels, Lymphatics, and Nerves

Blood vessels and lymphatics begin to develop early in gestation but do not evolve into those of adults until a few months after birth. Initially, these vessels form horizontal plexuses within the subpapillary and deep reticular dermis, which are interconnected by groups of vertical vessels. This vascular framework has been shown to be in place by 45 to 50 days EGA. By the fifth month of EGA, arterioles and venules are able to be distinguished. Capillary loops, which supply the developing epidermal appendages and maintain thermoregulation, are established at the time of rete ridge formation at the DEJ. During the period of embryonic and fetal development, this framework changes constantly, depending on many factors, including site of the body and presence of associated adnexal structures.

Accumulating evidence suggests that lymphatics originate from endothelial cells that bud from veins, and the pattern of embryonic lymphatic vessel development parallels that of blood vessels. Studies using newly discovered molecular markers specific for lymphatics have allowed a more detailed understanding of lymphatogenesis. As described in another section, defects in some of the genes encoding some of these proteins have been found to result in congenital disorders of the lymphatic system.

Nerves, like blood vessels and lymphatics, are also present in early embryonic skin and continue to develop after birth. Nerves begin as large trunks at the dermal-subcutaneous boundary that branch into fibers and extend into the dermis. They form networks distinct from the cutaneous vasculature, although they become more closely juxtaposed in the fetal dermis. At 70 days EGA, an arrangement of nerves that is similar to the adult configuration can be detected, although the density and final distribution of fibers continue to be modified until after birth. Sensory receptors such as the pacinian and meissnerian corpuscles begin to develop in the fourth month of EGA. Although meissnerian corpuscles are fully developed only after birth, pacinian corpuscles are completed and are found in plantar and palmar skin in neonates.

Clinical Relevance

Few human mutations have been identified that result in defective dermal specification and differentiation, most likely because such mutations would be incompatible with life. However, experiments in animal model systems have suggested that the genes *Lmx1b*, *Wnt7a*, and *En1* may be important in specifying dorsal and ventral (palmoplantar) mesenchyme as distinct tissues.

Restrictive dermopathy is a clinical entity in which the dermis is universally abnormal, now found to be caused by mutations in LMNA and ZMPSTE24.[33] Some mosaic conditions also show localized defects in dermal development, most notably focal dermal hypoplasia (Goltz syndrome), as well as Proteus syndrome. In Goltz syndrome, dermal hypoplasia exists in Blaschkoid patterns.[34] Proteus syndrome is characterized by subcutaneous masses, lipomas, capillary malformations, plantar and palmar hyperplasia, varicose veins, and asymmetric soft tissue and bony hypertrophy of the hands, feet, and limbs.[35]

Connective tissue disorders are caused by mutations in genes encoding differentiation products of mature dermal fibroblasts and other mesenchymal cells. Ehlers-Danlos syndrome has many clinical subtypes, caused by a variety of mutations in genes affecting collagen types I, III, and V, collagen-modifying enzymes, and other important structural proteins (Table 61-3).[36,37] Mutations in the encoding elastin can result in cutis laxa, characterized by loose, redundant skin, a syndrome that can be complicated by lung hypoplasia, diaphragmatic hernias, gastrointestinal diverticula, bladder diverticula, and emphysema.[38] Osteogenesis imperfecta, characterized by multiple fractures in utero, beaded ribs, crumpled humeri and femora, limb avulsion during delivery, kyphoscoliosis, blue sclerae, and easily bruisable skin, has been found to result from defects in collagen type I.[39] Marfan syndrome results from mutations in fibrillin, which codes for a vital component of the microfibrillar elastic tissue network, resulting in defects in ocular, cardiovascular, and musculoskeletal systems.[40]

Defects in angiogenesis have also been identified. Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome) is a vascular dysplasia that leads to telangiectases and arteriovenous malformations of skin, mucosa, and viscera. Mucosal involvement often results in epistaxis and gastrointestinal bleeding. Visceral involvement includes that of the lung, liver, and brain. Mutations in endgolin, as well as activin receptor-like kinase 1 have been shown to cause this disease.[41,42] TIE2 is a tyrosine kinase, mutations in which have been shown to result in a dominantly inherited syndrome of venous malformations.[43,44]

Work on lymphangiogenesis has uncovered genetic mutations in some forms of hereditary lymphedema. The most common form of primary hereditary lymphedema, Milroy disease, is caused by mutations in vascular endothelial growth factor receptor 3. This disease is characterized by severe edema, especially below the waist.[45] Mutations in FOXC2, a transcription factor, have been implicated in lymphedema-distichiasis syndrome, featuring late-onset lymphedema and a double row of eyelashes (distichiasis).[46]

DERMAL-EPIDERMAL JUNCTION

The DEJ is a key interface for inductive interactions during early skin organogenesis. Defects in this region are also responsible for many well-studied congenital blistering diseases, the group

TABLE 61-3

Ehlers-Danlos Syndrome Classification

Type	Clinical Findings	Inheritance	Gene Defect(s)
Classic (I/II)	Skin and joint hypermobility, atrophic scars, easy bruising	AD	COL5A1, COL5A2
Hypermobility (III)	Joint hypermobility, pain, dislocations	AD	COL3A1, TNXB
Vascular (IV)	Thin skin, arterial or uterine rupture, easy bruising, small joint hyperextensibility	AD	COL3A1
Kyphoscoliosis (VI)	Scoliosis, joint laxity, ocular fragility and retinal detachment, skin laxity, muscle hypotonia	AR	PLOD1
Arthrochalasia (VIIA, VIIB)	Joint hypermobility, mild skin laxity, bruising, scoliosis	AD	COL1A1, COL1A2
Dermatosparaxis (VIIC)	Severe skin fragility, cutis laxa, bruising	AR	ADAMTS2 (procollagen N-peptidase)
Other			
X-linked EDS (V)	Skin laxity, bruising	XL	Lysyl oxidase
Periodontitis (VIII)	Periodontitis, blue sclerae, atrophic scarring, bruisability	AD	?
Fibronectin-deficient (X)	Striae distensae, joint laxity, skin laxity, scarring, bruisability, bleeding (platelet) disorder (petechiae)	?	?
Familial hypermobility (XI)	Recurrent joint dislocation, joint laxity	AD	?
Progeroid	Progeroid facies, short stature, osteopenia, skin laxity, atrophic scarring, hypermobile joints	?	B4GALT7

AD, Autosomal dominant; *AR,* autosomal recessive; *EDS,* Ehlers-Danlos syndrome; *XL,* X-linked.

of diseases known as epidermolysis bullosa (EB). This zone includes the adhesive structural elements along the basal surface of the basal keratinocyte plasma membrane, as well as the extracellular matrix proteins of the basal lamina and the most superficial fibrillar structures of the papillary dermis.

The most primitive form of the DEJ can be seen between the epidermis and the dermis by 8 weeks EGA. Laminin, collagen type IV, heparan sulfate, and proteoglycans, components common to all basal lamina, can be found at this stage. The more specific molecules that make up the basal lamina of stratified epithelia, such as hemidesmosomal components, begin to be detected at the time when the presumptive epidermis begins to stratify. It is at this time that such proteins as integrins (α6 and β4) become properly localized to the DEJ, even though the expression of some can be detected at earlier times.

Clinical Relevance

As alluded to earlier, several congenital disorders characterized by severe blistering occur as a result of mutations in the genes encoding the components of this region (Table 61-4).[47] The severity of the disorder, the plane of tissue separation, and the involvement of noncutaneous tissues depend on the specific proteins affected by the mutations. These diseases carry with them a high degree of morbidity and mortality, and as such they are frequent candidates for prenatal testing.

TABLE 61-4

Disorders of the Dermal-Epidermal Junction

Location	Disorder	Defective Protein(s)
Basal cell adhesion complex	EB simplex	K5, K14
	EB with muscular dystrophy	Plectin
Basal lamina	Generalized atrophic benign EB	BPAG2 (collagen type XVII)
	EB with pyloric atresia	β4 subunit of α6 or β4 integrin subunits
	Junctional EB, lethal and less severe forms	Laminin 5 subunits
Superficial dermis	Dystrophic EB (dominant and recessive)	Collagen type VII

EB, Epidermolysis bullosa.

The most superficial of these phenotypes is EB simplex. Keratins 5 and 14, the proteins defective in this disease subtype, are expressed by basal keratinocytes. Because of loss of adhesion at the level of the basal keratinocyte, the epidermis is fragile, and bullae develop. Depending on the particular mutation, the blistering phenotype can be more or less severe. These blisters can involve the mucous membranes and can result in a hoarse voice. One specific subtype of EB simplex is associated with muscular dystrophy. The mutation for this disease is within a protein called plectin, involved in adhesion within the hemidesmosome.

Junctional EB variants form bullae within the lamina lucida, the superficial portion of the DEJ. Affected patients have been found to have defects in laminin 5. One form of junctional EB, the Herlitz variant, is more severe: In addition to blistering, laryngeal and respiratory edema can be associated with the disorder, as well as anemia and hypoproteinemia. The non-Herlitz variant is less life-threatening, although, in this case, bullae heal with atrophic scarring. Nails and hair can also be affected. Another subtype is associated with pyloric atresia, which is caused by mutations in the genes encoding α6 and β4 integrins.

The most disfiguring form of EB is termed dystrophic EB. Because the blisters in this variant affect the superficial dermis, with defects in collagen type VII, they heal with scarring. In the more severe forms, extensive scarring can lead to digital fusions and so-called mitten deformities of the hands and feet, flexion contractures, mucous membrane scarring, dental abnormalities, and hematologic disorders.

SKIN APPENDAGES

Skin appendages include hair, nails, and sweat and mammary glands. These structures are derived from both an epidermal component and a dermal component. The epidermis contributes the physical structure of the unit, whereas the dermis provides signals for the unit's differentiation. During embryogenesis, interactions between the dermis and the epidermis are critical for normal development of these structures, disruption of which often results in defects in the formation of skin appendages.

Hair

Between 75 and 80 days EGA, dermal signals instruct the basal cells of the scalp epidermis to cluster together at regularly spaced intervals. This initial group is known as the follicular placode or anlage.[48] Although the specific mediators of this first "dermal signal" are not definitively identified, CTNNB1 (encodes β-catenin) has been implicated as a candidate gene based on its molecular localization.

From the scalp, the follicular placodes develop ventrally and caudally and eventually cover the skin. The epidermal placodes then signal back to the underlying dermis to form a dermal condensate, which occurs at 12 to 14 weeks EGA. The communication between the epidermis and dermis at this stage and the morphogenetic development are considered to be a balance between placode promoters and placode inhibitors.[48] Wnt family signaling molecules are proposed to mediate placode promoting effects via the molecules LEF and β-catenin, as well as fibroblast growth factor, transforming growth factor-β2, Msx-1 and -2, and ectodysplasin A (EDA) and EDA receptor (EDAR). BMP family molecules, conversely, act as inhibitors of follicle formation. In model systems, ectopic expression of this family of molecules tends to suppress the formation of follicles. In mice, EDAR and β-catenin expression are required for expression of BMP4 and sonic hedgehog (Shh), a finding implicating these molecules in early follicular morphogenesis. Furthermore, EDAR may be important for lateral inhibition of cells surrounding the follicles.

The dermal papilla is believed to be formed as a result of epithelial cell instructions transmitted to the underlying mesenchyme. Molecular candidates for this signal include platelet-derived growth factor and Shh. The dermal cells, in turn, instruct the epidermal cells to proliferate and invade the dermis. The dermal cells differentiate into the dermal papilla; the involved epithelial cells become the inner root sheath and hair shaft of the mature follicle.

In addition to the widened bulge at the base of the mature hair follicle, two other bulges form along the length of the developing follicle. The uppermost bulge is the presumptive sebaceous gland, whereas the middle bulge serves as the site for insertion of the arrector pili muscle. Accumulating evidence also suggests that hair stem cells reside in the middle follicular bulge.[49,50] These important multipotent cells are able to differentiate into any of the cells of the hair follicle and to reconstitute the entire epidermis, which has been reported in cases of extensive surface wounds or burns.[50-53] Although bulge cells from the hair follicle are capable of adopting all these different cell fates, recent work suggests that under normal homeostatic conditions, these cells restrict their contributions to the hair follicle.[51,54]

By 19 to 21 weeks EGA, the hair canal has completely formed, and fetal scalp hair becomes visible. These hairs continue to lengthen until 24 to 28 weeks, at which time they shift from the active growth (anagen) phase to the degenerative phase (catagen), then to the resting phase (telogen), thus completing the first hair cycle. With subsequent hair cycles, hairs increase in diameter and coarseness. During adolescence, vellus hairs of androgen-sensitive areas mature to terminal-type hair follicles.

Sebaceous Glands

Sebaceous glands, as mentioned earlier, mature during the course of follicular differentiation, beginning 13 to 16 weeks EGA.[55] At this time, the presumptive sebaceous gland is the most superficial bulge in the developing hair follicle. As the sebaceous gland develops further, the outer cells differentiate and proliferate, giving rise to cells that accumulate lipids and sebum.[56,57] These differentiated cells ultimately disintegrate to release their contents into the upper portion of the hair canal. The production of sebum is responsive to hormonal influences, including maternal steroids during the second and third trimesters and at adolescence, a finding suggesting a factor contributing to the increased incidence of acne at this age.[58]

Nail Development

The earliest nail structures begin to appear on the dorsal digit tip at 8 to 10 weeks EGA, slightly earlier than the initiation of hair follicle development.[59] The first sign is the delineation of the flat surface of the future nail bed. The proximal nail fold is formed from a portion of ectoderm that buds inward at the proximal boundary of the early nail field. The presumptive nail matrix cells ultimately differentiate to become the nail plate; these cells are present on the ventral side of the proximal invagination. At 11 weeks EGA, the dorsal nail bed surface begins to keratinize. By the fourth month of gestation, the nail plate grows out from the proximal nail fold and completely covers the nail bed by the fifth month.[60]

Eccrine and Apocrine Sweat Gland Development

Eccrine glands begin to develop on the volar surfaces of the hands and feet, beginning as mesenchymal pads 55 to 65 days EGA.[2] By 12 to 14 weeks EGA, parallel ectodermal ridges overlay these mesenchymal pads. The eccrine glands arise from the ectodermal ridge. By 16 weeks EGA, the secretory portion of the gland becomes detectable. The dermal duct is complete by this time, but the epidermal portion of the duct is not canalized until 22 weeks EGA. Dermatoglyphics are formed by the curvilinear patterns of the epidermal ridges, and these become visible on the volar surfaces of the digits by 5 months EGA.

Interfollicular eccrine and apocrine glands, in contrast to volar glands, do not begin to bud until the fifth month of gestation. Apocrine sweat glands usually bud from the upper portion of the hair follicle. By 7 months EGA, the cells of the apocrine glands become distinguishable, composed of both clear cells and mucin-secreting dark cells. Apocrine glands become transiently functional during the third trimester, but eccrine glands are not functional until the postnatal period.

Only a few genes have been identified that encode critical regulators responsible for the development and differentiation of these structures. Mutations in either the EDA or EDAR gene, which encode a TNF-related ligand and its receptor, have been demonstrated in individuals with hypohidrotic and/or anhydrotic ectodermal dysplasia associated with markedly impaired sweating. Animal studies have implicated additional genes in sweat gland formation and function, including *En1* and *Wnt10b*.

Clinical Relevance

As has been described in some of the genetic diseases affecting development of the skin, genes that affect cutaneous appendage formation can also have effects on noncutaneous tissues; findings suggest an early role for these factors or a more global role in development and differentiation.

Certain genetic syndromes with prominent hair defects, often associated with other ectodermal appendageal abnormalities, have been identified; some of the responsible genes have been cloned. Menkes kinky hair syndrome is an X-linked recessive syndrome featuring pili torti (twisted hair) that is sparse and brittle with a "steel wool" quality. The skin is doughy, and patients have skeletal abnormalities and general failure to thrive. The defect for this disease is an ATP-dependent copper transporter, ATP7A.[61-63] Argininosuccinic aciduria is an autosomal recessive disorder, resulting from defective argininosuccinate lyase, that may present neonatally with trichorrhexis nodosa and short, broken scalp hairs, neurologic deficits including lethargy and mental retardation, hepatomegaly, and failure to thrive.[64,65] The mechanism responsible for the hair defects is not known.

Other defects occur in the structural proteins of hair, such as hair keratins. Monilethrix is caused by defects in hair cortex keratins 1, 3, and 6 (HB1, HB3, and HB6), which result in beaded hair and brittle nails.[66,67] Diseases such as pachyonychia congenita present primarily with cutaneous and ectodermal appendageal defects, including hyperkeratotic nails, palmoplantar keratoderma, follicular hyperkeratosis, and

steatocystoma multiplex. These defects have been found to be mutations in genes encoding typical "soft" keratin genes: K6a, K6b, K16, and K17.[68] Trichothiodystrophy, or Tay syndrome, presents as short, brittle hair with alternating dark and light "tiger-tail" bands as well as trichoschisis.[69] Nails are dystrophic, skin is ichthyotic, and patients exhibit photosensitivity, short stature, intellectual impairment, and decreased fertility. Two genes have been identified that are involved in DNA repair and identical to the genes mutated in the xeroderma pigmentosum complementation groups B and D. The reasons for the cutaneous manifestations of this disease are not clear, but the hair and nail phenotypes have been proposed to be a result of low cysteine content.

Mutations in critical developmental regulatory genes, such as those encoding DNA binding proteins and growth factors, have also been found in congenital syndromes affecting skin appendage formation.[1,44] Hypohidrotic ectodermal dysplasia, a defect in either EDA or EDAR, features sparse hair, nail dystrophy, as well as hypohidrosis or anhidrosis, which can lead to poor thermoregulation. In contrast, hidrotic ectodermal dysplasia, which presents with sparse hair, dystrophic nails, palmoplantar keratoderma, and tufting of terminal phalanges, is associated with defects in connexin 30. Mutations in p63 affect nail development in syndromes such as ankyloblepharon, ectodermal defects, and cleft lip and ectrodactyly-ectodermal dysplasia clefting.[70] Functional p63 is required for the formation and maintenance of the apical ectodermal ridge, an embryonic signaling center essential for limb outgrowth and hand plate formation. The secreted factor Wnt7a and the transcription factor Lmx1b are important for dorsal limb patterning, including nail formation. Nail-patella syndrome results from mutations in LMX1B. In contrast, the transcription factor En1 is required for ventral limb patterning and eccrine gland formation. Other regulatory molecules important in appendage development include the secreted factor Shh, which is required for hair follicle formation but not nail plate formation, and the transcription factor MSX1, which is required for tooth and nail formation. *Hoxc13* is an important homeodomain-containing gene for later stages of follicular and nail differentiation, at least in murine models.

PRENATAL DIAGNOSIS

Identification of the genes involved in early cutaneous development and determination of associated mutations responsible for disease have allowed prenatal diagnosis of life-threatening or debilitating cutaneous disease.[71] Some techniques have been devised for such testing, such as chorionic villous sampling, which can be performed at 8 to 10 weeks EGA, or amniocentesis, which can be done at 16 to 18 weeks EGA. These diagnostic procedures can be performed earlier and are associated with less fetal morbidity and mortality than the only previously available technique—fetal skin biopsy—performed 19 to 22 weeks EGA. Even though chorionic villous sampling and amniocentesis are less invasive, certain diseases, such as harlequin fetus, still require fetal biopsy for diagnosis because no definitive genetic mutations have yet been identified. Candidates for prenatal testing include fetuses with an affected sibling or family member.

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Steven B. Hoath

Physiologic Development of the Skin

Human skin is a complex structure with unusual functional diversity.[1] Topologically, the skin is continuous with the lung and intestinal epithelia. The lung and gut are generally viewed as exchange surfaces for gases and nutrients; the skin is more commonly considered a barrier.[2,3] The concept of an integumental barrier emphasizes the role of the skin as a protective boundary between an organism and a potentially hostile environment. This protective role is evident at birth as a fetus abruptly transitions from a warm, wet, sterile, protected sphere to a cold, dry, microbe-laden world filled with physical, chemical, and mechanical dangers. Focusing only on the barrier properties of the skin, however, minimizes the critical role of the skin in social communication, perception, and behavioral interactions. The skin, as the surface of an organism, is both a cellular and molecular structure as well as a perceptual and psychological interface.[4] This dual functionality befits a true boundary and must be kept in mind to fully appreciate the dynamic organization of the skin and its close kinship with the nervous system.

The skin also provides the physical scaffold that defines the form of an animal.[5] As a result of the action of the somatic musculature, a vertebrate's body continually changes shape. A wide variety of strategies have been devised by different animals to cope with the exigencies of different habitats (Table 62-1).[6] Arthropods have a largely inflexible body surface, covered with an exoskeleton. Many vertebrates, such as amphibians, live on land but are confined to humid or wet microhabitats. Reptiles and fish have a skin surface covered predominantly with scales; birds have evolved feathers; and most mammals are covered with a protective mantle of fur. In primates, humans are unique in possessing a nonfurred skin with a thick stratified interfollicular epidermis and a well-developed stratum corneum.[5,7]

The question of the presumptive advantage of losing a protective and insulating coat of fur has long intrigued evolutionary biologists and physical anthropologists.[8] Three of the most distinctive physical features distinguishing human beings are (1) a nonfurred skin surface; (2) a large, versatile, highly complex brain; and (3) opposable thumbs. The close embryologic connection between the epidermis and the brain (both are ectodermal derivatives) supports the contention that these peculiar

TABLE 62–1

Animal Body Covering

Type	Biologic Example
Simple membrane	Earthworm
Simple stratified epidermis, often mucus-secreting	Frog, toad
Exoskeleton	Insects, crustaceans
Scales	Reptiles, fish
Feathers	Birds
Fur	Most mammals (including all primates except humans)
Nonfurred complex stratified epidermis	
Thick interfollicular epidermis with separate functional strata and well-developed stratum corneum	Humans
Lipid droplets in stratum corneum	Dolphins

structural aspects of human development have co-evolved. We often overlook the direct participation of the skin in higher level functions, such as perception and behavioral interactions.[4,9] Cutaneous attributes form the basis for many readily observed biologic distinctions (age, race, sex) as well as multiple, overlapping sociocultural characteristics (tattooing, cosmetics, tanning). During recovery from illness, the skin forms a critical interface linking a patient with caregivers and a nurturing environment.

In this chapter, specific aspects of the physiologic development of fetal and neonatal skin are highlighted, focusing particularly on the development of the epidermal barrier and the functions subserved by the outermost layer of the skin, the stratum corneum. As relevant, new areas of skin biology pertinent to a fetus and a newborn are emphasized. Areas of active research and unanswered questions will be identified in hopes of spurring new investigation of this highly accessible but extraordinarily complex interface.

DEVELOPMENT OF THE EPIDERMAL BARRIER

Human epidermis consists of a number of renewing structures, including the interfollicular epidermis, the hair follicle, the sweat gland, and the sebaceous gland.[10] The bulk of human skin is composed of the dermis and consists of collagen and elastin fibers embedded in a hydrated matrix of glycosaminoglycans. Blood vessels and the majority of cutaneous nerve endings are present in the dermis. The cells of the dermis and subcutaneous fat derive from the embryonic mesoderm. In contrast, epidermal appendages, such as hair follicles, sweat glands, sebaceous glands, and the interfollicular epidermis, derive from embryonic ectoderm.

The epidermis is a composite tissue containing multiple cell types, including keratinocytes, melanocytes, Merkel cells, and Langerhans cells. Only the antigen-presenting Langerhans cells are of mesodermal origin. The epidermis is traditionally segmented into four separate structural and functional compartments (Figure 62-1): (1) the stratum basale, responsible for keratinocyte proliferation and epidermal renewal; (2) the stratum spinosum, consisting of tightly packed keratinocytes linked via desmosomal connections; (3) the stratum granulosum, responsible for barrier lipid synthesis and corneocyte production via programmed cell death; and (4) the stratum corneum, the anucleated outermost layer, which forms the physical interface with the environment.[11,12] The stratum corneum is markedly deficient in preterm human infants.[13-16] Protective functions of the stratum corneum are listed in Table 62-2.

Cornified Envelope and Epidermal Lamellar Body

During the latter part of gestation, epithelial surfaces at environmental interfaces undergo structural and functional changes, including synthesis of complex, proteolipid materials. These events are highly coordinated and can be influenced by prenatal hormone exposure, such as glucocorticoids given to a mother.[16-21] Skin development in a human fetus is separated into three stages that correlate with the major events in epidermal differentiation: stratification, follicular keratinization, and keratinization of the interfollicular epidermis.[10] The stratum corneum is generally formed after 23 to 24 weeks of gestation. The formation of a barrier to water loss and infection is a sine qua non for survival in the extrauterine environment. Figure 62-2 illustrates the complex stages in the assembly of the cornified envelope in human stratum corneum.[22,23] Of note, synthesis and secretion of epidermal barrier lipids occurs in the form of lamellar bodies. This process is similar to that occurring over a parallel time frame in the developing lung (Table 62-3).[16] Fusion of the intracellular lamellar body with the limiting plasma membrane results in extrusion of barrier lipids, which subsequently undergo extracellular organization providing the primary barrier to transepidermal water loss. The barrier lipids in the epidermis, unlike the lung, are generally devoid of phospholipids and consist primarily of free fatty acids, cholesterol, and ceramides (see Table 62-3).[2]

Covalent crosslinking of structural proteins and ceramides results in formation of a highly insoluble cornified envelope, typical of the mature mammalian stratum corneum.[22,23] Each corneocyte consists of a covalently crosslinked envelope, linked internally to structural proteins such as keratins and externally to other corneocytes via desmosomes, resulting in the assembly of a huge macromolecule.[12] The complexity and redundancy of the cornified envelope provide evidence for a highly organized and conserved molecular structure.[24,25] The cornified envelope, although only 10 nanometers thick and of uniform density, is highly insoluble, secondary to crosslinking by intracellular transglutaminases.[26] The isopeptide bond formed by these enzymes cannot be cleaved in vertebrate cells; therefore the resulting macromolecular protein complex is stable and essentially insoluble. This crosslinked assembly forms the scaffolding for the highly ordered lamellar lipid matrix. The very-low-birth-weight preterm infant lacks an effective epidermal barrier.[13,14] A better understanding of the mechanisms by which the stratum corneum forms in utero may offer insights and direction to therapeutic regimens aimed at epidermal barrier formation postnatally.

The Biology of Vernix

Scientifically, the development of epidermal barrier function has many similarities to surfactant production and lung development (see Table 62-3). Both epidermal keratinocyte and type II alveolar cell are lipid-synthesizing cells that secrete barrier lipids in the form of lamellar bodies. Both ultimately interface with a gaseous environment and both are under similar hormonal control at similar periods of development. A clear analogy exists between the mechanisms by which the lung develops a functionally mature epithelial surface ready for air adaptation and those by which the skin surface matures under total aqueous conditions for terrestrial adaptation to a dry environment. An unanswered question in epidermal biology, however, is by which mechanism the epidermal barrier forms under conditions of total aqueous immersion. Prolonged exposure of the skin surface to water in adults is harmful.[27] Growing evidence indicates that vernix caseosa, a complex proteolipid cellular cream, which forms primarily during the last trimester of gestation, interacts with the developing epidermis and facilitates

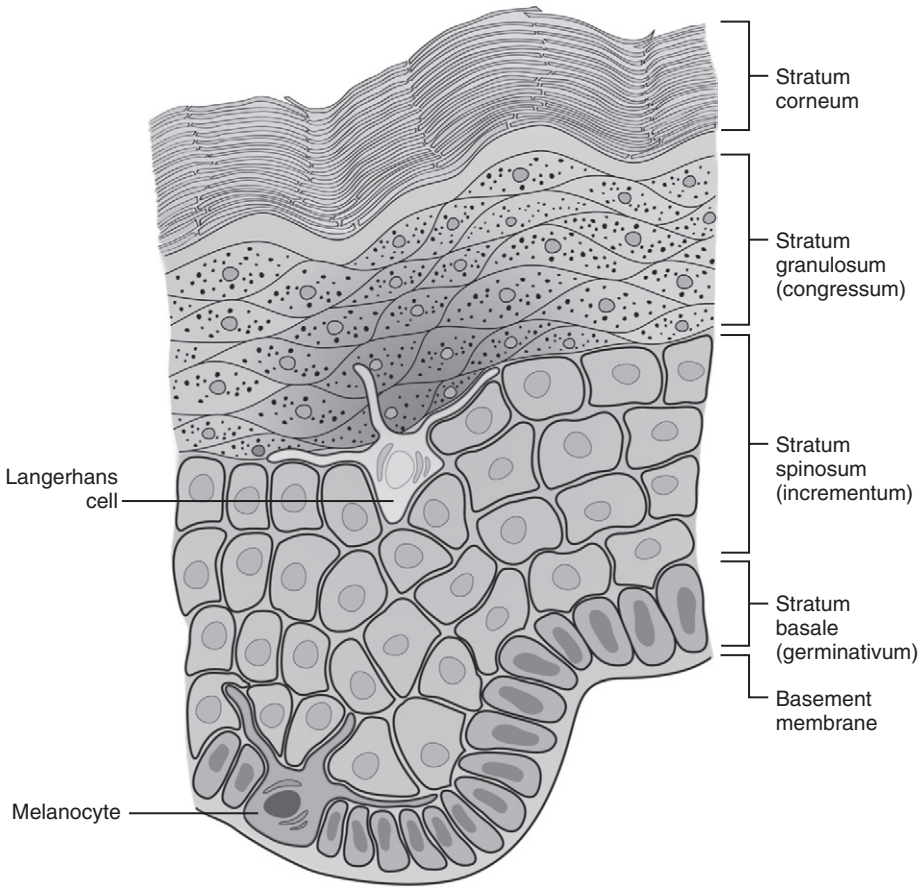


Figure 62-1 Organization of human epidermis. The interfollicular epidermis is traditionally considered a tetratomic identity incorporating four separate structural and functional epidermal strata. In humans, the epidermis between the hair follicles is much thicker than in furred animals. The epidermis is composed primarily of keratinocytes but includes other epidermal cells such as melanocytes, Langerhans cells, and Merkel cells (latter not shown). The outermost stratum, the stratum corneum, incorporates the transition from living, nucleated keratinocytes to terminally differentiated corneocytes. This diagram is schematic only and does not illustrate the important intercellular connections (desmosomes) converting single cells into integrated membrane structures. *(Modified from Hoath SB, Leahy DG: The human stratum corneum as extended, covalently cross-linked biopolymer: mathematics, molecules, and medicine,* Med Hypotheses *10:10, 2006.[12])*

TABLE 62–2

Protective Functions of the Stratum Corneum

Functions	Structural Basis	Biochemical Mechanisms
Mechanical integrity/resilience	Cornified envelope, cytosolic filaments	Crosslinked peptides (e.g., loricrin, keratin filaments)
Xenobiote defense	Lamellar bilayers, extracellular matrix	Acidic pH; free fatty acids; antimicrobial peptides
Antioxidant defense	Corneocytes and extracellular matrix	Keratins; sebaceous gland–derived vitamin E and other antioxidants
Cytokine signaling	Corneocyte cytosol	Storage and release of interleukins; serine proteases
Permeability barrier	Lamellar bilayers	Hydrophobic lipids
Hydration	Lamellar bilayers Corneocyte cytosolic matrix	Sebaceous gland–derived glycerol; filaggrin breakdown products (NMFs)
Waterproofing/repellency	Lamellar bilayers	Keratinocyte and sebum-derived lipids
Cohesion/desquamation	Corneodesmosomes	Acidic pH serine proteases
UV protection	Corneocyte cytosol	Structural proteins; urocanic acid; light scattering/absorption

Modified from Chuong CM, Nickoloff BJ, Elias PM, et al: What is the 'true' function of skin? *Exp Dermatol* 11:159, 2002.[5]
NMF, Natural moisturizing factor; *UV,* ultraviolet.

the in utero formation of the stratum corneum.[28,29] The macroscopic and phase contrast appearances of vernix are shown in Figure 62-3.

Recent studies in rodent and fetal human skin using the technique of methylene blue dye exclusion[30,31] have demonstrated the development of the epidermal barrier. Cellular tissue exposed to this dye turns blue, but an intervening cornified barrier prevents tissue staining. This technique offers a relatively straightforward method of determining the pattern and timing of permeability barrier formation. In rodents, epidermal barrier development begins dorsally and extends in a ventral direction during the sixteenth and nineteenth days of gestation

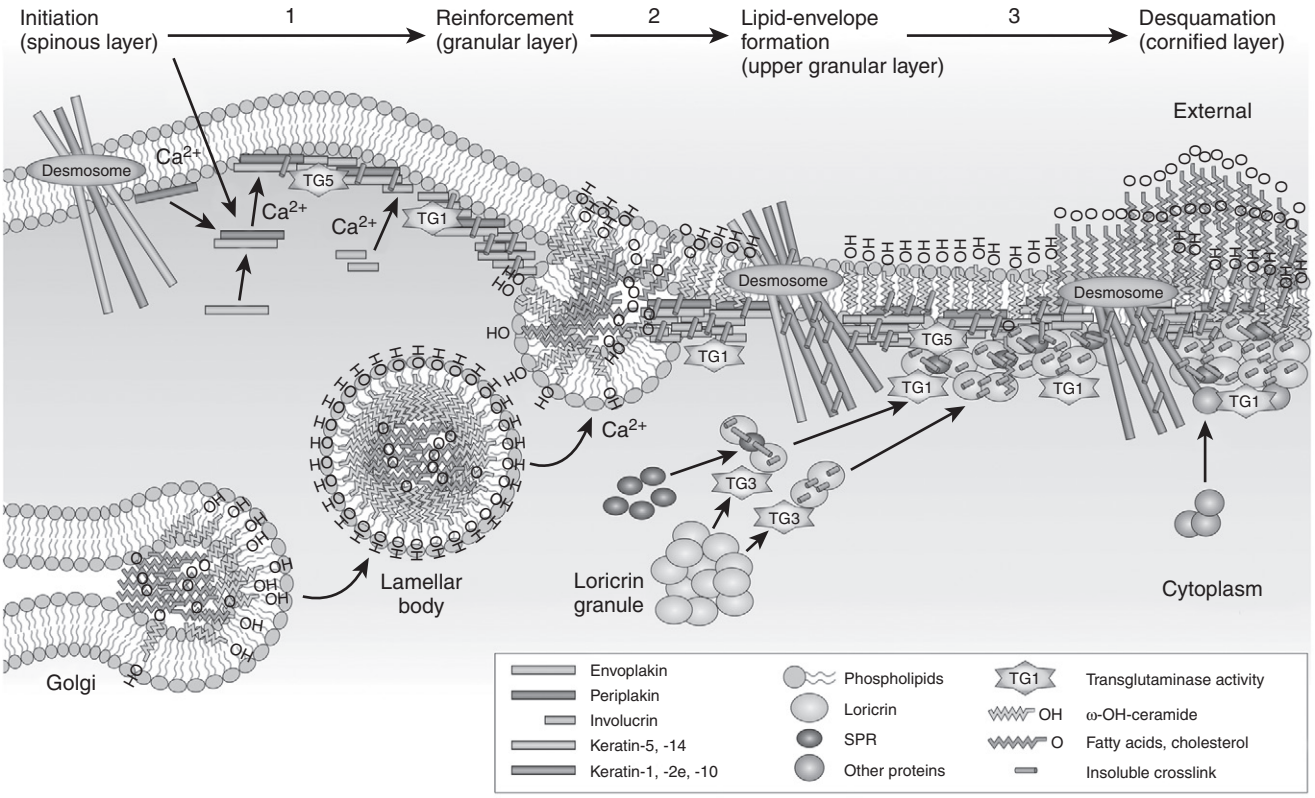


Figure 62-2 Stages in the assembly of the cornified envelope in human stratum corneum. 1, In the upper layers of the epidermis, rising levels of intracellular Ca^{2+} lead to expression of envoplakin, periplakin, and involucrin, as well as transglutaminase-1 with subsequent covalent crosslinking of the structural proteins along the inner surface of the plasma membrane. **2,** Synthesis, packaging, and extrusion of barrier lipids (free fatty acids, cholesterol, ceramides) in the form of lamellar bodies are accompanied by replacement of the plasma membrane with covalently bound ceramides that serve to interdigitate with and organize the extracellular lipids into the characteristic lamellar pattern. **3,** Covalent crosslinking of intracorneocyte proteins, including keratins, loricrin, and small proline-rich peptides, occurs via mediation of transglutaminase-1 and -3. The end product is the transformation of nucleated keratinocytes in the uppermost granular layer of the epidermis into a network of interlinked, terminally differentiated corneocytes embedded in a matrix of structured lipid lamellae, collectively called the stratum corneum. The stratum corneum provides the vital barrier to water loss and infection necessary for life after birth. *(Modified from Candi E, Schmidt R, Melino G: The cornified envelope: a model of cell death in the skin,* Nat Rev Mol Cell Biol *6:328, 2005.[22])*

TABLE 62–3

Comparison of Lamellar Bodies of Epidermal and Pulmonary Origins

	Epidermis	Lung
Cell type	Spinous and granular keratinocytes	Type II alveolar cell
Size/structure	Ovoid (0.25-0.5 micron) Disk-like lamellations	Spheroid (1.2-1.6 microns) Lamellated sacs with amorphous core
Lipid content	40% phospholipids 20% glycosphingolipids 20% free sterols 20% other neutral lipids	85% phospholipids 10% free sterols 5% other neutral lipids
Protein content	Acid phosphatase Glycosidases Proteases Lipases	Acid phosphatase Proteases Glycosidases Surfactant apoproteins
Function	Delivery of lipids to form extracellular matrix of stratum corneum Skin permeability barrier	Delivery of surfactant lipid to alveolar spaces Lower alveolar surface tension

in mice and rats, respectively.[31] This process leads to a slightly thicker stratum corneum over the back than the front in a late-gestation fetal rat.[32] Stratum corneum formation in a rat at birth requires approximately 3 cells/cm^2/s to undergo programmed cell death.[32] Corneocyte formation rate in humans has not been measured.

In humans, unlike rodents, which lack body hair at birth, the permeability barrier forms initially in the region of the pilosebaceous apparatus.[30] This finding strongly supports the hypothesis that vernix caseosa (in part a product of sebaceous secretions) participates in "waterproofing" the skin surface, thereby allowing cornification to occur initially in the area of the hair follicles and then over the interfollicular skin.[33] Standard skin culture systems are organizationally simpler than fetal skin and notably lack skin appendages. Such systems must be raised to an air-liquid interface to obtain adequate cornification.[34] A better understanding of vernix caseosa and fetal sebaceous gland physiology is biologically relevant and may be applicable to clinical situations in which the epidermal surface is inadequately developed, burned, or traumatized.

Sebaceous glands are found in the skin of all mammals except whales and porpoises.[35] Their primary function is the excretion of sebum, which is a complex mixture of relatively nonpolar

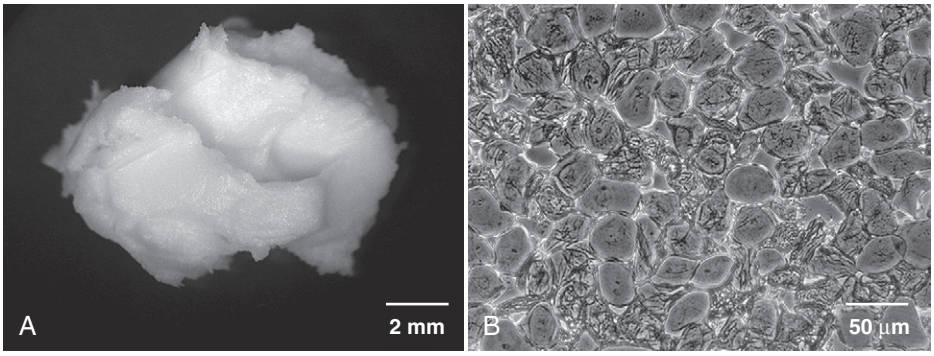


Figure 62-3 Vernix caseosa. A, Macroscopically, vernix is a thick, viscous, white paste. The phase contrast image of native vernix **B,** reveals a dense packing of fetal corneocytes. Vernix consists primarily of thin, hydrated, polygonal, terminally differentiated, fetal corneocytes embedded in a nonlamellar lipid matrix. The cells are heterogenous in size and structure. Many nuclear ghosts are evident. Scale bars are shown.

lipids, most of which are synthesized de novo by the glands.[36] Sebum provides a hydrophobic coating for the fur of mammals, which protects against overwetting. Sebaceous glands are multi-acinar, holocrine-secreting structures that occur in all areas of the skin except the palms and soles and only sparsely on the dorsal surfaces of the hand and foot.[37] The development of the sebaceous glands is closely related to the differentiation of hair follicles in the epidermis. In newborn infants, sebaceous glands are well formed, hyperplastic, and macroscopically visible over certain body areas, such as the nose. The close connection of vernix production in utero with the developing pilosebaceous apparatus supports a mechanism by which the surge in sebaceous gland activity during the last trimester of pregnancy leads to production of a thick, lipid-rich, hydrophobic film (the vernix caseosa) overlying the developing stratum corneum.[28,36,38] The hyperplasia of the sebaceous glands in term infants is putatively secondary to androgenic stimulation from the equally hyperplastic adrenal glands.[28] This hypothesis provides a working model that remains to be fully tested (Figure 62-4). It has the advantage of functionally integrating two disconnected facts—the mutual hyperplasia during the latter half of gestation of the sebaceous gland and the adrenal gland in a human fetus.

Other clinical observations are also explicable under this hypothesis. Obstetricians have long observed that the amniotic fluid becomes turbid during the last trimester of pregnancy.[39] In vitro, the addition of physiologically relevant amounts of pulmonary surfactant leads to emulsification and release of immobilized vernix. This finding is consistent with a mechanism by which vernix is progressively released from the skin surface after formation of an intact stratum corneum under the influence of lung-derived surfactant within the amniotic fluid.[39] The fetus subsequently swallows the detached vernix (see Figure 62-4). Measurements of the amino acids of vernix have demonstrated that it is rich in glutamine, a known trophic factor for the developing gut.[40,41] Further work is required to establish the degree to which epithelial surfaces "crosstalk" in preparation for birth. What emerges from the available data, however, is a coherent and intriguing glimpse of a new area in perinatal biology.

TRANSITION AT BIRTH

Few events in mammalian life are as physiologically abrupt as birth. As with other epithelial surfaces that interface with the environment after birth (lung, gut, and kidney), the skin must immediately perform multiple functions vital to the survival of the organism (Box 62-1).[14] Many of these functions, such as thermoregulation, were unnecessary before birth and largely performed by the placenta. Birth, however, marks a transition to a cold, nonsterile environment that includes high oxidative stress and exposure to ultraviolet light.

Physiologic mechanisms in the epidermis that contribute to formation of an adaptive environmental interface include activation of eccrine sweating, which is important for thermoregulation and bacterial homeostasis, and sebum production by hyperplastic sebaceous glands.[42] At birth, the epidermal barrier is essentially pH neutral, but it rapidly develops an acid mantle via mechanisms distinct from simple bacterial colonization.[43] Physiologically, the epidermis and its ultimate differentiation product, the stratum corneum, remain in balance via the reciprocal properties of renewability and self-cleaning, properties that reflect the distinct but tightly coupled processes of cornification and desquamation.[44] Recent data have indicated that transepidermal water flux is an important regulator of DNA and lipid synthesis within the epidermis.[45-47] Environmental humidity and maintenance of stratum corneum hydration are necessary for normal desquamation.[48,49] A preterm infant is vulnerable to disruption of all these physiologic mechanisms.

Prenatally, lipid synthesis is necessary for production of the vernix caseosa and formation of the interfollicular barrier lipids of the epidermis, which are required for successful postnatal adaptation. Postnatally, lipid synthesis and metabolism continue to subserve multiple functions (Table 62-4). Thus specific barrier lipids in the stratum corneum form the primary structural basis for the postnatal epidermal permeability barrier. The epidermis and the brain contain unusually high concentrations of ceramides.[50-52] This fact deserves further examination given the close embryologic and functional connection between these two ectodermal derivatives. Other skin lipids include sebum and triglycerides in adipose tissue. The latter functions primarily as a systemic energy reservoir in addition to providing cushioning and biomechanical support.

Water Loss, Temperature Control, and Blood Flow

Life in a dry, terrestrial environment necessitates protection against the constant dehydrating effects of air exposure. The formation of a barrier against transepidermal water loss is well known to be a direct function of gestational age.[53,54] As shown in Figure 62-5, transepidermal water loss decreases dramatically as term approaches, with levels in the near term infant equal to or less than adult values. This critical and vital postnatal function resides almost entirely in the outermost 20 microns of the skin surface—in the stratum corneum.[2] Heat exchange between the skin of an infant and the surrounding environment occurs via

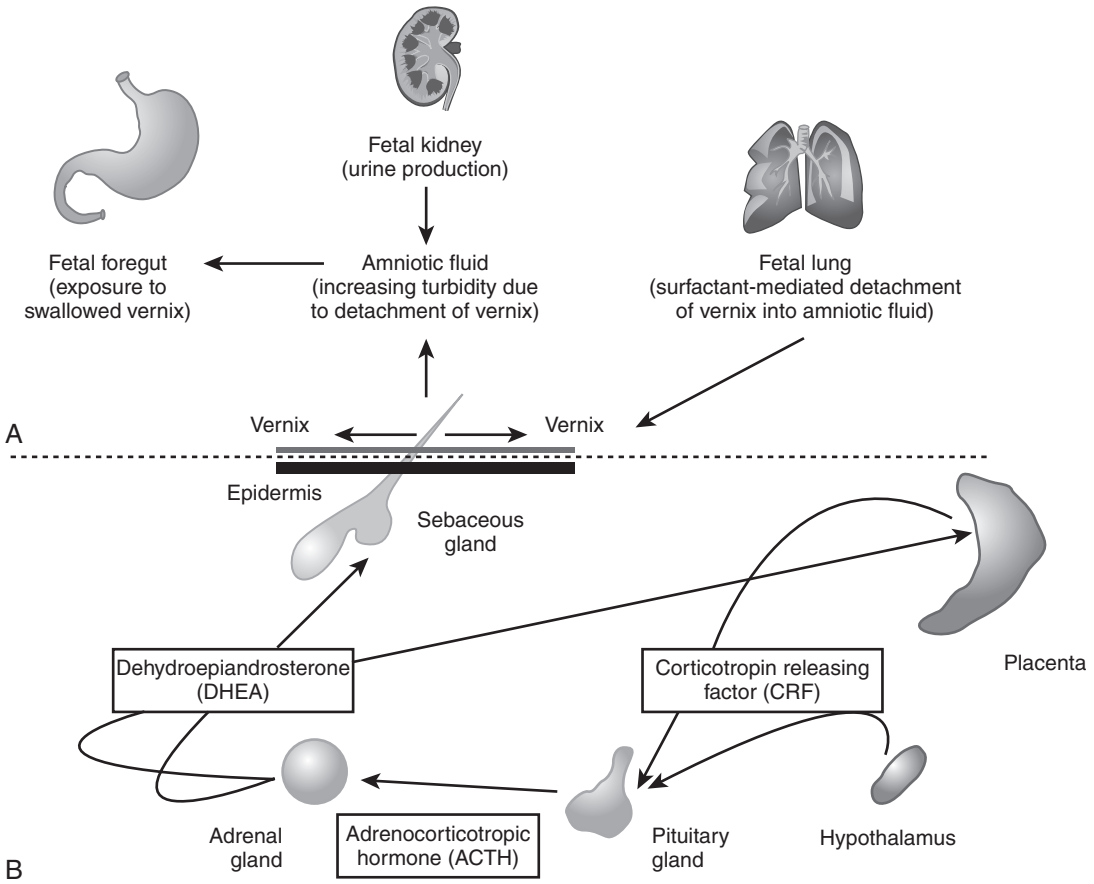


Figure 62-4 A, Proposed mechanism for pulmonary surfactant–mediated vernix detachment. The fetal kidney contributes significantly to amniotic fluid production. During the third trimester, vernix covers the developing epidermis and the fetal lung produces and secretes increasing amounts of pulmonary surfactant into the amniotic fluid. Vernix on the skin surface builds up and detaches into the surrounding milieu, leading to amniotic fluid turbidity.[39] Vernix within the amniotic fluid is subsequently swallowed by the fetus with potential effects on the fetal foregut and/or systemic absorption of vernix components. **B,** Endocrine-based model for vernix production and epidermal barrier maturation. In this working model, corticotropin-releasing factors from either the placenta or hypothalamus act to initiate adrenocorticotropic hormone release from the pituitary gland. Adrenocorticotropic hormone stimulation of the adrenal cortex promotes synthesis and release of androgenic steroids such as dihydroepiandrosterone (DHEA), which are subsequently converted enzymatically within the sebaceous gland to active androgens. Production of superficial lipid film (sebum) in the immediate vicinity of the hair follicle modulates the transepidermal water gradient with putative effects to facilitate cornification of the underlying epidermis.[31] Desquamation of corneocytes into the overlying lipid matrix results in formation of vernix. The recent finding by Ito et al[159] that human hair follicles synthesize cortisol and exhibit a functional equivalent of the hypothalamic-pituitary-adrenal axis raise interesting questions regarding local versus systemic control mechanisms in the schema. *(Modified and expanded from Hoath S, Pickens W: The biology of vernix. In Hoath SB, Maibach H, editors:* Neonatal Skin: Structure and Function, *2nd ed, New York, 2003, Marcel Dekker.[28])*

BOX 62-1

Multiple Physiologic Roles of the Skin at Birth

- Barrier to water loss
- Thermoregulation
- Infection control
- Immunosurveillance
- Acid mantle formation
- Antioxidant function
- Ultraviolet light photoprotection
- Barrier to chemicals
- Tactile discrimination
- Attraction to caregiver

evaporation, radiation, conduction, and convection.[55] Of these, evaporative heat loss is the primary mode of temperature instability in very-low-birth-weight preterm infants and is the primary mode of heat loss in all infants at the time of birth. It is important to distinguish between evaporative heat loss from standing water or amniotic fluid on the skin surface and evaporative heat loss secondary to a poor epidermal barrier, which occurs after delivery in very-low-birth-weight preterm infants. The second mode of heat and fluid loss is ongoing and is exacerbated in nonhumidified, radiant-heated environments.

Given the critical importance of limiting evaporative water and heat loss in temperature control, it is surprising how little attention is given to the role of the stratum corneum in thermoregulation. Most reviews of body temperature control assume the presence of this water-retentive envelope. As with many other functions, the strategic position of the stratum corneum is taken for granted and focus is placed on more central mechanisms. In a newborn infant, however, it is important to

TABLE 62–4

Sources, Composition, and Presumed Functions of Skin Lipids in Neonates

Source	Composition	Functions
Stratum corneum	Ceramides, cholesterol sulfate, neutral lipids (free and esterified sterols, free fatty acids, triglycerides)	Permeability barrier, cohesion/desquamation, antibacterial
Sebaceous glands	Triglyceride, wax/ sterol esters/squalene	Antibacterial Moisturization
Adipose tissue	Triglyceride	Systemic energy reservoir

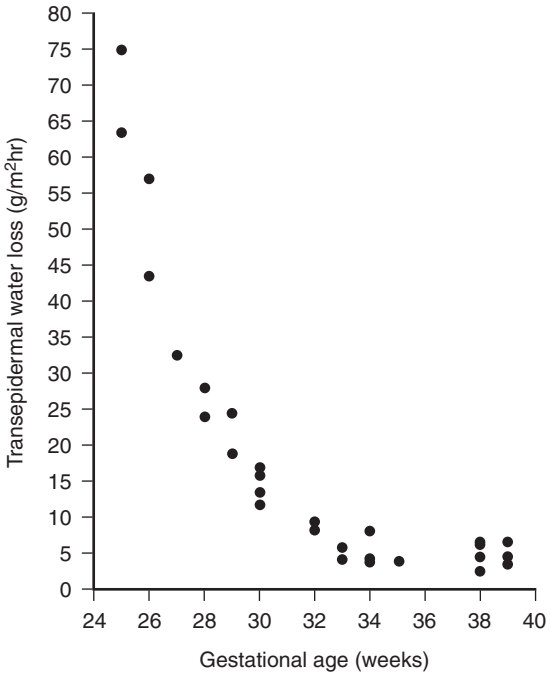


Figure 62-5 Transepidermal water loss as a function of gestational age. These data demonstrate the inverse relationship between transepidermal water loss (TEWL) and gestational age. Very-low-birth-weight preterm infants have extraordinarily high TEWL in the range of 50 to 70 g/m^2/hr. Term infants have low TEWL, in the range of 5 g/m^2/hr, which is comparable with or lower than adult values. *(Modified from Hammarlund K, Sedin G: Transepidermal water loss in newborn infants. III. Relation to gestational age,* Acta Paediatr Scand *68:795, 1979.[54])*

continually bear in mind the role of peripheral mechanisms in organizing, maintaining, and guiding central nervous system development.

The classic study by Adamsons et al[56] more than 40 years ago provides an example of the importance of the periphery in temperature control in newly born infants (Figure 62-6). Thus oxygen consumption, measured in term infants 4 hours after birth, is not a function of central body temperature as might be expected with warm-blooded endothermic organisms.[53] Systemic oxygen consumption is, rather, a function of heat exchange at the skin-environment interface. Central homeothermic mechanisms are not well developed at birth. A basic knowledge of skin-environment interactions, therefore, is key for infant caregivers. Preterm infants exhibit even more vulnerability to the environment owing to higher surface area to mass ratios, lower endogenous sources of heat generation such as brown adipose tissue, an incompetent epidermal barrier, and the inability to self-regulate with flexural positioning.[13]

The central control of body temperature also requires mechanisms such as eccrine sweating and vasodilatation/vasoconstriction, which are under autonomic nervous system control.[57] These mechanisms are generally not well established at birth. Newborn infants may exhibit vasoconstriction of the extremities (acrocyanosis) and may have wide fluctuations in red blood cell content (hematocrit) as well as blood volume. Peripheral cooling results in increased blood viscosity and decreased blood flow. After birth, considerable reorganization of the cutaneous vascular bed, with development of a subpapillary plexus in the first 3 months of life, occurs (Figure 62-7). The formation of a superficial vascular plexus is also associated with increased convolution of the undersurface of the epidermis (rete peg formation), which is a hallmark of the mature human epidermis. The development of the cutaneous vasculature and the role of growth factors, such as vascular endothelial growth factor have been reviewed elsewhere.[58]

Development of the Acid Mantle

A number of physiologic mechanisms are triggered at the time of birth, which persist into later life. Human skin, for example, is characterized by an acidic skin surface, the so-called acid mantle. At birth, the skin surface pH is relatively neutral and gradually becomes more acidic in the first few postnatal weeks (Figure 62-8). The first measurements of infant skin pH were performed by Taddei[59] in 1935, who reported surface skin pH in the range of 6.5 at birth. The use of alkaline soaps for infant bathing can markedly alter the development of the acid mantle, as can the local environment.[60] Acid mantle development is delayed in very-low-birth-weight preterm infants.[61] Surface skin pH measurements are generally higher under occluded sites, such as the diaper region.[62] Site-dependent differences in surface pH have also been reported in neonates.[63]

A number of different mechanisms for establishing the acid mantle have been proposed, including endogenous factors such as lactic acid from sweat, free fatty acid generation from metabolism of triglycerides in sebum, and metabolic byproducts of bacterial colonization.[43] Endogenous factors include the formation of lactic acid from epidermal cell metabolism and free fatty acid generation from phospholipid breakdown within the stratum corneum. Active proton pump mechanisms also have been proposed, as well as the controlled degradation of structural proteins such as filaggrin to form acidic byproducts such as urocanic acid.[43]

The presence of an acidic skin surface has advantages.[43] Proposed functions of the acid mantle include antimicrobial defense and maintenance of epidermal barrier integrity.[64] Enzymes involved in stratum corneum desquamation, which are pH sensitive, include cathepsin D, chymotryptic serine protease, and tryptic serine protease. Extracellular lipids involved in formation of the epidermal barrier are also processed via pH-sensitive enzymes, including beta-glucocerebrosidase. Thus the acid mantle is derived from multiple redundant sources and may regulate a number of processes essential for maintenance of a competent epidermal barrier. A newborn infant offers an exciting and accessible area for studying these multiple etiologic mechanisms and functions.

Bacterial Colonization and Skin Cleansing

Paramount in the many functions served by the skin is the prevention of infection. Before birth, a fetus resides in a putatively sterile environment. Colonization of newborn skin begins upon

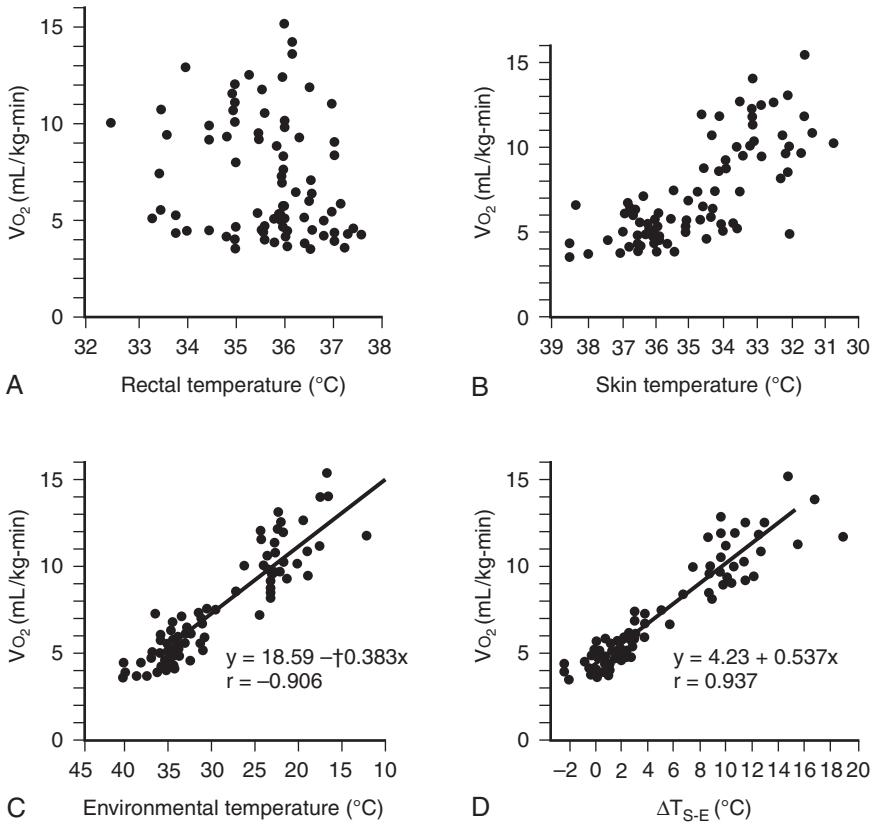


Figure 62-6 Oxygen consumption of term newborn human infants approximately 4 hours after birth. Systemic oxygen consumption correlates best with the skin-environment gradient, indicating the importance of peripheral heat flux in regulating body temperature in neonates. *(From Adamsons K, Gandy G, James L: The influence of thermal factors upon oxygen consumption of the newborn human infant,* J Pediatr *66:495, 1965.[56])*

first exposure to the external world. Infants born vaginally become colonized during passage through the birth canal, whereas infants born by cesarean delivery are typically sterile if the amniotic membranes were not ruptured before the onset of labor.[65] A list of the common skin-colonizing bacterial flora is given in Box 62-2.

Staphylococcus epidermidis, one of 13 species of coagulase-negative staphylococci, is the most common vaginal organism before birth and is ubiquitous in the environment. This organism rapidly colonizes the skin surface and is the predominate organism on the skin of most neonates.[66-70] In normal conditions, the resident flora of a neonate resembles that of adults after the first few weeks of life. These skin-colonizing flora are generally nonvirulent, stable in number, and only infrequently pathogenic given a normal epidermal barrier and immune properties.[71] In a very-low-birth-weight preterm infant, however, *S. epidermidis* is the most common cause of nosocomial sepsis.[71] This fact complicates the choice of appropriate skin care procedures and practices. Normally, commensal bacterial flora play a protective role, and recent evidence suggests that organisms such as *S. epidermidis* may up-regulate expression of antimicrobial peptides, such as human beta-defensin-2.[72,73]

The mechanisms leading to colonization of the skin are incompletely understood and involve a complex interplay among rapid growth of commensal organisms, the development of the acid mantle, local microenvironmental factors such as occlusion and humidity, and the choice of exogenous soaps and skin care practices.[73] There is a surprising lack of standard guidelines regarding optimal skin care in neonates.[74,75] Specific aspects of care include hand washing hygiene, bathing and choice of surfactants, regional skin care including the umbilical cord stump, and preparation of the skin before invasive procedures such as venipuncture.[74] Recently the use of alcohol-based disinfectants has been promoted as an alternative to routine hand washing.[76,77] It is important to recognize that such disinfectants have no cleansing function and will not remove soils on the skin surface. Recently, vernix caseosa has been reported to function as an endogenous skin cleanser that readily removes topical soils, such as carbon particles.[78] This finding is consistent with the view that the surface of a newly born infant possesses endogenous skin cleansing mechanisms (vernix, stratum corneum desquamation). These natural functions should be incorporated into the design of exogenous skin cleansing regimens.

Cutaneous Immunity

Given the plethora of bacteria and the multiple opportunities for superficial trauma, it is not surprising that the skin has a well-developed immune system.[5,79,80] Increasing evidence is available that innate immune function in term infants may be well developed, whereas such function in preterm infants is poor.[81] Once pathogenic bacteria or fungi have established adherence to the skin, a number of cutaneous mechanisms of host defense are called into play, including (1) the role of the stratum corneum as a mechanical barrier, (2) the establishment of an acid environment inhospitable for growth of pathogenic bacteria, and (3) the presence of metabolic products within the stratum corneum itself with antimicrobial activity.[82,83] Commensal organisms, such as the lipophilic corynebacteria, have

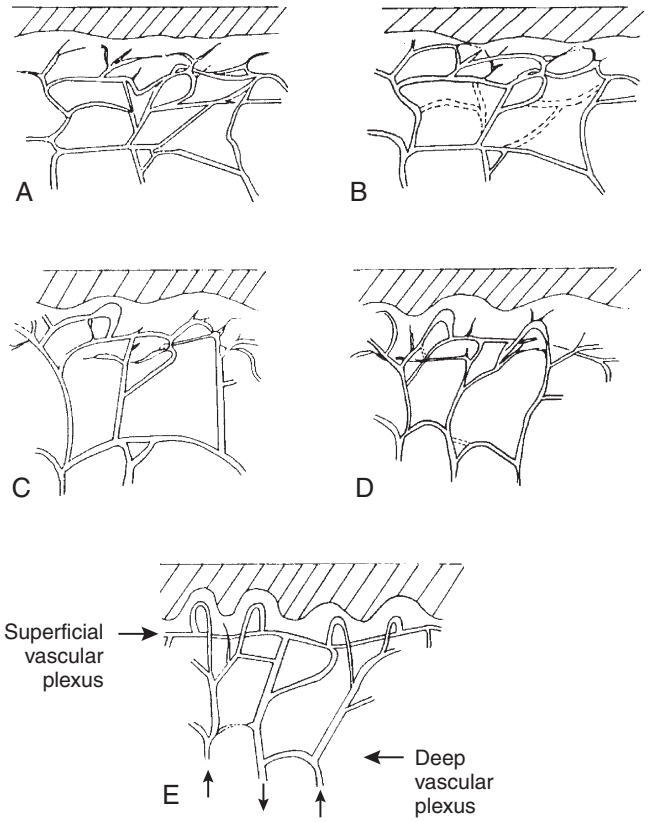


Figure 62-7 Diagrammatic representation of the blood supply at birth. A, The gradual development of papillary buds and organization of the subpapillary plexus from birth to 3 months of age **(B-D)** and the vascular pattern at 3 months of age **(E).** The *arrows* **(E)** show the direction of blood flow for arteriolar and venous systems. *(Modified from Perera P, Kurban A, Ryan T: The development of the cutaneous microvascular system in the newborn,* Br J Dermatol *82(Suppl 5):86, 1970.[157])*

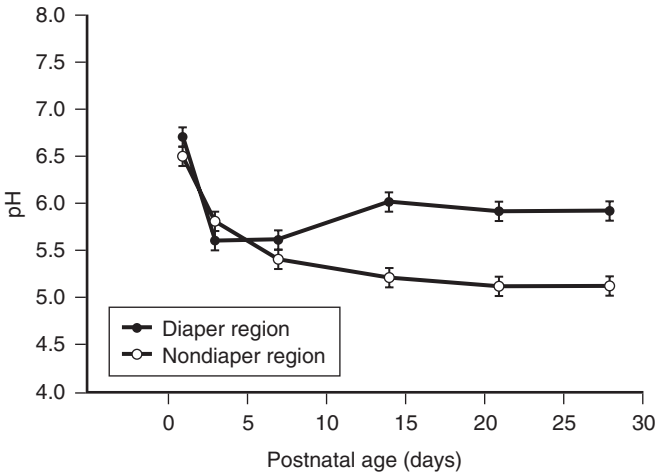


Figure 62-8 Postnatal development of the acid mantle in term newborn infants—effect on body site. Skin pH was measured using a flat surface electrode (Courage and Khazaka pH meter, model 900, Cologne, Germany) in male and female infants born 37 to 42 weeks of gestation. Values are means ± SE. *(Data from Visscher M, Munson K, Pickens W, et al: Changes in diapered and nondiapered infant skin over the first month of life,* Pediatr Dermatol *17:45, 2000.[62])*

BOX 62–2

Common Skin Flora Colonizing Newborn Skin

- Micrococcaceae
  - Coagulase-negative staphylococci
  - *Staphylococcus epidermidis*
  - *Staphylococcus hominis*
  - *Staphylococcus saprophyticus* (perineum)
  - *Staphylococcus capitis* (sebum-rich areas)
  - *Staphylococcus auricularis* (ear canal)
  - *Peptococcus* species
  - *Micrococcus* species
- Diphtheroids
  - Corynebacterium (moist intertriginous areas)
  - Brevibacterium (toe webs)
  - Propionibacterium (hair follicles, sebaceous glands)
- Gram-negative rods
  - *Acinetobacter* (moist, intertriginous areas, perineum)
  - Rarely *Klebsiella, Enterobacter, Proteus*
- Yeast
  - *Malassezia* species

Modified from Sidbury R, Darmstadt G: Microbiology. In Hoath SB, Maibach H, editors: *Neonatal Skin: Structure and Function*, New York, Marcel Dekker, 2003[73]; and Darmstadt G: Staphylococcal and streptococcal skin infections. In Harahap M, editor: *Diagnosis and Treatment of Skin Infections*, Oxford, Blackwell Scientific Publications, 1997.[71]

BOX 62–3

Antimicrobial Agents Produced in the Epidermis

- Complement system
- Defensins
- Cathelicidins
- Cytokines
- Chemokines
- Reactive oxygen species

Modified from Chuong CM, Nickoloff BJ, Elias PM, et al: What is the 'true' function of skin? *Exp Dermatol* 11:159, 2002.[5]

been reported to contribute to host defense by liberating antibacterial fatty acids from the triglycerides of sebum.[84] Cationic peptides may be elaborated in the skin with direct antimicrobial effects on the bacterial side of the cytoplasmic membrane.[85] A list of potential antimicrobial peptides in human epidermis is given in Box 62-3. Vernix caseosa has also been demonstrated to contain multiple antimicrobial peptides, including lactoferrin and lysozyme.[86-89]

Specific cell types within the epidermis play critical roles in host defense. In addition to terminal differentiation and formation of the stratum corneum, keratinocytes have been reported to internalize bacteria, leading to bacterial death and containment of infection.[90,91] The epidermis also contains migratory dendritic cells associated with antigen presentation.[92] These Langerhans cells are derived from the bone marrow and are located in the mid-to-lower epidermis, where they maintain a strategic position for immunosurveillance (Figure 62-9). Breach of the epidermal barrier leads to exposure of Langerhans cells to microorganisms and other potentially pathogenic material. After exposure, Langerhans cells migrate to regional lymph nodes. Their density and function within preterm epidermis have not been explored.

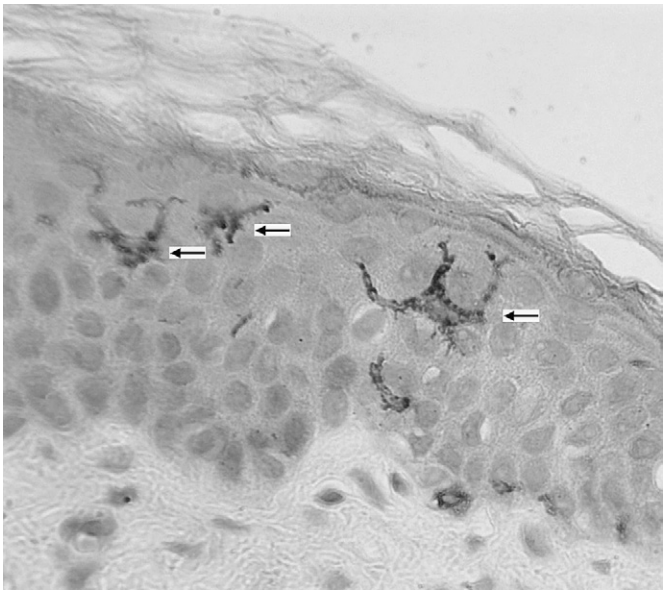


Figure 62-9 Immunolocalization of Langerhans cells in premature human epidermis (foreskin) by CD1a antibody staining (arrows). Langerhans cells are dendritic, antigen-presenting cells derived from the fetal bone marrow. They are typically located within the epidermis and function as a major component of the skin immune system. Skin specimen is from an approximately 32 week gestational age premature infant circumcised 1 month after birth.

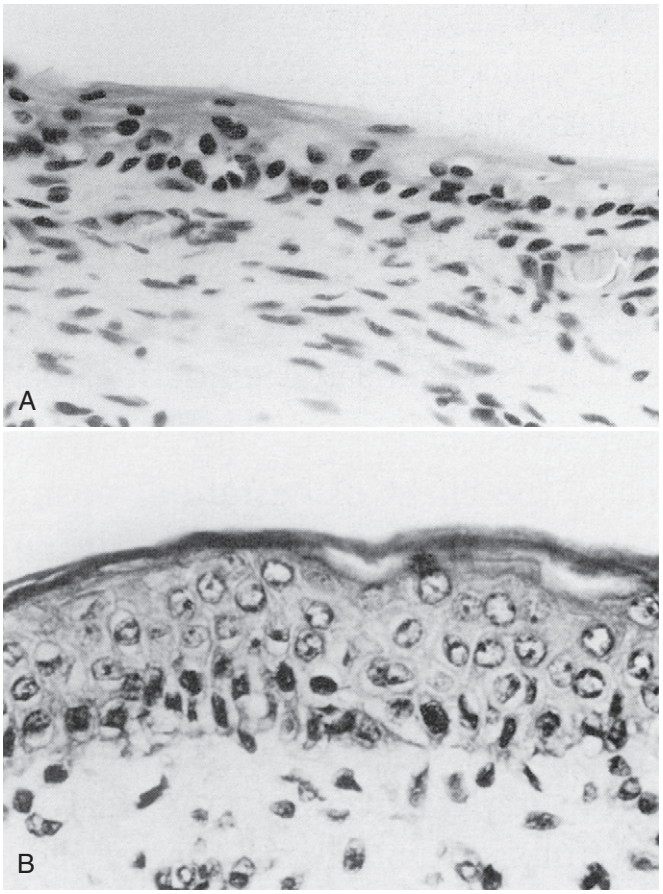


Figure 62-10 Rapid development of the epidermal barrier after exposure to the ambient environment (xeric stress) in very-low-birth-weight preterm infants. A, Epidermis of a 26-week-gestational-age infant on day 1 of life. No stratum corneum is present and the nucleated epidermis is thin. **B,** Epidermis of a 26-week-gestational-age infant on postnatal day 10. *(Reproduced from Rutter N: Percutaneous drug absorption in the newborn: hazards and uses,* Clin Perinatol *14:911, 1987.[158])*

PROBLEMS OF VERY-LOW-BIRTH-WEIGHT INFANTS

Ineffective Barrier

Without the prevention of water and heat loss provided by the stratum corneum, life in a terrestrial environment is impossible. Immaturity of the dermis and the cutaneous appendages poses problems of lesser magnitude for very-low-birth-weight infants. Exposure to air after birth in conjunction with other unknown stimulatory factors results in marked acceleration of barrier maturation (Figure 62-10). This effect is thought to be secondary to xeric stress imposed by the dry environment.[45,46] Similar effects are observed in cultured human skin, where lifting the culture system to an air-liquid interface is required for adequate barrier formation in vitro.[34] After preterm delivery, the normal slow intrauterine rate of epidermal barrier maturation is forgone. Birth of a very-low-birth-weight infant triggers immediate lipid and DNA synthesis, with subsequent cornification of the nucleated epidermal keratinocytes. The rapid transition from the sticky, wet, translucent skin surface of a very-low-birth-weight infant to the dry, opaque stratum corneum of an older preterm infant is familiar to infant caregivers. Such rapid formation of the stratum corneum often results in excessive desquamation and scaling several weeks after preterm birth. Measurements of transepidermal water loss and surface electrical capacitance have been used to track the rate of barrier formation after preterm birth.[53,93] Transepidermal water loss in immature infants (24 to 25 weeks gestation) decreases during the first few days after birth but remains elevated on the twenty-eighth postnatal day at 2 to 4 times the expected levels of 5 to 10 g/m^2/hr in term infants.[53] The etiology of the poor stratum corneum barrier in the infants is unclear but may relate to the extreme rapidity of barrier formation in this vulnerable population. Other skin conditions characterized by rapid epidermal turnover, such as atopic dermatitis and psoriasis, have similar high water loss rates.

The immature epidermal barrier of very-low-birth-weight infants is permeable to substances other than water. Care must be exercised to avoid toxic substances, which can enter the body via a transdermal route.[94,95] Respiratory gases can also move across the epidermal barrier under normal conditions. The immature barrier of a preterm infant, for example, allows the efflux of carbon dioxide[96] and the influx of oxygen[97] in a manner similar to that of water vapor. These findings support the contention that increasing ambient oxygen in a convective incubator during the first few postnatal days may lead to an appreciable augmentation of systemic oxygen. The rapid development of the stratum corneum, however, precludes long-term utility of this novel therapeutic approach.

Strategies for Epidermal Barrier Maturation/Repair

Therapeutic strategies for facilitating epidermal barrier development and protection of the preterm infant are listed in Box 62-4. Prematurity and infection are leading worldwide causes of neonatal morbidity and mortality.[98,99] Preterm infants are vulnerable to infection because of a poorly developed epidermal barrier combined with developmentally immature host defense mechanisms. The first week of life is a vulnerable period, particularly in developing countries, when 50% to 70% of fatal

BOX 62–4

Epidermal Barrier Repair Strategies

- Topical application of one or more nonphysiologic lipids (e.g., petrolatum)
- Topical application of mixtures of physiologic lipids (ceramides, cholesterol, free fatty acids) in appropriate molar ratios
- Topical dressings
- Vapor-permeable: Allow metabolic (repair) processes to continue in the underlying epidermis
- Vapor-impermeable (occlusive): Delay metabolic responses in the underlying epidermis

Modified from Chuong CM, Nickoloff BJ, Elias PM, et al: What is the 'true' function of skin? *Exp Dermatol* 11:159, 2002.[5]

and life-threatening neonatal illnesses occur, many of which are infectious complications associated with preterm birth.[74] Because the consequences of systemic infections are so devastating in premature and low-birth-weight infants, therapeutic options for preventing bacterial and fungal infections assume high priority as a means for improving neonatal outcomes.[98]

One of the earliest methods of improving outcome in preterm infants was the provision of a thermal neutral environment with increased ambient humidity.[100-102] Ambient humidity is inversely related to transepidermal water loss as a function of gestational age.[103] Increasing the humidity within a convective incubator, for example, creates a microenvironment in which transepidermal water loss is lessened. A similar microenvironment can be created under semipermeable membranes with the infant lying beneath radiant warming devices. High humidities, however, may result in "rain-out" with obscuration of an infant. Warm, wet environments are also associated with an increased risk for infection. Radiant warming devices increase transepidermal water loss and require a concomitant increase in fluid replacement rates. Recent studies have demonstrated that the increase in transepidermal water loss with infrared warmers is due entirely to the low humidity of the overlying air rather than any direct drying effect of the infrared radiation.[104]

Evaporation of amniotic fluid at the time of delivery coupled with an incompetent epidermal barrier may create severe fluid imbalances and heat loss in newborn infants. Vohra et al[105] studied the use of polyethylene films on premature infants in the delivery room. This study reported better temperature stability and a significant decrease in mortality in extremely-low-birth-weight infants treated with vapor-reducing occlusive membranes at birth. This approach highlights the "golden hour" concept of care originally developed for treatment of adult patients after stroke and myocardial infarction.[106] Unlike the latter events, however, the birth of a preterm infant often can be anticipated and prepared for with emergency personnel and equipment. Attention to environmental temperature, humidity, adhesive application, and the presence or absence of surface biomaterials such as vernix play a role in delivery room management. Therapeutic regimens should be tailored to begin in the delivery room with extension to the neonatal intensive care unit (NICU).

One innovative approach for enhancing epidermal barrier function is application of topical lipid-rich emollients to the skin surface.[107] These agents may contain a mixture of nonphysiologic lipids, such as petrolatum or a mixture of physiologic lipids, such as ceramides, cholesterol, and free fatty acids.[108] In addition to their ability to decrease transepidermal water loss and form a mechanical barrier to bacteria, the components of the emollient may serve as a source of material for active lipid metabolism in the epidermis.[109] In moderately preterm infants, petrolatum application to the skin surface has been reported to improve barrier function with concomitant reduction of the rate of nosocomial infections.[110] A large study of more than 1200 low-birth-weight preterm infants demonstrated an increase in late-onset nosocomial infections secondary to coagulase-negative staphylococci in extremely-low-birth-weight infants (500- to 750-g birth weight).[111] More therapeutic strategies involving topical emollient application intended to facilitate epidermal barrier development are needed.

In many developing countries, natural oils are applied topically after birth as part of a traditional oil massage regimen. A recent study by Darmstadt et al[112] examined the effects of a variety of naturally occurring vegetable oils on the skin barrier. The goal was to identify safe, inexpensive vegetable oils available in developing countries that might improve epidermal barrier function in very-low-birth-weight infants. When vegetable oils were applied to barrier-compromised skin of adult hairless mice, oils rich in linoleic acid enhanced epidermal barrier formation. In contrast, mustard oil, a topical emollient used routinely in newborn care throughout South Asia, had toxic effects on the epidermal barrier. The investigators noted that emollients containing a physiologic balance of epidermal lipids (3:1:1 molar ratio of cholesterol, ceramide, palmitate and linoleate) may be optimal for barrier repair.[108] Vegetable oils are readily available worldwide. Such oils provide a simple, inexpensive, and effective alternative for topical use. Clearly, this is a research area requiring further investigation.

Visscher et al[113] studied the effects of semipermeable films on human skin after a standardized superficial wound (removal of the stratum corneum by tape stripping), which mimics the immature epidermal barrier of the premature infant. Transepidermal water loss, skin hydration, rate of moisture accumulation, and erythema were measured. Wounds treated with semipermeable films had more rapid barrier recovery than either nonoccluded wounds or wounds under complete occlusion. This finding is consistent with a role for vapor permeability to regulate epidermal barrier formation.

Therapeutic strategies may result from a combination of individual methods optimized for a specific infant.[13] Prenatal steroid administration coupled with optimal delivery room management and a seamless transition to a controlled NICU environment using physiologic emollients and minimal adhesive injury may provide the optimal therapeutic interface between the preterm infant and the care environment.

Many questions remain unanswered. For example, the efficacy of prenatal glucocorticoid therapy to accelerate epidermal barrier maturation is well documented for rodent skin and for fetal lung maturation.[18-21,114] Studies in the neonatal clinical literature provide conflicting reports on the efficacy of steroids in mature fetal epidermis.[19] Similarly, the use of topical emollients has been questioned for very-low-birth-weight preterm infants.[111] Work thus far suggests that provision of a semipermeable membrane or dressing may be optimal for promoting epidermal maturation.[113] In contrast to petrolatum ointment, vernix is highly vapor permeable and may provide a more physiologically relevant prototype for development of a natural wound dressing for use in the low-birth-weight preterm infant. "Smart fabrics" with heat- and motion-activated transfer of topical emollients and wound-healing ointments to the skin surface have been developed for treatment of diaper rash.[115,116] An attractive concept is that clothing or bedding material, in addition to wound dressings, may contain physiologic emollients and growth factors for facilitation of epidermal barrier development. All these therapies draw attention to the role of the skin in environmental coupling and the close interconnection between the human skin surface and the caregiver.

THE SKIN AS A NEURODEVELOPMENTAL INTERFACE

In all vertebrates, touch is the first sense to develop, followed closely by vestibular or position sensing.[117] Human neonates, compared with neonates of other species, is relatively helpless in motor capabilities and relatively precocious in sensory capabilities.[118] Sensory and affective information is necessary for body orientation and the spatial organization required for proper motor output. Evaluation of sensory competency is difficult, however, and neonatal outcome studies typically use developmental scoring systems, which rely heavily upon tests of motor skills or behavior. The idea that the sensory system is precocious in early human development places the skin in a strategic location to affect subsequent development.[4] As an interface between the body and the environment, the skin links to the developing brain on the one hand and to external factors including light, heat, fabrics, and the interactions of caregivers and parents, on the other hand. Neonatal animals, such as kittens and rodents, have proved to be useful models to study the effect of sensory inputs on central nervous system development.[119-123] These models clearly suggest that sensory signals are required during critical developmental windows for proper central nervous system maturation. In humans, clinical studies have demonstrated various effects of tactile stimulation during infancy. Field et al[124] showed that tactile stimulation of hospitalized preterm infants results in greater weight gain and higher behavorial scores.

Cutaneous Receptors and Electrical Maturation

A useful concept for interpreting the functional role of the skin surface is the idea (derived from the engineering sciences) of a "smart material" interface.[125,126] The outermost layer of the epidermis, the stratum corneum, has many of the attributes of a smart material.[4,11] It is strategically positioned as part of a larger functional system and adapts readily to changes in the environment. This material is self-cleaning and self-assembling and possesses sensing and actuating properties putatively secondary to the piezoelectric and piezomechanical properties of the contained keratin filaments.[127] In its role as the limiting surface of the body, the stratum corneum simultaneously forms the perceived surface of the organism and the biologic boundary with the environment. Thus the stratum corneum and, by extension, the skin in general corresponds in a simple manner to the distinction between subject and object.[4]

Most textbooks on neurophysiology consider sensory signal processing to be mediated initially by specialized nerve endings (Figure 62-11). Nerve endings, however, never directly touch the environment. Interfacing with the environment is a function of the material in which the nerve endings are embedded.[4] This perspective applies an engineering view to the material properties of the skin surface[125] and is important for understanding innovative approaches to skin–central nervous system interactions as put forth by Ansel et al.[128] Thus the stratum corneum and the epidermal/dermal matrix are potential mediators of sensory signal processing. After birth, the physical properties of the epidermis change rapidly during adaptation to a gaseous environment.[93] Biophysical properties of the skin can be easily measured with noninvasive instrumentation.[129] These properties include skin viscoelasticity, hydrophobicity, thermal conductivity, color, transepidermal water loss, and pH. In particular, study of the electrical properties of the skin may be relevant for very-low-birth-weight preterm infants.[93] Table 62-5 shows the measured electrical resistance to direct current in human skin (adult) and various commonly used animal models.[130] Only newborn rats have an electrical resistance comparable with that seen in adult humans. By contrast, the electrical resistance of the epidermal barrier in very-low-birth-weight preterm infants is likely to be low owing to relative deficiency of vernix and stratum corneum.

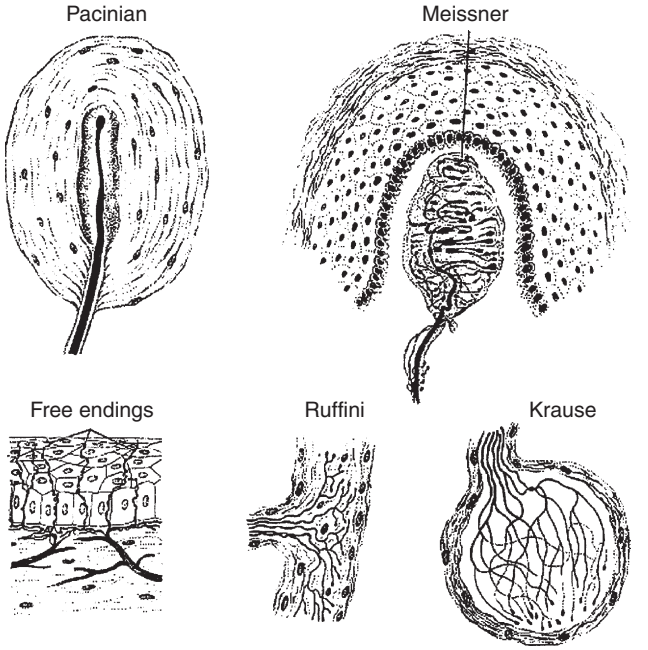


Figure 62-11 Types of cutaneous nerve endings. Pacinian corpuscles are deep cutaneous receptors for crude touch and vibration on both hairy and glabrous skin. Meissnerian corpuscles are superficially located, rapidly adapting nerve endings modulating light touch and vibration on primarily glabrous skin. Free nerve endings are associated with crude touch, tickle, and itch sensations. Ruffini organs are deep, slowly adapting structures for pressure sensing. The Krause end bulb is particularly associated with the perception of cold. Nerve endings typically are embedded in a material biomatrix and never touch the environment. The biomatrix surrounding the nerve endings directly contacts the environment.

TABLE 62–5

Electrical Resistance to Direct Current in Skin

Skin Specimens	Effective Resistance (kohms/cm^2)
Excised human skin	135 ± 54.0 (n = 40)
Newborn rat (day 0)	124 ± 24.2 (n = 18)
Infant rat (day 6)	27.7 ± 8.9 (n = 9)
Adult rat	28.3 ± 6.8 (n = 4)
Hairless mouse	9.3 ± 5.3 (n = 10)

Data from Shivanand P: Electrical and transport properties of neonatal rat skin [PhD thesis]. In *Division of Research and Advanced Studies,* College of Pharmacy, Cincinnati, Ohio, University of Cincinnati, 1995.[130]

Wakai et al[131] demonstrated the development of a high electrical impedance barrier in utero during the last trimester of pregnancy. During the first half of gestation, the amplitude of the fetal electrocardiogram can be measured directly from the surface of the maternal abdomen. After 26 to 27 weeks gestation, the amplitude gradually disappears, concomitant with the intrauterine development of an epidermal barrier consisting of vernix caseosa and the stratum corneum. Thus the gradual disappearance of the fetal electrocardiogram from the maternal skin surface is secondary to a film of high electrical impedance developing between fetus and amniotic fluid. This process presumably reflects growing fetal autonomy with electrical isolation of fetus from mother.

After birth, the electrical resistance of the skin of the term newborn is relatively high.[132] Surface electrical measurements are a common form of noninvasive monitoring in NICUs. New advances in monitoring, such as evoked potential testing of the brain, hold great promise for the assessment of multiple clinical conditions such as birth asphyxia.[133,134] In contrast to commonly performed electrocardiographic measurements, however, evoked potential testing of the electrical activity of the brain requires measurement of low amplitude voltages (microvolts versus millivolts), which are greatly affected by the skin-electrode contact surface.[130] It is common practice for electrical signals to be obtained after abrading the skin surface with a gritty contact paste. From a practical standpoint, the field of evoked potential testing in newborns could be improved by focus on the electrode/skin interface and the development of a seamless electrical contact, which avoids wounding the skin.

Skin as an Information-Rich Surface

Development of the immature central nervous system depends on sensory input during the immediate postnatal period. Experimental interference with several sensory modalities, such as vision, touch, and hearing, results in profound anatomic, functional, and biochemical impairment to the central nervous system structures that regulate such modalities. In newborn rats, tactile stimulation is an important regulator of somatic growth. Schanberg et al[121-123] have studied the link between tactile stimulation and the molecular regulation of internal cellular growth promoting enzymes, such as ornithine decarboxylase (ODC). ODC is a sensitive index of the maturation and growth of internal organs such as the heart, liver, and brain. Brain, liver, and heart ODC levels are decreased by 35%, 81%, and 53%, respectively, when rat pups are removed from their mothers for periods as short as 1 hour. Restoration of the pups to the litters rapidly normalizes enzyme activity. This normalization is specific for touch. Other potential mediators, such as nutrition, have no effect.

In other work, tactile stimulation of neonates influences circulating levels of lactate, an important energy substrate for brain metabolism.[119] In both newborn rats and human infants, lactate levels are high immediately after birth and decrease rapidly after the first few hours of age.[119,135] When newborn rat pups are removed from maternal contact and receive light tactile stimulation by means of rostral-caudal stroking with a camel hair brush (similar to the methodology established by Schanberg[122]), lactate levels increase significantly and the elevations persist for up to 30 minutes after cessation of the tactile stimulus. These same stimuli fail to elicit an increase in serum lactate at 1 week of age. These results are noteworthy because the brain of the early suckling rat uses lactate in preference to other metabolic fuels like glucose and 3-hydroxybutyrate.[136] Moreover, lactate levels increase without development of hypoxic metabolic acidosis. This experiment demonstrates, in an animal model, that sensory interaction between the organism and the environment is a potential regulator of the availability of cerebral energy substrates. Such studies have yet to be performed in humans.

The concept that human newborn infants is precocious in terms of sensory capabilities and relatively helpless in terms of motor capabilities[118] provides a conceptual framework within which to investigate the complex maturation of sensorimotor feedback loops. Preterm infants exhibit an age-dependent neurophysiologic response pattern similar to newborn rats insofar as the threshold for eliciting a motor response (flexor withdrawal) progressively increases with advancing gestational age (Figure 62-12).[137] This finding is usually attributed to increased inhibition of the reflex arc by central nervous system structures.

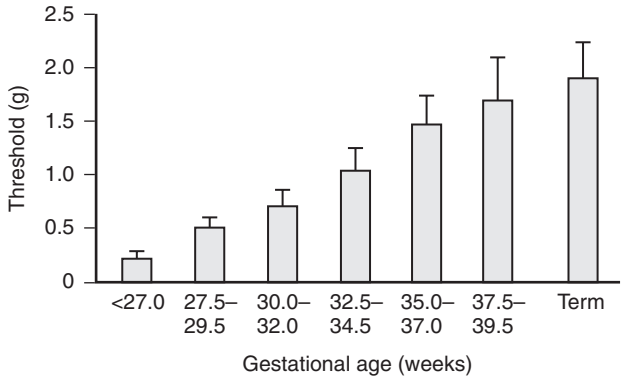


Figure 62-12 Threshold stimulus for eliciting flexor withdrawal in neonates of varying gestational age. Flexor withdrawal is elicited by stimulation of the foot with nylon filaments (von Frey hairs) of graded thickness. Preterm infants exhibit progressively lower thresholds for withdrawal than term infants. *(Data from Fitzgerald M, Shaw A, MacIntosh N: Postnatal development of the cutaneous flexor reflex: comparative study of preterm infants and newborn rat pups,* Dev Med Child Neurol *30:520, 1988.[137])*

The cutaneous flexor response is elicited by stimulation of the foot with nylon filaments (von Frey hairs) of graded thickness. In addition to maturation of central nervous system structures, the material properties of the interface (dermis, epidermis/stratum corneum) are changing concomitantly and may contribute to the effect observed in Figure 62-12. The accessibility of the skin surface and the availability of multiple biomedical instruments for noninvasive measurement[129] provide an open area for skin-based research in neonatology, with potential relevance for central nervous system organization and control. The concept of a smart material interface presumes that changes in the biophysical properties of the skin surface will have a direct influence on adaptive environmental interfacing and sensorimotor response loops.[4,9,126]

An area in which noninvasive monitoring of the skin surface may reveal important information on central nervous system response and behavioral state involves skin conductance measurements in newborn infants associated with eccrine (emotional/nonthermal) sweating.[57] Sweating is commonly viewed as secondary to the need for thermoregulation and evaporative cooling. It also occurs in response to arousal and pain in addition to temperature. This "emotional" sweating is easily measured from the palm or sole using a skin evaporimeter[138] but is usually estimated indirectly by measuring skin electrical conductance or resistance.[139] The presence of sweat within the eccrine ducts of the epidermis, and, to a lesser extent, the circumferential hydration of the stratum corneum surrounding the sweat glands, lowers the electrical resistance of the skin and raises its electrical conductance. Various instruments are now available for directly, simply, and noninvasively measuring skin surface conductance at the bedside. This technique has been widely used in psychological research for many years but has yet to be systematically applied in neonatology.

Figure 62-13 shows the increase in skin conductance after a painful stimulus.[139] In this case, a heel prick was administered to the foot, followed by skin conductance measurement over the sole of the other foot. Peak values are reached at approximately 1 minute after the heel prick. This response is characteristic of the change in skin conductance caused by arousal in a neonate. Confirmatory measurements also can be made using different techniques, such as transepidermal water loss. Figure 62-14 shows

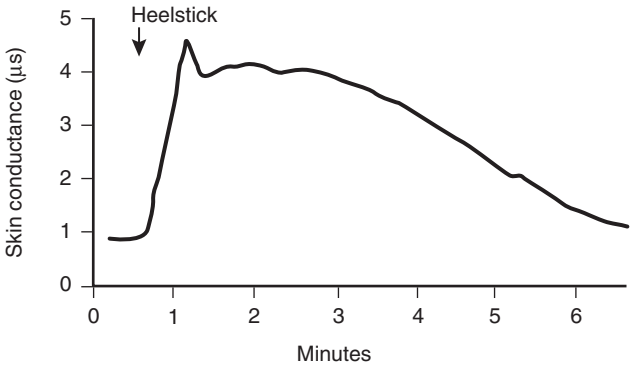


Figure 62-13 Effect of painful stimulus on skin conductance measurements in a term newborn infant. Skin conductance measured on the sole of the foot shows a sharp increase over the first minute in response to a heel prick on the other side. Values are in microsiemens (mcS). *(From Gladman G, Chiswick ML: Skin conductance and arousal in the newborn,* Arch Dis Child *65:1063, 1990.[139])*

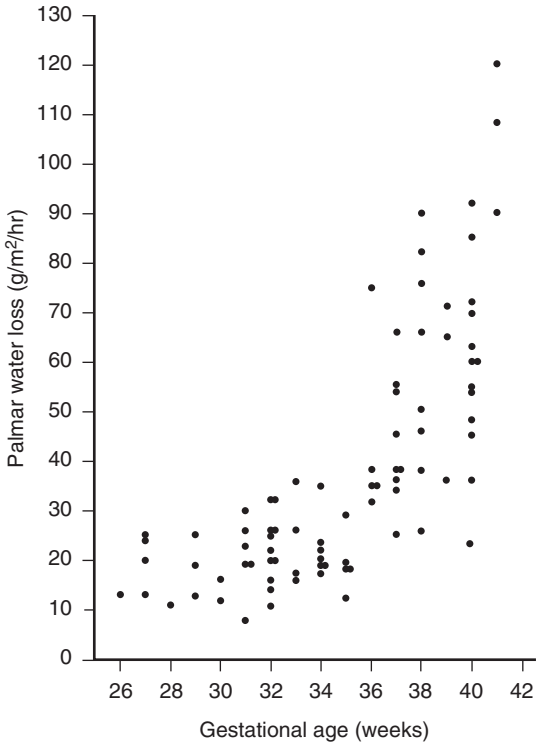


Figure 62-14 Palmar water loss in infants ranging from 25 to 41 weeks gestation, showing values at maximal arousal (vigorous crying). Results are displayed according to equivalent gestational age (gestation plus postnatal age). Values are 10 to 30 g/m^2/hr until the equivalent gestational age of 36 weeks is reached. Values then increase abruptly to 30 to 100 g/m^2/hr. *(From Rutter N: In Hoath SB, Maibach H [eds]:* Neonatal Skin: Structure and Function*, New York, Marcel Dekker, 2003.[57])*

palmar water loss in crying infants ranging from 25 to 41 weeks gestational age.[57,140] Of note, the profile for palmar water loss is *inversely* related to transepidermal water loss over the same age range (see Figure 62-4). This inverse relationship deserves further investigation. Recently, Storm[141,142] and Storm and Fremming[143] used conductance measurements of the skin surface to study behavioral state changes and developmental effects. This skin-based method, combined with sensory evoked potential testing, offers new techniques for assessment of central nervous system functioning in developing preterm infants.

Studies of epidermal barrier function may exhibit not only short-term responses secondary to autonomic nervous system activation of eccrine sweating, but also long-term consequences related to stress and glucocorticoid secretion. Denda et al[144,145] showed that stressful events such as immobilization, overcrowding, and abrupt change in physical environment can result in impaired epidermal barrier function in murine skin. Similarly, Garg et al[146] demonstrated a delay in recovery of epidermal barrier function after induced trauma in a group of psychologically stressed graduate students. These studies indicate a link between physiologic stress of the organism and epidermal barrier function. As presented earlier, a very-low-birth-weight preterm infant exhibits compromised barrier function up to 28 days after delivery.[53] Whether this increased transepidermal water loss is secondary to stress-related events is unclear but worthy of further investigation.

The idea of the skin as an information-rich surface can be extended to the intensive care or emergency room setting.[147,148] Indeed, cardiorespiratory and thermal monitoring, as well as transcutaneous blood gas measurements, are skin-based systems of clinical data retrieval. Given the ready accessibility of the skin surface and the plethora of noninvasive measurement techniques that have yet to be applied to the study of newborn physiology,[129] it seems reasonable to assume that a number of innovative and useful skin-based sensing systems will be designed for clinical decision making in the future. This concept extends Kligman's idea of "invisible dermatology"[149] to the NICU; early, objectively measured changes in the physical properties of the skin may herald illness in the term or preterm infant *before* such illness is apparent to the caregiver. De Felice et al,[150] for example, recently used skin colorimetry to provide a significant quantitative predictor of illness severity in hospitalized newborns. The work supports the hypothesis that noninvasive measurements of skin physical properties directly reflect the pathophysiologic state of infants. Such measurements can be used as objective adjuncts to clinical decision making and bedside assessment.

FUTURE DIRECTIONS

A better understanding of fetal and neonatal skin physiology and the targeted use of quantitative measures of skin structure and function is a laudable and needed goal for perinatal medicine.[44] Every patient care encounter involves the skin in some practical way: temperature regulation, electronic monitoring, bathing, infection control, touch and infant massage, skin-to-skin contact, pain management, catheter care, etc. Box 62-5 lists a number of roles of the skin in the provision of primary health care in neonatology. These functions are disease-independent but essential for the delivery of high quality care, whether the health care environment is hospital based or home based.

Importantly, the recognition of the skin as a primary care interface for patient care and parental satisfaction creates an opportunity for collaborative practice between nursing and medicine.[151] Many of the functions listed in Box 62-5 are scientifically complex and fall largely in the domain of nursing. The reciprocal coupling of the infant and the care environment is an assumption of fields such as "developmental care," which focus largely on brain maturation and neurodevelopment.[152-154] The dual nature of the skin as a boundary interfacing cellular and molecular domains with psychological and perceptual domains is consistent with this approach. A better understanding of skin development, especially the role of the epidermal barrier and

BOX 62–5

Aspects of the Skin as a Primary Care Interface

- Surface of interaction with soaps, surfactants, disinfectants, and bacteria
- Support for tapes and other adhesives
- Interface for bedding, clothing, and the environment
- Site of action of topical anesthetics and analgesics
- Surface for wound and ostomy care
- Barrier for transdermal drug delivery
- Site of most laboratory blood drawing
- Platform for percutaneous catheters
- Boundary for noninvasive monitoring and skin-based sensing techniques
- Medium of interaction in kangaroo care and massage therapy
- Basis for initial clinical evaluation of patient well-being (appearance)

Modified from Hoath S, Narendran V: Development of the epidermal barrier. *Neoreviews* 2:e269, 2001.[44]

infection control, has implications for improving infant care in developing countries.[155,156]

Finally, the critical role of the epidermal barrier, the plethora of antimicrobial peptides in innate immune function, and the early events in sensory transduction have scarcely been investigated in infants. Human skin is unique in all primates. Why? What is the connection between the skin and the brain? It is scientifically incontrovertible, for example, that the crosslinked envelope of the cornified epidermal cell is a single, extended, multigene molecule strategically located at the organism-environment interface.[12,22,23] This molecule forms the physical scaffolding of the stratum corneum and participates directly in multiple care interactions between a newborn and a caregiver. Successful postnatal transition of a very-low-birth-weight preterm infant requires the rapid formation and assembly of an organized, polymeric, molecular interface with the environment. These facts place the differentiated structure of the epidermis, an ectodermal derivative like the brain, front and center in the translation of molecular biology to clinical bedside care.

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SECTION XI

Fetal and Neonatal Cardiovascular Physiology

63

Brian S. Snarr, Andy Wessels, and Tim C. McQuinn

Cardiovascular Development

The morphologic development of the heart from its first appearance as a tube heart to a four-chambered structure with fully septated ventricles occurs between days 20 and 44 of human gestation (Carnegie stages 9 to 18).[1] The same program of development occurs much more rapidly in the mouse (days 8.5 to 14 of gestation) and chick (days 1.5 to 5.5).[2,3]

Externally visible cardiac development starts with formation of the heart tube in a position cranial to the anterior intestinal portal of the embryo, but many critical determinative events occur before that. The earliest contributions to cardiogenesis are made during gastrulation.

Gastrulation creates the three cell layers of the embryo—ectoderm, mesoderm, and endoderm—by migration of cells through the streak, a visible groove that begins at the caudal end of the embryo and grows toward the cranial end. At the cranial tip of the streak is a structure called the node, or Hensen node, in chick embryos.[4] The cranial-caudal (anterior-posterior) axis of the embryo is first made apparent by the streak, and the node is a critical element in right-left axis determination. Cells from the embryonic epiblast migrate to the streak and pass into the interior of the embryo to form the mesoderm.

Precardiac cells gastrulate as a relatively broad swath in which the most anteriorly gastrulating cells contribute to the most anterior portion of the primitive heart tube.[5,6] Precardiac cells enter the streak uncommitted as to their developmental potential and only become committed to their cell type or migratory pathways in the mesoderm after leaving the primitive streak.[5] Once in the blastocoele cavity, the precardiac cells move laterally to join the lateral plate mesoderm at the level of the node. The lateral plate mesoderm then splits into two layers, a splanchnic layer directly above the endoderm and a somatic layer directly below the ectoderm. The endoderm and hypoblast induce splanchnic mesodermal cells to enter the precardiac lineage through mechanisms known to include a combination of Wnt, fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and activin/nodal signaling pathways.[7]

The region of splanchnic mesoderm expressing precardiac markers is known as the heart-forming field.[8] Whether the earliest precardiac mesoderm consists of a single lineage or dual lineages is not entirely clear. Before 2001, the molecular markers most widely used to identify precardiac mesoderm (transcription factors such as NKX2-5, MEF2, HAND1 and HAND2, GATA4, and TBX5)[9-13] indicated that there were bilateral populations of precardiac cells that migrated to the midline and added myocardium sequentially to the venous pole of the heart. However, classic embryologic experiments in chick embryos suggested the presence of a population of myocardium that was added to the heart tube by migration from the arterial pole of the heart,[14] and recent molecular, morphologic, and genetic observations indicate that these cells originate from a distinct set of precardiac mesodermal cells now known as the second heart field (SHF), in distinction to the previously recognized first heart field (FHF).[15-18] Molecular markers for the SHF in the mesoderm include the transcription factors TBX1 and ISL1 as well as FGF8 and FGF10. However, it is not clear whether SHF and FHF cells pass through a common precursor lineage.[19] SHF-derived myocardium is the source of all the outflow myocardium of the primitive heart, whereas the left ventricular myocardium is derived entirely from the FHF. All other segments of the heart receive a mixed complement of FHF and SHF cells. SHF mesoderm also differs from current understanding of FHF mesoderm in its pluripotentiality; FHF cells exhibiting the premyocardial markers noted previously give rise only to myocardium, whereas SHF cells give rise to myocardium, vascular smooth muscle cells, and endothelial cells.

PRECARDIAC MESODERM AND THE AXIAL BODY PLAN

The cranial-caudal and left-right axial body plans are determined during gastrulation, and both are of major importance in establishing the molecular identity and the morphology of the cardiac segments. Left-right axis determination is a critical element of the vertebrate body plan,[20] and substantial impairment of cardiac structure and function occur in the absence of normal left-right axis determination, with the atria, atrioventricular (AV) junction, and outflow tract showing greater sensitivity than the ventricular chambers.[21-28] The signaling mechanisms identified include critical elements of cell movements and oriented ciliary motion in the node.[29-31] FGF influences this process through a role in ciliary growth,[32] and the ciliary movements lead to asymmetric calcium transients[33] as well as to asymmetric expression of nodal, sonic hedgehog, and lefty.[34-36] These signals in turn lead to asymmetric expression of the homeodomain transcription factor PITX2C.[25,27,28,37] As is true in general for developmental processes, most of the molecules involved in elaborating the left-right signaling process in mice have easily recognized counterparts in birds, *Xenopus* species, and other vertebrates and invertebrates. However, in an intriguing twist on that theme, some of the molecules required for left-sidedness in mice are determinants of right-sidedness in birds.[38] Many of the genes implicated in the mouse models are candidate genes for heterotaxies in the human population.[36]

The anterior-posterior (cranial-caudal) body axis is also established during gastrulation. Retinoid signaling pathways are critical to normal anterior-posterior axial patterning in general and cardiac development in particular.[39,40] Retinoids are also implicated in the regulation of SHF differentiation.[41] Failure of formation of atrial segments and systemic venous connections with the heart are observed in conditions of retinoid deficiency. Excess retinoids create cardiac malformations, often involving the outflow tract, and result in ventricular expression of several genes that are normally largely restricted to the atria at the equivalent stages of normal development.[42,43]

FORMATION AND GROWTH OF THE TUBE HEART

The precardiac mesodermal sheets on each side migrate to the midline cranial to the anterior intestinal portal, forming a horseshoe-shaped crescent (Figure 63-1). Precursors of the endocardium follow the same migratory pathways as the precardiac cells. As the precardiac cell masses move steadily to the midline, the endocardial cells begin to form a network of tiny channels that will coalesce into a complex endocardial network surrounded by a myocardial mantle. In normal development, the endothelial network quickly transforms into a single endothelial channel invested by a single myocardial mantle.

The tube heart is initially organized into three structural layers: a muscle layer two to three cell thick, an intermediate layer of acellular cardiac jelly, and a luminal passage formed by endothelial cells. The tube heart is a ventral structure and is initially tethered throughout its length on its dorsal aspect by the dorsal mesocardium. The dorsal mesocardium will regress in its center portion but remain attached at the arterial and venous poles as the heart grows in length and begins to curve into a C-shaped structure. As the length of the tube further increases, it assumes a more complex S-shaped bend. The tube is lengthened by migration of cells into both the arterial (SHF) and venous (FHF and SHF) poles of the heart (Figure 63-2).

Looping is a somewhat ill-defined term, referring in the most general sense to the bending of the heart tube before septation.[44] The initial bend results in a C-shaped structure, with the outer loop of the C to the right side of the embryo. As the heart elongates, the loop becomes more complex and has been described as S shaped (see Figure 63-1). Looping (in the context of this chapter) begins and ends with the AV junction connected solely to the left ventricle and the outflow tract connected only to the right ventricle.

The looped heart has an inner curvature and an outer curvature. By nature of the tight angulation of the inner curvature, the primitive atrial, AV, ventricular, and outflow segments are in close proximity for the remainder of cardiac development (Figure 63-3). The inner curvature is the site of the intricate morphologic events resulting in the right ventricle acquiring its inflow and the left ventricle its outflow—a morphologic process sometimes labeled *convergence*.

Concomitant with the realignment of the cardiac segments, the lumen of the heart begins to develop distinctive features along its length. While the atria and ventricles lose their cardiac jelly, an increase of cardiac jelly in the AV junction and the outflow tract develops into discrete mounds known as *endocardial cushions* (see Figure 63-3). The acellular jelly becomes populated by mesenchymal cells derived from various sources, the significance of which is discussed later. In both the AV junction and outflow tract, the cushions contribute to the propulsion of blood and protect against regurgitation.

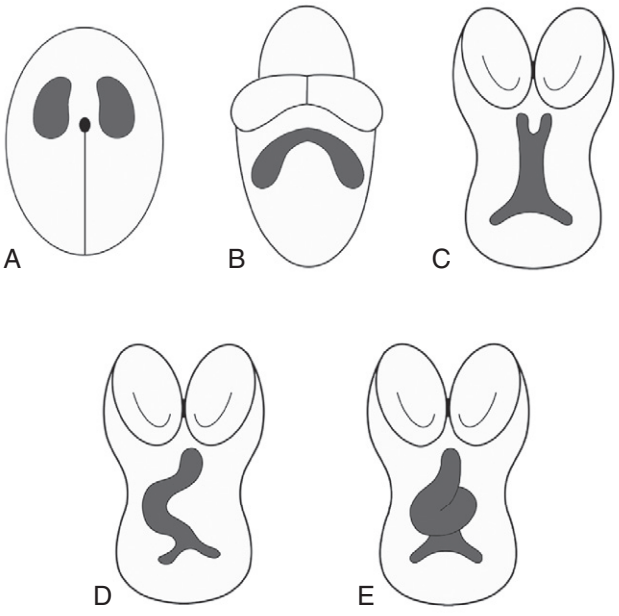


Figure 63-1 Schematic representing the early stages of heart development. **A,** Bilateral heart fields form from precardiac mesoderm after migration from the primitive streak. **B,** Formation of the cardiac crescent after fusion of the heart fields at the midline. **C,** The linear heart tube forms from fusion of the cardiac crescent. **D,** Regression of the dorsal mesocardium results in the formation of a C-shaped structure and permits further looping. **E,** The S-shaped primitive heart formed on completion of looping. *(Modified from Snarr BS, Kern CB, Wessels A. Origin and fate of cardiac mesenchyme,* Dev Dyn *237:2804-2819, 2008.)*

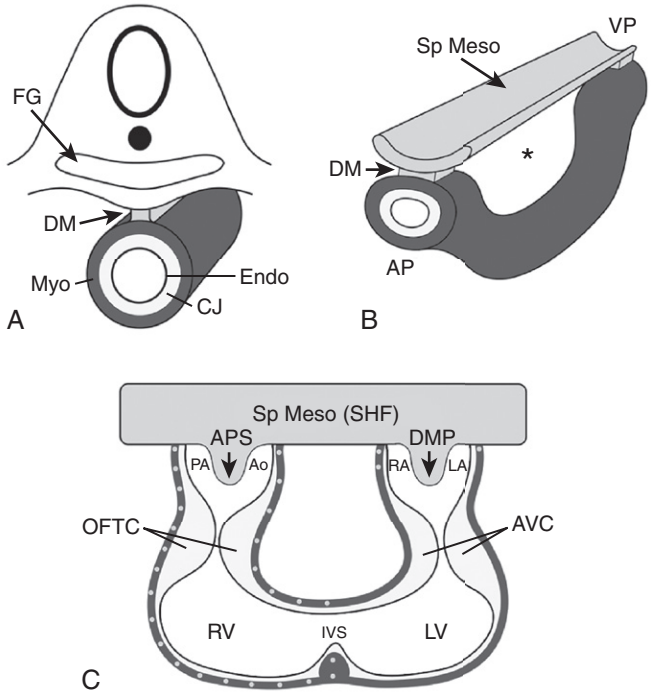


Figure 63-2 Involvement of the dorsal mesocardium in heart development. **A,** The heart tube is suspended along its length from the embryo by the dorsal mesocardium *(DM)*. **B,** After regression of the DM along the midsection *(asterisk)*, the heart tube remains connected to the embryo by the arterial and venous pole *(AP, VP)* attachments of the DM. The DM is continuous with the splanchnic mesoderm *(Sp Meso)* that lies ventral to the foregut *(FG)*. **C,** Cell populations derived from the Sp Meso, known as the second heart field *(SHF),* are added to the AP and VP of the heart through the DM attachments. These cells contribute to the developing aorticopulmonary septum *(APS)* and the dorsal mesenchymal protrusion *(DMP)*. *Ao,* Aorta; *AVC,* atrioventricular cushions; *CJ,* cardiac jelly; *Endo,* endocardium; *IVS,* interventricular septum; *LA,* left atrium; *LV,* left ventricle; *Myo,* myocardium; *OFTC,* outflow tract cushions; *PA,* pulmonary artery; *RA,* right atrium; *RV,* right ventricle. *(Modified from Snarr BS, Kern CB, Wessels A. Origin and fate of cardiac mesenchyme,* Dev Dyn *237:2804-2819, 2008.)*

On the luminal surface of the heart, the fold in the heart at the inner curvature results in a small muscular ridge inside the heart between the AV junction and the outflow tract called the *ventriculoinfundibular flange* or *ridge*. Other key landmarks are the two endocardial cushions of the AV junction and two endocardial ridges of the outflow tract. The inferior endocardial cushion is attached to the dorsal AV myocardium, and the superior endocardial cushion is attached to the ventral AV myocardium. As convergence proceeds, small right and left lateral AV endocardial cushions become visible.

Two outflow tract endocardial cushions form extended spiral ridges from the end of the truncus distally to the body of the right ventricle caudally. The parietal endocardial ridge makes contact with the right lateral endocardial cushion, which itself becomes continuous with the superior endocardial cushion. The septal endocardial ridge will make contact with the inferior endocardial cushion. As the endocardial cushions fuse during septation, they create an unbroken chain of cushion tissue extending from the AV junction to the distal extent of the myocardial outflow tract and as a simplified description can be thought of as a tissue zipper that septates the heart. The endocardial cushion origins of the outlet septum and much of the ventricular septum are not apparent on inspection of the mature heart owing to the replacement of cushion mesenchyme by myocardium, in the process known as *myocardialization*.[45]

During convergence, the angle of the inner curvature of the heart becomes even more acute. The AV endocardial cushions approach each other in the center of the lumen of the AV junction, and the right atrial–right ventricular AV junction forms by rightward expansion of the AV orifice. The outflow endocardial ridges, although unfused, define distinct aortic and pulmonary channels. The aortic channel moves leftward and anterior to its original position. Because of the combined rightward expansion of the AV orifice and the leftward movement of the aortic outflow tract, the acute angle of the inner curvature now defines the region where the AV junction and the outflow tract are in continuity. These same morphogenetic movements result in rotation of the outflow ridges to a plane that more closely parallels the growing muscular septum.

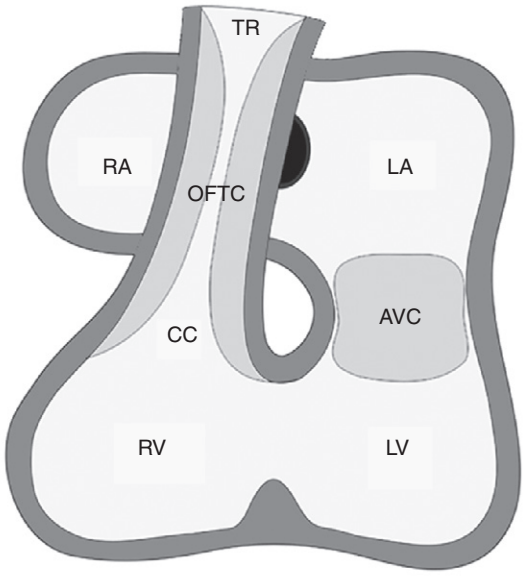


Figure 63-3 Schematic representation of the looped heart with chamber alignment in series. The cardiac jelly accumulates into discrete mesenchymal structures forming the endocardial atrioventricular cushions *(AVC)* and the outflow tract endocardial cushions *(OFTC)*. The *OFTC* may further be subdivided into proximal (conal cushions, *CC*) and distal (truncal ridges, TR) segments. *LA,* Left atrium; *LV,* left ventricle; *RA,* right atrium; *RV,* right ventricle. *(Modified from Snarr BS, Kern CB, Wessels A. Origin and fate of cardiac mesenchyme,* Dev Dyn *237:2804-2819, 2008.)*

MESENCHYMAL CONTRIBUTIONS TO ATRIAL AND VENTRICULAR SEPTATION

Septation of the atrial and ventricular chambers is dependent on the proper formation of a mesenchymal complex of endocardial cushion tissues that becomes continuous from the AV junction to the outflow tract. Multiple lines of evidence now show that important contributions to the cellular population of the cushions are made by both endothelial-to-mesenchymal transformation of the endocardial lining and extracardiac cell populations. In the outflow tract, this includes cell populations derived from the neural crest as well as anterior splanchnic mesoderm (SHF). The AV cushions receive a mesenchymal cell contribution from the persisting venous pole attachment of the dorsal mesocardium, which derives from posterior splanchnic mesoderm, also part of the SHF (see Figure 63-2, *C*). Studies to this point clearly suggest that failure to incorporate these extracardiac cell populations into the cushions results in abnormal septation.[46]

ATRIAL SEPTATION

Atrial septation and connection of the common pulmonary vein to the left atrium are closely related events in the normal heart.[23] Recent investigations in mouse, chick, and human embryos highlight the importance of the dorsal mesocardium to these events. At the site where the dorsal mesocardium is in continuity with the atrium (about the level of the lung buds), there is a protrusion of mesenchymal tissue into the atrium. This tissue has been recognized for many years and by a variety of names—most recently, the *dorsal mesenchymal protrusion*, the terminology adopted here. The dorsal mesenchymal protrusion extends ventrally into the common atrium, forming the mesenchymal base of the developing septum primum. From the earliest stages at which it can be recognized, the dorsal mesenchymal protrusion is continuous with two endocardium-derived cell populations: the mesenchymal cap that extends along the tip of the septum primum (anteriorly) and the inferior AV cushion (posteriorly). In contrast to its endocardium-derived cushion cell counterparts, the dorsal mesenchymal protrusion undergoes a mesenchymal-to-myocardial differentiation and forms the myocardial base of septum primum. The importance of the dorsal mesenchymal protrusion has gained recent attention as studies have suggested abnormal development of this structure is associated with the pathogenesis of complete AV septal defects (Figure 63-4).[46]

The initial (myocardial) portion of the primum septum is derived from left atrial myocardium, as determined by molecular markers distinguishing between left and right atrial myocardium.[23] Growth of primum septum occurs by myocardial lengthening, and as it grows, the mesenchymal cap, dorsal mesenchymal protrusion, and major AV endocardial cushions come into close proximity and fuse. Fusion of these structures closes the primary interatrial foramen. Before the closure of the primary interatrial foramen occurs, the foramen secundum forms in the body of the septum primum. In humans, this process is initiated by the appearance of small fenestrations that increase in number and size until they coalesce into a definitive foramen secundum.[23]

The septum secundum forms as an infolding of the atrial myocardium between the left venous valve of the sinus venosus and the primum septum. The septum secundum marks the site of left

atrial–right atrial myocardial boundary. Left atrial myocardium of the septum secundum exhibits left atrial molecular markers, whereas the right atrial myocardial surface of the septum secundum exhibits right atrial markers.[23,47]

GROWTH OF VENTRICULAR MYOCARDIUM AND DEVELOPMENT OF THE VENTRICULAR SEPTUM

Early formation of the muscular septum of the ventricles and enlargement of the ventricular chambers are closely linked processes.[48] The overall process of ventricular growth has been described as "ballooning" of specialized myocardium with left or right ventricular molecular identity toward the outer curvature of the heart, whereas along the inner curvature, "primitive" myocardium persists, which will make an important contribution to the development of the conduction axis of the heart.[49] Much of the patterning of the primitive and ventricular myocardial molecular identities is linked to the expression of Tbox gene family members such as TBX1, TBX2, TBX3, TBX5, and TBX20.[50-55]

In the chick heart, the primitive muscular septum is initially formed by a coalescence of trabeculae. In the mammalian heart, the primitive muscular septum appears to be the product of infolding of the compact myocardium produced by growth of the ventricular apices. Lineage studies provide indication that the myocardium of the muscular septum is of combined right and left ventricular origins,[56,57] although data have also been presented indicating that the ventricular septum is of exclusively SHF derivation.[58]

The primitive muscular interventricular septum is initially a crescent-shaped structure that extends at its posterior limit to the inferior endocardial cushion and at its anterior limit to the superior endocardial cushion. Part of the process of closure of the primary interventricular foramen consists of expansion of the superior and inferior endocardial cushions toward each other, where they will make contact and fuse at about 6 weeks gestation in the human. The mechanism of fusion of the endocardial cushions despite the continuous mechanical activity of the heart is not known but certainly deserves respect. Further growth of the interventricular muscular septum results in fusion of the crest of the septum with the fused cushion. The boundaries between the original muscular septum and endocardial cushion–derived portions of the septum become obscured by myocardialization, except for the membranous septum between the left ventricle and right atrium. Normal inlet septum development is primarily determined by interactions between the inferior endocardial cushion–derived tissue and the muscular ventricular septum, whereas the smooth anterior interventricular septum is derived from interactions between the superior endocardial cushion and the muscular septum. The membranous septum is believed to be the approximate site of final union between the muscular septum and the superior and inferior endocardial cushions.[59]

From this discussion, it becomes plausible to hypothesize general mechanisms that might result in the various clinical types of ventricular septal defect. Perimembranous and AV canal–type ventricular septal defects may be the result of failure of normal growth and fusion processes between cardiac mesenchymal tissues of the endocardial cushions and myocardial septal tissue. Defects limited to the muscular outlet septum occur in myocardium derived from myocardialization after fusion of the mesenchyme of the outlet cushions and may therefore represent a localized failure of outlet cushion fusion. Muscular ventricular septal defects between the ventricular bodies could be hypothesized to arise when the trabecular mass is inadequate in either volume or signaling ability to appropriately undergo fusion.

Semilunar valve leaflet development begins shortly after septation. As studied in mice, the initial process is outgrowth of unexcavated cusps of tissue corresponding to the future leaflets from the arterial surface of the distal endocardial ridges and intercalated cushions.[60] Valve sinuses are formed by active endothelial excavation of the outlet surface of the leaflets. The initial valve leaflets are thickened structures filled with an abundant extracellular ground substance densely populated with endocardial and neural crest–derived mesenchymal cells, bordered by a cuboidal endothelium on the arterial surface and a flattened, streamlined endothelium on the ventricular surface. After the sinuses are fully excavated, the leaflets remodel into the delicate fibrous tissue characterizing mature semilunar valves.[61] Valve remodeling is a slow process that may be histologically incomplete at the time of birth.[60,62] The molecular biology of semilunar valve development shares many features with AV valve development.[63]

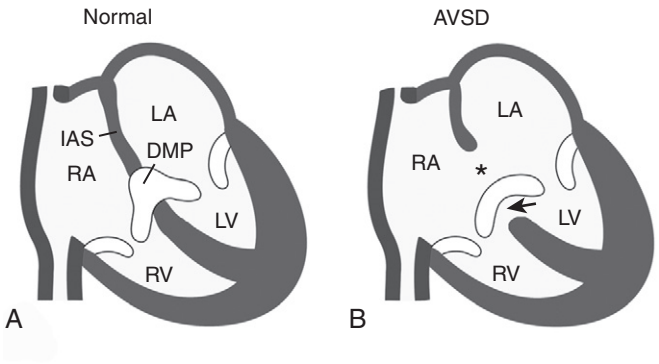


Figure 63-4 This diagram compares the anatomic features of a normal heart **(A)** to those of a heart with a complete atrioventricular septal defect *(AVSD)* **(B). A,** In the normal heart, the dorsal mesenchymal protrusion *(DMP)* forms a central component of the atrioventricular *(AV)* mesenchymal complex, a structure that serves as an anchor for both atrial and ventricular septa. **B,** In the heart with the AVSD, the DMP is missing, the AV mesenchymal complex is maldeveloped, there are communications between the atria *(asterisk)* and ventricles *(arrow)*, and there is a common AV valve.

ATRIOVENTRICULAR JUNCTION SEGMENT AND ATRIOVENTRICULAR VALVE DEVELOPMENT

Myocardial continuity among the AV junctional myocardium, atrial myocardium, and ventricular myocardium must be interrupted for the development of the fibrous crux of the heart and the correct function of the conduction system. This is accomplished by formation of a layer of fibrous insulation that will completely interrupt myocardial continuity between the AV myocardium and the ventricular myocardium, with the exception of the penetrating bundle of His specialized myocardium. In the mature heart, the remnants of AV junction myocardium become incorporated into the myocardium of the definitive atrium.[64]

Fibrous interruption of AV myocardial continuity results from the fusion of mesenchymal cell populations of the AV endocardial cushions with a mesenchymal cell population found in the AV sulcus on the external surface of the looped heart. AV sulcus mesenchymal cells are brought to the heart in the course of the epicardial cell migration, as discussed in greater detail later in this chapter in connection with coronary artery development (Figure 63-5). Morphologic studies suggest that the sulcus mesenchyme actively invaginates toward the base of the endocardial cushions; the mechanisms driving mesenchymal invagination and the parting of the myocardial layer are unknown. Interruption of myocardial continuity begins at 52 to 60 days gestation in the human heart and is normally "complete" by the 4th month of gestation.[64] Molecularly, a pathway linking FGF4 signaling to expression of scleraxis and transcriptional activation of tenascin

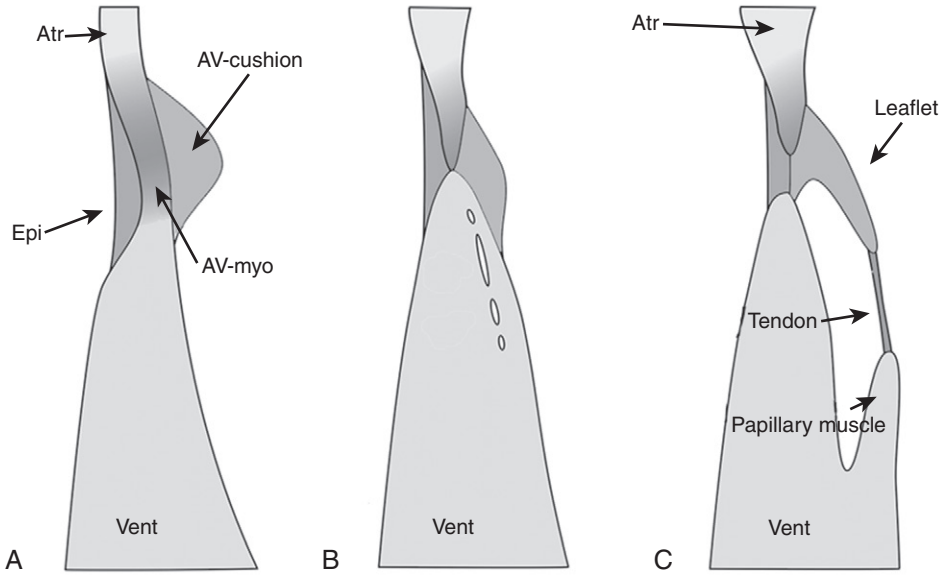


Figure 63-5 This schematic illustrates the accumulation of the subepicardial mesenchyme at the atrioventricular *(AV)* sulcus and its fusion with the AV cushion mesenchyme at the boundary of the AV myocardium *(AV-myo).* This process helps create an electrical insulation between myocardium of the atria and ventricles. *Atr,* Atrial myocardium; *Epi,* epicardium; *Vent,* ventricular myocardium. *(Modified from Snarr BS, Kern CB, Wessels A. Origin and fate of cardiac mesenchyme.* Dev Dyn *237:2804-2819, 2008; and Wessels A, Sedmera D. Developmental anatomy of the heart: A tale of mice and man,* Physiol Genom *15:165-176, 2003.)*

has been described in chick limb tendon[65] and is proposed to be active in normal chordae formation.[66-68]

As previously noted, there are four endocardial cushions in the AV junction. The superior and inferior cushions are the most prominent endocardial cushion masses from their first appearance, but the lateral endocardial cushions, which are visible only after Carnegie stage 17 (about 42 days), also have important contributions,.[64,69] A map of endocardial cushion contribution to definitive AV valve structure has been published (Figure 63-6).[46,70]

AV valve morphogenesis is one of the most prolonged aspects of human cardiac development. Recognizable elements of the tricuspid valve begin formation at 5 to 6 weeks gestation. The AV endocardial cushions are actively reconfiguring at this time, with fusion of the superior and right lateral AV endocardial cushions to each other. The conal cushions are also completing their fusion during this time. Despite the overall advanced stage of cardiac morphogenesis at this point, the tricuspid valve leaflets are still very primitive in appearance and not freely mobile. The inferior leaflet is fully delaminated by the end of the 8th week of gestation, the anterior leaflet by the 11th week, and the septal leaflet in the 12th week. The commissure separating the anterior and septal leaflets is not complete until the septal leaflet is fully delaminated.[71]

In development of the mitral valve, the inferior and superior AV cushions begin to fuse at roughly 5 weeks gestation; even before fusion, the enlarged trabecular complex that will evolve into the two mitral papillary muscles can be seen connecting the superior and inferior cushions. The left lateral cushion, the precursor to the posterior or mural leaflet, is visible by the 7th week of gestation. At about this time, initial delamination of the mitral valve structures becomes detectable and continues until the 10th week of development. Between the 10th and 14th weeks of development, myocardial elements of the leaflets are eliminated, the papillary muscles achieve their adult appearance, and chordae differentiate.[69]

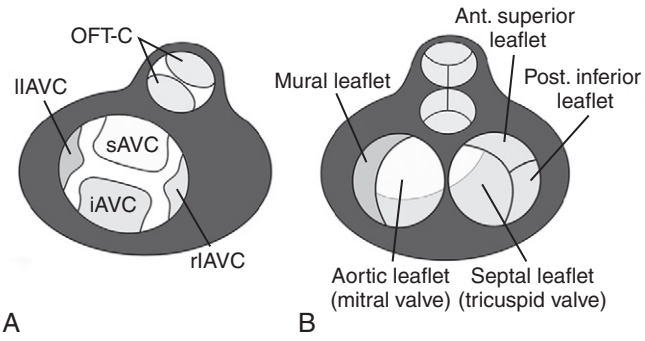


Figure 63-6 Fate map of the atrioventricular *(AV)* and outflow endocardial cushions. **A,** The major AV cushions consist of the superior and inferior AV cushions *(sAVC, iAVC).* The sAVC predominantly gives rise to the aortic leaflet of the mitral valve, whereas the iAVC gives rise to the septal leaflet of the tricuspid valve. The right lateral AV cushion *(rlAVC)* contributes to the anterior-superior and posterior-inferior leaflets of the tricuspid valve. The left lateral AV cushion *(llAVC)* contributes to the mural leaflet of the mitral valve. The outflow tract cushions *(OFT-C)* give rise to the semilunar valves. *(Modified from Snarr BS, Kern CB, Wessels A. Origin and fate of cardiac mesenchyme,* Dev Dyn *237:2804-2819, 2008.)*

DEVELOPMENT OF THE AORTIC ARCH

The embryonic arterial circulation is initially bilaterally symmetric and consists of multiple pairs of aortic arch vessels connecting the heart outflow to the paired dorsal aortae. Fusion of the originally separate right and left dorsal aortae into a single structure begins distally and progresses retrograde to the 7th somite. As development proceeds, the paired 1st, 2nd, 3rd, 4th, and

6th aortic vessels and their connections to the dorsal aortae undergo an intricate series of transformations. The 1st and 2nd aortic arch vessels regress, remaining patent only as capillary structures. The dorsal aorta between the 3rd and 4th aortic arch vessels (the carotid duct) regresses completely, leaving no remnant, resulting in the paired 3rd aortic arch vessels becoming the only source of blood flow from the aortic sac and truncus complex to the head of the embryo. The 3rd aortic arch vessels become the precursors of the definitive right and left common carotid arteries. The right dorsal aorta completely regresses at the site of dorsal aortic bifurcation; this leaves the right 4th aortic arch vessel to become a short stub connecting the right 7th intersegmental (future subclavian) artery to the aortic sac and truncus complex. The right 6th aortic arch vessel regresses fully. The left 6th aortic arch vessel becomes the ductus arteriosus connecting the pulmonary plexus–derived distal main pulmonary artery and left pulmonary artery to the left dorsal aorta at the junction of the left dorsal aorta and the left 4th arch vessel. The left dorsal aorta remains widely patent throughout its length but remodels so that the definitive left 4th aortic arch vessel, the ductus arteriosus, and the left 7th intersegmental artery (future left subclavian artery) all connect to the left dorsal aorta within a very short span.

The vertebral arteries are derived from anastomoses that form between the seven cervical intersegmental arteries. After continuity is established between the intersegmental arteries, their connections to the dorsal aorta regress, with the exception of the connection of the 7th intersegmental vessel (as noted previously, the 7th intersegmental artery becomes the subclavian artery), creating the subclavian origin of the definitive vertebral arteries.

NEURAL CREST AND MOLECULAR REGULATION OF AORTIC ARCHES

The neural crest is a transient structure in development with multiple critical roles in the formation of craniofacial structures, the autonomic nervous system, pigmentation, and endocrine function. The cells of the neural crest reside on the lateral margins of the neural plate, and the right- and left-sided populations are brought into apposition by the folding of the neural plate into the neural tube. The cells then disperse and migrate along precise pathways to their multiple destinations. Specific regions of the neural crest seed their cells through specific pathways to specific structures.[72] The neural crest cells that reach the outflow tract of the early heart migrate through the 3rd, 4th, and 6th aortic arch vessels. A role for neural crest in cardiac development was recognized in 1983 through the work of M. L. Kirby; the region of the neural crest contributing to cardiac and 4th aortic arch morphogenesis is sometimes called *cardiac neural crest*.[73] The patterning of the aortic arch arteries is greatly influenced by the migration of neural crest cells into the pharyngeal arches. Studies in animal models have shown that neural crest expression of a receptor for endothelin-1 (ET1) is necessary for interaction with endothelial cells of the arch arteries.[74] Definitive aortic arch formation is the product of complex signaling interactions among neural crest cells, SHF cells, and pharyngeal mesoderm.[54,73,75]

DEVELOPMENT OF THE PULMONARY VEINS

The pulmonary veins develop from coalescence of endothelial networks in the lung parenchyma. The earliest evidence of the formation of the common pulmonary vein in the embryo is the presence of a strand of endothelial cells in the dorsal mesocardium. The endothelial strand forms a lumen and passes through the mesenchyme of the dorsal mesocardium to enter the left atrial myocardium.[23] The common pulmonary vein therefore connects directly to the left atrium in normal development and does not represent an outgrowth of the atrial wall. Despite this, it is now widely recognized that the muscular layer of the proximal portions of the individual pulmonary veins is of a variably myocardial (as opposed to smooth muscle) phenotype that is clinically important in the etiology and management of atrial fibrillation.[76-78]

DEVELOPMENT OF SYSTEMIC VEINS

The embryonic systemic veins are also formed by vasculogenesis. Initially, there are three bilaterally symmetric venous drainages: the vitelline, umbilical, and cardinal venous systems. The vitelline veins drain the embryonic gastrointestinal tract and gut derivatives. The umbilical veins bring oxygenated blood to the heart from the placenta. The cardinal venous system returns blood from the embryonic head, neck, and body wall. All three of these drainages enter into the sinus venosus of the primitive heart tube. The adult venous pattern is established through a complex process of regression, remodeling, and replacement of the embryonic venous systems and their connections to the sinus venosus.[79,80]

The sinus venosus is initially bilaterally symmetric, with right and left "horns" that provide connection for the right and left common cardinal veins, umbilical veins, and vitelline veins. However, the connections of the left-sided cardinal, vitelline, and umbilical venous systems with the left horn of the sinus venosus normally regress. This results in the coronary sinus remaining as the primary structural derivative of the left horn of the sinus venosus in the normal fetal and postnatal heart. When embryonic venous connections with the left venous horn fail to regress, persistence of the left superior vena cava is observed. The right horn of the sinus venosus normally accommodates the entirety of the systemic venous drainage, except the portion from the heart returned through the coronary sinus. In addition, the portion of the mature right atrium between the orifices of the vena cavae is derived from the right horn of the sinus venosus.

The right and left vitelline veins are connected to each other through a plexus of veins that become the liver sinusoids. After the left vitelline vein loses connection with the left horn of the sinus venosus, it regresses. Therefore the entire venous system of the embryonic gut normally drains to the heart through the right vitelline vein. The connection of the right vitelline vein to the right sinus venosus persists into fetal and adult life as the terminal portion of the IVC.

The left umbilical vein also loses its connection with the left horn of the sinus venosus, but it is the right umbilical vein that regresses as a distinct structure. The left umbilical vein forms anastomoses with the ductus venosus (derived from the liver plexus of the vitelline veins). There are no derivatives of the embryonic umbilical venous drainage that connect to the heart or persist after the closure of the ductus venosus in adult life.

The cardinal venous system initially consists of bilateral anterior cardinal veins and bilateral posterior cardinal veins. Fusion of the anterior and posterior cardinal veins at the level of the sinus venosus forms the common cardinal veins. The left anterior cardinal vein loses its connection with the left horn of the sinus venosus, but a small remnant on the surface of the heart normally persists as a passage of coronary venous blood to the coronary sinus and is known as the oblique vein of the left atrium. Another portion of the left anterior cardinal vein persists as the left internal jugular vein. As the left anterior cardinal vein loses connection with the heart, it becomes connected to the right anterior cardinal vein through the intercardinal anastomosis that forms between the thyroid vein and the thymic vein; this connection will persist as the left brachiocephalic vein. The portion of the right anterior cardinal vein between the right atrium and the drainage of the left anterior cardinal vein proximally (through the intercardinal anastomosis) becomes the normal right superior vena cava.

The posterior cardinal veins are the only portion of the embryonic venous drainage that are destined to have a symmetric

fate. Both posterior cardinal veins will regress throughout most of their length and lose their direct connections with the sinus venosus. The posterior cardinal veins originally drain the body wall, gonadal, and renal structures. Their function in venous drainage of the body wall is supplanted by the supracardinal venous plexus, whereas the gonadal and renal venous drainage is captured by the subcardinal venous plexus.

The posterior cardinal, supracardinal, and subcardinal venous beds contribute the segments that form the definitive inferior vena cava (IVC) to the level of the vitelline vein–derived segment connecting to the right atrium. Remnants of the posterior cardinal veins in the fetal and adult circulation are limited to the most distal portion of the IVC (formed by anastomosis of the right and left posterior cardinal veins) and the common iliac veins. The posterior cardinal vein–derived IVC connects to the supracardinal segment of the IVC. The supracardinal venous system is the site of origin of the azygous and hemiazygous veins, which ordinarily connect to the IVC between the renal veins and the common iliac veins. The supracardinal segment of the IVC connects to the subcardinal segment of the IVC, which receives the drainage of the gonadal veins and renal veins before connecting to the vitelline venous channel into the heart.

The molecular pathways differentiating venous development from arterial development are becoming better understood. Key molecular pathways include VEGF and Notch signaling, ephrins and their receptors, and the transcription factor COUP-TF.[81-84] The lymphatic system develops as an outgrowth of the primitive venous circulation, with lymphatic markers that include PROX1, podoplanin, and VEGF-R3.[85-88]

EPICARDIAL CELL MIGRATION AND DEVELOPMENT OF THE CORONARY ARTERIES

Epicardial cells are not present in the tube heart, and an epicardial cell migration is essential for development of the epicardium, the coronary arteries, a major component of the fibrous crux, and the fibroblast population of the myocardium. The epicardium migrates to the surface of the heart from villous projections in the region of the sinus venosus in birds and from the septum transversum in mammals.[89-92] The precursor tissue is histologically and functionally complex and has been called the *proepicardial organ*.[93] It is a transitory embryonic structure that gives rise to the epicardial cell layer and is also a source of fibroblasts and coronary smooth muscle cells of the heart.

In the mouse and rat, villous processes arising from the septum transversum in close proximity to the heart appear to be the source of the epicardial cells. Small aggregates of cells appear on the surface of the ventricles and atria that faces the septum transversum. With time, the cells flatten over the surface of the myocardium and develop morphologic characteristics compatible with primitive epithelial cells. The flattening process also causes cells to occupy a greater surface area, bringing adjacent clusters of cells into contact with each other until a continuous sheet results. These events occur between days 9 and 11 of embryonic life; the villous projections are markedly diminished by day 10.5.[90,93]

As the epicardial cell layer forms, an extensive acellular extracellular matrix layer, similar to the cardiac jelly in the subendocardial space, appears between the epicardium and the myocardium. The mesenchymal cells that subsequently populate this space are derived from an epithelial-to-mesenchymal transformation of the epicardial cells. This process is regulated by expression of transcription factors such as Slug and Snail, as well as growth factors, including transforming growth factor-β.[94] Subepicardial mesenchymal cells are generally accepted to provide the precursors of cardiac fibroblasts, coronary vascular smooth muscle cells, and coronary endothelial cells. The subepicardial extracellular matrix accumulates to its greatest degree in the AV groove. In addition to being a key site for coronary vasculogenesis, the mesenchymal cells that come to populate the AV groove matrix will form the majority of the fibrous insulating ring between the atria and the ventricles (see Figure 63-5).[64]

The generation of subepicardial mesenchyme can be inhibited in chick embryos by perturbing epicardial precursor cell migration from the proepicardial organ, resulting in failure of development of both the epicardium and subepicardial space.[91] The normal development of the epicardium and subepicardial mesenchyme is also dependent on cell-cell interactions with the underlying myocardium. Gene knockout studies in mice show that the absence of expression of genes such as vascular cell adhesion molecule 1 *(Vcam1)* or transcription factor friend of GATA2 *(Fog2)* in the myocardium, or Wilms tumor suppressor 1 *(Wt1)* and α_4 integrin in the epicardium, will produce a similar phenotype of abnormal coronary development and in some models *(Vcam1, Fog2, Wt1)* ventricular myocardium hypoplasia.[46]

All of the cell populations arising from the epicardium are collectively known as epicardium-derived cells (EPDCs).[95] As identified by lineage markers, EPDCs migrate into the sulcus matrix and from there into the AV endocardial cushions. In addition to entering the AV cushions, EPDCs were also noted to migrate individually into the myocardium and subendocardium. Disruption of EPDC migratory contributions to the myocardium and endocardial cushions results in abnormalities of Purkinje fibers, the annulus fibrosis, the compact myocardium, AV cushions, and coronary vasculature.[91,95-97]

The subepicardial space is the site of origin of the vascular plexus of the coronary vessel precursors. Three sequential and overlapping phases of nutrient delivery to the myocardium during embryogenesis of the heart have been described.[98] The first phase is associated with the development of an extensive network of intratrabecular sinusoids lined by endocardial cells, through which nutrient flow to the myocardium is assumed to occur. The second phase is the development of a subepicardial plexus of endothelium-lined channels that penetrate the myocardium. At least some of these channels communicate with the intratrabecular sinusoids. The third stage is the regression and coalescence of the vascular subepicardial network into specific muscular arterial channels. Virtually as soon as the vessels are readily identifiable, they are noted to penetrate into the ventricular and atrial walls, where they establish a mid-myocardial network. The vessels spread to the ventral surface of the heart and follow the sulci (especially the AV sulcus) to the outflow tract, where they form a plexus in the myocardial sheath surrounding the truncus arteriosus. Coalescence of vessels and capillary outgrowth from the peritruncal plexus result in penetration of the wall of the aorta by the definitive proximal coronary arteries.[98,99]

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H. Scott Baldwin and Justin C. Grindley

Molecular Determinants of Embryonic Vascular Development

The establishment of the cardiovascular system represents an early, critical event essential for normal embryonic development. Although much of the attention to date has focused on factors involved in key events in cardiac development, such as myocardial differentiation and neural crest migration, the importance of normal vascular morphogenesis has recently become a central focus of cardiovascular research. Considerable new information has accumulated concerning the pivotal role of endothelial differentiation in establishment of the vascular tree. This chapter reviews the general mechanisms of vascular development in light of some of the new information on critical molecular interactions that has recently been delineated. Specific areas of focus include (1) initial differentiation of the endothelium and formation of the primary vascular plexus, (2) vascular heterogeneity and remodeling, and (3) epigenetic factors that play a primary role in vascular ontogeny. Also reviewed is some recent evidence demonstrating how all of these factors are involved in regulation of pulmonary vascular development.

ORIGIN OF ENDOTHELIAL CELLS

Early observers noted the initial formation of blood vessels outside the embryo in the blood islands of the yolk sac, which preceded intraembryonic vascular development. This led to the conclusion that all vessels within the embryo resulted from a direct extension of extraembryonic vessels.[1] This hypothesis was quickly refuted when parts of the embryo, separated from the yolk sac before vascular invasion, were shown subsequently to develop vascular channels.[2] This suggested the possibility of endothelial cell (EC) precursors intrinsic to the embryo. Perhaps the most significant breakthrough in the study of endothelial ontogeny came with the development of monoclonal antibodies that recognized a species-specific epitope on the cell membrane of quail ECs. For the first time, this antibody allowed definitive identification of ECs independent of potentially ambiguous morphologic criteria. Using one of these antibodies, QH1, several investigators have been able to demonstrate that vascular development commences in the embryo proper shortly after gastrulation, as individual presumptive ECs, or angioblasts, differentiate from the mesoderm. These cells soon connect into a vascular plexus that matures into the major vessels of the adult (see later discussion). These studies have documented that most mesodermal tissues contain endothelial precursors and thus have the capacity for vascular formation.[3,4] Similar studies using a battery of endothelium-specific antibodies have defined a similar pattern of endothelial differentiation and vascular development in the mouse.[5]

For more than 70 years, a central question in vascular development has been whether ECs and hematopoietic cells (HCs) share a common origin. In the yolk sac blood islands, and at certain sites within the embryo such as the developing aorta, hematopoiesis and EC differentiation occur side by side. Early observation of simultaneous HC and EC differentiation[6,7] led to the hypothesis that HCs and EC precursors (angioblasts) are derived from the same progenitor cell, the hemangioblast (the subject of several reviews[8-10]). Again, this question has been answered by the

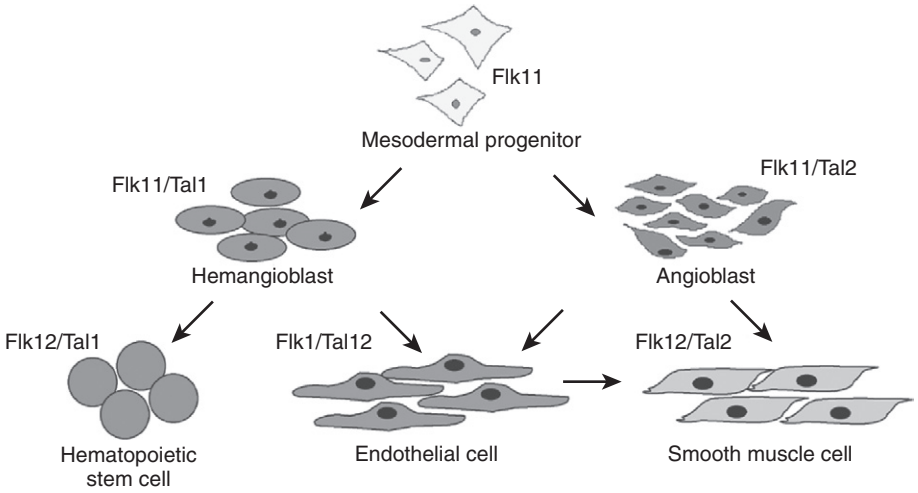


Figure 64-1 The common origins of endothelial and hematopoietic cells. ECs and HCs both derive from mesodermal progenitor cells that express the VEGF receptor VEGFR2 (Flk1). The hemangioblast is a Flk1-positive cell that also expresses the transcription factor TAL1/SCL (Flk1 positive/Tal1 positive) and can give rise to both HCs (down-regulating Flk1) or to ECs (down-regulating Tal1). ECs can also be derived from the angioblast, a precursor cell that, like the ECs themselves, is Flk1 positive but Tal1 negative. An additional cell-fate choice important for blood vessel formation is the production of SMCs versus ECs. Recent data suggest that both angioblasts and mature ECs can give rise to SMCs, which are Flk1 negative/Tal1 negative. *(Modified from Ema M, Faloon P, Zhang WJ, et al: Combinatorial effects of Flk1 and Tal1 on vascular and hematopoietic development in the mouse,* Genes Dev *17:380, 2003.)*

identification of molecular markers that define specific stages of mesodermal differentiation, most notably the vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2). VEGFR2-deficient mice do not develop blood vessels or yolk sac blood islands[11,12] and thus lack both endothelial and hematopoietic components. The combined EC and HC defects are consistent with a common molecular pathway governing EC and HC differentiation and support a direct lineage association between blood and endothelium. However, the combined defects could merely reflect a developmental arrest because loss of VEGFR2 function results in a relatively early embryonic demise.

To separate embryonic survival from the equation, VEGFR2-deficient embryonic stem cells have been used in both chimera and in vitro differentiation studies. These approaches have demonstrated that a single VEGFR2-positive cell can give rise to both endothelial and HCs and further suggest a requirement for VEGF/VEGFR2 signaling in hemangioblast survival, proliferation, or migration, as well as in differentiation.[8,12] Similar knockout, chimera, and in vitro studies suggest that the basic helix-loop-helix transcription factor TAL1/SCL (T-cell acute leukemia/stem cell leukemia) might play a concomitant role in differentiation of both the hematopoietic and EC lineages. TAL1/SCL-deficient animals have defective hematopoiesis, and TAL1/SCL-deficient cells fail to contribute to vascular remodeling. TAL1/SCL deficiency in embryoid bodies (differentiating cultures of embryonic stem cells) leads to defective hematopoiesis, accompanied by an increase in the percentage of cells expressing endothelial markers. Thus it was hypothesized that TAL1/SCL might have a biphasic action, acting in a common precursor to promote formation of HCs, but also acting within the endothelial lineage during angiogenesis.[8] TAL1/SCL is not expressed in VEGFR2-deficient embryos, suggesting a pathway in which expression of this transcription factor is downstream of a VEGF signal through VEGFR2.[13]

In an elegant series of experiments, Ema et al[13] delineated a combinatorial role for VEGFR2 and TAL1/SCL in vascular and hematopoietic development. By knocking-in TAL1/SCL into the VEGFR2 locus, they could separate TAL1/SCL activation from additional downstream effects of VEGFR2 action. They showed that TAL1/SCL partially rescues the in vivo endothelial defects seen in VEGFR2-null mutants but significantly improves hematopoietic differentiation in vitro and in vivo. This study (summarized in Figure 64-1) was able to identify definitively the hemangioblast as expressing TAL1/SCL (Tal1-positive) and the angioblast as nonexpressing (Tal1-negative). It was also able to confirm that angioblasts and definitive ECs could both give rise to smooth muscle cells (SMCs), as had been previously suggested by others.[14] Thus ECs, HCs, and SMCs have a common lineage in which cell-fate choices are governed in part by the combinatorial action of VEGFR2 and TAL1/SCL.

The Two Mechanisms of New Blood Vessel Formation

Following the differentiation and development of the EC populations, as described, a subsequent organization of these ECs into a complex but highly regulated system of arteries, capillaries, and veins occurs. Two different processes are thought to be involved in new blood vessel formation (Figure 64-2): *vasculogenesis*, which is the de novo organization of blood vessels by in situ differentiation of ECs from mesoderm; and *angiogenesis*, the budding and branching of vessels from preexisting vessels.[15-17]

Angiogenesis is the characteristic vascularization process for organs such as the brain and kidney; vasculogenesis is more prominent in the formation of the larger vascular network, including the dorsal aorta, blood islands, endocardium of the heart, and cardinal and vitelline vessels, as well as the liver. In some instances, vascular development may occur by simultaneous angiogenesis and vasculogenesis, which is characteristic of the developing lung.[18,19] Although angiogenesis can occur in both the embryo and the adult (e.g., wound healing, tumor vasculogenesis[20,21]), vasculogenesis is usually considered to be restricted to embryonic development. However, recently identified circulating ECs and EC precursors that can contribute to new blood vessel formation may represent exceptions to this temporal limit on vasculogenesis.[22] Despite this distinction between these two processes, the tasks that ECs subsequently perform in forming a new vessel are similar in both. Thus vasculogenesis and angiogenesis likely share many molecular components involved in cell-cell or cell-matrix signaling or in coordinated cell movement, organization, and adhesion.

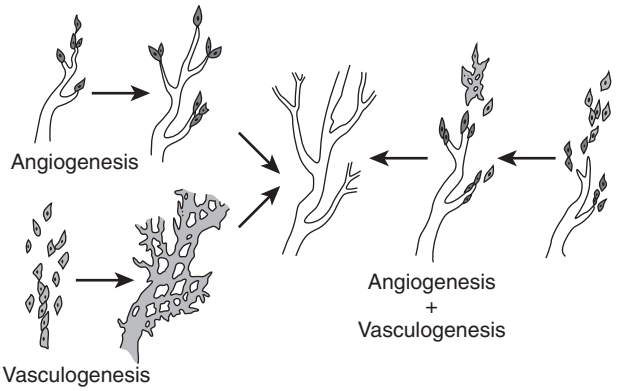


Figure 64-2 The basic mechanisms of vascular development in the embryo. *Angiogenesis* is the sprouting of new vessels from existing vessels, and *vasculogenesis* is the de novo differentiation of angioblasts from mesoderm and organization into a vascular plexus with remodeling into a definitive vessel. A third process may involve congruent angiogenesis and vasculogenesis.

Formation of a Primary Vascular Plexus

In vasculogenesis, ECs first assemble into vessel primordia, which lack a lumen, and then rearrange into endothelial tubes of polarized cells. Many such tubes then are joined into a polygonal array generating the primary vascular network. Recent work has identified key molecules involved in vessel assembly and morphogenesis. An early role for VEGF signaling is revealed by VEGFR1-deficient mice, which form angioblasts but fail to properly assemble ECs into tubes.[23] Inhibition of VEGF signaling using soluble VEGFR1 in quail embryos supports the requirement for VEGF signaling in this phase of vessel assembly.[24] Normally, the polygonal array of vessels is formed through the protrusive behavior of individual ECs, which adopt a bipolar shape and extend processes to link with neighboring ECs. In embryos injected with soluble VEGFR1, normal numbers of ECs are present, but they fail to exhibit protrusive behavior, resulting in a profound deficiency of vessels.[24]

In addition to signaling molecules, cell-cell contact and cell–extracellular matrix (ECM) interactions are important for early vascular morphogenesis. After the formation of vessel primordia, cells rearrange into tubes with lumens. Integrins, the best-characterized group of cell-ECM adhesion molecules, have been implicated in this phase of vasculogenesis, as well as in angiogenesis and vessel maintenance. Integrins are heterodimeric proteins that consist of an α-subunit noncovalently associated with a β-subunit. Currently, 8 different β- and 18 different α-subunits have been identified; however, members of the β1 and β3 family of integrin receptors, together with other cell adhesion family members, are thought to be particularly important in both vasculogenesis and angiogenesis.[25] The combination of specific α- and β-subunits determines ligand-binding specificity. For example, β1 in combination with α5 functions exclusively as a fibronectin receptor, whereas β1 in combination with α6 serves as a laminin receptor. Thus the ability of a cell to modulate adhesive characteristics is determined by both the types of integrins expressed and the absolute number of any given integrin.

Utilizing the monoclonal antibody CSAT, which interferes with ligand binding to the β1 integrins, Drake et al[26] were able to block formation of the lumen of the dorsal aorta in the early chick embryo. It is interesting that in these experiments, the early stages of vasculogenesis were not obviously affected in that angioblast differentiation and spatial organization into cords appeared normal. Only the final stages of tube formation were inhibited, suggesting that although β1 integrins are critical for final development of an endothelial tube, they are not involved in the initial commitment of mesoderm to EC lineage or in initial spatial organization. In results similar to those seen for injection of β_1 antagonists, αvβ3-blocking agents prevented lumen formation but not differentiation or spatial organization of the ECs.[27]

To assign roles in vascular development to specific subunits, integrins, or their ECM ligands requires much additional work, and it should be noted that inhibitor studies and gene inactivation studies have not always produced concordant results. A majority of αv-knockout mouse embryos die by midgestation, probably owing to placental defects, but around one fifth of the embryos survive until birth.[28] These embryos have vascular abnormalities and hemorrhaging that are unexpectedly restricted to intracerebral and intestinal vessels.[28] Similarly, β3-knockout mice are viable and fertile but have defects characteristic of the human bleeding disorder Glanzmann thrombasthenia, including cutaneous and gastrointestinal bleeding.[29] Thus, although both the αv and β3 gene-inactivations in mice have vascular defects, neither recapitulates the defects seen in avians in response to a specific αvβ3 inhibitor.

ANGIOGENESIS, REMODELING, AND VESSEL MATURATION

To generate a fully functional, arborized vascular tree, the primary vascular plexus must be extended and modified, and vessel maturation must occur. Angiogenesis generates new vessels from existing ones, either by a sprouting mechanism or by a nonsprouting (intussusception) mechanism in which columns of interstitial tissue are inserted inside the lumen of an existing vessel, dividing it into two or more new vessels.[30] During angiogenesis, the preexisting network of vessels with relatively uniform diameters is also extensively remodeled to generate a hierarchic arrangement of large and small vessels. These changes are accompanied by vessel maturation and stabilization, which depend upon the recruitment from mesenchyme of periendothelial mural cells, including pericytes and SMCs, as well as on the deposition of basement membrane. Interactions with periendothelial mural cells established during angiogenesis are critical for proper functioning of the vascular network. In particular, blood vessels located in different parts of the vascular tree, such as the elastic vessels, muscular arteries, resistance vessels, veins, and lymphatics, each require different physical properties. Many of these distinctions between vessels stem from local variation in ECM production by the mural cells.

In the last few years, considerable progress has been made in uncovering the molecular signals controlling angiogenesis, vessel remodeling, and maturation. This work has highlighted the involvement of several families of receptor tyrosine kinases (RTKs) and their ligands, shown schematically in Figure 64-3. The functions of selected RTKs and ligands in vascular development are illustrated in Figure 64-4. In addition to functioning during vasculogenesis, signaling by VEGF ligands and VEGF receptors is important for angiogenesis and regulates EC sprouting and survival (reviewed by Carmeliet and Collen[31]). Vascular development is particularly sensitive to VEGF dosage. Heterozygosity for a null mutation in the VEGF gene leads to multiple cardiovascular defects and embryonic lethality.[32,33] Consistent with a role in vessel sprouting, deficiencies occur in intersomitic vessels, in vessel in-growth to the neuroepithelium, and in the sprouting of head mesenchyme vessels. The vitelline vessels fail to fuse with the yolk sac, and an irregular primary plexus within the yolk sac is not remodeled to generate larger vessels.[32,33]

Specific inhibition of VEGF signaling with a soluble receptor and a conditional knockout of VEGF in which the gene is excised only in newborn mice further highlights the dependence on VEGF of vessels that form by angiogenesis.[34] In these animals, there is growth retardation and hypoplasia of multiple organs,

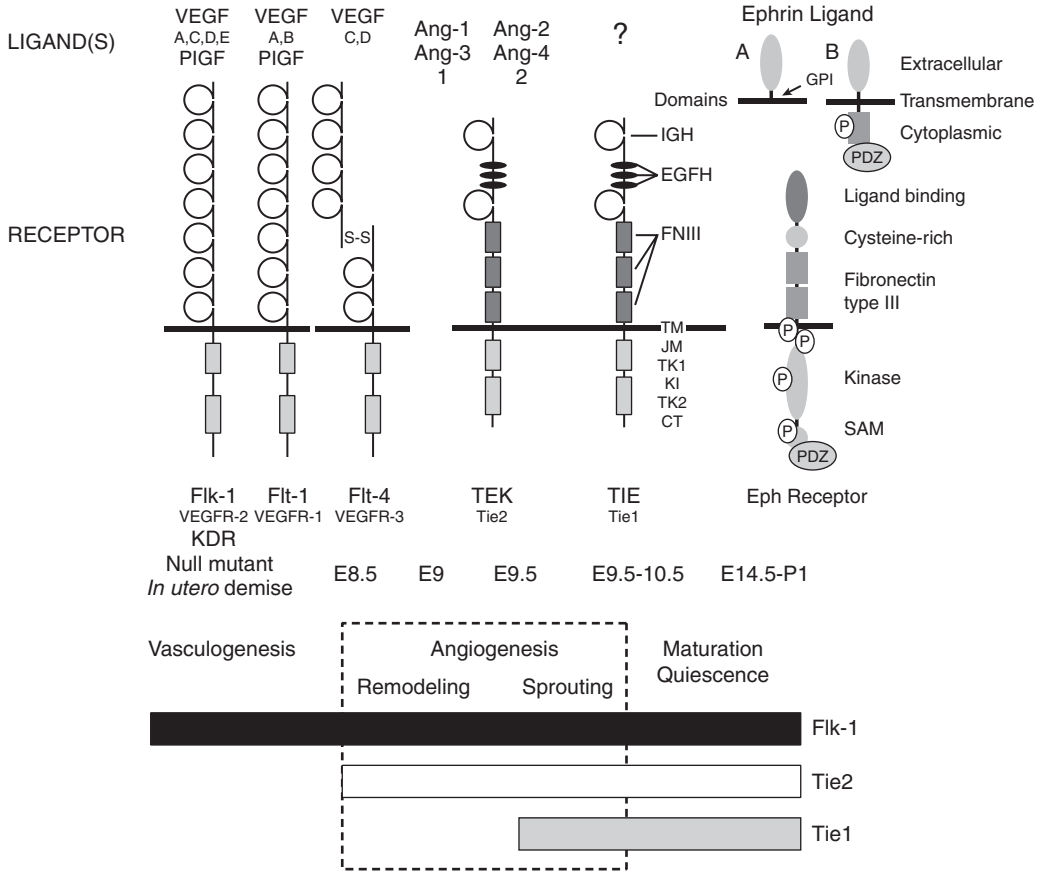


Figure 64-3 Major EC RTKs and growth factor ligands involved in vascular development. The structures of the receptors are shown schematically, and major protein domains have been indicated. (*Ang,* Angiopoietin; *Ang-1, Ang-2, Ang-3, Ang-4,* angiopoietin family of Tie2 ligands; *CT,* carboxyl-terminal tail; *EGFH,* epidermal growth factor homology domain; *FNIII,* fibronectin type III homology domain; *GPI,* glycosylphosphatidyl inositol anchor; *IGH,* immunoglobulin homology domain; *JM,* juxtamembrane domain; *KI,* kinase insert; *P,* phosphorylation site; *PDZ,* site of interaction with the PSD-95/Discs Large/ZO-1 [PDZ] class signaling proteins; *PlGF,* placental growth factor; *SAM,* domain that forms homodimers and may regulate receptor dimerization; *S-S,* disulfide bridge; *TK1* and *TK2,* tyrosine kinase catalytic domains; *TM,* transmembrane domain.) Also shown are the requirements for three RTKs relative to mouse gestation and phases of blood vessel development. Animals deficient in VEGFR2 (Flk1) fail to undergo vasculogenesis, indicating a very early requirement for this receptor. In contrast, Tie2 is not required for vasculogenesis but is necessary for subsequent angiogenesis. Tie1 appears to act later still, mainly in vessel maturation and vessel stability (quiescence).

which is associated with dramatic reductions in organ vascularization. In addition to reducing postnatal angiogenesis, the inhibition of VEGF signaling appears to cause loss of existing vessels because animals treated with soluble receptor exhibit a marked increase in EC apoptosis. Therefore VEGF acts as a survival factor for ECs in immature vessels. This survival function observed in the perinatal period does not appear to extend to mature vessels because treated adult animals did not exhibit organ abnormalities.

Although VEGF receptors such as VEGFR2 are expressed by the earliest EC precursors, other endothelium-specific RTKs are most prominently expressed at later phases of vessel development and are thought to act once initial vessel formation has occurred. Tie1 (tyrosine kinase with immunoglobulin-like loops and epidermal growth factor homology domains) and Tie2 are closely related endothelium-specific RTKs that are involved in angiogenesis and vessel maintenance. Tie2-deficient animals exhibit normal vasculogenesis but fail to remodel the primary vascular plexus correctly and are deficient in vessels that normally form by angiogenic mechanisms. A key defect is in the generation of small vessels from large ones by intussusception. Folds of interstitial tissue that normally invade vessels collapse to produce irregular, abnormally spaced vessel patterns.[35] The failure of proper tissue-fold formation apparently stems from a striking inability of Tie2-null vessels to recruit periendothelial cells to the vessel walls and into the tissue folds. The ECs themselves also exhibit abnormal morphology, being rounder than normal and often projecting into the vessel lumen.[35]

Ang-1 and Ang-2 (see Figure 64-3) are two members of a family of four structurally related Tie2 ligands.[36] Ang-1–null mice have a similar phenotype to Tie2 mutants, suggesting that this molecule is the main agonist ligand. Ang-1 is not expressed in EC cells but rather is produced in tissue adjacent to vessels in areas of angiogenesis, implying that the signals mediated by Ang-1/Tie2 are directed to ECs from their environment. The effect on mural cells indicates a critical place of Ang-1/Tie2 in reciprocal signaling between ECs and their surroundings because it suggests that the loss of Tie2 activity causes a disruption of some further signal produced by the EC cells. Ang-2, identified by its sequence similarity to Ang-1, was found in vitro to antagonize Ang-1 activation of Tie2, a role supported by in vivo overexpression studies that generated a phenotype similar to loss of function of Ang-1 or Tie2.[37] In vitro data and gene knockout experiments suggest a more complex picture, in which the function of Ang-2 is context-dependent and may be modulated by the bioavailability of VEGF.[38-40] Ang-2 appears to induce vessel regression in the absence of VEGF, but it can promote sprouting in the presence

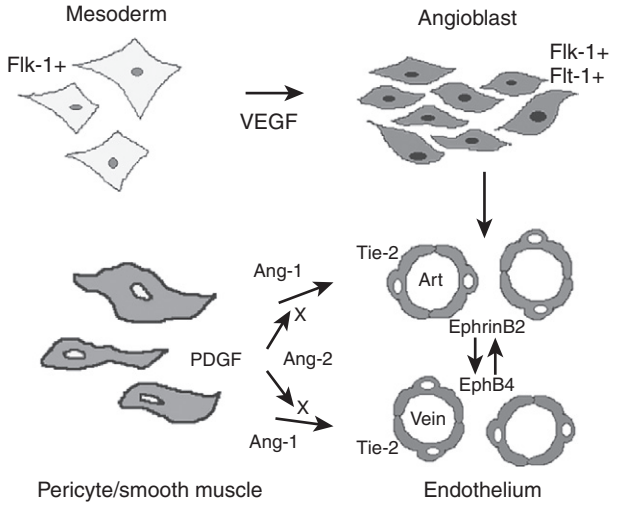


Figure 64-4 Key signals in the production of a vascular tree. Schematic of the cell types contributing to blood vessels, illustrating the action of selected signaling molecules. VEGF signals are involved in the differentiation of endothelial precursor cells (angioblasts) from mesoderm that contains progenitor cells that express VEGFR2 (Flk1). Angioblasts require VEGFR1 (Flt1) to assemble into endothelial tubes. The endothelium is patterned and remodeled. Patterning of vessels includes the expression of ephrin-B2 on arteries and Eph-B4 on veins. The Tie2 receptor, expressed on ECs, and its ligand Ang-1, expressed in adjacent tissues, are required for vessel remodeling and formation of new vessels by angiogenic mechanisms. Ang-2 can act as a negative regulator of these signals. Platelet-derived growth factor B (PDGF-B) is involved in the recruitment of periendothelial mural cells to vessels, which is necessary for vessel maturation and stability. *Art,* Artery.

of VEGF.[41] In blood vessels, Ang-2 functions in vessel regression, as shown by the failure of the normal programmed regression of the hyaloid vasculature of the eye in Ang-2–deficient mice. Conversely, the effect of Ang-2 on intestinal and dermal lymphatic ECs is proangiogenic, and loss of Ang-2 results in hypoplasia of these vessels.[38]

A necessary starting point for vessel regression (pruning of the vascular tree), sprouting (generating new branches), and intussusception (dividing vessels) is the destabilization of existing blood vessels. Work with additional RTKs and their ligands reveals the existence of mechanisms that stabilize ECs and highlights the importance of mural cells in vessel maintenance. Tie1 is an endothelium-specific orphan receptor (a receptor with no known ligand) closely related to Tie2. Tie1 is expressed in ECs and their precursors during development but is also expressed in most nonproliferating adult endothelium. Mice homozygous for null Tie1 mutations exhibit normal vascular development until midgestation. They then appear to suffer a loss of the microvasculature, accompanied by hemorrhage.[42] More detailed analysis of these animals suggests that the vascular density is actually higher than normal in many tissues because intussusception occurs more frequently.[35] This change appears due to the "hyperactive" behavior of Tie1-deficient ECs, which also compromises vessel integrity. Individual Tie1-null ECs project numerous extensions and filopodia and frequently have other defects in morphology, such as frequent intracellular and transcellular holes. Periendothelial mural cells associated with these defective vessels also exhibit a "hyperactive" phenotype.[35] Together these studies suggest that the normal function of Tie1 activity is to suppress the exploratory behavior of EC cells and their associated mural cells, thereby stabilizing vessels and preventing further vessel division.

Recruitment of periendothelial mural cells to new blood vessels is mediated by EC expression of platelet-derived growth factor B (PDGF-B), acting through its receptor PDGFR-β, which is expressed in the adjacent mesenchyme (reviewed by Betsholtz and co-workers[43]). Inactivation of either the PDGF-B or PDGFR-β genes in the mouse results in death in late gestation that is associated with dilation of the heart and blood vessels, rupture of capillaries, and widespread edema and hemorrhage.[44-47] Deficiencies in vascular SMCs and pericytes occur in many tissues, with the extent of deficiency correlating with the occurrence of vessel dilation and microaneurysms.[47] Absence of pericytes is linked to EC hyperplasia, with the walls of dilated vessels containing a higher than normal density of ECs. In the pericyte-deficient vessels of PDGF-B–null mice, ECs exhibit a "hyperactive" morphology that is reminiscent of the Tie1-null phenotype, with the most extreme disorganization of ECs occurring in microaneurysms.[48] Thus the recruitment of periendothelial cells mediated by PDGF-B and PDGFR- β appears to be a critical part of stabilizing both EC number and organization (see Figure 64-4).

Vascular Patterning: Not All Endothelial Cells Are Created Equal

Many differences between classes of vessels involve the vessel wall and stem from differential ECM production by the mural cells. It has long been postulated that the ECs themselves also form very distinct populations, with unique characteristics and perhaps unique origins. However strong molecular evidence for such distinctions has only recently been obtained, when localized expression of regulatory molecules, such as transcription factors, receptors, and ligands, revealed multiple levels of EC heterogeneity. In particular, distinct molecular signatures for ECs are located within arteries, veins, and lymphatic vessels and in the endocardium of the heart.[49-54]

The functional significance of such distinctions is exemplified by differences in the venous and arterial expression patterns of the RTK Eph-B4 and its transmembrane ligand ephrin-B2.[49,51] The Eph receptors, the largest described family of RTKs, bind membrane-bound ephrin family ligands. Members of the ephrin-B family of ligands can autophosphorylate upon receptor binding, thereby allowing bidirectional signaling during cell-cell interactions.[55] During embryogenesis, the Eph-B4 receptor is specifically expressed in ECs almost exclusively in developing veins, whereas its only known specific ligand, ephrin-B2, is expressed in ECs in arteries.[49-51] Homozygosity for null mutations in either ephrin-B2 or Eph-B4 results in defects in angiogenesis, but not in vasculogenesis, both in the yolk sac and in the embryo. In both cases, the primary vascular plexi are established, but there are abnormalities in remodeling that have an impact on both arteries and veins. Although both classes of vessels are disrupted, the venous system is more strongly affected in the Eph-B4–null embryos, and, conversely, the loss of ephrin-B2 has a greater impact on the arterial system.[49,51] The venous and arterial expression of this receptor-ligand pair is suggestive of a role in mediating interactions between the two classes of vessels. Consistent with this idea, normal intercalation between arteries and veins fails to occur in yolk sacs of ephrin-B2–deficient embryos.[49] Together with other data on the expression and function of Eph family members,[50] these results demonstrate that molecular differences between the arterial and venous ECs are established early in development. Moreover, these molecules are essential for the normal development of the vascular tree, likely mediating cell-cell interactions specific for subsets of the endothelium (see Figure 64-4).

The patterns of expression and localized functions of certain transcription factor genes reveal further levels of EC heterogeneity.

Notably the transcription factor gene, nuclear factor of activated T cells-1 (NFATc1), is expressed in the endothelial lining of the heart and the endocardium but not in other EC populations. In valve formation, ECs in the valve-forming region undergo an epithelial-mesenchymal transition, delaminating and entering the underlying endocardial cushion. During development, NFATc1 expression becomes restricted to the valve-forming regions of the endocardium. Thus NFATc1 expression defines an endothelial subpopulation with unique properties. A functional requirement for NFATc1 activity in endocardium is demonstrated by targeted disruption of the *NFATc1* gene. Mouse embryos homozygous for *NFATc1* mutations die in utero with congestive heart failure associated with the absence of aortic and pulmonary valve formation and with ventricular septal defects.[53,54]

The ECs of the lymphatic system are also distinct from other ECs at the molecular level. They exhibit higher levels of expression of several markers, including the transcription factor gene Prox1, the VEGF receptor VEGFR3, and the lymphatic endothelial hyaluronan receptor LYVE-1[52,56,57] (reviews of this subject are available[58,59]). The lymphatic system appears to develop through budding of the preexisting venous system.[60,61] Prox1 is expressed at sites where lymphatic vessels bud from veins, and its expression is subsequently restricted to the lymphatic system.[52] In Prox1-deficient–mice, the early budding of the future lymphatic vessels from the cardinal vein occurs normally, but this process arrests in such a way that the animals lack lymphatic ECs.[52] Failure of budding cells to express high levels of VEGFR3 or LYVE-1 suggests that Prox1 controls a program of lymphatic EC differentiation and is required to maintain the budding process.[52,58]

Our understanding of endothelial heterogeneity is extremely limited, and much remains to be discovered about the extent of such heterogeneity, its significance, and the cellular and molecular mechanisms by which it is acquired. A key question is, "How does such EC heterogeneity arise?" For example, do such differences reflect variation in the origin of ECs, or are they acquired by naive ECs through local environmental cues? Because localized expression of genes within the EC has only recently been described, few experiments have addressed this issue. A recent study of vessels in the skin, however, indicates that the arterial-venous identity of smaller vessels is defined at the time of vessel remodeling through local cues.

It has long been recognized that in peripheral tissues, nerves often run along blood vessels. The vascular system and peripheral nervous system are both complex branched structures, so this intimate association raised the question of whether one branched pattern determined the other, or whether they are independently responding to common cues from their surroundings (as reviewed by Shima and Mailhos[62]). In the embryonic chick skin, only some vessels are co-localized with nerves. Using the arterial and venous markers ephrin-B2 and Eph-B4 (described in an earlier section), Mukouyama et al[63] found that arteries but not veins ran along nerves (Figure 64-5). Mutations that eliminate or alter the patterns of peripheral sensory nerves caused corresponding alterations in arterial patterning, particularly in small-diameter vessels. Thus the pattern of small arteries appears to follow that of the sensory nerves. Expression of the arterial marker ephrin-B2 was found to be acquired during remodeling of the vascular plexus, around the time at which the patterns of vessels and nerves begin to correlate, suggesting that nerves might influence both arterial identity and vessel remodeling. Nerves and their associated Schwann cells are local sources of VEGF, and this factor can account for the ability of both neurons and Schwann cells to induce expression of the ephrin-B2 arterial marker from cultures of ephrin-B2–negative ECs. A specific marker of arterial identity thus appears to be induced by a local VEGF signal from sensory nerves. However, in these experiments the percentage of ECs that could be induced to express ephrin-B2 was never greater than 50%, perhaps also suggesting that only certain ECs are able to respond to this signal.[63]

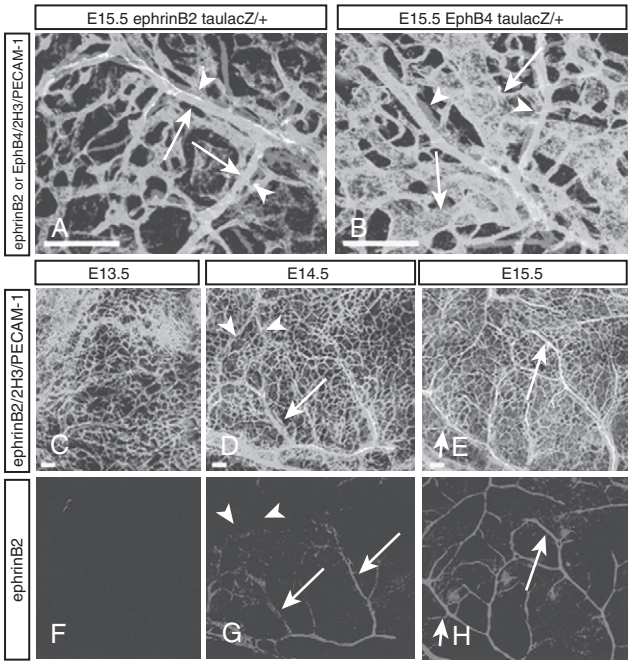


Figure 64-5 Peripheral nerves provide a template for arteriogenesis in the skin. Whole-mount triple immunofluorescence confocal microscopy was used to follow the differentiation of arteries (during embryogenesis; e.g., E15.5 = embryonic day 15.5) by ephrin-B2 in red **(A, C, D),** of veins by Eph-B4 immunolocalization in red **(B),** of nerves with 2H3 localization in green, and of endothelial cells with PECAM11/CD31 localization in blue. Arteries *(arrows)* are specifically aligned with peripheral nerves *(arrowheads)* **(A),** whereas veins *(open arrows)* show a more random distribution **(B).** In **C** to **H,** a time-course from mouse embryonic ages 13.5 days to 15.5 days (E13.5 to E15.5), it is apparent that nerve innervation of the skin *(green)* precedes arterial differentiation *(red)* of the primary vascular plexus *(blue). (Modified from Mukouyama YS, Shin D, Britsch S, et al: Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin,* Cell *109:693, 2002.)*

Epigenetic Factors Driving Vascular Development and Morphogenesis

In the mature vascular system, the extent of vascularization is exquisitely matched to the physiologic needs of the tissue. Moreover at different points in the vascular tree the properties of blood vessels, such as their number, diameter, and wall thickness, are matched to the flows and pressures in that part of the circulatory system. These relationships between vascular system structure and physiology are not the result of a rigid genetic program that predetermines the genesis and final properties of every blood vessel. Instead physiologic (epigenetic) factors exert a major influence over the form of the cardiovascular system. Epigenetic factors help explain some key questions that have arisen from observation of normal blood vessel morphogenesis and play important roles in cardiovascular malformations and disease. As discussed earlier, remodeling results in large vessels being created from a relatively uniform plexus of small vessels. What makes some vessels increase in size while others do not? Extensive vessel regression also occurs, so only a fraction

of the original capillaries within a primary plexus is present in the mature vascular network. What determines which vessels survive? Equally, new vessels are generated in angiogenesis by sprouting and intussusception. Why do new vessels form in one location and not another? What determines how many new vessels should be formed? Two epigenetic factors that play significant roles in vascular development are hemodynamic forces and hypoxia.

Hemodynamic Forces

The forces acting on a blood vessel due to blood flow can be resolved into at least two components (as reviewed by Davies[64]). Blood pressure acts perpendicular to the lumen surface, so changes in pressure stretch all components of the vessel wall (endothelium, smooth muscle, and ECM). Tangential to the lumen surface, the flowing blood generates a frictional force. This produces a shear stress, which mainly has an impact on the endothelium because these are the cells in contact with the fluid.[64] ECs in vivo are aligned in the direction of blood flow, and in vivo and in vitro experiments demonstrate that flow can cause ECs to alter their morphology to adopt this alignment (Figure 64-6). In fact ECs exhibit numerous responses to shear stresses, including alterations in morphology, mechanical properties, and gene expression (reviews are available in the literature[64,65]).

In model organisms, the manipulation of blood flow patterns can have a profound impact on cardiovascular development. For example, the vitelline veins in the avian yolk sac have been ligated to alter the hemodynamics of the developing embryo. Vitelline vein ligation alters the path through the heart adopted by blood flow originating from each region of the yolk sac.[66] Heart rate is also decreased, but there is a compensatory increase in cardiac output.[67] Although the ligation occurs at a site distant from the heart, these altered hemodynamics produce changes in cardiac morphology and disruption of the remodeling of the pharyngeal arch arteries. Typical outcomes include ventricular septal defects, semilunar valve malformations, and pharyngeal arch artery abnormalities.[66,67] Most recently, experiments in the zebrafish[68] further demonstrate that hemodynamic forces are epigenetic factors required for normal heart morphogenesis. Blocking either inflow or outflow to the heart results in similar malformed, hypoplastic hearts with poor differentiation of the valves. The similarity between these phenotypes suggests that shear stress, rather than blood pressure, is the main morphogenetic influence because obstructed inflow and outflow will both result in a reduced flow but are expected to have opposite effects on intracardiac pressure.[68]

During angiogenesis, local variation in hemodynamic forces likely exerts a major influence upon the remodeling of embryonic vessels,[30,69] particularly in determining which vessels are to be enlarged and in controlling the size they attain. For example, it is notable that the large yolk sac vessels develop only after there is significant flow when the connection with the intraembryonic vessels has been established. Defects in this connection are often associated with a failure of subsequent yolk sac remodeling.[32,33,70] Moreover, the vitelline vein ligation experiments described lead to altered remodeling of the capillary plexus, producing a new vein that bypasses the ligation.[66] Thus local changes of blood flow lead to alterations in the pattern of vessel remodeling.

The molecular basis of shear stress responses has yet to be fully characterized, but the wide variety of cellular and molecular responses observed is consistent with multiple structures acting as mechanosensors (see Figure 64-6) and consequently with a diversity of molecules acting in shear stress signal transduction.[64] Because the EC layer provides an "interface" between blood and tissue, it serves as the primary mechanotransducer. Variations

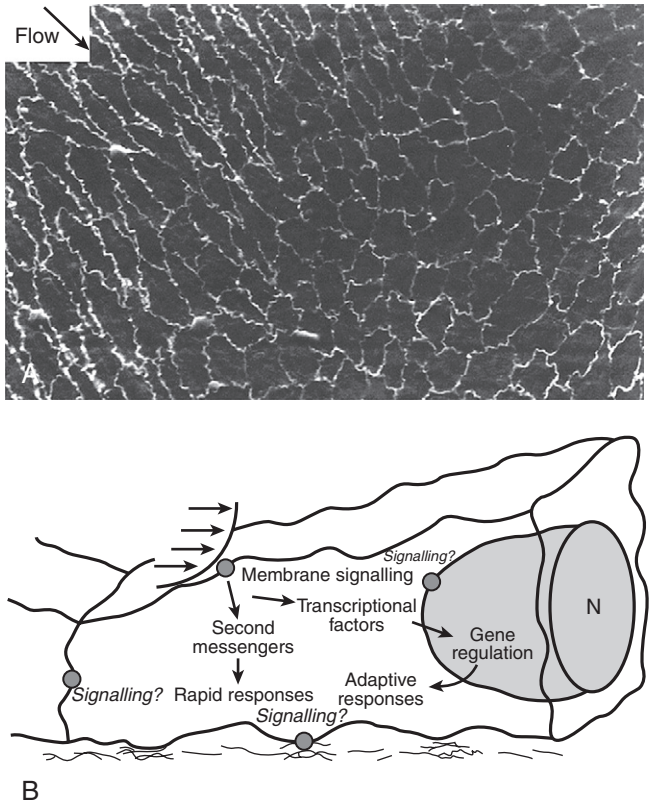


Figure 64-6 Shear stress is a major epigenetic determinant of vascular morphogenesis. **A,** ECs align themselves with direction of flow. **B,** Endothelial response to flow results in numerous alterations at the cell membrane, intercellular junctions, and basal lamina, which result in both primary and secondary alterations in gene regulation. Thus the EC serves as a "mechanotransducer" for flow within the cardiovascular system. *N,* Nucleus. *(Modified from Davies PF: Flow-mediated endothelial mechanotransduction,* Physiol Rev *75:519, 1995.)*

in flow result in altered levels of many vasoconstrictive and vasodilatory factors, including endothelin-1, prostacyclin, and nitric oxide.[64,69] These can certainly modulate vascular tone in response to hemodynamics, but such effects probably play roles late in vascular differentiation.[64,69]

Less is known about flow-responsive pathways in earlier vascular morphogenesis, but some key molecules in vascular development are also implicated in shear stress responses. For example, altered flow changes the EC cytoskeleton and the pattern of focal adhesions, the points of contact between ECs and ECM. Transmembrane integrins are prominent components of focal adhesions, where they bind extracellular adhesion proteins and link the ECM to the actin filaments of the cytoskeleton. New integrin binding to the ECM activates the small guanosine triphosphatase Rac1. Rac1 activation mediates cell alignment and orientation of filamentous actin stress fibers, as well as flow-induced stimulation of the transcription factor nuclear factor kappa B (NF-κB).[71] Thus EC integrins, important for vascular morphogenesis, also play a role in sensing shear stress.

One key transcriptional target of shear stress in ECs is PDGF-B, which is up-regulated by shear stress.[72] The up-regulation of the PDGF-B promoter is mediated by a shear stress–responsive element that is bound by NF-κB.[73] Because PDGF-B mediates recruitment of perivascular mural cells,[47] regulation of its expression by shear stress could link flow to stabilization of vessels during vascular development.

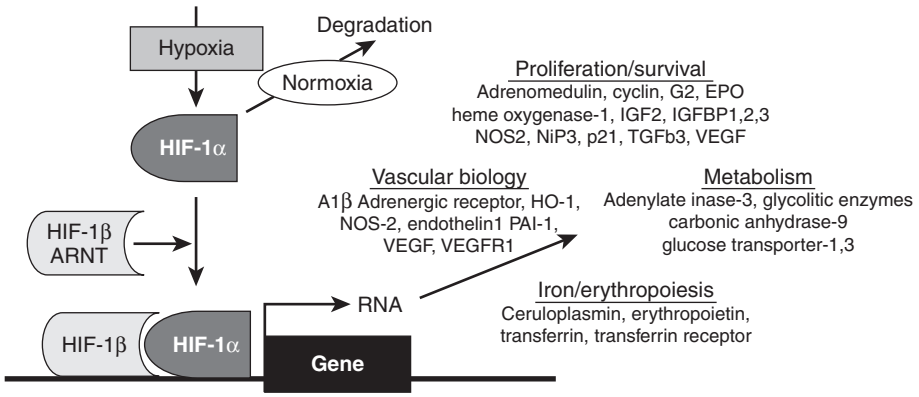


Figure 64-7 Hypoxia-mediated gene regulation through HIF-1α and HIF-1β. The level of HIF-1α is determined by the balance between hypoxia, which drives increased expression, and normoxia, which accentuates expression of signaling cascades, that result in degradation of HIF-1α. Under hypoxic conditions, HIF-1α and HIF-1β dimerize and then bind the hypoxia response element (HRE) in the regulatory region of several genes, resulting in transcription of numerous gene products.

Hypoxia

In healthy adults, angiogenesis is primarily limited to select sites such as the female reproductive system, but extensive angiogenic responses (neovascularization) can accompany disruptions in oxygen homeostasis, such as those that occur during tumor growth or in tissue ischemia.[21] Evidence for oxygen tension governing angiogenic processes in disease states has led to investigation of whether such mechanisms operate during the normal vascular development in utero. Consistent with this idea, multiple components of the signaling systems responsible for normal vascular development are regulated by hypoxia. Notably, VEGF and VEGF receptor 2 are both up-regulated in hypoxic conditions,[74,75] potentially enabling enhanced VEGF signaling to promote new blood vessel formation and/or promote survival of existing vessels. Additional hypoxia-responsive genes important for vascular morphogenesis include those encoding Tie1,[76] the angiopoietins Ang-1 and Ang-2,[77,78] and PDGF-B.[79] Many of these responses occur at the transcriptional level and are mediated by the hypoxia-induced transcription factor HIF-1, discussed in a later section. However, hypoxia also affects expression by other mechanisms, such as specific increases in VEGF messenger RNA stability.[80]

Recently some critical components of the oxygen-sensing pathways have been identified. Central to hypoxia-mediated control of gene expression is HIF-1. HIF-1 target genes up-regulated in hypoxia are involved in increasing O_2 delivery (via erythropoiesis, vascularization, or vasodilation) or are involved in metabolic adaptation to limited O_2 (principally glycolysis) (reviewed by Ikeda and associates[81]). The HIF-1 factor is a heterodimeric complex,[82] consisting of a regulatory α subunit and a constitutive β subunit, both of which are helix-loop-helix PAS domain proteins (Figure 64-7). The α subunits, Hif-1α and Hif-2α, confer the hypoxia responsiveness of the complex. Expression of HIF-1 increases exponentially with decreasing oxygen tension.[83] It is interesting that the major part of this up-regulation is achieved not through an increase in HIF-1α production in response to hypoxia, but rather through increased protein stability of the Hif-α subunits and diversion from the normal pathway of rapid protein degradation.[84,85]

With the exception of structures in direct contact with the atmosphere, such as the respiratory epithelium and outer layers of skin, the normal environment for most mammalian tissues is an O_2 concentration of 3% to 5%, making the tissues "hypoxic" relative to the 20% atmospheric O_2 concentration. The intrauterine environment is likely at a similar low oxygen tension because the optimum O_2 concentration for in vitro culture of early mammalian embryos is 5%, not 20%.[86] Embryonic tissues thus generally are developing at low oxygen tensions but also appear to contain regions of significant hypoxia. Using the hypoxia marker pimonidazole hydrochloride, Lee an colleagues[87] examined the spatiotemporal distribution of tissue hypoxia in the developing mouse embryo. They found multiple domains of local hypoxia that were not only closely correlated with the Hif-1α and VEGF immunoreactivity but also were mapped to locations where angiogenesis is known to occur, including the extraembryonic tissues, neural tissue, intersomitic regions, and ventricles of the heart.[87] These data support the idea that during embryogenesis there is local tissue hypoxia that could modulate blood vessel morphogenesis through induction of VEGF or other HIF-1 responsive genes.

The existence of embryonic tissue hypoxia, together with the oxygen-tension effects on regulators of vascular morphogenesis, has led to the idea that the proper morphogenesis of the vascular system *depends* upon the relatively hypoxic state of tissues in utero. Explant organ-culture models appear to confirm this idea because EC survival and development of the vasculature are sensitive to oxygen tension.[88] Additional evidence supporting an important role for hypoxia in the regulation of vascular development comes from analysis of animals with targeted deficiencies in the HIF-1 regulatory cascade. Mice homozygous for null mutations in the *Hif-1*α gene die at midgestation with pericardial effusion and myocardial hyperplasia. There are vascular malformations, including defects in yolk sac remodeling, interruptions in the intersomitic vessels, and a marked deficiency of cephalic vessels, accompanied by dramatic cephalic vessel dilation.[89,90] It is surprising, however, that the defective remodeling and vascular regression do not appear to be related to a deficiency in VEGF; they may instead by a consequence of extensive cell death in mesenchyme, probably of neural crest origin.[91] These studies also found that glucose deprivation was able to stimulate VEGF expression independently of HIF-1. Therefore, in regions of vascular insufficiency, VEGF up-regulation may be achieved either by a response to inadequate glucose supply or by a response to hypoxia through HIF-1.[91]

Another member of the hypoxia-inducible factor family, Hif-2α (also known as hypoxia-inducible factor-like/endothelial PAS domain protein 1), which is 48% similar to Hif-1α at the amino acid sequence level, can modulate expression of genes important for vascular development, including Tie2.[92] Mice deficient in Hif-2α die in utero and either exhibit a failure of vascular remodeling[93] or exhibit defects of catecholamine homeostasis coupled with heart failure.[94] Mice homozygous for a hypomorphic allele

of Hif-2α are viable and apparently develop normally, but they exhibit a diminished response to changes in oxygen tension.[95] In a mouse model of retinopathy of prematurity, exposure of young animals to 75% O_2 for 5 days causes obliteration of retinal vessels, which is followed by neovascularization when the animals are returned to room air and the retina experiences hypoxia owing to vascular insufficiency. After the transfer to room air, Hif-2α expression levels in the eye are approximately 20% of normal in the hypomorphic Hif-2α mutants, and erythropoietin levels are similarly reduced. The retinas of these animals do not undergo neovascularization and instead undergo retinal degeneration. Recombinant erythropoietin restores neovascularization in mutant retinas, suggesting overlap between Hif-2α functions in regulating oxygen delivery and in hypoxia-induced neovascularization.[95]

Two other components of the HIF-1 pathway, Hif-1β (also known as aryl hydrocarbon receptor nuclear translocator) and von Hippel-Lindau syndrome tumor suppressor (VHL), have been studied in the mouse by targeted gene disruption experiments. Deficiencies in these factors have been shown to have profound impacts on vascular development, generating defects that ultimately result in embryonic lethality.[96-100] Animals deficient for Hif-1β also have defective angiogenesis in yolk sac and branchial arch arteries.[97] Defects are also seen in the placenta, where there is a failure of the labyrinth to vascularize.[98,100] The mechanism by which Hif-1β deficiency disrupts placental vascular development has not yet been established, but there has been considerable progress toward identifying the specific cell types and molecules involved.

Studies in which Hif-1β–null ES cells are combined with wild-type cells, in chimeric embryos or with tetraploid embryos, demonstrate that Hif-1β function is required in the trophoblast cells rather than in the embryonic endothelial component of the placenta.[100] Placental levels of VEGF are unaltered in Hif-1β–null embryos, but VEGFR2 is significantly lower in the labyrinth endothelial, trophoblast, and giant cells.[99] Deficiency for VHL, which normally serves to "mark" the Hif-1α subunits for degradation, also results in embryonic lethality from defects in the development of the placental vasculature. On the maternal side, the capillary networks of the placenta form by angiogenesis, whereas on the embryonic side, the first capillaries develop in the allantois by vasculogenesis.[101] In VHL-deficient embryos, there is a lack of embryonic blood vessels within the placental labyrinth, which leads to the formation of a severely disrupted, hemorrhagic placental site.[96]

Certainly, VHL and Hif-1β may each have functions besides their contributions to HIF-1 action; nevertheless, the embryonic and placental defects observed in mice with deficiencies in four separate components of the HIF-1 hypoxia-response cascade (Hif-1α, Hif-2α, Hif-1β, and VHL) collectively provide strong genetic evidence for a role for oxygen tension as a regulator of cardiovascular development in utero. For the future, examining how the cellular sensing of oxygen tension is integrated with vascular morphogenesis can be expected to offer significant insight into how the developing vascular system is matched to the metabolic needs of embryonic tissues.

Development of Pulmonary Vessels

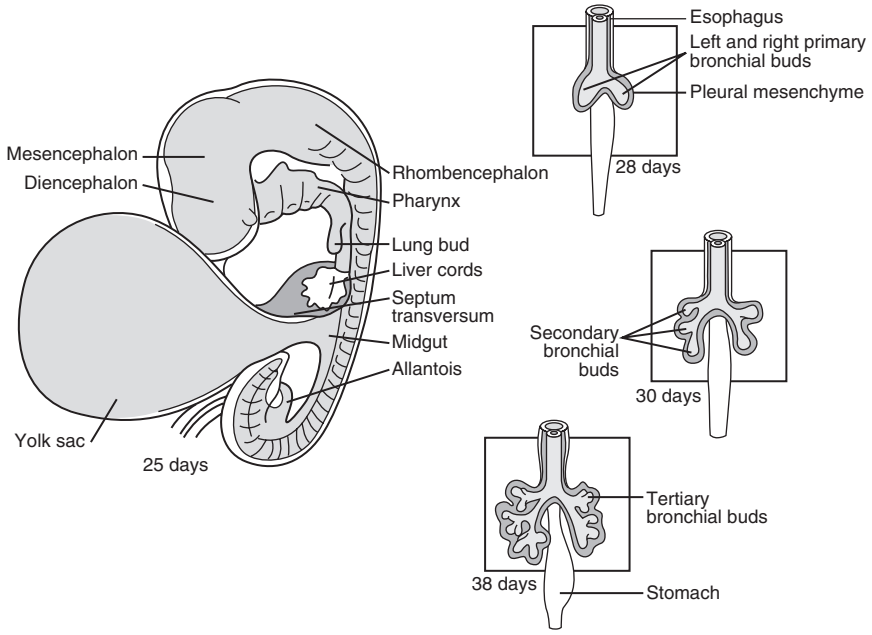
Although treatment of airway immaturity in the premature lung is a significant focus of clinical effort in neonatal intensive care units, the pathology associated with abnormal development of the pulmonary vascular bed has been one of the least-studied areas in cardiovascular biology. Establishment of a dual blood supply, considerable development after birth, and an apparent dependence on airway formation combine to make pulmonary vascular ontogeny an extremely challenging area of research. Much of what is known is currently descriptive in nature. Nevertheless, a renewed interest in this vascular bed,[102-106] accompanied by the use of new molecular-genetic tools, cellular markers, and culture models,[103,105,107-109] is beginning to provide answers to some long-standing questions and may provide novel strategies for therapeutic intervention.

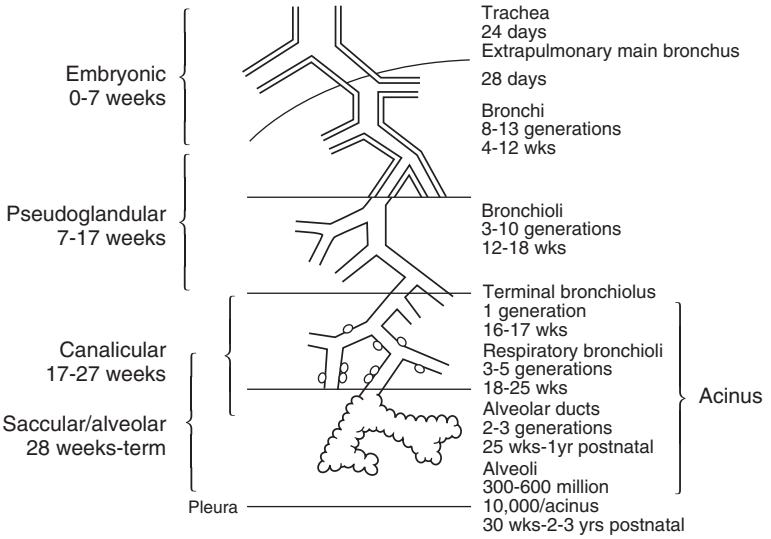
Based primarily on epithelial processes, lung development has been divided into four or five stages (Figure 64-8).[110,111] The embryonic stage (26 days to 6 weeks in humans) begins with the first appearance of lung buds as outpocketings of the foregut endoderm, surrounded by splanchnic mesoderm. The pseudoglandular stage (6 to 16 weeks) is characterized by rapid and extensive epithelial branching so that by the end of this stage, all preacinar airways are present. During the canalicular stage (16 to 28 weeks), the airways continue to branch, the mesenchyme thins, capillaries come to underlie the epithelium, and effective blood-gas barriers are first formed. The epithelium differentiates into type I and type II pneumocytes, and surfactant production begins. The saccular stage (26 to 36 weeks) features division of distal air spaces into smooth-walled structures known as saccules. In the alveolar stage (36 weeks onward), the saccules are remodeled into alveoli.

The lung epithelium is closely associated with developing vasculature from the earliest stage. Differentiation of a ventral vascular plexus into which a bud of foregut endoderm protrudes was first described in 1931 by Chang[112] and has since been observed by numerous investigators using a variety of animal models.[113] The arterial system of the lung is ultimately connected through the sixth pharyngeal arch, which becomes the pulmonary artery. The venous system of the lung connects directly to the left atrium. A major issue in past decades has been whether the early vascularization of the lung and its connection to the arterial and venous systems involves vasculogenesis, angiogenesis, or some combination of the two. Incorporated in this question is the origin of the ECs in the pulmonary vessels. Are their precursors endogenous to the lung, or do they immigrate into the lung from elsewhere?

Some of the debate about the relative contributions of angiogenesis or vasculogenesis in pulmonary vascular development may reflect the limitations of the individual techniques employed. Classic histologic analysis and methods that rely on the presence of a patent lumen, such as India ink injections and resin corrosion (Mercox) casts, tend to suggest penetration of the lung primordia by angiogenic mechanisms arising from major pharyngeal arch arteries.[18] On the other hand, using marker gene expression to identify individual ECs or precursors reveals more cell behavior that is not associated with a definitive vessel, behavior characterized as vasculogenesis.[103-105,113] Figure 64-9, *A,* shows a reconstruction[105] of thorax sections from a 34-day-old human embryo, illustrating the early vascular network of the lung primordia in relation to the heart and major vessels. The presumptive pulmonary arteries and pulmonary veins are already present and associate through a capillary plexus. Sections from transgenic mice expressing β-galactosidase in all ECs (Figure 64-9, *B*) reveal a similar continuity between the future arterial, venous, and capillary vessels.[103] In the periphery of the developing pseudoglandular-stage human or mouse lung, ECs assemble into local networks before the anastomosis with the intrapulmonary arteries.[18,106] Thus vasculogenesis is responsible for much of the early lung vascular development.[106]

At this stage, two classes of ECs can already be identified: those associated with smooth muscle actin–positive mural cells, and those that are not.[106] These differences reflect distinctions between capillaries and major vessels, suggesting that extensive differentiation of the lung vasculature begins very early in lung morphogenesis. Allografts of early pseudoglandular-stage mouse lungs under the kidney capsule have been shown to allow for extensive lung development and maturation to the saccular





Stage	Mouse	Human
Embryonic	E9-14.2	3-7 wk
Pseudoglandular	E14.2-16.6	7-17 wk
Canalicular	E16-17.5	17-27 wk
Saccular	E17.5-5d p.c.	24-38 wk
Alveolar	5-30 p.c.	36.5 yrs p.c.

Figure 64-8 The critical stages of lung development, with a comparison of equivalent developmental stages in both mouse and human embryos. These stages are based on branching morphogenesis and relation to alveolar development. Comparative stages in vascular development have not yet been fully defined. *(Modified from Larsen WJ, editor:* Human Embryology, *2nd ed. New York, Churchill Livingstone, 1997, p 138; and Hislop AA: Airway and blood vessel interaction during lung development,* J Anat *201:325, 2002.)*

stage, including formation of appropriate vasculature.[108] Genetic marking of host ECs revealed that all vessels within the lung were derived from the graft. Therefore at the time of grafting the explant contained all the ECs and angioblasts required for production of the complete saccular-stage vascular tree, independent of angiogenic contributions from the extrapulmonary vasculature. Connections with the host circulation were made, but host vessels never penetrated the graft (host to graft), implying that the lung tissue prevents other vessels from entering it while maintaining the intrinsic ability to establish angiogenic connections (graft to host) with the circulatory system.

It was previously thought that the canalicular stage represented a burst of vascular growth/differentiation, but recent studies relating EC number to lung protein content (lung size) refute

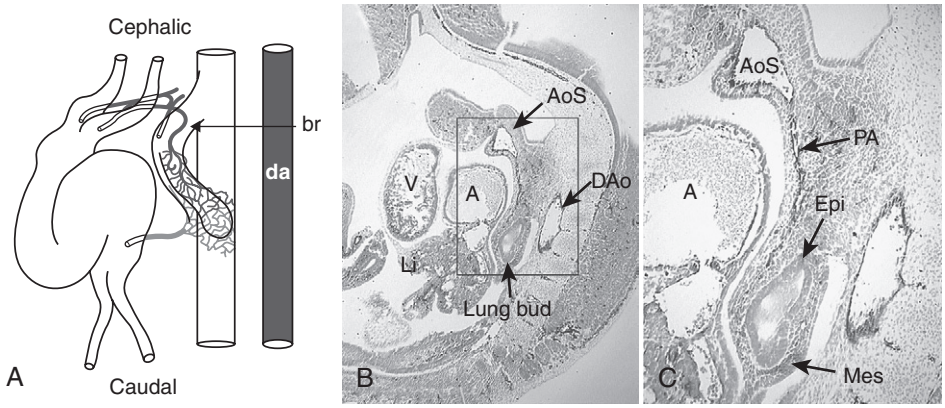


Figure 64-9 A, Diagram produced from a serial reconstruction of a 34-day human embryo, showing continuity of the lung bud capillary network with central vascular structures from the onset of lung development. Low-power **(B)** and high-power **(C)** magnification of a sagittal section through an embryonic day 10.5 mouse embryo of similar gestational age to that of the human embryo in **A.** ECs can be identified by β-galactosidase activity resulting from lacZ inserted into the Flk1 locus. The pulmonary artery *(PA)* can be seen connecting the aortic sac *(AoS)* and lung mesoderm *(Mes).* These examples document that in both human and mouse, development of the pulmonary vascular bed occurs in continuity with the major vascular structures of the embryo. *A,* Atrium of the heart; *br*, bronchus; *da,* dorsal aorta; *DAo,* dorsal aorta; *Epi*, epithelium of the lung bud; *Li,* liver; *V,* ventricle of the heart. *(Modified from Hall SM, Hislop AA, Haworth SG: Origin, differentiation, and maturation of human pulmonary veins,* Am J Respir Cell Mol Biol *26:333, 2002; and Schachtner SK, Wang Y, Scott Baldwin H: Qualitative and quantitative analysis of embryonic pulmonary vessel formation,* Am J Respir Cell Mol Biol *22:157, 2000.)*

this idea. It now appears that the pulmonary vasculature expands smoothly, rather than in a burst, so that the increase in the vasculature with gestation age largely mirrors the total growth of the lung.[103] Of importance in this period, however, is an apparent transition from vasculogenesis to angiogenesis. In particular, although undifferentiated mesenchyme in the early pseudoglandular lung is highly proliferative, the endothelium now exhibits a greater proliferative rate than the remaining mesenchyme, suggesting that the vascular network may be growing by angiogenesis as well.[105]

Lung vasculature development parallels the branching morphogensis of the lung epithelium (as reviewed by Hislop[114]), which suggests that epithelial and vascular development are somehow coordinated. This would likely demand active signaling between epithelium and ECs. Tissue recombination experiments with embryonic lung mesenchyme and epithelium confirm that the lung epithelium produces some factor(s) that promotes or maintains normal vascular development in lung mesenchyme.[109] Separating the lung mesenchyme and epithelium disrupts the normal spatial relationship between epithelium and endothelium, but recombining the separated epithelium and mesenchyme and culturing them together generates new EC patterns in appropriate positions relative to the epithelium. Furthermore, culturing lung mesenchyme in the absence of epithelium results in decreased mesenchymal proliferation and increased apoptosis, as well as a marked reduction in the number of ECs.[109]

The pulmonary ECs express VEGF receptors 1 and 2. These likely mediate signals from the nearby epithelium, which expressed VEGF ligand.[106,115-118] Early lethality of VEGF-null mutations has precluded use of these mice to study the dependence of pulmonary vascular development on VEGF, but other approaches have recently revealed the importance and complexity of VEGF signaling in the lung. Through alternative splicing, the VEGF gene produces multiple protein isoforms that differ in their abilities to bind cell-surface and ECM-associated heparan sulfate proteoglycans and the VEGF receptors (as reviewed by Neufeld and co-workers[119]).

In the mouse, there are at least three such isoforms: $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$, of which only $VEGF_{164}$ and $VEGF_{188}$ can bind heparan sulfate. These heparan sulfate-binding VEGFs are the predominant isoforms expressed in the developing lung.[117,118] Upon synthesis by the epithelium, VEGFs are localized to the subepithelial matrix. Heparin-bound $VEGF_{164}$ on beads implanted into pseudoglandular-stage lung explants induces neovascularization around the bead, suggesting that VEGF localized close to the distal epithelium helps direct the pattern of new blood vessel formation, linking epithelial and vascular morphogenesis.[117] Isoform-specific gene knockout experiments support this idea but also suggest that the heparan-binding VEGF isoforms may be uniquely required only for the microvessels of the pulmonary vascular tree. Mice engineered to produce $VEGF_{120}$, but not $VEGF_{164}$ or $VEGF_{188}$, survive to birth but exhibit pulmonary hypoplasia, with reductions in air spaces and multiple defects in pulmonary vasculature.[120] The diameter and branching patterns of the preacinar vessels are relatively unaffected, but animals producing only $VEGF_{120}$ have dramatically fewer small, but dilated, peripheral vessels (Figure 64-10). Blood-gas barriers are reduced in number, and intervening cells aberrantly separate the capillaries from the air space.[120] These data point to a requirement for epithelium-derived matrix-binding VEGF isoforms in the generation and proper juxtaposition of capillaries with alveolar type I cells.

Pulmonary vascular development depends upon the combinatorial action of multiple signals from the lung epithelium as well as inputs from other cell types, and the repertoire of signals is not limited to the VEGF signaling cascade. During branching morphogenesis, distal lung epithelium expresses high levels of the *Wnt7b* gene,[121] a member of the Wnt family of at least 19 mammalian growth factor genes (as reviewed by Moon[122] and Wodarz[123] and their colleagues). *Wnt7b*-deficient mice die at birth from respiratory failure associated with hypoplastic lungs that exhibit pulmonary hemorrhage. Such mice demonstrate deficiencies in the integrity of the vascular smooth muscle, including both vascular smooth muscle cell (VSMC) hypertrophy and apoptosis.[121] These observations suggest that a *Wnt7b* signal from the lung epithelium promotes mesenchymal proliferation in the early lung and later supports VSMC differentiation and survival. Together, the studies on the actions of VEGFs and *Wnt7b* indicate that the epithelium produces factors controlling

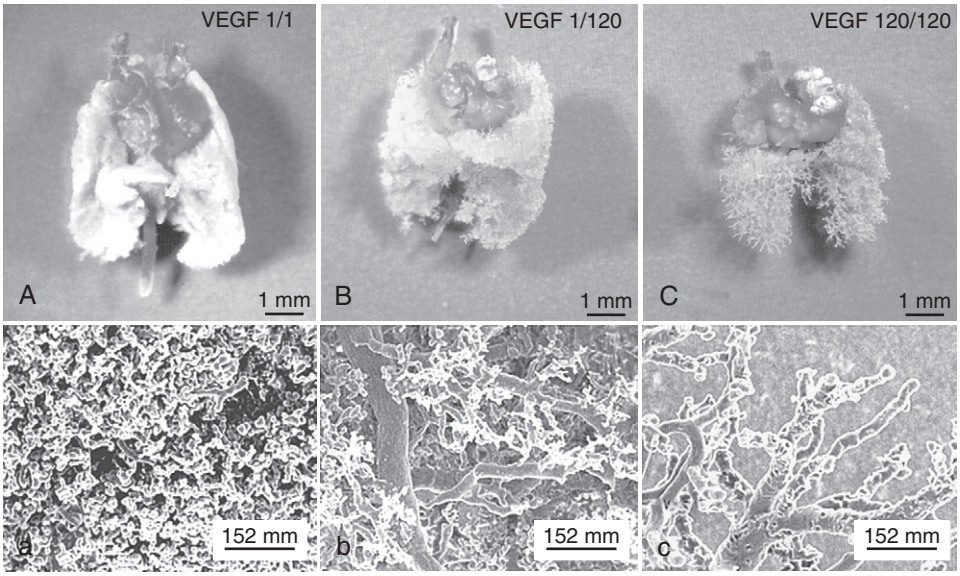


Figure 64-10 The impact of altered VEGF signaling on pulmonary vascular development. Lung resin corrosion (Mercox) casts **(A-C)** and scanning electron micrographs **(a-c)** from E17 wildtype **(A, a),** heterozygous $VEGF_{120/+}$ **(B, b),** or homozygous $VEGF_{120/120}$ mice **(C, c).** Replacement of all VEGF isoforms with $VEGF_{120}$ alone results in a marked, "dose-dependent" reduction in the number of small peripheral vessels without a noticeable impact on the preacinar vessels. *(Modified from Galambos C, Ng YS, Ali A, et al: Defective pulmonary development in the absence of heparin-binding vascular endothelial growth factor isoforms,* Am J Respir Cell Mol Biol 27*:194, 2002.)*

and supporting pulmonary vascular development at many levels that include:

- Promoting and localizing neovascularization
- Regulating vessel size
- Enabling proper association between capillaries and epithelium
- Regulating the behavior of VSMC on larger vessels

Conversely, reciprocal signaling likely occurs from the vessels to the lung epithelium because pulmonary vasculature influences epithelial morphogenesis. Interfering with VEGF signaling in the neonatal animal, normally a time of significant alveolargenesis, is an example of this phenomenon. Mice in which VEGF signaling has been blocked for the first 5 days of life have lungs that appear immature and exhibit a less complex alveolar pattern than normal, establishing that ongoing VEGF-mediated development or function or both of the vasculature is essential for normal alveolar morphogensis.[34]

Epigenetic factors like hypoxia and shear stress are almost certain to be important for pulmonary vascular development. An example from our own work on the role of oxygen tension in pulmonary vascular development is shown in Figure 64-11. Mouse lung explants cultured in 3% O_2 and 19% O_2 exhibit very different patterns of EC localization (revealed by whole-mount immunohistochemisty for the EC-specific adhesion molecule PECAM/CD31). In low-oxygen tension, comparable to that detected in utero, many PECAM-positive cells are present, and an extensive vascular network, including major vessels, is apparent. In contrast, the lungs cultured in 19% O_2 exhibit much lower levels of PECAM immunoreactivity, with most ECs present being located in clusters of small vessels near the periphery of the explant. Thus variation in oxygen tension profoundly alters EC survival and vascular patterning in developing lungs.

The relationship between oxygen tension and lung development has yet to be extensively studied at the molecular level, but recent genetic evidence suggests that the hypoxia-inducible factor Hif-2α regulates VEGF, which in turn is required for proper lung maturation, including the production of surfactant.[124]

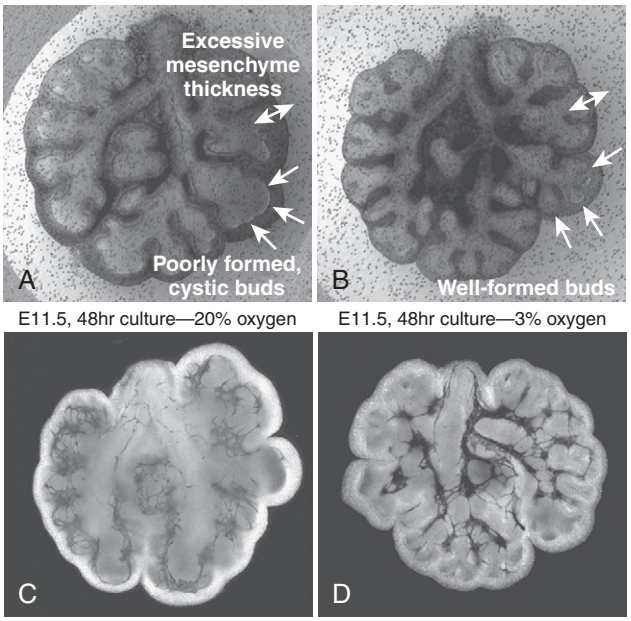


Figure 64-11 A comparison of E11.5 mouse lung buds grown under normoxia **(A, C)** or hypoxia **(B, D).** In normoxia, branching morphogenesis is diminished, with development of poorly formed cystic buds and excessive mesenchymal thickening compared with that seen in hypoxia. **C and D,** Whole-mount immunohistochemical staining with anti-PECAM/CD31 antibody to delineate vascular development. Under hypoxic conditions there is a marked accentuation in larger vessel development (in the regions surrounding the main bronchi) combined with an attenuation of endothelial differentiation surrounding the distal epithelial buds. These data suggest that hypoxia may not only affect endothelial proliferation and survival but also play a critical role in promoting the vascular remodeling required for more proximal large vessel formation.

Hif-2α–deficient animals that do not die in utero breathe irregularly at birth, are cyanotic, and succumb to severe respiratory failure within a few hours. Analysis of lung morphogenesis in these animals reveals that Hif-2α is required both before and after birth to establish an adequate lung microvasculature, to thin the alveolar septa, and to remodel the vessels juxtaposing the endothelium and type 1 pneumocytes. The lung epithelium is immature, and there is abnormally low production of surfactant phospholipids and surfactant proteins A, B, and D. The normal rise in pulmonary VEGF levels during late gestation fails to occur in Hif-2α–deficient animals, which suggests the possible involvement of VEGF in their lung pathology. Consistent with this idea, inhibiting prenatal VEGF signaling by intraamniotic injection of neutralizing antibodies to VEGFR2 results in a comparable level of lung immaturity. Conversely, respiratory distress resulting from premature delivery of mice can be alleviated by in utero or intratracheal administration of VEGF, which results in thinner alveolar septa and increased surfactant production.[124] Studies such as these reveal components of the complex interactions among oxygen tension, lung vascular development, and maturation of the lung epithelium, which may be disrupted by premature birth or oxygen therapy. Understanding such interactions has important clinical implications because disruption of the lung vasculature may contribute to poor outcomes in bronchopulmonary dyplasia, a chronic lung disease in infants that is typically associated with both premature birth and oxygen therapy for acute respiratory failure.[125]

Likewise, flow and resultant shear stress may play a significant role in pulmonary vascular development. Previous work utilizing in utero sheep models suggested that pulmonary blood flow represented only a small percentage of combined cardiac output (3.7% to 7%), with no significant increase in flow despite a dramatic increase in vascular density during the last trimester.[126] Therefore blood flow was not thought to be a primary modulator of pulmonary vascular growth. However, more recent studies on human fetuses suggest that at least 11% of the combined cardiac output from 11 to 41 weeks of gestation is directed to the lung.[127] Additional studies report a gestational increase in pulmonary blood flow from 13% to 25% of combined cardiac output by week 30 of gestation.[128] Similarly, in vitro studies suggest that flow to the pulmonary vascular bed may increase vascular morphogenesis.[129] Although the exact role of flow in pulmonary vascular development remains to be defined, it will likely play a significant role in defining optimal pulmonary development.

CONCLUSION AND FUTURE PROSPECTS

There has been an explosion of new information concerning the molecular regulation of vascular development. However, future efforts to characterize the molecular genetic pathways governing vascular morphogenesis in the lung and elsewhere will need to go beyond molecular characterization and examine the interplay of developmental genetics with shear stress, hypoxia, and other epigenetic phenomena during critical periods of development. This chapter has highlighted just a few of the molecular players that are involved in the production of the vascular system. Many more await discovery. The challenge ahead is not only to identify the interactions among molecules, cells, and environment that govern vessel morphogenesis, but also to build the growing knowledge of these interactions into useful models of vascular development that can help us understand vascular system defects and direct future therapeutic interventions.

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65 Cardiomyopathies in Infants and Children

Jeffrey A. Towbin, John Lynn Jefferies, and Matteo Vatta

Cardiomyopathies are major causes of morbidity and mortality, and, over the past 20 years, limited improvements in outcome have been reported.[1-3] However, improvement in the understanding of the major forms of cardiomyopathy has occurred over that time, in large part because of advances in genetics and genomics.[4,5] In addition, new forms of cardiomyopathy have been described and classified over that time frame, in large part because of our improved genetic-based understanding of heart muscle disease. Further, the improved understanding gained in heart muscle disease has led to understanding of similarities and differences in heart and skeletal muscle and their often overlapping clinical presentations.

A new classification scheme was recently developed for the cardiomyopathies in which five forms of disease were formally classified as distinct forms of cardiomyopathy.[6] These forms are dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, and left ventricular noncompaction (LVNC). These were further classified into genetic/inherited forms and acquired/noninherited forms.[6] All of these forms of disease occur in childhood, including at birth or in the first weeks of life in many cases, although arrhythmogenic right ventricular cardiomyopathy is almost always identified in young adults, and therefore this disorder will not be discussed herein.

To understand the mechanisms responsible for the development of the clinical phenotypes of cardiomyopathies, an understanding of normal cardiac structure is necessary.

NORMAL CARDIAC STRUCTURE

Cardiac muscle fibers are composed of separate cellular units (myocytes) connected in series.[7] In contrast to skeletal muscle fibers, cardiac fibers do not assemble in parallel arrays but bifurcate and recombine to form a complex three-dimensional network. Cardiac myocytes are joined at each end to adjacent myocytes at the intercalated disk, the specialized area of interdigitating cell membrane (Figure 65-1). The intercalated disk contains gap junctions (containing connexins) and mechanical junctions, composed of adherens junctions (containing N-cadherin, catenins, and vinculin) and desmosomes (containing desmin, desmoplakin, desmocollin, desmoglein). Cardiac myocytes are surrounded by a thin membrane (sarcolemma), and the interior of each myocyte contains bundles of longitudinally arranged myofibrils. The myofibrils are formed by repeating sarcomeres, the basic contractile units of cardiac muscle composed of interdigitating thin (actin) and thick (myosin) filaments (see Figure 65-1), that give the muscle its characteristic striated appearance.[8,9] The thick filaments are composed primarily of myosin but additionally contain myosin-binding proteins C, H, and X. The thin filaments are composed of cardiac actin, α-tropomyosin (α-TM), and troponins T, I, and C (cTnT, cTnI, cTnC). In addition, myofibrils contain a third filament formed by the giant filamentous protein, titin, which extends from the Z disk to the M line and acts as a molecular template for the layout of the sarcomere. The Z disk at the borders of the sarcomere is formed by a lattice

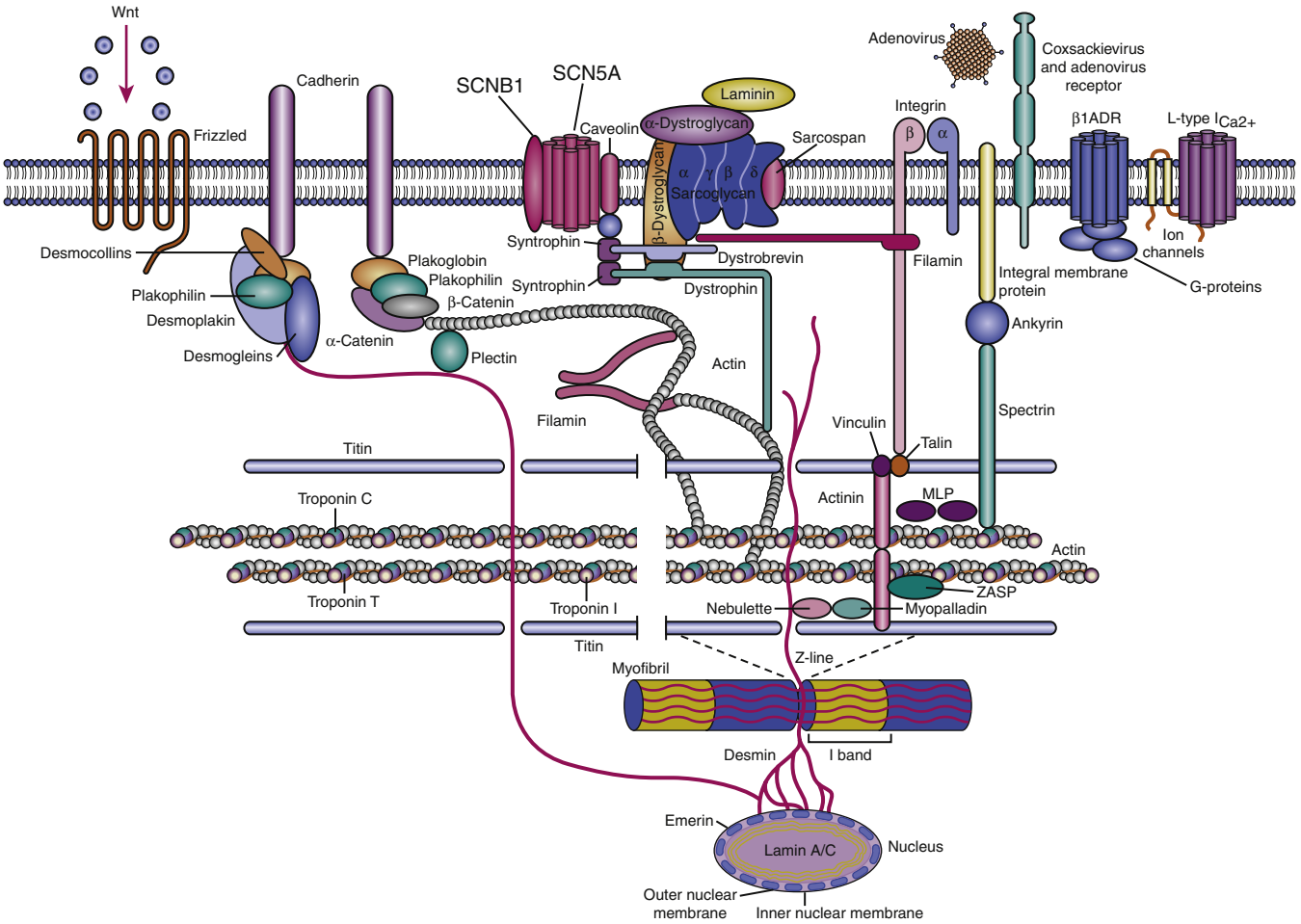


Figure 65-1 Cardiac myocyte cytoarchitecture. Schematic of the interactions between dystrophin and the dystrophin-associated proteins in the sarcolemma and intracellular cytoplasm (dystroglycans, sarcoglycans, syntrophins, dystrobrevin, sarcospan) at the carboxy-terminal end of the dystrophin. The integral membrane proteins interact with the ECM via α-dystroglycan–α_2-laminin connections. The amino-terminus of dystrophin binds actin and connects dystrophin with the sarcomere intracellularly, the sarcolemma, and ECM. Additional sarcolemmal proteins include ion channels, adrenergic receptors, integrins, and the Coxsackie and adenoviral receptor. Cell-cell junctions, including cadherins, the plakin, and other desmosomal family proteins, are also notable. Also shown is the interaction between intermediate filament proteins (i.e., desmin) with the nucleus. *MLP,* Muscle LIM protein.

of interdigitating proteins that maintain myofilament organization by crosslinking antiparallel titin and thin filaments from adjacent sarcomeres (Figure 65-2). Other proteins in the Z disk include α-actinin, nebulette, telethonin/T-cap, capZ, muscle LIM protein (MLP), myopalladin, myotilin, Cypher/ZASP, filamin, and FATZ.[8-10]

Finally, the extrasarcomeric cytoskeleton, a complex network of proteins linking the sarcomere with the sarcolemma and the extracellular matrix (ECM), provides structural support for subcellular structures and transmits mechanical and chemical signals within and between cells. The extrasarcomeric cytoskeleton has intermyofibrillar and subsarcolemmal components, with the intermyofibrillar cytoskeleton composed of intermediate filaments, microfilaments, and microtubules.[11,12] Desmin intermediate filaments form a three-dimensional scaffold throughout the extrasarcomeric cytoskeleton with desmin filaments surrounding the Z disk, allowing for longitudinal connections to adjacent Z disks and lateral connections to subsarcolemmal costameres.[12] Microfilaments composed of nonsarcomeric actin (mainly γ-actin) also form complex networks linking the sarcomere (via α-actinin) to various components of the costameres. Costameres are subsarcolemmal domains located in a periodic, gridlike pattern, flanking the Z disks and overlying the I bands, along the cytoplasmic side of the sarcolemma. These costameres are sites of interconnection between various cytoskeletal networks linking sarcomere and sarcolemma and are thought to function as anchor sites for stabilization of the sarcolemma and for integration of pathways involved in mechanical force transduction. Costameres contain three principal components: the focal adhesion-type complex, the spectrin-based complex, and the dystrophin/dystrophin-associated protein complex (DAPC).[13,14] The focal adhesion-type complex, composed of cytoplasmic proteins (i.e., vinculin, talin, tensin, paxillin, zyxin), connects with cytoskeletal actin filaments and with the transmembrane proteins α- and β-dystroglycan; α-, β-, γ-, and δ-sarcoglycans; dystrobrevin; and syntrophin. Several actin-associated proteins are located at sites of attachment of cytoskeletal actin filaments with costameric complexes, including α-actinin and MLP. The C-terminus of dystrophin binds β-dystroglycan (see Figure 65-1), which in turn interacts with α-dystroglycan to link to the ECM (via α_2-laminin). The N-terminus of dystrophin interacts with actin. Also notable, voltage gated sodium channels colocalize with dystrophin, β-spectrin, ankyrin, and syntrophins, and potassium channels interact with the sarcomeric Z disk and intercalated

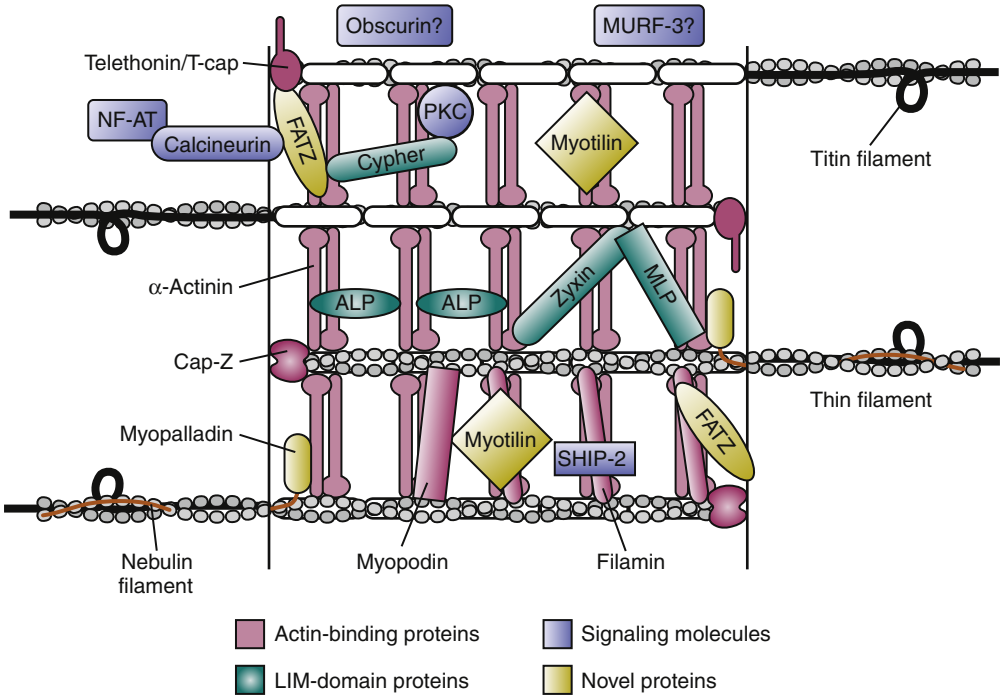


Figure 65-2 Z disk architecture. The Z disk of the sarcomere is composed of multiple interacting proteins that anchor the sarcomere. *(From Clark KA, McElhinny AS, Beckerle MC, Gregorio CC: Striated muscle cytoarchitecture: an intricate web of form and function,* Ann Rev Dev Biol *18:637-706, 2002. With permission.)*

disks.[15,16] Because arrhythmias and conduction system diseases are common in children and adults with DCM, this could play an important role. Hence, disruption of the links from the sarcolemma to ECM at the dystrophin C-terminus and those to the sarcomere and nucleus via N-terminal dystrophin interactions could lead to a "domino effect" disruption of systolic function and development of arrhythmias.

DILATED CARDIOMYOPATHY

DCM, the most common form of cardiomyopathy, is characterized primarily by left ventricular (LV) dilation and systolic dysfunction (Figure 65-3). Increasingly recognized, right ventricular function and diastolic function are also abnormal. In children, the annual incidence of DCM is 0.57 cases per 100,000 per year overall but is higher in boys than in girls (0.66 versus 0.47 cases per 100,000; $p < .001$), in blacks than in whites (0.98 versus 0.46 cases per 100,000; $p < .001$), and in infants (<1 year) than in children (4.40 versus 0.34 cases per 100,000; $p < .001$). The majority of children (66%) are thought to have idiopathic disease.[17] The mortality rate in the United States resulting from cardiomyopathy is greater than 10,000 deaths per annum, with DCM being the major contributor.[1] The total cost of health care in the United States focused on cardiomyopathies is in the billions of dollars, and only limited success has been achieved. To achieve improved care and outcomes in children and adults, understanding of the causes of these disorders has been sought.

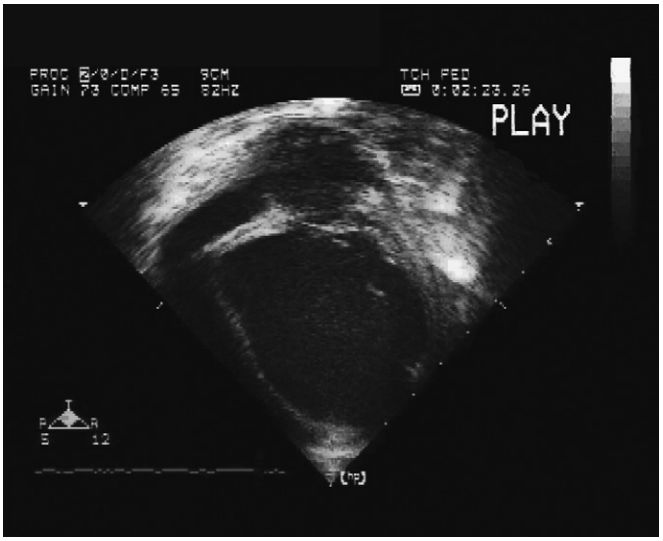


Figure 65-3 Echocardiography in DCM. Apical four-chamber view demonstrating a dilated left ventricle. In real time, the systolic function is depressed.

Etiologies of Dilated Cardiomyopathy

The clinical appearance of DCM, a dilated left ventricle with poor contractile performance, occurs for a variety of causes. These include genetic and acquired etiologies. Acquired forms of disease include myocarditis, usually the result of viral infection; chemotherapy-induced cardiomyopathy; intrauterine disorders such as placental insufficiency and nonimmune hydrops; and acute stress such as hypoxia. In addition, DCM can develop because of genetic-based disease. These genetic disorders include inherited forms of disease, cardioskeletal disease such as muscular dystrophies, and metabolic forms of disease.

Clinical Genetics of Dilated Cardiomyopathy

DCM was initially believed to be inherited in a small percentage of cases until Michels and co-workers[18] showed that approximately 20% of probands had family members with echocardiographic

evidence of DCM when family screening was performed. More recently, inherited, familial DCM has been shown to occur in 30% to 40% of cases[4] with autosomal dominant inheritance being the predominant pattern of transmission; X-linked, autosomal recessive, and mitochondrial inheritance is less common.

Molecular Genetics of Dilated Cardiomyopathy

DCM has become a popular target of research over the past 10 to 15 years, with multiple genes identified during that time period (Table 65-1). These genes appear to encode two major subgroups of proteins, cytoskeletal and sarcomeric proteins.[4] The cytoskeletal proteins identified to date include dystrophin, desmin, lamin A/C, δ-sarcoglycan, β-sarcoglycan, and metavinculin. In the case of sarcomere-encoding genes, the same genes identified for HCM appear to be culprits and include β-myosin heavy chain, myosin binding protein C, actin, α-TM, and cTnT. A new group of sarcomeric genes, those encoding Z-disk proteins, have also been identified and include Cypher/ZASP,[1,3,5] MLP, and α-actinin-2.[1,3,5] In addition, phospholamban and G4.5/tafazzin have also been reported. Another form of DCM, the acquired disorder viral myocarditis, has the same clinical features as DCM including heart failure, arrhythmias, and conduction block.[1,5] The most common causes of myocarditis are viral, including the enteroviruses (coxsackieviruses and echovirus), adenoviruses, and parvovirus B19, among other cardiotropic viruses.[1] Evidence exists that suggests that viral myocarditis and DCM (genetic) have similar mechanisms of disease on the basis of the proteins targeted.

Initial progress was made in understanding the familial forms of DCM by studying families with X-linked forms of DCM. Autosomal dominant forms of DCM began to unravel only over the past few years. In the case of X-linked forms of DCM, three disorders have been well characterized: X-linked cardiomyopathy (XLCM), which presents in adolescence and young adults; Barth syndrome, which is most frequently identified in infancy[19,20]; and Danon disease, which most typically presents with HCM with later-onset DCM, although this presentation can be variable.

X-LINKED CARDIOMYOPATHIES

X-Linked Dilated Cardiomyopathy

First described in 1987 by Berko and Swift[19] as DCM occurring in males in the teen years and early twenties with rapid progression from congestive heart failure (CHF) to death caused by ventricular tachycardia/ventricular fibrillation or transplantation, the condition in these patients is distinguished by elevated serum creatine kinase muscle isoforms (CKMM). Female carriers tend to have development of mild to moderate DCM in the fifth decade, and the disease is slowly progressive. Towbin and colleagues (1993)[21] were the first to identify the disease-causing gene and characterize the functional defect. In this report, the dystrophin gene was shown to be responsible for the clinical abnormalities, and protein analysis by immunoblotting demonstrated severe reduction or absence of dystrophin protein in the heart of these patients. These findings were later confirmed by Muntoni and associates[22] when a mutation in the muscle promoter and exon 1 of dystrophin was identified in another family with XLCM. Subsequently, multiple mutations have been identified in dystrophin in patients with XLCM.

Dystrophin is a cytoskeletal protein that provides structural support to the myocyte by creating a latticelike network to the sarcolemma.[23] In addition, dystrophin plays a major role in linking the sarcomeric contractile apparatus to the sarcolemma and ECM.[23-26] Furthermore, dystrophin is involved in cell signaling, particularly through its interactions with nitric oxide synthase. The dystrophin gene is responsible for Duchenne and Becker muscular dystrophy (DMD and BMD) when mutated as well.[27] These skeletal myopathies present early in life (DMD is diagnosed before age 12 years, and BMD is seen in teenaged males older than 16 years), and the vast majority of patients have DCM before the twenty-fifth birthday. In most patients, CKMM is elevated similar to that seen in XLCM; in addition, manifesting female carriers have disease late in life, similar to XLCM. Furthermore, immunohistochemical analysis demonstrates reduced levels (or absence) of dystrophin, similar to that seen in the hearts of patients with XLCM.

TABLE 65-1

Dilated Cardiomyopathy Genetics

Chromosome locus	Gene	Protein
Xp21.2	DYS	Dystrophin
Xq28	G4.5	Tafazzin
1q21	LMNA	Lamin A/C
1q32	TNNT2	Cardiac troponin T
1q42-43	ACTN	α-Actinin-2
2q31	TTN	Titin
2q35	DES	Desmin
5q33	SGCD	δ-Sarcoglycan
6q22.1	PLN	Phospholamban
10q22.3-23.2	ZASP/Cypher	ZASP
10q22-q23	VCL	Metavinculin
11p11	MYBPC3	Myosin binding protein C
11p15.1	MLP	Muscle LIM protein
14q12	MYH7	β-Myosin heavy chain
15q14	ACTC	Cardiac actin
15q22	TPM1	α-Tropomyosin

Murine models of dystrophin deficiency demonstrate abnormalities of muscle physiology based on membrane structural support abnormalities.[28] In addition to the dysfunction of dystrophin, mutations in dystrophin secondarily affect proteins that interact with dystrophin. At the amino-terminus (N-terminus), dystrophin binds to the sarcomeric protein actin, a member of the thin filament of the contractile apparatus. At the carboxy-terminus (C-terminus), dystrophin interacts with α-dystroglycan, a dystrophin-associated membrane-bound protein that is involved in the function of the DAPC, which includes β-dystroglycan, the sarcoglycan subcomplex (α-, β-, γ-, δ-, and ε-sarcoglycan), syntrophins, and dystrobrevins[29-31] (see Figure 65-1). In turn, this complex interacts with α_2-laminin and the ECM.[32] Like dystrophin, mutations in these genes lead to muscular dystrophies with or without cardiomyopathy, supporting the contention that this group of proteins is important to the normal function of the myocytes of the heart and skeletal muscles.[32,33] In both cases, mechanical stress[28] appears to play a significant role in the age onset–dependent dysfunction of these muscles. The information gained from the studies on XLCM, DMD, and BMD led us to hypothesize that DCM is a disease of the cytoskeleton/sarcolemma that affects the sarcomere,[34] a "final common pathway" of DCM.[35] We also have suggested that dystrophin mutations play a role in idiopathic DCM in males. This is supported when we showed that 3 of 22 boys with DCM had dystrophin mutations, and all were later found to have elevated CKMM as well.[36] In addition, eight families with DCM and possible X-linked inheritance were screened, and in three of eight families dystrophin mutations were noted. Again, CKMM was elevated in all subjects carrying mutations.[37]

Barth Syndrome

Initially described as X-linked cardioskeletal myopathy with abnormal mitochondria and neutropenia by Neustein and colleagues[38] and Barth and co-workers,[39] this disorder typically presents in male infants as CHF associated with neutropenia (cyclic) and 3-methylglutaconic aciduria.[40] Mitochondrial dysfunction

is noted on electron microscopy and electron transport chain biochemical analysis. Recently, abnormalities in cardiolipin have been noted.[41] Echocardiographically these infants typically have LV dysfunction with LV dilation, endocardial fibroelastosis, or a dilated hypertrophic left ventricle. In some cases these infants succumb because of CHF/sudden death, ventricular tachycardia/ventricular fibrillation, or sepsis caused by leukocyte dysfunction. The majority of these children survive through infancy and do well clinically, although DCM usually persists. In some cases, cardiac transplantation has been performed. Histopathologic evaluation typically demonstrates the features of DCM, although endocardial fibroelastosis may be prominent and the mitochondria are abnormal in shape and abundance.

The genetic basis of Barth syndrome was first described by Bione and colleagues,[42] who cloned the disease-causing gene, G4.5. This gene encodes a novel protein called tafazzin, whose gene product is an acyltransferase and results in cardiolipin abnormalities.[41] Mutations in G4.5 result in a wide clinical spectrum, which includes apparent classic DCM, hypertrophic DCM, endocardial fibroelastosis, or LV noncompaction.[4,43]

The final form of X-linked disease, Danon disease, will be discussed in the section on HCM caused by infiltrative disease.

AUTOSOMAL DOMINANT DILATED CARDIOMYOPATHY

The most common form of inherited DCM is the autosomal dominant form of disease.[1,4,5] These patients present as classic "pure" DCM or DCM associated with conduction system disease (CDDC). In the latter case, patients usually present in the third decade of life with mild CDDC that can progress to complete heart block over decades. DCM usually presents late in the course but is out of proportion to the degree of CDDC.[44] The echocardiographic and histologic findings in both subgroups are classic for DCM, although the conduction system may be fibrotic in patients with CDDC. In both groups of patients with DCM, ventricular tachycardia, ventricular fibrillation, and torsades de pointes occur and may result in sudden death.

Genetic heterogeneity exists for autosomal dominant DCM with more than 15 loci mapped for pure DCM and five loci for CDDC.[3] In the case of pure DCM, 10 genes have been identified to date, including three by our group (δ-sarcoglycan, α-actinin-2, ZASP),[4,5,45] as well as actin, desmin, troponin T, β-myosin heavy chain, titin, metavinculin, myosin binding protein C, α-tropomyosin, MLP, β-sarcoglycan, and phospholamban[4,46,47] (see Table 65-1).

The majority of genes identified to date encode either cytoskeletal or sarcomeric proteins. In the case of cytoskeletal proteins (desmin, δ-sarcoglycan, metavinculin, MLP), defects of force transmission are considered to result in the DCM phenotype, whereas defects of force generation have been speculated to cause sarcomeric protein–induced DCM.[4,34]

Cardiac actin is a sarcomeric protein that is a member of the sarcomeric thin filament interacting with tropomyosin and the troponin complex. As previously noted, actin plays a significant role in linking the sarcomere to the sarcolemma via its binding to the N-terminus of dystrophin, and the mutations in actin that resulted in DCM as described by Olson and associates.[48] appear to be directly involved in the binding of dystrophin. The DCM-causing mutations are believed to result by causing force transmission abnormalities. Further, actin interacts in the sarcomere with TnT and β-MHc, two other genes resulting in either DCM or HCM depending on the position of the mutation.[49] In the case of TnT and β-MHC, force generation abnormalities have been speculated as the responsible mechanism.

Desmin is a cytoskeletal protein that forms intermediate filaments specific for muscle.[12] This muscle-specific 53 kDa subunit of class III intermediate filaments forms connections between the nuclear and plasma membranes of cardiac, skeletal, and smooth muscle. Desmin is found at the Z lines and intercalated disk of muscle, and its role in muscle function appears to involve attachment or stabilization of the sarcomere. Mutations in this gene appear to cause abnormalities of force and signal transmission similar to that believed to occur with actin mutations.[50]

Another DCM-causing gene, δ-sarcoglycan is a member of the sarcoglycan subcomplex of the DAPC.[45,51] This gene encodes for a protein involved in stabilization of the myocyte sarcolemma, as well as signal transduction. Mutations identified in familial and sporadic cases resulted in reduction of the protein within the myocardium. In the absence of δ-sarcoglycan, the remaining sarcoglycans (δ, β, γ, Σ) cannot assemble properly in the endoplasmic reticulum.[52] Mouse models of δ-sarcoglycan deficiency demonstrate dilated HCM, sarcolemmal fragility, and disrupted vasculin smooth muscle, which leads to vascular spasm, including coronary spasm.[53,54] In addition, mutations in this gene lead to the phenotype of the cardiomyopathic Syrian hamster.[55] Other human mutations in δ-sarcoglycan cause a form of autosomal recessive limb girdle muscular dystrophy (LGMD2F) that rarely is associated with heart disease.

The final cytoskeletal protein-encoding gene, metavinculin, encodes vinculin and its splice variant metavinculin. Vinculin is ubiquitously expressed, and metavinculin is coexpressed with vinculin in heart, skeletal, and smooth muscle, with this protein complex localized to subsarcolemmal costameres in the heart where they interact with α-actinin, talin, and γ-actin to form a microfilamentous network linking cytoskeleton and sarcolemma. In addition, these proteins are present in adherens junctions in intercalated disks and participate in cell-cell adhesion. Mutations in metavinculin have been shown to disrupt the intercalated disks and alter actin filament crosslinking.[56,57]

Mutations in the sarcomere may produce HCM or DCM. In the latter case, abnormalities in force generation or transmission are thought to contribute to the development of this phenotype.[49] In addition to mutations in the thin filament protein actin, mutations in the thick filament protein-encoding gene β-myosin heavy chain have been shown to cause DCM with associated sudden death in at least one infant, as well as DCM in older children and adults.[49,58] Mutations in this gene are thought to perturb the actin-myosin interaction and force generation or alter cross-bridge movement during contraction. Mutations in cardiac troponin T, a thin filament protein, have been speculated to disrupt calcium-sensitive troponin C binding.[49,58] Mutations in phospholamban[59,60] have also been identified that further support calcium handling as a potentially important mechanism in the development of DCM. Interestingly, Haghihi and co-workers.[60] identified homozygous mutations causing DCM and heart failure, whereas heterozygotes had cardiac hypertrophy. Recessive mutation in troponin I is thought to impair the interaction with troponin T, and α-tropomyosin mutations have also been identified and were predicted to alter the surface charge of the protein leading to impaired interaction with actin.[61,62]

A recent area of interest for evaluation at the molecular level is the Z disk.[63] Knoll and co-workers[64] identified mutations in MLP and demonstrated that this results in defects in the interaction with telethonin.[64] Using mouse models, they also demonstrated that MLP acts as a stretch sensor and that mutant MLP causes defects in this activity. More recently, mutations in MLP in families and sporadic cases were described, and, in addition, abnormalities were identified in the T-tubule system[4] and Z-disk architecture by electron microscopy, which correlates with the histopathology seen in MLP-knockout mice.[65] This was further supported by the finding of reduced expression of MLP in chronic human heart failure.[66,67] In addition, mutations in α-actinin-2, which is involved in crosslinking actin filaments and shares a common actin binding domain with dystrophin, were also identified in familial DCM, which disrupts its binding

to MLP.[5] Vatta and associates[4] identified mutations in the Z band alternatively spliced PDZ-motif protein ZASP, the human homolog of the mouse Cypher gene that when disrupted leads to DCM.[68] Multiple mutations in this gene were identified in families and sporadic cases of DCM and with LVNC. This protein, which interacts with α-actinin-2, disrupts the actin cytoskeleton when mutated. Another gene, titin, which encodes the giant sarcomeric cytoskeletal protein titin that contributes to the maintenance of the sarcomere organization and myofibrillar elasticity, interacts with these proteins at the Z-disk/I-band transition zone.[69] Mutations have been identified in familial DCM as well.[70]

As seen in pure autosomal dominant DCM, genetic heterogeneity also exists for CDDC. To date, CDDC genes have been mapped to chromosomes 1p1-1q1, 2q14-21, 3p25-22, and 6q23. The only gene thus far identified was reported to be lamin A/C on chromosome 1q21, which encodes a nuclear envelope intermediate filament protein.[71,72]

Lamin A/C

The lamins are located in the nuclear lamina at the nucleoplasmic side of the inner nuclear membrane, and lamin A and C are expressed in heart and skeletal muscle.[73] Mutations in this gene were initially reported to cause the autosomal dominant form of Emery-Dreifuss muscular dystrophy,[74,75] which has skeletal myopathy associated with DCM and CDDC. It has also been found to cause a form of autosomal dominant limb girdle muscular dystrophy (LGMD1B), which is also associated with CDDC.[76] Multiple mutations have been identified in patients with CDDC, who, in some cases, had mildly elevated CK. This gene defect appears to be relatively common in patients with CDDC. The mechanism(s) responsible for the development of DCM and conduction system abnormalities and of skeletal myopathy is being determined.[77] Understanding may be aided by understanding how other genes that encode interacting proteins result in disease, such as thymopoietin.[78]

Muscle Is Muscle: Cardiomyopathy and Skeletal Myopathy Genes Overlap

Interestingly, nearly all of the genes identified for inherited DCM are also known to cause skeletal myopathy in humans and/or mouse models. In the case of dystrophin, mutations cause DMD and BMD whereas δ-sarcoglycan mutations cause LGMD2F. Lamin A/C has been shown to cause autosomal dominant Emery-Dreifuss muscular dystrophy and LGMD1B whereas actin mutations are associated with nemaline myopathy. Desmin, G4.5, α-dystrobrevin, Cypher/ZASP, MLP, α-actinin-2, titin, and δ-sarcoglycan mutations also have associated skeletal myopathy, suggesting that cardiac and skeletal muscle function is interrelated and that possibly the skeletal muscle fatigue seen in patients with DCM with and without CHF may be due to primary skeletal muscle disease and not only related to the cardiac dysfunction. It also suggests that the function of these muscles has a final common pathway and that both cardiologists and neurologists should consider evaluation of both sets of muscles.

Further support for this concept comes from studies of animal models. Mutations in δ-sarcoglycan in hamsters result in cardiomyopathy whereas mutations in all sarcoglycan subcomplex genes in mice cause skeletal and cardiac muscle disease. Mutations in other DAPC genes, as well as dystrophin in murine models, also consistently demonstrate abnormalities of skeletal and cardiac muscle function. Arber et al.[65] also produced a mouse deficient in MLP, a structural protein that links the actin cytoskeleton to the contractile apparatus. The resultant mice develop severe DCM, CHF, and disruption of cardiac myocyte cytoskeletal architecture. Murine mutations in titin, Cypher, α-dystrobrevin, desmin, and other all demonstrate cardiac and skeletal muscle disease. Finally, Badorff and colleagues[79] have shown that the DCM that develops after viral myocarditis has a mechanism similar to that of the inherited forms. Using coxsackievirus B3 infection of mice, the authors showed that the coxsackievirus B3 genome encodes for a protease (enteroviral protease 2A) that cleaves dystrophin at the third hinge region of dystrophin, resulting in force transmission abnormalities and DCM. In addition, Xiong and co-workers[80] showed that abnormal dystrophin increases susceptibility to viral infection and resultant myocarditis. Interestingly, a similar dystrophin mutation that affects the first hinge region of dystrophin in patients with XLCM was previously reported by our laboratory, demonstrating a consistent mechanism of DCM development, abnormalities of the cytoskeleton/sarcolemma and sarcomere. In addition, we have shown that N-terminal dystrophin is reduced or absent in hearts of patients with all forms of DCM (ischemic, acquired, genetic, idiopathic) and that reduction of mechanical stress by use of LV assist devices results in reverse remodeling of dystrophin and of the heart itself.[81,82]

HYPERTROPHIC CARDIOMYOPATHY

HCM is characterized by asymmetric or concentric wall thickening (Figure 65-4), hence the term hypertrophic heart disease.[83-85] However, the major impact of this disorder on human health is its predilection to be inherited; its reputation as the most common cause of sudden death in young, healthy, athletic individuals[86-89]; and its potential to develop heart failure.[90-92] In the latter case, heart failure can occur because of diastolic factors or can occur because of the development of systolic dysfunction, so-called burned-out HCM.[90-92] No matter what the cause of untoward clinical features, HCM plays a significant role in our health care monetary burden, as well as the untold price of the tragedies in affected families.

In the pediatric age range, the underlying etiologies responsible for the disease and the variable age range of onset differentiates further the childhood form of disease from the adult counterpart.[93] In very young children (i.e., less than 1 year of age), ventricular hypertrophy associated with systolic dysfunction is more the rule than the exception. In addition, overlap disorders in which HCM coexists with other atypical features are also more common during childhood, further confounding the presentations, treatments, and outcomes compared with adult disease.

Clinical Aspects of Hypertrophic Cardiomyopathy

HCM is a primary myocardial disorder with an autosomal dominant pattern of inheritance that is characterized by hypertrophy of the left (± right) ventricle with histologic features of myocyte hypertrophy, myofibrillar disarray, and interstitial fibrosis.[83-85] HCM is one of the most common inherited cardiac disorders, with a prevalence in young adults of 1 in 500.[85] Various names have been given to this disorder including hypertrophic obstructive cardiomyopathy and idiopathic subaortic stenosis. These names reflect "textbook" features of asymmetric septal hypertrophy and LV outflow tract obstruction. This description of the disease is based primarily on patients with severe symptoms seen in tertiary hospital referral centers. Epidemiologic studies now suggest that a wide spectrum of clinical manifestations of varying severity and prognosis is present in community populations. The first clinical description of HCM was reported in 1869[94] in France, and it was recognized to be a genetic disorder in the late 1950s. Since then, numerous clinical and pathologic studies of HCM have been performed. During the last 15 years, molecular genetic studies have given important insights into the pathogenesis of HCM and have provided a new perspective for the diagnosis and management of patients with this disorder.[95]

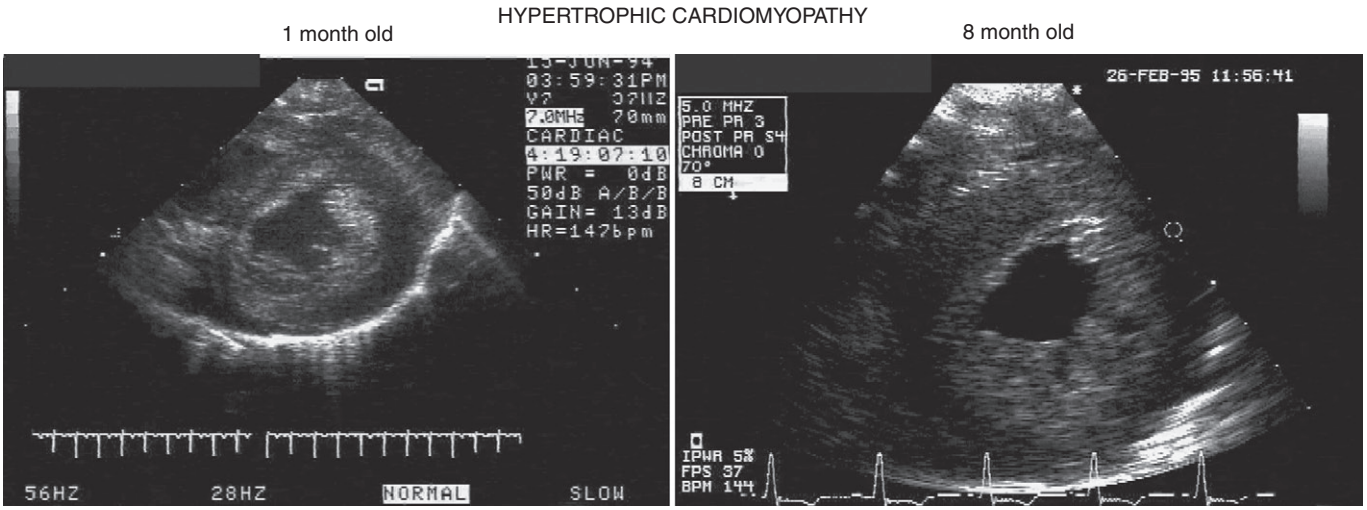


Figure 65-4 Echocardiography in HCM. *Left panel,* Parasternal short-axis echocardiogram. Concentric hypertrophy in a 1-month-old. Note the small LV chamber. *Right panel,* Similar view demonstrates worsening LV hypertrophy and a smaller chamber size at 8 months of age in the same child.

Genetics of Familial Hypertrophic Cardiomyopathy

The first gene for familial hypertrophic cardiomyopathy (FHC) was mapped to chromosome 14q11.2-q12 by using genome-wide linkage analysis in a large Canadian family.[96] Soon afterward, FHC locus heterogeneity was reported[97] and subsequently confirmed by the mapping of the second FHC locus to chromosome 1q3 and of the third locus to chromosome 15q2.[98,99] Carrier and associates.[100] mapped the fourth FHC locus to chromosome 11p11.2. Multiple other loci were subsequently reported, including loci on chromosomes[101-108] (Figure 65-5).

Gene Identification in Familial Hypertrophic Cardiomyopathy

The majority of the disease-causing genes identified to date code for proteins that are part of the sarcomere, which is a complex structure with an exact stoichiometry and multiple sites of protein-protein interactions.[95,109,110] These include three myofilament proteins: the β-myosin heavy chain (β-MyHC), the ventricular myosin essential light chain 1 (MLC-1s/v), and the ventricular myosin regulatory light chain 2 (MLC-2s/v); four thin filament proteins: cardiac actin, cTnT, cTnI, and α-TM; and one myosin-binding protein, the cardiac myosin binding protein C (cMyBP-C) (Table 65-2). Each of these proteins is encoded by multigene families that exhibit tissue-specific, developmental, and physiologically regulated patterns of expression.[111] The giant protein titin[105] and its interactive Z-disk protein, MLP, have also been identified.[106] In addition, the gene located on chromosome 7q3 associated with HCM and Wolff-Parkinson-White (WPW) syndrome was identified as AMP kinase (AMPK), which has been suggested to play a role in energy metabolism and cause infiltration of a glycogen-like substance similar to that seen in Pompe disease.[112-114]

Thick Filament Proteins

Myosin Subunits

Myosin is the molecular motor that transduces energy from the hydrolysis of adenosine triphosphate (ATP) into directed movement and that, by doing so, drives sarcomere shortening and muscle contraction. Cardiac myosin consists of two heavy chains (MyHC) and two pairs of light chains (MLC), referred to as essential (or alkali) light chains (MLC-1) and regulatory (or phosphorylatable) light chains (MLC-2), respectively.[111] The myosin molecule is highly asymmetric, consisting of two globular heads joined to a long rodlike tail. The light chains are arranged in tandem in the head-tail junction. Their function is not fully understood. Neither MLC type is required for the adenosine triphosphatase (ATPase) activity of the myosin head, but they probably modulate it in the presence of actin and contribute power stroke. Mutations have been found in the heavy chains and in the two types of ventricular light chains.

Concerning the heavy chains, the β isoform (β-MyHC) is the major isoform of the human ventricle and of slow-twitch skeletal fibers. It is encoded by MYH7. This gene appears to be the most commonly mutated HCM gene, and hot spots for mutations have been identified.[95,109,115] The majority of mutations are missense mutations located either in the head or in the head-rod junction of the molecule. On the basis of their structural location in the myosin head, the majority of mutations are likely to disrupt both mechanical and catalytic components of actin-myosin interactin, resulting in reduced force generation. Sarcomere assembly is also likely to be disrupted. Mutations in the light meromyosin domain have also been identified, and Blair and colleagues[115] speculated that HCM develops in this case because of abnormalities of myosin filament assembly or interactions with thick filament binding proteins.

The MLC isoforms are expressed in the ventricular myocardium and in the slow-twitch muscles and are the so-called ventricular myosin regulatory light chains (MLC-2s/v) encoded by MYL2 and the ventricular myosin essential light chain (MLC-1s/v) encoded by MYL3. The MLCs are thought to influence the mechanical efficiency of cross-bridge cycling and speed of contraction. It is believed that these proteins regulate power output via a calcium-dependent mechanism, and disruption leads to the HCM phenotype.

Myosin-binding Protein C

MyBP-C is part of the thick filaments of the sarcomere, being located at the level of the transverse stripes, 43 nm apart, seen by electron microscopy in the sarcomere A band. Its function is uncertain, but, for more than a decade, evidence has existed to indicate both structural and regulatory roles. Partial extraction of cMyBP-C from rat skinned cardiac myocytes and rabbit skeletal

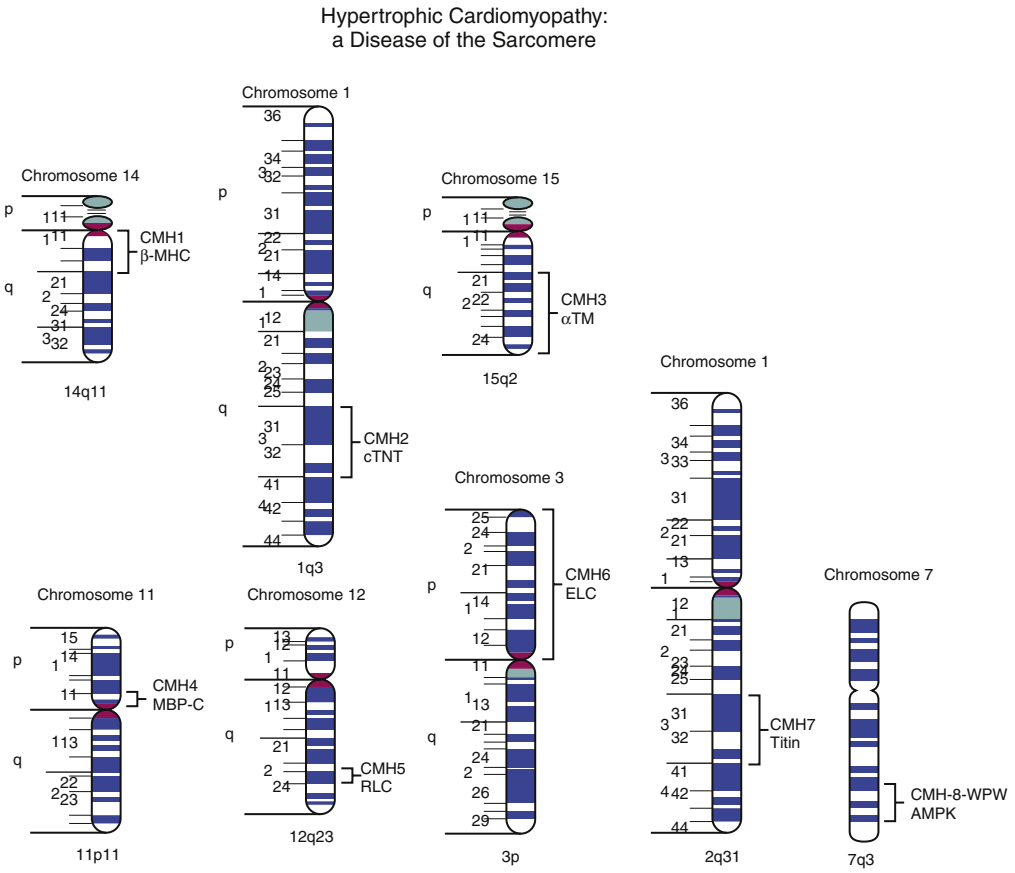


Figure 65-5 Chromosomal locus positions of the original sarcomere-encoding genes responsible for HCM. See Table 65-2 for the complete list of disease-causing genes and their locus positions.

TABLE 65-2

Hypertrophic Cardiomyopathy Genetics

Chromosome locus	Gene	Protein
Xq22	α-Gal	α-Galactosidase
Xq24	LAMP-2	Lysosomal-associated membrane protein 2
Xq28	G4.5	Tafazzin
1q32	TNNT2	Cardiac troponin T
1q42-43	ACTN	α-Actinin-2
2q31	TTN	Titin
3p21.2	MELC	Myosin essential light chain
3p25	CAV3	Caveolin-3
4q26	MYOZ2	Myozenin
6q13	MYO6	Unconventional myosin VI
7q31	AMPK	Adenosine monophosphate–kinase
10q22.3	ZASP/Cypher	ZASP
11p11	MYBPC3	Myosin binding protein C
11p15.1	MLP	Muscle LIM protein
12q23	MRLC	Myosin regulatory light chain
14q12	MYH7	β-Myosin heavy chain
15q14	ACTC	Cardiac actin
15q22	TPM1	α-Tropomyosin
17q12	TCAP	Telethonin
19p13.2	CTNNI	Cardiac troponin I

muscle fibers alters Ca^{2+}-sensitive tension,[116] and it has shown that phosphorylation of cMyBP-C alters myosin cross-bridges in native thick filaments, suggesting that cMyBP-C can modify force production in activated cardiac muscles. The cardiac isoform is encoded by the MYBPC3 gene.

Thin Filament Proteins

The thin filament contains actin, the troponin complex, and tropomyosin. The troponin complex and tropomyosin constitute the Ca^{2+}-sensitive switch that regulates the contraction of cardiac muscle fibers. Mutations have been found in α-TM and in two of the subunits of the troponin complex: cTnI, the inhibitory subunit, and cTnT, the tropomyosin-binding subunit.

α-TM is encoded by TPM1. The cardiac isoform is expressed both in the ventricular myocardium and in fast-twitch skeletal muscles.[117] It shares the overall structure of other tropomyosins that are rodlike proteins that possess a simple dimeric α-coiled-coil structure in parallel orientation along their entire length.[117] It is thought that some mutations in this gene could alter tropomyosin binding to actin.

cTnT is encoded by TNNT2. In human cardiac muscle, multiple isoforms of cTnT have been described that are expressed in the fetal, adult, and diseased heart and that result from alternative splicing of the single gene TNNT2.[118,119] The precise physiologic relevance of these isoforms is currently poorly understood. Mutations in this gene are predicted to influence the inhibitory regulatory effect of the tropomyosin-troponin complex.

cTnI is encoded by TNNI3. The cTnI isoform is expressed only in cardiac muscles.[120] Cooperative binding of cTnI to actin-tropomyosin is a unique property of the cardiac variant; it is thought that mutations disrupt the calcium-sensitive switch mediated by this protein, resulting in increased calcium sensitivity and reduced maximum tension.

α-Cardiac actin (ACTC) mutations also cause FHC. Mogensen et al.[104] identified mutations in a family with heterogeneous phenotypes, ranging from asymptomatic with mild hypertrophy to pronounced septal hypertrophy and LV outflow tract obstruction.

Z-Disk and Other Proteins

Mutations in titin[105] and MLP[106] have been identified as causes of HCM, suggesting the Z disk to be important in the development of HCM (see Figures 65-1 and 65-2). More recently, mutations in ZASP, telethonin (TCAP), α-actinin-2,[121] and myozenin/calsarcin[122] have also been demonstrated to be HCM causing. In addition, disease-causing genes with uncertain mechanisms have been identified including mutations in caveolin-3 and unconventional myosin VI. The functional abnormalities resulting in the clinical phenotype are currently unclear.[107,108]

Genotype-Phenotype Relations in Familial Hypertrophic Cardiomyopathy

The pattern and extent of LV hypertrophy in patients with HCM vary greatly even in first-degree relatives, and a high incidence of sudden death is reported in selected families. An important issue therefore is to determine whether the genotype heterogeneity observed in HCM accounts for the phenotypic diversity of the disease. However, the results must be seen as preliminary, because the available data relate to only a few hundred individuals, and it is obvious that although a given phenotype may be apparent in a small family, examining large or multiple families with the same mutation is required before drawing unambiguous conclusions. Several concepts have been published for mutations in the MYH7, TNNT2, and MYBPC3 genes. For MYH7, the prognosis for patients with different mutations has been shown to vary. For example, the R403Q mutation was thought to be associated with markedly reduced survival,[123] whereas some others, such as V606M, appeared more benign. The disease caused by TNNT2 mutations was reportedly associated with a 20% incidence of nonpenetrance, a relatively mild and sometimes subclinical hypertrophy, but a high incidence of sudden death that occurred even in the absence of significant clinical LV hypertrophy.[124,125] Mutations in MYBPC3, on the other hand, have been characterized by specific clinical features with a mild phenotype in young subjects, a delayed age at the onset of symptoms, and a favorable prognosis before the age of 40 years.[126-129] However, despite these assertions, the notion of mutation-specific clinical outcomes was challenged by Van Driest and colleagues,[130] who demonstrated that "benign" mutations were uncommon (5/253) and that patients with the mutations studied all had severe clinical disease.

Genetic studies have also revealed the presence of clinically healthy individuals carrying the mutant allele, which is associated in first-degree relatives with a typical phenotype of the disease. Several mechanisms could account for the large variability of the phenotypic expression of the mutations: the role of environmental differences and acquired traits (e.g., differences in lifestyle, risk factors, and exercise) and finally the existence of modifier genes and/or polymorphisms that could modulate the phenotypic expression of the disease. Significant results have been obtained thus far regarding the influence of the angiotensin I–converting enzyme (ACE) insertion/deletion polymorphism. Association studies showed that, compared with a control population, the D allele is more common in patients with HCM and in patients with a high incidence of sudden cardiac death.[131,132] It was also shown that the association between the D allele and hypertrophy was seen in the case of MYH7 R403 codon mutations, but not with MYBPC3 mutation carriers,[133] raising the concept of multiple genetic modifiers in HCM.

Therapy in Hypertrophic Cardiomyopathy

The mainstay of therapy in children with HCM has been pharmacologic approaches. The two major medication classes used include β-blockers and calcium channel blockers.[134-136] In small children, we have used propranolol as our drug of choice because of ease of access, liquid formulation, and low side-effect profile. Therapy in these children is monitored by heart rate response, with the goal being approximately 80 to 100 beats per minute. In older children, we typically treat with atenolol; in children with excessive hypertrophy and severe outflow tract obstruction, we occasionally consider use of combination therapy (β-blocker plus calcium channel blocker) although this is not without risk. However, the risk/benefit ratio must be determined for each patient.

When standard pharmacologic therapy fails, there are limited options, although the size of the child plays a role. In small children, myomectomy is the only proved option.[137,138] Again, this is not without risk. In older patients, pacing protocols have been used but are controversial.[139-141] In adults, alcohol septal ablation has been used, but this has not yet been championed in children because of the uncertainties associated with creating an infarct in a young individual regarding long-term outcome.[142-145]

In patients with syncope, ventricular arrhythmias, or other presumed high risk, implantable cardioverter-defibrillator implantation should be considered.[89] In some patients, pacing is necessary as well. Heart failure in HCM occurs because of either diastolic dysfunction or "burned-out" disease with resultant systolic dysfunction. In the latter case, therapy is similar to that used in patients with DCM. This includes use of ACE inhibitor plus β-blocker therapy, with or without diuretic and digoxin.[91] Enalapril and carvedilol are the most common ACE inhibitor/β-blocker combination used. In the case of diastolic dysfunction with heart failure and preserved systolic function, combination therapy with ACE inhibitors, diuretic, with or without angiotensin II–receptor blockers such as candesartan or losartan, is commonly used. In these patients, β-blockers, calcium channel blockers, and pacing are also considered, along with surgical relief.[91]

Finally, in children with metabolic or mitochondrial dysfunction underlying the HCM, metabolic therapies have been successful on occasion. Similar to the therapy in DCM caused by these deficiencies, carnitine, coenzyme Q10, riboflavin, and thiamine may be considered.[93]

Infiltrative Forms of Hypertrophic Cardiomyopathy

A variety of disorders that have apparent LV hypertrophy and features of HCM occur because of infiltrative disorders. The classic form of infiltrative disease in this category is Pompe disease, a disorder presenting in the first weeks of life typically.[93] More recently, other forms of infiltrative disease have been identified with later-onset disease, such as Fabry disease,[146] Danon disease,[147,148] and LV hypertrophy caused by mutations in AMP-activated protein kinase (AMP-K) encoded by the PRKAG2 gene.[112-114] These disorders, along with those caused by mitochondrial abnormalities and genetic dysmorphism syndromes such as Noonan syndrome and LEOPARD syndrome, are caused by abnormalities not primarily affecting the sarcomere.[93] Therapy is similar to that for the sarcomeric form of the disease, unless systolic dysfunction occurs. In this case, heart failure therapy should be instituted.

Pompe Disease (Type II Glycogen Storage Disease)

Genetic deficiency of acid α-1,4-glucosidase, an enzyme involved in the breakdown of glycogen to glucose, results in a wide clinical spectrum ranging from the rapidly fatal infantile onset of type II glycogen storage disease to a slowly progressive adult-onset myopathy. The infantile-onset form (Pompe disease) typically manifests during the first 5 months of life, and patients usually die before their second year.[149] This rare inborn error of glycogen metabolism occurs in fewer than 1 per 100,000 births. Massive glycogen accumulation occurs, leading to the clinical findings of enlarged tongue, striking hepatomegaly, hypotonia with decreased deep tendon reflexes, and cardiomyopathy (usually HCM) with CHF. The glycogen accumulation can be noted

histologically in the skeletal muscles, liver, and heart. Children usually succumb in the first 2 years of life. The diagnosis may be predicted from the pathognomonic electrocardiogram.[93,150] The disease has autosomal recessive inheritance; the gene coding for the lysosomal enzyme was originally mapped to chromosome 17 at subband 17q23-q25.[151]

Allelic variation at the acid α-glucosidase locus is presumed to be the most important factor in diversity of type II glycogen storage disease.[149,152] It has been shown that various combinations of homoallelic and heteroallelic mutant genotypes are the basis for this clinical heterogeneity. Zhong and colleagues[153] identified a missense mutation in one allele of a patient with Pompe disease. This base pair substitution resulted in a loss of restriction endonuclease sites, which allowed them to demonstrate messenger RNA expression deficiency from the second allele using polymerase chain reaction–amplified RNA. This was the first evidence of single–base pair missense mutations in patients with this disease. In addition to molecular analysis, the diagnosis can be made biochemically by analysis of α-glucosidase in blood lymphocytes or skin fibroblasts. Prenatal diagnosis is possible by amniocentesis or chorionic villus sampling by assay of α-glucosidase. Enzyme therapy is now possible and appears to reverse the cardiac phenotype.[154]

Fabry Disease

An X-linked recessive disorder with mild expression occasionally seen in carrier females, this entity is caused by deficiency of the enzyme α-galactosidase (α-Gal) and is found in 1 of 40,000 people. Young adults may be prone to renal failure and myocardial infarctions. Fabry disease usually has its onset in adolescence and usually manifests with sensations of burning pain in the hands and feet.[146,155] These sensations tend to be associated with fever, heat, cold, and exercise. With increasing age, multiple angiokeratoma become noticeable, especially around the umbilicus and genitalia. Corneal opacities are often noted. Progressive renal failure develops with age. Central nervous system manifestations include seizures and headaches, as well as hemiplegia associated with an increased risk of stroke. Primary cardiac manifestations in affected males are HCM and mitral insufficiency, and the diagnosis depends on echocardiography.[156-158] The LV myocardium and mitral valve tend to be areas of greatest storage of lipid material. On electrocardiogram, the PR interval is usually short. Deposition of sphingolipids in the coronary arteries leads to myocardial ischemia and infarction.[157,159]

The disease-causing gene was originally localized to the long arm of the X chromosome in the Xq22 region, and the full-length complementary DNA was isolated and sequenced,[160] demonstrating a 1393–base pair complementary DNA with a 60-nucleotide 5′ untranslated region and encoding a precursor peptide of 429 amino acids. The gene was found to contain seven exons; mutations and phenotypic correlation have been described.[161,162]

Nakao and colleagues[163] studied 1603 men by echocardiography and demonstrated significant LV hypertrophy in 230 subjects (14%), of whom 7 (3%) had proved α-Gal deficiency. These patients had concentric LV hypertrophy. Linhart et al.[164] studied 30 patients with Fabry disease and found 37% had concentric LV hypertrophy, 10% had asymmetric septal hypertrophy, and 3% had an eccentric pattern of hypertrophy. More recently, Sachdev and co-workers[165] identified five patients with HCM diagnosed after age 40 years and one with earlier-onset (younger than 40 years) HCM, all with low α-Gal activity. In five of the six patients, the hypertrophy was concentric whereas one had asymmetric hypertrophy. In one case, LV outflow tract obstruction was noted. Nonsustained ventricular tachycardia occurred in two patients, and another patient had second-degree atrioventricular block initially and then atrial fibrillation. Therefore it appears that these abnormalities in α-Gal activity result in some cases of HCM or "unexplained" LV hypertrophy.

Danon Disease

Danon disease is an X-linked dominant disorder characterized by intracytoplasmic vacuoles containing autophagic material and glycogen in cardiac and skeletal muscle cells, cardiomyopathy, and skeletal myopathy, with or without conduction defect, WPW syndrome, or mental retardation.[166] The underlying abnormality affects lysosomal function and is due to mutations in the lysosomal-associated membrane protein 2 (LAMP2).[167] The clinical phenotypic expression of Danon disease is variable. Charron et al.[168] screened 50 cases of HCM for LAMP2 mutations and identified mutations in two patients with HCM and skeletal myopathy. Both of these individuals presented during their teenage years, and other younger affected individuals in the family were also identified as young as 7 years of age. WPW syndrome and high-voltage QRS complexes on electrocardiogram were notable along with high creatine kinase plasma levels. In addition, late LV dilation and dysfunction occurred with symptoms of heart failure. Atrial and ventricular arrhythmias and conduction disease was notable along with death during their twenties. Visual acuity abnormality was also common, because of choriocapillary ocular atrophy. This disease appears to be underrecognized and may play a significant role in pediatric heart failure.

Adenosine Monophosphate–Activated Protein Kinase

AMP-K, encoded by the γ2 regulatory subunit of the PRKAG2 gene on chromosome 7q31,[101] is an enzyme that modulates glucose uptake and glycolysis.[169,170] Dominant mutations in this gene were first identified by Gollob and associates[112] and Blair and colleagues[113] in 2001 in subjects with HCM, WPW preexcitation, and atrioventricular block. The genetic locus on chromosome 7q3 was first described by MacRae and colleagues[101] in 1995 in families with HCM and WPW and was thought to be clinically different than in the patients with other forms of adult HCM. Blair and colleagues[113] made the case that mutations in AMP-K resulted in HCM because of compromise of energy production and utilization, but Arad and colleagues[114] provided evidence that this disorder is a form of glycogen storage disease. Cardiac pathology differed from that in other forms of HCM, with no myocyte and myofibrillar disarray seen but instead pronounced formation of vacuoles that were filled with glycogen-associated granules. The myocytes were enlarged, and interstitial fibrosis was minimal. Using a yeast system in which a similar enzyme is functional, Arad and co-workers[114] introduced the same mutations found in the patients and showed that the enzyme activity is persistent (i.e., does not turn off), leading to glycogen accumulation. The authors confirmed these findings by developing a murine model that mimics the human disorder.[171]

Energy-Dependent Forms of Hypertrophic Cardiomyopathy

Mitochondrial Cardiomyopathies

The human mitochondrial genome[172] is a small, circular DNA molecule that is maternally inherited. Mitochondrial DNA (mtDNA) encodes 13 of the 69 proteins required for oxidative metabolism and 22 transfer RNAs (tRNAs) and two ribosomal RNAs required for their translation. Because mtDNA has much less redundancy than the nuclear genome (in which essentially identical information is received from both parents), and tRNAs and ribosomal RNAs are present in multiple copies, the mitochondrial genome is an excellent target for mutations giving rise to human disease.[173-175] Mitochondria enjoy a symbiotic relationship with the cell. These subcellular organelles are dependent on nucleocytoplasmic mechanisms for most structural components but do contribute vital peptides that are central to cellular respiration. Mitochondria contain a permeable outer membrane and a highly restrictive inner membrane that guards the chemical

microenvironment of the matrix compartment. Adaptive mechanisms exist for the passage of large and small molecules across the inner membrane. Translocases shuttle monocarboxylic acids, amino acids, acyl-carnitine conjugates, small ions, and other metabolites in and out of the mitochondrial matrix. Energy is required for importation of proteins into the mitochondria because the nuclear gene-synthesized mitochondrial proteins are precursor molecules that require presequence cleavage. The 13 mtDNA genes are located in the respiratory chain[175-177] and include seven complex I subunits (ND1, 2, 3, 4L, 4, 5, and 6), one complex III subunit (cytochrome b), three complex IV subunits (COI, II, III), and two complex V subunits (ATPase 6 and 8). Coordination must exist between nuclear and mitochondrial genomes to permit assembly of the complex holoenzymes. Each cell contains numerous mitochondria, and each mitochondrion contains multiple copies of mtDNA. This genetic material derives exclusively from the female gamete, and any mutation must be passed from female parent to all progeny, male and female. The replicative segregation of mutant mtDNA copies within the cell determines whether this biologic disadvantage is expressed. In most mitochondrial disorders, patients carry a mix of mutant and normal mitochondria, a condition known as heteroplasmy, with the proportions varying from tissue to tissue and individual to individual within a pedigree in a manner correlating with severity of phenotype.[174,175]

Mitochondrial diseases often produce disturbances of brain and muscle function, presumably because these two organs are so metabolically active, and therefore the metabolic demand is high during growth and development.[178] Cardiac disease is most commonly seen with respiratory chain defects.[179,180] Ragged red fibers are present in muscle biopsy specimens almost invariably when the molecular defect involves mtDNA (except in infants).[173] These defects represent the genetics of ATP production. The diverse clinical syndromes associated with various respiratory chain complexes are thought to result from involvement of tissue-nonspecific (generalized) subunits in other cases and the residual enzyme activity in affected tissues.[181] The cardiac diseases associated with mitochondrial defects include both HCM and DCM, and LVNC.[182,183] No theory has thus far been advanced to explain the cause of these phenotypically different cardiac abnormalities. It is possible, however, that the dilated form occurs after an initial hypertrophic response (i.e., it is a burned-out dilated form of HCM).

Kearns-Sayre Syndrome

This mitochondrial myopathy is characterized by ptosis, chronic progressive external ophthalmoplegia, abnormal retinal pigmentation, and cardiac conduction defects, as well as DCM. Channer and associates[184] reported a case of rapidly developing progressive CHF and DCM requiring transplantation in a patient with Kearns-Sayre syndrome. Approximately 20% of patients with Kearns-Sayre syndrome have cardiac involvement, and the majority usually have conduction defects causing progressive heart block. These patients generally have large, heterogeneous deletions in the mitochondrial chromosome. Poulton and associates[185] showed germ line deletions of mtDNA in a family with Kearns-Sayre syndrome using polymerase chain reaction to amplify across the deletion, with primers flanking these deletions. The patient was shown to have a deletion in muscle mtDNA and at low levels in blood that was identical to that found in the mother and sister. The probands, however, had more deleted DNA, correlating with more severe symptoms. Other mutations have also been described.[186,187]

MERRF Syndrome

This syndrome is characterized by myoclonic epilepsy with ragged red muscle fibers (MERRF) and is caused by a single nucleotide substitution in tRNA LYS that apparently interferes with mitochondrial translation.[188,189] Shoffner and colleagues[190] showed an A to G transition mutation as the cause of the disease associated with defects in complexes I and IV. This abnormality causes decline in ATP-generating capacity, with onset of disease that includes cardiomyopathy. Other reports outline various disease-causing mutations.[191-193]

OVERLAP DISORDERS

Left Ventricular Noncompaction

This disorder has previously been considered to be a rare disease and has been identified by a variety of names including spongy myocardium, fetal myocardium, and noncompaction of the LV myocardium.[194-198] The abnormality is believed to represent an arrest in the normal process of myocardial compaction, the final stage of myocardial morphogenesis, resulting in persistence of multiple prominent ventricular trabeculations and deep intertrabecular recesses (Figure 65-6). This cardiomyopathy is somewhat difficult to diagnose unless the physician has a high level of suspicion during echocardiographic evaluation. In fact, on careful review of echocardiograms and other clinical data, it appears that LVNC is relatively common in children and is also seen in adults.[194,197] In the most recent American Heart Association/American College of Cardiology Cardiomyopathy Classification, LVNC for the first time was recognized as a formal form of classified cardiomyopathy.

Two forms of LVNC occur: (1) isolated noncompaction and (2) noncompaction associated with congenital heart disease such as septal defects (ventricular and/or atrial septal defect), pulmonic stenosis, and hypoplastic left heart syndrome, among others.[110,195-199] In the isolated form and the form associated with congenital heart disease, metabolic derangements may be notable.[183,196]

Clinical Features

LVNC most commonly presents in infancy with signs and symptoms of heart failure, but some patients are identified during later childhood, adolescence, or adulthood. Pignatelli and associates[194] recently reported the findings in 36 children identified over a 5-year period, with the median age at presentation being 90 days (range 1 day to 17 years). In this study, 40% of the children presented with low cardiac output or CHF, and only one child (3%) presented with syncope. The most common presenting symptom other than heart failure was asymptomatic electrocardiographic or radiographic abnormalities, with 42% being asymptomatic. In addition, 14% of children had associated dysmorphic features, and 19% of affected children had first-degree relatives with cardiomyopathy. Among the children with dysmorphic features, DiGeorge syndrome was diagnosed in one child and one child had congenital adrenal hyperplasia.

Genetics

When LVNC is inherited, it can be transmitted as an X-linked, mitochondrial, autosomal recessive, or autosomal dominant trait.[110] In approximately 20% to 30% of cases, familial inheritance has been identified. The X-linked form usually is associated with isolated noncompaction and a mutation in the G4.5 (tafazzin) gene located on chromosome Xq28.[199] This gene has also been identified in patients with Barth syndrome.

In autosomal dominant inherited cases, mutations in the Z-line protein encoding ZASP, located on chromosome 10q22, have been identified in isolated noncompaction,[200] and mutations in the gene encoding α-dystrobrevin, a cytoskeletal protein located on chromosome 18q12, have been identified in patients with noncompaction associated with congenital heart disease.[199] No genes have been identified thus far for autosomal recessive–inherited noncompaction whereas mutations in mtDNA have been seen in patients with noncompaction.[183,196]

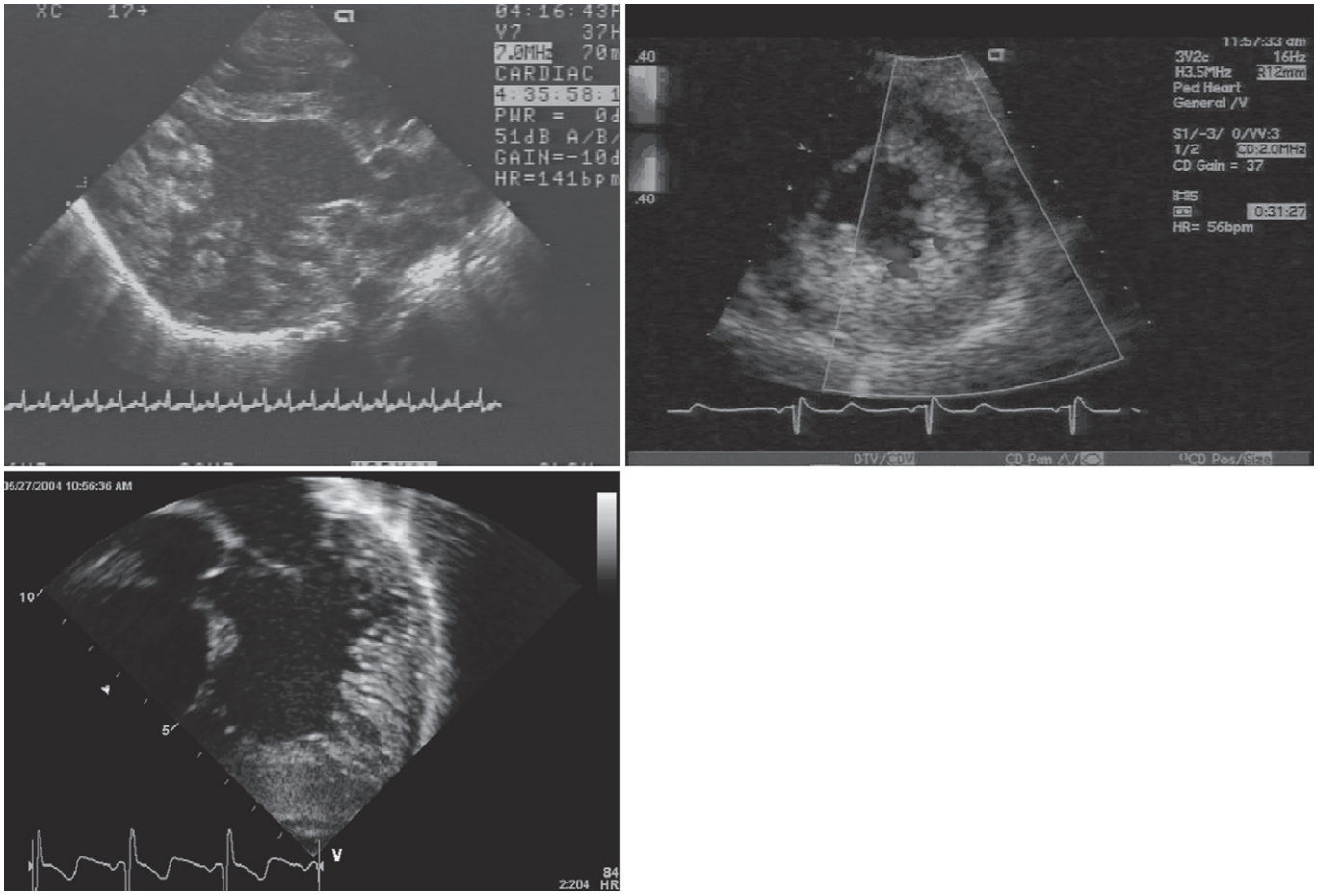


Figure 65-6 Echocardiographic features of left ventricular noncompaction (LVNC). *Top left panel,* A parasternal long-axis view demonstrates a hypertrophic posterior left ventricular (LV) wall with a moth-eaten pattern. The LV chamber is small. *Top right panel,* A parasternal short-axis view with color Doppler demonstrates hypertrophy of the LV apex with deep trabeculations filled with blood.

Therapy and Outcome

The specific therapy depends on the clinical and echocardiographic findings. In patients with systolic dysfunction and heart failure, anticongestive therapy identical to that used in patients with DCM is appropriate. In particular, ACE inhibitors such as captopril and enalapril, as well as β-adrenergic blocking agents such as metoprolol or carvedilol, are useful. Diuretics may also be needed. However, in those patients exhibiting findings more consistent with a HCM or diastolic dysfunction physiologic phenotype, β-blocker therapy alone with propranolol or atenolol is more appropriate. In patients with either of these forms of noncompaction with associated mitochondrial or metabolic dysfunction some investigators add a "vitamin cocktail" to the cardiac therapy with coenzyme Q10, carnitine, riboflavin, and thiamine commonly used alone or in combination.

In patients having associated congenital heart disease, appropriate therapeutic approaches may include simple pharmacologic therapy with diuretics for volume overload associated with left-to-right shunts, more complex pharmacologic therapy for patients with restrictive physiology and pulmonary hypertension, or invasive therapy with catheter intervention or surgical repairs, depending on the lesions. Intimate understanding of the cardiac function abnormalities, evidence of thrombi (which should be treated with anticoagulation), and the metabolic status of the patient must be attended to by the interventional cardiologist, cardiac anesthesiologist, and surgeon in approaching these patients invasively. In addition, cardiac rhythm disturbances need to be identified and therapies such as pacemakers, implantable defibrillators, and intracardiac ablations considered.

The clinical outcome of patients with noncompaction has been reported to be poor with death occurring because of heart failure or sudden death presumably arrhythmia related or stroke related because of embolization of LV thrombi.[195,198] However, Pignatelli and colleagues.[194] demonstrated a 5-year survival rate of 86%; when patients who had transplants were added, the 5-year survival free of death or transplantation was 75%.

CONCLUSION

The cardiomyopathies appear to occur because of disruption of "final common pathways." These disruptions may be due to purely genetic causes, such as mutations in a single gene that result in a dysfunctional protein, which leads to a domino effect of downstream protein interaction abnormalities and ultimately a phenotype. In other situations, multiple mutations in the same gene (compound heterozygosity) or in different genes (digenic heterozygosity) may lead to a phenotype that may be classic, more severe, or even overlapping with other disease forms. In some cases, different intersecting pathways may become disturbed, resulting in complex phenotypes. Further, acquired causes may play a role by causing disruption of these functional pathways. For instance, DCM results from disruption in the final common pathway linking the sarcomere and sarcolemma, and mutations in the affected genes are responsible for cardiac and

skeletal muscle dysfunction. The mechanisms of disease, which include disruption of the linkage caused by protein-protein interaction abnormalities that occur from dysfunctional proteins, as well as the interplay of other factors such as mechanical stress and stretch, are being elucidated in detail with the development of animal models of the human disease. Many of the genes identified are now clinically available in fee-for-service laboratories. Novel therapies have resulted from the improved understanding of this clinical phenotype, as noted earlier. Similarly, the genetic basis and mechanistic understanding of HCM as a disturbance of sarcomeric function have occurred over the past two decades, and genetic tests are clinically available. The development of novel targeted therapies has been somewhat slow in coming but is expected to develop in the near future. In the case of LVNC, the "new kid on the block," genetic understanding is in the early phases. The future of cardiomyopathy care is poised to shift in the next decade because of these new developments, as well as the growing science of stem cell therapy. Because children have "pure" disease states, unfettered by comorbidities, the dream of "cures" of muscle disease (cardiac, skeletal muscle) will likely be realized more fully in this population. We must move toward that goal.

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66

Arthur S. Pickoff

Developmental Electrophysiology in the Fetus and Neonate

In this chapter, the physiology of both impulse formation and conduction within the developing heart is discussed. It is now clear that there are significant age-related changes in the ionic currents responsible for the generation of the cardiac action potential as well as changes in the microscopic and macroscopic anatomic and neural substrates that govern the physiology of intracardiac conduction.

CARDIAC ACTION POTENTIAL

The cardiac action potential is the electrical event responsible for the generation of the cardiac impulse and conduction of the electrical impulse through the myocardium and specialized cardiac conduction system. It is based on a complex series of transmembrane ion fluxes that result in a net flow of electrical current across the cell membrane.[1,2] The cell membrane is a lipid bilayer and is thus highly impermeable to charged ions. The cardiac action potential is generated as a result of two important conditions. The first is an unequal distribution of electrically charged ions, most importantly sodium, potassium, and calcium ions, across the cell membrane (Table 66-1). These ion gradients are established and maintained largely as a result of the activity of energy-expending membrane ion pumps (sodium-potassium adenosine triphosphatase [ATPase]) and ion exchange complexes (the sodium-calcium exchanger). The second critical factor in the generation of the action potential is that under appropriate biophysical conditions, complex polypeptide pores known as *ion channels*, embedded within the cell membrane, are open, thereby allowing the passage of charged ions through the cell membrane. The detailed molecular structure of many of these ion channels is now understood (Figure 66-1, *A* to *C*).[3,4] The direction and magnitude of ion flow through these channels are determined by both ion concentration and electrical gradients, and also, in part, by physical properties inherent to the specific ion channel.

It is useful to consider the ionic events that determine the cardiac action potential before considering the influence of development on this process. The descriptions that follow are derived in part from intracellular microelectrode recordings of changes in the cellular transmembrane potential and also from voltage-clamp studies. At rest, the interior of the typical cardiac cell exhibits a negative electrical potential with respect to the extracellular space. For Purkinje fibers and atrial and ventricular myocytes, this transmembrane potential is approximately −90 mV. In the sinoatrial and atrioventricular (AV) nodes, the interior of the cell is somewhat less negative, approximately −60 mV (Table 66-2). This negative resting membrane potential is generated in part by the activity of the sodium-potassium ATPase pump. This

TABLE 66-1

Intracellular and Extracellular Ion Distribution in the Cardiac Cell

	Na^+,K^+	Cl^-	Ca^{2+}	
Extracellular	145 mM	4 mM	120 mM	2 mM
		cell membrane		
Intracellular	15 mM	145 mM	5 mM	10^{-4} mM

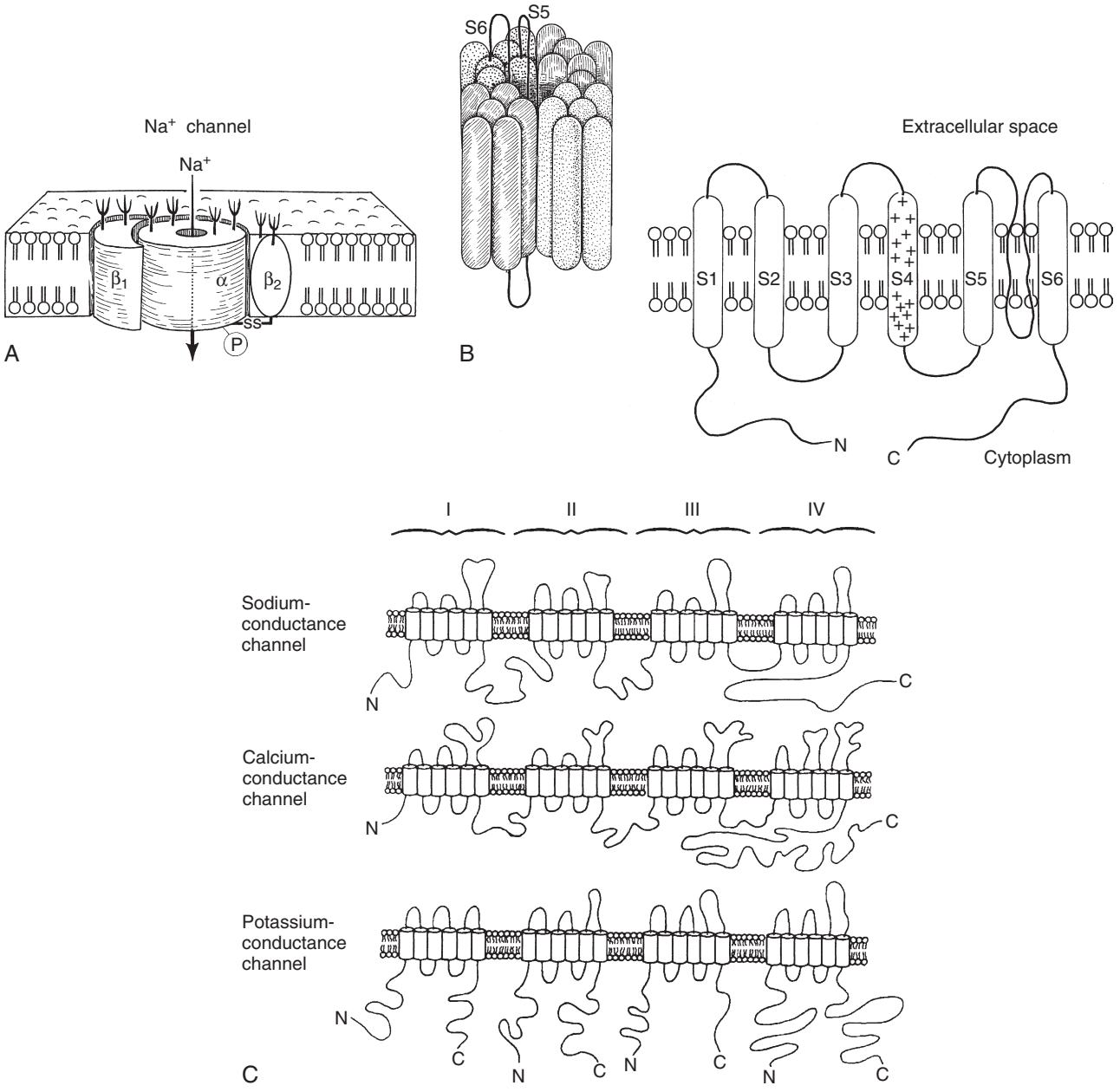


Figure 66-1 **A,** Three-dimensional representation of a sodium channel from mammalian brain. The ion channel consists of the α subunit, with two smaller protein subunits (β_1 and β_2) associated with the main channel. The β_1 subunit is found only in cardiac sodium channels and is thought to stabilize the channel. The treelike (cactus-like) structures are glycosylation sites. *P,* Phosphorylation sites; *SS,* disulfide bond; **B,** The ion channel is composed of four domains of a protein complex that consists of six separate transmembrane segments (S1 to S6). The topographic arrangement of one such domain is shown on the left. The peptide loops between S5 and S6 are thought to represent the central pore of the channel. Subunit S4 is a highly charged protein segment that is thought to serve as the voltage sensor of the ion channel. **C,** Diagrammatic representation of the sodium channel, calcium channel, and potassium channel. In sodium and calcium channels, there is covalent linkage of the four domains (I to IV). (Each domain consists of the six transmembrane segments S1 to S6 already described.) Potassium channels consist of single domains. There is no covalent linkage between domains. *(***A,** *Modified from Shib H-T. Anatomy of the action potential in the heart,* Tex Heart Inst J *21:30, 1994.* **B,** *From Brown AM: Ion channels in action potential generation.* Hosp Pract [Off Ed] *27:129, 1992. Illustration by Alan D. Iselin.* **C,** *Modified from Catterall WA. Structure and function of voltage-sensitive ion channels,* Science *242:50, 1988. Copyright 1988 American Association for the Advancement of Science.)*

energy-requiring pump maintains a high intracellular concentration of potassium ions and low intracellular sodium concentration relative to the extracellular space, by pumping two potassium ions into the cell for every three sodium ions extruded into the extracellular space. This unequal exchange rate of positive ions results in a net negative charge of the intracellular space. The most important factor responsible for the negative resting membrane potential of the cardiac cell is the relatively high permeability of the resting cell membrane to the potassium ion. This high permeability permits the movement of potassium

TABLE 66-2

Action Potential Characteristics in Cardiac Cells

	SA Node	Atrium	AV Node	His-Purkinje	Ventricle
Resting potential (mV)					
Action potential	250-260	280-290	260-270	290-295	280-290
Amplitude (mV)	60-70	110-120	70-80	120	110-120
Overshoot (mV)	0-10	30	5-15	30	30
Duration (msec)	100-300	100-300	100-300	300-500	100-200
$\dot{V}_{max}$ (V/sec)	1-10	100-200	5-15	500-700	100-200
Conduction velocity (m/sec)	<0.05	0.3-0.4	0.1	2-3	0.3-0.4

AV, Atrioventricular; mV, millivolts; msec, milliseconds; m/sec, meters per second SA, sinoatrial; $\dot{V}_{max}$, maximum rate of rise of the action potential phase 0 in volts per second (V/sec).

(Modified from Sperelakis N. Origin of the cardiac resting potential. In Berne RM, Sperelakis N (eds): *Handbook of Physiology, Vol 1. The Cardiovascular System*. Bethesda, MD, American Physiological Society, 1979, pp 187-267.)

ions down their concentration gradient (from the intracellular space into the extracellular space), thereby rendering the interior of the cell more negative with respect to the exterior. The relationship of resting membrane potential to the intracellular-extracellular potassium concentration gradient is described by the Nernst equation:

$$E = (RT/F) \log \left(K^+_{in} / K^+_{out} \right)$$

where E is the membrane potential, R is the gas constant, T is the temperature, F is the Faraday constant, and K^+_{in} and K^+_{out} represent the concentrations of potassium ion inside and outside of the cell.

When the resting potential of the cardiac cell is made less negative, either as a result of the cell's exhibiting spontaneous depolarization (automaticity) or as a result of an advancing wave of electrical current, the cell reaches a level of depolarization, called the *activation potential*. At that point, there is a sudden and rapid influx of positively charged sodium ions (in the case of atrial and ventricular myocytes and Purkinje fibers). This rapid influx of positively charged sodium ions rapidly depolarizes the cell to potentials close to +20 to +30 mV. This rapid phase of depolarization is commonly referred to as *Phase 0 of the action potential* (Figure 66-2). The maximal rate of rise of the action potential phase 0, $\dot{V}_{max}$, in ventricular and atrial muscle approaches 200 volts (V)/second; in Purkinje fibers, even higher rates of change in membrane potential are observed ($\dot{V}_{max}$ of approximately 500 V/second) (see Table 66-2). The amplitude and rate of rise of phase 0 of the action potential are key determinants of conduction velocity in the myocardium and specialized conduction system, with greater conduction velocities resulting from action potentials of greater amplitude and greater $\dot{V}_{max}$. The sudden influx of sodium ions, triggered by the change in membrane potential, results from the sodium channel's changing from a conformationally specific "rested" state to an active or "open" channel configuration[3] (Figure 66-3). A highly charged protein subunit (subunit S4; see Figure 66-1, *B*) of the sodium channel serves as the so-called voltage sensor. After the activation potential has been reached, the voltage sensor moves within the membrane, inducing a conformational change in the sodium channel and resulting in the opening of an "m" or activation gate. This, in turn, allows free passage of sodium ions. As the membrane potential becomes more positive, the sodium channel enters into a third distinct conformational state, the inactivated state, which results from closure of an "h," or inactivation, gate (see Figure 66-3). This inactivation gate may correspond to one of the intracellular peptide loops of the cardiac ion channel.[5,6] Activation and inactivation of the sodium channel occur rapidly, within a time course of a few milliseconds. Allowing for significant differences in the kinetics of channel activation and inactivation, other voltage-gated ion channels, such as calcium and potassium channels, are believed to function in a similar state-dependent fashion (Table 66-3).

After the completion of phase 0, there is often a short, rapid repolarization phase, which creates a notch in the action potential. This notch, which represents phase 1 of the action potential, and the resulting spike-and-dome appearance of the initial portion of the action potential plateau are most notable in epicardial ventricular muscle cells and in Purkinje fibers (Figure 66-4, *A*). There are two principal charge carriers responsible for this phase 1 notch. One is a transiently activated outward potassium current, referred to as I_{to}, and the second is an inward chloride current (I_{to2}).[7] The transient outward current is markedly reduced or absent in ventricular endocardium. It exhibits significant rate-dependent behavior (except possibly in humans) (see Figure 66-4, *B*).

The plateau of the action potential, phase 2 (see Figure 66-2), can persist for hundreds of milliseconds and reflects the delicate balance of both inward and outward currents on a high-resistance membrane. The most important of the inward currents during the action potential plateau is the inward calcium current. L-type ("long-lasting," "large"), and T-type ("tiny," "transient") calcium channels predominate in the heart.[8] The L-type calcium current ($I_{Ca\text{-}L}$) activates at membrane potentials of −55 to −30 mV and is the main charge carrier responsible for maintaining the action potential plateau during phase 2. Activation of $I_{Ca\text{-}L}$ is also highly dependent on intracellular calcium concentration.[9] T-type calcium channels, which activate at potentials more negative than those of the L-type channels, may primarily contribute to pacemaker activity in the heart.[8] The magnitude of $I_{Ca\text{-}L}$ is nearly 10-fold less than that of the sodium current. Activation and inactivation kinetics of $I_{Ca\text{-}L}$ are also an order of magnitude slower than those of the sodium channel (see Table 66-3).[10] The slow inward current, $I_{Ca\text{-}L}$, also represents the major current responsible for phase 0 of the action potential in the sinus and AV nodes (see Figure 66-2, *right side*) and is characterized by a very slow $\dot{V}_{max}$ of approximately 10 V/second. The action potentials recorded from these regions are, therefore, referred to as *slow response* action potentials. A small inward sodium current is also present during phase 2 of the action potential, termed the *window current*.

Balancing the inward depolarizing plateau currents is a family of repolarization currents, largely outward potassium currents, that drive the membrane potential back toward the resting potential (see Figure 66-2). Experiments with cloned pore-forming (α) and accessory (β) subunits of potassium channels have provided new insights into the relationships between molecular assembly and the functional expression of the major repolarization currents (I_K, I_{Kr}, I_{Ks}, I_{Kur}) in the mammalian heart.[11] The first major repolarizing current is referred to as the *delayed rectifier*, I_K. This large outward potassium current is termed *delayed* because of its slow time course of activation. I_K begins to activate relatively late during the action potential plateau, as the time-dependent slow calcium current $I_{Ca\text{-}L}$ begins to inactivate. In some species, including the human, there are two principal components of the delayed rectifier potassium current, a fast (I_{Kr}) and a slow (I_{Ks}) component.[12] Phase 3 of the action potential represents the phase of rapid repolarization of the cell toward the resting membrane potential. Phase 3 occurs as the result of decay in the inward calcium current and the activation of (several) outward potassium currents. The ion current responsible for the terminal portion of this phase of the action potential, as well as the current responsible for maintaining the resting potential, is an outward potassium current activated at negative membrane potentials, referred to as the *inward rectifier*, or I_{K1}.[13] An additional outward potassium

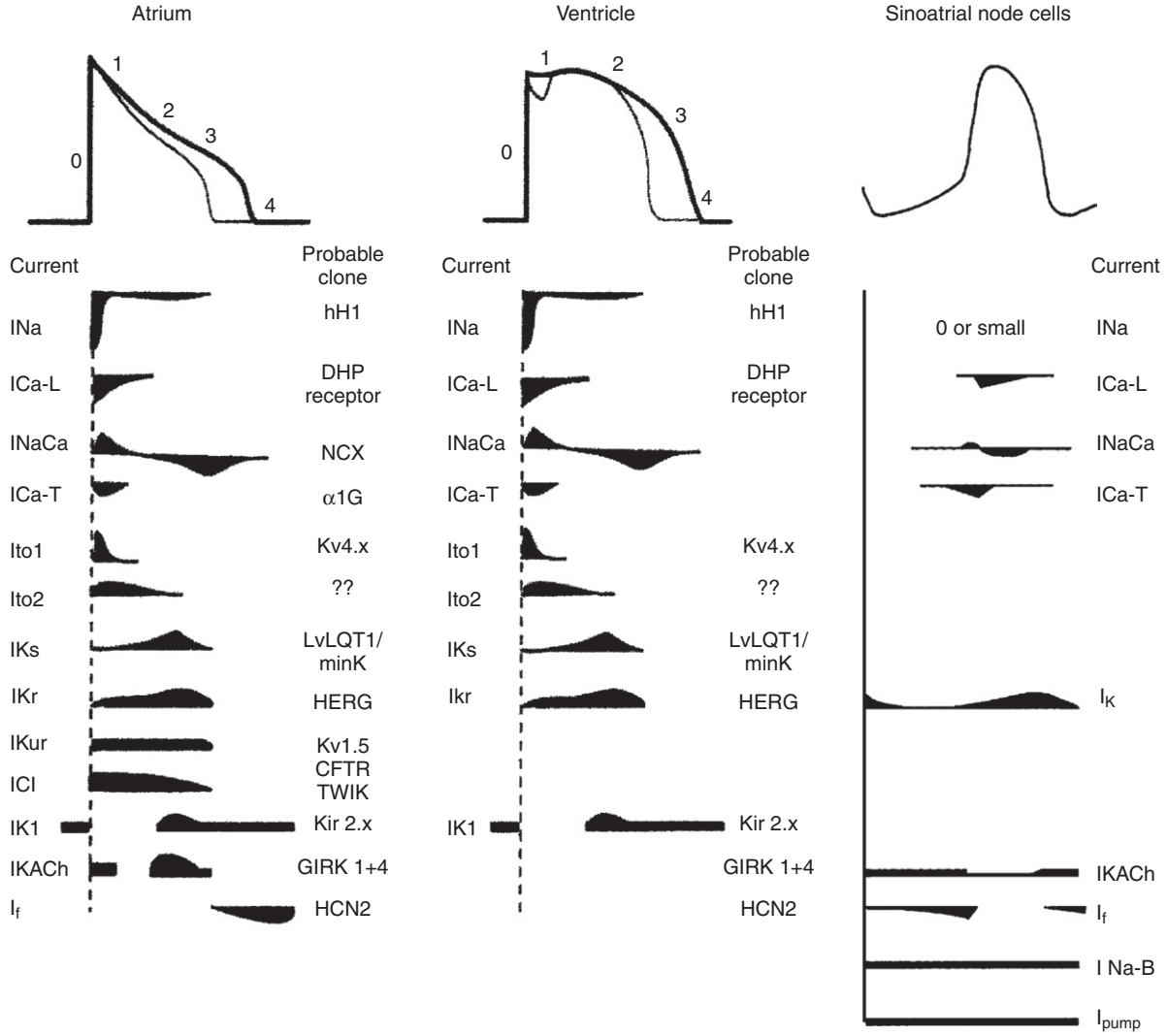


Figure 66-2 Diagrammatic representation of the atrial, ventricular, and sinus node action potential. (For atrial and ventricular action potentials, the bolded action potentials depict changes in action potential morphology that have been associated with the failing heart.) The major ion currents and ion pumps involved in the generation of the action potentials are depicted. For each of the currents, the direction of current flow is indicated (downward indicating an inward current, upward indicating an outward current) as well as the approximate time course of activation and inactivation. Gene products (Probable Clone) that are associated with each current are shown. Relative current amplitudes are *not* depicted. I_{Na} represents the inward sodium current; $I_{Ca\text{-}L}$, the large, long-lasting slow inward calcium current; $I_{Ca\text{-}T}$, the tiny, transient calcium current; and $I_{Na/Ca}$, the current generated by the action of the sodium-calcium exchanger. Outward potassium currents depicted include I_{K1}, the inward rectifier current; I_K, the delayed rectifier current; I_{to}, the transient outward current (of which there are two components); and, limited to the atrium, I_{Kur}. $I_{K(ACh)}$ represents the receptor-activated potassium channel (activated by acetylcholine and adenosine). I_f represents the "funny" pacemaker current, a current carried by both sodium and potassium. All these currents are discussed further in the text. Not discussed but presented here for completeness are I_{Cl}, a chloride current; I_{ump}, an ion pump current; and $I_{Na\text{-}B}$, an inward background current thought to be present in sinus node cells. *(Modified from Task Force of the Working Group on Arrhythmias of the European Society of Cardiology: The Sicilian gambit: A new approach to the classification of antiarrhythmic drugs based on their actions on arrhythmogenic mechanisms,* Circulation *84:1831, 1991, by permission of the American Heart Association; and from Fuster V, Alexander RW, O'Rourke RA:* Hurst's the Heart, *11th ed. New York, McGraw-Hill, 2004.)*

repolarization current called I_{Kur} ("ur" for "ultrarapid") is identified in the human atrium. This atrial current is a rapidly activating, non-inactivating potassium current.[14]

The repolarizing outward potassium currents described are the *major* currents involved in cardiac repolarization. There are other outward potassium currents that have been described that can become important under specific conditions. For example, the ATP-dependent potassium current, $I_{K(ATP)}$, is an outward current that is small under normal conditions; however, it activates strongly under conditions of depleted intracellular stores of ATP.[15] This outward current may become important under hypoxic conditions. The receptor-activated potassium channel, $I_{K(ACh)}$, is activated by exposure to either the parasympathetic agonist acetylcholine or to adenosine.[13] Other potassium channels that have been described include one that activates in response to elevated intracellular calcium concentrations ($I_{K(Ca)}$) and another that activates in response to increased intracellular sodium concentrations ($I_{K(Na)}$).[2]

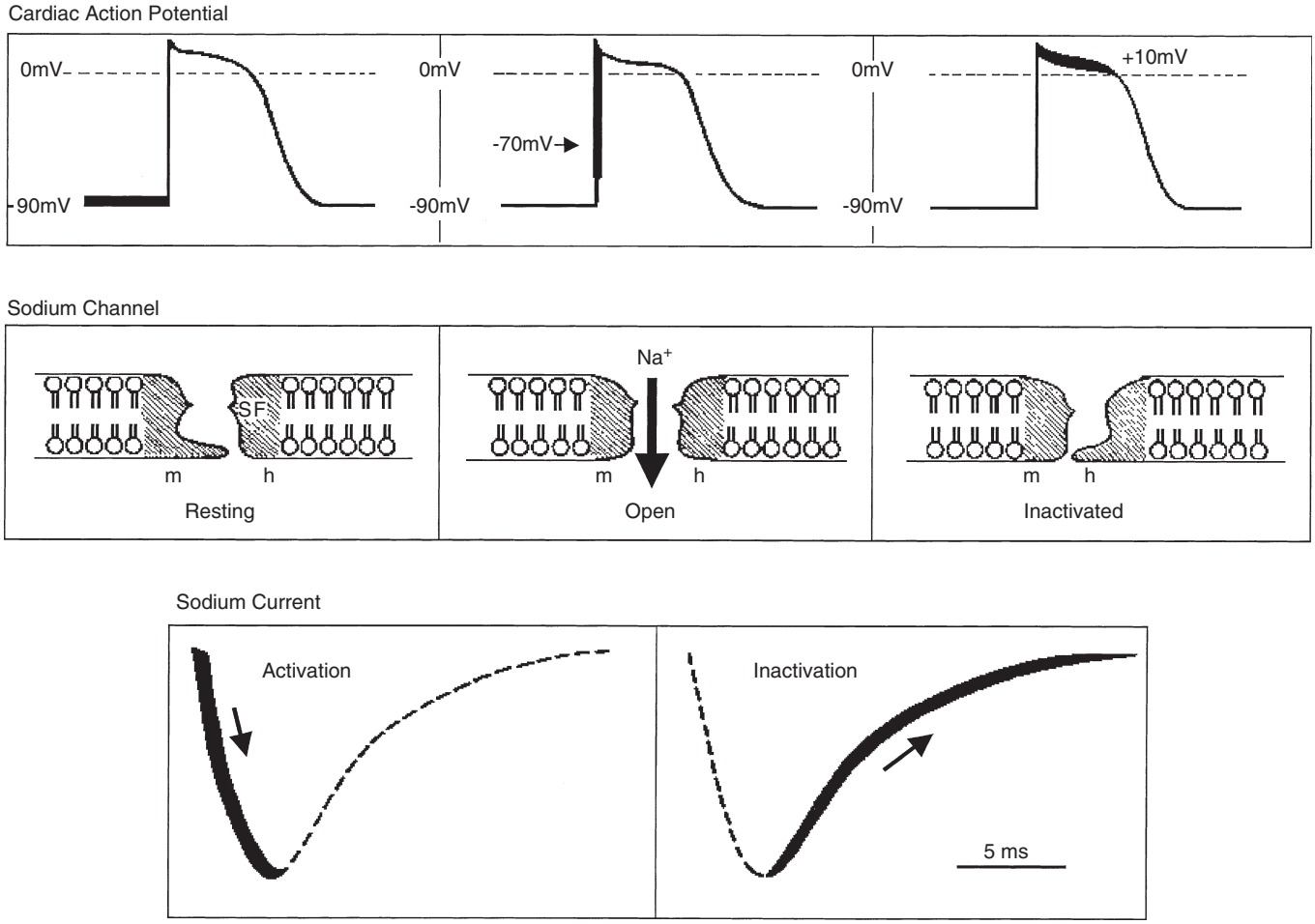


Figure 66-3 Three conformation states of the sodium channel. Illustration of the changes in transmembrane action potential *(top three diagrams),* changes in the conformational state of the sodium channel *(middle diagrams),* and corresponding activation and inactivation of the sodium current *(lower diagrams).* At rest, the membrane potential is approximately −90 mV *(left).* At this membrane potential, the sodium channel exists primarily in the rested state. The *m* gate of the sodium channel is closed. (*SF* denotes the selectivity filter of the ion channel.) As the cell membrane depolarizes (phase 0 of the action potential), the sodium channel changes to the open conformation, associated with opening of the *m* gate *(middle).* The sodium current rapidly activates because sodium ions can now pass through the ion channel. As the membrane potential becomes more positive, the sodium channel enters into its third conformational state, the inactivated state. This occurs as a result of closure of the inactivation gate *h (right).* The sodium current rapidly inactivates, or decays, back toward zero current.

TABLE 66-3

Comparison of Functional Characteristics of Three Different Ion Channels in Cardiac Tissue

	I_{Na}	$I_{Ca\text{-}L}$	I_K
Charge carrier	Sodium	Calcium	Potassium
Activation threshold (mV)	270-255	255-230	220
Magnitude (mA/cm^2)	0.9-1.8	0.035-0.1	0.003
Time constraint (T, msec)			
Activation	<1	<10	400-2400
Inactivation	<1	10-100	—

Modified from Table 22-3 in Zipes DP. Genesis of cardiac arrhythmias: seen in electrophysiological considerations. In Braunwald E (ed): *Heart Disease. A Textbook of Cardiovascular Medicine*, 4th ed. Philadelphia, WB Saunders, 1992: 599; Moak JP. Cardiac electrophysiology. In Garson A Jr, Bricker JT, McNamara DG (eds): *The Science and Practice of Pediatric Cardiology*. Philadelphia, Lea & Febiger, 1990, pp 294-317; Isenberg G, Klockner U. Calcium currents of isolated bovine ventricular myocytes are fast and of large amplitude. *Pflüger's Arch* 395:30, 1982; and Sanguinetti MC, Jurkiewicz NK. Two components of cardiac delayed rectifier K^+ current. *J Gen Physiol* 96:195, 1990.

$I_{Ca\text{-}L}$, L-type calcium channel; I_K, delayed rectifier current (the "s" or slow component); I_{Na}, sodium channel; mA/cm^2, milliamperes per squared centimeter; msec, milliseconds; mV, millivolts.

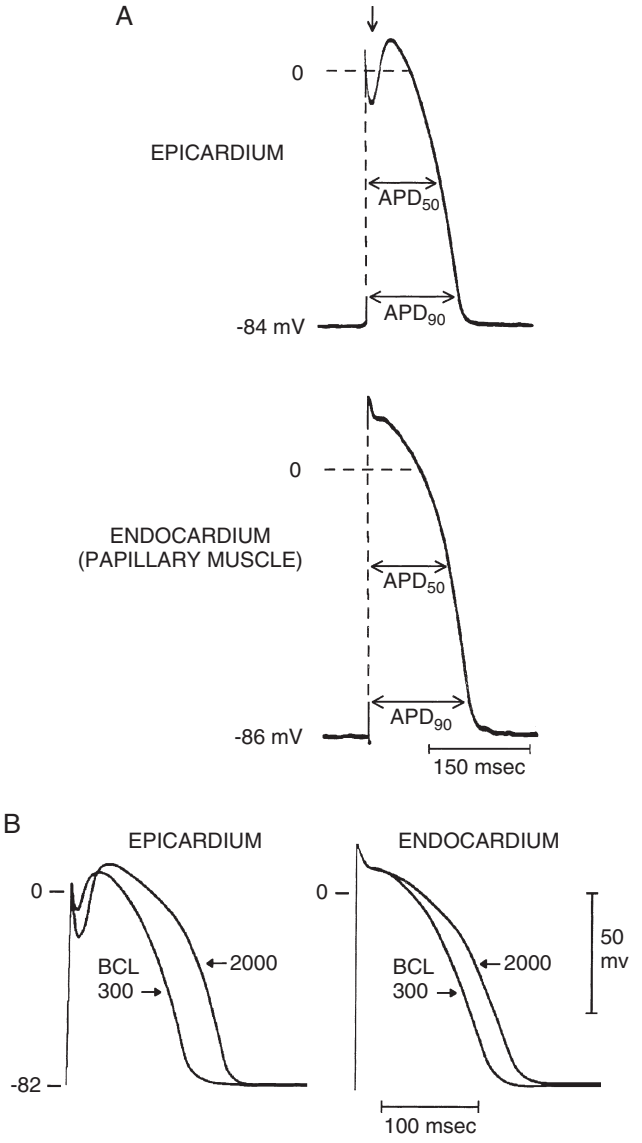


Figure 66-4 **A,** Representative action potentials from ventricular endocardium and epicardium. In the epicardium, there is a prominent spike-and-dome appearance of the action potential plateau *(arrow).* This spike-and-dome appearance is the result of a prominent transient outward current, carried by potassium, which is present in the epicardium and separates phases 1 and 2 of the action potential. This is much less pronounced in action potentials recorded from the endocardium. **B,** Demonstration of the rate-dependent behavior of the transient outward current in canine ventricular myocardium. In epicardium, decreasing basic cycle length (BCL) (i.e., increasing heart rate) from 2000 to 300 msec markedly diminishes the spike-and-dome appearance of the action potential plateau and causes marked shortening of action potential duration. This is due to rate-dependent reduction of the transient outward current at faster heart rates (due to incomplete recovery from inactivation). This is not seen in endocardium, where I_{to} is negligible, resulting in little or no change in action potential duration in response to increases in heart rate. (*Note:* The effects of the rate-dependent decreases in I_{to} on action potential duration are variable. In some species and preparations, action potential duration increases; in others, it decreases.) *(**A,** Modified from Litovsky SH, Antzelevitch C: Transient outward current prominent in canine ventricular epicardium but not endocardium,* Circ Res *62:116, 1988, by permission of the American Heart Association.* **B,** *From Litovsky SH, Antzelevitch C: Rate dependence of action potential duration and refractoriness in canine ventricular endocardium differs from that of epicardium: role of the transient outward current,* J Am Coll Cardiol *14:1053, 1989. Reprinted with permission from the American College of Cardiology.)*

Under normal conditions, spontaneous automaticity is generally confined to the pacemaker cells of the sinus node. In these cells, microelectrode recordings reveal a slow depolarization of the membrane potential during phase 4 of the action potential, from the maximal diastolic potential (MDP) toward the activation, or threshold potential (Figure 66-5). Atrial myocytes, AV junctional cells, and His-Purkinje cells can all exhibit spontaneous diastolic depolarization, although at significantly slower rates than the sinus node. The precise currents responsible for pacemaker activity are still not completely delineated and may vary from site to site. A decline in an outward potassium current (referred to originally as I_{K2}) was initially proposed as the major depolarizing force resulting in spontaneous automaticity. It is now believed that spontaneous automaticity may result from a combination of factors, including a decline in an outward potassium current (possibly a decline in I_K or in AV nodal cells, I_{K1}), increases in both L- and T-type calcium currents, and a transient background Na^+ current.[16,17] Another inward current, carried partly by potassium and partly by sodium and termed I_f ("f" for "funny"), has been described and is believed to be an important pacemaker current in relatively hyperpolarized cells.[18] It is not clear whether I_f has a significant role in mediating pacemaker automaticity under normal conditions in the sinus node, where MDPs of only −50 to −60 mV are achieved.

DEVELOPMENTAL CHANGES IN ACTION POTENTIAL MORPHOLOGY AND TRANSMEMBRANE ION CURRENTS

Changes in action potential morphology and corresponding changes in ion current physiology occur throughout the course of development, beginning in early embryonic life, in both mammalian and nonmammalian species. Although important species-related differences do exist, increases in membrane resting potential, action potential amplitude, $\dot{V}_{max}$, and action potential duration are most often noted during the course of development.

Resting Membrane Potential

Increases in the resting membrane potential during development have been reported in several species. The resting membrane potential in the chick embryo doubles from −35 mV to approximately −70 mV from day 2 to day 18.[19] Similar increases in resting membrane potential have been reported in fetal rat, dog, and guinea pig hearts.[20-22] In rat and dog hearts, further increases in the resting membrane potential occur postnatally. There are several likely mechanisms responsible for the increasing resting membrane potential noted with development. Increases in activity of the sodium-potassium ATPase pump have been reported with maturation. In the chick heart, a threefold increase in the activity of sodium-potassium ATPase has been reported between day 6 and day 20 of incubation.[23] Similar increases in sodium-potassium ATPase have been observed in guinea pig myocardium from fetal day 45 to neonatal days 21 to 25.[24] The increase in sodium-potassium ATPase activity noted during development may in part result from expression of different isoforms of the sodium-potassium ATPase pump.[25] Increased activity of the

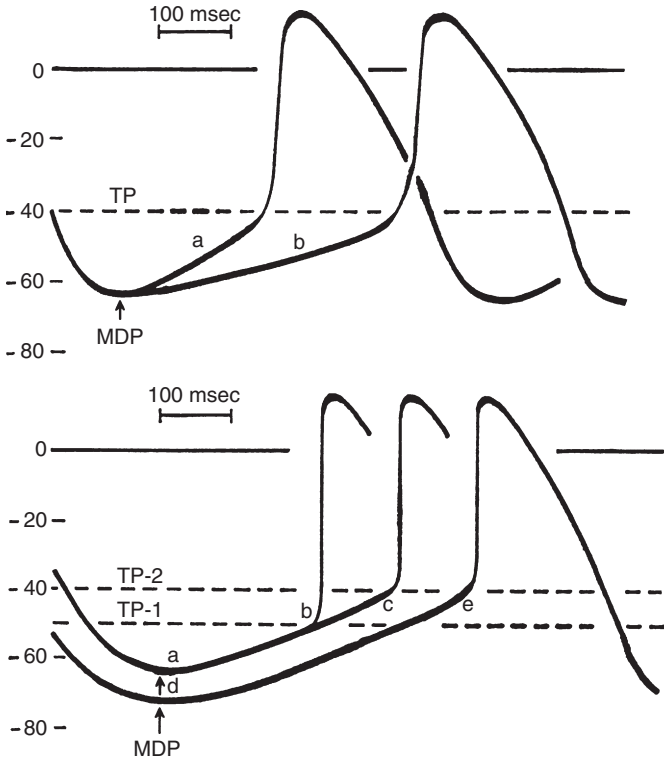


Figure 66-5 Mechanisms of modulation of cardiac pacemaker rate. *Top panel*, Two sinus node action potentials are illustrated. The threshold potential (TP) for both action potentials is −40 mV. The maximum diastolic potential (MDP) is approximately −60 mV. If the slope of spontaneous depolarization decreases (*b*), pacemaker firing rate is delayed. *Bottom panel*, Three sinus node action potentials are shown. A decrease in the threshold potential (i.e., less negative, TP-1 to TP-2) results in a delay in firing rate (*c* versus *b*). Pacemaker firing rate can also be delayed (*e* versus *c*) by an increase in the MDP (i.e., a more negative diastolic potential) (*a* versus *d*), with the slope of spontaneous depolarization and the threshold potential remaining constant. *(From Hoffman BF, Cranefield PF.* Electrophysiology of the Heart. *New York, McGraw-Hill, 1960, p 109.)*

sodium-potassium ATPase pump increases the concentration of potassium intracellularly and decreases the concentration of sodium. This results in a more strongly negative resting membrane potential, as discussed earlier in this chapter.

An increase in membrane permeability to potassium with development also contributes to the more negative resting potential observed with maturation. Specific increases in the current density of the inward rectifier current, I_{K1}, the main outward potassium current responsible for maintenance of the resting membrane potential, have been reported in the chick embryo, and in fetal and newborn rat and rabbit ventricular myocytes.[26-31] In the rat, the increase in I_{K1} may occur as a result of acquisition of a high-conductance potassium channel, one that is absent in the neonate.[31] In the human fetus, resting membrane potentials at midgestation (about 20 weeks) are similar to values reported in adult mammalian tissue.[32] In studies performed in atrial myocytes isolated from young infants (more than 2 months) undergoing heart surgery, the resting membrane potential is similar to that of the adult.[33] These studies suggest that in the human, developmental increases in resting membrane potential are not evident after midterm gestation. This does not preclude the possibility that increases in resting membrane potential might occur earlier in gestation.

Action Potential Upstroke, Phase 0

An increase in action potential phase 0 amplitude and $\dot{V}_{max}$ with maturation has been documented in several species. In the chick heart, $\dot{V}_{max}$ increases from 20 V/second at day 2 to more than 250 V/second at day 18.[19] In the rat, $\dot{V}_{max}$ increases 10-fold from day 10 of gestation to day 21 (term).[20] A threefold increase in $\dot{V}_{max}$ (200-550 V/second) occurs in the canine fetus Purkinje fiber action potential, from early fetal life to near term.[21] Similar trends are noted in guinea pig atrium and ventricle.[22] These increases in action potential amplitude and $\dot{V}_{max}$ are only partly the result of the increases in the resting membrane potential already described. Developmental changes in the structure or function of the ion channel or channels responsible for the upstroke of the action potential appear to contribute importantly to the developmental changes noted in the rate of rise of phase 0 of the action potential in the young embryo and fetus. In the early embryonic chick heart (before day 5), phase 0 of the action potential appears to depend on an ion channel that is not sensitive to the potent sodium channel blocker tetrodotoxin. Through day 8 of incubation, as $\dot{V}_{max}$ increases, a marked increase is noted in the sensitivity of phase 0 of the action potential to tetrodotoxin.[19] This suggests that in the early embryonic phase, the current responsible for the upstroke of the action potential is an embryonic sodium channel, one with slow kinetics, and one that is insensitive to tetrodotoxin. With maturation, an apparent switch occurs, with increased expression of a fast, tetrodotoxin-sensitive sodium channel. (Phase 0 of the action potential in the early chick embryonic heart is inhibited by calcium channel blockers, but sodium is still believed to be the dominant charge carrier.) An increasing sensitivity with maturation of phase 0 of the action potential to tetrodotoxin has also been reported in the fetal rat heart.[20] In the fetal mouse heart (fewer than 13 days' gestation), L-type calcium currents appear to play the dominant role in cardiac excitation, with sodium channel expression increasing only just before birth.[34] In contrast, in the canine heart, although increases in action potential amplitude and $\dot{V}_{max}$ also occur during fetal life, there appears to be no change in the apparent sensitivity of phase 0 to tetrodotoxin.[21] In summary, substantial increases in the amplitude and rate of rise of phase 0 of the action potential occur during avian embryonic and mammalian fetal life. Although increases in resting membrane potential undoubtedly contribute to this increase, developmental changes in the structure and function of cardiac ion channels, including the expression or function of accessory protein subunits, or changes in posttranslational modification of channel function, also likely contribute to the observed differences.

Phase 1 of the Action Potential

Developmental increases in the transient outward current, the outward current responsible for the spike-and-dome appearance of the action potential, have been described in several species. In rabbit ventricular cells, the current density of I_{to} in the neonate is about half that recorded in adult tissue.[29] The rate of inactivation and the rate of recovery from inactivation of I_{to} are both significantly faster (5 to 10 times) in neonatal cells. Because I_{to} displays rate-dependent behavior (decreasing as rate increases), developmental changes are observed in the response of action potential duration to pacing. In the adult rabbit, action potential duration increases, whereas in neonatal cells, little change in action potential duration is observed.[29,35] In rat ventricular myocytes, I_{to} appears to be absent in newborn myocytes and increases slightly between days 1 and 8. The most significant increases occur between 1 week of age and adulthood.[36] In canine ventricular myocytes, neither a notch nor spike-and-dome appearance in the action potential plateau is observed in epicardial myocytes until 2 months of age.[37] The spike-and-dome evolves with increasing postnatal age, reaching its full phenotypic expression only in adulthood. Maturation

of the spike-and-dome appearance correlates with the messenger RNA (mRNA) expression of genes associated with the transient outward current, specifically *KCNIP2* and *Kv4.3* (encoded by *KCND3*).[38] The maturation of the transient outward current also is associated with the development of the phenomenon of "cardiac memory," which refers to changes in repolarization that occur with and that persist after a period of chronic pacing, thought to represent a form of electrophysiologic remodeling. In human atrial cells, an absence of the typical spike-and-dome shape of the action potential has been reported in infant tissue, believed to reflect an absence of the transient outward current (Figure 66-6, *A*).[33] Although I_{to} has subsequently been identified in some cells isolated from young infants undergoing heart surgery,[39,40] significant changes in current density and in recovery kinetics have been reported with maturation (see Figure 66-6, *B*).[40] Most recently, changes that include a larger current density, faster inactivation, and slower recovery from inactivation in the neonate have been described and are believed to be a function of the differences in the relative expression of *KCNIP2* and *Kv4.3* (encoded by *KCND3*) in the neonate compared with the adult heart.[41] Thus, there appear to be consistent age-related increases in I_{to}, the transient outward current. The functional significance of the increase in the transient outward current is not fully understood, nor are the mechanisms that lead to the increased expression of the transient outward current in postnatal life, although augmentation of I_{to} may be linked to postnatal increases in oxygen tension.[42]

Action Potential Plateau and Repolarization, Phases 2 and 3

The slow inward calcium current ($I_{Ca\text{-}L}$) contributes importantly to the sustained depolarization that constitutes the action potential plateau. In the chick heart, nitrendipine (a calcium channel blocker) binding sites increase in number, beginning from day 3 of incubation, but channel affinity appears to be similar throughout development.[43] In day 11 chick embryos, calcium channels detected by nitrendipine binding studies are 100-fold more numerous than calcium channels determined by voltage-clamp studies.[44] These so-called silent calcium channels (present in ligand binding studies, absent from electrophysiologic studies) appear to become electrically active with further development. Kinetic changes in the behavior of the calcium channel have also been described in the chick heart with maturation. In voltage-clamp studies of very young (i.e., 3-day-old) chick embryos, the L-type calcium channel exhibits two distinct modes of firing: a bursting, transient mode and a long-lasting mode. With maturation, a significant decrease occurs in the long-lasting firing mode, and there is an increase in the bursting mode.[45] This *loss* of the long-lasting firing mode may contribute to the decrease in calcium current density that has been observed during chick embryonic development, despite an increasing number of calcium channels.[45,46] Furthermore, shifts occur in the relative contributions of L-type and T-type calcium channels to the calcium transient in early embryonic development. At embryonic day 5 in the chick heart, the relative contribution of the T-type current to the calcium transient is greatest. This dominant T-type current likely plays a significant role in excitation-contraction coupling in the very early ventricular myocardium (through Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum).[47] In the 10-day-old chick embryo (and 18-week human fetus), angiotensin II can be shown to differentially modulate the relative contribution of the T- and L-type calcium current, acting to increase the T-type and decrease the L-type current.[48] The relative contribution of the T-type current then declines, such that by embryonic day 11 to day 15, it is the L-type current that becomes dominant.[47]

In mammalian hearts, increases in nitrendipine binding sites have been described in fetal rat and mouse hearts.[49,50] In the fetal rat heart (18 days), novel non-L-type, non-T-type calcium channels have been described.[51] These novel channels inactivate faster than the typical L-type calcium channel, and they have a different steady-state inactivation curve (percentage of channels inactivated as a function of membrane potential). Thus, as in the chick heart, there appears to be a functionally different calcium channel present in the rat fetus, one that is less expressed with further development. Postnatal increases in the L-type calcium current has been described in the mouse heart and is believed to be dependent on postnatal completion of sympathetic innervation and the action of the sympathetic cotransmitter, neuropeptide Y.[52] Calcium current density in the rat heart is fivefold larger in cells isolated from neonates compared with those from adults.[53] In addition to the inward calcium current, an inward sodium current (the window current) appears to play a relatively greater role in maintaining the action potential plateau in the fetal rat myocyte.[54]

In contrast to the developing chick and rat heart, in which calcium current densities tend to decrease with maturation, in the rabbit progressive increases in myocardial calcium current

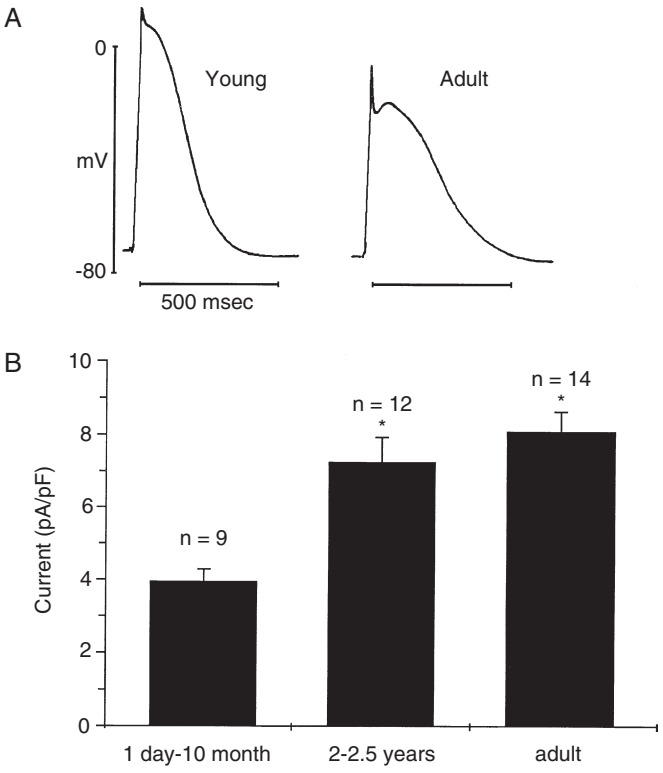


Figure 66-6 A, Comparison of human atrial action potentials. Note that in the adult atrial action potential, there is a prominent spike-and-dome appearance to the plateau phase of the action potential. This is presumed to reflect a prominent transient outward current (I_{to}) in the adult. This spike-and-dome shape of the atrial action potential is absent in the recording from a young infant. **B,** Relationship between age and transient outward current amplitude (I_{to}) in human atrial myocytes. Note that the transient outward current amplitude (pA/pF) is significantly less (*P <.05) in atrial cells isolated from infants less than 2 years of age. *(**A,** From Escande D, Loisance D, Planche C, et al: Age-related changes of action potential plateau shape in isolated human atrial fibers.* Am J Physiol *249:H843, 1985.* **B,** *From Crumb WJ Jr, Pigott JD, Clarkson CW: Comparison of I_{to} in young and adult human atrial myocytes: evidence for developmental changes,* Am J Physiol *268:H1335, 1995.)*

density have been described from fetal to adult life.[27] In addition to an increase in current density, significant changes in calcium channel kinetics also occur during development in the rabbit.[55] Inactivation of the calcium current occurs at less-negative membrane potentials in fetal myocytes compared with adult myocytes. In addition, recovery from inactivation occurs over a longer time course in immature cells. Because of the more prolonged time course of recovery from inactivation in immature cells, the calcium current becomes inhibited to a greater extent at faster stimulation frequencies in the fetus and newborn. This inhibition of the calcium current at faster heart rates may place the immature heart at a potential disadvantage for excitation-contraction coupling (a process that is dependent on transsarcolemmal calcium transport predominantly through the Na^+-Ca^{2+} exchanger but also to some extent through the L-type calcium channel).[56,57] Finally, it has been reported that calcium current density is equivalent in atrial myocytes of newborn and adult humans.[58]

TABLE 66-4

Developmental Variations in Voltage-Gated K^+ Currents in Mammalian Ventricular Myocytes

Current	Fetal	Neonatal	Adult	Species
I_{to}	ND	2	++++	Dog
		+	++++	Human
	+	+++	++++	Mouse
	ND	++	+++	Rabbit*
	+	++	++++	Rat
I_{Kr}	++	++	2	Mouse
	+	+	+	Dog
	ND	+	+	Rat
I_{Ks}	2	++	2	Mouse
	2	2 (<30 days)	++	Dog
	ND	2	2	Rat
I_K	+	++	++	Mouse
	+	++	++	Rat
I_{Kur}	ND	+	2	Rat

From Nerbonne JM. Regulation of voltage-gated K^+ channel expression in the developing mammalian myocardium. *J Neurobiol* 37:37, 1998; and Obreztchikova MN, Sosunov EA, Plotnikov A, et al. Developmental changes in IKr and IKs contribute to age-related expression of dofetilide effects on repolarization and proarrhythmia. *Cardiovasc Res* 59:339, 2003.

*Properties of rabbit I_{to} also change with age (Sánchez-Chapula et al., 1994).

+, Detectable; ++, moderate density; ++++, high density; *2,* not detected; *ND,* not determined.

Repolarization is the gradual activation of outward potassium currents during phases 2 and 3 that initiates and then drives the repolarization process. Marked developmental changes have been reported in the densities and distribution of the various K^+ repolarization currents over the course of development (Table 66-4).[11,59] In the rat, rabbit, and mouse, significant increases in I_K have been described from the fetal to neonatal period. In the rabbit, current density of I_K doubles postnatally through adulthood.[29] Interestingly, functional expression of I_{Kr} and I_{Ks}, the two main components of I_K, are only detectable in fetal or neonatal mouse hearts, and not in those of the adult.[60] In the canine heart, I_{Kr} but not I_{Ks} is expressed in the first 30 days of life, with both currents being observed in the adult myocardium.[61] I_{Kr} blockers, such as dofetilide, thus cause the greatest prolongation in action potential duration in the neonatal canine heart.

I_{Kur}, the ultrarapid component of the delayed rectifier I_K, which is detected only in the atrium of the adult dog, rat, and human heart, is also detected in neonatal rat ventricular myocytes.[62] I_{Kur} is absent in the immature mouse atrium.[63] Finally, an outward repolarization current unique to the newborn heart has been described in canine epicardial ventricular myocytes.[37] In neonatal myocytes, but not adult myocytes, an outward current is present (probably a potassium current) that rapidly activates with depolarization and shows little decay as a function of time, features qualitatively similar to I_{Kur} (Figure 66-7).

In summary, substantial changes have been described with maturation in action potential morphology and in the kinetics and densities of the individual ion currents that constitute the action potential. Allowing for some significant species-related and tissue site differences, increases in (1) the resting membrane potential, (2) the major inward cationic currents responsible for the upstroke of the action potential, (3) the plateau currents, and (4) many outward repolarization currents have been observed from early embryonic or fetal life. These maturational changes undoubtedly contribute to some important functional differences in impulse formation, conduction, and myocardial refractoriness that have been noted with development.

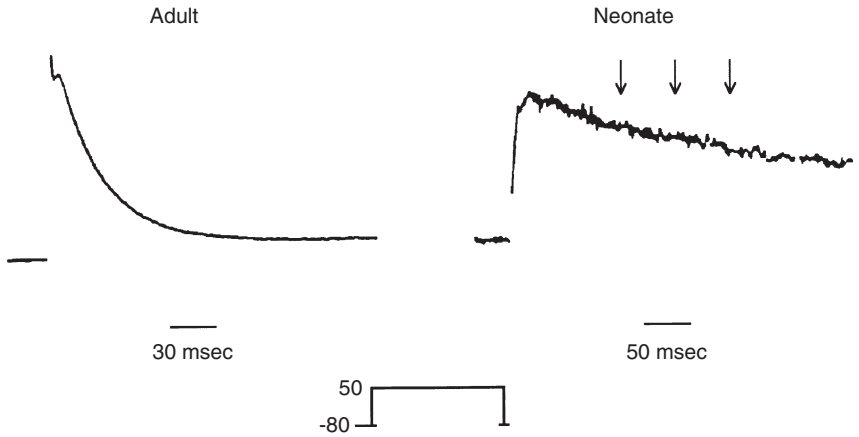


Figure 66-7 Comparison of outward currents recorded from ventricular myocytes isolated from adult and neonatal dogs. On the left, the transient outward current (I_{to}) is elicited in the adult myocyte in response to changing membrane voltage from −80 mV to +50 mV. The transient outward current is not recorded in neonatal cells. In the neonate, a unique outward current is elicited that rapidly activates and shows little decay over time. This current is not observed in adult myocytes. *(Modified from Jeck CD, Boyden PA. Age-related appearance of outward currents may contribute to developmental differences in ventricular repolarization,* Circ Res *71:1390, 1992, by permission of the American Heart Association.)*

IMPULSE FORMATION AND INTRAATRIAL AND ATRIOVENTRICULAR CONDUCTION IN THE EMBRYO, FETUS, AND NEONATE: MORPHOLOGIC AND PHYSIOLOGIC CONSIDERATIONS

In the chick embryo, spontaneous cardiac electrical depolarizations can be detected by voltage-sensitive dyes at about the seventh somite stage, well before contractions become evident.[64] These studies, as well as direct microelectrode recordings,[65] have demonstrated that, although contraction is first noted in the primitive ventricle of the chick embryo (somite stage 10), pacemaker activity evolves simultaneously in both atrial and ventricular sites. With subsequent development, pacemaker activity becomes confined to atrial sites. There is little information concerning the specific ion currents responsible for cardiac automaticity during development. Very early in embryonic life, before the development of true pacemaker ionic currents, "shuttling" of calcium in and out of the sarcoplasmic reticulum, through an inositol triphosphate–dependent mechanism, may be responsible for pacemaker activity at the earliest stages of development.[66] Embryonic stem cell–derived cardiomyocytes depend on a functional ryanodine receptor to differentiate into contracting myocytes with a progressively increasing beating rate.[67] Clusters of murine embryonic stem cells that in vitro demonstrate spontaneous depolarization ("pacemaker nodes") emit calcium signals to adjacent contractile cells. These "pacemaker nodes" are characterized by the expression of the genetic molecular marker *GATA6*.[68] It has been suggested that the so-called funny inward pacemaker current, I_f, which normally activates at hyperpolarized membrane potentials, may also contribute importantly to cardiac automaticity in very early embryonic life.[69] I_f is prominent in ventricular cells of 3-day-old chick embryos and is virtually absent from cells isolated from 17-day-old embryos. In the mouse heart, expression of I_f is also very high in ventricular cells early in development (10.5 days postcoitus) and diminishes in later developmental stages (13 to 16 days postcoitus).[70] In the newborn rabbit, sinus node automaticity can be shown to be due in part to a small, slowly inactivating, tetrodotoxin-sensitive inward sodium current. This current completely disappears shortly after birth.[71,72] Postnatal differences in sinus node L-type (but not T-type) calcium current density and kinetics have also been identified in rabbit sinus node.[73]

The genetic and environmental factors that contribute to pacemaker formation are largely unknown. It is known that cardiac mesenchymal tissues that are destined to form specific regions of the embryonic heart beat at predetermined, characteristic rates.[74] Tissue explanted from regions destined to form the sinoatrial region beats at a faster intrinsic rate than tissue destined to form the primitive ventricle.[75] Genetically determined differences in ion channel populations and kinetics of ion channel function are believed to be important in determining these programmed beating rates. That environmental cues can also modulate pacemaker development and function is demonstrable, in that transplanting mesenchymal tissue from "fast" sinoatrial regions into "slow" regions results in a gradual decrease in the rate of spontaneous firing of the transplanted tissue.[76] Physical contact between myocardial cells and nonmyocardial elements also may contribute to the induction of pacemaker cell aggregates.[77] In vivo, looping of the heart may bring the sinoatrial region of the heart into close contact with nonmyocardial mesenchyme and thereby induce formation of the sinoatrial pacemaker. In the human embryo, a sinus node is first identified shortly after the looping process is completed. The sinus node is an epicardial structure; it is positioned at the junction of the superior vena cava and the right atrium. It is relatively larger in the fetus than in the adult and is horseshoe shaped rather than having the spindle shape observed in the adult.[78]

Conduction of the Impulse From Sinus Node to Atrioventricular Node

The existence of specialized "internodal" conduction pathways within the right atrium, linking the sinus and AV nodes, has been proposed.[79] These putative specialized conduction pathways are located laterally, along the terminal crest of the right atrium, and medially, along the posterior and anterior rims of the fossa ovalis. Although such internodal pathways have been demonstrated histologically and by immunochemistry (including in the developing heart[80,81]), a lack of true specialized cell types and lack of insulation from surrounding myocardium have been cited by some researchers as evidence against true specialized internodal conduction pathways.[82] Studies of HNK-1 (Leu-7) antigen expression patterns in the embryonic rat heart provide evidence that connections between the sinus and AV nodes do exist and that they are derived from the embryonic sinoatrial junction. These connections will, by necessity, join the sinus and AV nodes and are in anatomic proximity to the putative special internodal tracks.[83] More recently, in the developing human heart, three internodal pathways have been identified connecting the sinus node and AV node after incorporation of the sinus venosus into the atrium. These studies, which also employed the HNK-1 antibody to track development of the cardiac conduction system, suggest that these internodal tracks (as well as portions of the posterior left atrial wall surrounding the pulmonary veins) are derivatives of the embryonic sinoatrial ring.[84]

Electrophysiologic studies of intraatrial conduction have also yielded conflicting results. In the dog and rabbit atrium, Spach and colleagues[85] were unable to find electrophysiologic evidence of specialized internodal conduction pathways. Other studies of the canine atrium, however, have reported the identification of three distinct bundles of myocardium that appear to serve as specialized inputs to the canine AV node. These myocardial fibers are electrically active even when atrial contractility is abolished by exposure to high levels of extracellular potassium, and they fire at the same rate as the sinus node.[86]

Whether or not true specialized internodal pathways exist, intraatrial conduction tends to propagate as waves of excitation within the atrium along directions that roughly follow the anatomic courses of the putative internodal pathways. This preferential conduction, along the terminal crest and the anterior and posterior interatrial septum, may be the result of a more uniform, longitudinal alignment of atrial myocytes in these anatomic locations, rather than being the result of specialized conduction pathways.[87]

Electrophysiologic differences in atrial action potential morphology and intraatrial conduction have been described during the course of development. In the adult dog, atrial action potential durations tend to decrease as recordings are made at sites more distal to the sinus node. Action potential durations in the newborn, in contrast, tend to be much more uniform throughout the atrium.[88] Atrial action potential durations also tend to be significantly shorter in the newborn rat, dog, and human compared with the adult (Figure 66-8, *A*).[33,88,89] In the mouse atrium, action potential durations are dramatically shorter in the neonate, a result of postnatal upregulation of outward K^+ currents (other than IKur, which as previously stated is absent in the newborn atrium).[63] Shorter action potential durations result in shorter refractory periods in the neonatal atrium compared with in the adult, which may facilitate the development of intraatrial reentry (see Figure 66-8, *B*).[90,91] It is possible that the occurrence of "benign" atrial flutter, observed at times in the fetus and newborn with an otherwise structurally normal heart, results in part from these shorter atrial refractory periods.[90,92] Finally, in addition to developmental changes in the electrophysiology of the developing atrial myocyte, growth of nonmyocardial elements within the atrium is believed to contribute to developmental differences

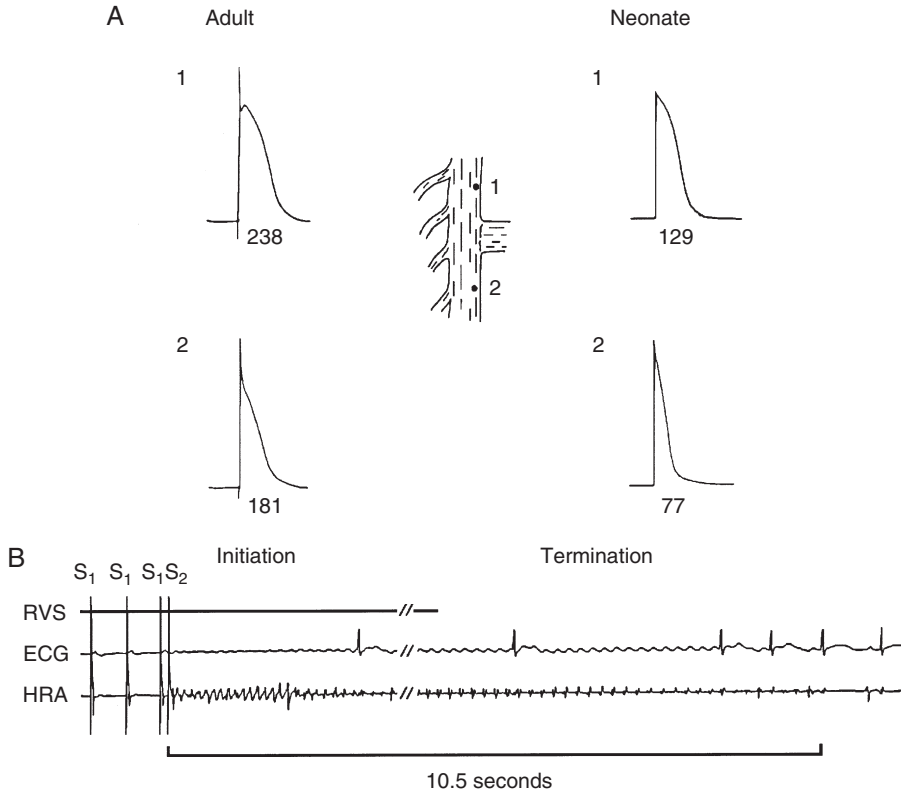


Figure 66-8 A, Atrial action potentials recorded from roughly similar locations along the terminal crest in adult and newborn dogs. Note that neonatal action potentials are characterized by little or no action potential plateau and shorter action potential durations (indicated beneath each action potential, in milliseconds) compared with the adult. **B,** The effect of premature atrial simulation during vagal stimulation in the newborn dog. RVS indicates the period of stimulation of the right vagus nerve; S_1 refers to a basic paced drive train; S_2 refers to a single premature atrial paced beat. Surface electrocardiogram lead II and intracardiac recordings of atrial activity from the right atrium (HRA) are shown. Note that with the introduction of the premature beat (S_2), a long train of atrial fibrillation-flutter is induced. (Similar but shorter runs of atrial fibrillation-flutter are induced in the atrium of the newborn in the absence of vagal stimulation.) *(**A,** Modified from Spach MS, Dolber PC, Anderson PA: Multiple regional differences in cellular properties that regulate repolarization and contraction in the right atrium of adult and newborn dogs.* Circ Res *65:1594, 1989, by permission of the American Heart Association.* **B,** *Modified from Pickoff AS, Stolfi A: Modulation of electrophysiological properties of neonatal canine heart by tonic parasympathetic stimulation,* Am J Physiol *258:H38, 1990.)*

noted in propagation patterns within the atrium. For example, in the neonatal heart, impulses conduct directly from the superior rim of the terminal crest to the adjacent atrial myocardium, whereas in the adult, atrial conduction proceeds inferiorly first, before activation of the myocardium adjacent to the superior terminal crest.[88]

Atrioventricular Conduction *Before* the Formation of the Specialized Atrioventricular Conduction System

Delay between contraction of the chick atrium and contraction of the ventricle is first noted 50 hours after conception (20th somite stage).[93,94] This emulation of AV nodal function occurs before the development of the AV conduction system. Microcellular recordings from the AV canal region at this stage of development demonstrate the presence of slow response-type action potentials. The slow rate of rise of the action potential upstroke functionally corresponds to this zone of slow conduction. It is likely that AV canal cells are the mediators of AV delay in the early chick embryo.[95] These cells, however, can be shown *not* to contribute to the formation of the definitive AV node in later development.[96] Similar findings have been reported in mammalian hearts. In the prelooped, preinnervated rat heart, AV delay also appears to originate in cell populations corresponding to the endocardial cushions or AV canal.[97] Although the endocardial cushions stain positive for acetylcholinesterase in these preinnervated hearts (and adjacent myocardium lacks this cholinergic enzyme),[98] it is not clear that this intrinsic cholinergic system contributes to the observed AV delay. Finally, in the rat embryo, simulation of His-Purkinje system function is also observed before formation of the true AV conduction system. Shortly after looping, a synchronization of ventricular wall motion is observed, coupled with a 10-fold increase in conduction velocity.[97] These changes are highly correlated with expression of the major gap junction protein, connexin 43.[99] Gap junctions are the specialized protein channels that connect adjacent myocytes (Figure 66-9, *A* to *C*).[100] These channels allow not only the passage of electrical current through a low-resistance pathway from cell to cell but also the exchange of larger molecules between cells. The expression of different isoforms of the connexin proteins form gap junctions with different functional characteristics. Expression of connexin isoforms is highly regulated throughout cardiac development.[101] In the mouse heart, connexin 40 is diffusely up-regulated through embryonic day 14, at which time its expression becomes largely confined to the atrium, whereas connexin 43 becomes the dominant isoform expressed in the ventricular myocardium.[99,102] Expression of a third isoform, connexin 45, is largely confined to the specialized cardiac conduction system (see Figure 66-9, *D*). A similar pattern of differential connexin expression is observed in the human

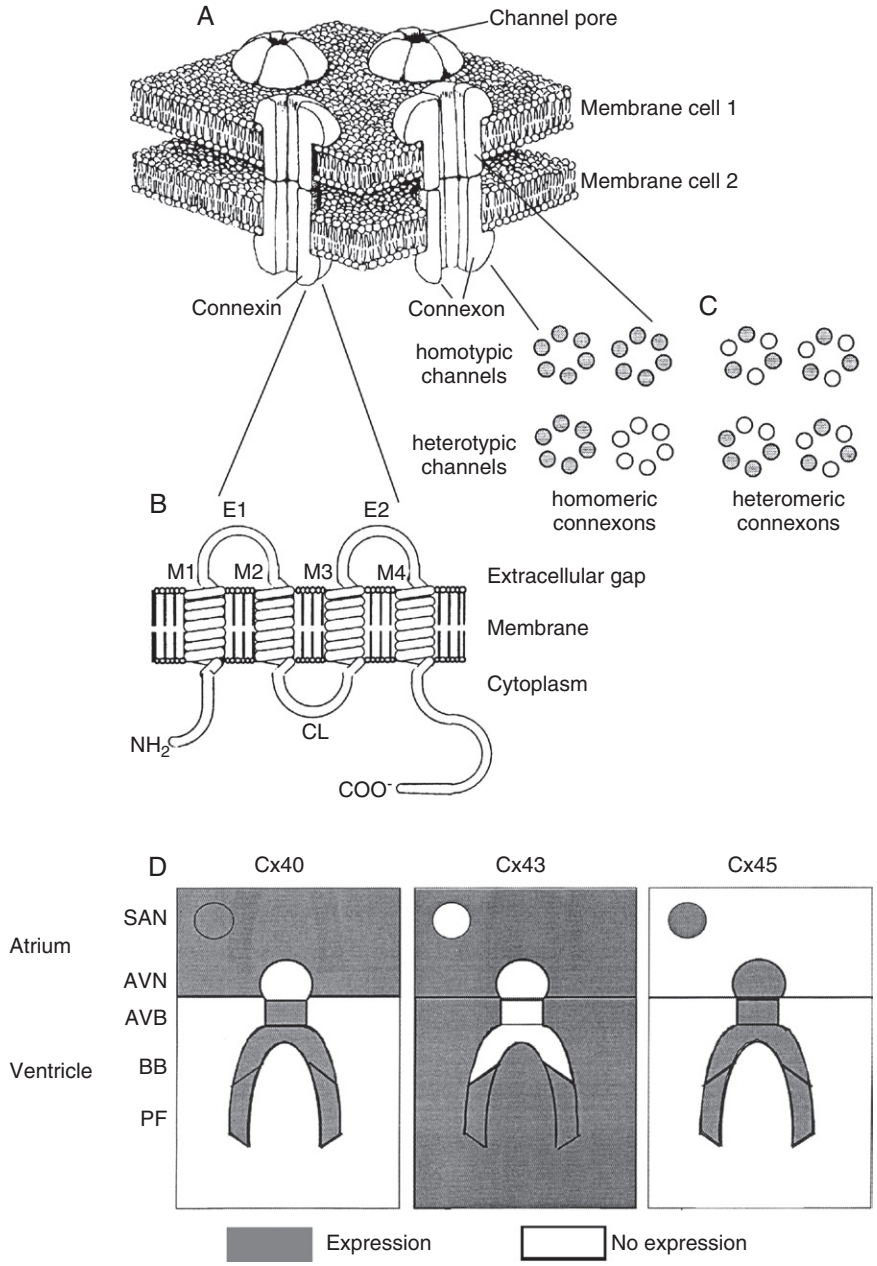


Figure 66-9 Ultrastructure of cardiac gap junctions. Gap junctions occur as "plaques" of multiple intercellular channels. **A,** Portion of a gap junction plaque demonstrating the structure of the intercellular channels, which join cell membranes from two adjacent cardiac myocytes (membrane cell 1, membrane cell 2). Each cell membrane contributes half of the channel structure, called a *connexon*. Each connexon consists of six connexin proteins. **B,** Secondary structure of a single connexin protein. **C,** Connexons formed from identical sets of connexins are termed *homomeric connexons*. Those formed from dissimilar connexin isoforms are termed *heteromeric connexons*. These then combine to form either homotypic or heterotypic intercellular channels. **D,** Expression of the major connexin isoforms, Cx40, Cx43, and Cx45, in the mammalian myocardium and specialized conduction system. AVB, His bundle; BB, bundle branches; AVN, atrioventricular node; PF, Purkinje fibers; SAN, sinoatrial node. *(From van Veen TA, van Rijen HVM, Opthof T: Cardiac gap junction channels: Modulation of expression and channel properties,* Cardiovasc Res *51:217, 2001.)*

fetal heart.[103] The differential regulation of expression of connexins within the developing heart may be altered by factors such as exposure to hypoxia and tumor necrosis factor-α.[104,105] The critical role of connexins for normal cardiac conduction, as well as normal cardiac development, is demonstrated by reports of abnormal, sometimes lethal, intracardiac conduction abnormalities, and of abnormal anatomic development, in hearts of mice with absent or diminished expression of specific connexins.[106,107]

In the developing embryo, the newer regions of fast conduction remain flanked by persisting zones of slow conduction, including the AV canal region and the outflow tract. These regions are characterized by continued expression of both atrial and ventricular myosin heavy chain isoforms and very scarce gap junctional protein.[108] With completion of septation, ventricular activation in the chick embryo switches from an "immature" base-to-apex activation pattern to the "mature" apex-to-base activation pattern.[109] This switch in activation pattern occurs gradually, rather than

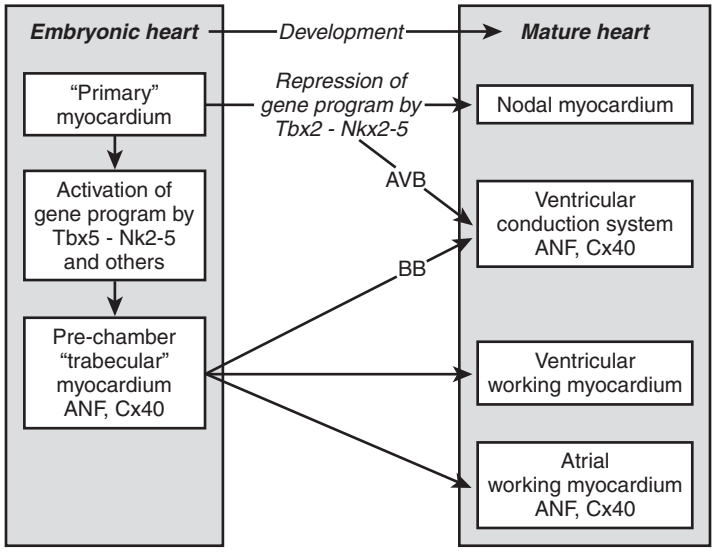


Figure 66-10 The primary myocardium of the heart tube is of a nodal phenotype. The SA node, AV node, and His bundle are derived from this embryonic primary myocardium. The ventricular conduction system (i.e., the bundle branches) and myocardium of the ventricles are derived from the prechamber trabecular myocardium of the embryo. ANF and Cx40 are expressed in those elements of the conduction system that are formed through the trabecular, or secondary, myocardium, whereas ANF and Cx40 expression is repressed by Tbx and Nkx2.5 in components derived from the primary myocardium. See text for further discussion. *(From Moorman AFM. Christoffels VM. Development of the cardiac conduction system: A matter of chamber development. In Chadwick DJ, Goode J (eds):* Novartis Foundation Symposium 250. Development of the Cardiac Conduction System. *Chichester, UK, Wiley and Sons, 2003, pp 25-43.)*

abruptly.[110,111] Two-dimensional optical mapping has permitted the elucidation of "breakthrough" foci in ventricular activation in the course of development. These foci roughly correspond to regions of precursors of the cardiac conduction system.[111,112] In contrast, in the mammalian heart, completion of septation may not be associated with such a switch in ventricular activation sequence.[113]

Formation of the Specialized Atrioventricular Conduction System

The true specialized AV conduction system, including the AV node, bundle of His, and right and left bundle branches, forms after the looping process is completed. Multiple immunohistochemical markers have been employed to delineate and track the development of the specialized conduction system, including in the human heart. Although many of these molecular markers are also markers of neural tissue (e.g., ganglion nodosum [GLN], HNK-1), the conduction system is *not* derived from neural (i.e., ectodermal) precursors. Rather, the conduction system is derived from multipotential mesenchymal precursors. The sinus node, AV node, and bundle of His are derivatives of the embryonic primary myocardium, whereas the more distal ramifications of the conduction system, or bundle branches, are formed from derivatives of embryonic secondary myocardium, specifically, the "prechamber" trabecular myocardium, a domain that is genetically distinct from the adjacent embryonic compact myocardium (Figure 66-10).[108,114] Derivatives of the secondary myocardium are characterized by the expression of atrial natriuretic factor (ANF), connexin 40 and connexin 43, a genetic program not present in derivatives of the embryonic primary myocardium.[114] Cardiomyogenic precursor cells may be induced by regional cues to express genetic programs that promote differentiation into cells of the specialized cardiac conduction system. These regional cues, or inducers, may include neural crest cells, which migrate during development into the region of the central cardiac conduction system, and epicardium-derived cells, which may influence the differentiation of the more distal ramifications of the Purkinje network.[115,116] Neural crest cells entering the heart through the arterial poles may contribute to the differentiation of the bundle branches, whereas neural crest cells entering through the venous pole may have a role in the induction of sinus node and AV nodal cell development.[117] Recruitment of mesenchymal tissue into the specialized cardiac conduction system may also involve induction by cues originating from nearby developing coronary vasculature.[118] Mounting evidence points to endothelin-1 as a crucial signaling molecule governing the transformation of cardiomyogenic precursor cells into cells of the specialized cardiac conduction system.[115,119]

The transcriptional regulation of the differentiation of cardiomyogenic precursor cells into cells of the specialized cardiac conduction system is the subject of intense investigation. Several transcription factors, including the homeodomain transcription factor *Nkx2.5,* the zinc finger factor GATA6, members of the T-Box family (*Tbx2, Tbx 3,* and *Tbx5*), and Hop (homeodomain only protein, acting downstream from *Nkx2.5*) appear to be critical in the differentiation, formation, and function of the specialized cardiac conduction system.[120-122] *Nkx2.5* is preferentially expressed in the developing cardiac conduction system. Precise regulation of *Nkx2.5* expression is essential for normal development of Purkinje fibers in the developing chick embryo.[123] Mutations of this critical transcription factor in humans result in congenital heart defects as well as conduction disturbances and arrhythmias.[124,125] In some patients, mutations of *Nkx2.5* are also associated with a cardiomyopathy, characterized by overgrowth of trabecular ventricular muscle.[126] In transgenic mouse models, *Nkx2.5* insufficient mice develop conduction and electrophysiologic abnormalities that are in part the result of the marked hypoplasia of the AV node and His-Purkinje system.[127] How the aforementioned transcription factors direct the differentiation of the heart and cardiac conduction system is illustrated in Figure 66-10. ANF is a highly specific marker for the developing working myocardium. Furthermore, the promotor region of

the *ANF* gene contains multiple binding sites for several cardiac transcription factors important in the development and function of the cardiac conduction system, including *Nkx2.5, GATA,* and *Tbx.*[127] It is hypothesized that chamber-specific, working myocardium gene expression is repressed in the nodal myocardium (sinus node, AV node, and His bundle) secondary to an inhibition of ANF expression, as a result of the binding of *Tbx2* and *Nkx2.5* to the ANF promotor region.[124,128] Elements of the cardiac conduction system derived from the prechamber trabecular myocardium (the bundle branches) do express ANF as well as connexin 40 *(Cx40).* The result is that with differentiation, the specialized conduction system expresses a molecular pattern that is distinct from the surrounding atrial or ventricular myocardium.[117,129,130]

In humans, recent immunohistochemical studies have demonstrated that the entire AV conduction system forms from a single ring of specialized tissue. This ring of tissue can be identified by its binding of a specific monoclonal antibody raised against chicken GLN antigen (Figure 66-11, *A*). The region can be tracked throughout the development of the AV conduction system.[131] In the human embryo (Carnegie stage 14), the original GLN ring surrounds the interventricular foramen, which connects the primitive left and right ventricles (see Figure 66-11, *B*). At this early stage of development, just after looping of the heart has occurred, the atrium is connected only with the left ventricle. With further development (Carnegie stages 15 to 17), rightward expansion of the GLN ring is observed, as the atrium expands rightward to form the tricuspid orifice (see Figure 66-11, *C*). Thus, the GLN ring always outlines the pathways of blood that flow into the primitive right ventricle. As development of the interventricular septum proceeds (Carnegie stages 18 to 23), the lower rim of the original GLN ring is raised superiorly. After contact between the rising interventricular septum and the lesser curve of the looped heart, the AV node and proximal bundle of His are formed at the medial posterior aspect of the rightward expansion of the GLN ring, with the bundle of His in continuity with the right and left bundle branches (see Figure 66-11, *D*).

In the normal heart, the AV node is positioned anatomically within the triangle of Koch. This triangle is formed by the septal leaflet of the tricuspid valve and the tendon of Todoro, forming two arms of the triangle, and the coronary sinus orifice, defining

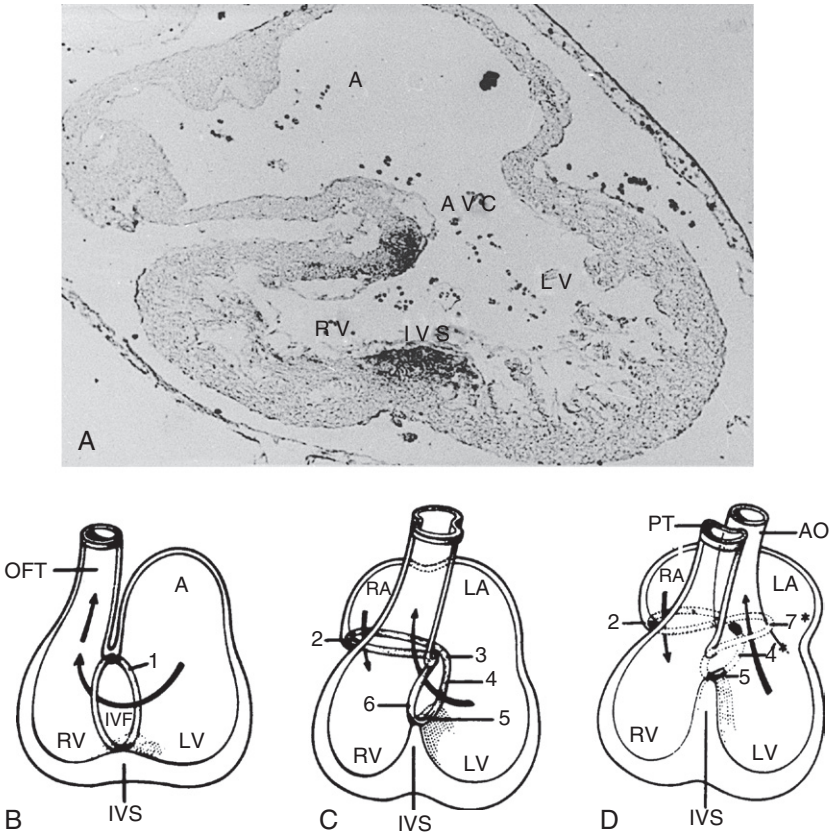


Figure 66-11 **A,** Immunohistochemical staining of the embryonic precursor of the atrioventricular (AV) conduction system in the human heart (Carnegie stage 14). A single ring of tissue, stained for the ganglion nodosum (GLN) antigen, is seen surrounding the interventricular foramen. It is from this single ring of tissue that the entire AV conduction system forms. This includes the AV node, His bundle, and right and left bundle branches. AVC, atrioventricular canal. **B-D,** Formation of the AV conduction system. **B,** The single GLN ring surrounds the interventricular foramen (IVF) in the looped heart (Carnegie stage 14). At this stage of development, the primitive atrium connects solely with the left ventricle (LV). **C,** At Carnegie stages 15 to 17, there is rightward expansion of the GLN ring, as the right atrium (RA) and tricuspid orifice form over the right ventricle (RV). Note the interventricular septum (IVS) rising from the floor of the ventricles. The AV node forms at position 3, the His bundle at position 4, and the bundle branches at position 5. **D,** At Carnegie stages 18 to 23, there is further development of the IVS. Involution of much of the original GLN ring now occurs, leaving the newly formed AV conduction system. *(From Wessels A, Vermeulen JLM, Verbeek FJ, et al: Spatial distribution of "tissue-specific" antigens in the developing human heart and skeletal muscle. III. An immunohistochemical analysis of the distribution of the neural tissue antigen G1N2 in the embryonic heart; implications for the development of the atrioventricular conduction system.* Anat Rec *232:97, 1992, by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons.)*

the base of the triangle. The AV node is positioned at the apex of the triangle. Histologically, the node consists of a loose or transitional zone and a compact region.[132] In hearts from some neonates, extensions from the compact zone of the AV node have been observed and in the past have been considered by some as possible substrates of electrical instability and a possible cause of sudden death.[133] These extensions are now believed to more likely represent normal variants.[134] From the compact node, the AV bundle arises, piercing the central fibrous body of the heart and branching into the right and left bundle branches. The left bundle branch is located in a subendocardial position and spreads in a fanlike fashion over the left ventricular septal surface. The right bundle branch runs an intramyocardial course within the right septal surface, is more cordlike, and emerges onto the endocardial surface of the right ventricle beneath the medial papillary muscle, descending to the apex of the right ventricle along the moderator band.

Physiology of Atrioventricular Conduction

Earlier studies of AV conduction in the developing mammal suggested that the AV node and specialized conduction system of the younger animal offered little or no protection of the ventricles against rapid atrial rates. In young goats, pigs, and puppies, it was reported that the refractory period of the AV conduction system (which determines how closely two impulses can be conducted) was shorter than the refractory period of the ventricle. Thus, rapid atrial rates were reported to conduct down the specialized conduction system to the ventricles, causing ventricular fibrillation in young goats and pigs.[135,136] In newborn calves, little or no increase in AV nodal conduction time was noted during closely coupled electrical stimulation of the atrium.[137] Collectively, these studies suggested that the neonatal AV node was ineffective as a filter.

More recent studies have challenged these conclusions. In the intact, anesthetized newborn dog, intracardiac electrographic recordings have demonstrated that, when corrected for inherent differences in heart rate, the neonatal AV node does in fact act as an effective filter.[138] The refractory period of the AV node in the neonate is significantly longer than the ventricular refractory period, and the AV node serves as the primary site of conduction slowing in both neonates and adult animals. Microelectrode recordings within the neonatal and adult rabbit AV node have also demonstrated little developmental difference in the electrophysiologic characteristics of the AV node.[139] Normal AV conduction delay has been demonstrated by magnetocardiography and fetal echocardiography in fetal sheep and in human fetuses at about 20 weeks of gestation.[140-143] In humans, there are few differences in the AV nodal conduction times of young infants and adults.[144] Thus, the neonatal AV node may act as more of an effective filter than was suggested by earlier studies.

In the dog, the electrophysiology of the specialized conduction system has been studied from the time of implantation to birth.[21] Conduction velocities increase during the course of development. This increase in conduction velocity is due in part to maturational changes in Purkinje fiber action potential characteristics (increases in resting membrane potential and increases in amplitude and $\dot{V}_{max}$). Changes in the architecture of the developing Purkinje fiber, however, likely contribute to the increase in conduction velocity as well. Increases in Purkinje fiber diameter and cell-to-cell contacts have been described in the developing canine conduction system.[145] In addition to an increase in cell size, gap junctions "redistribute" with maturation. In newborn ventricular myocytes, gap junctions are located over the entire cell surface (ends and sides), whereas in the adult, an end-to-end arrangement is observed (Figure 66-12). Both the increase in cell size and the redistribution of gap junctions contribute to the significant increase in $\dot{V}_{max}$ observed in conduction along the transverse (but not longitudinal) direction in the adult heart.[146]

Maturational changes in His-Purkinje conduction characteristics other than conduction velocity have also been described in the canine conduction system. In the newborn dog, the initial site of activation of the ventricles appears to be a right ventricular site rather than the left aspect of the interventricular septum, as in the adult.[147] Retrograde conduction, the ability to conduct an impulse from the ventricle to the atrium, appears more common in the neonatal heart.[148] Finally, action potential durations along the His-Purkinje system are more uniform in the neonate than in the adult, in whom there is an abrupt increase distally in the action potential duration of the Purkinje fiber, at the level of the distal false tendon.[149] This abrupt increase in action potential duration at the site of insertion of the Purkinje fiber into the ventricular muscle has been interpreted as functioning as a "physiologic gate," serving as a protector against closely coupled, rapid impulses.[149,150] This physiologic gate appears to be absent in the neonatal heart, rendering the neonatal ventricle *potentially* susceptible to the rapid conduction of supraventricular impulses (Figure 66-13).

Autonomic Innervation-Modulation of Cardiac Automaticity and Conduction

In considering developmental cardiac electrophysiology, it is also necessary to consider maturation of the autonomic nervous system and its effect on the electrophysiologic properties of the heart. Changes in autonomic tone profoundly affect pacemaker impulse generation as well as AV conduction and myocardial refractoriness.

Parasympathetic Nervous System

In the chick heart, evidence of cholinergic innervation, assessed by the histochemical detection of choline acetyltransferase (CAT) (the enzyme responsible for the synthesis of acetylcholine), is first noted about 3 days after fertilization. Progressive increases in CAT, and in the uptake of the parasympathetic neurotransmitter precursor choline, occur with further development. Choline uptake peaks about10 days after fertilization, just before the onset of ganglionic transmission.[151] In the mammalian heart, muscarinic receptors are present before actual innervation of the heart. Acetylcholinesterase is also demonstrable before innervation in the developing rat, rabbit, and human heart.[152] Progressive age-related increases in acetylcholine synthesis are demonstrable in the atria of rats. Cholinergic innervation of the heart becomes denser in the regions of the sinus and AV nodes and throughout the atria. In the human newborn, moderate cholinesterase activity is found in association with the conduction system, mostly in association with the sinus and AV nodes.[153] Little or no cholinesterase staining of the bundle branches is observed in the newborn infant. Postnatal maturation of innervation to include the remainder of the conduction system occurs, reaching a maximal density in childhood.[154,155] Intracardiac ganglia undergo significant postnatal morphologic changes in the human, with increases in the area occupied by both intracardiac ganglia and neurons occurring with age.[156] Furthermore, developmental changes in the expression and function of ion channels of intracardiac neurons have been described, with an inwardly rectifying potassium channel being identified in adult, but not neonatal, rat intrinsic cardiac neurons.[157]

Muscarinic receptor density has been found to be significantly higher in the myocardium of fetal rat and sheep hearts compared with in the adult. The production of phosphoinositol (a secondary muscarinic messenger) is also greater after muscarinic receptor activation in the immature heart.[158] Muscarinic receptor subtypes may differ in the immature heart. In addition to the dominant cardiac cholinergic M2 receptor found in the adult heart, M1 subtypes may be found in the neonatal heart. These receptors may subserve different physiologic responses to

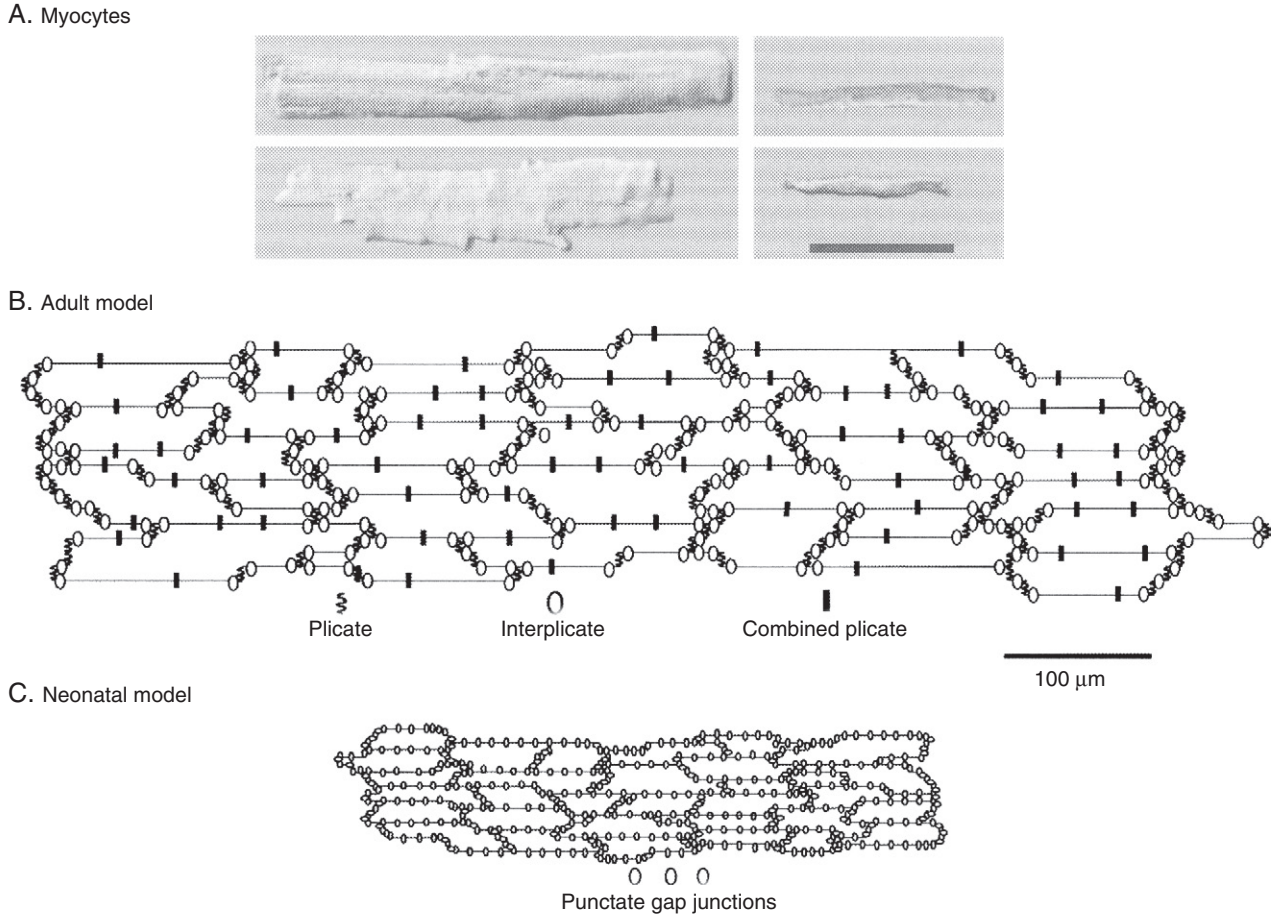


Figure 66-12 Ventricular myocyte dimensions and gap junction distributions in the adult and newborn dog. **A,** Relative size of adult compared with newborn ventricular myocytes. Adult myocytes are significantly larger than newborn myocytes. (Bar measurement = 50 μm). **B,** Two-dimensional multicellular model of adult ventricular myocytes. Three different gap junction morphologies are illustrated (plicate, interplicate, and combined plicate). Note that the location of most gap junctions in the adult (all of the plicate and interplicate types) are confined to the ends of the ventricular myocytes. **C,** Two-dimensional multicellular model of neonatal ventricular myocytes. A diffuse end and side distribution of gap junctions is illustrated. *(From Spach MS, Heidlage JF, Dolber PC, Barr RC. Electrophysiological effects of remodeling cardiac gap junctions and cell size,* Circ Res *86:302, 2000, by permission of the American Heart Association.)*

acetylcholine stimulation. The presence of a different subpopulation of muscarinic receptors in the neonate may explain the paradoxical increase in automaticity observed in neonatal ventricular cells in response to low concentrations of acetylcholine, a response not seen in adult cells.[159]

The primary actions of acetylcholine on cardiac ion channels include an increase in outward potassium currents ($I_{K(ACh)}$ and I_{K1}) and an inhibition of the inward calcium current, $I_{Ca\text{-}L}$.[159-162] These primary effects are mediated through a membrane-bound, pertussis toxin–sensitive, inhibitory G_i protein and a pertussis toxin–insensitive protein, G_q (Figure 66-14, *A*). In sinus and AV nodal tissue, increases in outward potassium currents result in hyperpolarization of the cell. This increase in membrane potential, coupled with muscarinic inhibition of the inward calcium current, accounts for the slowing of the cardiac pacemaker rate that is characteristic of muscarinic stimulation. It has also been shown that acetylcholine inhibits I_f, the funny pacemaker current, which also contributes to cardiac pacemaker slowing by acetylcholine.[163] Increases in outward potassium currents account for the marked shortening of atrial action potential duration and the marked shortening of atrial refractoriness observed with muscarinic stimulation in atrial tissue. In the rat atrium, acetylcholine-induced outward potassium current density progressively increases from 12 days gestation to 20 days postnatally (and then decreases in adulthood as cell capacitance [i.e., cell size] increases).[164] Inhibition of the inward calcium current, primarily within the nodal or N region of the AV node, reduces the amplitude and rate of rise of the slow-response AV nodal action potential. This accounts for the slowing of conduction through the AV node. The refractoriness of the AV node is also markedly increased by muscarinic stimulation. In ventricular myocardium, a slight but significant increase in ventricular refractory periods occurs with vagal stimulation. Evidence is increasing that this is a direct effect of acetylcholine.[165] The precise ionic basis for this effect is not well understood.

Maturation of parasympathetic control of cardiac electrophysiology is evident from studies of heart rate variability, as well as the effects of muscarinic blockade, vagus nerve transection, and vagal nerve stimulation in the course of development. In the human fetus, the increase in heart rate observed in response to a maternal dose of atropine increases with advancing gestation.[166] In the premature infant (32.3 ± 1.3 weeks), analyses of the respiratory component of heart rate variability, a surrogate of vagal activity, suggest that respiratory vagal activity increases over the first postnatal month.[167] In the fetal rabbit, cutting the vagus nerve causes a smaller increase in heart rate than is observed in the adult.[168] In the fetal lamb, vagal stimulation causes a greater slowing of the heart rate as gestation progresses.[169] Continued maturation

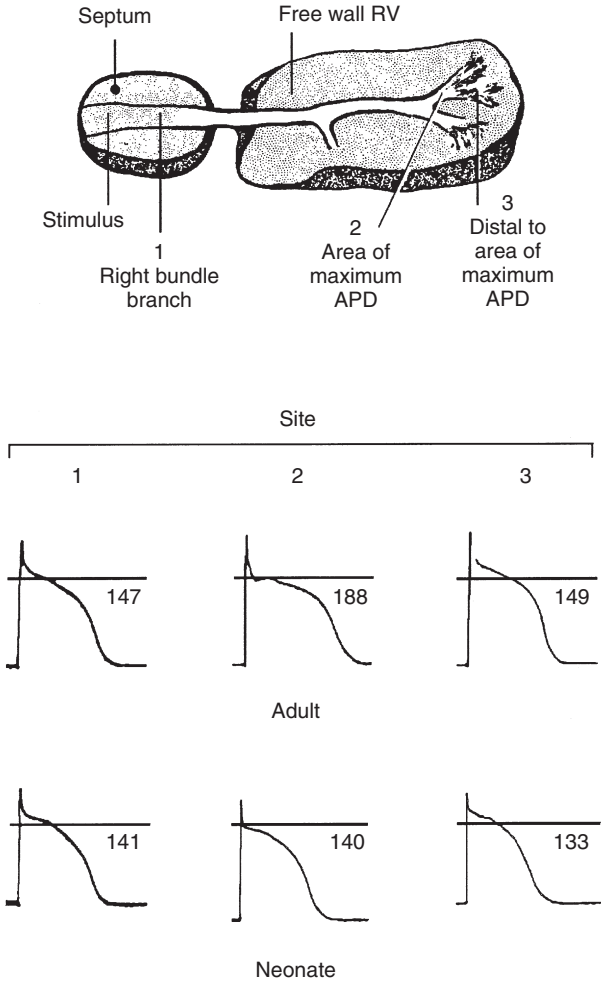


Figure 66-13 Physiology of the specialized ventricular conduction system in the neonatal and adult dog. Intracellular microelectrode recordings of transmembrane action potentials are shown along the length of the right bundle branch, including distal ramifications. Site 1 corresponds to the proximal right bundle branch, site 2 just proximal to distal ramifications, and site 3 distal ramifications. In the adult dog, there is an abrupt increase in action potential duration (APD) noted at site 2 (increase from 147 to 188 msec, site 1 to site 2). This abrupt increase in action potential duration functions as a physiologic gate, blocking the conduction of closely coupled impulses to the ventricle. This abrupt increase in action potential duration is not present in the neonate. Therefore the physiologic gate is inoperative in the neonate. *(Diagram of right bundle branch and right ventricle free wall is from Myerburg RJ, Stewart JW, Hoffman BF. Electrophysiological properties of the canine peripheral A-V conducting system.* Circ Res *26:361, 1970, by permission of the American Heart Association. Action potentials are modified from Untereker WJ, Danilo P Jr, Rosen MR. Developmental changes in action potential duration, refractoriness, and conduction in the canine ventricular conducting system,* Pediatr Res *18:53, 1984.)*

of parasympathetic responses is evident in many species postnatally. In the puppy, the magnitude of the response of heart rate to long trains of vagal nerve stimulation increases postnatally.[170] The response to brief trains of vagal stimuli, which experimentally mimics how the vagus actually fires in vivo, also changes with development. In the adult, the degree of prolongation of sinus cycle length (as well as the degree of prolongation of AV nodal conduction) progressively *increases* as a brief vagal train is delivered progressively later in the cardiac cycle. This typical phase-response relationship is not observed in the newborn (Figure 66-15). Full maturation of the response to brief vagal stimuli is not observed until about 1 to 2 months of age.[171,172] Thus, although parasympathetic responses and reflexes are clearly present in utero and in the preterm infant, it is incorrect to consider the parasympathetic nervous system as being fully mature at birth. Significant maturation occurs in both the magnitude and type of responses elicited by parasympathetic stimulation in postnatal life.

Sympathetic Nervous System

Evidence of functional β-adrenergic receptor modulation of calcium channel currents is observed in the early mammalian heart, before the time of sympathetic innervation.[173] However, in many mammalian species, including the human, rat, and dog, it is believed that the sympathetic nervous system is not as well developed as the parasympathetic nervous system at the time of birth. Rapid maturation occurs within the first postnatal months. Immunohistochemical studies of the specialized cardiac conduction system of the newborn human and dog heart have demonstrated nerve fibers reactive to dopamine β-hydroxylase (putative sympathetic neurons) mostly in the sinus and AV nodes, principally in association with small blood vessels, and only in small numbers.[153,174] Evidence of sympathetic innervation of the atrium, sinus, and AV nodes, as well as the ventricular epicardium, is first observed at midgestation and progressively increases, reaching full maturity only after 2 months of age. In the dog, there is no sympathetic innervation of the AV bundle noted at birth, but this region also is fully innervated by 2 months of age. These histochemical studies of the mammalian cardiac conduction system complement earlier reports of increasing catecholamine content with maturation in the hearts of fetal and newborn dogs, as well as other mammalian species.[175-177]

Maturation of sympathetic innervation is associated with profound changes in the electrophysiologic responses of the developing heart to catecholamine stimulation. In Purkinje fibers of neonatal dogs, exposure to the α-adrenergic agonist phenylephrine increases the rate of spontaneous firing in a dose-dependent fashion.[178] This α-adrenergic-mediated *increase* in automaticity is not observed in Purkinje fibers from adult animals in whom α-adrenergic stimulation *decreases* automaticity. It has been shown that the conversion of the chronotropic response to α-adrenergic stimulation from the neonatal response (increase in automaticity) to the adult response (decrease in automaticity) is contingent on the process of sympathetic innervation. In rat myocytes that are cocultured with sympathetic nerves, it can be shown that a specific pertussis toxin–sensitive 41-kDa guanosine triphosphate-binding protein is acquired that links the α-adrenergic receptor to the sodium-potassium pump, increasing MDP and thus causing a corresponding decrease in automaticity.[179,180] Two separate subtypes of α_1-adrenergic receptors appear to mediate the positive and negative chronotropic responses. One subtype is antagonized by an analogue of clonidine and is linked to the neonatal positive chronotropic response. The other subtype is linked to the adult negative chronotropic response.[181] In the neonatal rat, α_1-adrenergic activation increases the L-type calcium current, $I_{Ca\text{-}L}$, one of the currents responsible for automaticity. This α_1-adrenergic-mediated increase in calcium current is not observed in the adult heart. This suggests that there is also a developmentally determined switching of the coupling of α_1-adrenergic receptors with respect to calcium channels during development.[182]

In contrast to the cardiac electrophysiologic effects of parasympathetic stimulation, which exhibit rapid kinetics, the kinetics of sympathetic stimulation are more prolonged. The maximal effects of adrenergic stimulation on heart rate, myocardial refractoriness, and conduction tend to develop over several seconds, in

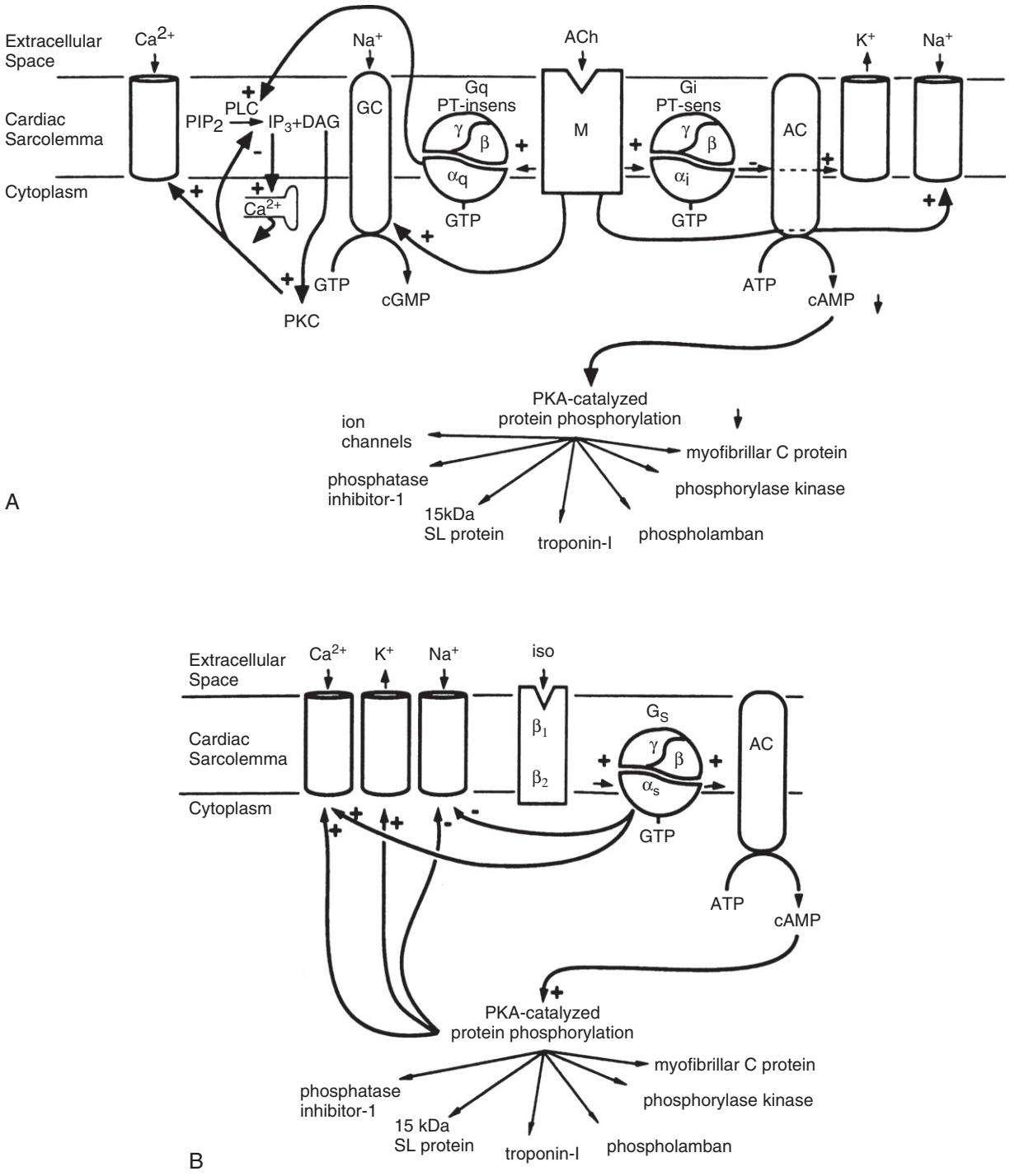


Figure 66-14 A, Schematic of cholinergic receptor-effector coupling. The muscarinic receptor is coupled to a pertussis toxin–sensitive inhibitory G protein (G_i). When activated, the α_i-subunit of the G protein dissociates and causes an inhibition of adenylate cyclase (AC) and a fall in intracellular cyclic adenosine monophosphate (cAMP) levels, with a resultant decrease in protein kinase A (PKA) phosphorylation of cellular proteins, including cardiac ion channels. It is by this mechanism that muscarinic stimulation decreases the inward calcium current. Direct activation of potassium channels by the G protein is also known to occur. There is also a pertussis toxin-insensitive G protein (G_q) that is linked to the muscarinic receptor. This G protein stimulates phospholipase C production, which stimulates the production of diacylglycerol (DAG) and 1,4,5-trisphosphate (IP_3). DAG stimulates protein kinase C (PKC), which phosphorylates and stimulates sarcoplasmic reticulum receptors, causing intracellular release of calcium. IP_3 directly stimulates release of calcium from the sarcoplasmic reticulum. **B,** Schematic of β-adrenergic receptor-effector coupling. The β -adrenergic receptor is coupled to the stimulatory trimeric G protein, G_s. In the presence of guanosine triphosphate (GTP), this G protein dissociates, and the α_s subunit results in direct activation of adenylate cyclase (AC). This increases intracellular cAMP and stimulates protein kinase A. This enhances phosphorylation of cellular proteins, including cardiac ion channels. *(From Fleming JW, Wisler PL, Wantanabe AM. Signal transduction by G proteins in cardiac tissues,* Circulation *85:420, 1992, by permission of the American Heart Association.)*

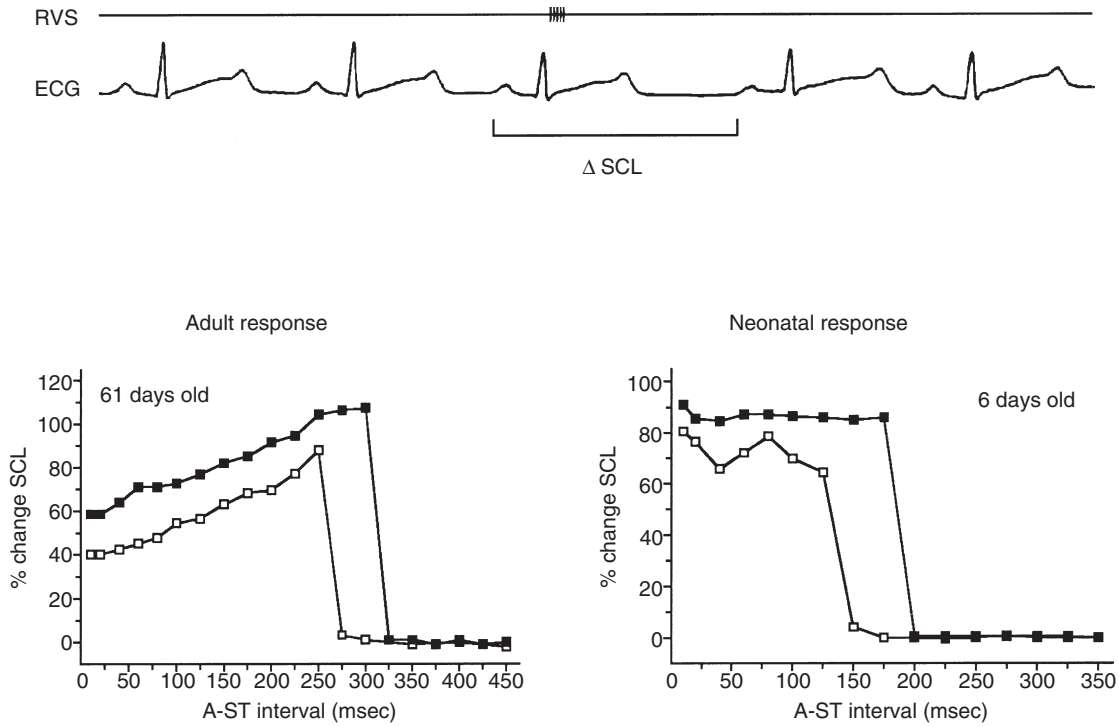


Figure 66-15 The response of heart rate to brief vagal stimulation. A short train of vagal stimulation is delivered once, at varying times, within the cardiac cycle. The effect of each brief, critically timed vagal train on sinus cycle length is determined, and a phase-response curve is generated. On the left, a typical adult-type vagal phase response curve is shown, obtained in experiments performed on a 2-month-old puppy. As the vagal train is delivered at progressively later times after the last atrial depolarization (A-ST interval), the change in sinus cycle length (percentage change of sinus cycle length [SCL]) evoked by that vagal stimulation train progressively increases. Eventually the vagal train is delivered too late in the cardiac cycle to affect sinus cycle length. On the right, a typical phase-response curve in a newborn is shown. In contrast to in the older puppy, in the neonate, the vagal response remains flat as the vagal train is advanced through the cardiac cycle. *Closed squares* indicate right vagal stimulation (RVS); *open squares* are left vagal stimulation (LVS). *(Phase response curves are from Pickoff AS, Rios R, Stolfi A, Wang SN. Pickoff AS, et al. Postnatal maturation of the response of the canine sinus node to critically timed, brief vagal stimulation,* Pediatr Res *35:55, 1994.)*

contrast to the nearly instantaneous effects of vagal stimulation. The dominant electrophysiologic actions of sympathetic stimulation in the heart are mediated by the β_1 receptor, although β_2 receptors do coexist on the cardiac myocyte. The main electrophysiologic effects of β-adrenergic stimulation include an increase in sinus node automaticity, which is in part mediated by an increase in the rate of diastolic depolarization (phase 4 of the action potential). There is also an increase in the MDP of the sinus node, which serves to increase the activity of the I_f pacemaker current. In the AV node, conduction velocities are increased and refractory periods are shortened; the increases in AV nodal action potential amplitude and $\dot{V}_{max}$ account for the increase in conduction velocity. In the myocardium, β-adrenergic stimulation increases the height of the action potential plateau by increasing the inward calcium current; it also increases the speed of repolarization by increasing outward potassium currents. Overall, these effects tend to shorten myocardial refractoriness. The increase in MDP, observed in the sinus node and in the working myocardium, is likely caused by an adrenergic-mediated increase in the activity of the sodium-potassium pump. These primary effects of β-adrenergic stimulation are mediated through a membrane-bound stimulatory guanine nucleotide binding protein, G_s (see Figure 66-14, *B*). The effects of sympathetic stimulation on the heart of the newborn and young infant are qualitatively similar to those in the adult. The degree of change in heart rate with sympathetic stimulation, however, is considerably less in the young puppy compared with the adult.[170] The increase in response to sympathetic stimulation with maturation is probably multifactorial. As was mentioned previously, the density of sympathetic innervation continues to increase in many species postnatally. Furthermore, the stimulatory effect of β-agonists on adenylate cyclase increases postnatally in many mammalian species.[183-186] In rabbit ventricular cells, it has been reported that the postnatal increase in β-adrenergic-stimulated cyclic adenosine monophosphate is more related to a reduction in inhibition of adenylate cyclase by the inhibitory G protein, G_i, with maturation, rather than to a change in the β receptor–adenylate cyclase complex.[186]

Several complex interactions between the two branches of the autonomic nervous system have been described.[187] These interactions involve complex presynaptic and postsynaptic mechanisms that allow for fine regulation of autonomic modulation of the electrophysiologic and mechanical properties of the heart. One classic interaction is the "accentuated antagonism" interaction between the sympathetic and parasympathetic nervous systems. With respect to heart rate and contractility, the effect of a given vagal stimulus becomes progressively greater as the level of background sympathetic tone increases.[187,188] At the presynaptic level, it is believed that a cholinergic inhibition of norepinephrine release from sympathetic nerve endings contributes to the antagonism of sympathetic effects. At the postsynaptic level, the precise basis for interaction between the sympathetic and parasympathetic nervous systems is still somewhat speculative but may involve a muscarinic-mediated stimulation of phosphatases (enzymes), which results in a reversal of the adrenergic-stimulated phosphorylation of key intracellular proteins, such as the calcium channel.[189] Nitric oxide, a derivative of L-arginine, has also been implicated as a mediator of the cholinergic inhibition of β-adrenergic responses.[190,191]

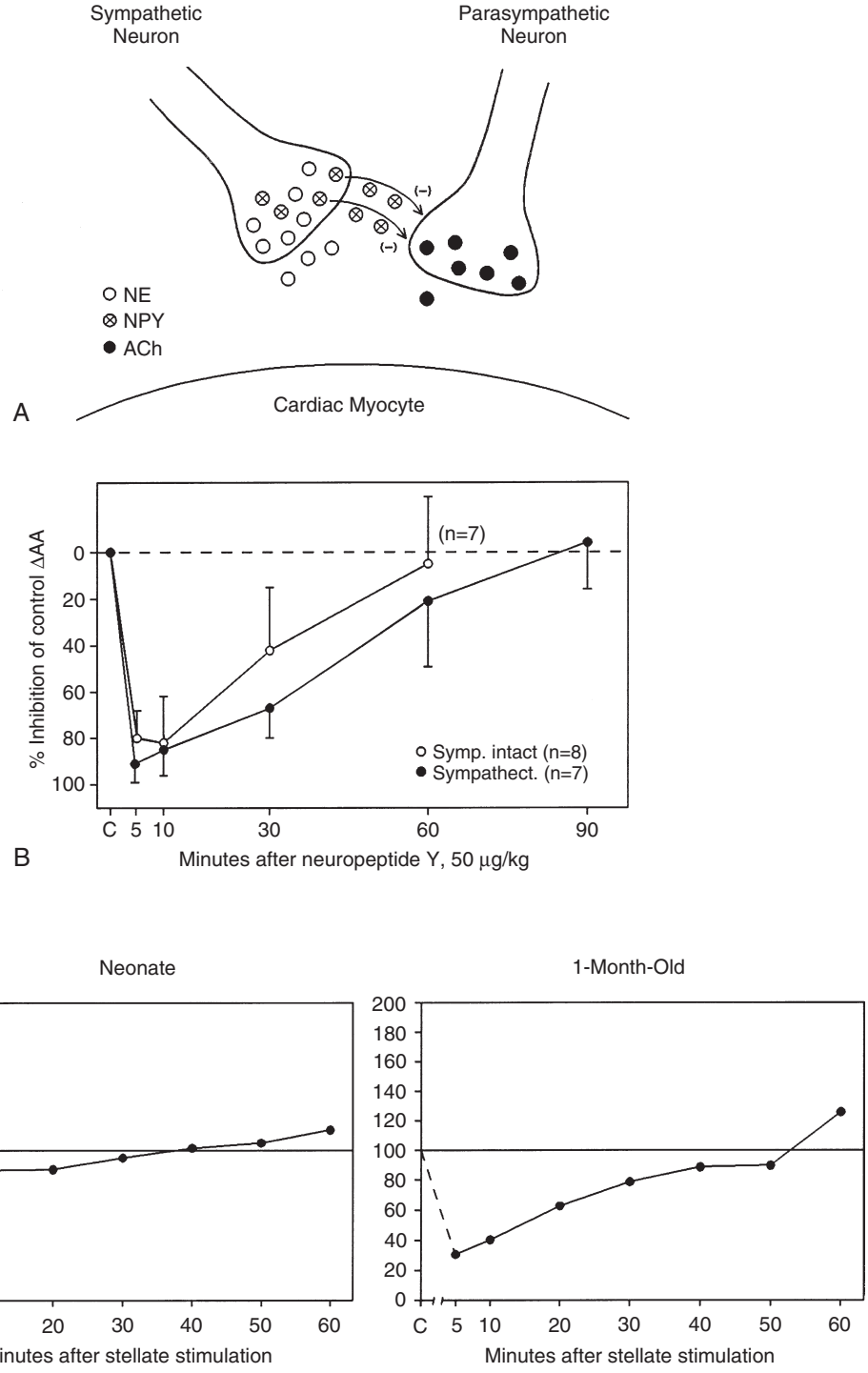


Figure 66-16 **A,** Illustration of the neuropeptide Y (NPY)-mediated sympathetic-parasympathetic interaction. NPY is stored within neuronal vesicles, along with norepinephrine (NE), in sympathetic nerve endings. When released, NPY can bind to receptors on the parasympathetic nerve terminals and inhibit the release of acetylcholine (ACh). **B,** The effects of an intravenous injection of NPY (50 μg/kg) on the vagal response of heart rate to vagal stimulation in sympathetically intact and sympathectomized newborn dogs. In both groups, NPY causes a profound (nearly 100%) and long-lasting (nearly 60 minutes) inhibition of the change in sinus cycle length caused by vagal stimulation (percentage inhibition of control ΔAA). C, control (pre-NPY administration). **C,** The effects of right stellate ganglion stimulation (10 Hz, 5 minutes) on cardiac vagal chronotropic responses in young puppies. In these graphs, 100% represents the control (i.e., prestellate stimulation) vagal response. In the neonate *(left),* stellate stimulation causes little change in the magnitude of elicited vagal responses. In contrast, in the 1-month-old puppy *(right),* 5 minutes of stellate stimulation causes a profound, long-lasting inhibition of the cardiac vagal response. This occurs, presumably, as a result of the release of NPY during stellate stimulation. *(***A** *and* **C,** *From Rios R, Stolfi A, Campbell PH, Pickoff AS.* Cardiovasc Res *31:E96, 1996, with permission of Elsevier Science-NL.* **B,** *From Yamasaki S, Stolfi A, Mas MS et al. Rapid attenuation ("fade") of the chronotropic response during vagal stimulation in the canine newborn: Evidence for a prominent neuropeptide Y effect,* Circ Res *69:406, 1991, by permission of the American Heart Association.)*

There have been few studies of interactions between the two branches of the autonomic nervous system during development. In isolated canine Purkinje fibers, Moak and colleagues[192] reported that acetylcholine attenuated the isoproterenol-induced shortening of action potential duration in adult cells. In neonatal (i.e., 1 to 10 days old) Purkinje cells, in contrast, action potential duration lengthened after exposure to isoproterenol. Furthermore, this effect was not modified by exposure to acetylcholine. This was interpreted to indicate that accentuated antagonism is not present in the neonate. In somewhat older (e.g., 35 days) animals, however, prominent sympathetic-vagal interactions in the control of heart rate can be demonstrated, with sympathetic effects becoming more attenuated with higher levels of background vagal stimulation.[193] With respect to AV nodal conduction, an interaction between the sympathetic and parasympathetic nervous systems has been demonstrated in young puppies, but one with dominance of the sympathetic, not parasympathetic, nervous system.[194] Recent studies of beat-to-beat variability in AV nodal conduction in the premature infant during the first postnatal month (measured as variability in the PR interval of the surface electrocardiogram) also provide evidence for accentuated sympathetic modulation of AV nodal conduction.[167] Further studies characterizing interactions between the branches of the autonomic nervous system in the developing heart are needed.

It is known that in addition to the classic autonomic neurotransmitters, acetylcholine and norepinephrine, a family of "neuropeptides" also appears to innervate the human heart. These peptides include neuropeptide Y, vasoactive intestinal peptide, calcitonin gene–related peptide, somatostatin, and substance P.[195] These neuropeptides exist, either colocalized with the classic neurotransmitters within neurons of the sympathetic or parasympathetic nervous system or confined to neurons that appear to be separate from the two major branches of the autonomic nervous system. In the fetal heart, peptide immunoreactive nerves first appear at about 10 weeks gestation, or 3 weeks after the appearance of cardiac ganglia and nerves.[196] Neuropeptide Y–containing nerves are the dominant peptide-containing nerves in the heart.[197] In the early fetal heart, neuropeptide Y is initially localized within the atria. With further development, innervation of the ventricles becomes evident. Vasoactive intestinal peptide and somatostatin appear localized primarily within the atrium and are first observed at 10 to 12 weeks gestation. Substance P and calcitonin gene–related peptide do not appear in the fetal heart until somewhat later, at 18 to 24 weeks of gestation. In the newborn canine heart, neuropeptide Y and vasoactive intestinal peptide are both found in higher concentrations in the atria than ventricles, and for both peptides, concentrations in the first weeks after birth are higher than in the adult.[198]

Although the precise function and mechanisms of action of this peptidergic neurotransmission system remain to be fully elucidated, it is clear that at least two of these peptides, vasoactive intestinal peptide and neuropeptide Y, may exert significant direct or indirect (or combined) effects on cardiac automaticity and conduction. Vasoactive intestinal peptide can be shown to increase sinus node automaticity directly and to enhance AV nodal conduction in both the adult dog[199,200] and the newborn.[201] In the adult dog, vasoactive intestinal peptide shortens atrial refractoriness,[200] an effect not observed in the neonatal atrium.[201] Vasoactive intestinal peptide may be responsible for the acceleration in heart rate observed with vagal stimulation in the presence of autonomic blockade.[202] Neuropeptide Y appears to function as a sympathetic cotransmitter, being localized with norepinephrine within adrenergic neurons.[203,204] Neuropeptide Y release from sympathetic nerves inhibits release of acetylcholine from adjacent parasympathetic nerve endings, thus representing another type of autonomic sympathetic-parasympathetic interaction (Figure 66-16, *A*). This interaction explains the profound and prolonged attenuation of vagal responses that is observed after a period of antecedent stellate ganglion stimulation in the adult dog.[205] In the neonatal dog, intravenous neuropeptide Y causes a profound and long-lasting inhibition of cardiac vagal response (see Figure 66-16, *B*).[206] Stellate stimulation, however, causes only a small inhibition of cardiac vagal response in the neonate, suggesting that the neuropeptide Y–sympathetic–parasympathetic autonomic interaction is immature at birth. The magnitude of the neuropeptide Y–autonomic interaction increases dramatically within the first postnatal month, probably as a result of the general postnatal maturation of adrenergic innervation (see Figure 66-16, *C*).[207] Further research is warranted to elucidate the role of peptidergic transmission in the modulation of cardiac electrophysiology throughout development.

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67

Marlene Rabinovitch

Developmental Biology of the Pulmonary Vasculature

This chapter discusses the fetal, neonatal, and postnatal changing morphology of the developing pulmonary vascular bed. Studies have focused on structural and functional alterations in endothelial cells during postnatal development and have addressed the phenotypic heterogeneity of the vascular smooth muscle cells in the perinatal period.There are new insights into mechanisms regulating endothelial migration and angiogenesis, smooth muscle cell proliferation, hypertrophy, and migration.These studies have also provided novel therapeutic targets whereby progression of pulmonary vascular disease may be retarded or prevented and regression induced.

MORPHOLOGY OF THE DEVELOPING PULMONARY CIRCULATION IN THE FETUS

Early embryologic studies have shown primitive pulmonary vessels from the sixth aortic arch in the 5-week human embryo. These vessels supply the upper poles of the right and left lung. The lower poles are supplied by a pair of intersegmental arteries arising from the dorsal aorta that penetrate upward through the diaphragm.At this stage the developing lung parenchymal blood vessels are largely localized to the interlobular septa. Between 5 and 8 weeks gestation it is likely that numerous paired dorsal intersegmental arteries supply the emerging blood vessels that are developing alongside the branching bronchi in the lung parenchyma. As the true central pulmonary arteries form from the aortopulmonary trunk and anastomose with the intrapulmonary arteries, the primitive pulmonary arteries arising from the aorta as well as the primitive intersegmental arteries involute. By 9 weeks gestation the bronchial system has formed. With each airway generation is an accompanying artery, in addition to numerous supernumerary arteries that are also produced. By 16 weeks gestation the number of preacinar airways and accompanying arteries is complete. Thereafter, as the acini develop (terminal bronchioli, respiratory bronchioli, alveolar ducts, and alveoli), so do the accompanying and supernumerary arteries.[1,2]

Abnormal maturation or maturational arrest in pulmonary arterial development is reflected in functional derangement that can appear in the newborn period. For example, persistent pulmonary hypertension of the neonate has been reported in association with pulmonary arterial maturational arrest at the fifth week gestation (Figure 67-1).[3] Infants with pulmonary atresia and ventricular septal defect frequently have persistence of the intersegmental arteries (aortopulmonary collaterals) (Figure 67-2).[4] These vessels can serve as the sole source of blood supply to a lobe or lobar segment.Alternatively, a dual circulation with intersegmental arteries anastomosing to arteries can be traced back to a central pulmonary artery origin. In these infants there can

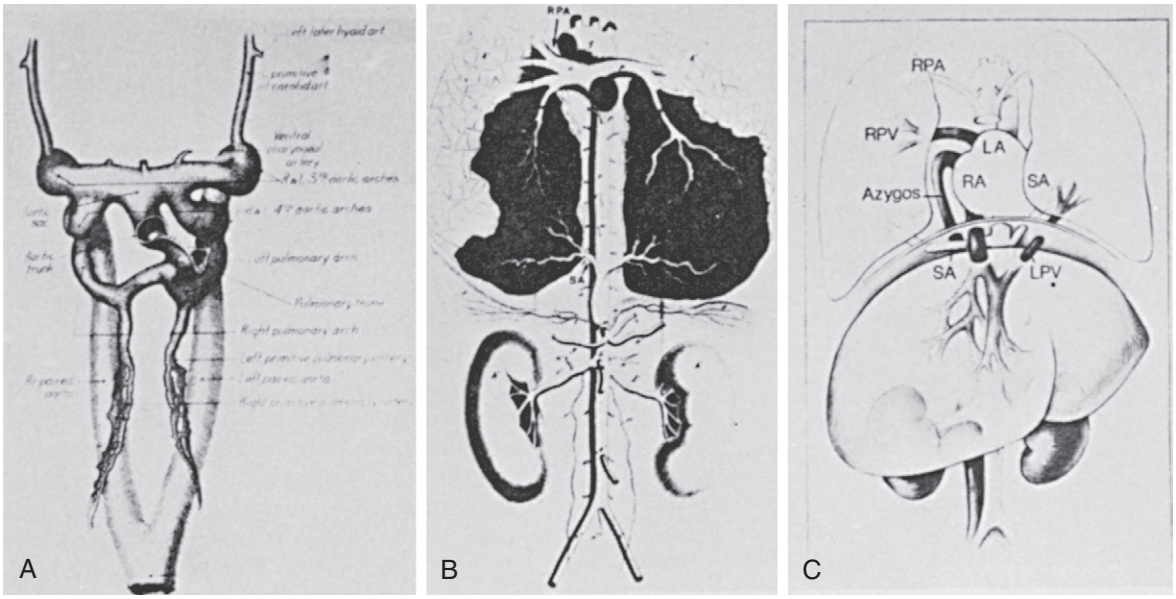


Figure 67-1 A, Early embryologic development shows normal disparity between the large main pulmonary artery and narrow right and left branch pulmonary arteries at 5 weeks. **B,** Only the arterial system is shown.The bilateral systemic arteries arising from a single trunk supply the lower lobes. **C,** An infant with persistent pulmonary hypertension and developmental arrest at 5 weeks gestation. *LA,* Left atrium; *LPV,* left pulmonary vein; *RA,* right atrium; *RPA,* right pulmonary artery; *RPV,* right pulmonary vein; *SA,* systemic arteries. *(***A,** *From Congdon ED:Transformation of the aortic-arch system during the development of the human embryo,* Contrib Embryol *14:47, 1922;* **B,** *from Maugars A:* J Med Chir Pharm Paris *3:453, 1802;* **C,** *reprinted by permission of the publisher from Goldstein JD, Rabinovitch M, Van Praagh R, et al: Unusual vascular anomalies causing persistent pulmonary hypertension in a newborn,* Am J Cardiol *43(5):962, 1979. Copyright 1979 by Excerpta Medica, Inc.)*

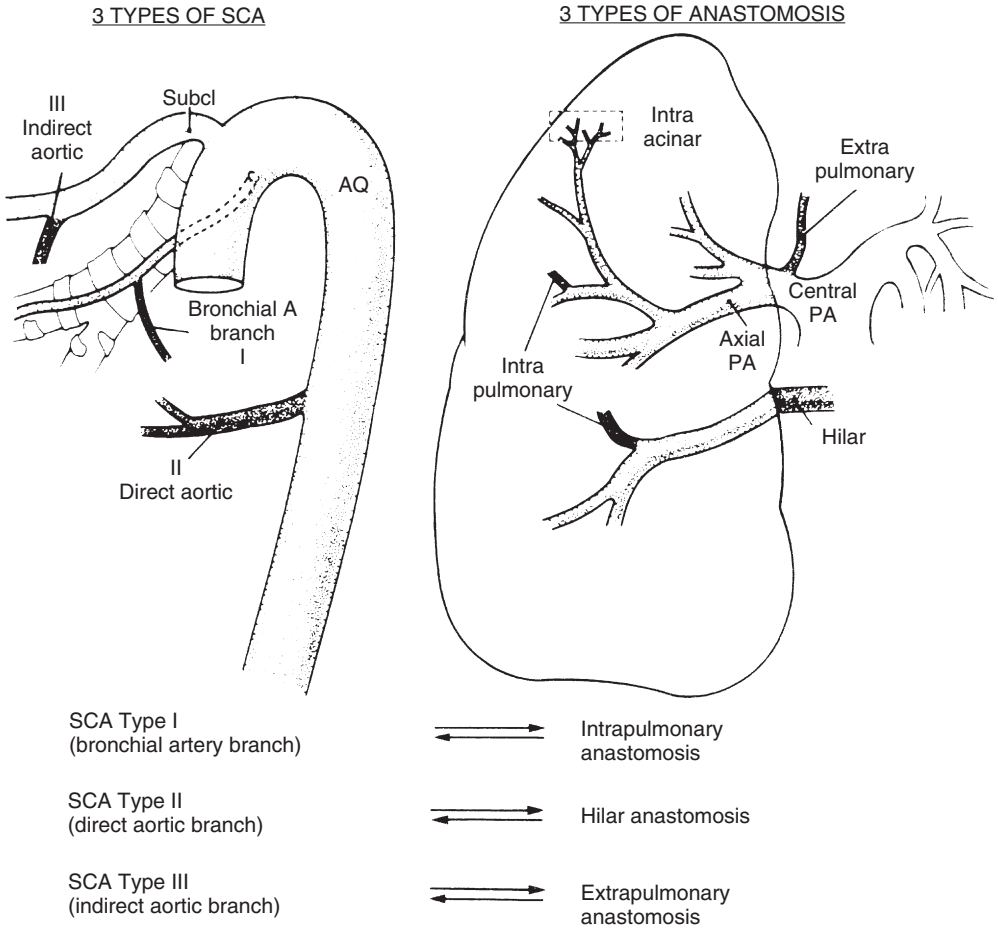


Figure 67-2 The three types of systemic collateral artery (SCA) and the three types of anastomosis with the pulmonary artery (PA). The characteristic pattern of anastomosis for each type of SCA is given. *AO,* Aorta; *SUBCL,* subclavian. *(From Rabinovitch M, Herrera-deLeon V, Castaneda AR, et al: Growth and development of the pulmonary vascular bed in patients with tetralogy of Fallot with or without pulmonary atresia,* Circulation *64(6):1234, 1981.)*

also be multiple indirect aortopulmonary collaterals arising from branches of aortic branches (e.g., from subclavian, intercostals, and coronary arteries). Because these indirect collaterals can be observed in the absence of direct collaterals, they probably represent the sequelae of pulmonary atresia when it occurs later in development. Finally, in some cases of pulmonary atresia, there is supply by anastomotic vessels, which arise from true bronchial arteries.

Another morphologic pulmonary vascular abnormality is observed with absence of the pulmonary valve.[5] In those patients who present with severe respiratory problems from birth, abnormal branching of the vessels has been observed. Tufts of intersegmental pulmonary arteries are seen arising in weeping willow or squidlike fashion, encircling and compressing the intrapulmonary airways (Figure 67-3).

Experimental evidence shows that branching morphogenesis of the large preacinar airways provides cues to regulate branching morphogenesis of the preacinar arterial tree, whereas the intra-acinar vessels regulate the development of the alveoli (this is discussed later).

Microscopic Features

The normal morphologic development of the pulmonary circulation has also been studied at the microscopic level. In the fetus, the preacinar arteries and those at the terminal bronchiolus level are muscular, whereas the intra-acinar arteries (i.e., those accompanying respiratory bronchioli) are partially muscular (surrounded by a spiral of muscle) or nonmuscular. Arteries at alveolar duct and alveolar wall levels are nonmuscular. The preacinar arteries are thick walled and change little in wall thickness relative to external diameter throughout the fetal period. Experimental studies[6] suggest that the immediate postnatal period is characterized by rapid recruitment of small alveolar duct and wall vessels that appear to be functionally and structurally closed in the prenatal period. There is also progressive dilation of muscular arteries. Within a few days the smallest muscular arteries (<250 μm) dilate, and their walls thin to adult levels; by 4 months of age this process has included the largest pulmonary arteries at the hilum. As intra-acinar arteries at the various airway levels increase in external diameter, muscle is said to extend peripherally (i.e., it is observed in arteries located more peripherally within the acinus). At first nonmuscular arteries become partially muscular and later they become fully muscularized. For example, in infancy, vessels at alveolar duct level are still largely nonmuscular, but in childhood they become partially muscularized and in the adult they are fully muscularized.[7] Alveolar wall arteries remain largely nonmuscular, even in the adult.

Clinical as well as experimental studies have suggested that the muscularization of these peripheral pulmonary arteries may be related to the differentiation of pericytes as well as to the recruitment of fibroblasts.[8,9] Arteries proliferate through the neonatal period and early infancy, accompanying the proliferation of alveoli; the alveoli-arteries ratio can therefore be used

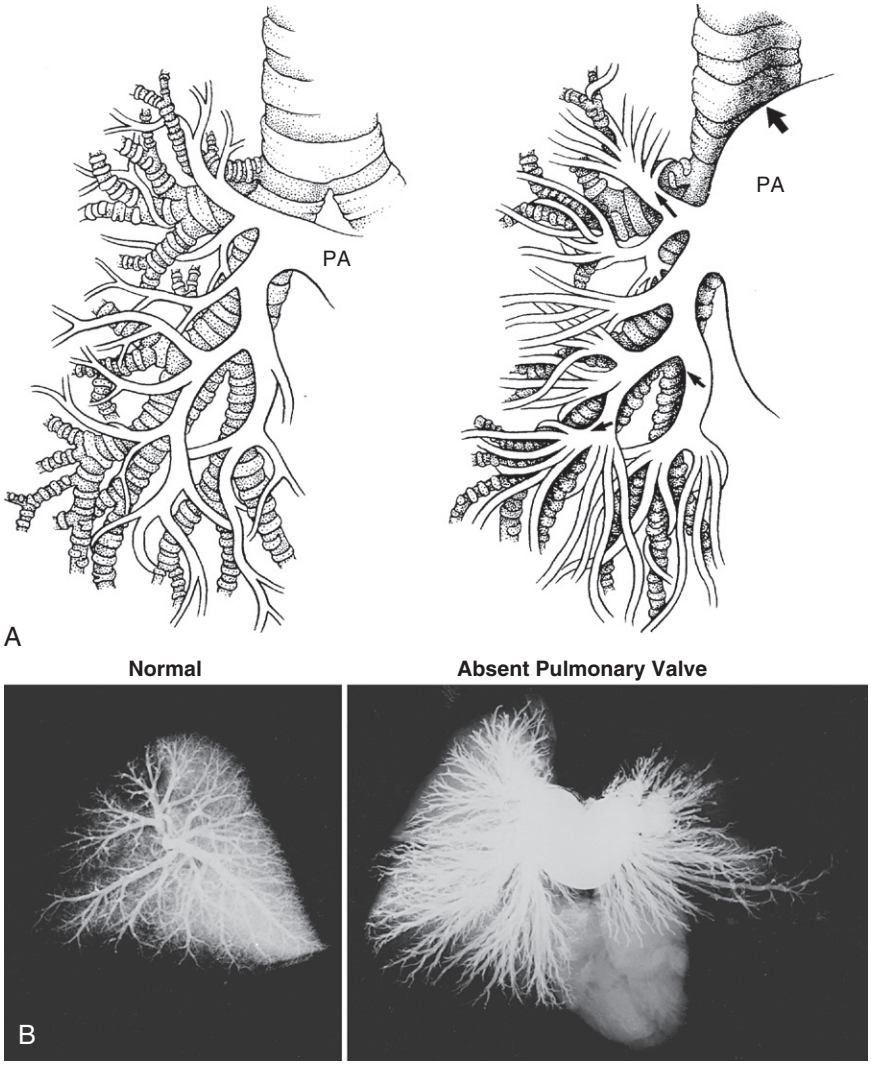


Figure 67-3 A, Diagrammatic representation of normal pulmonary artery (PA) branching and the abnormal pattern seen in cases of absent pulmonary valve syndrome—tufts of vessels emerging at the segmental artery level that entwine the bronchi. *Arrows* denote large right pulmonary artery compressing right mainstem bronchus. **B,** Postmortem arteriograms from a 4-day-old normal infant *(left)* and from a 4-day-old patient with absent pulmonary valve syndrome associated with a ventricular septal defect and D-transposition of the great arteries *(right),* showing tufts of vessels in both lungs. *(Reprinted by permission of the publisher from Rabinovitch M, Grady S, David I, et al: Compression of intrapulmonary bronchi by abnormally branching pulmonary arteries associated with absent pulmonary valves,* Am J Cardiol *50(4):804, 1982. Copyright 1982 by Excerpta Medica, Inc.)*

as a measure of numerical arterial growth. The alveoli-arteries ratio actually decreases from the newborn value of 20:1 to the value of 8:1, which is achieved first in early childhood and persists (Figure 67-4). The growth and development of the pulmonary circulation are also likely influenced by the trophic effects of neuropeptides[10] released from nerve endings, as well as from neuroendocrine bodies[11] associated with accompanying airways (Figure 67-5).

Experimental studies have indicated how changes in connective tissue, especially elastin and collagen,[12,13] and cellular arrangement[14] govern the normal adaptation to postnatal life. The endothelial cells begin to flatten because of increased deposition of elastin in peripheral arteries, forming an intact elastic lamina. In the central pulmonary arteries similar changes occur, and elastin remodeling is prominent in the subendothelium in the early neonatal period,[13] whereas elastin synthesis, as judged by messenger RNA (mRNA) levels, appears to be less prominent in the outer media and adventitia (Figure 67-6). It has also been shown experimentally that there are smooth muscle cells with differing proliferative potentials and that, in the neonatal period, the specialized functions of selective subgroups of smooth muscle cells, characterized by expression of cytoskeletal proteins, become even better defined.[15] The metavinculin-positive smooth muscle cells are relatively resistant to proliferation. Moreover, the high proliferative potential demonstrated in neonatal bovine pulmonary artery smooth muscle cells is reflective of a difference in activation of protein kinase C.[16]

Cellular Mechanisms

The cellular and molecular mechanisms that regulate fetal growth and development of the vasculature are being studied by a number of groups,[17] with extensive investigation into the expression in early vascular development of cell adhesion molecules, such as vascular cell adhesion molecule 1 (V-CAM-1)[18] and platelet cell adhesion molecule (PECAM)[19] (Figure 67-7) in addition to the β_1[20] and β_3 family of integrin receptors, especially $\alpha_v\beta b_3$.[21] These molecules appear to direct interactions between endothelial and

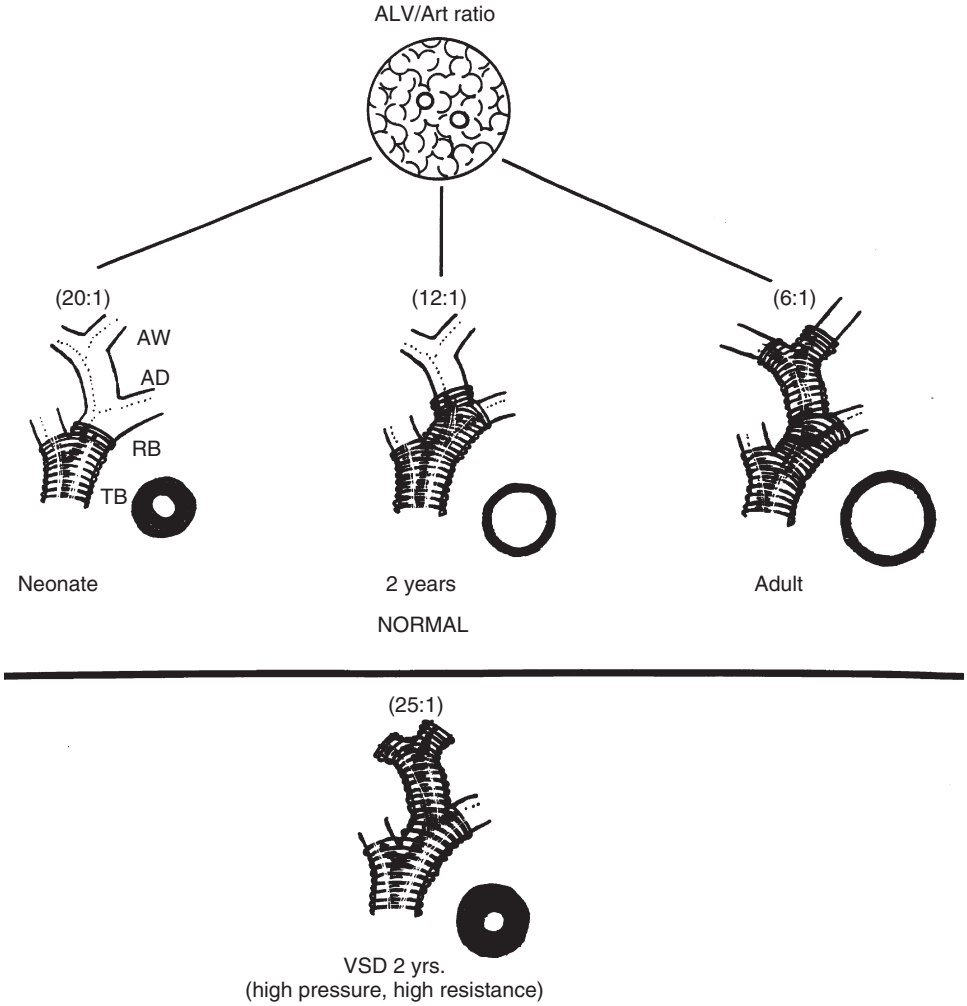


Figure 67-4 Schema showing peripheral pulmonary arterial development through morphometric changes: extension of muscle into peripheral arteries, percent wall thickness, and artery number (alveolar/arterial ratio), as they relate to age. *Upper panel*, Normal development. *Bottom panel*, Abnormalities in all three features in a 2-year-old child with a hypertensive ventricular septal defect (VSD). *ALV/Art,* Alveolar-arterial; *AD,* artery accompanying an alveolar duct; *AW,* artery accompanying an alveolar wall; *RB,* artery accompanying a respiratory bronchiolus; *TB,* artery accompanying a terminal bronchiolus. *(From Rabinovitch M, Haworth SG, Castaneda AR, et al: Lung biopsy in congenital heart disease: a morphometric approach to pulmonary vascular disease,* Circulation *58:1107, 1978.)*

smooth muscle cells, and the extracellular matrix and perturbations of these interactions lead to malformation of the pulmonary and systemic arteries. The β_1 family of integrins bind fibronectin; the β_3 family binds fibronectin as well as a host of matrix molecules, but especially tenascin, osteopontin, and vitronectin, some of which have been shown to govern vascular cell migration (e.g., fibronectin)[22-25] and proliferation (e.g., tenascin).[26] In addition, a variety of growth factors also appear to be responsible for the orderly growth and branching morphogenesis of blood vessels. These include vascular endothelial growth factor (VEGF),[27] particularly VEGF A and D,[28] and angiopoietin,[29] as well as acidic and basic fibroblast growth factor (FGF-1 and FGF-2).[30] Evidence shows that VEGF production by epithelial cells is necessary for angiogenesis but that blockade of VEGF A induction of angiogenesis represses epithelial proliferation, suggesting that blood vessel formation regulates alveolar formation.[31-33] More studies have characterized the endothelial contribution to alveolar septae as being regulated by hepatocyte growth factor[34] (Figure 67-8). In addition, endothelial nitric oxide synthase (eNOS) also appears to regulate distal vascular and alveolar growth based on studies in transgenic mice with eNOS deleted.[35]

It has also been suggested that growth factors promote the induction of stromal derived factor-1,[36] a chemokine for endothelial cells bearing the receptor CXCR4, which is also induced. The functions and interactions between members of the Wingless family (Wnts) and bone morphogenetic proteins (BMPs) in regulating lung branching morphogenesis have been studied by a number of groups.[37-40] Our work in endothelial and smooth muscle cells indicates that signaling interactions downstream of the receptors for these ligands may be critical in regulating endothelial and smooth muscle cell survival, proliferation, and migration. Wnt7b,[41] has specifically been implicated in vascular development, particularly those features that regulate investment of smooth muscle cells around the endothelial framework (Figure 67-9).

Growth factors are regulated in their function by specific tyrosine kinase receptors, such as flt-1 and flk-1, tek, and tie,[42-44] as well as the activin receptor–like kinase (Alk)-1[45] and bone morphogenetic protein receptors. Alk-1 has been implicated in the later stages of vasculogenesis, and a mutation is associated with hereditary hemorrhagic telangiectasia,[46] whereas bone morphogenetic protein receptor (BMPR) II mutations are found in primary pulmonary hypertension.[47] Using a flk-1 reporter construct,

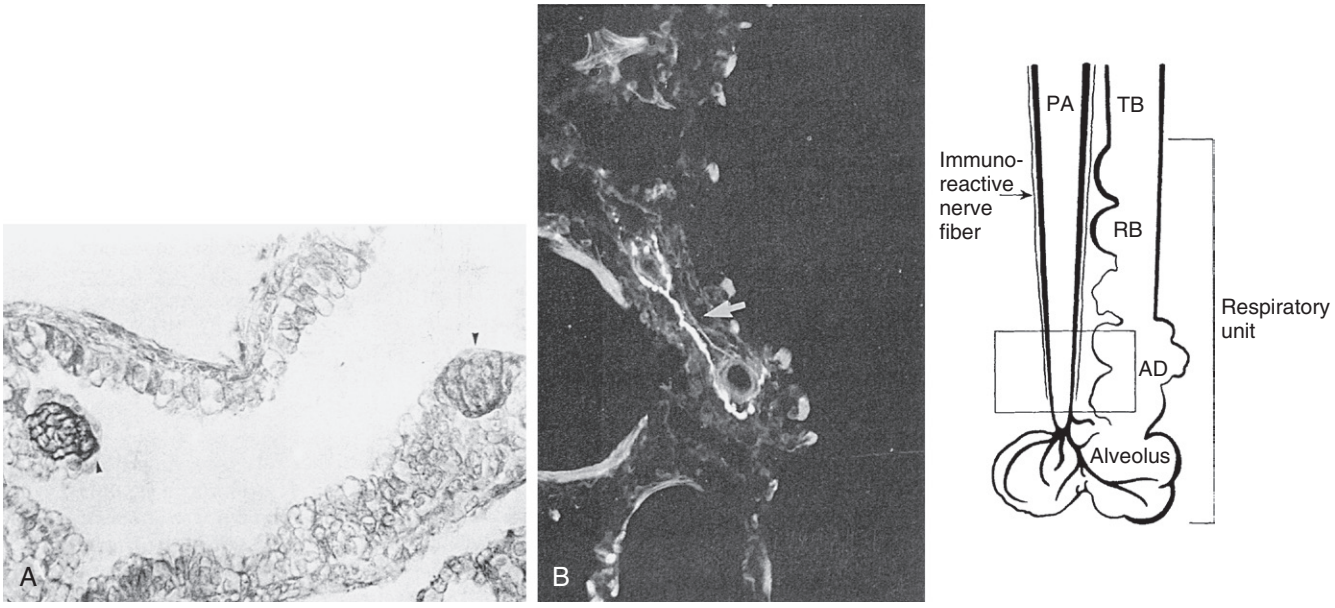


Figure 67-5 **A,** Neuroepithelial bodies *(arrowheads)* are seen as dark-staining regions (immunoreactive for serotonin) in the airway of a newborn infant. **B,** Tyrosine hydroxylase immunoreactive perivascular nerve fibers *(arrow)* at the adventitial-medial border of an alveolar duct artery in a child age 2.5 years. Diagram on the *right* shows terminal bronchiolus *(TB)* and airways of respiratory unit accompanied by an innervated pulmonary artery *(PA). AD,* Alveolar duct; *RB,* respiratory bronchiolus; square indicates area shown in **B.** *(***A,** *From Rabinovitch M: Pathophysiology of pulmonary hypertension. In Emmanouilides GC, et al [eds]:* Moss and Adams' Heart Disease in Infants, Children, and Adolescents Including the Fetus and Young Adult. *Baltimore, Williams & Wilkins, 1995, pp 1659-1695. Original supplied by E. Cutz, Hospital for Sick Children;* **B,** *from Allen KM, Wharton J, Polak JM, et al: A study of nerves containing peptides in the pulmonary vasculature of healthy infants and children and of those with pulmonary hypertension,* Br Heart J *62:353, 1989.)*

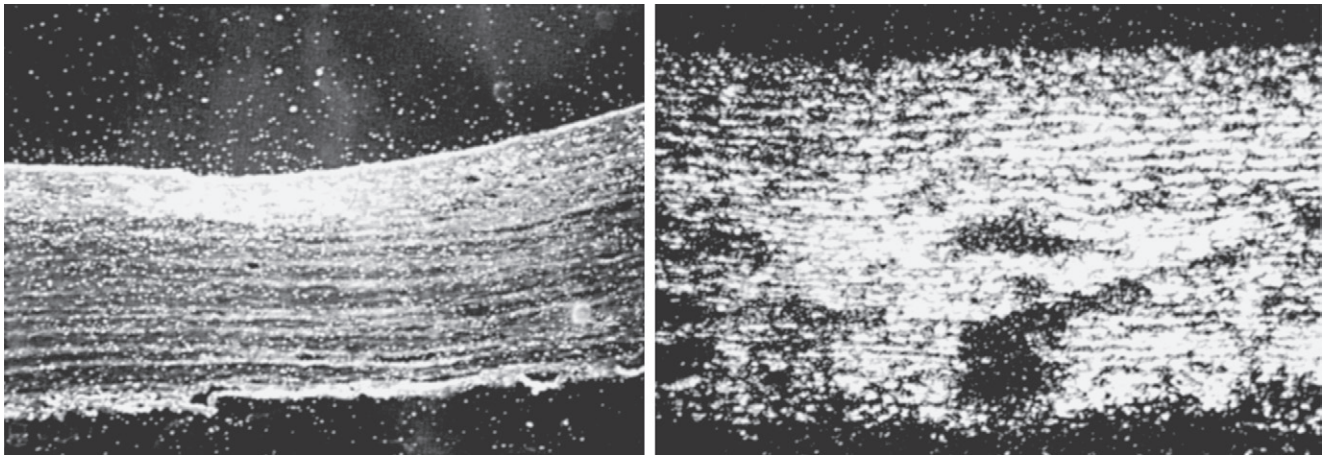


Figure 67-6 In situ hybridization localization of tropoelastin messenger RNA (mRNA) in control and hypertensive vessels from neonatal calves. White staining over areas indicates tropoelastin mRNA labeling. In normotensive vessels *(left),* labeled cells (^{35}S-labeled T66-T7) were confined to the inner media. Minimal signal is noted in the outer vessel wall. In vessels from hypertensive animals (14 days of hypoxia) *(right),* intense autoradiographic signal was observed throughout the media, albeit in a patchy distribution. *(From Prosser IW, Stenmark KR, Suthar M, et al: Regional heterogeneity of elastin and collagen gene expression in intralobar arteries in response to hypoxic pulmonary hypertension as demonstrated by in situ hybridization,* Am J Pathol *135:1073, 1989.)*

Schachtner and colleagues[48] found lung vascular development at all stages of lung growth. Notch and Jagged1 interaction are associated with early stages of lung vascular development.[49] The transcription factors TAL1[50] and Foxp,[51] are implicated in vasculogenesis from precursor cells of the hematopoietic lineage.

Matrix components (especially elastin and collagen) are regulated by insulin-like growth factor-1[52,53] and transforming growth factor-β (TGF-β).[54] Insulin-like growth factor-1 and its receptor act in concert with VEGF to stimulate fetal vascular growth through the same intracellular signaling pathways.[53] Lack of elastin leads to extensive proliferation of smooth muscle cells in pulmonary arteries. The regulation of growth factor interaction with cell surface molecules is also determined by the balance between proteases and antiproteinases. Plasmin, thrombin,[55,56] and elastases, including leukocyte and endogenous vascular elastase,[57] have been shown to release growth factors from storage

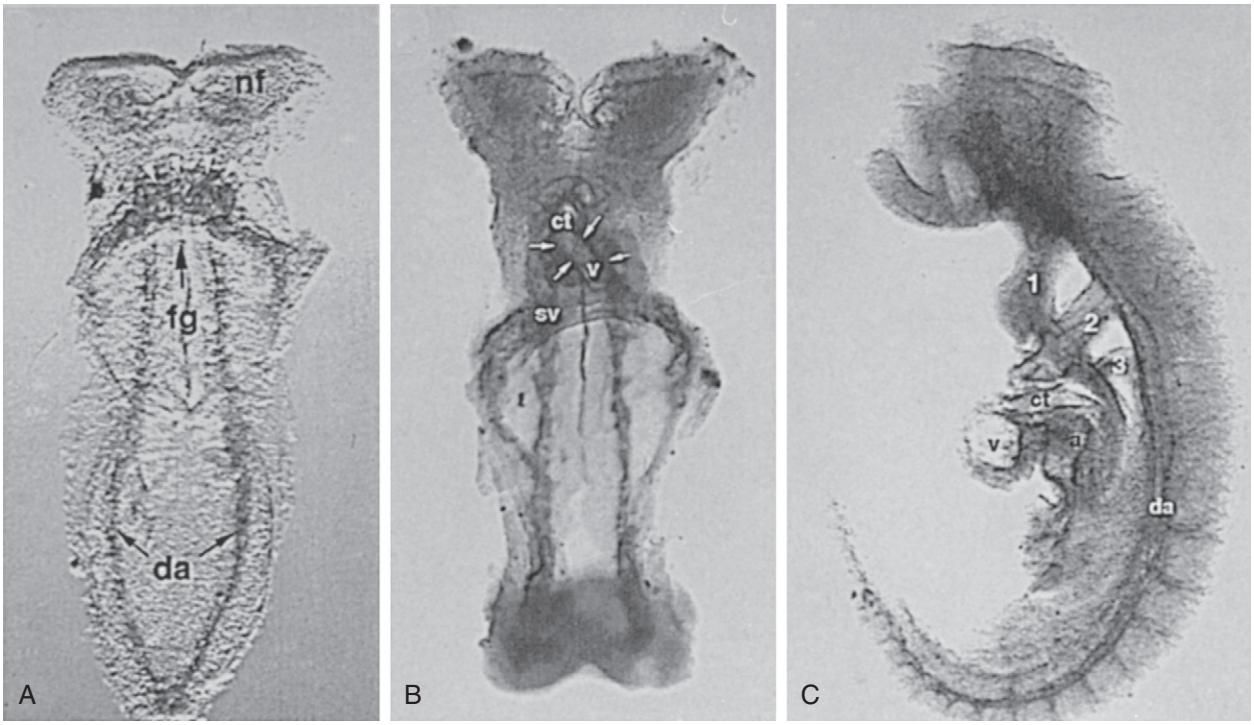


Figure 67-7 Early vascular development in the mouse embryo as defined by whole-mount in situ hybridization with platelet cell adhesion molecule (PECAM)-1 (CD31) riboprobes. **A,** Day 7.5 mouse embryo (four somites) showing an organized bilateral dorsal aorta *(da)* with initial formation of a vascular plexus in the cardiogenic crescent just cranial to the developing foregut *(fg).* **B,** Sequential organization of the endocardial cell of the vascular plexus *(arrows)* in the 8.5-day embryo (six to eight somites) into a lumen forming the sinus *(sv),* ventricle *(v),* and conotruncus *(ct).* **C,** Definitive organization of endothelial cells into the vascular template for the embryo, including clearly defined endocardium of the atrium *(a),* ventricle *(v),* and conotruncus *(ct)* as well as the pharyngeal arches 1 to 3 and dorsal aorta *(da). (From Baldwin HS: Early embryonic vascular development,* Cardiovasc Res *31:E34, 1996, with permission of Elsevier Science-NL.)*

sites in the extracellular matrix in an active form. In addition, a variety of endogenously expressed inhibitors of proteinases and elastases, such as plasminogen activator inhibitor[58] and elafin,[59] control growth and development, and other classes of molecules are known to control angiogenesis, such as angiostatin, a molecule derived from plasminogen.[60]

The expression of vasoactive peptides in the lung during development also may play a role in the development of the pulmonary vasculature. Endothelin has been associated with cell proliferation and NO with the suppression of smooth muscle cell growth,[61] and the early expression of NOS,[62] and it likely regulates vasculogenesis in addition to vascular tone.

ABNORMAL LUNG GROWTH

Underdevelopment of the lung parenchyma and associated pulmonary vasculature[1] is associated with congenital diaphragmatic hernia,[63] hypoplastic or dysplastic lungs, scimitar syndrome, and oligohydramnios secondary to renal agenesis and dysplasia. Pulmonary hypoplasia is also a feature of prematurity, absence of the phrenic nerve, asphyxiating thoracic dystrophy, rhesus isoimmunization, and, experimentally, amniocentesis[64] and smoking.[65]

Reversible or irreversible pulmonary artery hypertension and right-to-left shunting from birth can be a direct result of derangement or hypoplasia of the pulmonary vascular bed, or both. In addition to the structural changes in the vessels, heightened pulmonary vascular resistance also can be attributed to impaired gas exchange (hypoxia, hypercapnia). In infants with congenital diaphragmatic hernia, reversal of pulmonary hypertension has been achieved with the use of vasodilators,[66] extracorporeal membrane oxygenation (ECMO) (or high-frequency oscillation),[67] and NO alone or in combination with phosphodiesterase inhibitors.[68] Infants with hypoplastic lungs that had been refractory to nitric oxide before ECMO have demonstrated a beneficial effect from NO after ECMO. The expectation is that the reduction in pulmonary artery resistance will stimulate regression of vascular changes and maturation in growth of the pulmonary arteries.

Although the degree of arterial muscularity may predict whether the pulmonary vascular bed is reactive, in studies of infants who succumbed after attempt at repair of congenital diaphragmatic hernia, a striking decrease in the number of alveoli and associated arteries was the major determinant of mortality (Figure 67-10). This is probably why attempts to reverse severe pulmonary hypertension with NO have been less successful in this group of patients. Studies in experimental models of diaphragmatic hernia in lambs have indicated that endothelin A receptor blockade and endothelin B receptor–stimulated might reverse pulmonary hypertension[69] and that soluble guanylate cyclase activity is reduced.[70] It was also shown that vitamin A might stimulate lung maturation in a nitrogen-induced model of congenital diaphragmatic hernia.[71] Inflation of the lungs in utero via occlusion of the trachea has been proposed to help mature the lung.[72,73] Platelet-derived growth factor (PDGF) when dysregulated (e.g., increased in response to hypoxia) may contribute to aberrant vascular development.[74]

Future strategies to improve lung growth may come from understanding underlying transcription factor pathways that are repressed, such as the Sonic hedgehog pathway.[75] Studies

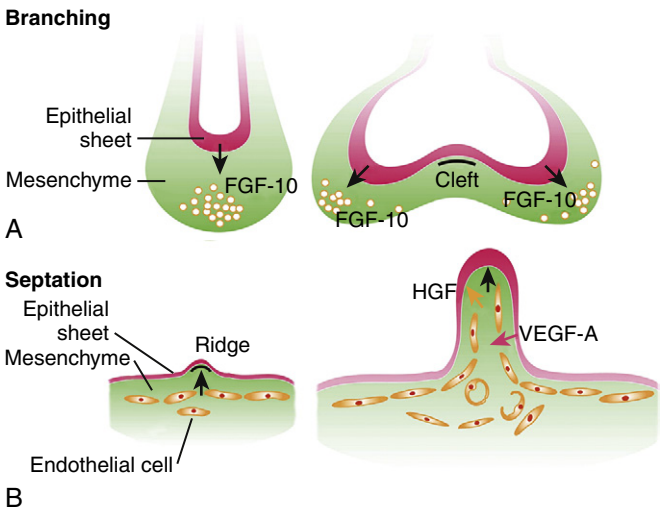


Figure 67-8 Model for septation in comparison to branching morphogenesis. **A,** During branching morphogenesis, epithelial tubes grow toward a chemoattractive source such as fibroblast growth factor (FGF)-10 in the mesenchyme. On redistribution of the chemoattractive source, the epithelial tube bifurcates to grow toward the new sources and a cleft is formed at the previous tip, causing branching. **B,** During septation, the epithelial sheet is pushed up to form a ridge or crest by underlying endothelial cells. Capillary angiogenesis stimulated by epithelium-derived vascular endothelial growth factor-A (VEGF-A) coupled with growth of epithelial cells induced by endothelium-derived paracrine factors such as hematocyte growth factor (HGF) causes formation and growth of the septae. *(From Yamamoto H, Jun Yun E, Gerber HP, et al: Epithelial-vascular cross talk mediated by VEGF-A and HGF signaling directs primary septae formation during distal lung morphogenesis,* Dev Biol *308(1):44-53, 2007.)*

have also focused on the possible contribution of resident, bone marrow–derived, or engineered stem cells.[76,77] The expansion of these cells is repressed by hyperoxia as is the expansion of endothelial progenitor cells.[78,79]

In lungs studied postmortem from infants with pulmonary hypoplasia or dysplasia, a reduced number of arteries is observed, appropriate to the reduced number of airways (see Figure 67-10). The arteries are also generally small but not incompatible with the size of the lung. Also, although the arteries, both centrally and peripherally, may be more muscular than normal, as in congenital diaphragmatic hernia, there may be hypoplasia of the pulmonary musculature, as in renal agenesis. Dysplasias of the lung associated with persistent pulmonary hypertension of the newborn (e.g., alveolar capillary dysplasia) (Figure 67-11)[80] are so refractory to treatment that a biopsy should probably be done to guide clinical management. That having been said, the pathology is similar to that seen in transgenic mice with deletion of endothelial NOS.[35] In unpublished studies Stewart and colleagues have injected progenitor cells producing eNOS into the lungs of the embryonic mice that lack eNOS with the expectation that this might lead to normal realignment of arteries, veins, and capillaries. Experimental studies carried out in newborn lambs and rabbits have shown that heparin can stimulate remodeling of the pulmonary circulation.[81] Accelerated maturation of the pulmonary circulation was achieved by inducing an increase in the number of peripheral pulmonary arteries relative to alveoli. This therapeutic strategy might prove clinically useful in inducing the growth of peripheral arteries and thereby reducing pulmonary vascular resistance.

Another factor that contributes to arrest in arterial and subsequently alveolar development is mechanical ventilation as studied in the fetal lamb[82] and more recently in the neonatal mouse.[83] In the latter study it was shown that those genes that regulate arterial and alveolar growth are suppressed by mechanical ventilation. It is interesting that dysregulaton of elastin with mechanical ventilation appears to play a critical role in impairing the formation of the distal lung vasculature and alveoli.

FAILURE TO REVERSE ELEVATED PULMONARY VASCULAR RESISTANCE

Perinatal stress (e.g., hemorrhage, hypoglycemia, aspiration, or hypoxia) may result in failure of dilation of normally muscular vessels and left ventricular dysfunction, both contributing to persistent pulmonary hypertension. The use of inhaled NO in clinical studies,[84] L-arginine in experimental studies,[85] gene transfer of endothelial NOS,[86] and inhibition of phosphodiesterases[87] have proved effective in lowering pulmonary artery pressure.

Because increased production of endothelin may underlie the pathophysiology of persistent pulmonary hypertension of the newborn,[88] the use of endothelin receptor blockade or endothelial-converting enzyme inhibition[89] may prove beneficial.[90]

INDUCTION OF VASCULAR ABNORMALITIES

In Utero

Structural studies of the lung at postmortem in fatal cases of meconium aspiration suggest that antecedent pulmonary vascular abnormalities exacerbated the postnatal pulmonary hypertension.[91] The most striking feature is the presence of muscle in arteries, small and peripheral in location and normally nonmuscular. The muscle cells are surrounded by darkly stained elastic laminae, suggesting that they formed several weeks before death and therefore in utero. Clinical studies have suggested a relationship between maternal ingestion of prostaglandin synthetase inhibitors and subsequent persistent pulmonary hypertension.[92] However, a large population of women take aspirin or indomethacin during pregnancy, and a low incidence of persistent pulmonary hypertension occurs in their newborn infants. Conversely, in the majority of infants with persistent pulmonary hypertension, no history of maternal ingestion of these compounds can be documented. Experimental studies in lambs have shown that prostaglandin synthetase inhibitors constrict the ductus arteriosus in utero. Chronic indomethacin treatment in pregnant rats produces structural changes in the pulmonary vascular bed of the newborn.[93] Thus it seems likely that, in an occasional susceptible human fetus, there may be a relationship between prostaglandin synthetase inhibitors and persistent pulmonary hypertension.

Chronic maternal hypoxemia in experimental guinea pigs has not reproduced the structural and physiologic changes of pulmonary hypertension.[94] In lambs the clinical syndrome has been produced by administration of a cytokine associated with inflammation (i.e., tumor necrosis factor-α).[95] Relatively short periods of hypoxia in the fetal lamb result in sustained elevation of pulmonary artery pressure and structural changes in the pulmonary arteries. In utero closure or constriction of the ductus arteriosus has proved to simulate the structural changes and the initial hemodynamic profile of persistent pulmonary hypertension.[96,97] An intriguing observation between increased superoxide generation and pulmonary hypertension has been established in this model,[98] suggesting a potential for treatment with superoxide dismutase.[99] This also helps explain the refractoriness of this particular group of patients to NO[100] and is in keeping with the elevated levels of soluble guanylate cyclase and phosphodiesterase.[101]

Although the mechanism is not established, changes in the regulation of production of vasodilators and vasoconstrictors may be

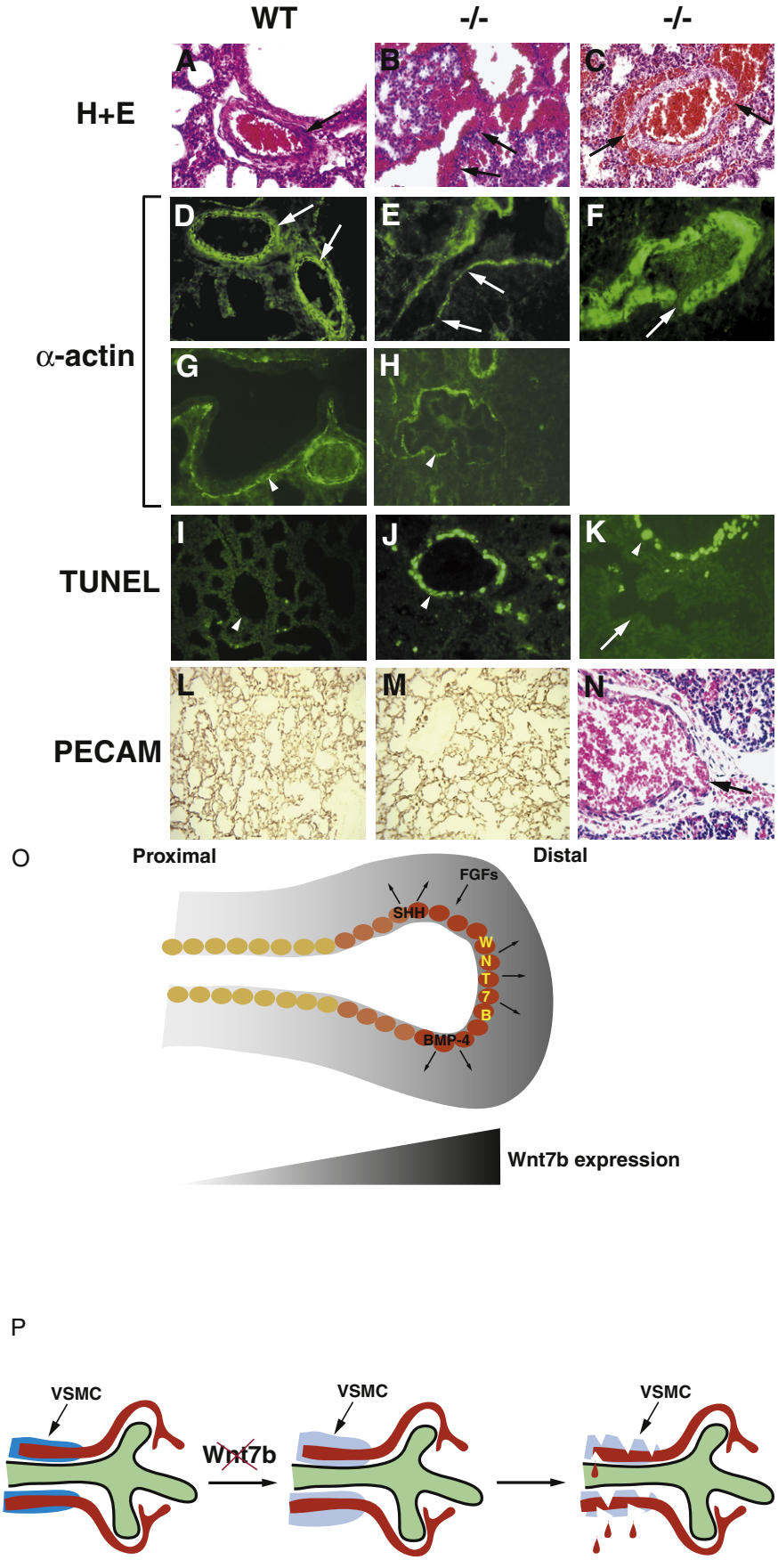


Figure 67-9 Defective smooth muscle integrity in Wnt7b$^{lacZ-/-}$ embryos and mice. Close examination of blood vessels in wild-type (**A,** *arrow*) and Wnt7b$^{lacZ-/-}$ P0 neonates (**B** and **C**) reveals several breaches in the vessel wall in Wnt7b$^{lacZ-/-}$ mice. In some instances, very little of the structure of the wall is left (**B,** *arrows*), whereas in others a thicker vessel wall with several ruptures is observed (**C,** *arrows*). Staining of sections with an antibody against smooth muscle α-actin shows robust staining surrounding blood vessels in wild-type neonates (**D,** *arrows*). Smooth muscle α-actin staining shows reduced staining, suggesting degradation of smooth muscle surrounding some vessels in Wnt7b$^{lacZ-/-}$neonates (**E,** *arrows*), whereas other vessels show frank breaches in the hypertrophic vessel wall **(F).** Bronchial smooth muscle appears normal in both wild-type (**G,** *arrowhead*) and Wnt7b$^{lacZ-/-}$neonates (**H,** *arrowhead*).TUNEL staining shows an increase in TUNEL-positive cells in the smooth muscle of the blood vessel wall in Wnt7b$^{lacZ-/-}$neonates (**J,** *arrowhead*) but not in bronchial smooth muscle (**K,** *arrow*).This is not observed in wild-type littermates (**I,** *arrow*). PECAM staining reveals a normal endothelial network in wild-type **(L)** and Wnt7b$^{lacZ-/-}$ neonates **(M).** Many large blood vessels showed rupture of the smooth muscle layer, with herniation of the intact endothelial cell layer (**N,** *arrow*). **O,** A model for the role of Wnt7b in lung development. Wnt7b is expressed at the distal tips of the airway epithelium in a pattern similar to that observed with BMP4 and overlapping that of SHH. In addition,Wnt7b is expressed in an increasing gradient from the proximal-to-distal airway epithelium. FGFs are expressed in the mesenchyme and are known to regulate epithelial branching and proliferation. However, because BMP-4 and SHH expression are unchanged in Wnt7b$^{lacZ-/-}$ embryos and Wnt7b expression is unchanged in *Shh*-null mice,Wnt7b regulates mesenchymal proliferation and differentiation through a unique pathway. **P,** Lung vasculature is composed of both endothelium *(red)* and vascular smooth muscle (VSMC, *blue*), and develops in parallel with the airways *(green)*. Loss of Wnt7b function results in defects in vascular smooth muscle differentiation and/or survival, leading to a hypertrophic response (change from *dark blue* to *light blue*), degradation of the vessel wall, and eventual rupture of the weakened vessels. *BMP,* Bone morphogenetic protein; *FGFs,* fibroblast growth factors; *H+E,* hematoxylin and eosin (stain); *PECAM,* platelet cell adhesion molecule; *TUNEL,* terminal deoxynucleotidyl transferase dUTP nick end labeling. *(From Shu W, Jiang YQ, Lu MM, et al:Wnt7b regulates mesenchymal proliferation and vascular development in the lung,* Development *129(20):4831, 2002.)*

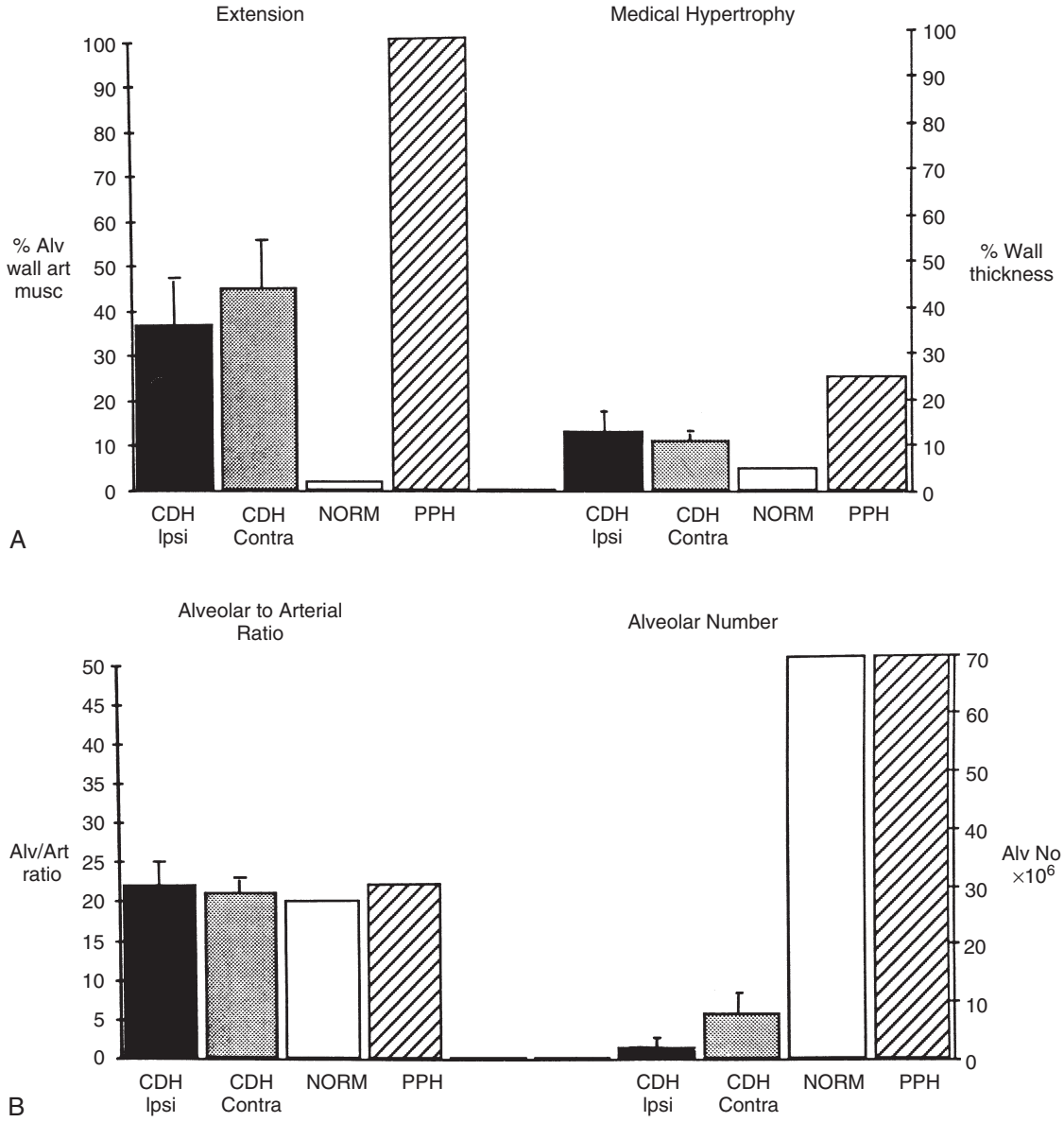


Figure 67-10 A, Morphometric data from nine patients with congenital diaphragmatic hernia (CDH), compared with published values in normal neonates and in infants with idiopathic persistent pulmonary hypertension of the newborn (PPH). Infants with CDH had greater smooth muscle extension into peripheral arteries and increased medial hypertrophy than normal infants but less than infants with PPH. **B,** Morphometric data from 6 infants with CDH compared with normal neonates and infants with PPH. Alveolar/arterial ratio was similar to that in normal infants, but total alveolar number was severely reduced in both ipsilateral and contralateral lungs. *(From Bohn D, Tamura M, Perrin D, et al: Ventilatory predictors of pulmonary hypoplasia in congenital diaphragmatic hernia, confirmed by morphologic assessment,* J Pediatr *111(3):423, 1987.)*

pertinent. That is, because NO can induce angiogenesis and endothelin may be a mitogen for smooth muscle cells, one might speculate that increased production of endothelin or reduced production of nitric oxide in utero might lead to an increase in muscularity of peripheral pulmonary arteries and a reduction in their number.

As previously discussed, smooth muscle cells with differences in potential for proliferation and for production and accumulation of extracellular matrix components may also be differentially responsive to stimuli that perturb the pulmonary vasculature. Conversely, structural remodeling of the pulmonary circulation affects the responsivity to vasodilator stimuli. For example, increased adventitial thickening, as observed in hypoxia-induced neonatal pulmonary hypertension, may prevent the access of NO to the vascular smooth muscle cells.[102] An alternative pathway that might be equally important in reducing vascular tone as well as in suppressing the propensity to vascular inflammation is the heme oxygenase pathway.[103]

ALTERED POSTNATAL PULMONARY VASCULAR DEVELOPMENT

Hypoxia, hyperoxia, toxins, or alterations in pulmonary blood flow also affect the postnatal structural development of the pulmonary vascular bed in infancy and childhood. We have shown in clinical studies that high flow and pressure induce increasing pulmonary hypertension in association with progressive vascular abnormalities.[104] First, there is extension of muscle into peripheral, normally nonmuscular arteries (morphometric grade A). This is followed by

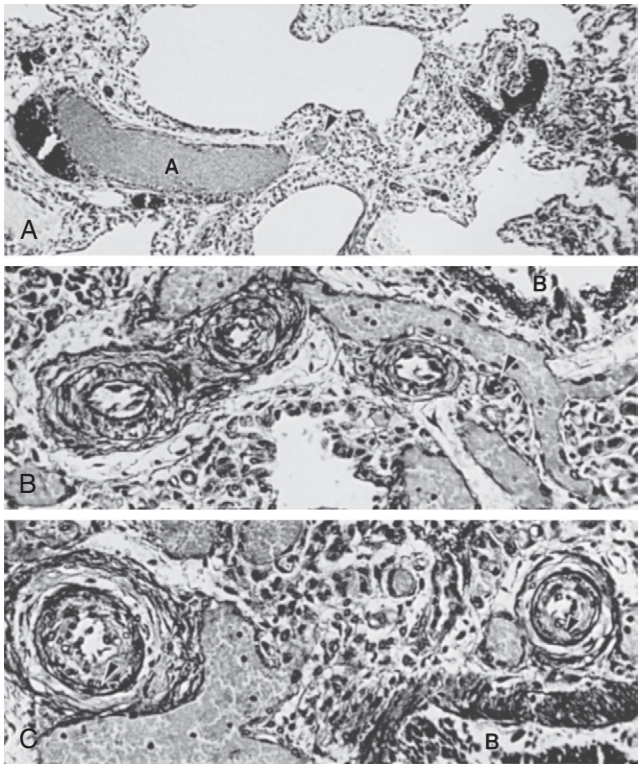


Figure 67-11 Lung micrographs from the left **(A)** and right **(B, C)** lungs of a patient with alveolar capillary dysplasia. **A,** Barium distends the lumen of a preacinar artery *(A)* but does not enter the anomalous vein to the left of the artery. Intra-acinar arterial branches that contain barium are identified *(arrowheads),* but intra-acinar veins and venules are distended with red blood cells, presumably forced ahead of the barium by the postmortem angiogram. Air spaces are lined by cuboidal epithelium, and no luminal capillaries are seen, all vessels lying centrally in the air space walls. (Hematoxylin-eosin, ×90.) **B,** Intra-acinar pulmonary arteries showing medial muscular thickening that forms a continuous layer even in the smallest branch *(arrowhead),* which is 20 μm in external diameter. Media is demarcated by external and internal elastic laminae, stained black in this elastic stain. A bronchiole is identified *(B).* (Elastic–van Gieson, ×220.) **C,** Intra-acinar arteries, the smaller measuring 60 μm in external diameter, with concentric intimal fibrosis; the latter is overlaid by *arrowheads* that mark the internal elastic laminae. The media is narrow in these branches, but note that the lumen (containing festooned endothelial cells) is the same size as in the similar-sized arteries seen in *B.* (Elastic–van Gieson, ×220.) *(From Cullinane C, Cox PN, Silver MM, et al: Persistent pulmonary hypertension of the newborn due to alveolar capillary dysplasia,* Pediatr Pathol *12:499, 1992. Reproduced with permission. All rights reserved.)*

medial hypertrophy of muscular arteries (grade B = Heath-Edwards grade I)[105] and reduced arterial concentration (morphometric grade C), associated with increased pulmonary artery pressure and resistance.[104] There is also the concomitant development of neointimal formation. This is initiated by cellular changes (Heath-Edwards grade II), which further progress to occlusive fibroproliferative lesions (grade III) and culminate in plexiform networks of obstructed and dilated vessels (grade IV). The potential for reversibility of these changes is dictated by their severity; for example, grade I-B almost always regresses following removal of the abnormal hemodynamic stimulus, whereas the loss of arteries (grade C) and the higher Heath-Edwards grades generally regress or remain functionally insignificant only if the hemodynamic insult is relieved in early infancy (first 8 months of life).[106]

To investigate the cellular mechanisms that control the pulmonary vascular abnormalities in congenital heart defects, we applied electron microscopy to the study of lung biopsy tissue. Endothelial changes were observed on scanning and transmission electron microscopy, suggesting a potential for altered interaction with circulating blood elements, such as platelets and leukocytes.[107] Functional abnormalities also were reflected in increased production of von Willebrand factor.[108,109] In addition, alterations in the subendothelium, reflected in fragmentation of elastin, suggested that an elastolytic enzyme might be stimulating the remodeling process.[107]

EXPERIMENTAL PATHOPHYSIOLOGY OF PULMONARY HYPERTENSION

We have shown experimentally in infant rats that exposure to low oxygen (equivalent to 10% Fio_2) or hyperoxia (80% to 100% Fio_2), or to the toxin monocrotaline induces abnormal muscularization of peripheral arteries, medial hypertrophy of muscular arteries, and reduced arterial relative to alveolar density.[1] Although the pulmonary vascular abnormalities induced by changes in ambient oxygen may be largely reversible, monocrotaline-induced changes progress in the adult animal but not in the infant. Hyperoxia is especially associated with a failure of normal lung compliance and growth, whereas monocrotaline induces only alveolar abnormalities when given to neonatal rats.

Experimentally we have related the induction of structural changes in pulmonary arteries to the increased activity of the endogenous vascular elastase (EVE).[110,111] Increased EVE is observed in rat pulmonary arteries several days after exposure to hypoxia or injection of monocrotaline. A second increase in elastase activity is seen in adult animals injected with monocrotaline but not in infant rats or adult or infant hypoxic rats in which there is potential for regression. A cause-and-effect relationship between elastase and the pathophysiology of pulmonary arterial changes and hypertension is further demonstrated in studies in which a variety of serine elastase inhibitors proved effective in preventing development or progression of pulmonary vascular changes and pulmonary hypertension.[110,112]

The induction of elastase activity appears to be related to the functional changes associated with the perturbed endothelium, which results in loss of the barrier function. We speculated and subsequently showed in vitro that serum factors or endothelial factors that might gain access to the subendothelium when the barrier is lost can induce release of a serine elastase from pulmonary vascular smooth muscle cells.[113] This elastase can liberate biologically active smooth muscle cell mitogens, such as basic fibroblast growth factor (FGF) (FGF-2), from extracellular matrix stores.[57]

The resulting smooth muscle cell hyperplasia contributes to the hypertrophy of the arterial wall. Release of other growth factors, such as TGF-β, which can induce increased synthesis of elastin and collagen, might also add to the thickening. We have shown further that there is also induction of the matrix glycoprotein tenascin, which interacts cooperatively with growth factors, such as epidermal growth factor and basic FGF, in inducing the proliferative response in the vessel wall[114] (Figure 67-12). Having shown that hyperoxia-induced pulmonary hypertension and altered lung compliance in newborn rats could also be largely prevented with the use of elastase inhibitors,[115] we initiated a clinical trial with promising results in preventing and reducing the severity of bronchopulmonary dysplasia in premature infants.[116] Experimental studies by our group have also shown that elastase inhibitors can reverse established pulmonary vascular disease produced by the toxin monocrotaline, including muscularization of distal arteries, medial hypertrophy of muscular arteries, and loss of small vessels (Figure 67-13).

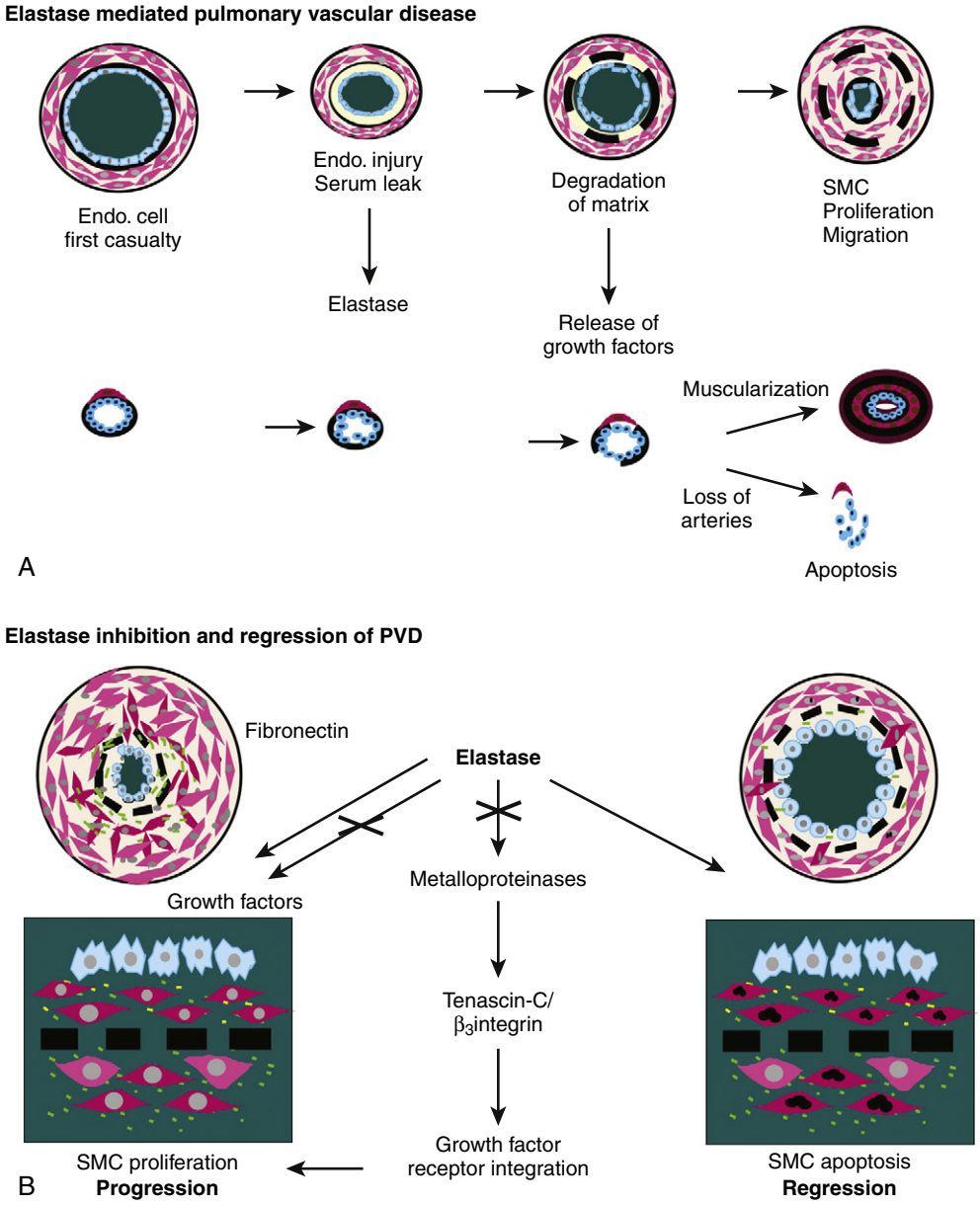


Figure 67-12 Schema describing relationship of elastase to pulmonary vascular disease. We speculated, based on our studies in cultured cells and in experimental animals, as to how a stimulus could induce activity of an elastolytic enzyme and how this might stimulate the remodeling process. In response to an injurious stimulus, the first "casualty" is the endothelial cell. As a result of structural and functional alterations in endothelial cells, some of the barrier function would be lost, allowing a leak into the subendothelium of a serum factor normally excluded from this region. The serum factor could induce activity of an endogenous vascular elastase (EVE). This enzyme released from precursor or mature SMCs would activate growth factors normally stored in the extracellular matrix in an inactive form, which are known to induce smooth muscle hypertrophy and proliferation and increases in connective tissue (CT) protein (e.g., collagen and elastin) synthesis. The growth factors could also result in the differentiation of precursor cells to mature smooth muscle related to the muscularization of normally nonmuscular small peripheral arteries. Elastase activity could also contribute to the loss of small arteries. **B,** As a result of elastase activity, a proteolytic cascade could be amplified through the activation of matrix metalloproteinases. We have shown that proteolysis of the matrix leads to the induction of tenascin-C, which in clustering to β_3 integrins results in the clustering and activation of growth factor receptors that send important survival as well as growth signals to the cells. Continued elastase activity causes migration of SMCs in several ways. The elastin peptides or degradation products of elastin can stimulate fibronectin, a glycoprotein that is pivotal in altering smooth muscle cell shape and in switching them to the motile phenotype. Inhibition of elastase activity could lead to smooth muscle cell apoptosis and regression of vascular disease. *(From Rabinovitch, M: Pathobiology of pulmonary hypertension,* Annu Rev Pathol Mech Dis *2:369-99, 2007.)*

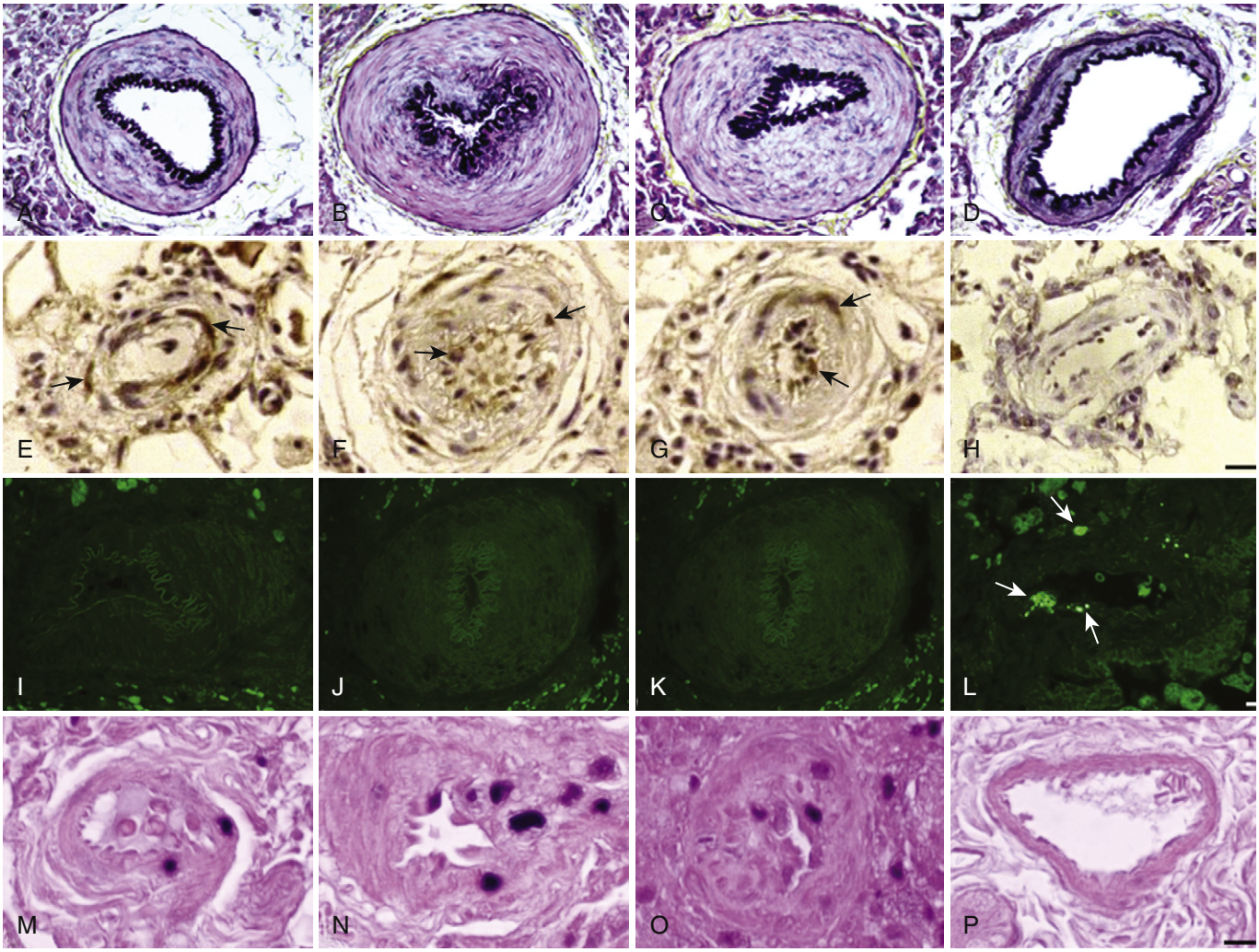


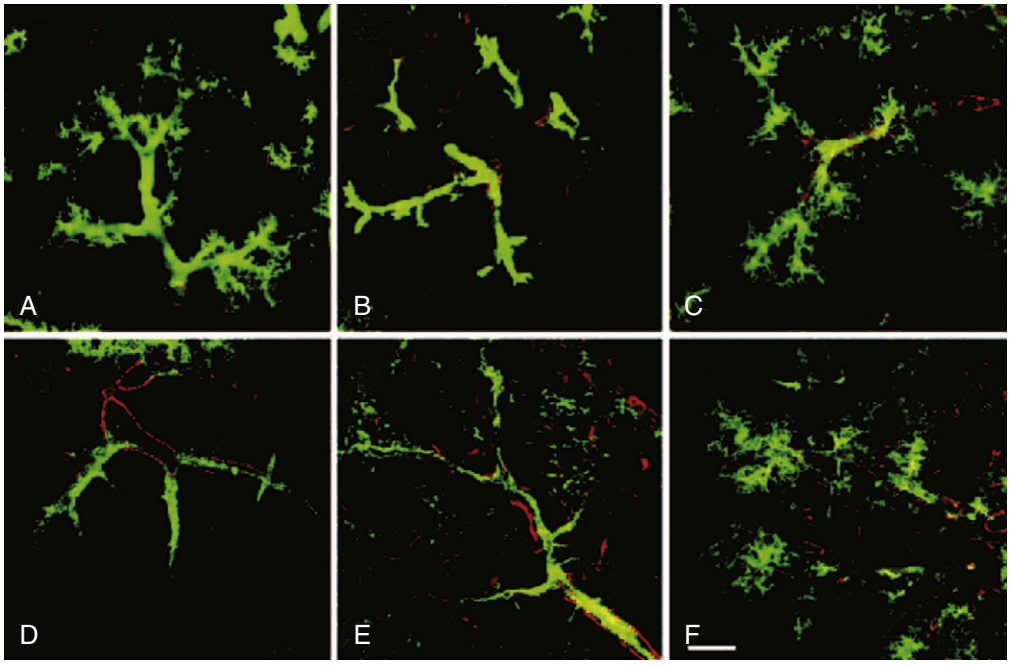
Figure 67-13 Cellular mechanism responsible for reversal of pulmonary artery muscularity. Elastase inhibition arrests tenascin-C accumulation and proliferation and induces apoptosis and loss of extracellular matrix (such as elastin). **A–P,** Days refer to time after injection of monocrotaline: A,E,I,M, day 21; B,F,J,N, day 28; C,G,K,O, day 28; D,H,L,P, day 28. **A–D,** Saline-perfused pulmonary arteries stained with Movat pentachrome stain. **E–H,** Pulmonary arteries after tenascin-C immunohistochemistry. *Arrows* indicate positive brown peroxidase staining. **I–L,** In situ TUNEL assays identifying apoptosis. *Arrows* indicate TUNEL-positive vascular cells. **M–P,** Proliferating vascular cells, shown by immunohistochemistry for proliferating cell nuclear antigen (PCNA); dark nuclei are PCNA-positive cells. *(From Cowan KN, Heilbut A, Humpl T, et al: Complete reversal of fatal pulmonary hypertension in rats by a serine elastase inhibitor,* Nat Med *6:698-702, 2000)*

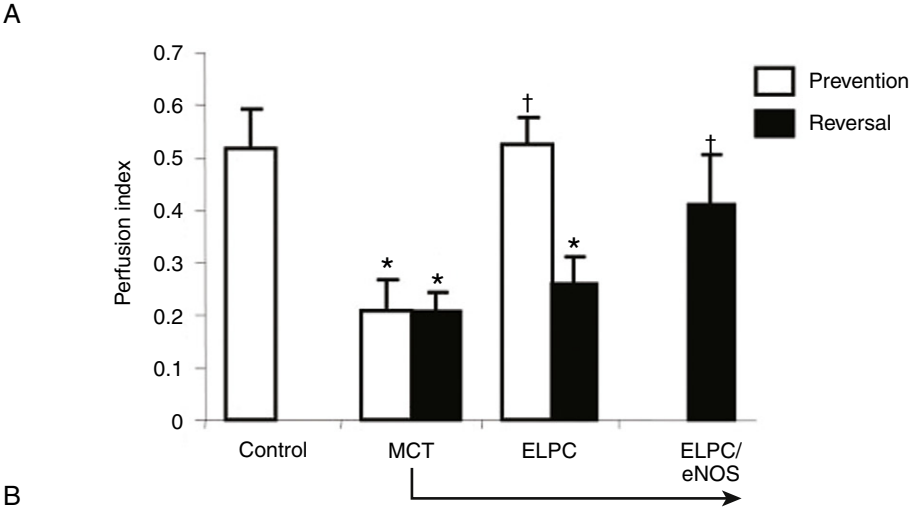
Based on subsequent knowledge that the effect of elastase was critical in maintaining survival signals through epidermal growth factor receptors (EGFRs), we blocked EGFR.[117] In these studies we were able to show reversal of progressive pulmonary arterial hypertension (PAH) that was sustained even 1 month after cessation of treatment. More recently, the use of a PDGF receptor blocker to reverse PAH in this experimental model[118] has prompted a case report showing the successful use of the tyrosine kinase inhibitor Imatinib (Gleevec) in a patient with advanced pulmonary vascular disease.[119] This report has prompted several clinical trials. Others have used simvastatin to reverse PAH in a monocrotaline-pneumonectomy model[120] or endothelial eNOS gene therapy in association with endothelial progenitor cell administration (Figure 67-14).[32,121] This study is important in showing recovery of the distal vasculature using this strategy.

Gene therapy with survivin[122] or angiopoietin-1,[123] as well as inhibition of the serotonin transporter (SERT),[124] and administration of adrenomedullin[125] and the rho kinase inhibitor, fasudil[126] have been used to reverse experimental pulmonary vascular disease. Potassium channel dysfunction is implicated in the pathogenesis of pulmonary vascular disease,[127] and gene therapy restoring K channel function has been used effectively to suppress[128] and reverse experimentally induced pulmonary vascular abnormalities. Our ability to prevent progression or reverse advanced pulmonary vascular disease in patients and restore the normal growth of blood vessels may require further identification of the cause of the smooth muscle cell and fibroblast proliferative response and the initial endothelial cell susceptibility to apoptosis.[129-132]

DECREASED GROWTH OF THE PULMONARY VASCULAR BED

Reduced pulmonary blood flow and pressure may also result in reduced growth of the pulmonary vascular bed with relative hypomuscularity of vessels that are decreased in size and number. This can be further aggravated by suppressed growth of the





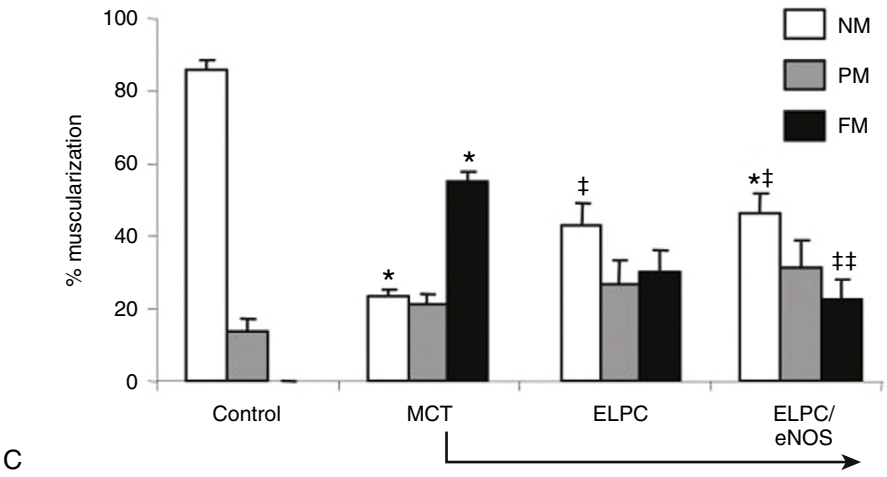


Figure 67-14 **A,** Representative confocal projection images of lung sections perfused with fluorescent microspheres *(green)* suspended in agarose (i.e., fluorescent microangiography) and immunostained for α-smooth muscle actin *(red)*. Normal filling of the microvasculature was observed in control rats *(A),* whereas rats treated with MCT showed a marked loss of microvascular perfusion and widespread precapillary occlusion 21 days *(B)* and 35 *(D)* days after MCT injection. In the prevention model, animals receiving ELPCs displayed improved microvascular perfusion and preserved continuity of the distal vasculature *(C).* In the reversal model, eNOS-transduced ELPCs dramatically improved the appearance of the pulmonary microvasculature *(F),* whereas progenitor cells alone resulted in more modest increases in perfusion and little noticeable reduction in arteriolar muscularization *(E).* Summary data for pulmonary microvasculature perfusion **(B)** for animals treated in the prevention *(white bars)* and reversal *(shaded bars)* protocol. **C,** Proportion of small pulmonary arterioles that are nonmuscularized *(NM),* partially muscularized *(PM),* or full muscularized *(FM)* in the various treatment groups of the reversal protocol. *(From Zhao YD, Courtman DW, Deng Y, et al: Rescue of monocrotaline-induced pulmonary arterial hypertension using bone marrow-derived endothelial-like progenitor cells: efficacy of combined cell and eNOS gene therapy in established disease,* Circ Res *96(4): 442-50, 2005.)*

lung parenchyma, which can also be a feature of low pulmonary blood supply.[4]

PULMONARY VENOUS ABNORMALITIES

The pulmonary vascular abnormalities that are caused by pulmonary venous hypertension are the result of mitral valve or left ventricular dysfunction and anomalous pulmonary venous return. They largely regress following correction of the anatomic or physiologic abnormality, although in the initial postoperative period the abnormally muscularized vasculature[133,134] may be reactive. Individual stenoses of the extraparenchymal and particularly of the intraparenchymal pulmonary veins are difficult to correct with current surgical or interventional cardiologic approaches. We created a neonatal piglet model of progressive pulmonary venous obstruction and showed that there was altered compliance of the veins, which was reflected in an increase in pulmonary arterial pressure.[135] This preceded the elevation in pulmonary venous pressure, which occurred later when there was evidence of some compromise of the vessel lumen by the intimal lesion. At this stage, there was a disproportionate increase in collagen in the vessel wall, which likely further contributes to the hemodynamic abnormality and the resistance to successful intervention.

CLINICAL IMPLICATIONS

The reduced growth of the pulmonary circulation may pose a contraindication for surgery using the Fontan principle.[133] Also, consideration of patients for cavopulmonary anastomoses in preparation for a final-stage Norwood operation must take into account evidence of regression of the left-sided obstructive lesion–induced vascular changes, that is, the increased muscularity of pulmonary arteries and veins that are associated with heightened pulmonary vascular reactivity. Future studies aimed at accelerating the structural maturation of the lung to induce both dilation and recruitment of small vessels may prove of benefit in allowing surgical correction of even complex congenital heart disease in the newborn infant.

CONCLUSION

Increasing knowledge of the genes that control vascular cell phenotype and differentiation and their regulation in development will lead to new and fruitful directions in preventing or reversing abnormal structural development of the lung.

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Development of the Gastrointestinal Circulation in the Fetus and Newborn

The rapid cellular growth of the intestine in fetal life requires sufficient substrate for energy and a means for clearance of metabolic waste. This is accomplished by a rapid and proportionate growth of the intestinal circulation. Although anatomic and histologic studies have been performed on salvaged human fetal intestines, lack of availability of samples and autolysis has precluded detailed and systematic studies of the physiology of the developing gastrointestinal circulation. Therefore much of the information presented in this chapter is derived from controlled studies in animals. In addition, the most reliable data involve only that portion of the alimentary tract below the diaphragm, namely, the gastrointestinal and colonic circulation.

Several principles of gut development have an impact on intestinal blood flow. The cephalic portion of the embryonic intestinal tract develops and matures more rapidly than do caudal areas.[1] As the gut intestinal lining rapidly multiplies, the embryonic ileum is obliterated and eventually recanalizes. Although the intestine begins as a straight tube, differential growth rates result in the contrasting calibers of various gut segments and in the rotation and final positioning of various components.[2]

The arterial supply to the intestine does not develop in isolation but forms in response to the rapid growth of this organ. The majority of the intestinal tract mucosa, along with the liver parenchyma and pancreas, is derived from endoderm. However, the connective tissue and muscular components are derived from splanchnopleuric mesoderm. Oral and anal epithelium is derived from the ectoderm of the stomatodeum and proctodeum, respectively. Progressing from the germ cell stage, the intestine is divided into three primary portions: the foregut, midgut, and hindgut.[2-4] The foregut includes all structures distal to the tracheal diverticulum from the esophagus through the first half of the duodenum. The midgut is composed of structures distal to the second portion of the duodenum, including the jejunum, ileum, and proximal two thirds of the transverse colon. The hindgut consists of the distal transverse colon and the proximal two thirds of the anal canal.

DEVELOPMENT OF INTESTINAL CIRCULATION

Early somite embryos have an extensive vascular network on the yolk sac. In the process of vasculogenesis, vascular endothelial precursor cells (angioblasts) migrate to the location of future vessels, coalesce into cords, differentiate into endothelial cells and ultimately form patent vessels. Vascular endothelial growth factors and their receptors have been identified. Erythropoietin has been shown to stimulate vasculogenesis in neonatal rat mesenteric microvascular endothelial cells.[5] Unpaired ventral branches of the dorsal aorta (vitelline arteries) pass to the yolk sac, allantois, and chorion. The network drains by way of the vitelline veins to the heart. During the fourth week the primitive gut is formed as the dorsal portion of the yolk sac is incorporated into the embryo. Three vitelline arteries persist to supply the foregut (celiac artery), midgut (superior mesenteric artery), and hindgut (inferior mesenteric artery).[2]

The vessels distributed to the foregut fuse and form a single vessel, the celiac artery. With a downward migration of the viscera, the aortic attachment of the celiac artery moves caudally. It divides into the hepatic artery, splenic artery, and left gastric branches to supply the stomach and duodenum. The liver, pancreas, and related mesodermal spleen receive their blood supply from these branches. At the vascular division between the foregut and midgut is the anastomosis between the superior pancreaticoduodenal and the inferior pancreaticoduodenal arteries.[4]

The embryonic vitelline arteries fuse and form a superior mesenteric trunk, which reaches the midgut by passing through the mesentery. Terminal branches of this trunk supply the yolk sac. The various branches distal to the intestine are obliterated when the ileum separates from the yolk sac and vitelline stalk. However, the superior mesenteric artery remains and supplies the intestinal circulation from the second part of the duodenum through the proximal two thirds of the transverse colon.[3,4]

The ventral branches of the aorta supplying the hindgut fuse to form a single inferior mesenteric trunk. Its final distribution includes the distal one third of the transverse colon and the entire descending colon and sigmoid. The inferior mesenteric trunk anastomoses with the middle colic artery, which is a branch of the superior mesenteric artery. Furthermore distal to this is an anastomosis to branches of the inferior and middle rectal arteries from the internal iliac trunk.[2,3]

VENOUS DRAINAGE OF THE GASTROINTESTINAL TRACT

The venous drainage of the intestine is much more variable than is the arterial blood supply. The low pressure venous drainage in the embryo is plexiform, and therefore patency is based somewhat on blood flow. In addition, blood has a tendency to seek the most direct route of flow because of hydrodynamic factors.

The vitelline veins initially pass along each side of the anterior intestinal portal vein. They form an anastomosing plexus around the developing duodenal loop in the substance of the septum transversum. Cords of liver cells extend into the septum transversum and divide the vitelline plexus into the primitive hepatic sinusoids. Anterior stems of the vitelline veins enter the primitive sinus venosus. As the stomach, duodenum, and small intestine elongate and rotate from their original midsagittal position to the adult position, blood flow develops along the most direct route to the liver cutting from one vitelline vein to another through the connecting plexus. Thus the portal venous system does not develop in a spiral fashion around the developing intestine but instead evolves short and straight with the duodenum and intestine around it.[2,3]

In embryos of less than 5-mm crown-rump length (<5 weeks), blood from the paired umbilical veins passes into the liver, at which point it communicates with the vitelline sinusoids. At a 7-mm crown-rump length (33 to 34 days), the right umbilical vein atrophies and disappears.[2] As a result, all the placental blood entering the embryo enters through the left umbilical vein and

empties into the hepatic sinusoids. As the right side of the sinus venosus elaborates, an enlargement of the hepatic sinusoidal communication occurs between the right hepatocardiac channel and the left umbilical vein forming the ductus venosus.[4] Thus blood entering from either the umbilical or vitelline systems can pass by way of the ductus venosus to the right atrium or into the liver sinusoids. The venae advehentes, connecting the umbilical and vitelline systems to the hepatic sinusoids, become the branches of the portal vein in the liver. In addition, the venae revehentes connecting the sinusoids to the right hepatocardiac channel become the tributaries to the hepatic veins. After birth the left umbilical vein and the ductus venosus are obliterated and become the ligamentum teres and the ligamentum venosum, respectively.[3]

THE INTESTINAL MICROCIRCULATION

The microscopic anatomy of the intestinal circulation varies from species to species. In rabbits and humans, villus arterioles ascend from the submucosal arterioles into the villus. As the arteriole reaches the villus tip, it divides into a diffused capillary network, which then drains into a centrally located villus venule originating in the distal one third of the villus.[6] In most mammals, the capillaries of the intestinal villus are fenestrated. Just as the villus and the microvillus are exposed to a large solute and water load, so the villus capillaries must be capable of handling the absorbed materials. The endothelium of the capillary facing the epithelium is very thin and usually contains fenestrae, with the greatest number at the villus tips and in the crypts.

Water and solutes are transported into the capillary by a number of different pathways (Figure 68-1). Low-molecular-weight nonpolar substances and lipid-soluble substances such as oxygen and carbon dioxide may cross directly through the cell membrane. The bulk of absorbed materials passes through fenestrae, which are numerous circular openings of up to 30 nm in the capillary endothelium and may be either opened or diaphragmed.[6] At least 60% of the fenestrae have a diaphragm. Although the porosity is not known, these diaphragms limit the movement of molecules. Much less commonly, water and solutes move slowly by pinocytosis. In this process, vesicles are formed that move through the cell to the opposing side before releasing their contents. On rare occasions, transendothelial channels may be formed when several vesicles simultaneously open and bridge the cell. These occurrences are relatively infrequent and do not have a major impact on total absorptive capacity. Intercellular junctions are impermeable to solutes of 2-nm diameter in the arteriole and capillary but may play a limited role in venule absorption.

CONTROL OF BLOOD FLOW

Numerous factors impinge on the maintenance of intestinal blood flow. They can be grouped into at least four major categories: cardiovascular status, neural control, humoral substances, and local control.[7-9] Although not much data are available specifically regarding the fetal intestine in these areas, certain principles can be inferred to be operative in the fetus.

Cardiovascular Function

Adequate perfusion of any tissue in the body is dependent on the maintenance of sufficient cardiac output and mean arterial pressure. Reduction in cardiac output or systemic arterial pressure within physiological range leads to compensatory vasodilation of the regional circulation to maintain the tissue blood flow relatively constant, the process termed as pressure-flow autoregulation. The autoregulation of intestinal blood flow guarantees the preservation of continuous blood flow to the intestine while large fluctuations are occurring in the arterial perfusion pressure.[8,10] Although superior mesenteric circulation autoregulation has been well established in canine, porcine, and feline circulations, the mechanism for this regulation is controversial.[11] The capacity to autoregulate the intestinal blood flow is a function of gestational maturation: however, in the presence of profound hypotension, net vasoconstriction occurs and intestinal blood flow decreases. In newborn swine, moderate systemic arterial hypoxemia causes vasodilation and increase in gut perfusion, whereas severe hypoxemia (PO_2 <40 mm Hg) causes vasoconstriction and gut ischemia.[12] Intestinal perfusion and oxygenation is profoundly reduced in puppies made polycythemic by exchange transfusion. Fortunately few infants have polycythemia and most escape intestinal damage. Both of these factors are influenced by blood volume. The proportion of cardiac output destined for the intestine is altered in newborns with cyanotic congenital heart disease and vascular malformations, such as coarctation of the aorta, which limits blood flow to the intestinal tract. In the fetus, systemic circulatory needs are maintained even with severe left ventricular obstruction. However, fetal asphyxia and other fetal problems can be associated with reduced myocardial contractility and tricuspid regurgitation, limiting cardiac output and consequently reducing intestinal flow.

Intestinal oxygen uptake and blood flow increase dramatically after birth to sustain rapid growth of the mucosa and oxidative demands of enteral nutrition (secretion, absorption, motility).[11] Postnatally, the splanchnic circulation accounts for 20% of cardiac output; however, splanchnic blood flow increases 30% to

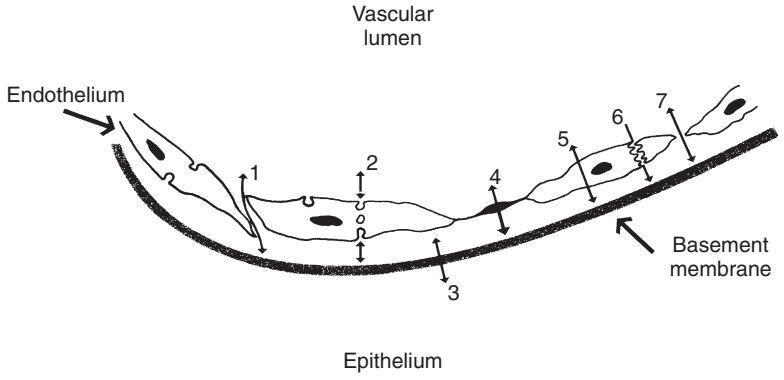


Figure 68-1 Transport pathways in intestinal capillaries. *Arrow* represents potential direction of movement of fluid and solids. (1) Intercellular junction, (2) pinocytosis, (3) basement membrane, (4) fenestrae with diaphragms, (5) cell membrane, (6) transendothelial channel, and (7) open fenestrae.

130% after a meal, known as *postprandial hyperemia*.[10] Glucose and solubilized long-chain fatty acids increase jejunal blood flow. In preterm infants, dietary mixed nucleotides increased the superior mesenteric artery blood flow velocity, peaking at 30 minutes postprandial and having a sustained effect for 90 minutes.[13] Bile and bile salts may double ileal blood flow. Volatile fatty acids (acetic, butyric, propionic) derived from fermentation of undigested carbohydrates by microflora increase colonic blood flow and serve as a metabolic substrate for colonic enterocytes.[8]

Neural Controls

The gastrointestinal tract is extensively innervated by the autonomic and enteric nervous system.[7,11] Enhanced sympathetic tone elicits arterial smooth muscle contraction, thereby raising intestinal vascular resistance and decreasing blood flow. This effect is mediated through the α-adrenergic receptors in the vascular smooth muscle. However, this vasoconstrictor response is short lived, and decreased vascular resistance and improved blood flow rapidly return by stimulation of α-adrenergic receptors.[9,12,14] Thus sympathetic stimulation may produce a variable response (Box 68-1). Although acetylcholine causes vasodilation through an endothelium-dependent mechanism,[14-16] the effects of acetylcholine on intestinal blood flow are also variable. Acetylcholine has the ability to initiate intestinal smooth muscle contraction, which can impede blood flow by increasing transmural pressure.

Marginal tissue oxygenation antagonizes the sympathetic response by inducing vasodilation. Furthermore, intestinal wall relaxation resulting from sympathetic stimulation decreases the resistance of the vessels and allows increased blood flow to the stomach and intestines. The adrenal medullary catecholamines and angiotensin II, formed locally in response to renal renin, constrict vascular smooth muscle in the gastrointestinal circulation and therefore decrease local blood flow.[17,18] This effect may be temporary, however, and can be readily overridden by local metabolic control.

The majority of extrinsic nerves innervating the intestine are afferents. They contain two important polypeptides, substance P and calcitonin gene-related peptide (CGRP), which are released by noxious chemical and mechanical stimulation. These agents induce local vasodilation and contribute to neurogenic inflammation by mast cell degranulation and altering vascular permeability.[19]

BOX 68-1

Agents That Increase Intestinal Blood Flow

Acetylcholine[14,17,19,41]; motlin[8,10,20]
Adenosine[8]; neurotensin[10]
Adrenergics[16]; nitric oxide[24,25,27,42]
Isoproterenol
Salbutamol peptide hormones
Cholecystokinin[19-21]
Aminophylline[1]; gastrin[19-21]
Glucagon[43,44]
Bradykinin[21,45]; vasoactive intestinal polypeptide[46]
Calcium antagonists[47]; platelet-activating factor (directly)[15,40]
Carbon dioxide[14]; potassium low dose[16,20,23]
Dietary nucleotides[13]; prostaglandins[8,45,48]
PGD2, PGE1, PGE2
Histamine[17,43]
Serotonin[8]
Magnesium[16,22]
Methionine-enkephalin[46]; substance P[18]

Humoral Controls

Numerous humoral substances have been demonstrated to alter gastrointestinal blood flow in experimental animals. Some are circulating substances, whereas others are released locally. Boxes 68-1 and 68-2 summarize the agents that have been well demonstrated to increase or decrease intestinal blood flow. Gastrointestinal hormones released during various phases of digestion may dilate the gastrointestinal circulation. Glucagon and cholecystokinin increase both intestinal blood flow and pancreatic flow.[19-21] Gastrin specifically increases blood flow to the gastric mucosa.[19,21]

Local Controls

The local extracellular environment is altered by the increased metabolic activity of the gastrointestinal parenchymal cells, for example, during digestion.[10] An increased metabolic demand may lead to relative tissue hypoxia and consequently the release of many vasodilators, for example, K^+, Mg^{2+}, histamine, polypeptides (bradykinin, vasoactive intestinal polypeptide), prostaglandins, carbon dioxide, and adenosine.[8,14-16,22,23] Cells with an increased metabolic rate are likely to produce greater quantities of dilator metabolites, which ensure the provision of more substrate by increasing blood flow.

Role of Nitric Oxide

Basal vascular resistance, which determines the regional gastrointestinal blood flow, depends upon the delicate balance between the constrictor and dilator forces which are acting upon the vascular smooth muscle. Nitric oxide is the major dilator which counterbalances the constrictive effect of the endothelin 1 and the intrinsic contractile response of smooth vascular muscle. Under basal conditions, nitric oxide is continuously produced by endothelial cell in presence of nitric oxide synthase (constitutive isoform) from L-arginine. It relaxes vascular smooth muscle by decreasing cytosolic free calcium via increased cGMP.[24,25] In rats, acute administration of a nitric oxide synthase inhibitor (L-NNA) reduces basal intestinal blood flow.[26] Rate of nitric oxide production can increase markedly following chemical or mechanical stimulation of nitric oxide synthase (induced or stimulated isoform). Methylene blue, a guanylate cyclase inhibitor, abolishes intestinal vasodilation resulting from administration of sodium nitroprusside, a nitric oxide donor.[27] After ischemia with reperfusion, exogenous nitric oxide sources (SIN-1, CAS-754, and nitroprusside) and L-arginine reduce mucosal barrier dysfunction without improving intestinal blood flow, suggesting that nitric oxide may have modulating effect on microvascular permeability.[28]

The fetal circulation has an increased basal production of nitric oxide compared with the adult.[29] In late gestation sheep fetus,

BOX 68-2

Agents That Decrease Intestinal Blood Flow

Adrenergics[8,16]; peptide hormones
Dopamine angiotensin II[8]
Epinehprine vasopressin[21,49]
Methoxamine
Norepinephrine physostigmine[8]
Phenylephrine
Potassium high dose[16,21]
Calcium[16,23,47]
Endothelin[35,36]; prostaglandin F2a[41]
5-Hydroxytryptamine[1]; somatostatin[50]
Leukoterine D4[38,39]; thromboxane A2[48]
Methacholine[44]; tyramine[1]

nitric oxide was shown to play important role in contributing to low vascular resistance and increased blood flow throughout the gastrointestinal tract, as well as in redistribution of intestinal blood flow between intestinal segments.[30] Inhibition of endogenous nitric oxide in midgestation fetal sheep results in substantial blood flow reduction across all segments of gastrointestinal tract, implying that nitric oxide plays a major vasodilator role at such an early stage of development of gastrointestinal circulation.[31] Sustained inhibition of nitric oxide synthase in utero results in vasoconstriction of fetal blood vessels, but no evidence of injury to the gastrointestinal tract exists.[32] A lack of nitric oxide synthesis appears to be causative in neonatal hypertrophic pyloric stenosis,[33] but this effect is mediated by the neuronal not the endothelial isoform of nitric oxide synthase based on gene knockout studies.[34]

Endothelin 1

Endothelin 1 is a vasoactive and mitogenic polypeptide synthesized and secreted by endothelial cells.[15] It acts on specific receptor ET_A on vascular smooth muscle, causing sustained powerful vasoconstriction, whereas binding to ET_B receptor on endothelial cell causes release of nitric oxide with resultant vascular relaxation. Although cord blood levels are higher than any other values reported in humans, little information is available about its role in the splanchnic circulation of the fetus and newborn.[35] Hypoxia and shear stress stimulates endothelin 1 secretion.[36] Preterm neonates born to mothers with pre-eclampsia have increased circulating levels of endothelin 1 and reduced superior mesenteric artery blood flow velocity, possibly due to fetal hypoxia.[37]

Local inflammatory mediators

Systemic or local activation of polymorphonuclear cells has been demonstrated to be a component of the pathophysiology of a variety of altered blood flow states. Activated neutrophils adhere to vascular endothelium and may damage or occlude them. Secondary release of vasoconstrictors (leukotrienes) and substances that increase permeability (platelet-activating factor, leukotrienes) may further compromise tissue perfusion.[38,39]

Platelet-activating factor (PAF) is a phospholipid synthesized by many types of cells including endothelium, macrophages, and granulocytes.[27] Although PAF is a vasodilator, it may cause paradoxical vasoconstriction and ischemic injury to the intestine as a consequence of its ability to activate leukocytes. PAF-activated leukocytes may plug postcapillary venules and release vasoconstrictors, for example, leukotriene C_4.[39]

CLINICAL CONSIDERATIONS

Virtually no study has examined the human fetal and neonatal intestinal blood flow in vivo. It may be inferred from various animal models that some of the clinical conditions encountered in critically ill neonates may result from compromise of the gastrointestinal circulation.[6] Limitation of blood flow as local tissue metabolism increases may result in malabsorption. The early-onset form of necrotizing enterocolitis (in the first week of life) is often associated with perinatal asphyxia, arterial catheterization, or the polycythemia-hyperviscosity syndrome.[40] In each of these conditions, poor tissue perfusion may result in ischemic necrosis of the intestine.

With gene expression techniques it may be possible to trace the ontogeny of systems regulating the gastrointestinal circulation (neural and humoral) in the developing human and experimental animals. These approaches may circumvent the technical problems of directly assessing the splanchnic circulation in the perinatal period. Insights drawn from these molecular approaches may guide improvements in the therapeutic management of the fetus and newborn.

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69 Physiology of Congenital Heart Disease in the Neonate

Thomas J. Kulik

If one juxtaposes a drawing of the heart and great arteries—four chambers and a few large blood vessels—with a depiction of the cascade of biochemical events that follow, say, a growth factor binding to its receptor, it may seem that the physiology of congenital heart disease ought to be child's play. It is not, for several reasons key to understanding the physiologic responses to congenital heart lesions.

A comprehensive understanding of the cardiovascular physiology of a neonate with a cardiac lesion requires consideration of *many factors.* The first factor pertains to key variables (myocardial function; intravascular volume; cardiac and vascular transmural pressures, flows, and compliances; and pulmonary venous oxygen [O_2] content)[1] needed to describe the physiology of a neonate with a cardiac lesion. However, any description taking into account only these variables, although complex, would be incomplete because cardiac lesions affect somatic physiology in various ways (e.g., sympathetic nervous system activation) that cannot be specified from these variables alone. Indeed, alterations in somatic physiology linked to altered hemodynamics account for many of the clinical manifestations of cardiac lesions.[2,3] Hence, the net physiology observed is a result of the interaction of many elements: (1) cardiac and vascular anatomy; (2) hemodynamic variables related to cardiovascular function; (3) pulmonary function; (4) O_2 carrying capacity and O_2 release from the blood; and (5) myriad other factors, such as O_2 consumption (Vo_2), autonomic nervous system, and endocrine, lymphatic, and other organ system function.

The physiology of such lesions is complex for other reasons as well. With cardiac structural anomalies, *more than one type* of physiologic derangement will often be operative. For example, with a large patent ductus arteriosus (PDA) the left ventricle (LV) may have both an increased volume load and reduced myocardial perfusion, the latter resulting from decreased aortic diastolic pressure. In addition, the consequences of multiple anatomic cardiac abnormalities may be *additive, or,* conversely, *they may balance each other*. Finally, subtle differences in physiology may result in very different clinical phenotypes in anatomically identical patients. For example, the arterial O_2 saturation in neonates with D-transposition of the great arteries after balloon atrial septostomy is variable and unpredictable, related to subtle differences in physiology.[4]

Second, physiologic variables change. Some factors that affect cardiovascular physiology may vary over months (especially those related to anatomic changes, e.g., pressure-related remodeling of pulmonary blood vessels), whereas others may change significantly over seconds or minutes (e.g., alterations in pulmonary vascular tone). The physiology that attends a given cardiac defect may actually be a spectrum of physiologies.

Finally, the precise elucidation of a given patient's hemodynamics may be difficult or impossible, even with "simple" anatomic lesions. Measurement of relevant pressures, blood flows, and ventricular function is often not feasible, especially when invasive studies are not warranted. Although information derived from noninvasive evaluation is usually sufficient for design of therapy, situations exist in which important information is difficult or impossible to obtain. For example, in patients with reduced pulmonary blood flow (Qp) and an anatomic restriction to flow, it can be difficult to determine to what extent flow is limited by the anatomic restriction versus increased pulmonary vascular resistance (PVR).

This chapter focuses on congenital cardiovascular *anatomic* lesions; nonanatomic abnormalities (e.g., arrhythmia, intrinsic myocardial muscle dysfunction) are not discussed. It is also beyond the scope of this chapter to examine the biology of specific elements related to cardiovascular function (e.g., cardiac mechanics) or to discuss structural alterations caused by cardiac lesions (e.g., ventricular hypertrophy with pressure overload). Essential to understanding of congenital cardiac malformations is an appreciation of normal fetal and neonatal physiology,[5,6] the physiology and pharmacology of the pulmonary circulation,[7-9] the general principles of cardiac hemodynamics,[10] and the anatomy of congenital cardiac lesions.

HOW CONGENITAL HEART LESIONS ALTER THE CLINICAL PHENOTYPE OF THE NEONATE

A description of the hemodynamic characteristics of an anatomic cardiac lesion is only characters on a page unless the impact of the abnormal hemodynamics on the greater physiology of the organism is considered. Leaving aside that a congenital heart

lesion may occasion the need for neonatal surgery, and ignoring the important issue of the impact of abnormal hemodynamics on cardiovascular growth and remodeling, a congenital anatomic lesion may affect a neonate in three ways.

No Appreciable Effect

An anatomic lesion may be such a minor perturbation of normal structure (e.g., a small ventricular septal defect [VSD]) that it may *never* have any appreciable physiologic consequences. In other cases, a lesion destined to later have significant impact may have few manifestations in the neonate because of the physiologic characteristics of the newborn. The classic example is that of a large VSD, which may have little effect initially but causes congestive heart failure (CHF) at a few weeks of age. The clinical phenotype changes partly because the relatively high neonatal PVR falls over time, increasing left-to-right shunting and hence the LV volume pumped,[11] and partly because of other changes, such as the normal postnatal decline in hematocrit.[12] Lesions that cause higher than normal right ventricular (RV) pressure (as long as the pressure is no higher than roughly systemic), for example, pulmonary stenosis, usually have little impact on ventricular function in the newborn because the neonate's RV is well adapted to pumping at systemic pressure.[6] Ductal-dependent left-sided obstructive lesions (e.g., severe coarctation of the aorta) may also have only subtle clinical manifestations in the first few days of life if the ductus remains widely patent.

Reduction of Systemic Oxygen Transport

Congenital cardiac lesions may reduce systemic arterial blood O_2 tension *(hypoxemia)* or *systemic blood flow* (Qs) and thus decrease systemic O_2 transport (Systemic O_2 transport [SOT] = Arterial O_2 content × Qs).[13]

Reduction of Systemic Oxygen Transport Resulting From Hypoxemia

Hypoxemia may be the only manifestation of some lesions (e.g., tetralogy of Fallot), or it may be present with defects that also cause reduced Qs (e.g., severe Ebstein anomaly). It appears that the neonate can tolerate a low P_{O_2} better than the mature organism,[14] and moderate hypoxemia, as an isolated abnormality, generally has little obvious effect on the neonate. Increased O_2 extraction, redistribution of blood flow to organs of high O_2 need, and perhaps increased Qs help to maintain systemic V_{O_2} with hypoxemia.[15,16] For example, when conscious lambs less than 1 week old were subjected to acute alveolar hypoxia (fraction of inspired O_2 [F_{IO_2}] = 0.12; arterial P_{O_2} = 35 mm Hg) no fall in V_{O_2} or acidosis occurred.[16] More severe hypoxia (F_{IO_2} <0.10; arterial P_{O_2} approximately 25 mm Hg) caused V_{O_2} to fall, but it was not associated with much acidosis in conscious newborn lambs.[15,16] Multiple variables (e.g., hematocrit, half-saturation O_2 pressure [P_{50}] of hemoglobin, environmental temperature, Qs, V_{O_2}, and whether the organism is spontaneously ventilating) determine the physiologic impact of hypoxemia.[13-16]

The quantitative relationship between hypoxemia and organ dysfunction or damage in the *human* neonate is unknown. One study found an F_{IO_2} of 0.15 to cause V_{O_2} to fall significantly in human infants, albeit without obvious detrimental effect.[17] Perhaps the clearest evidence that neonates can tolerate substantial hypoxemia with modest or no long-term consequences comes from experience treating infants with D-transposition of the great arteries, a lesion that almost always causes considerable hypoxemia, especially before balloon septostomy is performed. One hundred twenty-nine neonates with this lesion were operated on in the first week of life at the Children's Hospital in Boston; the mean lowest preoperative arterial P_{O_2} was approximately 24 mm Hg. Even though these infants had undergone a period of substantial hypoxemia (before operation), the hospital survival rate for these patients was more than 98%.[18] Neurologic evaluation at 4 years of age revealed that only 4% of these patients had moderate or severe neurologic abnormalities; average intelligence quotient scores were lower than average, but not much (94 vs. 100).[19] Furthermore, it is likely that some or all of the observed decrement in neurologic function was related to aspects of the patient's treatment and clinical course distinct from the hypoxemia.

Reduction of Systemic Oxygen Transport Resulting From Reduction of Systemic Blood Flow

Congenital heart lesions may also decrease SOT by decreasing Qs. As is the case for hypoxemia, modest reductions of Qs may not affect tissue oxygenation. For example, Fahey and Lister[20] found that Qs could be reduced to approximately 42% of the resting value before V_{O_2} fell in the conscious 2-week-old lamb; increased fractional extraction of O_2 from the blood maintained O_2 delivery with decreased Qs. Most congenital cardiac lesions that reduce Qs do so by one or more of three mechanisms: (1) abnormally low systemic ventricular output, (2) abnormal arterial connection between the heart and peripheral tissues, and (3) recirculation of previously oxygenated blood to the lungs (see later). Few quantitative data regarding Qs in the neonate with heart disease are available, in part because of the difficulty in measuring this variable.

Neurohumoral Effects of Abnormal Hemodynamics

Symptoms can occur in infants with a heart lesion not attended by a reduction in SOT. For example, infants with CHF resulting from a large VSD generally have Qs within the normal range[11,21,22] (but see references 12 and 23). The symptoms of "CHF" are a consequence of *neurohumoral alterations* related to the physiologic abnormality, not altered SOT.[2,3,24] These alterations help to maintain adequate blood pressure and tissue perfusion in part by augmenting myocardial contractility (sympathetic nervous system activation)[25,26] and by increasing systemic vascular resistance (SVR; activation of the sympathetic nervous system, the renin-angiotensin-aldosterone system,[24,27,28] and release of vasopressin). Ventricular output is also increased (via the Frank-Starling principle) through fluid retention (and therefore increased ventricular filling) caused by activation of the renin-angiotensin-aldosterone system and other mechanisms.[2,3,24]

These factors help to maintain adequate SOT, but they also have *unfavorable consequences*. For example, vasoconstrictors increase SVR and therefore ventricular afterload; the clinical result is decreased peripheral perfusion. Increased catecholamines also contribute to failure to thrive by increasing V_{O_2}.[29] Fluid retention causes edema,[30] and it increases lung water and the work of breathing. It is often these secondary manifestations of abnormal cardiovascular function, caused by the attendant neurohumoral activation, rather than critically reduced SOT per se, that affect infants with congenital cardiac lesions.

PHYSIOLOGY OF SHUNTING AND SINGLE-VENTRICLE PHYSIOLOGY

Multiple formulas are useful for calculating flows and resistances relevant to understanding intracardiac shunting, but because space constraints prohibit delineation and derivation of most of them here the reader is referred to the literature.[10] However, a few concepts are especially important and are briefly covered.

Pulmonary Vascular Resistance

The *resistance to blood flow through the lungs* is an important determinant of the physiologic effects (ventricular work and systemic O_2 saturation) of many cardiac lesions with

shunting. A useful, albeit simplified[31] way to conceive of PVR is PVR = (PAP − LAP)/Qp, where PAP is mean pulmonary arterial pressure (millimeters of mercury), LAP is mean left atrial pressure (millimeters of mercury), and Qp is pulmonary blood flow (which, for pediatric patients, is usually indexed—liters per minute per square meter). SVR is calculated in an analogous way. Because PVR is primarily a reflection of resistance to flow through the pulmonary microcirculation, it is sometimes referred to as pulmonary *arteriolar* resistance. The calculated PVR can be low, but total resistance to flow through the lungs and into the heart can be high, if the patient has obstruction to systemic ventricular inflow (e.g., because of mitral stenosis) or poor systemic ventricular compliance. The concept of *total pulmonary resistance* (Total pulmonary resistance = Mean PAP/Qp) takes into account all resistance to flow from the central pulmonary arteries to the ventricle, although the term is misleading because some of the resistance to flow resides outside the lungs.

PVR is much higher than SVR in the fetus,[8,32] but PAP normally decreases to approximately one-half systemic arterial pressure in the first 24 hours of life (presuming the ductus does not remain widely patent), and it is at essentially mature levels by 1 to 2 weeks after birth.[33] The postnatal decline in PVR is considerably slower with cardiac lesions that maintain elevated PAP (e.g., a large VSD or PDA).

Ratio of Pulmonary to Systemic Blood Flow

The *Qp/Qs ratio* provides an estimate of the volume pumped by the ventricles and an estimate of the Qp (and hence of PVR if the PAP is known). It can be calculated by using blood oximetry values: $Qp/Qs = (Sao_2 - Mvo_2)/(Pvo_2 - Pao_2)$, where Sao_2 is the systemic arterial O_2 saturation, Mvo_2 is the mixed venous O_2 saturation, Pvo_2 is the pulmonary venous O_2 saturation, and Pao_2 is the pulmonary arterial O_2 saturation. The superior vena caval O_2 saturation is generally taken as representative of the Mvo_2 when there is left-to-right intracardiac shunting. This formula can be used to calculate Qp/Qs with any sort of lesion, presuming that the relevant O_2 saturations can be measured. This calculation can be simplified in certain situations; for example, if there is no right-to-left shunt, then $Pvo_2 = Sao_2$. Likewise, if there is no left-to-right shunt, then $Pao_2 = Mvo_2$. It cannot be used when multiple sources of pulmonary or systemic flow are present, with each source having a different O_2 saturation. For example, with right-to-left ductal shunting, O_2 saturations in the ascending aorta or transverse arch and descending aorta will differ, making the calculation of Qp/Qs virtually impossible.

Effective Versus Ineffective Blood Flow

Left-to-right shunting occurs when blood that has traversed the lungs is recirculated to them without having first crossed the systemic capillary bed. Such pulmonary flow is sometimes termed *ineffective,* because little additional O_2 is picked up with more than one passage through the lungs. (Pulmonary flow composed of systemic venous blood is *effective* pulmonary flow.) Ineffective Qp represents volume pumped by the heart to no useful end, at least as far as systemic O_2 transport is concerned (see later). In an analogous fashion, blood that has traversed the lungs before being pumped to the systemic circulation is considered *effective* Qs, whereas systemic venous blood that enters the systemic arterial circulation without passage through the lungs (right-to-left shunting) is *ineffective* Qs. The concepts of effective and ineffective blood flow are useful because they help in thinking about complex cardiac physiology, such as that observed with D-transposition of the great arteries (see later).

Left-to-Right Shunting at the Ventricular or Great Artery Level

Because the ventricle pumps only blood that enters it during diastole, left-to-right shunting at the ventricular or great artery level imposes a volume load on the *systemic* ventricle (LV in an otherwise normal heart). (Actually, with a large VSD, because some pulmonary venous blood enters the RV during diastole, the RV volume pumped is also somewhat increased.[34,35])

If the connection between the systemic and pulmonary circulations (e.g., VSD or PDA) is large enough so that the systolic pressure in the two circuits is equal, the communication is said to be *unrestrictive*. With an unrestrictive defect, the *Qp/Qs is primarily determined by the ratio of total pulmonary resistance to SVR*. (With a small defect, the pressure difference between the two ventricles and the size of the communication are usually the primary determinants of the shunt magnitude.) However, not only PVR but also *all* resistances to flow affect Qp/Qs; that is, relevant variables include not only the cross-sectional area of the pulmonary microcirculation but also the size of the central pulmonary arteries and large pulmonary veins, any impediment to ventricular filling (such as obstruction to ventricular inflow), and the compliance of the ventricle. Because the systemic and pulmonary microvascular resistance can change substantially with physiologic, pharmacologic, and other perturbations,[8,36] the Qp/Qs can vary considerably.

As a relevant aside: in an anatomically normal heart, PAP is a function of Qp and total pulmonary resistance. However, the *systolic PAP will always be essentially the same as the ventricle or artery to which it is connected* (assuming that the connection is unobstructed), regardless of the volume of flow through the lungs or the resistance to flow. Therefore, with an unrestrictive VSD or PDA, systolic PAP will be essentially the same as aortic pressure regardless of the magnitude of flow or PVR. In neonates with unrestrictive ventricular or great vessel communications large shunts generally develop as their PVR falls after birth, but the systolic PA pressure remains elevated. High PVR with unrestrictive communications is reflected by a low Qp/Qs, not the pulmonary arterial systolic pressure per se. (Pulmonary arterial *diastolic* pressure is, however, a reflection of PVR with an unrestrictive VSD.) Thus pulmonary arterial systolic hypertension and increased PVR are related but distinct concepts (see Figure 69-1).

Other factors besides PVR and SVR also influence cardiovascular physiology with ventricular or great artery–level shunting lesions. For example, Jarmakani and colleagues[37] demonstrated that infants younger than 2 years old with a PDA had greater LV end-diastolic pressure and end-diastolic wall stress than patients with a VSD and comparable magnitude of shunt. The reason for this is unclear, but it may be related to differences in LV stroke work with the two lesions.[37] *Diastolic runoff* into the pulmonary arteries with connections between the aorta and pulmonary artery may decrease aortic diastolic pressure and hence may reduce coronary perfusion.[38] *Streaming* of blood can affect whether pulmonary venous blood ends up in the aorta or in the pulmonary artery. For example, with double-outlet RV, the location of the VSD (subpulmonary vs. subaortic) will influence the volume of *effective* Qp (with a subpulmonary VSD, pulmonary venous blood tends to be recirculated to the lungs rather than find its way into the aorta).[39] Left-to-right shunting from the *LV to the right atrium* can occur, mostly with atrioventricular canal defects. The magnitude of an LV-to-RA shunt is primarily determined by the effective size of the LV–right atrial communication; PVR and right atrial pressure have no appreciable role.

Left-to-Right Shunting at the Atrial Level

Left-to-right shunts across an atrial septal opening increase the volume of blood pumped by the pulmonary ventricle (the RV in an otherwise normal heart). With a small defect, left atrial mean

pressure is usually substantially different from that of the right atrium, and shunting is primarily the result of this pressure difference. Given a large defect (in which the mean atrial pressures are essentially the same), the magnitude of shunting is generally ascribed to the relative compliances of the left atrium–pulmonary veins–LV and the right atrium–vena cava–RV: blood tends to flow into the venous chamber or ventricle that most readily accepts it (see Figure 69-1).[40] This finding explains the presence of left-to-right shunting in older patients, because the RV is thinner than the LV and is more compliant. Because substantial atrial shunting can occur in the neonate,[41,42] whose RV and LV presumably have similar compliances, Rudolph[43] proposed that such shunting may be a function of *differential ejection* of blood from the two ventricles, given that RV afterload is less than that of the LV. Left-to-right atrial shunting also occurs *with partial anomalous pulmonary venous connection* to the right side of the circulation. Assuming that there is no defect in the atrial septum, the shunt magnitude is determined by the fraction of Qp that goes to the lobes of the lungs drained by the anomalously connected veins, which is a function of the total resistance to blood flow through those lobes relative to lobes with normal venous connection to the heart. It is unusual for atrial level shunts in *children*—even if the Qp is very large—to be associated with significant pulmonary hypertension. However, in the neonate, the capacity for flow-related dilation or recruitment in the pulmonary vascular bed is apparently limited, and large *atrial* level shunts (e.g., unobstructed total anomalous pulmonary venous connection) are often accompanied by considerably increased PAP.[44-47]

Right-to-Left Shunting

Right-to-left shunting occurs when systemic venous blood enters the systemic arterial circulation without having passed through the pulmonary microcirculation. Isolated right-to-left shunting is unusual (tetralogy of Fallot being the obvious exception), and in most congenital heart defects with right-to-left shunting (especially if the ductus is patent) a left-to-right shunt is also present.

Determinants of Systemic Arterial Oxygen Saturation With Right-to-Left Shunting

Whenever there is right-to-left shunting, *systemic arterial O_2 saturation* is primarily a function of four variables:

1. The *fraction of systemic arterial blood composed of systemic venous blood.* No matter how complex the pattern of intracardiac shunting is, with right-to-left shunting a certain fraction of the aortic blood will not have crossed the lungs, and this results in a reduced systemic O_2 saturation. The greater the fraction of systemic arterial blood that did not pass through the lungs, the lower the arterial O_2 saturation.
2. *Mixed venous O_2 saturation*. The O_2 saturation of venous blood that reaches the aorta will obviously affect the O_2 saturation of the aortic blood. Because the mixed venous O_2 saturation reflects what O_2 is left in the blood after it has crossed the systemic capillary bed, this value is influenced by the following:
   a. Hematocrit. The greater the O_2 carrying capacity of the blood, the greater the SOT will be, and, given a fixed quantity of O_2 unloaded to the tissue, the greater the venous O_2 saturation.
   b. Qs. Given a fixed amount of O_2 unloaded into the tissues, the greater the quantity of O_2 transported, the greater the venous O_2 saturation will be.
   c. V_{O_2}. The greater the quantity of O_2 consumed, given a fixed amount transported, the lower the venous O_2 saturation will be.
3. *Pulmonary venous O_2 saturation*. For obvious reasons, the lower the pulmonary venous O_2 saturation, the lower the systemic arterial O_2 saturation will be.
4. *The O_2 dissociation characteristics of hemoglobin*. With a leftward shift of the O_2 dissociation curve, *less* O_2 is unloaded from hemoglobin at any given P_{O_2}. Therefore, for any given end-capillary P_{O_2}, the mixed venous saturation will be a function of the P_{50} of the hemoglobin. With a right-to-left shunt, a leftward shift of the curve will increase the mixed venous O_2 saturation and therefore will *increase* the systemic arterial saturation.

Systemic Oxygen Transport With Right-to-Left Shunting

SOT and O_2 delivery to the tissues are not necessarily proportional to arterial O_2 saturation with *right-to-left* shunting lesions. For example, a leftward shift in the hemoglobin O_2 dissociation curve will increase the arterial O_2 saturation with right-to-left shunting lesions; however, this increase in saturation does not imply an increase in O_2 delivery to the tissue, because less O_2 is released at any given tissue P_{O_2}. In addition, with admixture lesions, systemic O_2 transport actually *declines* as arterial O_2 saturation increases beyond a certain point (see later). *Maximum* V_{O_2} = maximum O_2 uptake in the lungs, which is a function of effective Qp, the gas exchange properties of the lung, and the O_2 capacity of the blood. Hence, if effective Qp is severely reduced, SOT will be compromised, regardless of Qs or any other factors.

Isolated Right-to-Left Shunting

Isolated right-to-left shunting occurs at the *ventricular level* when a VSD is present and resistance to Qp (resulting from obstruction at the level of the RV outflow tract or points beyond) is greater than resistance to flow into the systemic vascular bed, thus causing a shunt of desaturated (RV) blood into the aorta. Right-to-left *ductal* shunting occurs when total pulmonary resistance is greater than SVR, which is most often the result of increased PVR but can also be caused by pulmonary venous or left atrial hypertension. There is also right-to-left ductal shunting when the descending aorta is mostly or entirely supplied by the RV, such as severe coarctation of the aorta. In such cases, however, there is almost always a component of left-to-right shunting—at the atrial or ventricular level—as well. The magnitude of right-to-left shunting with ventricular or arterial communications is determined by the ratio of resistance to flow into and through the pulmonary circuit relative to the systemic circuit. Because SVR and PVR (and sometimes even the degree of RV outflow tract obstruction, as in tetralogy of Fallot) may fluctuate with time, the magnitude of right-to-left shunting is variable. Right-to-left shunting across an atrial defect most commonly results from a reduction in RV compliance, usually because of RV hypertrophy or hypertension (Figure 69-1).

Bidirectional Shunting

Both left-to-right shunting and right-to-left shunting are present in various cardiac lesions. For example, a patient with severe aortic stenosis (and severely reduced LV ejection) with an open ductus arteriosus will often have a left-to-right atrial shunt, as well as a right-to-left shunt across the ductus to the aorta. Right-to-left atrial shunting can also occur simultaneously with a left-to-right ventricular shunt (e.g., with an atrioventricular septal defect).

With total anomalous connection of the pulmonary veins, all pulmonary veins connect to the systemic veins, coronary sinus, or right atrium; thus a fraction of both the pulmonary and systemic venous return crosses the atrial septum to supply the LV. The variables that influence Qp (the mixture of systemic and pulmonary venous blood that finds its way into the RV) and Qs (the mixture of systemic and pulmonary venous blood entering the LV) likely include the size of the atrial opening,[47] the relative ventricular compliances, and possibly any preferential streaming of venous

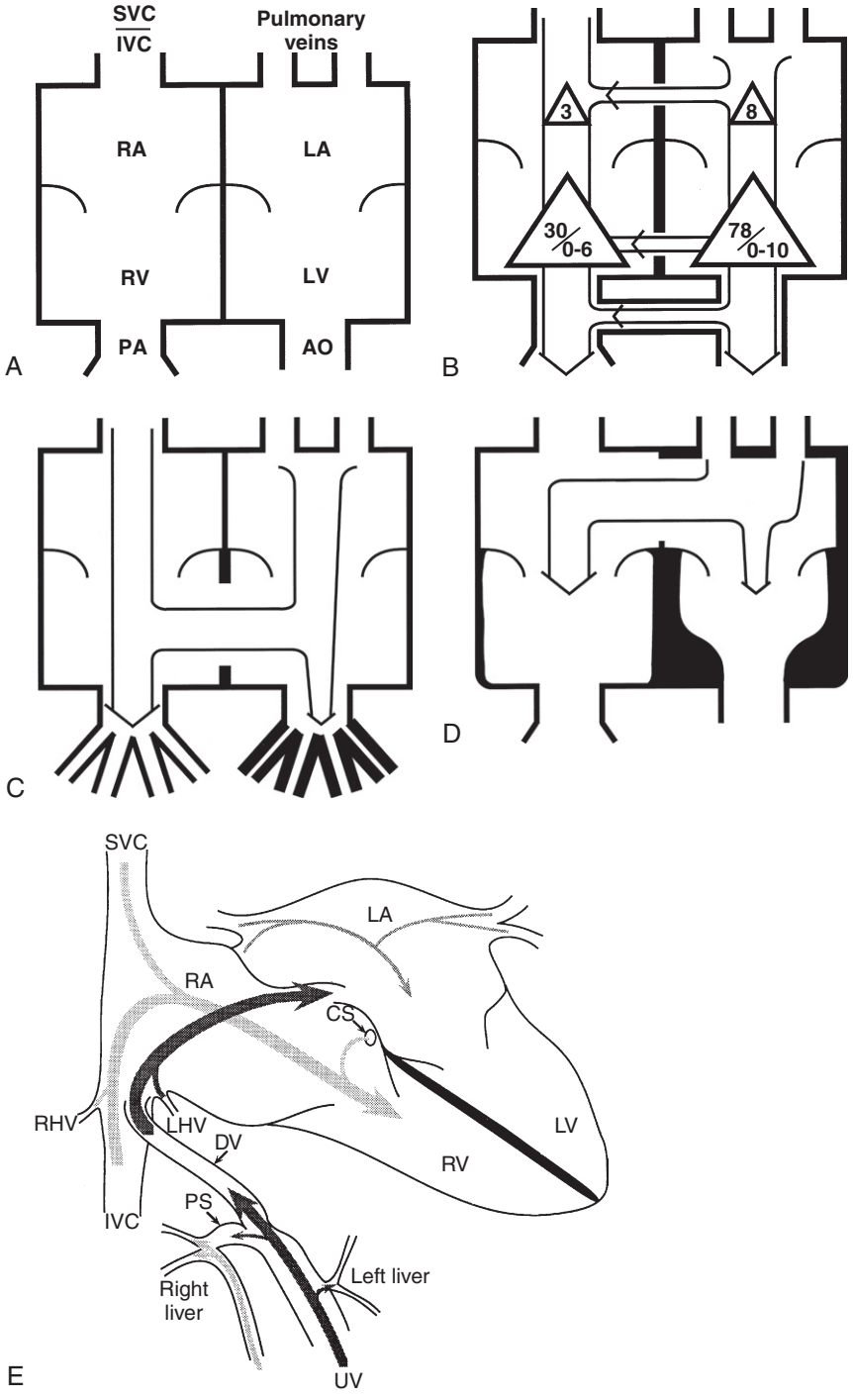


Figure 69-1 Factors that influence where blood goes with structural heart lesions. A, General scheme of box diagrams. **B,** Blood moves from high pressure to low. With relatively small communications, shunting at the atrial, ventricular, and great vessel levels can result from a pressure gradient between communicating structures. **C,** When two vascular beds differing in resistance to flow are connected to a source of flow, more blood finds its way into the lower- than into the higher-resistance circuit. With an unrestrictive VSD and low PVR, systolic pressures are essentially the same in both the aorta and the pulmonary artery, yet there is more flow to the pulmonary artery. **D,** When two chambers differing in resistance to filling (compliance) are connected to a source of flow, more blood finds its way into the chamber with the greater compliance. **E,** Streaming can influence the chamber or vessel to which the blood flows. As depicted here, in the fetus umbilical venous blood preferentially crosses the foramen ovale, and therefore the most highly oxygenated blood tends to go to the organs of greatest need. Streaming can also take place at the ventricular and great vessel level. *AO,* Aorta; *CS,* coronary sinus; *DV,* ductus venosus; *IVC,* inferior vena cava; *LA,* left atrium; *LHV,* left hepatic vein; *LV,* left ventricle; *PA,* pulmonary artery; *PS,* portal sinus; *RA,* right atrium; *RHV,* right hepatic vein; *RV,* right ventricle; *SVC,* superior vena cava; *UV,* umbilical vein. Numbers in *triangles* indicate pressure. *(***E,** *From Teitel DF, Cassidy SC:* Moss and Adams' Heart Disease in Infants, Children, and Adolescents*, vol 1, ed 5, Baltimore, 1995, Williams & Wilkins, 1995, p 49.)*

blood. Whether streaming of venous blood actually affects Qp per se is unclear, but it can affect the arterial O_2 saturation[44,47] because pulmonary venous return from the inferior vena cava (with infradiaphragmatic total anomalous pulmonary venous connection) can preferentially stream across the foramen ovale into the left atrium. With a total anomalous pulmonary venous connection (assuming no other communication between the pulmonary and systemic arterial circuits), PVR only affects the pattern of intracardiac shunting insofar as it influences RV pressure and therefore RV compliance and systolic function. (Another physiologic consequence of obstructed pulmonary venous connection is pulmonary edema, which interferes with gas exchange and reduces lung compliance.) The shunting physiology with cerebral arteriovenous malformations is similar in many ways[46] (see Figure 69-2).

Admixture Lesions

Cardiac lesions that cause complete mixing of systemic and pulmonary venous blood are sometimes termed *admixture lesions*. All malformations with a single functional ventricle (e.g., hypoplastic left heart syndrome, tricuspid atresia) have this physiology, as well as some two-ventricle defects (e.g., tetralogy of Fallot with pulmonary atresia). Actually, there can be preferential streaming of pulmonary or systemic venous blood into one or the other great artery in some lesions with substantial mixing of pulmonary and systemic venous blood (e.g., truncus arteriosus), but, unless the quantity of "streamed" blood is significant, the physiology is best understood as admixture.

Determinants of Systemic Arterial Oxygen Saturation With Admixture Lesions

With admixture lesions, systemic arterial O_2 saturation is a function of all the variables outlined earlier. The fraction of aortic blood composed of pulmonary venous blood is determined by the *Qp/Qs:* because all the pulmonary venous blood mixes with all the systemic venous blood, the ratio of "blue" and "pink" blood will influence aortic O_2 saturation. For lesions in which the systemic and pulmonary arterial circuits are in unrestricted communication, the Qp/Qs is primarily determined by the ratio of total pulmonary resistance to SVR. When mixing of venous blood occurs at the atrial level and systemic and pulmonary arterial circulations are separate (e.g., total anomalous pulmonary venous connection), Qp/Qs may be largely determined by other factors (see earlier) (see Figure 69-2).

Systemic Arterial Oxygen Saturation Versus Systemic Oxygen Transport in Admixture Lesions

The variables that determine systemic arterial O_2 *transport* in admixture lesions (usually more relevant to tissue oxygenation than arterial O_2 saturation per se) are more complex. At any given ventricular output, every milliliter of blood pumped to the lungs is one less milliliter that goes to the systemic circulation. Therefore, as the Qp/Qs (and systemic arterial O_2 saturation) *increases*, Qs *falls*. Systemic O_2 transport is therefore a function of total ventricular output, hematocrit, pulmonary venous O_2 saturation, Vo_2, and Qp/Qs; the relationship between Qp/Qs and systemic O_2 transport is sigmoidal[48–50] (Figure 69-3).

Transposition Physiology

With D-transposition of the great arteries, the aorta arises from the RV and the pulmonary artery from the LV. Because systemic and pulmonary venous blood enters the right and left atria, respectively, the systemic and pulmonary circulations function in parallel rather than in series. Systemic venous blood, largely depleted of O_2, entering the RV is pumped out again to the body, whereas oxygenated pulmonary venous blood is ejected into the lungs.[51] Without any communication between the two circuits, all blood flow would be ineffective, and life would cease shortly after the placenta was disconnected from the systemic circulation. As it happens, communications between the systemic and pulmonary circuits usually exist at one or more levels (atrial septum, ventricular septum, ductus arteriosus) and, if of sufficient size, usually allow for enough *mixing* of systemic and pulmonary venous blood for adequate systemic arterial O_2 saturation (Figure 69-4). (Note, however, that rarely is a communication at the ductal or ventricular level alone sufficient—atrial level mixing is almost always required for adequate arterial O_2 saturation.)

The essence of mixing is (in a steady-state system) that for every milliliter of systemic venous blood that crosses from the right side of the heart, or the aorta, into the left side of the heart or pulmonary artery (and then into the lungs, constituting effective Qp), 1 mL of pulmonary venous blood must cross from the left side of the heart into the right side of the heart and into the aorta (becoming effective Qs). Were more blood to flow in one direction than the other, one of the two circulations would become depleted of blood. That is not to say that the volumes of blood pumped by the LV and RV need be equal, just that the average amount of blood crossing from left to right must equal the amount moving from right to left.[51] Indeed, whereas in the neonate with this lesion the LV volume pumped is similar to that of a normal neonate, by approximately 6 months of age it is considerably greater than normal.[52,53]

Echocardiographic studies have shown that infants with D-transposition and an atrial septal opening have shunting from the left atrium to the right atrium during systole and right-to-left blood flow during diastole.[54] The factors determining mixing have not been precisely determined, although it is clear that Qp is strongly positively related to the aortic O_2 saturation.[55] Thus the presence of a PDA increases mixing by increasing Qp.[56] Conversely, increased PVR in neonates with D-transposition can markedly reduce the aortic saturation.[57] The number of sites of mixing also plays a role, with arterial O_2 saturation increasing with the number of mixing sites.[55]

Whereas cyanosis is usually the key physiologic feature of the newborn with this lesion (although some neonates with D-transposition and a large VSD may be pink), CHF can develop in the neonate with D-transposition and a large PDA or VSD.[56,58] With a large VSD, the pattern of blood flow has not been fully elucidated, but there appears to be increased volume pumped by both ventricles,[59] and the Qp/Qs can be quite large.

Single-Ventricle Physiology

Multiple factors affect the physiology of neonates with complete mixing of systemic and pulmonary venous blood and communication at the level of the ventricle or great arteries (sometimes referred to as *single-ventricle physiology*). This physiology actually pertains to some lesions with two ventricles (e.g., truncus arteriosus or tetralogy of Fallot with pulmonary atresia), as well as with as the single ventricle. (With single-ventricle physiology, it is unclear whether the number of ventricles materially affects the hemodynamics, at least in the neonate. It may seem that two separate pumping chambers would be better than one at providing SOT in this context, but I am aware of no relevant data in this regard.)

1. With single-ventricle physiology (and therefore some fraction of Qp is ineffective), *volume work is increased for the ventricle* and can be considerable (see later and Figure 69-2).
2. In patients with a left-sided obstructive lesion and an unrestrictive PDA (e.g., hypoplastic left heart syndrome), the Qp tends to be very large, especially as PVR falls in the perinatal period, and hence for *Qs tends to be reduced*, as discussed earlier. This is less the case for right-sided obstructive ductal-dependent lesions (e.g., pulmonary atresia with intact ventricular septum) because the ductus is usually restrictive.

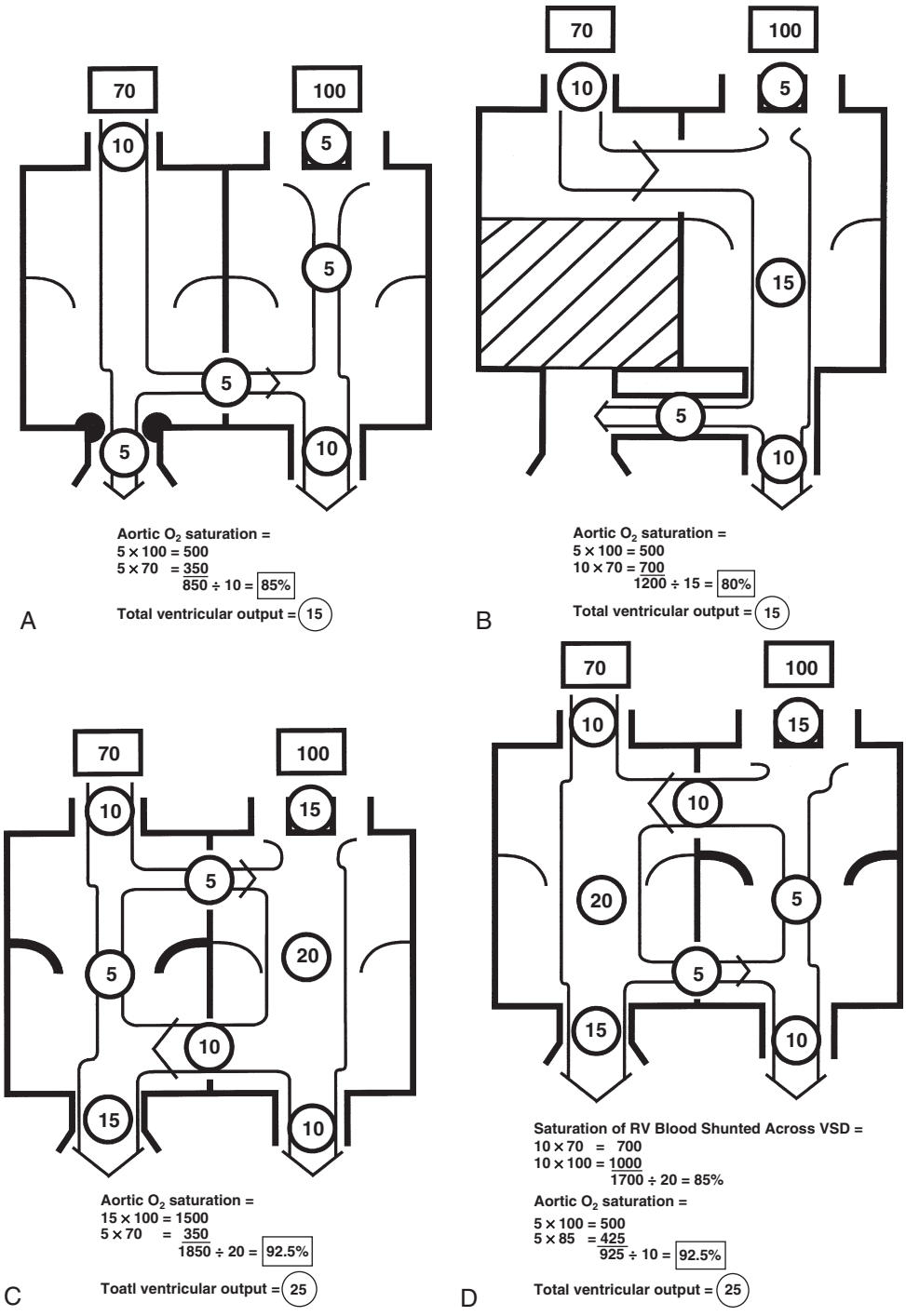


Figure 69-2 Patterns of intracardiac blood flow and ventricular output with right-to-left shunting. This illustration aims to provide a conceptual understanding of the relationship among intracardiac shunting, ventricular output, and oxygen saturation, but it is a simplified view relative to cardiac physiology; for example, given a fixed oxygen consumption, it would be unrealistic to expect systemic venous saturation to remain constant as SOT varies. Numbers in *boxes* indicate blood O_2 saturation; numbers in *circles* indicate the relative amount of blood flow. **A,** With an isolated right-to-left shunt (e.g., tetralogy of Fallot), all pulmonary blood flow is effective, and combined ventricular output is normal. **B,** With admixture physiology, there is ineffective pulmonary blood flow, and therefore systemic arterial saturation is less, for a given amount of ventricular output, than for a lesion with isolated right-to-left shunting. **C** and **D,** With bidirectional shunting, equal volumes of right-to-left shunting at either the atrial or ventricular level will result in equal systemic arterial oxygen saturation and equal ventricular output. The relationship between systemic arterial oxygen saturation and ventricular output depends on the volume of ineffective pulmonary blood flow (left-to-right shunt). *RV,* Right ventricular; *VSD,* ventricular septal defect.

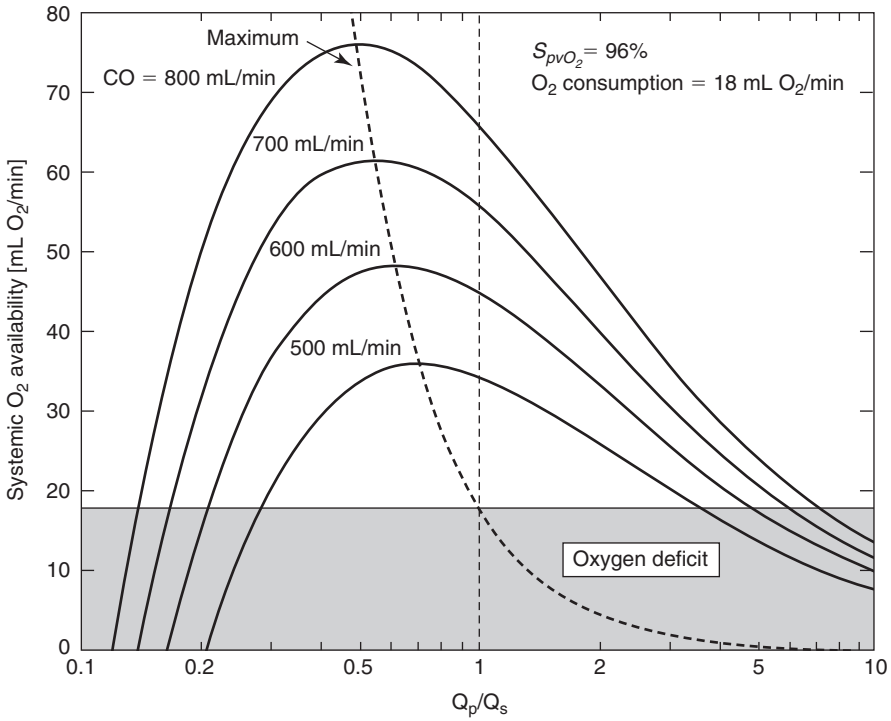


Figure 69-3 The relationship between systemic oxygen availability (transport) and the ratio of pulmonary to systemic blood flow (Qp/Qs) with cardiac admixture lesions. Barnea and colleagues[48] used a mathematical model to determine SOT for a patient with a single ventricle, with the aorta and pulmonary artery in communication, assuming the oxygen capacity of the blood to be 22 mL oxygen per deciliter, pulmonary venous oxygen saturation ($Spvo_2$) to be 96%, and oxygen consumption to be 18 mL oxygen per minute (the normal mean value for a 3-kg neonate). SOT is a function of both cardiac output and the ratio of pulmonary to systemic blood flow. Maximum SOT occurs with a Qp/Qs of <1, a value lower than that typical of most patients with hypoplastic left heart syndrome[86] and probably most patients with a "single-ventricle" lesion. *(From Barnea O, Austin EH, Richman B, et al: Balancing the circulation: theoretic optimization of pulmonary/systemic flow ratio in hypoplastic left heart syndrome,* J Am Coll Cardiol *24:1376, 1994.)*

3. With single-ventricle physiology, *arterial O_2 saturation is less* than normal, also contributing to a tendency to reduced SOT.
4. Lesions with aortic diastolic runoff into the pulmonary circuit can have *decreased diastolic perfusion pressure* and therefore decreased myocardial perfusion.[38] Diastolic retrograde flow away from the mesenteric arteries, perhaps in combination with reduced SOT for the reasons noted earlier, also appears to increase the risk for necrotizing enterocolitis.[60]

The concurrence of these factors, especially in infants with high Qp, often leads to CHF and sometimes even substantially reduced SOT. For example, many babies with truncus arteriosus have severe CHF and even die in the neonatal period,[61] presumably because of the combination of large ventricular volume load, cyanosis, and reduced myocardial perfusion.

HOW CONGENITAL HEART LESIONS AFFECT CARDIOVASCULAR PHYSIOLOGY IN THE NEONATE: GENERAL MECHANISMS

Because one's ultimate aim is to understand not general principles of physiology but rather individual patients, it is necessary to link these general principles with specific cardiac lesions. However, given the large number of distinct malformations, it is not feasible to describe the physiologic characteristics of each one. Even describing various classes of lesions (e.g., defects with left-to-right shunting) is unsatisfactory, because lesions within a class vary significantly in physiologic detail. The approach taken here is to describe several *characteristics of the efficient cardiovascular system,* to indicate how a heart with a given malformation *lacks* one or more of these characteristics, and the resulting physiologic consequences.

Adequate Capacity to Pump Blood to the Lungs and Body

The capacity to supply blood requires one or two pumping chambers of adequate size, with acceptable rhythm and systolic and diastolic function, in communication with the greater and lesser circulations. Only one ventricle is required for fetal development and postnatal survival, and inadequate pumping capacity resulting from a lack of ventricular capacity is rare in neonates with congenital heart disease. Far more commonly, an adequate pump is not connected to the systemic (e.g., the RV in hypoplastic left heart syndrome with ductal closure) or pulmonary circulation (e.g., the LV in pulmonary atresia with intact ventricular septum with ductal closure). Whereas modest degrees of ventricular dysfunction are not uncommon in neonates with cardiac lesions (especially those who have severe hypoxia or ischemia), frankly inadequate systolic or diastolic ventricular function resulting from intrinsic abnormality of the myocardium is rare. The infant with a coronary artery anomaly, such as anomalous origin of the left coronary artery[62] or pulmonary atresia with intact ventricular septum and coronary artery stenoses,[63] can have ventricular dysfunction resulting from myocardial ischemia, but these patients are very uncommon.

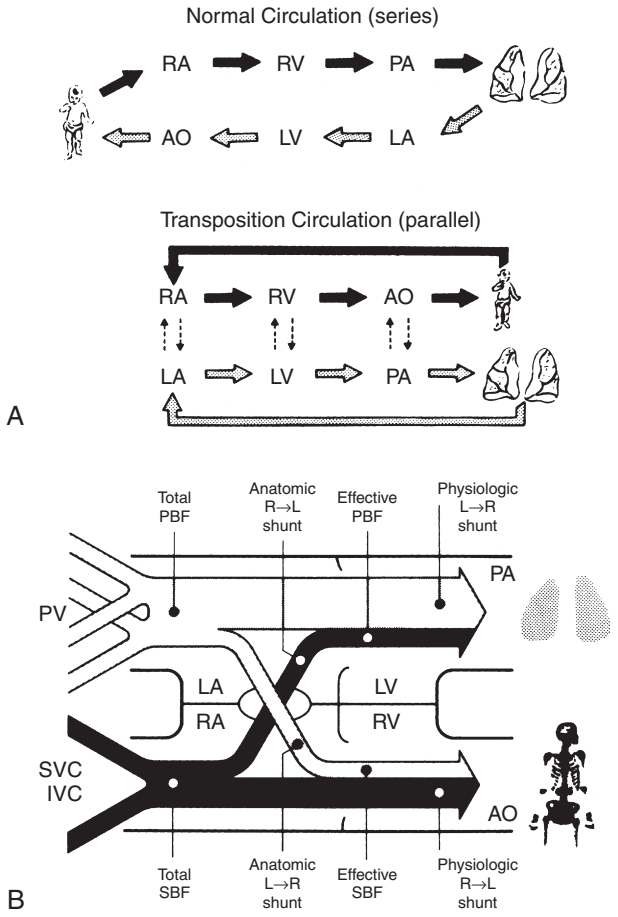


Figure 69-4 A and **B,** Intracardiac blood flow with D-transposition of the great arteries. Most pulmonary and systemic blood flow is ineffective. Systemic oxygen delivery depends on the passage of blood from the right side of the heart into the lungs (effective pulmonary blood flow) and the passage of an equal amount from the left side of the heart to the aorta (effective systemic blood flow). *AO,* Aorta; *IVC,* inferior vena cava; *LA,* left atrium; *LV,* left ventricle; *PV,* pulmonary vein; *RA,* right atrium; *RV,* right ventricle; *SBF,* systemic blood flow; *SVC,* superior vena cava. *(From Paul MH, Wernovsky G: Transposition of the great arteries.* Moss and Adams' Heart Disease in Infants, Children, and Adolescents, *vol 2, ed 5, Baltimore, 1995, Williams & Wilkins, 1995, pp 1166-1170.)*

Appropriate Afterload

The term *afterload* is used here to denote the resistance against which the heart pumps. Although convenient, this is, for a variety of reasons, a crude way to think of afterload (e.g., resistance to ventricular ejection imposed by small blood vessels has a different physiologic effect than that caused by semilunar valve stenosis).

Effect of Increased Afterload on Cardiovascular Physiology

The amount of blood ejected by the ventricle is inversely related to the magnitude of afterload against which it ejects.[64,65] Although the normal heart can to some extent maintain its output in the presence of *acutely* increased afterload,[65,66] this reserve is limited, especially for a dysfunctional ventricle. Because the neonatal heart has less contractile reserve than the more mature one,[67] this is especially pertinent. Furthermore, because the major determinant of myocardial V_{O_2} is pressure work,[68,69] the ventricle's ability to respond to an increase in afterload is crucially dependent on coronary perfusion pressure[70]; when increased afterload occurs with normal or decreased aortic pressure (e.g., severe valvar aortic stenosis), myocardial O_2 supply is especially likely to be inadequate.

Chronically increased afterload causes myocardial hypertrophy, which is usually adequate to maintain ventricular systolic function. Whereas hypertrophy is an adaptive response insofar as it normalizes wall stress,[71] *ventricular compliance* is reduced by hypertrophy, manifested in *increased atrial pressure*. Left atrial hypertension can cause pulmonary edema, or left-to-right shunting if there is an atrial opening, and "reflex" pulmonary vasoconstriction,[72,73] thereby increasing PAP beyond that accounted for by the elevation in LAP alone. Pressure overload of the RV can also cause increased right atrial pressure and systemic venous congestion. However, because the foramen ovale is usually nonrestrictive to right-to-left flow in the neonate, a reduction in RV filling is usually reflected by a right-to-left atrial shunt, rather than by systemic venous hypertension. Finally, *subendocardial ischemia* from severe chronic pressure overload can lead to endocardial fibrosis and to papillary muscle dysfunction or infarction and hence mitral regurgitation.[74]

Congenital Cardiac Lesions Causing Increased Afterload

The effects of increased afterload are clearly manifest in neonates with severe valvar *aortic stenosis*.[74] LV output is reduced,[75] sometimes so much so that adequate systemic perfusion requires maintaining patency of the ductus to allow the RV to contribute to Qs. Left atrial hypertension often causes a considerable left-to-right atrial shunt through the foramen ovale, pulmonary edema, and pulmonary arterial hypertension. Whereas relief of the increased afterload by balloon dilation of the aortic valve results in an immediate decrease in (but not normalization of) LAP,[76,77] LV systolic function remains abnormal for some time—at least days and probably weeks—after dilation.[78] *Severe coarctation of the aorta* in the neonate also causes reduction in LV systolic and diastolic function and output, as well as left-to-right atrial shunting. PAP is markedly increased, largely resulting from left atrial hypertension.[79]

Because the fetal RV operated at (and is therefore well adapted to) systemic pressure,[6] *increased RV pressure* does not appear to have a measurable impact on RV systolic performance in a neonate, as long as it is approximately at or lower than systemic blood pressure. However, neonates with *severe valvar pulmonary stenosis* with suprasystemic RV pressure will have reduced RV output and an atrial right-to-left shunt.[80] Right-to-left atrial shunting, often enough to cause substantial arterial desaturation, may persist for weeks after reduction of RV afterload by balloon dilation, a reflection of the relatively slow pace of normalization of RV compliance.[81]

Effect of Decreased Afterload on Cardiovascular Physiology

Because pressure is a function of vascular resistance and flow, inappropriately *low* SVR will cause hypotension unless Qs is increased. Lower-than-normal SVR (resulting from dilation of systemic arterioles) occurs in some settings (e.g., with sepsis), but it does not seem to be an inherent feature of congenital cardiac malformations. In lesions in which the systemic circulation is in unrestricted communication with the venous (e.g., arteriovenous malformation)[46] or pulmonary (e.g., large VSD or PDA) circulation, outflow resistance to systemic ventricular ejection is reduced (at least if PVR is lower than SVR, in the case of the latter). This does not generally have a clinically significant impact on blood pressure because total blood flow into the systemic and pulmonary circuits is usually sufficient to maintain normal blood pressure. However, the relatively low blood pressure associated with a large PDA in the premature infant[82,83] suggests that in these cases total (systemic plus pulmonary) flow is insufficient to compensate for the low total vascular resistance, resulting from inclusion of the pulmonary circuit (see later).

All the Blood Pumped by the Heart Moves Toward the Systemic Capillaries

In other words, the valves do not leak, and there is no left-to-right shunt. Valvar regurgitation and left-to-right shunting result in an inefficient circulation because the heart pumps volume that does not participate in gas exchange or SOT. Lumping together these multiple causes of volume overload is in one sense a crude approach, because these lesions perturb cardiac mechanics and biology differently. For example, acute mitral regurgitation increases LV systolic and diastolic wall tension much less than aortic regurgitation does.[84] Furthermore, as noted previously, left-to-right shunting at the ventricular level affects the LV differently than ductal level shunting does. The virtue of considering these defects together is that their physiologic impact is in many ways very similar (increased ventricular volume load, ventricular dilation, CHF).

Effect of Valve Regurgitation on Cardiovascular Physiology

Regurgitation of either the semilunar or the atrioventricular valves increases the volume pumped by the relevant ventricle. The magnitude of semilunar valve regurgitation is influenced by the degree of valve deformity, the heart rate, and the vascular resistance of the relevant circulation. Mild regurgitation has minimal effects on patient physiology, but more severe volume overload may be attended by symptoms of CHF, increased atrial pressure, and even reduced Qs. *Aortic valve regurgitation,* by reducing coronary perfusion pressure, may also compromise myocardial perfusion.[38] *Pulmonary valve* regurgitation may be more pernicious in the neonate relative to the older patient because PVR is likely to be higher in the newborn.

Effect of Left-to-Right Shunting on Cardiovascular Physiology

As with valve regurgitation, left-to-right shunts of sufficient size cause symptoms of CHF. Profound reduction in Qs, as may be seen with severe aortic stenosis, is unusual with left-to-right shunting lesions. More modest compromise in Qs may occur with a large left-to-right shunt, although there are few quantitative data regarding neonates.

Other physiologic perturbations, although not a direct consequence of the shunting per se, may attend lesions that cause left-to-right shunting: (1) with a large communication between the aorta and pulmonary artery, diastolic pressure and hence myocardial perfusion may be reduced[38]; and (2) shunting lesions attended by increased PAP are associated with decreased lung compliance.[85]

How Much Volume Reserve Does the Neonatal Heart Have?

The neonatal ventricle is capable of pumping considerably more blood than normal under certain circumstances. For example, Tabbutt and co-workers[86] measured the Qp/Qs in 10 anesthetized and hemodynamically stable neonates with hypoplastic left heart syndrome; they found that the mean Qp/Qs was approximately 3.4:1. Because neither actual Qp nor actual Qs was measured, the output from the (single) RV could not be determined. However, if one assumes that Qs was only 50% of usual (likely an underestimate), the single ventricle (RV) in these patients pumped 2.2 times as much blood as normal.

Data from animals are conflicting regarding how much extra volume can be pumped without significantly compromising Qs. In one study of mechanically ventilated preterm newborn lambs, relevant blood flows were measured with the ductus arteriosus open and closed; an approximately 60% increase in LV output resulting from opening the ductus was associated with a significant fall in Qs.[87] A similar study, however, showed no significant fall in Qs even with a 100% increase in LV output with opening of the ductus arteriosus.[88] Both studies were conducted over an hour or two and may not be reflective of long-term physiology. The newborn *human* with a PDA can have an LV output greater than 150% of normal,[89-92] but information regarding Qs in these neonates is very limited. Data from a few neonates with a PDA suggest that LV output of more than three times normal may be generated with Qs greater than 3 L/m^2/minute,[89,91] although accurate measurement of Qp in this setting is very difficult. Infants (generally >1 month old) with a VSD may have an LV output of three to four times normal, assuming that normal cardiac output = 4.2 ± 1.2 L/m^2/minute,[93] while still maintaining Qs greater than 3 L/m^2/minute.[11,21,22]

Congenital Cardiac Lesions With Valve Regurgitation

Atrioventricular valve regurgitation is uncommon in the neonate, although it can occur as an isolated lesion, with cardiac malformations,[74,94,95] or in association with papillary muscle dysfunction resulting from neonatal asphyxia.[96] Aortic regurgitation is exceedingly unusual (absent surgical intervention on the aortic valve). With absent pulmonary valve syndrome (tetralogy of Fallot with absent pulmonary valve), there is severe pulmonary regurgitation, but it is unclear precisely how this affects cardiac physiology because these patients also have a large VSD and RV outflow tract obstruction. With this condition, clinical phenotype is usually primarily determined by large airway abnormalities.[97]

Congenital Cardiac Lesions With Left-to-Right Shunting

Most cardiac lesions in the neonate have at least a component of left-to-right shunting. An isolated *VSD* causes left-to-right shunting, although, for the reasons noted earlier, symptoms of CHF usually do not develop for a few weeks. However, premature babies with a large VSD can have symptoms within 1 to 2 weeks of age, perhaps in part because PVR declines more rapidly than in the term infant.[32] A large PDA is usually associated with a large left-to-right shunt, especially in the premature infant.

It is also very unusual, but well described,[42] for left-to-right atrial level shunting through an isolated *atrial septal defect* to cause symptoms in infants. Left-to-right atrial shunting across the foramen ovale may be substantial in neonates with left-sided obstructive lesions (e.g., aortic stenosis, coarctation).[98] (This shunting is actually very helpful, because the left ventricle may have a limited capacity to accept pulmonary venous return in these lesions—especially with critical aortic stenosis and hypoplastic left heart syndrome—and without an atrial opening pulmonary venous pressures are therefore high and pulmonary blood flow limited.)

All Systemic Venous Blood Passes Through the Lungs Before Being Pumped to the Systemic Circulation

In other words, there is no right-to-left shunting. Actually, in the neonate, mildly to moderately reduced systemic arterial O_2 saturation may have little detectable impact on physiology; the unfavorable effects of chronic hypoxia (e.g., reduced exercise capacity, polycythemia, brain abscess, stroke, and other neurologic symptoms) are rarely manifest in the neonate. However, it is unclear where to draw the line between moderate hypoxemia (with little or no physiologic impact) and severe hypoxemia (which impairs organ function) in the neonate. As previously noted, other determinants of SOT (especially Qs and hematocrit) and Vo_2 undoubtedly influence what level of hypoxemia is compatible with aerobic metabolism, and there must be intraindividual variation as well. It seems clear that an arterial Po_2 in the mid-20 mm Hg range (and probably even somewhat lower) can be tolerated for at least hours or days in most patients, presuming that other factors influencing SOT are acceptable. A lack of metabolic acidosis is often taken as an indication that SOT (and hence Po_2)

is acceptable, but whether a lack of acidosis with hypoxemia correlates with a lack of long-term sequelae (especially central nervous system dysfunction) has not been clearly established.

Congenital Cardiac Lesions With Right-to-Left Shunting

Hypoxemia is usually the primary or sole physiologic manifestation of most lesions with *reduced Qp* (e.g., tetralogy of Fallot or severe valvar pulmonary stenosis). When some or all Qp is provided by a ductus in a patient with a right-sided obstructive lesion, there will be ineffective Qp and therefore increased volume pumped by the ventricle, but this volume load is usually modest, presumably because the ductus is somewhat restrictive with those lesions. Patients with lesions with *admixture physiology* and an unrestrictive communication between the systemic and pulmonary circulations (e.g., single-ventricle lesions without significant pulmonary stenosis) usually have only mild hypoxemia (presuming that PVR falls normally in the postnatal period), but they commonly manifest CHF resulting from increased ventricular volume load. Single-ventricle lesions with some limitation of Qp resulting from increased PVR or anatomic restriction (e.g., tricuspid atresia with a restrictive VSD) can be well balanced and may have adequate arterial O_2 saturation without CHF.

Systemic and Pulmonary Venous Blood Readily Enters the Ventricles

In other words, there is no anatomic obstruction to ventricular filling. The heart can eject only what it receives, and ventricular filling is largely a function of transmural filling pressure. Abnormalities that impair flow into the heart may reduce ventricular output, but more commonly adequate output is maintained at the expense of venous hypertension, which causes third spacing of fluid (edema, effusions). This is a serious liability, especially in neonates, and tends to beget more of the same: edema and effusions increase mean airway pressure, compress abdominal venous return, or directly compress the heart, leading to the accumulation of yet more fluid.

Congenital Cardiac Lesions With Restriction of Ventricular Filling

Isolated obstruction to ventricular inflow is very unusual in the neonate; when the tricuspid or mitral valve is small, the corresponding ventricle is usually reduced in volume. When filling of the RV is impeded (e.g., pulmonary atresia with intact ventricular septum), right-to-left flow across the foramen ovale is rarely obstructed; hence, right atrial pressure is usually normal. With mitral stenosis or atresia and hypoplasia of the LV (hypoplastic left heart syndrome), the atrial septal opening is usually large enough so that pulmonary venous return is not significantly obstructed, although the size of the opening often becomes smaller over the first few weeks of life. Occasionally, the atrial septal opening is small, with the resulting left atrial hypertension and increased total pulmonary resistance, causing a reduction in Qp. Pulmonary edema further reduces systemic arterial saturation by interfering with gas exchange and by reducing lung compliance. Qs, however, may actually be increased, because systemic venous return to the RV is not obstructed, and the increased total PVR reduces the fraction of RV output that goes to the lungs.

Isolated mitral stenosis with adequate LV size is rare in the neonate.[99] Obstruction to pulmonary venous return can occur with *total anomalous pulmonary venous connection,* cor triatriatum, and stenosis of the individual pulmonary veins, the latter two being very rare. Pulmonary arterial hypertension, a reduction of RV compliance and output, and pulmonary edema are the primary physiologic manifestations.

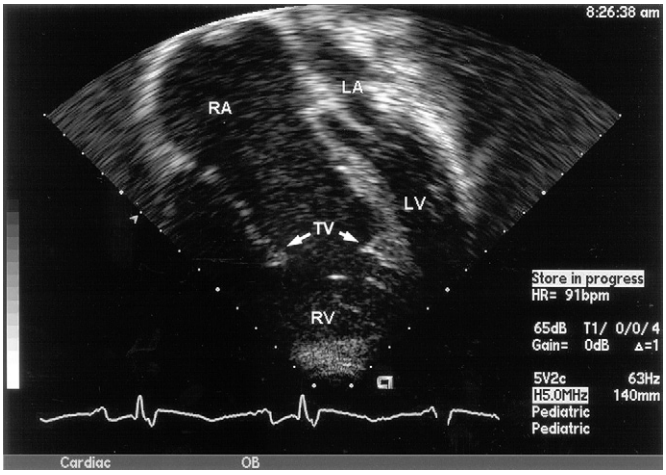


Figure 69-5 A cardiac ultrasound scan of a patient with Ebstein anomaly. The dilated right-sided structures impinge on the much smaller left ventricle *(LV). LA,* Left atrium; *RA,* right atrium; *RV,* right ventricle; *TV,* tricuspid valve.

Rarely, one massively enlarged ventricle encroaches on the other and inhibits ventricular filling, such as LV encroachment by the right atrium or RV with a severe Ebstein anomaly[95] (Figure 69-5). Other impediments to ventricular filling exist, such as pericardial effusion, that are not congenital malformations per se.

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Neural Regulation of Blood Pressure During Fetal and Newborn Life

Control of circulatory function is mediated through interacting neural, hormonal, and metabolic mechanisms acting at both central and local levels. The central nervous system in particular is critical for cardiovascular homeostasis including the maintenance of blood pressure within normal limits.[1,2] Autonomic tone to the heart and blood vessels is continuously modulated by an array of peripheral sensors, including arterial baroreceptors and chemoreceptors located in the aortic arch and carotid sinus, as well as mechanoreceptors located in the heart and lungs.[3] Brain cardiovascular centers, located between afferent and efferent pathways of the reflex arc, integrate a variety of visceral and behavioral sensations allowing for a wide range of modulation of specific autonomic, cardiovascular, and endocrine responses. While these basic mechanisms exist in the fetus and newborn, differential rates of maturation of these systems influence the ability of the developing animal to maintain adequate blood pressure and organ blood flow.

Arterial pressure displays natural oscillations within a physiologic range, the degree of which is similar in fetal and postnatal life.[4-7] In the adult, ganglionic blockade increases arterial pressure variability,[4,8] suggesting that a component of arterial pressure lability is peripheral or humoral in origin and is buffered by autonomic functions. In contrast, ganglionic blockade in term fetal sheep significantly attenuates heart rate and arterial pressure variability.[6] Changes in sympathetic tone as recorded from the renal sympathetic nerve have been shown to correlate positively with the fluctuations in heart rate and arterial pressure.[6] Oscillations in basal sympathetic tone appear to be related to changes in the behavioral state of the fetus.[9-11] Although fetal electrocortical and sympathetic activity have not been recorded simultaneously, heart rate, arterial pressure, and catecholamine levels are highest during periods of high-voltage, low-frequency electrocortical activity.[10-15] Other physiologic parameters, including organ blood flows, regional vascular resistances, and cerebral oxygen consumption are also dependent on electrocortical state and likely reflect changes in autonomic activity.[12,16,17]

Tonic discharge of spinal vasoconstrictor neurons is an important regulator of vasomotor tone early in development. However, the contribution of the autonomic nervous system on cardiovascular homeostasis changes with maturation. Both α-adrenergic and ganglionic blockade produce greater decreases in blood pressure in term fetal lambs than in preterm fetal lambs.[18,19] The hypotensive effect following α-adrenergic and ganglionic blockade is less in newborn lambs than in term fetuses and continues to decline with postnatal development.[20] These findings suggest that sympathetic tone is high late in fetal life and is important in the maintenance of fetal arterial pressure. The influence of the parasympathetic system on resting heart rate appears to increase with maturation.[21] There is evidence of choline acetyltransferase and muscarinic acetylcholine receptors in the human heart early during development.[22] In utero fetal tachycardic responses to atropine have been demonstrated at 15 to 17 weeks gestation, before the bradycardic responses from β-blockers.[22] Cholinergic blockade produces no consistent effect on heart rate in premature fetal sheep, a slight increase in heart rate in term fetuses, and the greatest effect in lambs beyond the first week of life.[19,20] Central cholinergic modulation of cardiovascular function also appears during fetal development. Acetylcholine receptors are present in the brain during the first trimester,[23] and intracerebroventricular administration of carbachol increases blood pressure in fetal sheep associated with increased neuronal c-fos expression in central cardiovascular centers.[24]

THE ARTERIAL BAROREFLEX

Arterial baroreceptors are major sensing elements of the cardiovascular system.[1,2] Acute changes in vascular stretch related to arterial pressure modify the discharge of afferent baroreceptors fibers located in the carotid sinus and aortic arch.[2] Afferent fibers located in the vagus and glossopharyngeal nerves carry signals to the lower brain stem, primarily to the nucleus of the solitary tract. Secondary fibers project to the midbrain and other regions

of the brain stem. From these centers, efferent parasympathetic and sympathetic activity is modulated. This, in turn, influences outflow, heart rate, myocardial contractility, and peripheral vascular resistance, and it buffers changes in arterial pressure.[25,26] Studies in sheep, which to date serve as the most common model for studying integrative developmental cardiovascular physiology, demonstrate that the arterial baroreflex is functional during fetal and postnatal life.[7,21,27,28] Investigators disagree, however, about the magnitude of the influence of these reflexes on controlling heart rate and arterial pressure early in development. Early studies indicate that the threshold for baroreceptor activity is above the normal range of arterial pressure during fetal and neonatal life and that baroreceptors may not be loaded during fetal life.[29,30] However, studies demonstrating that sinoaortic denervation produces marked fluctuations in fetal arterial pressure and heart rate[7,28] suggest that the arterial baroreflex plays an important role in maintaining cardiovascular homeostasis. Single fiber recordings of carotid sinus and aortic depressor nerve baroreceptor afferents in fetal and newborn animals demonstrate carotid sinus nerve activity is phasic and pulse synchronous and that activity increases with a rise in arterial or carotid sinus pressure.[31-33] Basal discharge of baroreceptor afferents does not change during fetal and postnatal maturation despite a considerable increase in mean arterial pressure during this time,[31] indicating that baroreceptors reset during development and continue to function within the physiologic range for arterial pressure.[34] The sensitivity of carotid baroreceptors to increases in carotid sinus pressure is greater in fetal than in newborn and 1-month-old lambs[31] and in newborn compared with adult rabbits.[34] These finding suggest that reduced heart rate responses to changes in arterial pressure during fetal life are not due to underdeveloped afferent activity of baroreceptors but to differences in central integration, efferent parasympathetic nerve activity, or end organ responsiveness.

The mechanisms regulating the changes in sensitivity of the baroreceptors early in development have not been investigated, but may be similar to those proposed in the adult.[35-37] In younger animals, the carotid sinus is more distensible, resulting in an increase in the degree of mechanical deformation of nerve endings and ultimately in a greater strain sensitivity.[35] Alternatively, mechanosensitive ion channels[36,38]—such as the degenerin/epithelial sodium channel (DEG/ENaC) that may operate at the receptor membrane to cause hyperpolarization of the endings—and substances released from the endothelium including prostacyclin[39] and nitric oxide[40-42] may modulate baroreceptor activity during development. Blockade of cyclooxygenase with indomethacin reduces carotid baroreceptor sensitivity in newborn but not adult sheep.[43] Along these lines, prostaglandin E_2 and I_2 levels within carotid sinus tissues are six-fold higher in newborn than adult sheep.[43] Whether such influences on baroreceptor activity are present during fetal development has not been investigated.

The arterial baroreflex during fetal and postnatal maturation has been investigated primarily by examining the relationship between the increase in arterial pressure and the fall in heart rate.[28,30,44-46] Baroreflex control of fetal heart rate is dominated by changes in cardiac vagal tone, although integrity of the reflex is dependent upon both sympathetic and parasympathetic pathways.[47] Several studies describe a relatively reduced heart rate response to alterations in arterial pressure in fetal and newborn animals and in human infants.[30,48-50] Using reflex bradycardia in response to increased blood pressure by balloon inflation, Shinebourne and colleagues[48] found that baroreflex activity is present as early as 60% of completed gestation in fetal lambs and that the sensitivity of the reflex increased up to term. Additional studies in sheep[49] and other species[51,52] have similarly found increasing baroreflex sensitivity with postnatal age. For example, reflex bradycardia in response to carotid sinus stimulation is absent during the first week of life in the piglet although vagal efferents exert a tonic action on the heart at this stage of development.[51] Age-related changes in heart rate in response to phenylephrine are also greater in 2-month-old piglets than in 1-day-old animals.[52]

Other studies suggest that the sensitivity of the cardiac baroreflex is in fact greater in the fetus than in the newborn and that it decreases with maturation.[46,53] Recent studies in the equine fetus at 60% and 90% of completed gestation demonstrate that cardiac baroreflex sensitivity decreased with advancing gestational age.[54] Recordings of heart rate and efferent renal sympathetic nerve activity (RSNA) in response to increases and decreases in blood pressure in fetal, newborn, and 4- to 6-week-old sheep demonstrate that baroreceptor activity regulates sympathetic outflow as well as heart rate during fetal life; that functional baroreflex control of RSNA and heart rate shifts toward higher pressures during development; and that the sensitivity of the RSNA baroreflex function curve is greater early in development and decreases following the transition from fetal to newborn life.[55] Interestingly, studies during postnatal life have shown that baroreflex controls heart rate and that sympathetic nerve activity is impaired with senescence,[56] an effect that may contribute to the development of hypertension.

Several reasons for reported differences in the sensitivity of baroreflex function early in development are apparent. Interspecies variability exists in the maturation and functional innervation of sympathetic and parasympathetic function, including maturity of the central and efferent components of the reflex.[49,52,57] Differences in methodologies, including the use of anesthesia, may also contribute to variances in results.[28,46,58] Some investigators looked at the heart rate response to a rise in blood pressure using either phenylephrine[28,46] or aortic balloon inflation.[46,48] Others derived complete sigmoidal baroreflex curves for heart rate and efferent sympathetic activity.[55] The use of anesthesia in several studies might also have altered baroreflex mediated responses in adults.[58]

Resetting the Arterial Baroreflex

Resetting the arterial baroreflex is defined as a change in the relationship between arterial pressure and heart rate or between pressure and sympathetic and parasympathetic nerve activities.[36,37] A number of studies demonstrate that the sensitivity of the baroreflex changes with maturation, and shifts—or resets—toward higher pressures.[55,56,59] This shift occurs during fetal life, is present immediately after birth, and continues with postnatal maturation, paralleling the naturally occurring increase in blood pressure.[60] The mechanisms regulating developmental changes in baroreflex sensitivity and controlling the resetting of the baroreflex are poorly understood. Changes in the relationship between arterial pressure and sympathetic activity or heart rate occur at the level of the baroreceptor itself (peripheral resetting) and from altered coupling within the central nervous system of afferent impulses from baroreceptors to efferent sympathetic or parasympathetic activities (central resetting).[36] Other factors that may modulate changes in arterial baroreflex during development are (1) changes in the levels of locally produced factors such as nitric oxide, (2) circulating hormones and neuropeptides such as angiotensin II (ANG II), vasopressin (AVP), and serotonin, and (3) activation of additional neural reflex pathways.[36,42,61,62]

In adults, adaptation or resetting of the baroreflex during acute or chronic increases in arterial pressure is modulated by both peripheral and central mechanisms.[36] Early in development, however, short-term increases in blood pressure do not alter the arterial baroreflex.[55] Based on observation, this failure of resetting seems to result in part from the absence of the arterial pressure-baroreceptor activity relation to adapt to increased pressure. With a sustained increase in arterial pressure, activity of the aortic depressor nerve, which contains afferent fibers from aortic arch

baroreceptors, remains elevated and fails to decrease, or escape, from constant baroreceptor stimulation. These findings suggest that factors other than the maturational increase in blood pressure influence the ontogenic changes in the arterial baroreflex.

Baroreflex Function in the Human Neonate

Information regarding the ontogeny of baroreflex function in the human infant is limited. Most commonly, the head-up tilt test has been used to unload arterial baroreceptors and measure heart rate and blood flow responses. Several investigators report the ability of the healthy term or preterm infant to demonstrate a consistent response of the heart rate to tilting, although reports have been conflicting.[63-65] Using venous occlusion plethysmography, Waldman and colleagues[65] found in healthy preterm and term infants that 45-degree head-up tilting produced on average a 25% decrease in limb blood flow, suggestive of an increase in peripheral vascular resistance, although no significant tachycardia was observed. A longitudinal study in preterm infants of spontaneous baroreflex sequences (hypertension/bradycardia and hypotension/tachycardia) found the baroreflex to be functional in premature infants (less than 30 weeks gestation) and that the sensitivity of baroreflex increases with postnatal age.[66] Baroreflex sensitivity values of premature infants upon reaching term postmenstrual age were less than that observed in term infants, suggesting ex utero maturation delays development of the reflex. A study of term infants at birth and again at 2 to 4 months of age demonstrates the sensitivity of the heart rate response to head tilt is lost at the later time point.[67] Loss of the heart rate response to tilt may reflect changes in autonomic input to the heart or increased capacity of vascular responsiveness to postural challenge and hypotension. Findings of Galland and colleagues[68] suggest that term, small-for-gestational–age infants exhibit higher resting sympathetic tone than appropriately grown term infants but relatively immature autonomic control of heart rate.

Spectral analysis, which offers the ability to quantify and characterize small spontaneous beat-by-beat variations in heart rate has been used in human adults,[69] infants,[70-72] and fetuses[73] to evaluate the contribution of the autonomic nervous system in maintaining cardiovascular homeostasis. Fetal electrocardiogram tracings have shown that younger fetuses have a greater total energy of the power spectrum compared with more mature fetuses, consistent with the evolution of a stable and mature autonomic nervous system.[73] Maturational changes in the power spectra of heart rate variability have also been shown by comparing preterm to term infants.[70-72] There is a progressive decline in the low-frequency/high-frequency power ratio associated with both increasing postnatal and gestational age, indicating an increase in parasympathetic contribution to control of resting heart rate with maturation. Clairembault and colleagues[72] found that changes in the high-frequency component of the spectrum were greater at 37 to 38 weeks, suggesting a steep increase in vagal tone at this age. In a cross-sectional study of infants between 28 and 42 weeks postmenstrual age (approximately 3 to 4 days postnatal age), Andriessen and colleagues[70] found increases in R-R interval fluctuations and baroreflex sensitivity (using low-frequency transfer gain) with advancing postmenstrual age. Using atropine to block muscarinic receptors, this same group demonstrated in preterm infants that vagal tone is present at birth and parasympathetic influence on baroreflex control of heart rate is significant.[74]

We performed a longitudinal study using power spectral analysis to characterize developmental changes in sympathovagal balance in response to arterial baroreceptor unloading in preterm infants beginning at 28 to 30 weeks postconceptional age.[75] The low-frequency/high-frequency ratio did not change with head-up postural change in infants at 28 to 30 weeks post conceptional age, although the low-frequency component of the spectrum increased with head-up tilt with increasing postnatal age. These findings support maturational changes in neural regulation of cardiac function occurring in the early postnatal period.

The Cardiopulmonary Reflex

In older children and adults, extracellular fluid volume remains remarkably constant despite day-to-day variations in dietary intake of salt and water.[76] The integrity of this system is essential to preserve circulatory performance and to ensure appropriate fluid and electrolyte homeostasis. Contrary to the steady-state condition of extracellular fluid volume in the adult, significant changes in total body water and in the partition of body water between intracellular and extracellular compartments occur during fetal and postnatal development.[77,78] It is likely that the changes in body water during development are, in part, linked to ontogenic changes in the sensing and effector mechanisms regulating extracellular fluid volume, including the cardiopulmonary baroreflex. Cardiopulmonary receptors are sensory endings located in the four cardiac chambers, in the great veins, and in the lungs.[79] The volume sensors mediating reflex changes in cardiovascular and renal function are believed to be primarily those residing in the atria[80,81] and the ventricles,[79] with the ventricular receptors being of utmost importance during decreases in cardiopulmonary pressures.[79,82,83] The majority of ventricular receptor vagal afferents are unmyelinated C-fibers that can be activated by exposure to chemical irritants (chemosensitive) and changes in pressure or strength (mechanosensitive receptors).[84,85] These receptors have a low basal discharge rate, which exerts a tonic inhibitory influence on sympathetic outflow and vascular resistance[79] and regulates plasma AVP concentration.[86] Interruption of this basal activity results in increases in heart rate, blood pressure, and sympathetic nerve activity, whereas activation of cardiopulmonary receptors results in reflex bradycardia, vasodilation, and sympathoinhibition.[79]

Initial characterization of the cardiopulmonary reflex during the perinatal and neonatal periods was performed by chemostimulation of cardiopulmonary receptors.[52,87,88] These studies demonstrated that the heart rate, blood pressure, and regional blood flow responses to stimulation of chemosensitive cardiac receptors were smaller during early development than later in life and absent in premature fetal lambs[87] and in piglets less than 1-week old.[88] Stimulation of cardiopulmonary receptors with volume expansion had no effect on basal renal nerve activity in the fetus, but significantly reduced RSNA in newborn and 8-week-old sheep.[89,90] However, the decrease in RSNA in response to volume expansion was totally abolished in sinoaortic denervated newborn lambs but was not affected by sinoaortic denervation in 6- to 8-week-old sheep.[91] These results suggest that cardiopulmonary reflexes are immature early in life and that stimulation of sinoaortic baroreceptors plays a greater role than cardiopulmonary mechanoreceptors in regulating changes in sympathetic activity in response to expansion of vascular volume.

Cardiopulmonary mechanoreceptors also respond to reductions in blood volume by eliciting reflexes that influence systemic hemodynamics, although these effects are developmentally regulated. Hemorrhage produces a significant decrease in arterial blood pressure without accompanying changes in heart rate in fetal sheep at less than 120 days gestation, whereas blood pressure remains stable and heart rate increases in near-term fetuses.[92] However, other investigators[93,94] found the hemodynamic response to hemorrhage to be similar in immature and near-term fetuses, with reductions in both heart rate and blood pressure. Inhibition of vagal afferents during slow and non-hypotensive hemorrhage blocks the normal rise in plasma AVP but does not alter the rise in plasma renin activity in near-term fetal sheep.[93]

When input from cardiopulmonary receptors is removed by section of the cervical vagosympathetic trunks, the decrease in

fetal blood pressure in response to hemorrhage is similar to that in intact fetuses,[95] whereas vagotomy with sinoaortic denervation enhances the decrease in blood pressure.[93] Therefore it is likely that activation of fibers from the carotid sinus (arterial baroreceptors and chemoreceptors), but not vagal afferents (cardiopulmonary baroreceptors and chemoreceptors), are involved in the maintenance of blood pressure homeostasis during fetal hemorrhage. Cardiopulmonary receptors also appear to have a diminished role in early postnatal life as reflex changes in newborn lamb RSNA during non-hypotensive and hypotensive hemorrhage are dependent upon the integrity of arterial baroreceptors but not cardiopulmonary receptors.[96]

The RSNA responses to vagal afferent nerve stimulation are similar in sinoaortic denervated fetal and postnatal lambs,[97] suggesting that delayed maturation of the cardiopulmonary reflex is not secondary to incomplete central integration of vagal afferent input. On the other hand, the decreased sensitivity of the cardiopulmonary reflex early in development in the face of a sensitive arterial baroreflex response (as outlined above) is intriguing. One may suggest that there is a competing interaction between these two reflexes during development. In support of this hypothesis, studies in adults[98,99] have shown that activation of arterial baroreceptors may impair the reflex responses to activation of cardiopulmonary receptors.

Peripheral Chemoreflex Responses

Acute hypoxemia is a physiologic stressor that evokes integrated cardiovascular, metabolic, and endocrine responses. These responses, mediated by peripheral chemoreceptors located in the aortic arch and carotid bodies are developmentally regulated during fetal life.[100-102] In fetal lambs, the cardiovascular response to acute hypoxemia is eliminated by carotid but not aortic chemodenervation.[103] The fetal cardiovascular responses to acute hypoxemia include transient bradycardia, an increase in arterial blood pressure resulting from increase in peripheral vascular resistance, and a redistribution of blood flow in favor of the cerebral, myocardial, and adrenal circulations.[104,105] The bradycardia is mediated by parasympathetic efferents while the initial vasoconstriction results from increased sympathetic tone.[102,106] The release of circulating factors such as AVP, neuropeptide Y (NPY) and catecholamines serve to maintain peripheral vasoconstriction while heart rate returns toward basal levels.[102,107] Fletcher and colleagues[108] examined cardiovascular responses to transient (1 hour) hypoxia in fetal sheep at three distinct ages (125 to 130, 135 to 140, more than 140 days of gestation, and term [145 days]). Close to term, there was an increase in the magnitude and persistence of bradycardia and the magnitude of the femoral constrictor response to hypoxemia, associated with greater increases in neuropeptide Y (NPY) and AVP levels. Irrespective of gestational age, the responses positively correlated to basal cortisol concentrations, suggesting the normal prepartum increase in fetal serum cortisol levels may mediate the gain of the neural and endocrine responses to hypoxemia.

Although chemoreceptors are active and responsive in the fetus and newborn, studies in sheep and human infants suggest that chemoreceptor sensitivity and activity is reduced immediately after birth.[100,109] This decreased sensitivity persists for several days until the chemoreceptors adapt and reset their sensitivity from the low oxygen tension of the fetus to the higher levels seen postnatally.[100,109] The mechanisms involved with this resetting are not known, although the postnatal rise in PaO_2 appears crucial because raising fetal PaO_2 produces a rightward shift in the response curve of carotid baroreceptors to differing oxygen tension.[110] Holgert and colleagues[111] hypothesized that developmental changes in dopamine turnover within the carotid body contribute the postnatal resetting of the arterial chemoreceptors, whereas others suggest differences in intracellular calcium mobilization during hypoxia may be an important component of chemoreceptor maturation.[112]

The cardiovascular responses to acute fetal hypoxemia undergo adaptation and depend on the prevailing intrauterine condition.[104,113] Chronically hypoxic fetuses (baseline $Pa\text{-}O_2$ 17.3 ± 0.5 mm Hg) displayed greater increases in arterial blood pressure and femoral vascular resistance than control fetuses (baseline PaO_2 22.9 ±1.0 mm Hg) in response to acute hypoxia.[104] Exposure to hypoxia for a limited period of time (hours to days) may have a sensitizing effect on the chemoreflex, whereas sustained hypoxia (days to weeks) appears to have a desensitizing effect.[113] The degree of preexisting hypoxia may also affect whether chemoreflex responses are attenuated or enhanced.[114] The mechanisms or factors regulating these adaptations are unclear. In the chick embryo, hypoxia increases sympathetic nerve fiber density and neuronal capacity for norepinephrine synthesis.[115] Thus, augmented efferent pathways may contribute to the enhanced responses. On the other hand, recordings from carotid chemoreceptors in chronically hypoxic kittens demonstrate blunted responses to acute decreases in PaO_2 relative to control animals.[116] It is therefore possible that with prolonged hypoxia, blunting of the chemoreflex responses may be related to afferent mechanisms.

Developmental changes in the chemoreflex responses are likely related to carotid body sensor mechanisms as well and end-organ responsiveness. The sensitivity of carotid body glomus cells from 1- to 3-day-old rats to hypoxia is decreased compared to that in 11- to 14-day-old rats.[117] Carotid body afferent discharge is also regulated by a large number factors other than arterial PaO_2, including hypercapnia, acidosis, hyperkalemia, hypoperfusion, and circulating hormones.[118] The purine nucleoside adenosine appears to play an important role in chemoreceptor mediated responses as adenosine receptor blockade abolishes hypoxia-induced bradycardia and hypertension in fetal sheep.[119,120] Treatment with an adenosine receptor antagonist or carotid sinus denervation prior to acute fetal hypoxemia also prevents an increase in plasma epinephrine and markedly reduces the increase in plasma norepinephrine.[121,122] Thus adenosine receptor blockade may act via chemoreceptor-dependent mechanisms to abolish circulatory and adrenergic responses to acute hypoxemia in fetal sheep, although chemoreflex-independent mechanisms also likely exist. In postnatal animals, adenosine increases carotid body afferent discharge, whereas hypoxia-induced increases in afferent activity are attenuated by adenosine receptor antagonists.[123]

Sympathetic Activity at Birth

Activation of the sympathetic nervous system plays an important role in the hemodynamic adjustments at birth, including changes in heart rate, peripheral vascular resistance, and a redistribution of blood flow.[124,125] Marked increases in circulating catecholamine levels occur with birth,[126,127] and sympathetic ganglionic blockade depresses arterial pressure and cardiac function, including heart rate and cardiac output, in newborn (1- to 3-day-old) lambs.[128] Renal sympathetic nerve activity increases nearly 250% following delivery of term fetal sheep by cesarean section and parallels the rise in arterial pressure and heart rate.[60] Delivery appears to produce near maximal stimulation of renal sympathetic outflow since further increases cannot be elicited by unloading of arterial baroreceptors.[60] Reflex inhibition of this increase in RSNA could not be achieved by arterial baroreceptor stimulation, as seen in fetal and 3- to 7-day-old lambs,[55] suggesting that central influences exist that override the arterial baroreflex and that the maintenance of a high sympathetic tone is vital during this transition period. A similar pattern of baroreceptor reflex inhibition has been well described in adult animals as part of the defense reaction.[129]

Factors mediating the large increase in sympathetic outflow at birth are unclear. In utero ventilation studies of fetal sheep have shown that rhythmic lung inflation increases plasma catecholamine concentrations although there are no consistent effects on blood pressure or heart rate.[130,131] Fetal RSNA increases only 50% during in utero ventilation, while in utero oxygenation and removal of the placental circulation by umbilical cord occlusion produces no additional effect.[132] Thus lung inflation and increased arterial oxygen tension appear to contribute little to the sympathoexcitation process at birth. The increases in heart rate, mean arterial blood pressure, and RSNA following delivery are similar in intact and sinoaortic denervated plus vagotomized fetal lambs,[133] suggesting that afferent input from peripheral chemoreceptors and mechanoreceptors contribute little to the hemodynamic and sympathetic responses at delivery.

On the other hand, change in environmental temperature at birth may play an important role in the sympathoexcitatory response at birth. Cooling of the near-term fetus both in utero and in exteriorized preparations results in an increase in heart rate, blood pressure, and norepinephrine concentrations, consistent with sympathoexcitation.[134,135] In contrast, exteriorization of the near-term lamb fetus into a warm water bath does not produce the alterations in systemic hemodynamics or catecholamine values typically seen at birth.[135] Fetal cooling, but not ventilation or umbilical cord occlusion, also initiates nonshivering thermogenesis via neurally mediated sympathetic stimulation of brown adipose tissue.[136] In utero cooling of fetal lambs produces an increase in RSNA of similar magnitude to that seen at delivery by cesarean section,[65] suggesting that cold stress plays an important role in the activation of the sympathetic nervous system at birth. These changes occur before a decrease in core temperature and are reversible with rewarming, suggesting that sensory input from cutaneous cold-sensitive thermoreceptors rather than a response to a change in core temperature is mediating the response.

Multiple brain centers are involved in autonomic control of the systemic circulation in adults. Sympathetic outflow is controlled not only by the medulla oblongata,[137] but also by higher centers, especially the hypothalamus,[138, 139] allowing for a wide range of modulation. In fetal sheep, electrical stimulation of the hypothalamus evokes tachycardia and a pressor response, which are attenuated by α-adrenoreceptor blockade.[140] Stimulation of the dorsolateral medulla and lateral hypothalamus in the newborn piglet similarly increases blood pressure and femoral blood flow.[52] Because the responses to hypothalamic stimulation are lost during stress (hypoxia, hypercapnia, hemorrhage), whereas those elicited from the medulla are not, some investigators have proposed that the hypothalamus exerts little influence of cardiovascular function until later in postnatal development.[52] However, other studies suggest forebrain structures are vital for normal physiological adaptation following the transition from fetal to newborn life.[132] Increases in heart rate, mean arterial blood pressure, and RSNA, which normally occur at birth, are absent in animals subjected to transection of the brain stem at the level of the rostral pons before delivery. These data suggest that supramedullary structures are involved in mediating the sympathoexcitation seen at birth. Ablation of the paraventricular nucleus of the hypothalamus also attenuates the postnatal increase in sympathetic outflow and alters baroreflex function.[141] Thus hypothalamic structures appear intimately involved in the regulation of circulatory and autonomic functions during the transition from fetal to newborn life.

The hemodynamic and sympathetic responses at birth are markedly different in prematurely delivered lambs (85% of completed gestation) compared to those delivered at term.[142] Postnatal increases in heart rate and blood pressure are attenuated, whereas the sympathoexcitatory response, as measured by RSNA, is absent.[142] This impaired response occurs despite the fact the descending pathways of the sympathetic nervous system are intact and functional at this stage of development, as demonstrated by a large pressor and sympathoexcitatory response to in utero cooling.[142] Antenatal administration of glucocorticoids augments sympathetic activity at birth in premature lambs and decreases the sensitivity of the cardiac baroreflex.[142] The mechanisms for antenatal glucocorticoid administration augmenting cardiovascular and sympathetic responses at birth are unclear, although stimulation of the peripheral renin-angiotensin system and activation of systemically accessible AT_1 receptors are not involved.[143]

Humoral Influences

Angiotensin II

The renin-angiotensin system is active in the fetal and perinatal periods.[144-146] During embryonic and early fetal life, the primary function of the renin-angiotensin system may be to regulate organ growth and development.[147,148] Only later during fetal development does the renin-angiotensin system become involved in modulating cardiovascular function and renal hemodynamics. In sheep, fetal plasma levels of renin are elevated when compared with adults.[149,150] In healthy children, plasma renin activity is elevated during the newborn period, declines rapidly in the first year of life, and continues to gradually decline until adulthood.[151-153] In preterm infants, plasma renin activity is markedly elevated and has close inverse relationship to postconceptual age.[154]

A large number of studies also report that administration of inhibitors of ANG II, including angiotensin converting enzyme inhibitors and ANG II subtype 1 receptor blockers, decrease fetal and newborn arterial blood pressure.[13,59,146,155-157] Fetal plasma renin activity and plasma ANG II concentration increase after aortic constriction, hypotension, and blood volume reduction.[144,158] Conversely, a rise in arterial blood pressure and volume expansion reduces plasma renin activity in fetal and newborn animals.[159,160] The vasopressor response and renal vascular reactivity to exogenous ANG II are less in fetal lambs than in adult sheep.[161,162] Factors explaining the higher activity of the renin-angiotensin system early in development and the decreased sensitivity to ANG II have not been explored in detail, although differences in localization and expression of the ANG II receptor subtypes or activation of second messenger systems contribute to this effect.

In addition to their direct effects on neural function, baroreceptors and chemoreceptors regulate the release of vasoactive hormones, including ANG II.[61,163] Changes in the levels of circulating hormones, in turn, influence neural regulation of cardiovascular function. For example, in the sheep fetus, a rise in arterial blood pressure produced by ANG II administration produces either attenuated or no reflex cardiac slowing.[161,164] The bradycardic and sympathoinhibitory responses to a given increase in blood pressure are less for ANG II than for other vasoconstrictor agents.[62] The site of action of ANG II in regulating baroreflex function in the fetus is not known. In the adult, ANG II facilitates activation of sympathetic ganglia and enhances the release of norepinephrine at the neuroeffector junction.[165] Within the central nervous system, ANG II stimulates sympathetic outflow and alters baroreceptor reflexes by acting on ANG II type 1 (AT_1) receptors located within the hypothalamus, medulla, and circumventricular organs.[166,167]

Endogenous ANG II participates in regulating arterial baroreflex responses early during development. The absence of rebound tachycardia after reduction in blood pressure by angiotensin converting enzyme (ACE) inhibitors is well described in fetal and postnatal animals,[155] as well in human adults and infants.[168] In the newborn lamb, angiotensin converting enzyme inhibition or ANG II type 1 (AT_1) receptor blockade decreases RSNA and heart rate and resets the baroreflex toward lower pressure.[62,169]

Resetting of the reflex is independent of changes in prevailing blood pressure. Lateral ventricle administration of an ANG II type 1 (AT_1) but not AT_2 receptor antagonist also lowers blood pressure and resets the baroreflex toward lower pressure in newborn and 8-week-old sheep at doses that have no effect when given systemically.[169]

Arginine Vasopressin

In addition to ANG II, arginine AVP plays an important role in maintaining cardiovascular homeostasis during fetal and postnatal development. Plasma AVP concentrations in the fetus are increased by multiple stimuli, including hypotension, hemorrhage, hypoxemia, acidemia, and hyperosmolality.[163,170-173] Graded infusions of AVP increase fetal blood pressure and decrease fetal heart rate in a dose-dependent manner.[174-176] AVP responses to hypotension are partially mediated by arterial baroreceptors, whereas the contribution of carotid or aortic chemoreceptors to the AVP response to hypoxia is limited.[122,177-179] Although AVP provides an important component of the cardiovascular response to acute stress, it appears to have little impact on basal fetal circulatory regulation. Blockade of AVP receptors in fetal sheep has no measurable effects on arterial blood pressure, heart rate, or renal sympathetic nerve activity in fetal sheep or newborn lambs.[43,180-182] However, AVP receptor inhibition impairs the ability of the fetus to maintain blood pressure during hypotensive hemorrhage and reduces the catecholamine response.[183]

In several adult species, AVP modulates parasympathetic and sympathetic tone and baroreflex function.[61,181,182] Administration of AVP evokes a greater sympathoinhibition and bradycardia than other vasoconstrictors for a comparable increase in blood pressure.[61,184] This modulation of the baroreflex has been attributed to AVP enhancing the gain of the reflex as well as resetting the reflex to a lower pressure.[61,184] While AVP increases fetal arterial pressure and decreases heart rate in a dose-dependent manner,[175,185] sequential increases in plasma AVP in fetal and newborn sheep does not alter baroreflex control of RSNA and heart rate in response to acute changes in blood pressure.[43] Endogenous AVP also appears to have little effect on baroreflex function early during development. Peripheral administration of a V_1-receptor antagonist has no measurable effects on resting hemodynamics in fetal sheep or on basal arterial blood pressure, heart rate, RSNA, or baroreflex response in newborn lambs.[43,180] This lack of baroreflex modulation by AVP may facilitate the pressor response to AVP in fetuses and newborns during stressful situations such as hypoxia and hemorrhage. In this way, AVP could play a particularly important role in maintaining arterial pressures during these states early in development.

The role of central AVP in maintaining hemodynamic homeostasis in the developing animal has not been extensively studied. Under basal conditions, fetal AVP levels are tenfold higher in the cerebrospinal fluid than in plasma, suggesting AVP contributes to central regulation of autonomic function.[186] Intracerebroventricular infusion of AVP produces significant decreases in mean arterial blood pressure and heart rate in newborn lambs although no reflex changes in RSNA are seen.[187] Interestingly, intracerebroventricular administration of AVP increases RSNA in 8-week-old sheep, demonstrating that the role of AVP receptors within the CNS in regulation autonomic function is developmentally regulated.[187] The changes in blood pressure and heart rate are completely inhibited by administration of a V_1 antagonist, suggesting the central cardiovascular effects of AVP are mediated by V_1 receptors, as has been reported in mature animals.[188]

Corticosteroids

Adrenal steroids are vital for normal physiological development. Fetal adrenalectomy attenuates the normal gestational age-dependent increase in blood pressure that occurs in late gestation, while cortisol replacement produces a sustained increase in fetal blood pressure.[153,189,190] Antenatal exposure to exogenous glucocorticoids increases fetal postnatal arterial blood pressure by enhancing peripheral vascular resistance and cardiac output without altering heart rate.[191-193] The mechanisms accounting for the increase in fetal vascular resistance are not clear. Although direct effects of glucocorticoids on vascular reactivity likely exist, antenatal glucocorticoids also modify autonomic and endocrine functions. Increases in fetal blood pressure and vascular resistance following betamethasone treatment occur despite marked suppression of circulating vasoconstrictors, including catecholamines, ANG II, and AVP.[142,178,192,194-196] Circulating neuropeptide Y concentration, which may provide an index of peripheral sympathetic activity, is increased following fetal exposure to dexamethasone.[197] Glucocorticoid treatment accelerates postnatal maturation of brain catecholaminergic-signaling pathways in rats and enhances renal sympathetic nerve activity in prematurely delivered lambs.[142,198-202]

Endogenous production of cortisol is important for normal maturational changes in autonomic and baroreflex function. Adrenalectomized sheep fail to display the normal postnatal increase in RSNA, but the response is restored by cortisol replacement.[116,203] Restoring circulating cortisol levels to the prepartum physiological range shifts the fetal and immediate postnatal heart rate and RSNA baroreflex curves toward higher pressure without altering the slope of the curves.[203] Antenatal administration of betamethasone decreases the sensitivity of baroreflex-mediated changes in heart rate in fetuses and lambs while resetting baroreflex control of heart rate and RSNA toward higher pressures.[142,197,200,204] In response to acute hypoxia, fetuses exposed to glucocorticoids display prolonged bradycardia and attenuated plasma catecholamine and AVP responses.[197] Taken together, these findings indicate that glucocorticoids modify autonomic and endocrine control of cardiovascular function during development. These effects may even persist well after cessation of exposure.[197]

Nitric Oxide

Endogenous NO contributes to regulation of fetal blood pressure and organ specific vascular resistance as acute blockade of NO production causes an immediate rise in blood pressure and umbilico-placental resistance, and it decreases heart rate, renal blood flow velocity, and plasma renin concentration.[205,206] In addition to its direct effect on vascular tone, there is evidence to suggest NO affects vascular function through interactions with central and peripheral autonomic function.[207] Studies in adult animals demonstrate that NO modulates sympathetic activity via its inhibitory actions on baroreceptor afferents and brain regions involved in regulating sympathetic activity.[207] NO also appears to function as a central neurotransmitter and acts within the brain to regulate fetal arterial blood pressure. Administration of the NO donor nitroglycerin into the fourth cerebral ventricle of the ovine fetus decreases mean arterial pressure, whereas blocking NO synthase in the fourth ventricle increases fetal blood pressure.[208] Inhibition of endogenously produced NO increases blood pressure in 1- and 6-week-old lambs to similar extents although the concomitant decreases in heart rate are greater in the young lamb, suggesting NO[42] may contribute to maturation changes in baroreflex function during this period. Developmentally regulated changes in the expression of NO synthase isoforms in the brain stem, which is known to occur in the fetus, may play a role in baroreflex function during fetal and early postnatal development.[42,209]

Conclusion

Understanding the mechanisms regulating cardiovascular function in the fetus and newborn is of great importance, particularly as these mechanisms relate to the physiologic adaptations

occurring with the transition from fetal to newborn life. Failure to regulate arterial pressure, peripheral resistance, and blood volume may lead to significant variations in organ blood flow and substrate delivery, resulting in ischemic or hemorrhagic injury. There is abundant evidence to suggest that autonomic regulatory mechanisms, including baroreceptors and chemoreceptors, are important modulators of blood pressure and circulatory function in the developing fetus and newborn. Humoral and endocrine factors also act directly and indirectly to regulate vascular tone and cardiac function. A large number of other cardiovascular regulatory factors, including opioids, natriuretic peptides, and prostenoids act centrally and peripherally to regulate systemic hemodynamics, and these have not been addressed. Further work is needed to determine the role of these factors and their interactions during relevant pathophysiological conditions. A more complete understanding of neurohumoral control of cardiovascular function early in life will result in the development of new therapeutic strategies to prevent complications during the perinatal period.

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Lucy R. Green and Mark A. Hanson

Developmental Effects on Fetal Circulation

The fetus depends on its cardiovascular system for growth and development. Vascular growth is closely linked to tissue growth, and the fetal heart must develop in relation to the venous return (preload), primarily from the umbilical vein, and the similar arterial pressures in the pulmonary trunk and the aortic arch (afterload). This matching of cardiovascular function to growth is known to be particularly important in late gestation, when cardiovascular redistribution and changes in tissue metabolism occur in response to placental insufficiency (reviewed in References 1 and 2). Whether such processes operate in early gestation is not known.

Current knowledge of late gestation fetal cardiovascular control is based on the concept that the mechanisms of such control will be best revealed when the system is challenged with a stimulus and the resulting response measured. The stimulus most widely employed is hypoxia, which has permitted a fairly complete picture of a temporal and functional hierarchy of reflex, endocrine, and local mechanisms operating during acute hypoxia.[3] Such mechanisms change in the course of gestation,[4,5] in the face of repeated acute or sustained hypoxia,[6-8] and in species adapted to life in the hypoxia of altitude.[9-12] However, whether such mechanisms operate in response to changes in fetal nutrition (e.g., glucose, amino acid, or micronutrient provision) is now beginning to be understood. This chapter addresses this question in the context of what is known of the programming effects of prenatal nutrition on cardiovascular development and fetal circulatory control mechanisms.

EPIDEMIOLOGIC OBSERVATIONS

Considerable evidence has now accumulated from a variety of studies that small or disproportionate size at birth is associated with increased risk of cardiovascular disease. The studies of Barker and colleagues[13] in adult men and women showed that the standardized mortality ratio for cardiovascular disease (CVD) decreased in a graded manner across the normal birth weight range. Numerous studies have linked low birth weight to the metabolic syndrome[14] or to components of the syndrome (defined as fasting plasma glucose more than 6 mmol/L; blood pressure more than 130/85 mm Hg; fasting plasma triglycerides greater than

1.7 mmol/L; plasma high-density lipoprotein cholesterol less than 1.1 mmol/L; (in men) waist measurement greater than 102 cm). A metaanalysis of the blood pressure effect suggests that a 1-kg increase in birth weight is associated with a decrease of 2 mm Hg in systolic blood pressure in later life.[15] Inevitably, the size and nature of these studies vary enormously and the effect of study size has been questioned[16] because larger studies (where birth weight is more likely to have been self-reported than actually measured) show weaker associations between birth weight and blood pressure than smaller studies. Nonetheless, the direction of the effect (small size at birth predicting higher blood pressure in later life) is not under question even if the magnitude of the effect is debated. Thus a major part of an individual's predisposition to cardiovascular disease appears to be established during development.

Birth weight is a poor proxy for fetal growth, and of course, blood pressure is a poor proxy for cardiovascular function or tissue perfusion. For these reasons it is vital that physiologic studies are conducted to understand the mechanisms underlying programming of cardiovascular function by life events in utero. Moreover it is highly unlikely that appropriate intervention measures to prevent the progression to disease (i.e., the metabolic syndrome) in susceptible individuals can be developed without an understanding of the mechanisms underlying the development of the disease. This may have enormous implications for public health; calculations based on the epidemiologic studies conducted in Finland[17] suggest that if all male offspring could be prevented from being thin at birth and thin and short at 1 year of age, the incidence of coronary heart disease (CHD) would have been halved. The phenomenon exists in developing societies across the normal birth weight range, which averages 1 kg less than in developed societies. Probably those at greatest risk throughout the world belong to transitional societies, in which, for example, children from agrarian societies in the developing world move to cities as adolescents. These considerations may explain the epidemic in CHD and in type 2 diabetes that is developing, for example, in the Indian subcontinent.[19] It has been proposed that developmental responses (e.g., of the fetus) might give offspring a survival advantage in the postnatal environment in which it predicts that it will live. By definition such predictive adaptive responses occur within the normal range of fetal growth and development, and they must use processes by which development is influenced by the prenatal environment in all pregnancies (e.g., maternal constraint).[20] A greater risk for disease may result when the postnatal environment does not match that which was predicted prenatally.[21]

GENES VERSUS ENVIRONMENT

Many reasons exist for believing that the effects just described are not purely genetic in origin; the most obvious is that changes in the incidence of the metabolic syndrome within a generation cannot be due purely to genetic (heritable) traits. Moreover, the maternal and paternal genetic contributions to fetal growth (see later discussion) and to outcome measures such as systolic blood pressure in adulthood, are highly dissimilar; in a study of adults in Preston, United Kingdom, systolic pressure was inversely related to maternal but not to paternal birth weight.[22] In pursuing the mechanisms underlying programming of cardiovascular function in relation to growth, it is therefore important to consider the interaction between the genome and the early environment in determining a phenotype that is susceptible to disease. This argument is substantiated by the discovery of numerous processes that modify genomic DNA, gene expression, and thus the production of the proteins that confer the phenotype. These include not only single-nucleotide polymorphisms, DNA methylation, and changes in histone structure that alter gene expression but also the many transcription factors that act to modify gene transcription and the processes that control the half-life of mRNA.

A good example of the interaction between genotype and the early environment is provided by studying peroxisome proliferator-activated receptor-gamma (PPAR-γ), a nuclear hormone receptor that mediates the effect of synthetic and endogenous ligands on the modulation of gene expression. Polymorphisms in the PPAR-γ2 gene are associated with type 2 diabetes. Moreover, the well-documented link between low birth weight and insulin resistance is seen only in subjects with PPAR-γ gene polymorphism.[23] Dennison and colleagues[24] have shown a similar relationship between the programming of osteoporosis, known to be inversely related to birth weight, and a polymorphism in the vitamin D receptor gene.

The processes most likely to be involved in mediating the maternal versus paternal genomic effects on development involve imprinting. Of the range of genes that are imprinted, the *H19/IGF2* cluster has received particular attention.[25] The insulin-like growth factor II (IGF-II) peptide, which is paternally expressed, is a potent stimulator of fetal cell division and differentiation—showing how the paternal genome drives growth in various key tissues, such as the fetal liver and growth and nutrient transport in the placenta.[26] The type II IGF receptor, which is maternally expressed, serves as a clearance receptor that modulates IGF-II peptide action in the tissues—showing how the maternal genome can down-regulate growth in accordance with the maternal body habitus. This observation is consistent with the concept of maternal constraint of fetal growth, which was highlighted by the pioneering studies of Walton and Hammond in which Shire horses were crossed with Shetland ponies.[27] It is therefore clear that the trajectory of growth in early life is determined by maternal and environmental factors acting to regulate the gene expression of the early embryo. First trimester growth in the human sets the trajectory for later fetal growth and predicts risk of low birth weight.[28] Similar epigenetic processes are now believed to provide a mechanism whereby undernutrition during development can influence gene expression and cardiovascular or metabolic phenotype into mature adulthood (see "Mechanisms").[29] Of importance, these processes involve not simply imprinted genes but a much wider range of the genome, which appears to be under graded epigenetic control throughout development.[30]

ENVIRONMENTAL STIMULI

In investigating the interaction between gene and environment it is important to take a lifespan perspective in considering the influence of maternal diet, body composition, social status, smoking, the effect of the tubal and uterine fluid environment on the early embryo, the development of the placenta, fetal adaptations, and postnatal effects which may exacerbate the problem.

Timing

Each organ has a timetable for development, so it is not surprising that the timing of environmental stimuli will influence the effects on offspring phenotype. In adults who were in utero at the time of the Dutch Winter Famine (an abrupt onset 5-month period of malnutrition in 1944 to 1945), exposure during early gestation was associated with increased incidence of CHD[31,32] and increased blood pressure response to stress,[33] and exposure to famine in midgestation was associated with microalbinuria in adults, possibly indicative of hyperfiltration injury as a result of fewer nephrons.[34] Mid- to late gestation undernutrition was associated with reduced glucose tolerance in adulthood.[35] Sheep are widely used as an animal model for studying the programming phenomenon, not least because they share similarities with humans in that the full complement of cardiomyocytes and renal glomeruli are formed prenatally. Studies support the concept that the early gestation

nutrient environment is important in determining later cardiovascular control,[36-39] whereas nutrition in the late gestation period may be important in determining later metabolic function and endocrine control.[40-42] The rat model has been widely used for studying programming phenomena (reviewed by Brawley[43] and Armitage[44] and their associates). Most previous studies with this model involved administration of the diet for the whole of pregnancy (approximately 21 days), and now a range of studies shows that offspring of rats fed a low-protein diet during pregnancy have altered endothelial function (e.g., the degree of relaxation to the endothelium dependent vasodilator acetylcholine). However Kwong and colleagues[45] have also shown that administration of a low-protein diet to rat dams in the first 4 days of gestation (i.e., preimplantation) produces blastocysts that have altered allocation of cells to the trophectoderm and inner-cell mass and to offspring with permanently increased blood pressure in adult life. Recent work suggests that feeding rat dams this low-protein diet exclusively during oocyte maturation leads to altered behavior and cardiovascular control in postnatal life.[46] Further observations in the mouse have shown that embryos transferred to recipient females mice developed elevated blood pressure in adult life.[47] Apart from demonstrating the importance of the early preimplantation embryonic environment in the programming of subsequent phenotype, these studies raise important questions about the long-term effects of reproductive technologies increasingly used in humans.[48] Additional evidence suggests that even prepregnancy maternal nutrient environment can influence cardiovascular function.[49,50]

Nutrients

The relative contribution of specific macro- or micronutrients in determining the balance between supply and demand during fetal growth has not yet been resolved. The fetal requirements in late gestation for the amino acid glycine are greater than any other amino acid, even though this amino acid is not normally considered to be essential. Glycine provision, with the availability of the micronutrient cofactor folate, is important in a range of biological processes that may alter cardiovascular function (Figure 71-1). It is involved in the synthesis of DNA and RNA that is important for cell growth and differentiation. It provides a source of methyl groups for the methylation of CpG dinucleotides, which silences gene transcription and thus the transition from genotype to phenotype.[29] An example of such methylated genes with an important role in the pathogenesis of cardiovascular disease is the estrogen receptor-α, which is linked to risk of atherosclerosis. Finally, glycine is important in the interconversion of homocysteine to methionine, such that its deficiency (or that of vitamin B_{12}) leads to accumulation of homocysteine in the plasma and associated vascular damage. The offspring of low protein–fed rat dams show elevated plasma homocystine levels. In rats, the addition of glycine, or indeed folate, to the low-protein diet prevents the elevation of blood pressure in the offspring and the development of vascular dysfunction in these offspring.[51,52] In the liver, hypomethylation of glucocorticoid receptor (GR) and PPAR-α promotors was also prevented by addition of folic acid to the diet.[29]

The major provision of fetal glycine is not by direct transport from the mother. Placental glycine production from the conversion of serine by the serine hydroxymethyltransferase is now thought to be important in sheep, but not in humans, in whom serine hydroxymethyltransferase activity is low.[53] The serine itself enters the placenta from the maternal and fetal circulation via system A and ASC transporter systems.[54,55] Placental size relates not only to fetal growth but also to blood pressure in later life.[56] However, relatively few studies have been performed on placental transport function in relation to fetal growth and outcome.[26] It is of interest that placental system A activity increases with reductions in birth weight in the normal range[57] although an association exists between reduced system A activity and intrauterine growth retardation.[58] A deeper understanding of the complex mechanisms by which amino acids are transferred is crucial.[59]

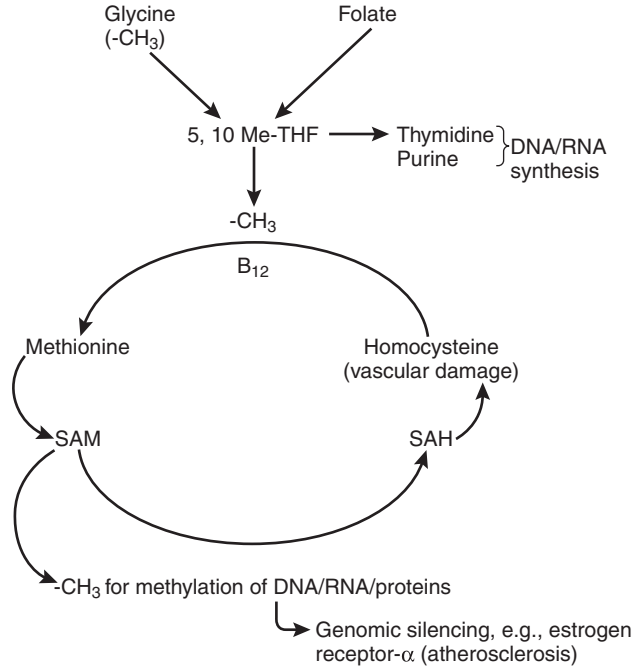


Figure 71-1 Diagram to illustrate the importance of glycine and folate in the processes that may underlie atherosclerosis. *B_{12},* Vitamin B_{12}; *-CH_3,* methyl group; *Me-THF,* methyl tetrahydrofolate; *SAH, S*-adenosyl-L-homocysteine; *SAM, S*-adenosyl-L-methionine.

FETAL ADAPTIVE RESPONSES

The incidence of singleton pregnancies in sheep allows the comparison of fetal growth patterns with human pregnancies, and their size and tolerance to surgery permits cardiovascular and growth measurements to be made during several weeks of gestation. However, studies of the fetal adaptations to perturbed nutrition are relatively few in number. The well-established "brain-sparing" cardiovascular phenomenon, in which a higher percentage of the combined ventricular output is directed to the brain at the expense of the developing fetus, has been demonstrated extensively in hypoxic fetuses. Brain-sparing is associated in the human fetus with late gestation asymmetric growth retardation, and the supposition has been that, if such fetuses are not hypoxic, the response is to reduced nutrition. The rapid phase of the vasoconstrictor component of the response to hypoxia is mediated by the carotid bodies; therefore it is of great interest that these chemoreceptors are also responsive to a reduction in plasma glucose in adults,[60] and recent data suggest that the differential fetal organ growth effects of maternal undernutrition is dependent on carotid sinus innervation.[61] Understanding of the fetal redistribution requires consideration of effects on individual organs, and one of particular interest is the developing fetal liver. Blood supply to the liver comes from a range of sources, primarily the oxygenated and highly nutritious blood from the umbilical vein. However, this blood entirely supplies the left lobe of the liver and, to a lesser extent, the right lobe, which also receives blood from the hepatic portal veins and the hepatic arteries (which is less well oxygenated and nutritious). A proportion of oxygenated umbilical blood is shunted through the

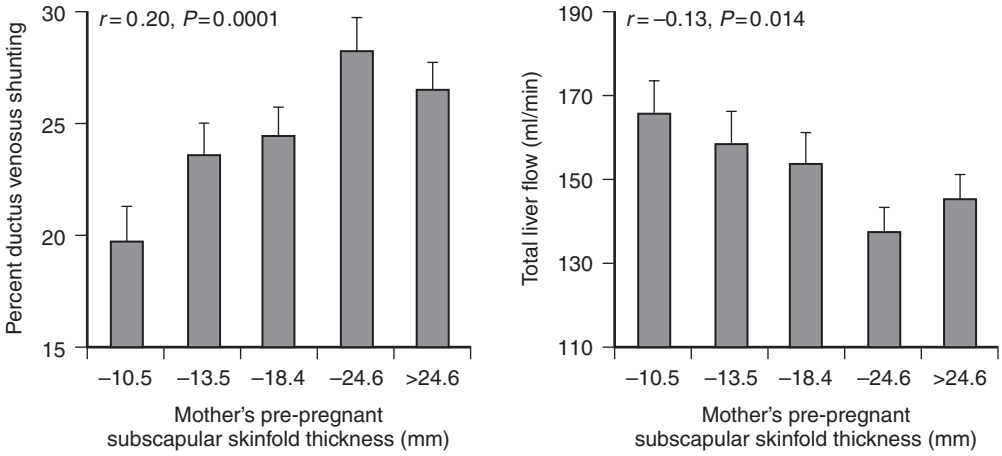


Figure 71-2 Data from 381 fetuses at 26 weeks gestation showing lower ductus venosus shunting and greater total liver blood flow in those mothers who had lower skinfold thicknesses before pregnancy. Maternal subscapular skinfold thickness is grouped into strict fifths of the distribution for presentation purposes; correlation analyses are based on continuously distributed variables. *(From Haugen G, Hanson M, Kiserud T, et al: Fetal liver-sparing cardiovascular adaptations linked to mother's slimness and diet. Circ Res 96,12, 2005.)*

ductus venosus towards the foramen ovale and through into the left atrium. The degree of shunting of this blood changes during gestation and is altered by challenges such as acute hypoxemia.[62] It is now known that the degree of shunting is also increased in late gestation fetuses of mothers with a high prepregnancy body mass index (BMI) or skinfold thickness[63] (Figure 71-2) and that low maternal weight gain is associated with preferential blood supply to the fetal left liver lobe and with higher birth weight and neonatal ponderal index.[64] This therefore fits with the notion that women with greater fat stores, or with altered endocrine status leading to increased body fat mass, produce fetuses with a more rapid trajectory of growth, at least in early gestation. If this growth imposes demands on the developing placenta that cannot be met, fetal cardiovascular redistributions must occur.

The extent to which concomitant changes in cardiovascular control and growth result from perturbations in the fetal nutrient supply line is unclear. Reduced maternal body condition at mating results in an elevated fetal blood pressure trajectory in late gestation fetuses[65] and the normal developmental increase in arterial pressure during late gestation in sheep is perturbed in small fetuses.[66] Restricted fetal nutrient supply by removal of placental caruncles before conception perturbs cardiovascular development and function in late gestation fetuses—with or without growth restriction.[67] Moreover, mild-moderate (15% to 30%) reduction in maternal nutrient intake for the first half of gestation lowered blood pressure in late gestation fetuses,[68] impaired vascular function in vitro[69] and enhanced the increase in peripheral vascular resistance in the hind limb in response to acute hypoxia,[36] with no change in fetal organ or body weight. Preliminary data (unpublished) in these fetuses indicate a redistribution of combined ventricular output in late gestation because baseline femoral blood flow tends to be lower and carotid blood flow tends to be elevated. Indeed recent data indicate that the prevailing glycemic environment (unknown etiology) of the fetus can influence its cardiovascular response to acute hypoxia[6] and that lower circulating blood glucose in late gestation is associated with greater vasoconstrictor activity in isolated vessels.[70] In addition, the fetus can make immediate cardiovascular adaptations to hypoglycemia, including a decrease in blood flow to the femoral bed, and this response is heightened by previous exposure to a restricted maternal diet around implantation.[71] Specific early-gestation moderate (30%) reduction of maternal protein intake markedly exacerbated the vascular dysfunction of isolated fetal resistance vessels[72] (Figure 71-3), indicating the importance of nutrient balance (see previous). Cleal and colleagues[73] recently observed that 50% maternal nutrient restriction for 31 days after conception increases fetal thymus and pancreas weight at midgestation. Undernutrition of a greater intensity leads to decreased fetal size, but the response depends on the rate at which growth is occurring: The growth of a rapidly growing fetus is slowed, whereas slow-growing fetuses fail to respond.[74] Such fetuses may have adapted to an earlier gestation insult, such as periconceptional undernutrition,[75] thereby protecting them from a subsequent late gestation challenge. These observations suggest that cardiovascular adaptations may be able to buffer changes in growth when faced with an altered nutrient supply, but it may be that when the upper limit in this buffering capacity has been reached, a change in growth will result. However, the same investigators also observed that undernutrition in early gestation did not alter size at birth but did cause a faster growth rate in the first 12 weeks after birth.[73] This concept fits with the epidemiologic observations that adult blood pressure is inversely related in a graded manner across the normal birth weight range[15] (see previous) but also that postnatal growth needs to be considered. Recent studies show that either periimplantation or late gestation maternal undernutrition decrease late gestation fetal myofiber density and that this is associated with decreased capillary density (Figure 71-4), and in the late restricted group with altered molecular markers of glucose uptake,[76] these changes are specific to the muscle bed. Overall these studies reinforce the idea that timing of undernutrition is important and highlight that to understand the link between growth and blood supply, mechanisms at the tissue level need to be considered along with hemorrheic control. Thus fetal adaptations, in the face of altered partitioning of nutrients between the maternal body, the placenta, and the fetus, may be beneficial in customizing fetal growth and development to the supply of oxygen and nutrients, but they may have long-term sequelae postnatally (see previous).

Prenatal and Postnatal Environmental Mismatch

Fetal growth and cardiovascular responses may be part of a survival strategy, or they may be of no immediate benefit, but rather be predictive adaptations in expectation of the postnatal environment. Current concepts suggest that, if these predictions are not met, and the pre- or postnatal nutritional environments are

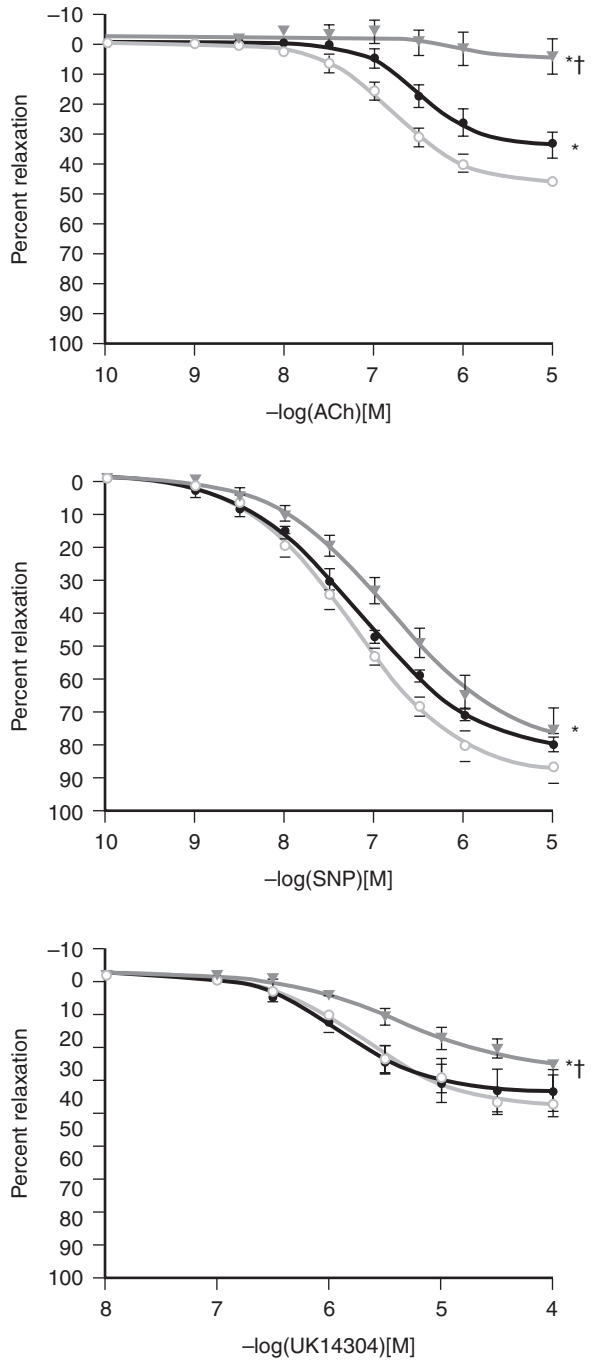


Figure 71-3 Vasorelaxation to acetylcholine (ACh), sodium nitroprusside (SNP) and UK14303 (α_2-adrenoceptor agonist) in femoral arteries of fetuses from control and nutrient restricted ewes. Data are expressed as a percentage of initial preconstriction. Values are mean ± SEM in control (○), 70% global (●), and 70% protein (▲) fetuses. *$p < .05$ (ANOVA), significantly different from control; † $p < .05$ (ANOVA), significantly different from global group. *(From Nisbina H, Green LR, McGarrigle HH, et al: Effect of nutritional restriction in early pregnancy on isolated femoral artery function in mid-gestation fetal sheep,* J Physiol *553:637, 2003.)*

mismatched, the risk of disease increases.[77] Indeed the window of opportunity for programming of cardiovascular function in early life does not stop at birth. The Helsinki study[78] showed that the effect of low ponderal index at birth on the risk of CHD in adulthood in men was amplified by increasing BMI during the first 12 years of life. Considerable interest exists about the effects of obesity in childhood (which in western societies may be 20% of children younger than 12 years of age and in the United Kingdom may affect 1 in 4 children younger than 16 years by 2050[79]) on risk for later disease. In addition, rat studies show that severe undernutrition during fetal life programs offspring, which are hyperphagic, obese, and insulin-resistant and demonstrate reduced physical activity.[80,81] This suggests a compounding of fetal programming effects by postnatal adaptations. In female sheep, early postnatal nutrient restriction (12 weeks of moderate intensity) improved later glucose handling through increased insulin sensitivity.[41] In men, this postnatal undernutrition altered cardiovascular function, but not if they were also exposed to a poor nutritional environment in early gestation and the effects of prenatal undernutrition of cardiovascular dysfunction were not seen when the early gestation nutrition was also poor.[38,39] In rats, dietary manipulation to minimize the mismatch between pre- and postweaning nutrition minimizes endothelial dysfunction, low heart rate, and the disruption of mechanisms regulating appetite and energy expenditure in offspring.[82,83] In sheep and rats, a greater pre- and postnatal dietary mismatch worsened the liver function and metabolic profile in offspring,[84] and in rats decreased lifespan.[85] In pigs, the coronary atherosclerotic effects of a high-fat diet were prevented by previous feeding of a similar diet to the pregnant mother.[86] Together, these studies support the concept that a greater mismatch between the pre- and postnatal nutrient environment increases the risk of later disease, including of the cardiovascular system, in an individual.

THE TRANSDUCTION OF ENVIRONMENTAL STIMULI

Cardiovascular adaptations of the mother to pregnancy provide another potential route whereby dietary or endocrine stressors may be transmitted to her fetus. The increase in blood volume and cardiac output during pregnancy[87] involves a redistribution of cardiac output in favor of the reproductive tract. In humans this is mediated primarily by a dramatic decrease in resistance in the spiral arteries supplying the endometrium, but there is an additional component of vasodilatation in the uterine bed provided by vascular endothelial growth factor/nitric oxide (NO) mechanisms.[88] Ahokas and colleagues[89] observed that with dietary restriction in the rat dam (50%), maternal liver blood flow was maintained at the expense of the pregnant uterus. In the low protein–diet rat model, Itoh and colleagues[90] found an impaired vasodilator response to vascular endothelial growth factor in late pregnancy uterine arteries in vitro. Innes and colleagues[91] recently reported a negative association between a women's birth weight and the risk of gestational diabetes mellitus. This effect persists after adjusting for gestational age and current BMI. Similarly, in hypertensive rat offspring of protein restricted dams, Brawley and colleagues[92] found a blunting of mesenteric vasodilator responses to acetylcholine. This effect clearly persists in the female offspring when they become pregnant, despite their adaptations to pregnancy, and even though they had not been exposed to any dietary challenge during postnatal life or pregnancy itself (unlike their mothers).[93]

Considerable work has been conducted on the mechanisms by which dietary imbalance in pregnancy may be transmitted to the fetus. Unraveling the relative contribution of the key mechanisms is likely to be complicated. Evidence is emerging to suggest that the fetal cardiovascular system may be able to respond to changes in nutrients other than oxygen and that this may be coordinated by the carotid chemoreceptors, which mediate rapid fetal cardiovascular adaptations to hypoxia and are responsive to reductions in circulating plasma glucose.[60,61,94] Thus common mechanisms of detection of changes in substrate (e.g., glucose) and oxygen may exist. Evidence from cultured rat hepatocytes indicates that regulation of glucagon receptor, insulin receptor, and L-type

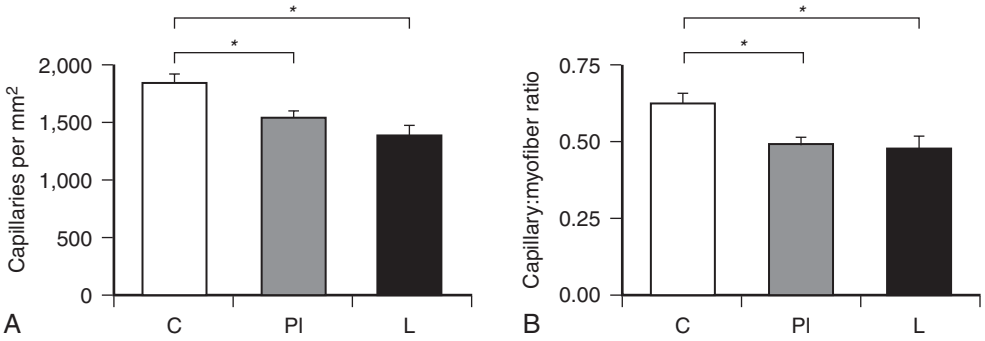


Figure 71-4 Capillary density and capillary:myofiber ratio in the triceps brachii muscle of late gestation fetuses. **A,** Capillary density and **B**, capillary:fiber ratio in control (*C*; $n = 6$), peri-implantation restricted (*PI*; $n = 9$), and late restricted (*L*; $n = 6$) groups. $^*p < .05$. *(From Costello PM, Rowlerson A, Astaman NA, et al: Peri-implantation and late gestation maternal undernutrition differentially affect fetal sheep skeletal muscle development,* J Physiol *586:2371, 2008.)*

pyruvate kinase gene expression by glucose and oxygen may be due to cross-talk between hypoxia inducible factor-1 (HIF-1) and glucose-responsive transcription factors at the glucose response element and hypoxia response element.[95] Otherwise, at the most basic level, a change in the quantity of a dietary component may have direct implications on the amount of substrate that is available for fetal metabolism, but maternal body composition (see previous) and maternal metabolic demands as well as placental metabolic demands have to be taken into account. An additional consideration is of the multiple genes and proteins that may respond simultaneously to altered nutrition—analogous to the 14-protein "stimulon" induced by glucose depletion in *Escherichia coli.*[96] As the available evidence indicates, the expression of a variety of genes, implicated in cardiovascular control and growth during fetal life, can be modulated by the direct action of nutrients; for example, IGF-II gene expression in cultured beta cells is regulated by glucose.[97] During fetal life, glucose appears to regulate *IGF-2* directly, but its effect on IGF-I is likely to be mediated by insulin.[98] Glucose activation of plasminogen activator inhibitor-1 gene expression in cultured rat vascular smooth muscle cells,[99] and of the osteopontin gene,[100] may be of relevance to vascular complications in patients with diabetes. High glucose stimulates angiotensinogen gene expression in rat kidney proximal tubular cells, mediated in part by the generation of reactive oxygen species.[101]

Currently much interest exists in fetal exposure to increased levels of glucocorticoid from the standpoint of the clinical use of steroids in pregnancy but also the potential "stress" experienced concomitant with altered nutrition. Glucocorticoids reduce fetal growth[102] and impact on the human platelet antigen hypothalam-pituitary-adrenal (HPA) axis of the developing fetus itself,[103] and administration in specific early gestation periods lead to postnatal hypertension, which persists into mature adulthood.[104] Glucocorticoids may mediate effects of altered maternal nutrition including via interaction with the expression of other genes,[105,106] NO signaling pathways,[107] and effects on DNA methylation.[108] Although some evidence is available that nutrient restriction in guinea-pigs elevates maternal plasma cortisol[109] and a high-fat diet in rats is associated with stimulation of the HPA axis,[110] but recent findings suggest that maternal cortisol is lower during undernutrition in early gestation,[73,111] and it has not been shown unequivocally in animals that altered maternal nutritional balance alters the level of plasma glucocorticoid in fetal life.[112,113] Even in the absence of such an elevation in maternal glucocorticoid the fetus may be exposed to greater levels of steroid as a result of a reduction in placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2).[114] In addition, at least in the hypertensive offspring of rat dams fed a low-protein diet, the tissue levels of GR are elevated and 11β-HSD2 activity is reduced, thereby exposing the tissues to greater levels of active steroid. Corresponding effects on 11β-HSD1 are not seen (reviewed in[115]). Finally, evidence for increased action of glucocorticoid tissues comes from the observation that glucocorticoid-responsive gene expression (e.g., Na^+,K^+-adenosine triphosphatase) is altered in programmed offspring.[115] The effect of gestational protein restriction on GR protein expression is greatest in the kidney This develops postnatally from 2 to 12 weeks, with similar effects seen in the liver and lung but not in the heart. In addition, despite no evidence of elevated late gestation fetal cortisol levels (at least in the sheep), fetal HPA axis responsiveness is altered after early gestation maternal nutrient restriction—although it is reduced, rather than being enhanced as predicted.[36] However, these observations are coupled to a reduction in GR messenger RNA in the pituitary in the "programmed" late gestation sheep fetuses,[116] and the implied reduction in feedback inhibition of the HPA axis is consistent with the hyperresponsiveness of the HPA axis seen in offspring from this sheep cohort at 3 months of age[36] and in adult men who were of a low birth weight studied in the Hertfordshire cohort.[117]

MECHANISMS

Several decades of work have contributed to current understanding of the mechanisms that underlie fetal cardiovascular control and growth under baseline conditions and during hypoxemia; such mechanisms include carotid chemoreflex mechanisms, hormonal factors (e.g., catecholamines, arginine vasopressin, adrenocorticotropic hormone, cortisol, angiotensin, the IGF system), and local mechanisms such as NO and endothelin-1.[3] The extent to which similar mechanisms mediate the fetal response to altered nutrition has not yet been fully elucidated. Likely candidates include components of the HPA axis, renin-angiotensin system and the IGF axis. Similar to the HPA axis (see previous), evidence exists that the fetal and postnatal renin-angiotensin system (RAS)[118-122] and IGF axis[123] are "programmable" by altered substrate supply during pregnancy. Indeed evidence is emerging that gestational dietary effects on angiotensin receptor expression in rats may be mediated via altered methylation in the promoter region.[124] The role of the kidney is uncertain because although studies in rats show that gestation-long protein restriction[125] or pharmacologic doses of dexamethasone[126] in early gestation sheep leads to postnatal hypertension and reduced nephron number, recent data find no evidence that periods of undernutrition in early or late gestation alter kidney structure or function in late gestation.[127,128] Fetal cardiovascular control (see earlier under "Fetal Adaptive Responses") and growth may be related in a graded manner in the face of altered nutrient supply. It is therefore conceivable that there are common mechanisms

integrating their control—a concept that is consistent with the dual roles for substances like AngII, IGF-I, and cortisol in cardiovascular control and growth.[3]

Ongoing postnatal studies in animals focus on the mechanisms by which programming alters peripheral vascular function. The observation that small resistance vessel responses to endothelium-dependent vasodilators, such as acetylcholine and bradykinin, are impaired in offspring of rat dams fed a low-protein diet during gestation[92] are supported by our finding that both basal and acetylcholine-stimulated NO release is reduced in the mesenteric arteries of such offspring.[92,129] Using global undernutrition during pregnancy, rather than the low-protein diet, Franco and colleagues[130] have shown that the adult offspring have reduced endothelial NO synthase activity and mRNA in the aorta. Feeding rat dams a diet high in soy isoflavones improves endothelial function and increases endothelial NO synthase and antioxidant gene expression in offspring.[131] Estrogen, acting via its α receptor, has a well-established vascular protective role mediated in part by its effects on NO production.[132] Endothelial responsiveness mediates flow-mediated vasodilatation in many vascular beds, and this can be studied in humans by techniques such as forearm plethysmography.[133] Such methods have demonstrated reduced flow-mediated vasodilatation with lower birth weight in adults[134] and an effect that is comparable to that of smoking. Similarly, endothelium-dependent vasodilator responses in the leg are reduced in obese persons with type 2 diabetes compared with control subjects.[135] Disorders of endothelial function predispose affected persons to atherosclerosis and hypertension. In adult sheep, vasoconstrictor tone after peri-implantation undernutrition was mediated in part by increased expression of myosin light-chain kinase expression, and the effect was minimized when the mismatch between the prenatal and postnatal nutrient environments was minimized.[39]

Mounting evidence suggests that epigenetic alterations may underlie many of the persistent effects of changes in the early nutrient environment on later cardiovascular phenotype. In sheep, depletion of B vitamins (B_{12} and folate) and methionine within normal ranges from the periconceptional diet of ewes led to widespread altered DNA methylation in the liver of adult offspring with associated elevated blood pressure.[136] In rat offspring evidence exists that hypomethylation of specific hepatic genes by a low-protein maternal diet can be prevented by folate supplementation.[29] It may be of great importance that grandchildren of the mothers exposed to a dietary impairment during pregnancy also show impaired vasodilator function.[93] This defect may depend more on endothelium-derived hyperpolarizing factor than on NO-mediated mechanisms and in this respect is similar to other models of hypertension in the rat.[137] This is potentially an extremely important observation because it demonstrates that programmed effects on vascular function can be passed in a transgenerational fashion, even in the absence of additional dietary or other stressors. Recently, similar transgenerational programming of risk for diabetes has been demonstrated for the male lineage.[138] The mechanisms underlying such transmission are not known, although it is of interest that the DNA methylation processes that mediate imprinting are known to be passed at least from mother to offspring,[139] and altered methylation of rat hepatic gene promotors (e.g., PPAR-α, GR) induced in the F1 generation by a maternal low-protein diet are transmitted to the F2 generation.[140] The implications of such findings for medicine are extensive, not least for the use of family linkage analysis in tracing the origins of diseases such as CHD.

CONCLUSION

The formation of the Developmental Origins of Health and Disease Hypothesis has made it necessary to take a new perspective on the mechanisms underlying fetal cardiovascular control. Moreover repeated demonstration of the association between size at birth and adult cardiovascular disease necessitates looking more closely at the link between cardiovascular control and growth during fetal life. A number of candidate mechanisms have been suggested from work in the field of fetal hypoxia, and in fact certain components of the renin-angiotensin system, HPA axis, and IGF system share physiologic endpoints in the regulation of cardiovascular control and growth. Work has begun on determining whether fetal growth and cardiovascular control are related in a graded fashion in the face of altered nutrition; this research is likely to be crucial to elucidation of fetal programming. The emergence of new concepts that it is the degree of mismatch between the pre- and postnatal nutrient environments that determines cardiovascular disease risk will be key to understanding the longer-term implications of such fetal adaptations and to the development of future intervention strategies to prevent disease in later life.

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John P. Kinsella and Philip W. Shaul

Physiology of Nitric Oxide in the Developing Lung

Since the recognition of nitric oxide (NO) as an important endogenous mediator of vasorelaxation in 1987,[1,2] its role in the regulation of pulmonary vasomotor tone has been intensively investigated. Because the successful transition from fetal placental dependence to survival at birth requires that pulmonary vascular resistance (PVR) rapidly declines and pulmonary blood flow increases,[3-6] the role of endogenous NO production in the fetal and newborn lung circulation has received considerable attention over the last 15 years. In particular, the physiology of NO-mediated vasoregulation in the fetal and neonatal lung has been of keen interest to developmental lung biologists because of the life-threatening clinical disorder of persistent pulmonary hypertension of the newborn (PPHN). Moreover, laboratory and clinical studies have also shown that, in addition to its effects on reducing pulmonary artery pressure, other beneficial effects of inhaled NO (iNO) may include improvements in ventilation-perfusion matching; decreasing lung inflammation and oxidant stress; and favorably modulating angiogenesis, growth, and extracellular matrix deposition in the immature lung. Thus considerable interest also exists in the potential role of iNO in premature newborns with respiratory failure. In this chapter we review the physiologic role of endogenous NO production in the fetal and transitional pulmonary circulation, the normal ontogeny of NO synthase (NOS) isoform expression in the fetal lung and alterations observed in animal models of PPHN and bronchopulmonary dysplasia (BPD), the effects of iNO on the lung of the term and preterm newborn, and the current clinical role of iNO.

ENDOGENOUS NITRIC OXIDE: FETAL AND TRANSITIONAL VASOREGULATION

Vasomotor tone in the fetus is modulated by both vasoconstrictor and vasodilator substances. The role of vasodilator substances such as NO has been of particular interest because of its role in

the marked pulmonary vasorelaxation that must occur at birth to assure a successful transition from fetal to neonatal life. NO was recognized as a potent vasodilator as early as 1979,[7] and in 1980 Furchgott and Zawadzki[7a] reported that acetylcholine-induced vasorelaxation was dependent on an intact endothelium through the elaboration of an endothelial-derived relaxing factor (EDRF) that diffused to the subjacent vascular smooth muscle.[1] In 1987 investigators from two separate laboratories reported that the biologic activity of EDRF was identical to that of NO or an NO-containing substance. Palmer and colleagues[1] induced the release of EDRF from porcine aortic endothelial cells in culture and compared the effects on superfused aortic strips with those of NO in solution. They found that the effects of EDRF were indistinguishable from those of NO. Ignarro and colleagues[2] used a bioassay cascade superfusion technique with intrapulmonary arteries and veins, identified EDRF pharmacologically and chemically as NO, and found that EDRF and NO produced similar vasorelaxation and were inhibited by common antagonists. Ignarro and colleagues[2] also recognized that NO was inactivated by combining with hemoproteins and speculated that hemoglobin could trap endogenously produced NO that diffused into the vascular lumen, thus preventing any "downstream" vasorelaxation by this paracrine mediator.

In mammalian cells, NO is produced from the terminal guanidino nitrogen of L-arginine on its conversion to L-citrulline by the enzyme NOS in a reaction that requires molecular oxygen.[8] NOS is a family of enzymes that includes three major isoforms known as neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS).[9-15] The three isoforms, nNOS, iNOS, and eNOS, are also referred to as NOS-I, NOS-II, and NOS-III. The activity of nNOS and eNOS is calcium dependent, whereas the activity of iNOS is calcium independent.[10] nNOS and iNOS are primarily found in the soluble fraction, whereas eNOS is primarily in the membrane fraction, particularly in specialized signaling microdomains on the plasma membrane called caveolae.[16] All NOS isoforms are homodimers of subunits that range between 130 and 160 kDa.[17] nNOS and eNOS expression were first thought to be primarily constitutive in nature, whereas the expression of iNOS is initiated by cytokines.[10,18]

The recognition that the endogenous production of this EDRF-NO mediator could be competitively blocked by modified L-arginine analogues prompted early experiments into the effects of NO in the fetal and transitional pulmonary circulation. The earliest studies were performed in fetal lambs because the relatively large size of the ovine fetus allows for instrumentation with catheters to monitor vascular pressure and electromagnetic or ultrasound probes to monitor blood flow. Moreover, the ovine fetus is a suitable candidate for such experiments because operating on the fetus does not induce parturition, as commonly occurs in primate models. Abman and colleagues[19] performed the first experiments on the role of EDRF in the ovine model, demonstrating that endogenous EDRF-NO production modulates basal pulmonary vascular tone in the late-gestation fetus and that pharmacologic NO blockade inhibits endothelium-dependent pulmonary vasodilation. These investigators also showed that pharmacologic NO blockade attenuates the rise in pulmonary blood flow at delivery, thus implicating endogenous NO formation in postnatal adaptation after birth and linking this laboratory observation to the life-threatening clinical condition of PPHN (Figure 72-1). In addition, experiments using this ovine model showed that increased fetal oxygen tension augments endogenous NO release,[20,21] and the increase in pulmonary blood flow in response to rhythmic distention of the lung and high inspired oxygen concentrations are mediated in part by endogenous NO elaboration.[22]

Of interest, the major NOS isoform responsible for modulation of basal pulmonary vascular tone in the fetus and pulmonary vasodilation at birth may not be eNOS (NOS type III). Studies have shown that the iNOS isoform (NOS II) is expressed in airway epithelium and vascular smooth muscle in the late-gestation ovine fetus.[23] Moreover, in the late-gestation ovine fetus, selective inhibition of iNOS increases basal PVR, reduces shear stress–induced pulmonary vasodilation, and attenuates NO-mediated pulmonary vasodilation at birth.[24-26]

In contrast to the condition in the mature fetus, indirect evidence exists that *endothelium-dependent* pulmonary vasodilation in the premature pulmonary circulation is diminished until late in gestation. Acetylcholine and increased oxygen tension cause marked pulmonary vasodilation in the late-gestation ovine fetus, but dilator responses to these stimuli are decreased in the premature fetus.[27-29] Because acetylcholine and oxygen-induced fetal pulmonary vasodilation are partly mediated by endogenous NO formation, it appears that endogenous NO activity increases with advancing gestational age.[20-22] Similarly, no differences were found in relaxation of ovine fetal pulmonary artery rings compared with adult rings when both were exposed to an endothelium-independent vasodilator (sodium nitroprusside), but maturational changes in vasorelaxation were observed with endothelium-dependent vasodilators.[30]

NITRIC OXIDE SYNTHASES IN THE DEVELOPING LUNG

The cellular distribution of the three NOS isoforms has been evaluated in parallel, isoform-specific immunohistochemical analyses in developing ovine lung. In third-trimester fetal lung eNOS was found in vascular endothelium as expected, but it was also markedly abundant in bronchial and proximal bronchiolar epithelium. eNOS was not detected in terminal or respiratory bronchiolar epithelium or in alveolar epithelium. Similar to eNOS, iNOS protein was detected in bronchial and proximal bronchiolar epithelium but not in alveolar epithelium. However, iNOS was also expressed in terminal and respiratory bronchioles. nNOS protein was found in epithelium at all levels including the alveolar wall. In addition, positive immunostaining was found for iNOS and nNOS in airway and vascular smooth muscle. The cellular distribution of all three isoforms was similar in fetal, newborn, and adult ovine lung, and the observations made in the epithelium by immunohistochemistry were confirmed by isoform-specific reverse transcriptase–polymerase chain reaction assays and reduced nicotinamide adenine dinucleotide phosphate diaphorase histochemistry.[25] More recent studies in lungs from late-gestation fetal baboons have yielded similar findings, demonstrating that nNOS, eNOS, and iNOS are primarily expressed in the airway epithelium in the fetal primate.[31] Thus the three NOS isoforms are abundant and commonly expressed in proximal lung epithelium, and they are differentially expressed in distal lung epithelium. All three isoforms may be important sources of pulmonary NO throughout lung development.

Maturational changes in NOS isoform expression in the lung have also been delineated. The ontogeny of eNOS and nNOS expression was first determined in lungs from fetal and newborn rats. Both eNOS and nNOS proteins were detectable in 16-day fetal lung; they increased four-fold and three-fold, respectively, to maximal levels at 20 days gestation (term = 22 days); and they fell after birth (1 to 5 days). In parallel with the findings for eNOS protein, eNOS messenger RNA (mRNA) levels assessed with use of semiquantitative reverse transcriptase–polymerase chain reaction were increased from 16 to 20 days gestation and fell after birth. In contrast, nNOS mRNA abundance declined during late fetal life and rose after birth. Findings by reverse transcriptase–polymerase chain reaction were confirmed by Northern analyses. These observations indicate that eNOS and nNOS gene expression are developmentally regulated in rat lung, with maximal eNOS and nNOS protein expression near term. Furthermore, the regulation of pulmonary eNOS may primarily involve alterations in transcription or mRNA stability, whereas nNOS expression in

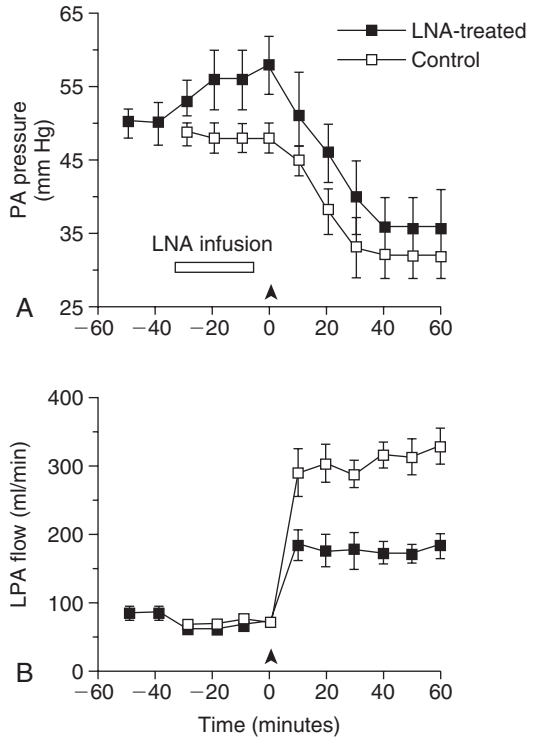


Figure 72-1 Inhibition of endogenous NO production attenuates the decline in PVR associated with birth-related events. *(From Abman SH, Chatfield BA, Hall SL, McMurtry IF: Role of endothelium-derived relaxing factor activity during transition of pulmonary circulation at birth,* Am J Physiol Heart Circ Physiol *259:H1921-H1927, 1990.)*

the maturing lung may also be mediated by additional posttranscriptional processes.[32] The developmental regulation of eNOS expression has also been discerned in ovine fetal lung. eNOS is present in the pulmonary vasculature of the premature ovine fetus as early as 43 days gestation (29% of term gestation[33]), with maximal eNOS mRNA and protein expression observed early in the third trimester.[34] Differences in the timing of eNOS upregulation in the sheep and rat may be related to the temporal features of lung development in the two species, with a greater proportion of maturation occurring after birth in the rat.[35]

To better understand the regulation of pulmonary NO production in the developing primate, maturational changes in NOS isoform expression and enzymatic activity have recently been determined in the proximal lung of third-trimester fetal baboons from 125 to 175 days gestation (term = 185 days) (Figure 72-2). A marked increase occurred in total NOS enzymatic activity from 125 to 140 days gestation because of elevations in nNOS and eNOS expression, whereas iNOS expression and activity were minimal. Total NOS activity was constant from 140 to 175 days gestation, and during the latter stage (160 to 175 days gestation) a dramatic fall in nNOS and eNOS was replaced by a rise in iNOS. Studies done within 1 hour of delivery at 125 or 140 days gestation revealed that the principal increase in NOS during the third trimester is associated with an elevation in exhaled NO levels, a decline in expiratory resistance, and greater pulmonary compliance.[18] These cumulative observations indicate that normal developmental increases occur in pulmonary NOS expression and NO production during the third trimester that may serve to optimize not only pulmonary vascular but also airway and parenchymal function in the immediate postnatal period.

Alterations in NO production and NOS expression have been evaluated in animal models of perinatal pulmonary vascular disease, with particular emphasis on eNOS action and expression. Chronic hypoxia is an important causal factor in the development of pulmonary hypertension in the perinatal period, and in newborn piglets chronic hypoxia attenuates acetylcholine-stimulated (eNOS dependent) pulmonary vasodilation, marked diminution occurs in both basal and acetylcholine-stimulated pulmonary NO production, and eNOS expression is attenuated.[36-38] In studies of the direct effects of varying oxygen exposure on isolated ovine fetal pulmonary artery endothelial cells, eNOS mRNA and protein expression were both attenuated by three-fold with hypoxia.[39] These findings suggest that decreased eNOS expression and resultant diminution in pulmonary NO production may contribute to the pathogenesis of hypoxic pulmonary hypertension in the newborn.

NOS expression has also been evaluated in a rat model of congenital diaphragmatic hernia (CDH). The development of CDH can be induced in approximately 50% to 60% of rat fetuses after maternal ingestion of the herbicide nitrofen early in gestation. Studies in this model have revealed that eNOS protein abundance is decreased by 42% in the lung ipsilateral to the hernia compared with lungs from control fetuses exposed to nitrofen but lacking CDH. Immunoblots for factor VIII–related antigen indicate that this is not related to alterations in endothelial cell density, and studies of nNOS protein reveal that this finding is unique to the endothelial isoform of NOS. eNOS mRNA abundance evaluated by reverse transcriptase–polymerase chain reaction assay is also decreased in CDH lung, falling to 22% of control levels.[40] These observations indicate that attenuated eNOS expression may contribute to PPHN in infants with CDH.

Potentially important observations have also been made in a lamb model of neonatal pulmonary hypertension created by ligation of the fetal ductus arteriosus 10 to 20 days before delivery. NO-dependent relaxation is attenuated in intrapulmonary arteries from these animals.[41] In parallel, eNOS protein expression is decreased by one half in the hypertensive lung, and nNOS expression is unaltered. Similarly, NOS enzymatic activity is decreased 45% to 75% in the hypertensive lung. Paralleling the declines in eNOS protein and NOS enzymatic activity, eNOS mRNA abundance is also decreased in the hypertensive lung.[42,43] Thus pulmonary eNOS gene expression is attenuated in the lamb model of neonatal pulmonary hypertension. As such, evidence exists of diminished NOS function in multiple models of PPHN in multiple species.

EXOGENOUS (INHALED) NITRIC OXIDE AS A SELECTIVE PULMONARY VASODILATOR

NO in its natural form exists as a gas, prompting its application to clinical medicine. The first clinical experiments with iNO gas were unrelated to its effects on pulmonary vasodilation. On the basis of the fact that iNO gas diffuses into the pulmonary circulation much faster than carbon monoxide, Borland and colleagues[44] tested the hypothesis that iNO could serve as a measure of diffusing capacity of the alveolar-capillary membrane in 1983. After the discovery that EDRF was actually NO, Higenbottam and colleagues[45] studied the effects of iNO on pulmonary vasodilation in adults with primary pulmonary hypertension in the cardiac catheterization laboratory. At a platform presentation during the American Thoracic Society meeting in 1988, they reported that, when compared with prostacyclin, iNO caused equivalent reductions in PVR, but without systemic vascular effects.[45]

These observations captured the interest of physiologists studying the newborn pulmonary circulation because, after birth and the initiation of air breathing, PVR rapidly declines and pulmonary blood flow markedly increases. These changes are essential for the normal transition to extrauterine life, and the potential of a selective pulmonary vasodilator was appealing, because available alternatives for the treatment of PPHN caused adverse systemic vascular effects.

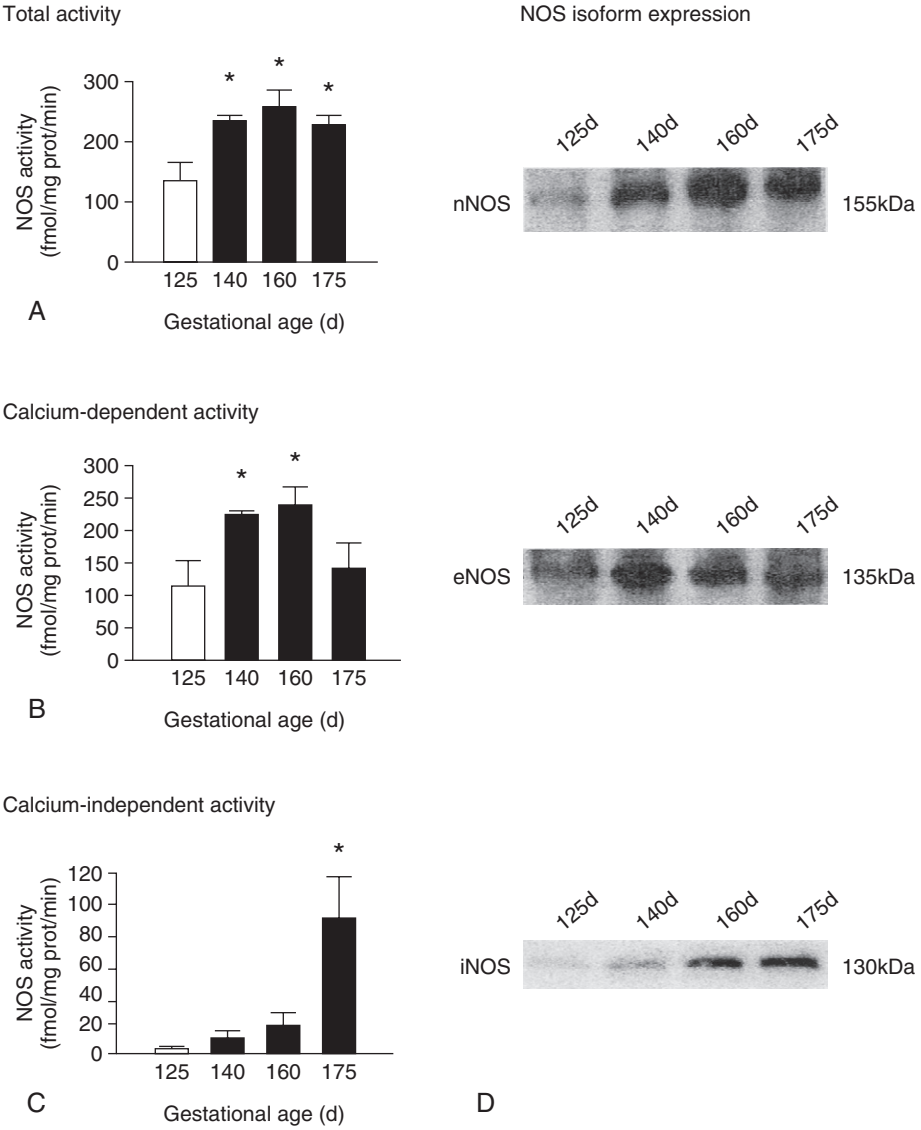


Figure 72-2 A-D, NOS enzymatic activity and NOS isoform expression in proximal baboon lung are developmentally regulated during the third trimester. *(From Shaul PW, Afshar S, Gibson LL, et al: Developmental changes in nitric oxide synthase isoform expression and nitric oxide production in fetal baboon lung,* Am J Physiol *283:L1192-L1199, 2002.)*

In 1991, prompted by the observations of Higenbottam and colleagues,[45] investigators commissioned an industrial gas manufacturer to combine NO with nitrogen to yield an NO concentration of 450 ppm, based on calculations designed to deliver 20 ppm NO within a continuous-flow, time-cycled, pressure-limited neonatal ventilator with a bias gas flow rate of 10 L/minute. The concentration of 450 ppm was chosen to balance environmental risk from sudden, complete tank evacuation against the obligate decrease in Fio_2 within the ventilator circuit during the administration of iNO. The first experiments of iNO in the late-gestation newborn lamb tested the effects at doses of 5, 10, and 20 ppm (based on the chronic ambient levels considered to be safe for adults by regulatory agencies in the United States[46]). Using vascular flow probes and radiolabeled microspheres to measure pulmonary and systemic blood flow, these investigators found that iNO caused potent, selective, and sustained pulmonary vasodilation in the late-gestation newborn lamb (Figure 72-3).[47] Moreover, cerebral blood flow and cerebral metabolic rate for oxygen were not affected by NO inhalation.[48] Frostell and colleagues[49] showed that iNO caused pulmonary vasodilation in adult animals during acute hypoxia and during pharmacologically induced pulmonary hypertension (U46619), and Roberts and colleagues[50] demonstrated that iNO reversed hypoxic pulmonary vasoconstriction in the juvenile lamb with respiratory acidosis. Corroborating studies in other animal models supported the observations that iNO is a selective pulmonary vasodilator at low doses (≤20 ppm),[51-54] and studies using a model of PPHN in which marked structural pulmonary vascular changes are induced by prolonged fetal ductus arteriosus compression demonstrated that, despite the loss of endothelium-dependent vasodilation, the response to iNO remained intact.[55,56]

The effects of iNO in the premature subject have also been of interest to translational physiologists. However, the premature fetus is characterized by both structural and functional pulmonary parenchymal and vascular immaturity. Thus separating the relative effects of pulmonary immaturity and its effects on lung inflation has been difficult because the pulmonary vascular, parenchymal, and airway responses of the premature lung to mechanical ventilation are uniquely different from those in the term subject. For example, surfactant deficiency compromises

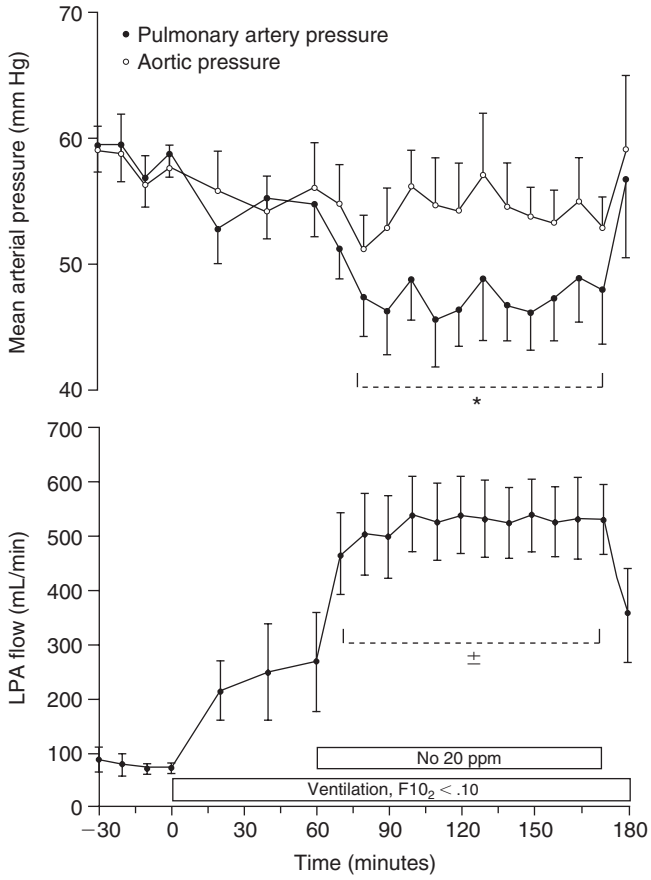


Figure 72-3 iNO causes potent, selective, and sustained pulmonary vasodilation in the term newborn lamb. *(From Kinsella JP, McQueston JA, Rosenberg AA, Abman SH: Hemodynamic effects of exogenous nitric oxide in ovine transitional pulmonary circulation,* Am J Physiol *1992;262:H875-H880.)*

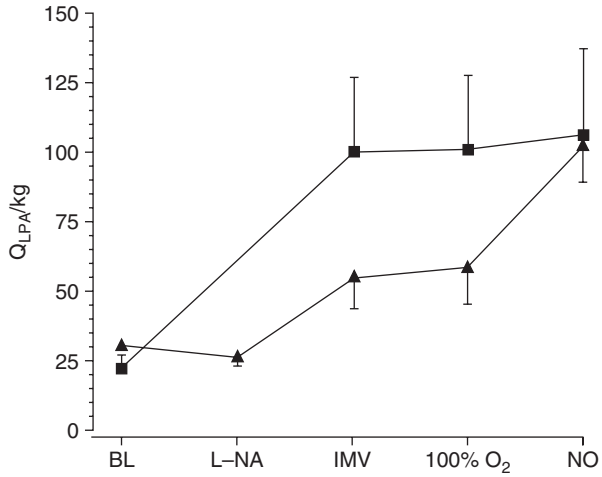


Figure 72-4 Inhibition of endogenous NO production in the premature lamb attenuates the increase in blood flow associated with birth-related events. Low-dose iNO increases pulmonary blood flow to normal levels. *(From Kinsella JP, Ivy DD, Abman SH: Ontogeny of NO activity and response to inhaled NO in the developing ovine pulmonary circulation,* Am J Physiol *267:H1955-H1961, 1994.)*

the normal increase in lung volume caused by tidal volume ventilation in extremely premature lambs. Although changes in PVR after delivery are mediated in part through the elaboration of vasoactive mediators by the pulmonary vascular endothelium, decreased lung volume exerts mechanical effects on the pulmonary circulation, which increases pulmonary vascular tone. The early observations of Dawes and colleagues[3] predicated the notion that the premature pulmonary vascular bed insufficiently dilated at birth and that this suboptimal vasodilation contributed to respiratory distress syndrome (RDS). Dawes and colleagues delivered four extremely premature lambs (76% to 83% of term gestation) and noted that the most immature animals had minimal reduction in PVR during tidal volume ventilation for 2 minutes after delivery, in contrast to the marked decrease in PVR noted in term animals. Indeed, pulmonary hypoperfusion was implicated in the evolution of "idiopathic" RDS.[57] However, after the seminal observations of Avery and Mead[58] and Fujiwara and colleagues,[59] the contribution of surfactant deficiency to respiratory failure in the premature newborn became the pivotal pathophysiologic focus.

Surfactant therapy has dramatically improved the outcome of infants born prematurely. However, surfactant treatment alone does not consistently cause sustained improvement in oxygenation in extremely premature lambs. Jobe and colleagues[60] found only transient improvement in oxygenation in response to exogenous surfactant in lambs delivered at 120 days gestation (82% of term), and repeated doses were less predictably effective. Walther and colleagues[61] showed that repeated doses of exogenous surfactant caused sustained improvement in oxygenation in lambs at 128 days gestation (87% of term), but serial surfactant replacement in more immature lambs at 118 days gestation (80% of term) did not modify the course of respiratory deterioration. These observations support the suggestion that other problems of prematurity (e.g., structural lung immaturity or altered vascular responses to dilator stimuli) contribute to respiratory failure in RDS. It is possible that persistent hypoxemia after surfactant replacement in the most immature subject is caused by pulmonary hypertension associated with decreased endogenous pulmonary vasodilator production.

After delivery, the fall in PVR in the premature lamb in response to birth-related events (rhythmic lung distention and increased inspired oxygen) is maximal with tidal volume mechanical ventilation and lung inflation alone, and little pulmonary hemodynamic response to high inspired oxygen occurs.[62] However, when endogenous NO production is blocked during delivery of the premature lamb, the normal increase in pulmonary blood flow associated with mechanical ventilation and lung inflation is markedly attenuated, and subsequent treatment with iNO increases pulmonary blood flow to the same level as in control animals (Figure 72-4).

The premature lamb has been extensively studied as a model of severe RDS.[63] Survival with exogenous surfactant treatment and mechanical ventilation at delivery varies depending on the gestational age of the lamb and the type of surfactant given.[60,64] In very immature lambs (115 days gestation), gas exchange worsens and PVR increases during mechanical ventilation beyond 60 to 90 minutes after birth, despite treatment with exogenous surfactant at delivery.

Several studies have examined the effects of iNO in the premature lamb with hyaline membrane disease. Skimming and colleagues[65] treated premature lambs (126 to 127 days gestation) with iNO at 5 and 20 ppm for brief periods and showed improvement in oxygenation and PVR. In another study, intermittent mandatory ventilation over a 2-hour period in the extremely premature ovine fetus (115 days gestation, 78% of term) resulted in progressive worsening of gas exchange and increased PVR. After 2 hours of ventilation, brief NO treatment lowered PVR and improved gas exchange.[66] Moreover, early and continuous treatment with iNO

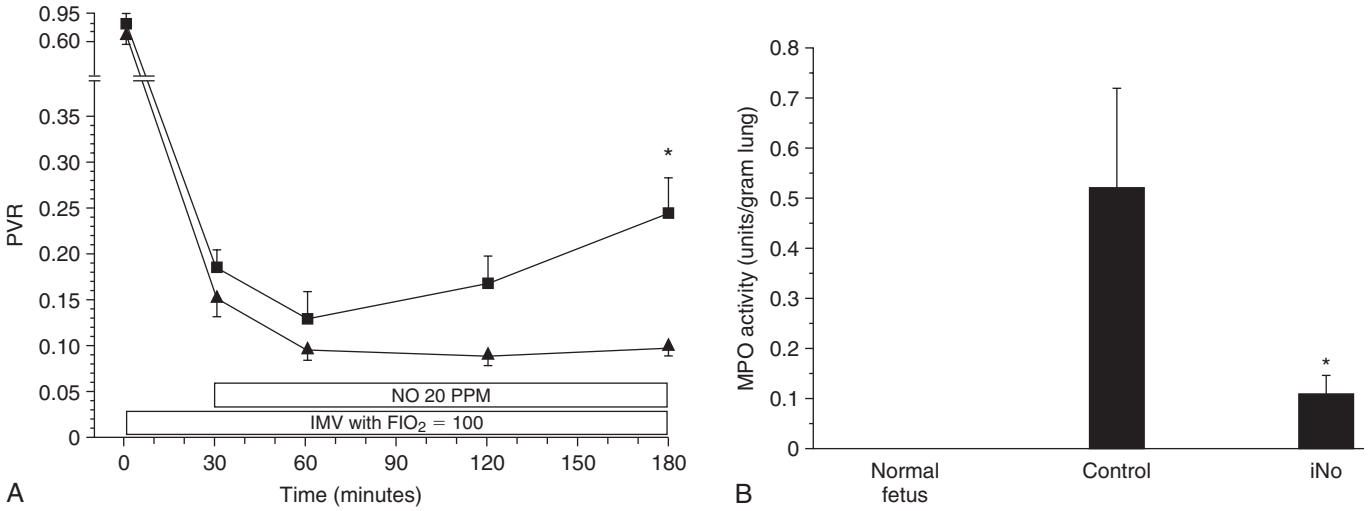


Figure 72-5 A, Low-dose iNO causes sustained pulmonary vasodilation in mechanically ventilated premature lambs with RDS. *(From Kinsella JP, Parker TA, Galan H, et al: Effects of inhaled nitric oxide on pulmonary edema and lung neutrophil accumulation in severe experimental hyaline membrane disease,* Pediatr Res *41:457-63, 1997.)* **B,** Low-dose iNO decreases lung neutrophil accumulation in the premature lamb with RDS.

(20 ppm) caused sustained improvement in gas exchange and pulmonary hemodynamics over 3 hours of mechanical ventilation in the extremely premature lamb. Lung recruitment strategies using high-frequency oscillatory ventilation (HFOV) have been shown to augment the response to low-dose iNO in premature lambs with RDS, emphasizing the critical role of adequate lung inflation during inhalational vasodilator therapy.[67]

Another reason iNO in the premature subject has been of interest is that, in addition to its effects on pulmonary hemodynamics and gas exchange during inhalation, endogenous NO has been shown to regulate vascular permeability and neutrophil adhesion in the microcirculation.[68] Although reports suggest that iNO may decrease capillary permeability in acute lung injury,[69,70] the immature lung is particularly susceptible to lung protein leak during mechanical ventilation.[71] This proteinaceous pulmonary edema causes atelectasis and ventilation-perfusion abnormalities, worsening gas exchange, and increasing PVR. Increasing pulmonary blood flow with iNO in the premature subject could also have potential adverse effects. For example, conditions associated with increased pulmonary blood flow in the premature infant (patent ductus arteriosus [PDA]) cause increased lung neutrophil accumulation.[72] The effects of iNO on pulmonary vascular permeability and lung leukosequestration in premature lambs with RDS have been reported.[73] In premature lambs delivered at 78% of term, low-dose iNO (5 ppm) increased pulmonary blood flow and improved gas exchange without increasing pulmonary edema, and decreased lung neutrophil accumulation (Figure 72-5). Sequestration of neutrophils in the lung is an early step in a complex inflammatory response mediated through the elaboration of oxy radicals, proteases, phospholipases, and lipid compounds.[74] Therapies that reduce neutrophil accumulation in the lung in hyaline membrane disease could potentially modify the early inflammatory process that amplifies acute lung injury and contributes to the development of chronic lung disease.[75]

In addition to the acute effects of iNO on pulmonary vasodilation, lung inflammation, and oxidant stress, evidence is increasing that impaired endogenous NO production contributes to the pathogenesis of BPD. For example, lung nNOS expression is decreased in the primate model of BPD (Figure 72-6), lung eNOS abundance is decreased in both the primate and ovine models of BPD (see Figure 72-6),[76,77] and mice genetically deficient for eNOS have abnormal distal lung architecture.[78,79] The potential

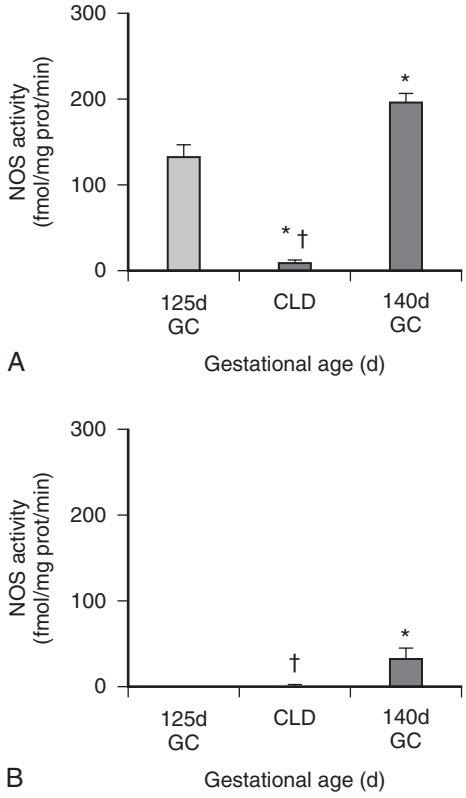


Figure 72-6 nNOS **(A)** and eNOS **(B)** are both decreased in the primate model of BPD. *(Modified from Afshar S, Gibson LL, Yuhanna IS, et al: Pulmonary NO synthase expression is attenuated in a fetal baboon model of chronic lung disease,* Am J Physiol Lung Cell Mol Physiol *284:L749-L758, 2003.)*

for iNO to modulate the evolution of lung injury in animal models of BPD has been the focus of recent studies, providing further experimental rationale for the role of iNO in premature subjects.[80] Lin and colleagues[81] found that hyperoxia exposure of neonatal rats inhibited lung vascular growth and impaired

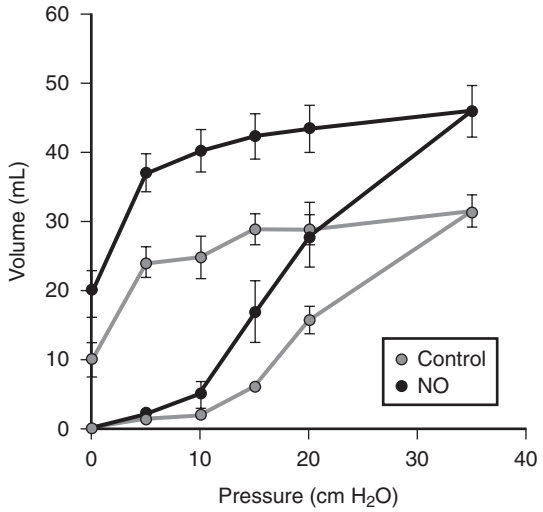


Figure 72-7 iNO improves pulmonary function in the primate model of BPD. Postmortem pressure-volume curves were performed after 14 days of control treatment or iNO administration. *(Modified from McCurnin DC, Pierce RA, Chang LY, et al: Inhaled NO improves early pulmonary function and modifies lung growth and elastin deposition in a baboon model of neonatal chronic lung disease,* Am J Physiol Lung Cell Mol Physiol *288:450-459, 2005.)*

alveolarization and that treatment with iNO after neonatal hyperoxia enhanced late lung growth and improved alveolarization in this model of BPD. McCurnin and colleagues[82] studied the effects of iNO in a baboon model of BPD over the first 14 days of life. They found that iNO improved pulmonary function (Figure 72-7) and favorably altered lung growth and elastin deposition (Figure 72-8) in mechanically ventilated premature baboons with evolving BPD. The tempering of elastin deposition with iNO was related to decreased elastin gene activation and fewer alveolar myofibroblasts (see Figure 72-8). In chronically ventilated premature lambs, Bland and colleagues[83] found that iNO administration preserved structure and function of airway smooth muscle and enhanced alveolar development. Thus compelling evidence suggests that endogenous NO plays a vital role in pulmonary vascular and alveolar development in the immature lung and that low-dose iNO may have beneficial effects on both the acute and chronic perturbations that are associated with the pathogenesis of BPD in the premature newborn.

CLINICAL EFFECTS OF INHALED NITRIC OXIDE IN THE TERM NEWBORN WITH PERSISTENT PULMONARY HYPERTENSION OF THE NEWBORN

Early studies showed that iNO therapy caused marked improvement in oxygenation in term newborns with PPHN.[84-89] Multicenter randomized clinical studies subsequently confirmed that iNO therapy reduces the need for extracorporeal membrane oxygenation treatment in term neonates with hypoxemic respiratory failure.[90,91] Extensive supportive data have demonstrated its overall safety and efficacy, as evidenced by its recent approval by the U.S. Food and Drug Administration.

PPHN[92] is a syndrome associated with diverse neonatal cardiac and pulmonary disorders that are characterized by high PVR causing extrapulmonary right-to-left shunting of blood across the ductus arteriosus and/or foramen ovale.[93] Extrapulmonary shunting caused by high PVR in severe PPHN can cause critical hypoxemia that is poorly responsive to inspired oxygen or pharmacologic vasodilation. Vasodilator drugs administered intravenously, such as tolazoline and sodium nitroprusside, are often unsuccessful because of systemic hypotension and an inability to achieve or sustain pulmonary vasodilation.[94,95] Thus the ability of iNO therapy to selectively lower PVR and decrease extrapulmonary venoarterial admixture accounts for the acute improvement in oxygenation observed in newborns with PPHN.[96]

The first studies of iNO treatment in term newborns reported initial doses that ranged from 80 ppm[76] to 6 to 20 ppm (Figure 72-9).[77] The rationale for doses used in these clinical trials was based on concentrations that had previously been found to be effective in animal experiments by the same investigators.[47,50] Roberts and colleagues reported that brief (30 minutes) inhalation of NO at 80 ppm improved oxygenation in patients with PPHN, but this response was sustained in only one patient after NO was discontinued.[76] In the second report, rapid improvement in oxygenation in neonates with severe PPHN was also demonstrated, but this was achieved at lower doses (20 ppm) for 4 hours.[77] This study also reported that decreasing the iNO dose to 6 ppm for the duration of treatment provided sustained improvement in oxygenation. The relative effectiveness of low-dose iNO in improving oxygenation in patients with severe PPHN was corroborated in a study by Finer and colleagues.[97] Acute improvement in oxygenation during treatment was not different with doses of iNO ranging from 5 to 80 ppm.

Because of its selective pulmonary vasodilator effects, iNO therapy is an important adjunct to available treatments for term newborns with hypoxemic respiratory failure. However, hypoxemic respiratory failure in the term newborn represents a heterogeneous group of disorders, and disease-specific responses have clearly been described. For example, patients with extrapulmonary right-to-left shunting (PPHN) show acute improvement in oxygenation when PVR becomes subsystemic during iNO therapy, and patients with predominantly intrapulmonary shunting (e.g., RDS) have less dramatic responses.

Several pathophysiologic disturbances contribute to hypoxemia in the newborn infant, including cardiac dysfunction, airway and pulmonary parenchymal abnormalities, and pulmonary vascular disorders. In some newborns with hypoxemic respiratory failure, a single mechanism predominates (e.g., extrapulmonary right-to-left shunting in idiopathic PPHN), but more commonly several of these mechanisms contribute to hypoxemia. For example, in a newborn with meconium aspiration syndrome, meconium may obstruct some airways, decreasing V/Q ratios and increasing intrapulmonary shunting. Other lung segments may be overventilated relative to perfusion and cause increased physiologic dead space. Moreover, the same patient may have severe pulmonary hypertension with extrapulmonary right-to-left shunting at the ductus arteriosus and foramen ovale. Not only does the overlap of these mechanisms complicate the clinical management, but the tendency for time-dependent changes in the relative contribution of each mechanism to hypoxemia requires continued vigilance as the disease progresses. Therefore understanding the relative contribution of these different causes of hypoxemia becomes critically important as the inventory of therapeutic options expands.

Considering the important role of parenchymal lung disease in many cases of PPHN, pharmacologic pulmonary vasodilation alone would not be expected to cause sustained clinical improvement.[98] The effects of inhaled NO may be suboptimal when lung volume is decreased in association with pulmonary parenchymal disease.[99] Atelectasis and air space disease (pneumonia, pulmonary edema) will decrease effective delivery of iNO to its site of action in terminal lung units. In PPHN associated with heterogeneous (patchy) parenchymal lung disease, inhaled NO may be effective in optimizing ventilation-perfusion matching by preferentially causing vasodilation in lung units that are well ventilated. The effects of inhaled NO on ventilation-perfusion matching appear to be optimal at low doses (<20 ppm).[100]

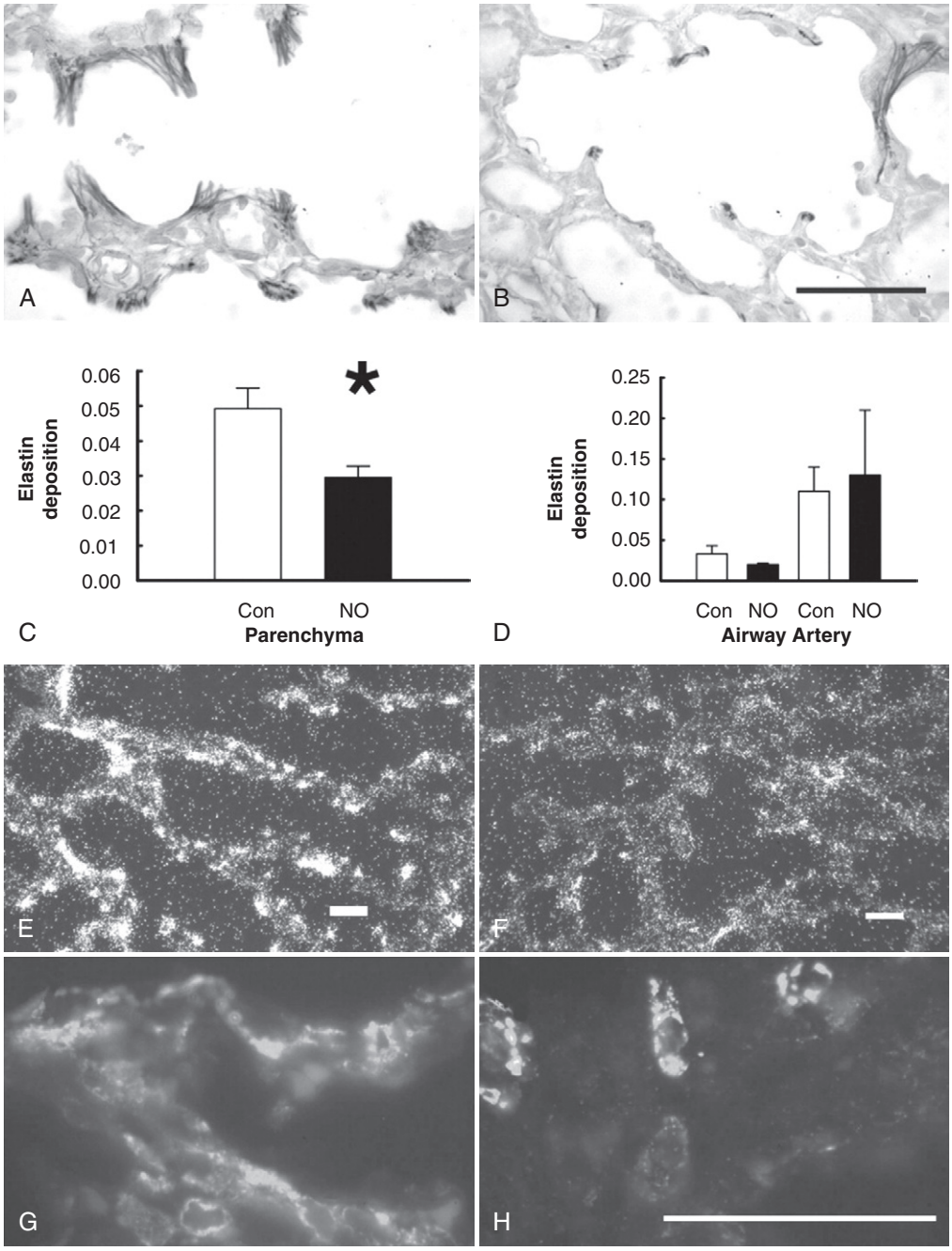


Figure 72-8 Elastin deposition in the primate model of BPD is tempered by iNO. Hart's staining of control **(A)** and iNO-treated lungs **(B),** and quantification of elastin deposition in the parenchyma **(C)** and airways and arteries **(D).** In situ hybridization for elastin mRNA in control **(E)** and iNO-treated lungs **(F),** and immunofluorescence for the alveolar myofibroblast marker α-smooth muscle actin in control **(G)** and iNO-treated lungs **(H).** *(From McCurnin DC, Pierce RA, Chang LY, et al: Inhaled NO improves early pulmonary function and modifies lung growth and elastin deposition in a baboon model of neonatal chronic lung disease,* Am J Physiol Lung Cell Mol Physiol *288:450-459, 2005.)*

However, in cases complicated by homogeneous (diffuse) parenchymal lung disease and underinflation, pulmonary hypertension may be exacerbated because of the adverse mechanical effects of underinflation on PVR. In this setting, effective treatment of the underlying lung disease is essential (and sometimes sufficient) to cause resolution of the accompanying pulmonary hypertension.

Along with iNO treatment, other therapeutic strategies have emerged for the management of the term infant with hypoxemic respiratory failure. Patients not responding to iNO can show marked improvement in oxygenation with adequate lung inflation alone.[78] High success rates in early studies were achieved by withholding iNO treatment until aggressive attempts were made to optimize ventilation and lung inflation with mechanical ventilation. These early studies demonstrated that the effects of iNO may be suboptimal when lung volume is decreased in association with pulmonary parenchymal disease, for several reasons. First, atelectasis and air space disease (pneumonia, pulmonary edema) may decrease the effective delivery of iNO to its site of action in terminal lung units. Second, in cases complicated by severe lung disease and underinflation, pulmonary hypertension

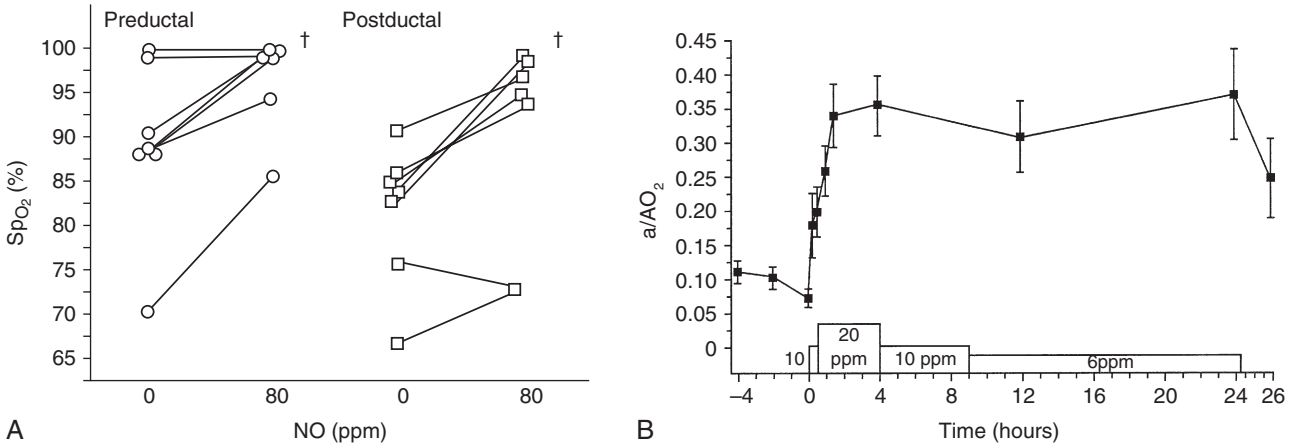


Figure 72-9 A, Brief treatment with 80 ppm iNO caused acute improvement in newborns with PPHN. *(From Roberts JD, Polaner DM, Lang P, Zapol WM. Inhaled nitric oxide in persistent pulmonary hypertension of the newborn,* Lancet *340:818-819, 1992.)* **B,** Treatment with low-dose iNO for 24 hours caused sustained improvement in oxygenation in newborns with PPHN. *(From Afshar S, Gibson LL, Yuhanna IS, et al. Pulmonary NO synthase expression is attenuated in a fetal baboon model of chronic lung disease,* Am J Physiol Lung Cell Mol Physiol *284:L749-758, 2003.)*

may be exacerbated because of the adverse mechanical effects of underinflation on PVR. Third, attention must be given to minimize overinflation to avoid inadvertent positive end-expiratory pressure and gas trapping that may elevate PVR from vascular compression. This commonly complicates the management of infants with asymmetric lung disease or airways obstruction as observed in meconium aspiration syndrome.

In newborns with severe lung disease, HFOV is frequently used to optimize lung inflation and minimize lung injury.[101] In clinical pilot studies using iNO, we found that the combination of HFOV and iNO caused the greatest improvement in oxygenation in some newborns who had severe PPHN complicated by diffuse parenchymal lung disease and underinflation (e.g., RDS, pneumonia).[102,103] A randomized, multicenter trial demonstrated that treatment with HFOV + iNO was often successful in patients who did not respond to HFOV or iNO alone in severe PPHN, and differences in responses were related to the specific disease associated with the complex disorders of PPHN (Figure 72-10).[78] For patients with PPHN complicated by severe lung disease, response rates for HFOV + iNO were better than HFOV alone or iNO with conventional ventilation. In contrast, for patients without significant parenchymal lung disease, both iNO and HFOV + iNO were more effective than HFOV alone. This response to combined treatment with HFOV + iNO likely reflects both improvement in intrapulmonary shunting in patients with severe lung disease and PPHN (using a strategy designed to recruit and sustain lung volume, rather than to hyperventilate) and augmented NO delivery to its site of action. Although iNO may be an effective treatment for PPHN, it should be considered only as part of an overall clinical strategy that cautiously manages parenchymal lung disease, cardiac performance, and systemic hemodynamics.

Pharmacologic augmentation of the iNO response may also prove to be effective in some patients with PPHN. Inhaled NO causes pulmonary vasodilation by stimulating soluble guanylate cyclase and increasing cyclic guanosine monophosphate (cGMP) content in vascular smooth muscle. Smooth muscle cGMP content is further regulated by cGMP-specific phosphodiesterase 5 (PDE5), which inactivates cGMP by hydrolysis.[104] Whether the inability to sustain cGMP contributes to the failure of some patients with PPHN to respond or to sustain improved oxygenation during iNO therapy is uncertain. Early clinical experience with dipyridamole, which has PDE5 inhibitory activity, has been variable. Although dipyridamole may enhance the

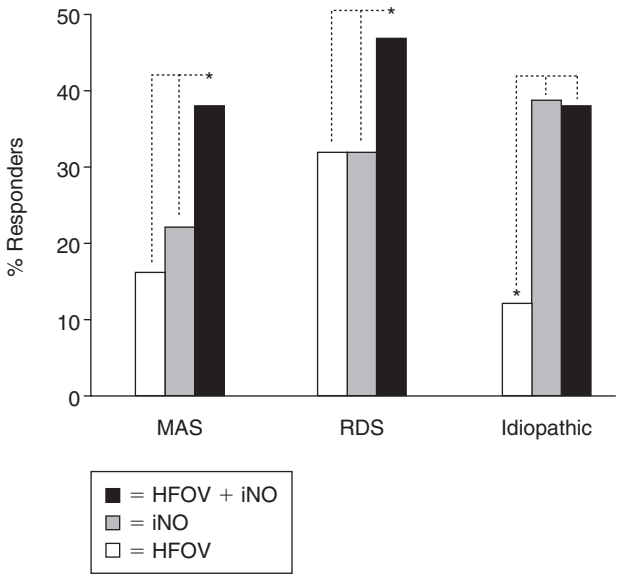


Figure 72-10 Lung recruitment with HFOV augments the response to iNO in patients with severe parenchymal lung disease. *(From Han RN, Babaei S, Robb M, et al. Defective lung vascular development and fatal respiratory distress in eNOS deficient mice: a model of alveolar capillary dysplasia?* Circ Res *94:1115-1123, 2004.)*

response to iNO in some patients,[105] its effects are variable and are not selective for the pulmonary circulation.[106] Further studies with more selective PDE5 antagonists (such as sildenafil) may lead to novel clinical strategies to enhance the treatment of pulmonary hypertension with iNO.[107] Moreover, other agents may enhance endogenous NO activity and potentially augment the effects of iNO, such as superoxide dismutase. Laboratory studies have shown that intratracheal administration of recombinant human CuZn superoxide dismutase causes pulmonary vasodilation and improves gas exchange during mechanical ventilation in animal models of pulmonary hypertension.[108,109] However, the independent and combined effects of superoxide dismutase and iNO have not been tested in a clinical trial in newborns with PPHN.

INHALED NITRIC OXIDE IN PREMATURE NEWBORNS

In the premature human neonate, severe respiratory failure is often the result of surfactant deficiency, and treatment with exogenous surfactant can cause dramatic improvements in oxygenation.[110] However, surfactant therapy results in suboptimal responses in up to 50% of human newborns with RDS.[111] Echocardiographic studies in human premature infants with RDS have shown that pulmonary hypertension may complicate the course of severe RDS.[57,112-115] Subsequent studies have also shown that the association of pulmonary hypertension with severe RDS leads to increased mortality despite surfactant therapy.[116] Walther and colleagues[116] performed echocardiographic studies in premature neonates with fatal RDS and found that severe pulmonary hypertension was an early and consistent finding in premature neonates who subsequently died. The frequency of severe pulmonary hypertension complicating RDS in premature neonates is unknown. However, the National Institute of Child Health and Human Development (NICHD) Neonatal Research Network recently reported that respiratory causes of early death in premature neonates remain a significant problem despite the availability of surfactant therapy.[117] Among 2867 infants with birth weights <1500 g born between March 1993 and February 1994, a mortality rate of 19% (553) was reported. For 26% (146) of these premature neonates, the major cause of death was pulmonary related (RDS, chronic lung disease).

These observations suggest that factors other than surfactant deficiency may contribute to the cardiopulmonary failure associated with prematurity. Multiple mechanisms may be involved, including the production of pulmonary vasoconstrictor agents or diminished endogenous vasodilator formation causing increased PVR and extrapulmonary right-to-left shunting of blood across the ductus arteriosus and foramen ovale. Surfactant inactivation may also occur in severe RDS, caused by alveolar-capillary leak and pulmonary edema.[118,119] Surfactant inactivation results in atelectasis, disturbances in ventilation-perfusion matching, and intrapulmonary shunting.

Treatment of pulmonary hypertension and ventilation-perfusion mismatching in newborn infants has been limited by the lack of a selective pulmonary vasodilator. Nonselective pulmonary vasodilators have been used in RDS complicated by pulmonary hypertension, but their use has been marked by lack of responsiveness and inability to sustain vasodilation. Also, adverse systemic vascular effects may occur, and the risks of systemic hypotension are of particular concern in premature subjects, because of markedly reduced cerebral blood flow in the first days after delivery.[120,121] Moreover, vasodilators delivered to the lung by intravenous administration may worsen ventilation-perfusion matching by increasing blood flow to both well-ventilated and poorly ventilated areas of the lung.[100]

As described earlier, convincing evidence now exists that low-dose (≤20 ppm) iNO delivered directly to the pulmonary circulation by inhalation causes potent, selective, and sustained pulmonary vasodilation in premature animals. Because of the inhalational route of delivery, low-dose iNO also improves matching of ventilation with perfusion within the lung by vasodilating vessels associated with well-ventilated lung units and favorably modulates lung inflammation and vascular-alveolar maturation.

Preliminary studies in human premature neonates with severe hypoxemic respiratory failure supported the potential role of low-dose iNO as adjuvant therapy. Low-dose inhaled NO caused marked improvement in oxygenation in a premature neonate with group B streptococcal sepsis and severe pulmonary hypertension, allowing reduction in ventilator pressure and inspired oxygen concentration with complete clinical recovery.[122] Peliowski and colleagues[123] studied eight premature neonates with severe hypoxemia associated with prolonged oligohydramnios and suspected pulmonary hypoplasia who showed marked improvement in response to iNO therapy. Five patients survived in this trial, three with severe intracranial hemorrhage (ICH). Skimming and colleagues[124] also reported a dose-response study in premature infants, and concluded that 5 ppm was as effective as 20 ppm in improving oxygenation. In a small, unmasked, randomized trial of iNO (20 ppm) and dexamethasone treatment, no differences were found in survival, chronic lung disease, or ICH between iNO-treated infants and controls.[125] In another report, Van Meurs and colleagues[126] described a dose-response study of iNO in 11 premature newborns and noted that 5 ppm was as effective as 20 ppm. Seven (64%) of these infants had ICH, and five (45%) had ICH of grade 3 to 4. However, when the authors compared these results with the NICHD Neonatal Network database (for historical controls matched for severity of illness), the incidence of ICH in premature newborns not treated with iNO was identical (64%). One of the most concerning potential risks of iNO treatment for premature neonates is the prolongation in bleeding time described at NO doses of 30 to 300 ppm.[127] Endogenous NO inhibits platelet adhesion to the vascular endothelium, and this effect may be related to the formation of stable *S*-nitrosoproteins.[128,129] However, in a small study in premature infants given treatment with inhaled NO, no change in bleeding time occurred.[130] These observations illustrate the limitations of toxicity determinations without appropriately designed clinical trials.

The largest trials of iNO therapy in premature newborns reported to date include the single-center study of Schreiber and colleagues[131] and the multicenter trials of Van Meurs and colleagues,[132] Ballard and colleagues,[133] and Kinsella and colleagues.[134] All of these studies were randomized, controlled, and masked but have key differences in patient population, disease severity, dose and duration of therapy, and other factors.

Schreiber and colleagues[131] randomly assigned 207 infants to treatment with iNO or placebo. The main finding of the trial was a reduction in the incidence of BPD and death by 24% in the iNO group. These benefits appeared to accrue predominantly from the subset of newborns with relatively mild respiratory failure (oxygenation index [OI] <6.94). However, in addition to apparent pulmonary benefit caused by low-dose iNO, these authors also reported a 47% decrease in the incidence of severe ICH and periventricular leukomalacia (PVL). Moreover, in a subsequent report, the same group showed that the early decrease in ICH and PVL associated with iNO treatment manifested in improved neurodevelopmental outcome on follow-up examinations of this population.[135] In this follow-up study, 138 children (82% of survivors of the randomized controlled trial) were evaluated for neurodevelopmental outcome at 2 years of age. In the group given treatment with iNO in the newborn period, 24% had abnormal outcomes (defined as cerebral palsy, blindness, hearing loss, or one score of less than 70 on the Bayley Scale of Infant Development II), in contrast to 46% in the control group.

Van Meurs and colleagues[132] enrolled 420 newborns (401 to 1500 g birth weight) in a multicenter randomized controlled trial. Although the focus of this study was on premature newborns and the major outcome measure was BPD, the design of the trial was similar to that of the previous NINOS trial in which term newborns were enrolled and acute changes in oxygenation determined continued treatment with study gas. That is, an acute dose-response study was performed, and only patients who showed significant improvement in Pa_{O_2} continued to receive study gas. In striking contrast to other studies, the average duration of iNO treatment was only 76 hours. Overall, they found no difference in the incidence of death or BPD between the iNO and control groups. However, in post hoc analyses, infants with birth weight >1000 g showed a reduction in death or BPD after treatment with iNO (50% iNO vs. 69% control). But a worrisome outcome was suggested in a post hoc analysis of newborns weighing ≤1000 g. This analysis showed an increased risk

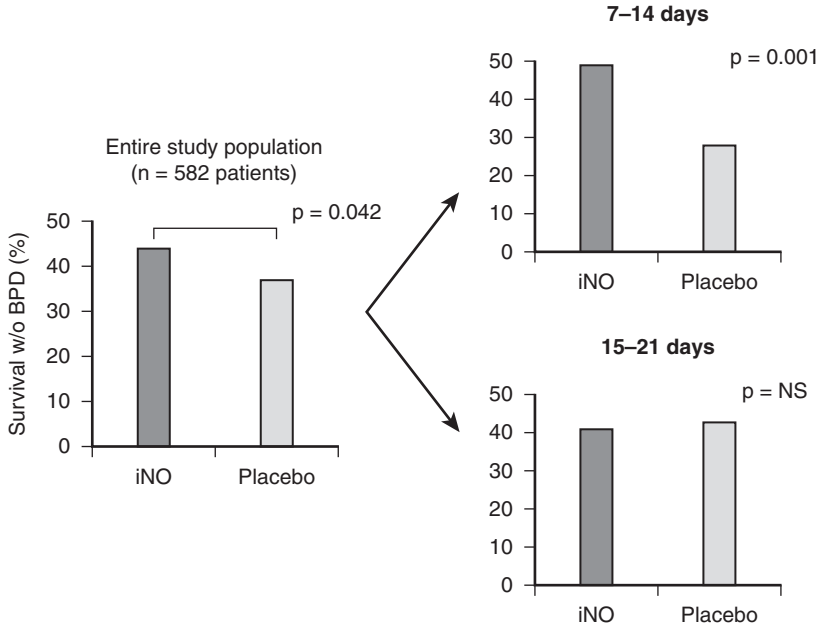


Figure 72-11 Effects of iNO on survival without BPD for infants enrolled between 7 and 21 days of age. As shown, iNO increased survival without BPD in infants who were treated before 14 days of age. *(From Ballard RA, Truog WE, Cnaan A, et al: Inhaled nitric oxide in preterm infants undergoing mechanical ventilation,* N Engl J Med *205:343-353, 2006.)*

of ICH or PVL (43% iNO vs. 33% control). However, as noted in an editorial by Martin and Walsh,[136] baseline ultrasound examinations were not performed, and it cannot be determined whether these very severely ill infants had ICH before iNO was initiated. Indeed, the severity of illness of infants in this trial of Van Meurs and colleagues[132] was also markedly different from the study of Schreiber and colleagues.[131] In the Van Meurs trial, the mean OI at enrollment for the iNO group was 23, compared with the median OI of 7.3 in the Schreiber study. This suggests that the degree of illness based on the severity of respiratory failure may be related to iNO safety and efficacy in this population; however, an increased risk of ICH or PVL was not observed in a previous trial of iNO in premature newborns with severe hypoxemic respiratory failure (OI = 30).[22] Other differences between these two trials may offer insights into the disparate outcomes, including the duration of iNO treatment (3 days vs. 7 days), birth weight (839 g vs. 992 g), and gestational age (26 weeks vs. 27.4 weeks). Thus Van Meurs and colleagues enrolled smaller, more immature infants with severe respiratory failure who were treated relatively briefly with iNO, making direct comparisons between these two trials problematic.

The results of the two largest randomized, controlled, and masked trials of iNO treatment in premature newborns have been reported. Ballard and colleagues[39] randomly assigned 582 premature newborns with birth weights of 500 to 1250 g who required ventilatory support between 7 and 21 days of age. Infants were given treatment with study gas for a minimum of 24 days and had an estimated OI of 7. They found that the incidence of survival without BPD was increased in the iNO treatment group (43.9%) compared with controls (36.8%) (p = .042). A major finding of this trial was that the benefit of BPD reduction derived almost entirely from the subset of patients enrolled between 7 and 14 days, suggesting that early treatment is important to prevent BPD (Figure 72-11). No differences were seen between the iNO and control groups in adverse events, including medical or surgical treatment of PDA, which is consistent with the lack of negative impact on ductal closure in the primate studies.[82] In addition, no differences were seen between the groups in ICH incidence; however, infants were enrolled after the first week of life. Thus this trial does not help inform the debate about iNO effects on brain injury in the premature newborn.

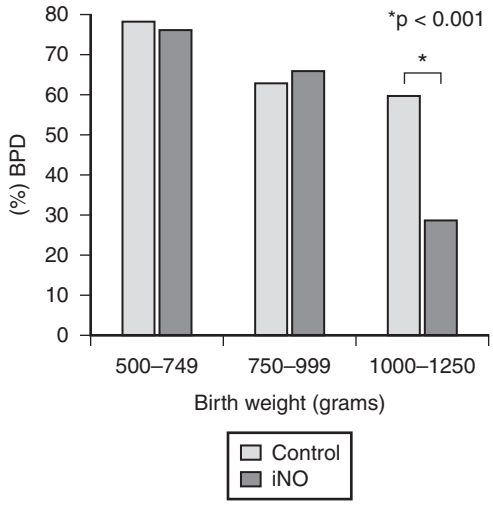


Figure 72-12 Effects of early treatment of preterm infants with low-dose iNO on BPD incidence by strata. Although no difference was seen on the incidence of BPD for the entire study group, this treatment strategy reduced BPD in infants >1000 g. *(From Kinsella JP, Cutter GR, Walsh WF, et al. Early inhaled nitric oxide therapy in premature newborns with respiratory failure,* N Engl J Med *205:354-364, 2006.)*

In the second trial, 793 premature newborns with birth weights of 500 to 1250 g and requiring mechanical ventilation in the first 48 hours of life were randomly assigned to treatment with 5 ppm iNO or placebo gas and given treatment for 21 days or until extubation.[40] Overall, no difference was found in the incidence of death or BPD between groups; however, iNO therapy reduced the incidence of BPD for infants with birth weight >1000 g by 50% (p = .001) (Figure 72-12). Low-dose iNO therapy reduced the incidence of PVL (p = .048), as well as the combined end points

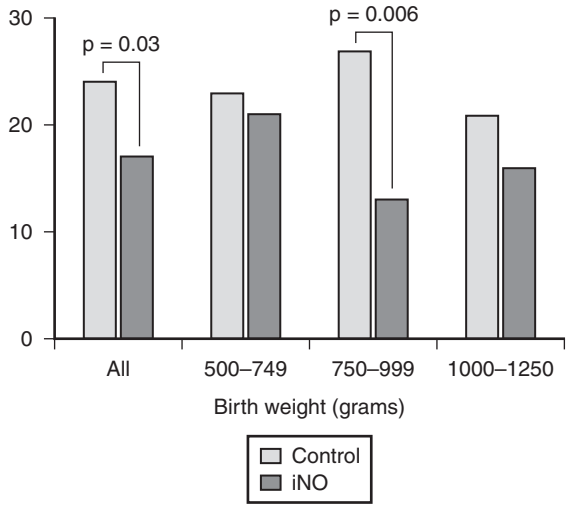


Figure 72-13 Effects of early treatment with low-dose iNO on brain injury (grade 3-4 ICH, PVL, ventriculomegaly) in preterm infants according to birth weight strata. iNO reduced ultrasound findings of brain injury for the overall group, with the largest effect in the 750- to 999-g strata. *(From Kinsella JP, Cutter GR, Walsh WF, et al. Early inhaled nitric oxide therapy in premature newborns with respiratory failure,* N Engl J Med *205:354-364, 2006.)*

of ICH, PVL, and ventriculomegaly for the entire study population (p = .032) (Figure 72-13). INO therapy did not increase the incidence of adverse events, including mortality, ICH, PVL, pulmonary hemorrhage, and PDA treatment in any subgroup. In this trial no relationship was found between OI and brain injury risk, in contrast to the findings of Van Meurs and colleagues.[132] Mechanisms through which iNO therapy might provide neuroprotection in the premature newborn are uncertain and warrant further study. Based on laboratory studies, several possibilities exist that include modulation of circulating cells (including neutrophils, monocytes, and platelets) that may occur during NO exposure as they transit the pulmonary circulation. Alternatively, iNO-induced down-regulation of lung-derived cytokines may also reduce distant organ injury.[137-139] Another possible mechanism may relate to distal delivery of NO or NO-related metabolites through the systemic circulation through red blood cell– or protein-mediated pathways.[75,140]

SUMMARY

The role of endogenous NO in pulmonary vasoregulation and modulation of pulmonary vascular homeostasis has been extensively investigated. Beginning with the observation that NO is the "endothelium-derived relaxing factor," translational physiology studies and clinical experimentation led to the approval of iNO as the first selective pulmonary vasodilator for the treatment of PPHN in term human newborns. Effective lung recruitment and vigilant management of cardiac performance appear vital to the effective use of iNO.

The available evidence from clinical trials suggests that low-dose iNO may be safe and effective in reducing the risk of death or BPD for a subset of premature newborns, in particular infants with birth weights >1000 g. A neuroprotective effect of iNO has been demonstrated in large randomized controlled trials, but the relationship of disease severity and ICH or PVL risk is uncertain. Treatment of premature newborns with respiratory failure between 7 and 14 days after birth appears to be safe and effective in reducing the incidence of BPD. Finally, early concerns about the potential adverse effects of iNO on surfactant function and PDA risk have been effectively eliminated with the cumulative results of both nonhuman primate studies and human clinical trials; however, routine use of iNO in premature newborns should await the results of follow-up studies from the largest clinical trials.

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73 Mechanisms Regulating Closure of the Ductus Arteriosus

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The ductus arteriosus represents a persistence of the terminal portion of the sixth branchial arch. During fetal life, the ductus arteriosus serves to divert blood away from the fluid-filled lungs toward the descending aorta and placenta. After birth, constriction of the ductus arteriosus and obliteration of its lumen separates the pulmonary and systemic circulations. In term infants, obliteration of the ductus arteriosus takes place through a process of vasoconstriction and anatomic remodeling. In the preterm infant, the ductus arteriosus frequently fails to close. The clinical consequences of a patent ductus arteriosus (PDA) are related to the degree of left-to-right shunt through the PDA with its associated change in blood flow to the lungs, kidneys, and intestine.

INCIDENCE

Pulsed Doppler echocardiographic assessments of term infants indicate that functional closure of the ductus has occurred in almost 50% by 24 hours, in 90% by 48 hours, and in all by 72 hours. The rate of ductus closure is delayed in preterm infants, however; essentially all healthy preterm infants of 30 weeks gestation or greater will have closed their ductus by the fourth day after birth. Respiratory distress syndrome (RDS) delays ductus closure; however, in most infants who are 30 weeks gestation or greater, the actual impact of RDS on ductal shunting may be less than commonly assumed (only 11% are still open after 4 days). On the other hand, preterm infants of less than 30 weeks gestation with severe respiratory distress have a 65% incidence of persistent PDA.

The recent introduction of exogenous surfactant therapy has altered both the incidence and the presentation of the PDA. Although surfactant has no effect on the contractile behavior of the ductus, its effects on pulmonary vascular resistance lead to an earlier clinical presentation of the left-to-right shunt in preterm animals[1,2] and humans.[3,4] Infants who are small for gestational age,[5] receive magnesium before birth,[6] have late-onset septicemia,[7] or receive excessive fluid administration during the first days of life also are more likely to have development of a clinically symptomatic PDA.[8] On the other hand, nonwhite infants and those who receive antenatal glucocorticoids have a reduced risk.[9,10]

REGULATION OF DUCTUS ARTERIOSUS PATENCY

In the term infant, closure of the ductus arteriosus occurs in two phases: (1) "functional" closure of the lumen within the first hours after birth by smooth muscle constriction and (2) "anatomic" occlusion of the lumen over the next several days because of extensive neointimal thickening and loss of smooth muscle cells from the inner muscle media.

Balance Between Vasoconstriction and Vasorelaxation

Patency of the fetal ductus arteriosus is regulated by both dilating and contracting factors. The ductus normally has a high level of intrinsic tone during fetal life.[11] The factors that promote ductus constriction in the fetus have yet to be identified. The intrinsic ductus tone is due to components that are both dependent on and independent of extracellular calcium.[11] Permeabilized smooth muscle from the fetal ductus is significantly more sensitive to the contractile effects of calcium than is smooth muscle from the aorta and the pulmonary artery.[12] Endothelin-1 also appears to play a role in producing the basal tone of the ductus.[13]

The factors that oppose ductus arteriosus constriction in utero are better understood. The elevated vascular pressure within the ductus lumen (caused by the constricted pulmonary vascular bed) plays an important role in opposing ductus constriction.[14] The fetal ductus also produces several vasodilators that oppose the ability of the intrinsic ductus tone to constrict the vessel. Vasodilator prostaglandins appear to be the dominant vasodilators that oppose ductus constriction in the later part of gestation.[15] Inhibitors of prostaglandin synthesis constrict the fetal ductus both in vitro and in vivo. Prostaglandin E_2 (PGE_2) is the most potent prostaglandin produced by the ductus[16,17] and appears to be the most important prostanoid to regulate ductus patency. The response of the ductus to PGE_2 is unique among blood vessels in that it is extraordinarily sensitive to this vasodilating substance. PGE_2 produces ductus relaxation by interacting with several of the PGE receptors (EP2, EP3, and EP4).[18] In the ductus, all three of the EP receptors participate in vasodilation by activating adenylate cyclase.[18] The increased intracellular concentrations of cyclic adenosine monophosphate inhibit the sensitivity of the ductus's contractile proteins to calcium.[12] Inhibitors of phosphodiesterase (the enzyme that degrades cyclic adenosine monophosphate) prevent ductus constriction in utero.[19] In addition, one of the EP receptors (EP3) also relaxes the ductus smooth muscle by opening adenosine triphosphate potassium channels, which hyperpolarize the muscle and inhibit ductus tone.[18]

Both isoforms of the enzyme responsible for synthesizing prostaglandins (cyclooxygenase [COX]-1 and COX-2), are expressed in the fetal ductus.[20] Depending on the species, both nonselective (e.g., indomethacin) and selective cyclooxygenase inhibitors constrict the ductus. In the fetal mouse, COX-2 appears to be primarily responsible for the prostaglandins that dilate the ductus,[21] whereas, in the fetal sheep, both COX-1 and COX-2 play a role in ductus patency.[20]

In addition to the prostaglandins that are made within the ductus, the fetal ductus is also under the influence of circulating concentrations of PGE_2. Circulating concentrations of PGE_2 appear to be of placental origin.[22] Circulating concentrations of PGE_2 (approximately 1 nmol/L) in the late-gestation fetal lamb are close to those that produce maximal relaxation of the ductus.[23] PGE_2 concentrations are particularly high in the fetus because of the reduced pulmonary clearance of circulating prostaglandins resulting from the low fetal pulmonary blood flow.[24]

Nitric oxide (NO) is made by the fetal ductus arteriosus and appears to play an important role in maintaining ductus patency in rodent fetuses early in gestation.[15] Although NO is also made in the ductus of larger species, its importance in maintaining ductus patency under normal in utero conditions has not been conclusively demonstrated[25] (see later for the role of NO in fetuses exposed to indomethacin tocolysis and in premature newborns).

Carbon monoxide relaxes the ductus arteriosus, and both hemoxygenase-1 and -2 (the enzymes that make carbon monoxide) are found within the endothelial and smooth muscle cells of the ductus. Under physiologic conditions the amount of carbon monoxide made by the ductus does not seem to affect ductus tone; however, in circumstances where its synthesis is upregulated, for example, endotoxinemia, it may exert a relaxing influence on the ductus.[26] Little evidence exists to suggest that adenosine or β-adrenergic stimulation plays a significant role in ductus patency.[27]

Although pharmacologic inhibition of prostaglandin synthesis produces ductus constriction in utero, genetic interruption of either prostaglandin synthesis (i.e., homozygous combined COX-1 and COX-2–null mice[21]) or prostaglandin signaling (i.e., homozygous EP4 receptor–null mice[28]) does not produce ductus closure in utero. In contrast to what would be expected from the pharmacologic inhibition studies, both genetic interruptions produce newborn mice that fail to close their ductus after birth.[21,28] The mechanisms through which the absence of prostaglandin stimulation alters the normal balance of other vasoactive factors in the ductus have yet to be elucidated. The COX-null mice do have increased nitric oxide production.[29,30] However, inhibitors of nitric oxide production do not increase the rate of ductus closure in the COX-null mice.[31] Stimulation of the prostaglandin receptor EP4 increases hyaluronic acid content in the ductus and promotes intimal cushion formation and ductus closure (see later). Interruption of EP4 signaling decreases intimal cushion formation, which may contribute to delayed luminal occlusion after birth.[32] It is interesting to note that pharmacologic inhibition of prostaglandin synthesis in human pregnancy also is associated with an increased incidence of PDA after birth.[33] However, this appears to be due to the ability of indomethacin to produce ductus constriction in utero. Ductus constriction in utero produces ischemic hypoxia, increased nitric oxide production, and smooth muscle cell death within the ductus wall. These factors prevent the ductus from constricting after birth and make it resistant to the constrictive effects of postnatal indomethacin.[34,35]

After delivery several events promote ductus constriction in the term newborn: (1) an increase in arterial Po_2, (2) a decrease in blood pressure within the ductus lumen (caused by the postnatal decrease in pulmonary vascular resistance), (3) a decrease in circulating PGE_2 (caused by the loss of placental prostaglandin production and the increase in prostaglandin removal by the lung), and (4) a decrease in the number of PGE_2 receptors in the ductus wall.[18] Although the newborn ductus continues to be sensitive to the vasodilating effects of NO, it loses its ability to respond to PGE_2.[36,37] All of these factors promote ductus constriction after birth.

The postnatal increase in arterial Pao_2 plays an important role in ductus constriction. Oxygen's mechanism of action is still unknown. Although neural and hormonal factors possibly contribute to ductus closure under physiologic conditions, they do not mediate oxygen-induced vessel closure. Oxygen appears to constrict the ductus arteriosus by a mechanism that involves smooth muscle membrane depolarization and by a mechanism that is independent of membrane potential.[38] A cytochrome P450 hemoprotein that is located in the plasma membrane of the vascular smooth muscle cells appears to act as a receptor for the oxygen-induced events in the ductus.[39,40] In larger species, oxygen appears to constrict the ductus arteriosus primarily by a mechanism that involves smooth muscle depolarization with subsequent calcium entry through voltage dependent, L-type calcium channels[41] (Clyman, unpublished observations). Oxygen leads to K channel inhibition,[42,43] which depolarizes the ductus smooth muscle cells. Several O_2-sensitive K^+ channels have been found in the fetal ductus. These vary with species and gestational age and may account for the differing sensitivity of the ductus to oxygen throughout gestation.[44,45] In smaller species oxygen increases calcium entry through a mechanism that depends on membrane depolarization and one that is independent of membrane depolarization (possibly acting through store-operated calcium channels and/or through increased calcium sensitization by Rho kinase).[38,46-48]

Oxygen also increases the formation of the potent vasoconstrictor, endothelin-1.[49] However, the role of endothelin-1 in postnatal ductus closure has been questioned,[13,43,50,51] and the pathways through which oxygen alters membrane potential are still unclear.[42,52]

In contrast to the term ductus, the premature ductus is less likely to constrict after birth. This is due to several mechanisms. The intrinsic tone of the extremely immature ductus (<70% of gestation) is decreased compared with the ductus at term.[11] This may be due in part to the presence of smooth muscle myosin isoforms with a weaker contractile capacity in the immature ductus.[53,54] In addition, calcium entry through L-type calcium channels appears to be disrupted under hypoxic conditions in the immature ductus. The potassium channels that promote ductus relaxation also change during gestation (e.g., from K_{Ca} channels [which are not regulated by the postnatal increase in oxygen tension] to K_V channels [which can be inhibited by oxygen in sheep] [Clyman, unpublished observations]).[44,55] Premature infants have elevated circulating concentrations of PGE_2, which may play a significant role in maintaining ductus patency during the first days after birth. This is due to the decreased ability of the premature lung to clear circulating PGE_2.[24] In addition, during episodes of bacteremia and necrotizing enterocolitis, circulating concentrations of PGE_2 reach the pharmacologic range and are often associated with reopening of the ductus arteriosus.[7] The most important mechanism that prevents the preterm ductus from constricting after birth is its increased sensitivity to the vasodilating effects of PGE_2 and NO.[56] The increased sensitivity is not due to an increased number of PGE_2 receptors but rather to enhanced coupling between the receptors and the downstream signaling pathways leading to increased intracellular cyclic adenosine monophosphate.[57] As a result, inhibitors of prostaglandin production (e.g., indomethacin, ibuprofen, and mefenamic acid) are usually effective agents in promoting ductus closure in the premature infant. It follows that drugs interfering with NO synthesis or function also could become useful adjuncts, especially in situations where indomethacin has proved to be ineffective.[58]

The endogenous factors that alter the ability of the preterm ductus to constrict with advancing gestation are unknown. Prenatal administration of vitamin A has been shown to increase both the intracellular calcium response and the contractile response of the preterm ductus to oxygen.[59] During normal fetal development, an increase in circulating cortisol occurs near the end of gestation. Elevated cortisol concentrations in the fetus have been found to decrease the sensitivity of the ductus to the vasodilating effects of PGE_2[60]; consistent with these findings, prenatal administration of glucocorticoids causes a significant reduction in the incidence of PDA in premature humans and animals.[10,60-64] The postnatal administration of glucocorticoids also has been shown to reduce the incidence of PDA.[65] However, postnatal glucocorticoid treatment also seems to increase the incidence of several of the other neonatal morbidities.[66]

Anatomic Closure—Histologic Changes

In the term newborn progressive intimal thickening and fragmentation of the internal elastic lamina occur after delivery. As the intima increases in size, it ultimately forms mounds that occlude the already constricted lumen. The increase in intimal thickening is due (1) to migration of smooth muscle cells from the muscle media into the intima and (2) to proliferation of luminal endothelial cells. The process of intimal cushion formation starts with the accumulation of hyaluronic acid (HA) below the luminal

endothelial cells. This is accompanied by the loss of laminin and collagen IV from the basement membrane of the endothelial cells and their subsequent separation from the internal elastic lamina. The hygroscopic properties of HA cause an influx of water and widening of the subendothelial space; this creates an environment well suited for cell migration. Accompanying the increase in HA is an increase in fibronectin and chondroitin sulfate in the neointimal space.[67] The endothelial and smooth muscle cells of the ductus arteriosus differ from those of the adjacent vessels in their rate of HA accumulation and their ability to form neointimal cushions. The increase in HA appears to be due to increased production of transforming growth factor β in the ductus.[68] Prostaglandins, acting through the EP4 receptor, also appear to play a critical role in HA production in the ductus.[32]

HA makes ductus smooth muscle cells migrate faster than aortic smooth muscle cells. The potentiating effect of HA on ductus smooth muscle cells is mediated through an HA-binding protein (RHAMM). Ductus smooth muscle cells synthesize more RHAMM than aortic smooth muscle cells and concentrate it at the leading edges of the cells. Antibodies against RHAMM will reduce the migration of ductus smooth muscle cells to the level found in aortic smooth muscle cells.[69]

Ductus smooth muscle cells also secrete more fibronectin and chondroitin sulfate than those of the aorta or pulmonary artery.[70] This does not appear to be due to transforming growth factor β. Fibronectin plays an important role in facilitating ductus smooth muscle cell migration. When fibronectin production in the ductus is inhibited, intimal cushion formation is blocked.[71] On the other hand, fibronectin has no effect on the migration of aortic smooth muscle cells. The increased production of chondroitin sulfate appears to have no direct effect on either ductus or aortic smooth muscle cell migration.[72]

Ductus arteriosus smooth muscle cells use a family of cell surface receptors, called integrins, to interact with, adhere to, and migrate through the extracellular matrix that surrounds them. When smooth muscle cells of the ductus are in a quiescent, contractile state, they express the same integrins on their cell surface as smooth muscle cells of the aorta. However, when ductus smooth muscle cells of the inner muscle media begin to migrate into the subendothelial space, two new integrin complexes appear on their cell surface: the αvβ3 and the α5β1 receptors. The αvβ3 integrin is a promiscuous receptor that interacts with most extracellular matrix glycoproteins and is essential for migration of ductus smooth muscle cells in vitro. The α5β1 integrin binds exclusively to fibronectin and mediates the potentiating effects of fibronectin on ductus smooth muscle cell migration. During the process of migration, ductus smooth muscle cells secrete laminin, which also has an important promigratory role.

Intimal cushion formation in the ductus is also associated with striking alterations in elastin fiber assembly. In contrast to the aorta, where formation of well-developed elastic laminae are seen between layers of muscle cells, smooth muscle cells of the ductus muscle media are surrounded by thin and fragmented elastin fibers. Smooth muscle cells in the neointima are surrounded by even fewer elastin fibers.[73] The disruption of normal elastin fiber assembly in the ductus does not appear to be due to increased elastase activity or decreased tropoelastin production. Rather, it appears to be due to a developmental mechanism that reduces insolubilization of elastin and prevents formation of intact elastic laminae. Vascular smooth muscle cells synthesize a 67-kDa elastin-binding protein (EBP) that is central to the assembly of soluble tropoelastin molecules into a mature matrix of insoluble elastic fibers.[74] The 67-kDa EBP binds the hydrophobic tropoelastin molecule intracellularly, escorts it through the smooth muscle cell's secretory pathways, and attaches it to the cell's surface. When galactosugars come in contact with the lectin-binding site of the 67-kDa EBP, the bound tropoelastin is released from the cell membrane. Coordinated presentation of galactosugars regulates the orientation and proper alignment of tropoelastin for cross-linking during normal elastin fiber assembly. On the other hand, excess galactosugars, from other matrix elements, may compete with this process and lead to abnormal assembly (see later).[75]

Ductus smooth muscle cells have less 67-kDa EBP on their cell surface when compared with aortic smooth muscle cells.[76] As a result, ductus smooth muscle cells deposit little insoluble elastin when compared with aortic smooth muscle cells. In addition, the increased amounts of chondroitin sulfate in the ductus may dissociate tropoelastin from the cell surface (through its galactosugar side chains); this would further interfere with elastin fiber assembly.[76]

The exact relationship between impaired elastin assembly and smooth muscle migration into the neointima is still open for speculation. Ductus smooth muscle cells are able to migrate through elastin membranes that restrain aortic smooth muscle cell migration. Treatment of aortic smooth muscle cells with chondroitin sulfate causes the release of the 67-kDa EBP from the aortic cell's surface and enables them to migrate through elastin membranes at the same rate as ductus smooth muscle cells.[77] In addition, tropoelastin fragments may act as a chemoattractant for smooth muscle cells.[78] Thus mechanisms appear to exist that link elastin fragmentation with formation of ductus intimal cushions. Conversely, in some genetic forms of PDA, the elastic laminae of the ductus appear abnormally well developed and similar to those in the aorta; when this occurs, intimal cushions fail to develop.[73]

Relationship Between Vasoconstriction and Anatomic Closure

In term animals, loss of responsiveness to PGE_2 shortly after birth prevents the ductus arteriosus from reopening once it has constricted.[36,37] This is due, in part, to the decreased synthesis of PGE_2 receptors in the ductus. Both the loss of vasodilator regulation and the anatomic events that lead to permanent closure appear to be controlled by the degree of ductus smooth muscle constriction. Experimental models that alter the ability of the ductus to constrict at term also prevent the normal histologic changes that occur after birth.* Constriction produces ischemic hypoxia of the vessel wall.[81] In the term newborn ductus, the ischemic hypoxia that accompanies constriction occurs even before luminal flow has been eliminated and depends on the presence of intramural vasa vasorum.[82] With advancing gestation, the thickness of the ductus wall increases in size to a dimension that requires the presence of intramural vasa vasorum to provide nutrients to its outer half. These collapsible, intramural vasa vasorum provide the ductus with a unique mechanism for controlling the maximal diffusion distance for oxygen and nutrients across its wall. In the term newborn, ductus constriction obliterates vasa vasorum flow to the outer muscle media, which turns the entire thickness of the muscle media into a virtual avascular zone. The profound ischemic hypoxia that follows the compression of the vasa vasorum inhibits local production of PGE_2 and NO, induces local production of growth factors (e.g., transforming growth factor β and vascular endothelial growth factor), and produces smooth muscle apoptosis in the ductus wall. Vascular endothelial growth factor plays a critical role in the migration of the ductus smooth muscle cells into the neointima and in the proliferation of intramural vasa vasorum.[83] After postnatal ductus constriction, several genes known to be essential for vascular remodeling (HIF1α, VCAM-1, E-selectin, IL-8, MCSF-1, CD154, IFNγ, IL-6, and TNFα) are increased in the ductus wall. In addition, VLA4-positive monocytes and macrophages (CD68 and CD14 positive) adhere to the ductus wall. The inflammatory response that follows postnatal ductus constriction may be as necessary for ductus remodeling because the extent of neointimal remodeling

*References 14, 21, 28, 71, 79, 80.

is significantly correlated with the degree of mononuclear cell adhesion.[84]

In preterm infants, the ductus frequently remains open for many days after birth. Even when it does constrict, the premature ductus frequently fails to develop profound hypoxia and anatomic remodeling. The preterm infant seems to require a greater degree of ductal constriction than the term infant to develop a comparable degree of hypoxia. In contrast with the term ductus, the thin-walled preterm ductus does not depend on intramural vasa vasorum to provide oxygen and nutrients to its wall. The absence of intramural vasa vasorum leaves the preterm ductus without a mechanism to rapidly increase the diffusion distance across its wall during postnatal constriction. As long as any degree of luminal patency exists, the thin-walled preterm ductus fails to become profoundly hypoxic and fails to undergo anatomic remodeling after birth. As a result, the preterm ductus requires complete cessation of luminal flow before it can develop the same degree of hypoxia as found at term. Once the preterm ductus develops the same degree of hypoxic ischemia as the term ductus, most of the anatomic changes seen at term will occur.[11,58] However, if the premature ductus does not develop the profound degree of ischemic hypoxia that is necessary to induce anatomic remodeling, it will not develop smooth muscle cell death and will remain essentially fetal in appearance. Without anatomic remodeling, the preterm ductus is still susceptible to vessel reopening.

In contrast to the term newborn ductus, where all of the PGE_2 EP receptors are down-regulated after birth, the preterm newborn ductus increases its synthesis of the dominant PGE_2 receptor, EP4, and continues to respond to PGE_2 after birth. In addition to its persistent responsiveness to PGE_2, the premature ductus synthesizes increased amounts of other vasodilators (e.g., NO, tumor necrosis factor α, and interleukin 6) after birth in response to the postnatal ductus wall hypoxia.[83,84] As a result, a change occurs in the relative balance of the vasodilators that maintain ductus patency after birth. Ductus patency becomes less dependent on prostaglandin generation and more dependent on other vasodilators during the first weeks after birth. In addition, adenosine triphosphate concentrations are significantly altered in the preterm ductus, even when the ductus remains patent after birth. The decreased adenosine triphosphate concentrations prevent the immature neonatal ductus from contracting as vigorously as the fetal ductus.[85] These postnatal changes could explain why the effectiveness of indomethacin wanes with increasing postnatal age.[86,87] In premature animals and humans, the combined use of a nitric oxide synthase-inhibitor and indomethacin produces a much greater degree of ductus constriction than indomethacin alone.[58,88] It follows that drugs that interfere with NO synthesis could become useful, especially in situations where indomethacin has been found to be ineffective.[58]

HEMODYNAMIC AND PULMONARY ALTERATIONS

The pathophysiologic features of a PDA depend both on the magnitude of the left-to-right shunt and on the cardiac and pulmonary responses to the shunt. Important differences exist between immature and mature infants in the heart's ability to handle a volume load. Immature infants have less cardiac sympathetic innervation. Before term, the myocardium has more water and less contractile mass. Therefore in the immature fetus the ventricles are less distensible than at term and also generate less force per gram of myocardium (even though they have the same ability to generate force per sarcomere).[89] The relative lack of left ventricular distensibility in immature infants is more a function of the ventricle's tissue constituents than of poor muscle function. As a result, left ventricular distention resulting from a large left-to-right PDA shunt may produce a higher left ventricular end-diastolic pressure at smaller ventricular volumes. The increase in left ventricular pressure increases pulmonary venous pressure and causes pulmonary congestion.

Studies in preterm lamb and human newborns[90,91] have shown that, despite these limitations, preterm newborns are able to increase left ventricular output and maintain their "effective" systemic blood flow, even with left-to-right PDA shunts equal to 50% of left ventricular output. With shunts greater than 50% of left ventricular output, "effective" systemic blood flow falls, despite a continued increase in left ventricular output. The increase in left ventricular output associated with a PDA is accomplished not by an increase in heart rate, but by an increase in stroke volume.[90,91] Stroke volume increases primarily as a result of the simultaneous decrease in afterload resistance on the heart and the increase in left ventricular preload. Despite the ability of the left ventricle to increase its output in the face of a left-to-right ductus shunt, blood flow distribution is significantly rearranged. This redistribution of systemic blood flow occurs even with small shunts.[90] Blood flow to the skin, bone, and skeletal muscle is most likely to be affected by the left-to-right ductus shunt. The next most likely organs to be affected are the gastrointestinal tract and kidneys. These organs receive decreased blood flow because of a combination of decreased perfusion pressure (resulting from a drop in diastolic pressure) and localized vasoconstriction. These organs may experience significant hypoperfusion before any signs of left ventricular compromise exist.[91,92] This decrease in organ perfusion may contribute to some of the morbidities that have been associated with ductus patency: decreased feeding tolerance, necrotizing enterocolitis, and decreased glomerular filtration rate.[86,93,94] Reversal of aortic flow during diastole has been found to be a good predictor of necrotizing enterocolitis.[95]

The decreased ability of the preterm infant to maintain active pulmonary vasoconstriction may be responsible in part for the earlier presentation of a "large" left-to-right PDA shunt.[96,97] In addition, therapeutic maneuvers (e.g., surfactant replacement) that lead to a more rapid drop in pulmonary vascular resistance can exacerbate the amount of left-to-right shunt in preterm infants with RDS and lead to pulmonary hemorrhage.[4,98] Randomized, controlled trials have shown that early ductus closure decreases the incidence of significant pulmonary hemorrhage.[86,99,100]

The factors responsible for preventing plasma fluid and protein from moving into the lung interstitium and from the interstitium into the air spaces have been described elsewhere in this text. With a wide-open PDA, the pulmonary vasculature is exposed to systemic blood pressure and increased pulmonary blood flow. Because the premature infant with RDS frequently has low plasma oncotic pressure and increased capillary permeability, increases in microvascular perfusion pressure that result from a PDA may increase interstitial and alveolar lung fluid. Leakage of plasma proteins into the alveolar space inhibits surfactant function and increases surface tension in the immature air sacs,[101] which are already compromised by surfactant deficiency. The increased Fio_2 and mean airway pressures required to overcome these early changes in compliance may be important factors in the association of PDA with chronic lung disease.[86,102,103] However, these changes in pulmonary mechanics appear to occur only after several days of exposure to a PDA. Although it is true that preterm animals with a PDA have increased fluid and protein clearance into the lung interstitium, resulting from an increase in pulmonary microvascular filtration pressure, a simultaneous increase in lung lymph flow appears to eliminate the excess fluid and protein from the lung. This compensatory increase in lung lymph acts as an "edema safety factor," inhibiting fluid accumulation in the lungs. As a result, no net increase occurs in water or protein accumulation in the lung and no change occurs in pulmonary mechanics.[1,86,104-106] This delicate balance between the PDA-induced fluid filtration and lymphatic reabsorption is consistent with the observation, made in human infants, that closure of the ductus arteriosus, within the first 24 hours after birth, has

no effect on the course of the newborn's hyaline membrane disease. However, if lung lymphatic drainage is impaired, as it is in the presence of pulmonary interstitial emphysema or fibrosis, the likelihood of edema increases dramatically. After several days of lung disease and mechanical ventilation, the residual functioning lymphatics are more easily overwhelmed by the same size ductus shunt that could be accommodated on the first day after delivery. As a result, it is not uncommon for pulmonary edema and alterations in pulmonary mechanics to develop in infants with a persistent PDA at 7 to 10 days after birth. In these infants, improvement in lung compliance occurs after closure of the PDA.[107-109]

The factors responsible for these alterations in pulmonary mechanics have recently been examined in a premature baboon model (McCurnin, Yoder, Seidner, and colleagues, unpublished results). These animals were delivered at 67% term gestation (equivalent to 26 weeks human gestation) and were given surfactant and mechanically ventilated for 2 weeks. One group was treated with a cyclooxygenase inhibitor to close the ductus whereas the other group had a persistent PDA for 2 weeks. No differences were found in surfactant secretion, epithelial protein permeability, or increase in surfactant inhibitory proteins when the preterm baboons were exposed to an open PDA for 2 weeks after birth. Although numerous changes occurred in the genes that regulate inflammation and tissue remodeling in the preterm lung after birth, the presence of an open ductus did not appear to alter the expression pattern of these pro-inflammatory genes or of the genes involved in lung remodeling. The animals with an open ductus had both an increased amount and an altered distribution of water in their lungs. In contrast with the term lung, which mobilizes fluid rapidly after birth, the preterm lung mobilizes lung fluid much more slowly. A persistent PDA led to a small but significant increase in lung water at 2 weeks after delivery. Closure of the PDA with a cyclooxygenase inhibitor, such as indomethacin or ibuprofen, produced increased expression of epithelial sodium channels and increased removal of fluid from the alveolar compartment. In addition, pharmacologic closure of the PDA was associated with improved lung development. In contrast to preterm animals with an open ductus, where an arrest in alveolar development (the hallmark of the new "bronchopulmonary dysplasia") was noticeable by 2 weeks after birth, pharmacologic closure of the PDA led to improved alveolarization. The same improvements in pulmonary mechanics and alveolar surface area were not observed after surgical ligation of the PDA.[110] Ductus ligation, although eliminating the detrimental effects of a PDA, appears to introduce its own set of problems in the preterm newborn.[111]

Not all of the changes associated with a PDA are necessarily detrimental to the immature infant with RDS. Persistence of the left-to-right shunt maintains an elevated Pa_{O_2} in the presence of atelectasis. Decreases in systemic arterial O_2 content have been observed after PDA closure, despite the absence of any alterations in pulmonary mechanics. This phenomenon is due to recirculation of oxygenated arterial blood through lungs that are not fully expanded.[90,112]

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74 Regulation of Umbilical Blood Flow

S. Lee Adamson, Leslie Myatt, and Bridgette M. P. Byrne

OVERVIEW

Oxygen, carbon dioxide, nutrients, metabolic wastes, and hormones are transported by blood flowing between the placenta and the fetal body in the umbilical cord. Normal fetal growth and development depend on ever-increasing umbilical blood flow to meet the growing demand for exchange with the maternal circulation. Growth of the placenta with the corresponding creation of new vascular channels provides an important mechanism for the long-term regulation of umbilical blood flow.[1,2] However, the focus of this chapter is the vasoactive regulation of umbilical blood flow, which is accomplished by numerous mediators that influence vascular resistance in umbilicoplacental circulation. Umbilical blood flow regulation plays a role in maintaining fetal growth and central arterial pressure, in perfusion-perfusion matching within the placenta, and in the closure of the umbilical vessels at birth.

The umbilicoplacental circulation is critically important for the normal growth and development of the fetus. This interesting vascular bed has many unique features. For instance, the umbilical artery and vein are extremely long and muscular, and the resistance of the placental microcirculation is low. This means that a significant proportion of the total vascular resistance in this circulation resides in the umbilical vessels themselves. Under normal in utero conditions, the umbilicoplacental circulation appears to be nearly maximally dilated, yet the umbilical vessels are highly vasoactive and, unlike most large vessels, are capable of constricting so vigorously that the lumen is obliterated (e.g., at birth). The umbilical artery is sensitive to many circulating vasoconstrictors but has the unusual feature of being refractory to active vasorelaxation by the cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) pathways.[3,4] Perhaps the absence of active relaxation contributes to the vasospasm of the umbilical vasculature after birth. How the umbilical vessels are maintained in a nearly fully relaxed state throughout pregnancy is an interesting unanswered question.

This chapter begins with a brief description of the anatomy of the umbilicoplacental circulation and the sites of blood flow regulation in this bed. The important functions of blood flow regulation and the factors involved are then reviewed, after a brief outline of the major experimental methods that have been used to obtain this information. Finally abnormalities in umbilical blood flow regulation in intrauterine growth restriction (IUGR), a common and serious cause of mortality and morbidity in human pregnancy, are described.

ANATOMY OF THE HUMAN UMBILICOPLACENTAL CIRCULATION

The umbilical arteries in the human arise from the internal iliac arteries (Figure 74-1), then curve upward along either side of the bladder. At the umbilicus, they enter the umbilical cord, where

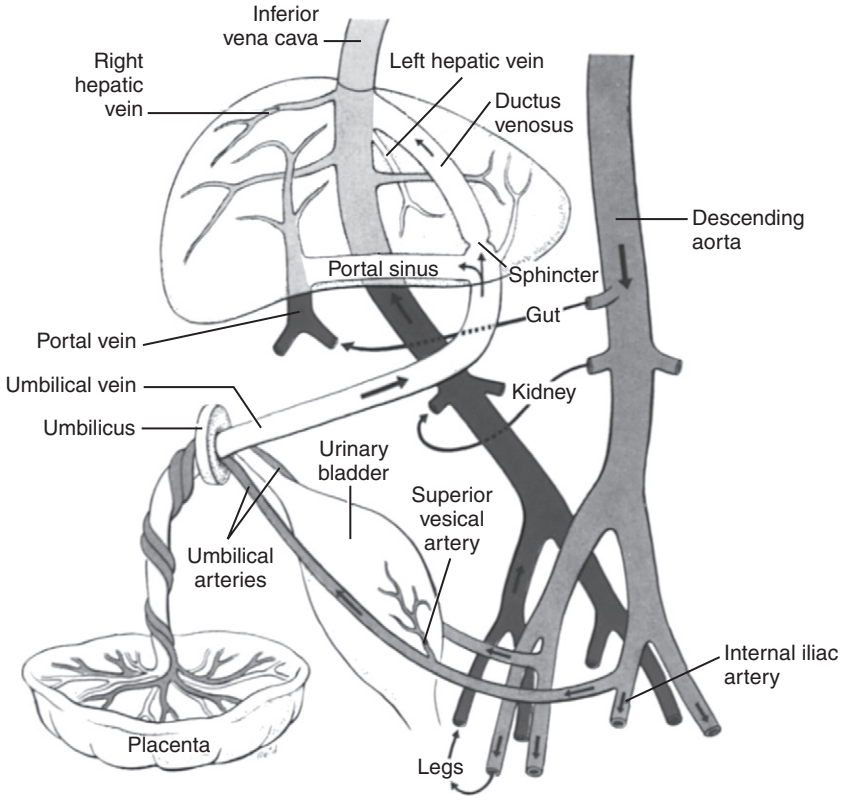


Figure 74-1 A simplified scheme of the human umbilicoplacental circulation. The umbilical arteries arise from the internal iliac arteries, then curve upward to the umbilicus on either side of the bladder. After emerging from the umbilicus, the arteries form a spiral around the umbilical vein before branching into the chorionic arteries on the surface of the placenta. Villous arteries penetrate the surface of the placenta then branch further, ultimately reaching the gas exchange surfaces in the terminal placental villi. Villous veins return blood from the placenta to the chorionic veins, visible on the surface of the placenta; blood then enters the umbilical vein in the umbilical cord. Within the abdomen, the umbilical vein enters the liver, where flow passes to the inferior vena cava either through the ductus venosus or the hepatic circulation. Not drawn to scale. *(Modified from Moore KL, Persaud TVN:* The Developing Human, *ed 5, Philadelphia, WB Saunders, 1993, p 344.)*

the arteries form a loose spiral around a single umbilical vein. The umbilical vessels are embedded in Wharton jelly within the umbilical cord, which is approximately 60 cm long at term.[5,6] The umbilical arterial wall is atypical because it has a high wall thickness relative to its lumen diameter, the vascular smooth muscle cells form several layers that differ in cellular orientation from circular near the outside to longitudinal near the lumen, and no internal elastic lamina separates the smooth muscle from the endothelial cells lining the lumen of the vessel.[7,8] The thick arterial muscle wall is sparsely supplied by vasa vasorum.[8,9] The umbilical arteries are joined by Hyrtl anastomosis near the surface of most placentas (approximately 96%) so that arterial pressure is equalized near the placental surface.[5,6]

The umbilical arteries branch at the fetal surface of the discoid human placenta to form many radially oriented chorionic arteries located on the surface of the chorionic plate. Distal branches of the chorionic arteries penetrate the fetal surface of the human placenta to supply 60 to 70 villous arteries, which in turn supply 10 to 40 cotyledons.[10] Cotyledonary units are separated by decidual septa arising from maternal tissue. Within the villous trees, the arteries continue branching to ultimately form arterioles within the intermediate villi. Exchange with maternal blood in the intervillous space of the cotyledons occurs primarily across the fetal capillaries of the terminal villi.

Veins within the villi merge progressively to return blood from the placenta to the chorionic veins on the fetoplacental surface. Blood then enters a single umbilical vein. The wall of the umbilical vein is highly muscular with circularly oriented smooth muscle cells arranged in concentric sheets loosely connected by ground substance.[7,8] Venous blood is then returned to the fetal inferior vena cava through the parallel arrangement of the ductus venosus and the hepatic vessels. The component of umbilical blood flow bypassing the hepatic circulation through the ductus venosus decreases from approximately 30% to 40% at midgestation to between 15% and 20% at term.[11,12]

HEMODYNAMICS OF UMBILICAL BLOOD FLOW

Umbilical arterial blood flow consists of a mean blood flow component (i.e., the mean flow over the cardiac cycle) with a superimposed pulsatile component (i.e., the phasic flow within a cardiac cycle). In terms of the net transport of substances to and from the placenta, it is the mean blood flow component that is of primary importance. The sites and mechanisms regulating these two components of arterial blood flow differ; for this reason they are considered in separate sections of this chapter.

Mean Blood Flow

Mean umbilical blood flow (Q_{Um}) depends on the pressure gradient that drives flow from the iliac artery in humans (BP_A) to the inferior vena cava (BP_{IVC}), and on the total vascular resistance of the serially arranged segments of the umbilicoplacental pathway (R_{tot}). Total vascular resistance represents the sum of the resistances in the umbilical artery and its branches (R_{UmA}), in the placental microcirculation (R_{Pl}), in the umbilical vein and

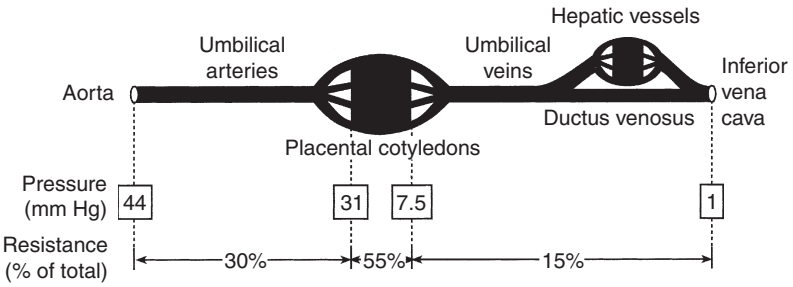


Figure 74-2 Distribution of blood pressures and vascular resistances within the sheep umbilicoplacental circulation near term (128 days gestation). Flow through this circulation is approximately 200 mL/min/kg. *(Data from Adamson SL, Whiteley KJ, Langille BL: Pulsatile pressure-flow relations and pulse-wave propagation in the umbilical circulation of fetal sheep,* Circ Res *70:761, 1992.)*

its branches (R_{UmV}), and in the parallel arrangement of the ductus venosus (R_{DV}) and hepatic circulation (R_H). Each of these segments is a potential site for regulation of mean blood flow through this bed.

$$Q_{Um} = (BP_A - BP_{IVC}) / R_{tot}$$

$$R_{tot} = R_{UmA} + R_{P1} + R_{UmV} + (1/R_{DV} + 1/R_H)^{-1}$$

In most systemic vascular beds, large arteries and veins contribute little to the total vascular resistance of the circulation and therefore are not important regulators of organ blood flow. However, the umbilicoplacental circulation is unusual in that the umbilical artery and vein are extremely long and muscular, and the resistance of the placental microcirculation is very low. In sheep, although the largest fraction of the total vascular resistance resides in the placental microcirculation (approximately 55%), almost half the total resistance resides in the umbilical vessels and their major branches, and in the ductus venosus–hepatic circulation[13,14] (Figure 74-2). Human umbilical vessels are perhaps four times longer than in sheep, so it is possible that the human umbilical vessels make an even larger contribution to total umbilicoplacental vascular resistance. The greater prevalence of Hyrtl anastamoses in humans (approximately 96%) than sheep (approximately 50%) may be caused by the greater need for pressure equalization at the placenta as a result of the much greater length of the umbilical artery in humans.

Pulsatile Blood Flow

The pulsatile component of umbilical arterial blood flow is driven by the pulsatile component of pressure at the entrance to the umbilical artery. The pulse amplitude of umbilical arterial flow depends on the pulse amplitude of arterial pressure and on the so-called vascular input impedance that is primarily dependent on the attributes of the umbilical artery, its radius, wall thickness, and wall stiffness.[15] Umbilical input impedance is also influenced by the properties of the chorionic and stem arteries, but it is entirely independent of the properties of the vessels past the point where all pulsations generated by the heart have been attenuated (i.e., past the arterioles). The pulsatile component of flow is not important for transport because there is no net motion when it is averaged over the cardiac cycle; even so, this pulsatility may be important in promoting endothelial nitric oxide production and maintaining low placental vascular resistance, as suggested by bypass experiments in fetal sheep.[16] It is also of interest clinically because the pulse amplitude of the flow velocity waveform in the umbilical artery is the numerator in the so-called pulsatility index, as well as being an important factor in other indices of pulsatility. Indices of pulsatility are elevated in the umbilical artery of fetuses with IUGR that are at highest risk for poor outcome.[17] The pulse amplitude of umbilical arterial flow tends to decrease with distance along the umbilical cord due to viscous losses in the blood and vessel wall. This attenuation results in a progressive decrease in indices of pulsatility when blood velocity waveforms are obtained at more distant sites along the cord.[18]

Atrial contraction of the heart generates pulse waves that are transmitted away from the heart into the venous system causing brief negative deflections in the predominantly forward-moving blood in the veins in association with the cardiac cycle. This effect can cause pulsatililty in the blood velocity waveform of the umbilical vein and ductus venosus. Although such pulsatility is normal in early human pregnancy, when propagation distances are short, pulsatility in the umbilical vein and ductus venosus typically is not observed in late pregnancy unless the fetus has cardiovascular pathology resulting in augmentation of the strength of atrial contraction or of the efficiency of pulse wave propagation in the venous system.[12] Wave propagation into the umbilical vein and ductus venosus is increased by dilation of the ductus venosus alone or in combination with constriction of the umbilical vein, such as may occur during fetal hypoxia or hemorrhage.[12]

METHODS USED TO STUDY UMBILICAL BLOOD FLOW REGULATION

Ultrasound Imaging

Pulsed Doppler ultrasound imaging provides a sensitive and noninvasive means of detecting the velocity of red blood cells in the umbilical artery or vein in humans[19] and other species including sheep,[19] guinea pigs,[20] and mice[21] (Figure 74-3). The shift in the frequency of the echo relative to the transmitted Doppler sound wave is directly proportional to the velocity of the red blood cells that generate the echo. For accurate velocity determination, angle correction is required if the angle between the transmitted wave and the direction of red blood cell motion exceeds approximately 20 degrees. Measurement of this angle can be problematic in the umbilical artery because of its spiral path but can be reasonably accurate in the much straighter intraabdominal umbilical vein. Blood velocity can be used to calculate blood flow by multiplying by vessel cross-sectional area determined from blood vessel diameter measurements. Flow per kilogram of fetal body weight can be calculated using an estimate of fetal body weight determined from ultrasound measurements of fetal abdominal and head circumferences.[22-24]

Perfused Placenta in Vitro

Early studies investigated the vasoactivity of the human umbilicoplacental circulation by perfusing the entire placenta through the umbilical cord after delivery at a fixed flow rate and then recording the change in perfusion pressure induced by vasoactive agents delivered into the umbilical artery.[25] Obtaining a completely undamaged (i.e., leak-free) placenta was difficult, however,

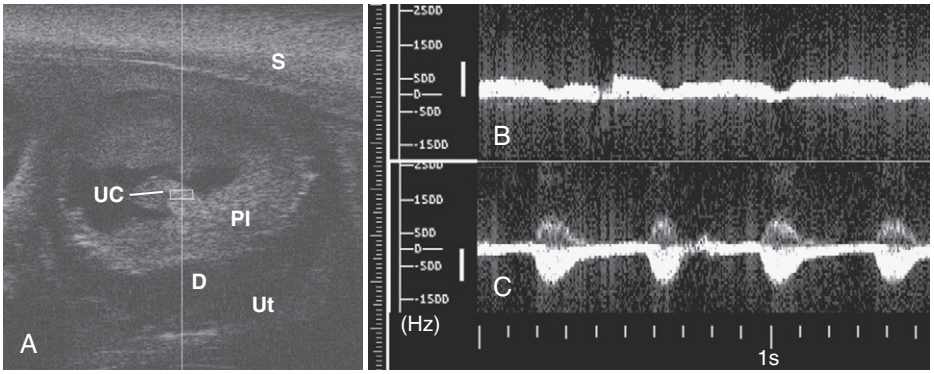


Figure 74-3 High-frequency ultrasound technology permits Doppler blood velocity measurements in the umbilical cord throughout embryonic and fetal development in the mouse. This, combined with the potential for exploring gene function in genetically modified mice, makes it an attractive new model for future research. **A,** The placenta *(Pl)* and umbilical cord *(UC)* of a mouse embryo at day 10.5 of gestation. The anesthetized pregnant mouse was imaged transcutaneously using 40 MHz ultrasound. The locations of the maternal skin *(S),* decidua *(D),* and uterus *(Ut)* are indicated. The *box* shows the location of the Doppler sample volume on the umbilical cord. By moving the sample volume slightly, the Doppler blood velocity waveform was obtained separately in the umbilical vein **(B)** and umbilical artery **(C)** of the embryo. The smallest division on the *scale bar* in **A** is 100 μm. The *vertical white bar* along the *y* axis in **B** and **C** shows a Doppler shift frequency of 0 to 1000 Hz. *(Unpublished data from the work of Zhou YQ, Adamson SL, 2002.)*

so most current work perfuses a single peripheral placental cotyledon through cannulas inserted into a chorionic artery and vein on the fetal placental surface and through two or more cannulas inserted into the corresponding intervillous space through the maternal placental surface. This method, originally developed to study placental transfer,[26] has been used to investigate vasoactivity. The cotyledon is perfused at a fixed flow rate so that an increase in perfusion pressure in the fetal chorionic artery indicates an increase in fetoplacental vascular resistance. The preparation is viable for 12 hours or more of perfusion.[27]

Umbilicoplacental Vessels in Vitro

The vasoactivity of segments, rings, or strips of larger vessels in the human umbilicoplacental circulation has been extensively studied in vitro. In early studies, endothelial cell damage may have occurred before the importance of the endothelium in regulation of smooth muscle tone was recognized. The vasoactivity of vessel strips or rings is studied by recording muscle tension with a strain gauge, whereas the vasoactivity of a vessel segment is detected by perfusing the segment at a constant rate and recording changes in perfusion pressure.[28] The vasoactivity of umbilical and chorionic vessels, as well as villous stem arteries, and of their second- and third-order branches has been studied using these methods.[29] The production of vasoactive substances by placental vessels can be studied by determining the effect of placental vessel effluent on a strip of isolated smooth muscle used as a detector tissue. The degree of contraction or relaxation of the detector muscle provides a bioassay for vasoactive substances.[30] Differences in vasoactivity between different segments of the umbilicoplacental circulation have been observed[31,32] (see Table 74-1).

Implanted Instrumentation

Fetal sheep are most commonly used for studies requiring implanted instrumentation, although similar studies are possible in other species such as goats and baboons. For studies on the umbilicoplacental circulation, chronic instrumentation is preferred to avoid the elevated umbilicoplacental vascular resistances observed in acutely instrumented fetal sheep under halothane anesthesia (approximately a 2.5-fold increase).[13,33] Mean blood pressure is measured using fluid-filled catheters implanted in arteries or veins that are coupled to strain-gauge pressure transducers outside the animal. Most studies measure only the total pressure gradient across the umbilicoplacental circulation (aorta to inferior vena cava) so that only total vascular resistance is determined. However, several studies have measured pressure gradients across individual segments of the umbilicoplacental circulation, enabling determination of segmental vascular resistance.[13,33-35] These studies have revealed large differences in vasoactivity among the umbilical arteries, veins, microcirculation, ductus venosus, and hepatic circulation in fetal sheep. Mean blood flow is measured using electromagnetic transducers or ultrasound transit-time transducers implanted around the common umbilical artery or vein[36,37] or by using labeled microspheres,[38] Fick's principle (e.g., with antipyrine),[39,40] or dye dilution methods.[41] It is now recognized that antipyrine is not an ideal tracer for flow studies because it inhibits prostaglandin synthesis and has measurable physiologic effects in fetal sheep.[42]

IMPORTANCE OF UMBILICAL BLOOD FLOW REGULATION

The umbilicoplacental circulation is nearly maximally dilated[35] and the fetal biventricular cardiac output is near maximal under basal conditions in the near-term fetal sheep.[43] Thus umbilical blood flow can be increased little by further dilating this vascular bed to reduce the resistance to flow or by increasing the driving pressure for flow by increasing cardiac output (hence mean arterial pressure). Nevertheless, umbilical blood flow can be increased by increasing vascular resistance in the fetal body to increase mean arterial pressure and thereby direct a larger proportion of the biventricular cardiac output to the placenta. In practice, however, umbilical blood flow is remarkably constant. During episodes of breathing activity in fetal sheep, fetal oxygen consumption increases and Pa_{O_2} decreases significantly, yet umbilical blood flow and mean arterial pressure remain essentially constant.[44] Umbilical blood flow also changes little when the fetal sheep changes sleep state.[45] Even when the sheep fetus is made hypoxemic, umbilical blood flow remains nearly constant in fetuses near term.[46] This has led to the impression that the umbilicoplacental circulation is passive. Nevertheless, short-term regulation of vascular resistance does occur in this vascular bed, and it plays important physiologic roles in optimizing placental exchange by matching regional maternal and fetal

blood flows, in maintaining mean arterial blood pressure during hemorrhage, and in causing complete closure of this vascular bed at birth. In addition, abnormalities in vasoactive control of the umbilicoplacental circulation may contribute to the etiology of IUGR. Each of these roles is described in detail later in the chapter.

Support of Fetal Growth

Umbilical blood flow gradually increases as the fetus grows and placental exchange requirements increase. In humans, umbilical blood flow increases in direct proportion to the increase in fetal body weight so that flow expressed relative to fetal weight remains approximately constant at 110 to 125 mL/min^{-1}/kg^{-1} during the last third of pregnancy.[23,47-49] Human fetal biventricular cardiac output is approximately 450 mL/min^{-1}/kg^{-1}, and it remains approximately constant over this gestational age range.[24,50] Thus umbilical blood flow represents approximately 30% of fetal biventricular cardiac output.[24]

In IUGR, fetuses fail to achieve their full growth potential for a variety of reasons, including infection, drug abuse, maternal malnutrition, and placental insufficiency caused by increased umbilicoplacental resistance or unknown etiology. The fetus appears to respond to inadequate placental transfer of nutrients or oxygen by restricting growth to conserve limited resources for more essential functions, thereby maintaining viability. Umbilical blood flow and umbilical blood flow per kilogram estimated fetal body weight are lower than normal in growth-restricted fetuses.[49,51] Early work suggested that umbilical blood flow in the sheep fetus normally exceeds that required so that there is a margin of safety in umbilical flow. This was based on evidence that acute reductions in umbilical blood flow caused an increase in oxygen extraction and no change in fetal oxygen consumption until flow was reduced by more than 40% to 50%.[52,53] However, a 30% to 40% reduction in umbilical blood flow that was maintained for 10 to 14 days reduced fetal growth and oxygen consumption, suggesting little margin of safety in umbilical blood flow with respect to fetal growth.[54,55]

Perfusion-Perfusion Matching to Optimize Maternal-Fetal Exchange

To maximize exchange, highly perfused regions on the maternal side of the placenta should be matched with well-perfused regions on the fetal side. This matching, analogous to ventilation-perfusion matching in the lung, makes teleologic sense, and this mechanism is supported by evidence from experiments in the perfused human placental cotyledon and in fetal sheep. Human cotyledons in vitro are usually perfused through the maternal intervillous space with media that have been equilibrated with 95% O_2 and 5% CO_2, which results in a perfusate Po_2 that exceeds 500 mm Hg. A large reduction in maternal perfusate Po_2 (to approximately 25 mm Hg) results in a rapid vasoconstrictive response in the fetal placental vasculature; an effect that is rapidly reversed by increasing maternal perfusate Po_2.[56] Hypoxic vasoconstriction of the fetal placental microcirculation has been postulated to mediate perfusion-perfusion matching in the human placenta,[56] and it appears to be mediated by an hypoxia-induced decrease in nitric oxide production.[57]

In sheep, regional maternal and fetal blood flows measured using radioactive microspheres within cotyledons are correlated under baseline conditions.[58] When maternal blood flow to a placental region is reduced (e.g., by ligating maternal vessels or by embolizing with microspheres) fetal blood flow to the corresponding region decreases within 24 hours.[59] The mechanisms that increase umbilicoplacental vascular resistance in response to a decrease in maternal placental blood flow in sheep are not fully understood. However, they may be mediated by hypoxic vasoconstriction, as occurs in ventilation-perfusion matching in the lung. In the fetal sheep near term, fetal hypoxia induced by maternal inhalation of a low-oxygen mixture causes only a small increase in umbilicoplacental vascular resistance (e.g., 20%).[14,60] Some evidence suggests that hypoxic vasoconstriction in the placenta may beis reversed by enhanced β-adrenergic activity caused by fetal hypoxia.[61] The small increase in umbilicoplacental resistance during fetal hypoxia is caused specifically by a more than 100% increase in resistance of the umbilical vein and hepatic circulation, which is mediated by the α-adrenergic system.[14,60,62] In preterm fetal sheep (at approximately 95 days gestation), fetal hypoxia causes a larger (e.g., 50%) increase in umbilicoplacental resistance.[63] The site and the mechanism of vasoconstriction in these preterm fetuses are not known. It is possible that locally mediated hypoxic vasoconstriction within the placenta is not so effectively reversed by systemic hypoxic responses in preterm fetuses.

Maintenance of Fetal Arterial Blood Pressure

The umbilicoplacental circulation appears to play a role in maintaining central arterial blood pressure during hemorrhage in fetal sheep. During mild hemorrhage (approximately 15% loss of fetal blood volume), vascular resistance of the umbilicoplacental circulation increases to a similar extent as the fetal body (20%) so that the proportion of biventricular cardiac output directed toward the placenta remains unchanged.[64] With life-threatening, severe hemorrhage (approximately 40% loss of fetal blood volume), however, vascular resistance in the umbilicoplacental circulation increases 260%, thereby helping to maintain central arterial pressure.[65] Resistance increases significantly in all segments: the umbilical arteries and placenta (+250%), the umbilical veins (+390%), and the hepatic circulation (+540%), and nonsignificantly in the ductus venosus (+190%).[65] Umbilicoplacental vasoconstriction may be mediated by increased circulating concentrations of catecholamines, angiotensin II (AT II), and vasopressin, individually or in combination.[65-68]

Factors affecting the exchange of water between the maternal and fetal circulations at the placental interface could influence fetal blood volume and hence play a role in maintaining fetal arterial blood pressure. The net transfer of water across the placenta is presumably controlled by the balance of osmotic and hydrostatic pressures across placental capillary walls, as in other microcirculatory beds. It has been proposed that a decrease in placental capillary blood pressure may promote the movement of water from the maternal to the fetal circulation, thereby increasing fetal blood volume and thus fetal arterial blood pressure.[69] In experiments in sheep, however, when pressure in the umbilicoplacental circulation was decreased 33% by partially occluding the fetal distal aorta for 2 weeks, hematocrit did not decrease, suggesting that no increase occurred in either plasma volume or central arterial blood pressure.[54] Thus it remains unknown whether alterations in water transport caused by physiologic alterations in placental capillary pressure play an important role in maintaining fetal arterial blood pressure.

Closure of Umbilicoplacental Circulation at Birth

In humans, umbilical blood flow rapidly decreases after delivery of the fetus to less than 20% of the normal fetal value[23] by 40 to 60 seconds after delivery.[70,71] This is accompanied by significant decreases in umbilical artery and vein diameters within 120 seconds.[72] The factors responsible for the rapid constriction of the umbilical vessels and the rapid increase in umbilicoplacental vascular resistance at birth are poorly understood.

It is possible that the sudden exposure of the umbilical cord after birth to a cool, more highly oxygenated room air environment plays a role. Cooling human umbilical vein segments from

37° C to 20° C caused vasoconstriction mediated by increased prostaglandin and thromboxane synthesis.[73] Cooling in this temperature range also caused constriction of human umbilical artery rings, but the contraction was transient, lasting less than 5 to 10 minutes,[74] which suggests this mechanism may not be important in the sustained closure of umbilical vessels at birth. Similarly, an increase in oxygen tension elicits a transient vasoconstriction of the umbilical artery, which is mediated by increased thromboxane synthesis.[74-76] An increase in oxygenation also enhances the contractile effects of bradykinin and serotonin[28,75,76] but not endothelin-1[31] on the human umbilical artery. Nevertheless, umbilical artery and vein diameters decrease markedly within 2 minutes after delivery despite no significant increase in arterial or venous pO_2.[72] Thus factors other than an increase in oxygenation (contraction precedes it) and cooling (contraction is transient) are likely involved in the rapid constriction of the umbilical vasculature at birth.

Local mediators produced by cells in the vicinity of the umbilical vessels may be the primary source of vasoactive agents that effect closure of these vessels after birth.[74] Serotonin in particular may have sufficient potency and efficacy and a sufficiently long duration of action (longer than 2.5 hours) to effect cord closure.[74] Alternatively vasoconstriction may be stimulated by mechanical stretch of the cord.[77] Umbilical vessels may be prone to vasospasm due to the absence of active vasodilation by cAMP or cGMP pathways.[3,4]

FACTORS THAT REGULATE UMBILICAL BLOOD FLOW

Mean blood flow through the umbilicoplacental circulation depends on the total pressure gradient driving flow and the sum of the resistances in each segment of this vascular bed (see Figure 74-2). Vasoactive factors, as well as changes in heart rate, can indirectly affect umbilical blood flow by altering the central blood pressures that drive flow through this circulation. However, umbilical flow can be affected more directly by alterations in vascular resistance within the umbilicoplacental vascular bed.

Vasoactive Mediators

The effects of numerous vasoactive mediators on different segments of the human umbilicoplacental vasculature, as reported by numerous groups of investigators, are summarized in Table 74-1; relevant studies are referenced in the table. Of note, vasodilator activity typically is evaluated in vessels that have been preconstricted with substances such as AT II, the prostaglandin $PGF_{2\alpha}$, or thromboxane. The mediators are listed in alphabetical order in the table because it is difficult to divide vasoactive substances into extrinsic (humoral) or intrinsic (local, paracrine-autocrine) categories; substances that act locally postnatally (e.g., PGE_2, endothelin) circulate in relatively high concentrations in the fetus[115,153] and may therefore have humoral effects. Similarly, other classic hormones (e.g., corticotropin-releasing hormone) are produced in the placenta[108,109] and may therefore have local effects. It is also difficult to divide them into dilators and constrictors because some mediators may cause vasoconstriction in one segment but vasodilation in another (e.g., histamine), and others usually considered to be vasodilators cause vasoconstriction in this bed (e.g., acetylcholine, bradykinin).

Observation of a vasoactive effect for a specific exogenous mediator, as identified in Table 74-1, does not necessarily mean that the mediator has an important physiologic role in controlling placental perfusion. Further evidence for such a role thus would include detection of the mediator in cord blood and the presence of local sites of synthesis within the umbilicoplacental circulation, as well as the presence of receptors. This information also has been included in Table 74-1. A role for endogenous production of a mediator also can be shown by blocking its synthesis or receptors and observing that this elicits a change in vascular resistance. Studies that have taken this approach are summarized and referenced in Table 74-2.

The effects of vasoactive mediators on the human umbilicoplacental vasculature usually are studied using isolated tissues in vitro that were collected after delivery at term. However, it is important to keep in mind that responses may differ from those observed in vivo (umbilical arteries, for example, may not fully relax in vitro[156]), and that responses may also change during gestation. For example, relative to umbilical arteries collected after preterm delivery (less than 35 weeks), arteries collected after delivery at term (38 weeks and beyond) constrict much less vigorously in response to AT II but more vigorously to vasopressin, norepinephrine, and PGE_2.[74]

Innervation

Except in the immediate vicinity of the umbilicus, adrenergic or cholinergic nerve fibers are not detectable in the human placenta or in human or sheep umbilical cord vessels.[5,8] By contrast, the intrafetal portion of the human umbilical artery is innervated,[5] as is the ductus venosus in sheep.[157] This innervation apparently exerts little influence over ductus venosus blood flow under basal conditions[157] or during increased α-adrenergic activity induced by hypoxia in fetal sheep[62,158] so the function of this innervation is unknown. Similarly, a significant neural role in control of the intrafetal portion of the umbilical artery has not been demonstrated to date. The absence of innervation of extrafetal umbilical cord vessels and the placenta indicates that these vessels are not under neural control.

Wall Shear Stress

Changes in the flow rate or viscosity of fluid moving through a vessel alters the wall shear stress (i.e., viscous drag) imposed on the endothelial cells lining the vessel lumen. Shear stress is an important stimulus for the tonic secretion of vasodilators, such as nitric oxide, from endothelial cells.[159,160] In the perfused human cotyledon, alterations in endothelial shear stress effected by alterations in flow appear to alter endothelial nitric oxide release.[161] Furthermore, in small placental arteries, flow-induced vasodilation is observed, and this effect is blocked by nitric oxide synthase inhibitors.[162] Flow-induced vasodilation may not occur in umbilical arteries, however, because the smooth muscle cells of these vessels exhibit little or no relaxation response to nitric oxide.[3,128] Alternatively, other mediators could regulate this response.[160]

UMBILICAL BLOOD FLOW IN INTRAUTERINE GROWTH RESTRICTION

Umbilical blood flow and umbilical blood flow per kilogram of estimated fetal body weight are lower than normal in human fetuses with IUGR,[49,51] indicating that either the vascular resistance of the umbilicoplacental circulation is high or the driving pressure for flow is low. In addition, many fetuses with IUGR have umbilical arterial blood velocity waveforms that are highly pulsatile.[49,163] Diastolic blood velocity in these fetuses may be reduced, absent, or even reversed (i.e., blood returns toward the heart) compared with the normally high diastolic velocities recorded in the umbilical artery in the latter part of normal pregnancy.[164]

Fetuses with IUGR, especially those with highly pulsatile umbilical arterial velocity waveforms, are often hypoxemic,[165,166] but hypoxemia itself does not increase blood flow pulsatility in the umbilical artery when induced experimentally in fetal sheep.[167,168] High pulsatility can be induced, however, by increasing the vascular resistance of the placental microcirculation.[169-171] In IUGR, elevated placental vascular resistance may be caused by structural abnormalities of the fetoplacental vasculature[172-176] or by

TABLE 74-1

Vasoactive Mediators of the Human Umbilicoplacental Circulation

Mediator	Detected in Cord Blood	Receptor Localization Sites	Local Production Sites	VASOACTIVITY					
				Umbilical Artery	*Chorionic Artery*	*Stem Artery/ Arteriole*	*Perfused Cotyledon*	*Chorionic Vein*	*Umbilical Vein*
Acetylcholine[a][29,30,74,78-80]			Pl	+		0	0		
Adenosine[81-83]				−			−		
Adrenomedullin[84-87]		Pl	Pl			−	−		
Angiotensin II[b][32,74,88-91]		Pl		+/0	+	+	+		
ANP[c][92-97]		Pl	Pl	−			−		
BNP[c][93,98]			Amnion				−		
Bradykinin[32,74,76,79,99,100]				+	+	+	+/0		
Carbon monoxide[101-105]			Pl				−		
CGRP[d][106,107]					−		−		
CRH[e][108-113]			Pl		−		−		
Cortisol[114]							0		
Endothelin-1[f][31,115-125]		UmA, Pl	Pl	+	+	+	+	+	+
Histamine[g][74,79,126]			Mast cells, basophils	+			−		
Leukotrienes[100,127]							+		
Nitric oxide[3,30,128-133]			UmA, UmV, Pl, ChA, SA	0/−			−		
Norepinephrine[74,83,96,100,134-136]				+			+/0		+
Neuropeptide Y[h][74,121]				+/0					
Oxytocin[74,136-138]				+	+	+		+	+
PGE_2 and $PGF_{2\alpha}$[74,91,100,139-141]			UmA, Pl	+			+		+
PGI_2[i][100,135,142-146]			Pl, UmA, UmV, ChV, ChA		−	−	−	−	−
PTH[j][107,147]			C				−		
PTHrP[j][107,147]		Pl							
Serotonin[29,31,31,32,74,76,91,100,135]			Platelets	+	+	+	+/0		+
Substance P[148]						−			
Thromboxane[k][81,91,100,144,146,149]		Pl	UmA, UmV, Pl	+		+	+		+
Urotensin-1[113]							−		
Urocortin[113,150-152]			D, Pl				−		
Vasopressin[74,136-138]				+	+	+		+	
VIP[143,148]					−	−			

[a]Acetylcholine does not stimulate release of nitric oxide from umbilical artery or umbilical vein segments.[30]

[b]Angiotension II (AT II) response is likely mediated by AT_1 receptors in the placenta because the AT_2 subtype is not detectable.[88] Vasoconstrictive response is greatest in villous stem arterioles, moderate in chorionic arteries, and nearly absent in umbilical artery near term.[32,74,91] Preterm umbilical arteries (<35 weeks) do not vasoconstrict in response to AT II.[74]

[c]BNP (brain natriuretic peptide) is a more potent vasodilator of perfused cotyledons than ANP (atrial natriuretic peptide).[93]

[d]CGRP (calcitonin gene–related peptide) mediated vasodilation in the perfused cotyledon appears to be independent of nitric oxide synthesis and mediated by both CGRP-1 and CGRP-2 receptors.[107]

[e]CRH (corticotropin-releasing hormone) acts by way off the nitric oxide/cyclic guanosine monophosphate pathway[110] to vasodilate the placenta cotyledon at physiologic concentrations.[112]

[f]Subthreshold doses of endothelin-1 increase the sensitivity of the umbilical artery to other vasoconstrictors.[125] Bolus doses cause prolonged vasoconstrictive responses (45 to 60 minutes) in the perfused cotyledon.[122]

[g]Histamine's vasodilator effects may be mediated, at least in part, by endothelial cell–derived relaxing factor (presumably nitric oxide).[30]

[h]Neuropeptide Y constricts umbilical arteries collected at term but not when collected preterm.[74,121]

[i]PGI_2 release by the perfused cotyledon is increased during angiotensin II–mediated vasoconstriction[146], thereby attenuating the vasoconstrictive response to angiotensin II.[142]

[j]PTHrP (parathyroid hormone–related peptide) is ~100 times more potent than PTH (parathyroid hormone) as a vasodilator of the perfused placental cotyledon.

[k]Or the thromboxane analogue U46619.

Cb, Chorionic vein; *ChA,* chorionic artery; *D,* decidua; *PGE,* prostaglandin E; *PGF,* prostaglandin F; *Pl,* placenta; *SA,* stem artery; *UmA,* umbilical artery; *UmV,* umbilical vein; *VIP,* vasoactive intestinal peptide; +, construction; −, relaxation; +/0, little or no constriction; 0/−, little or no relaxation.

abnormalities in placental vasoconstrictor tone.[177,178] Of interest, perfused cotyledons of such fetuses do not require significantly higher pressures to achieve the same perfusion rate, indicating that their vascular resistances are near normal in vitro.[110,179] Therefore, if elevated pulsatility of umbilical arterial velocity waveforms in these fetuses is caused by elevated resistance in vivo, then elevated vascular resistance appears to be mediated by vasoactive factors or at a site not represented in the perfused cotyledon in vitro.[15,179] The possibility that resistance is elevated within the umbilical cord, hepatic circulation, or ductus venosus in at least in some cases of IUGR warrants further consideration.[180]

Pulsatility of umbilical arterial velocity waveforms can be improved by bed rest,[181,182] maternal hydration,[182] or oxygen administration,[183] further suggesting that, at least in some cases, altered vasoactive tone rather than anatomic abnormalities of the umbilicoplacental circulation is involved. Some evidence does support abnormalities in vasoactive mediators in pregnancies with IUGR. For instance, vasoconstriction of the placental vasculature may be augmented by AT II, endothelin-1, or by the proinflammatory cytokine, tumor necrosis factor-α, all of which are elevated in the umbilical cord blood of fetuses with IUGR.[177,184,185] Alternatively, elevated circulating AT II levels may be offset by reduced protein level, binding capacity, and affinity of the angiotensin receptor AT_1 in placentas affected by IUGR.[88,186] Vasoconstriction may also be enhanced by decreased expression of heme oxygenase-2 and thus the reduced production of the

TABLE 74-2

Effects of Inhibitors of Vasoactive Mediators on the Human Umbilicoplacental Circulation

Vasoactive Substance	Inhibitor	Response
Cyclooxygenase	Indomethacin	No effect on basal tone of umbilical vein, perfused placental cotyledon, or stem villous arterioles[29,100,142,154]
Nitric oxide synthase	L-NAME	Increases basal tone and sensitivity to vasoconstrictors in perfused placental cotyledons[79,155]
Angiotensin II	Saralasin	Inhibits angiotensin II constriction of perfused placental cotyledons[89,90]
Heme oxygenase	Zinc protoporphyrin	Increases basal tone in perfused placental cotyledons[105]

L-NAME, Nitro-L-arginine methyl ester.

vasodilator carbon monoxide by the endothelium in placentas with IUGR.[101] Thromboxane and vasoconstrictor prostaglandins do not appear to be involved as production of these mediators is not enhanced in perfused placental cotyledons from fetuses with IUGR.[187] Vasocontrictive influences may be offset, at least in part, by a four-fold increase in cord blood concentrations of the vasodilator corticotropin-releasing hormone,[188-190] the activity of which is mediated by nitric oxide[110] and by increased expression of endothelial nitric oxide synthase in placental villous vessels.[191]

SUMMARY AND CONCLUSION

Many potentially important mechanisms regulate umbilicoplacental vascular resistance in vivo. Evidence from relevant studies supports an important physiologic role for the local production of corticotropin-releasing hormone and nitric oxide in promoting the normally low vascular resistance of the umbilicoplacental circulation. Many factors also are capable of vasoconstricting the umbilicoplacental circulation; endothelin-1 and thromboxane are among the most potent. Local alterations in resistance in the placental microcirculation may play a physiologic role in matching flow with the corresponding flow on the maternal side of the placenta. In addition, vasoconstrictors may play a critical physiologic role in the closure of the umbilicoplacental circulation after birth. In fetuses with IUGR, abnormalities in levels or in responsiveness to vasoactive factors may increase umbilicoplacental vascular resistance, thereby reducing umbilical blood flow and resulting in impaired fetal growth.

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Karel Maršál

Fetal and Placental Circulation During Labor

PHYSIOLOGIC BACKGROUND

A sufficient maternal blood supply to the placenta is of utmost importance to the fetus during pregnancy and labor. It has long been recognized that uterine contractions diminish uteroplacental blood flow. Contractions of myometrium compress the vessels traversing the uterine wall and increase intrauterine pressure, thus influencing the intervillous space pressure as well. The intrauterine pressure during labor usually ranges from 25 to 100 mm Hg. In contrast, the mean pressure in small arteries is 70 to 95 mm Hg and only 15 mm Hg at the capillary venous end. Compression and even collapse of the myometrial vessels during labor is thus probable. Even a slight reduction in the diameter of an artery results in reduction of flow, because resistance of flow is inversely proportional to the fourth power of the vessel radius according to the Poiseuille law. Obviously, during a contraction, the uterine veins are affected first, and the restricted venous outflow results in a reduction of the pressure gradient over the placenta. With use of radioangiographic technique in pregnant women, it has been shown that the maternal blood flow to the placenta decreases markedly during contraction.[1] Similarly, in the rhesus monkey, diminished arteriolar jets have also been demonstrated.[2] Flow to the intervillous space, however, appears to remain constant.

The intermittent decrement in the blood flow during myometrial contractions has been found to be inversely related to the increase in intrauterine pressure.[3-6] During uterine relaxation after a contraction, an increase in the blood flow—a reactive hyperemia—has been observed,[4,6] which compensates for the decreased oxygen delivery during the preceding contraction.

With the progress of labor, the peak intrauterine pressure during each contraction increases, and the time-averaged blood flow in the uterine artery diminishes[7] (Figure 75-1). Woodbury and co-workers[8] described a "maternal effective placental arterial pressure," defined as the arterial pressure minus the pressure within the uterus (which opposes the inflow of maternal blood). To ensure the perfusion of the placenta, the maternal central blood circulation responds to contractions by increasing both blood pressure and cardiac output.[9,10]

The circulation of a healthy fetus in uncomplicated labor usually remains unaffected. The umbilical circulation is relatively

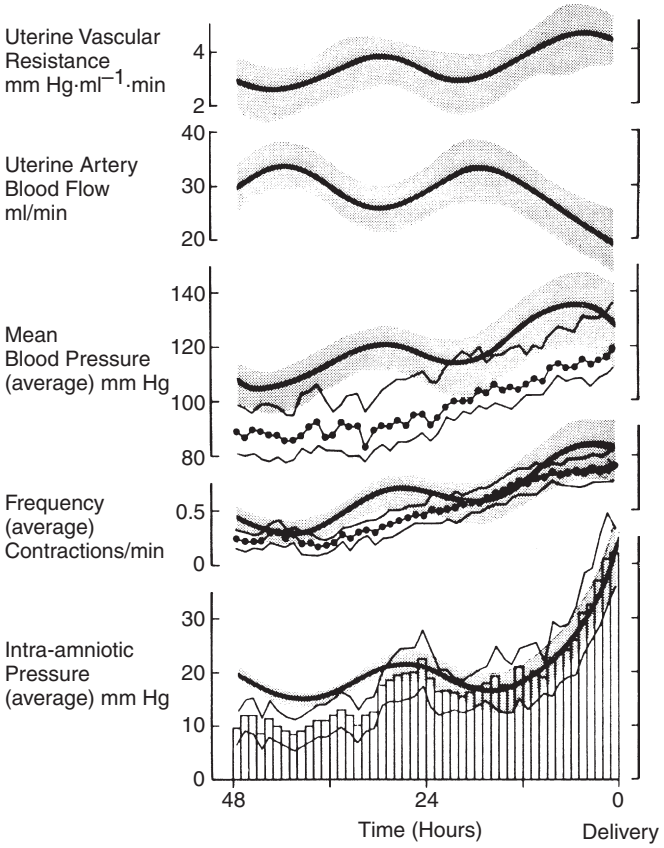


Figure 75-1 Composite drawing showing the incorporation of the circadian changes of uterine dynamics into the process of labor and delivery. The open bars of intraamniotic pressure and the solid points depicting frequency of contractions and mean blood pressure represent data obtained from five patients monitored continuously during the last 48 hours of pregnancy. Each bar and point represent the mean of hourly average values. The thin solid lines delineate the 95% confidence intervals. The heavy solid lines of intraamniotic pressure, frequency of contraction, mean blood pressure, uterine artery blood flow, and uterine vascular resistance were calculated from continuous recordings made in 15 monkeys during the 48 hours terminating in spontaneous labor and delivery. The shaded areas represent the 95% confidence intervals determined for individual mean values. Uterine vascular resistance was calculated by the formula: aortic blood pressure minus intraamniotic pressure divided by uterine artery blood flow. *(From Harbert GM Jr: Circadian changes in uteroplacental blood flow. In Rosenfeld CR, editor:* The uterine circulation. Reproductive and perinatal medicine, *vol X, Ithaca, NY, Perinatology Press, 1989, p 157.)*

unreactive and does not seem to respond to the changes in intrauterine pressure or to the short-term changes in the maternal placental blood flow during contractions. During normal labor, uterine contractions are not of sufficient magnitude to negatively affect gas exchange over the placenta. Therefore they do not endanger the fetus. In labor with a pathologic course, in which uteroplacental blood flow is diminished, fetal hypoxemia can develop. In that situation, the fetus reacts with changes in heart rate, blood pressure, and blood flow.[11] Uterine contractions can also cause a direct compression of the umbilical cord with restriction of the umbilical blood flow, leading to changes in the fetal circulation.

METHODS OF RECORDING HUMAN FETAL AND UTEROPLACENTAL BLOOD FLOW

Previously, most of our knowledge of uteroplacental and fetal circulation was based on animal experiments, in which invasive methods have been used. Approximately 20 years ago, a noninvasive method making use of Doppler ultrasound was introduced in the field of perinatal medicine and has made it possible to obtain data on blood flow in human pregnancies.[12,13]

Flow Probe Techniques

Measurement of the uterine, uteroplacental, or fetal blood flow using a flow probe technique (measuring the flow in a single vessel by evaluating changes caused by the blood flow in either the electromagnetic field or the ultrasound passage time) requires an application of the probe on an exposed vessel. Only in exceptional circumstances has the electromagnetic method been used on a human uterine artery during laparotomy for hysterectomy in pregnancy.[14]

Radioangiography

The radioangiographic technique, with injection of contrast medium followed by serial x-ray exposures, has made it possible to establish the time of appearance and disappearance of contrast dye in various parts of the uteroplacental circulation.[1] However, absolute flow cannot be determined, and, because of the high radiation hazard to the fetus, this method is not acceptable for use in humans.

Placenta Scintigraphy

Radioactive isotopes (^{24}Na, ^{133}Xe) can be injected into either the myometrium or the intervillous space, and the time rate of washout can then be determined by external measurement. This gives a semiquantitative measure of the maternal placental blood flow. To circumvent the disadvantage of the method's invasiveness, placenta scintigraphy has been developed that uses an intravenous injection of a radionuclide tracer (^{99m}Tc, ^{133}Xe, or ^{113m}In) and external measurement of accumulation or disappearance of radioactivity over the placenta.[15] Clinical application of this method is limited because it cannot be used for continuous measurement of the changes in placental blood flow over time, and only the anterior placenta can be examined. The most serious drawback of the method is exposure of the fetus to ionizing radiation.

Thermistor Method

The relative uterine blood flow can be evaluated by placing a preheated thermistor pearl into cervical tissue and then recording heat dispersal.[16] This method has the disadvantages of being invasive and of measuring flow in the cervix and lower segment of uterus and not directly in the placenta.

Doppler Ultrasound Method

Physical Principle

The Doppler ultrasound technique makes it possible to estimate blood velocity and blood flow in maternal and fetal vessels in a noninvasive way during late pregnancy and labor. The first report on the detection of flow signals from the umbilical artery was published in 1977 by FitzGerald and Drumm.[13] In recent years, the Doppler velocimetry has become a method of choice for evaluation of the uteroplacental and fetal circulation.

According to the Doppler principle, wave energy is reflected by a moving reflector with a wavelength (frequency) that is

different from the emitted wavelength. The change in frequency (Doppler shift) is proportional to the velocity of the reflector. In the situation of blood flow measurement, ultrasound with a frequency of 1 to 10 MHz is transmitted to the tissue and reflected by the moving red cells within the vessel. The frequency of the received ultrasound is higher than the emitted frequency when the blood is moving toward the transducer, and it is lower when the blood is moving away from the transducer. The Doppler shift (f_d) is defined by the formula:

$$f_d = 2 \cdot f_0 \cdot V \cdot \cos\theta / c$$

where f_0 is the ultrasound frequency, V is the blood velocity, θ is the angle between the ultrasound beam and the bloodstream direction, and c is the velocity of ultrasound in tissue. The Doppler shift frequencies are within the range of audible sound. They can be analyzed, for example, by the fast Fourier transform and displayed as a Doppler shift spectrum (Figure 75-2). From the spectrum, the mean and the maximum velocity can be estimated and further processed.

Doppler ultrasound can be used in four modes: continuous-wave Doppler ultrasound (CW), pulsed-wave Doppler ultrasound (PW), color flow imaging, or power Doppler imaging. In the first mode, CW Doppler ultrasound is continuously transmitted by one piezoelectric crystal, and the reflected ultrasound is received by another crystal. Signals of blood flow are obtained from all vessels traversed by the ultrasound beam. The CW Doppler instruments are technically simpler than are the instruments of the other Doppler modes and thus are usually cheaper. However, the application of CW Doppler ultrasound is limited because of the lack of range resolution.

In the PW mode, one single piezoelectric crystal is used for transmission and reception of the ultrasound bursts. By changing the time delay between the transmission and reception of signals, it is possible to determine the range within the tissue from which the Doppler-shifted signals are received. In other words, it is possible to choose a specific vessel for recording of blood velocity signals. The PW ultrasound is usually combined with imaging ultrasound (linear array or sector real-time scanner) to locate and identify the vessel of interest.

The color flow imaging enables a color coding of the received Doppler signals, usually red for the blood flow toward the transducer and blue for the flow in the opposite direction. The color signals are then superimposed on the two-dimensional real-time image. Color flow imaging facilitates detection of flow even in very small vessels, for example, fetal cerebral vessels or maternal spiral arteries. It is often combined with PW Doppler ultrasound for quantification of flow velocity.

The amplitude of the received Doppler shift signals can be used to characterize the signal power that is proportional to the number of blood cells moving within the investigated area, thus reflecting the perfusion of examined organs. The power Doppler signals can also be color coded and displayed in the two-dimensional image. The power Doppler mode allows detection of blood flow with low velocity; however, the information is nondirectional. In obstetric applications, this mode has been used for evaluation of perfusion of fetal organs, for example, lungs[17] or placenta.[18] The power Doppler signals can also be collected from a tissue volume and presented in three dimensions. However, the three-dimensional display of flow has not yet been used for quantitative analyses.

Volume Flow Estimation

The information on the time-averaged mean velocity *(V)* obtained from a specific vessel and corrected for the cosine θ can be used for calculation of the volume flow *(Q)* in milliliters per minute according to the formula:

$$Q = V \cdot d^2 \cdot \pi / \cos\theta \cdot 4$$

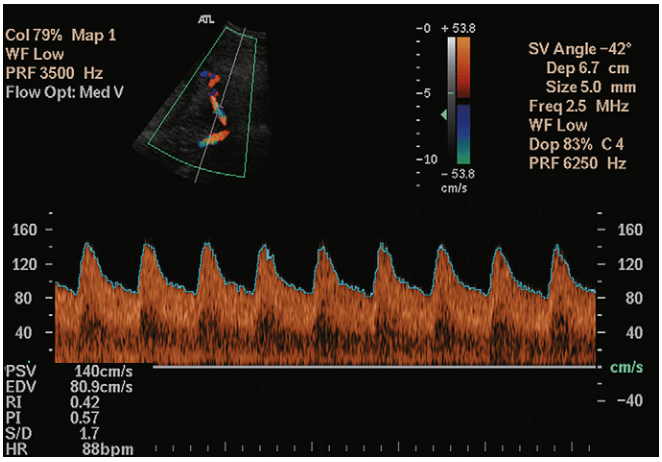


Figure 75-2 Doppler shift spectrum recorded from the uterine artery in a term pregnancy. The high proportion of diastolic flow velocity indicates a very low resistance to flow in the uteroplacental circulation.

This assumes that the diameter of the vessel *(d)* is known for the calculation of the cross-sectional area of the vessel. Originally, the method has been applied for estimations of volume flow in large fetal vessels, that is, abdominal aorta and umbilical vein.[12]

The measurement of vessel diameter using the two-dimensional ultrasound image is relatively inaccurate. The estimation of the volume blood flow also requires knowledge of the insonation angle and uniform insonation of the vessel for reliable estimation of the mean velocity. Because of all these possible sources of error, this method has not found wide application.[19] Nevertheless, it can be expected that further technical development will enable a more reliable estimation of flow. A certain revival of this method has occurred, and measurements of flow also in vessels of small caliber, for example, maternal uterine arteries and umbilical arteries, have been reported.[20,21] Nevertheless, the reported variability in the resulting values was of similar magnitude as in original reports 30 years ago and is, obviously, a combination of the physiologic variability and method errors.

Velocity Waveform Analysis

The maximum blood velocity (i.e., the envelope of the Doppler spectrum) recorded from an artery can be analyzed for its waveform and characterized by various indices (Figure 75-3). These indices are angle independent. The diameter of the vessel need not be known for waveform analysis, and the maximum velocity is easier to record than is the mean velocity. Thus a number of errors involved in the estimation of blood flow are eliminated. However, this method does not directly reflect flow, and the interpretation of results is not always obvious.

The diastolic part of the flow velocity waveform is mainly influenced by the peripheral vascular resistance; an increase in the resistance lowers the diastolic velocity and, consequently, increases the values of the waveform indices described in Figure 75-3. However, the waveform and its indices are also influenced by cardiac performance, blood pressure, vessel wall properties, and the viscosity of blood.

A simple semiquantitative evaluation of the waveform recorded from the umbilical artery or fetal descending aorta with regard to the presence or lack of the end-diastolic flow (blood flow classes) has proved useful in the identification of fetuses with growth restriction and fetuses at risk for intrauterine asphyxia[22] (Figure 75-4). In the cerebral vessels of hypoxic fetuses, an increase in

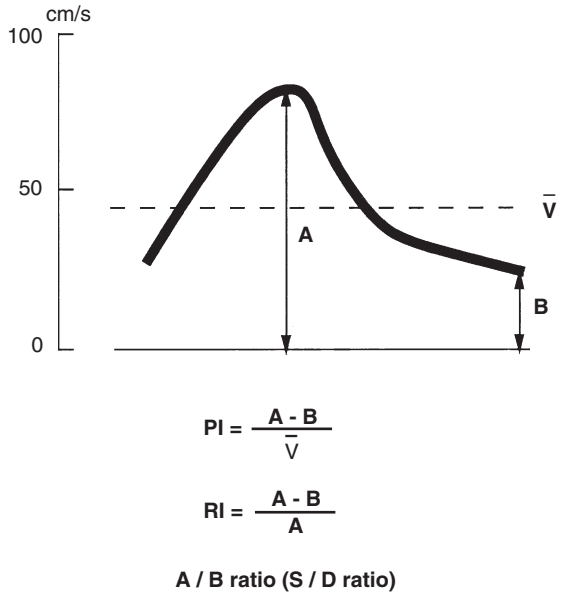


Figure 75-3 Schematic drawing demonstrating waveform analysis of the fetal arterial maximum blood velocity. *A (S),* Peak maximum velocity; *B (D),* minimum diastolic velocity; $\bar{V}$, mean velocity over the cardiac cycle; *PI,* pulsatility index according to Gosling and King[107]; *RI,* resistance index according to Pourcelot[108]; A/B ratio according to Stuart and colleagues.[109]

diastolic velocity can be observed as an expression of decreased resistance in the cerebral vascular bed and redistribution of blood flow (brain-sparing phenomenon).

The resistance to flow in the uterine artery is reflected in the pulsatility index (PI) of the uterine artery waveform. With increasing resistance, for example, in cases with intrauterine growth restriction and/or preeclampsia, the PI increases and a notch in the early diastole can occur. A bilateral notch in the uterine artery persisting after 26 weeks gestation has been shown to be associated with increased risk for development of preeclampsia and intrauterine growth restriction. For clinical use, a scoring system (uterine artery score) has been designed to characterize the resistance in both uterine arteries.[23]

SAFETY ASPECTS OF USING DOPPLER ULTRASOUND IN PREGNANCIES

Ultrasound with high intensity levels has known biologic effects, for example, thermal effects and cavitation. Therefore it is necessary that the users be constantly alert to the possibility of adverse effects of diagnostic ultrasound.[24] Hitherto, no harmful effects of ultrasound on human tissues have been found at the intensities used for diagnostic purposes, and epidemiologic follow-up studies have failed to elicit any evidence of adverse effects of exposure to diagnostic ultrasound in utero. Nevertheless, it is important that users of the Doppler ultrasound be aware of the output ultrasound energy of the equipment they are using for examining pregnancies. Some of the Doppler devices can produce considerable output energy exceeding the upper limit for

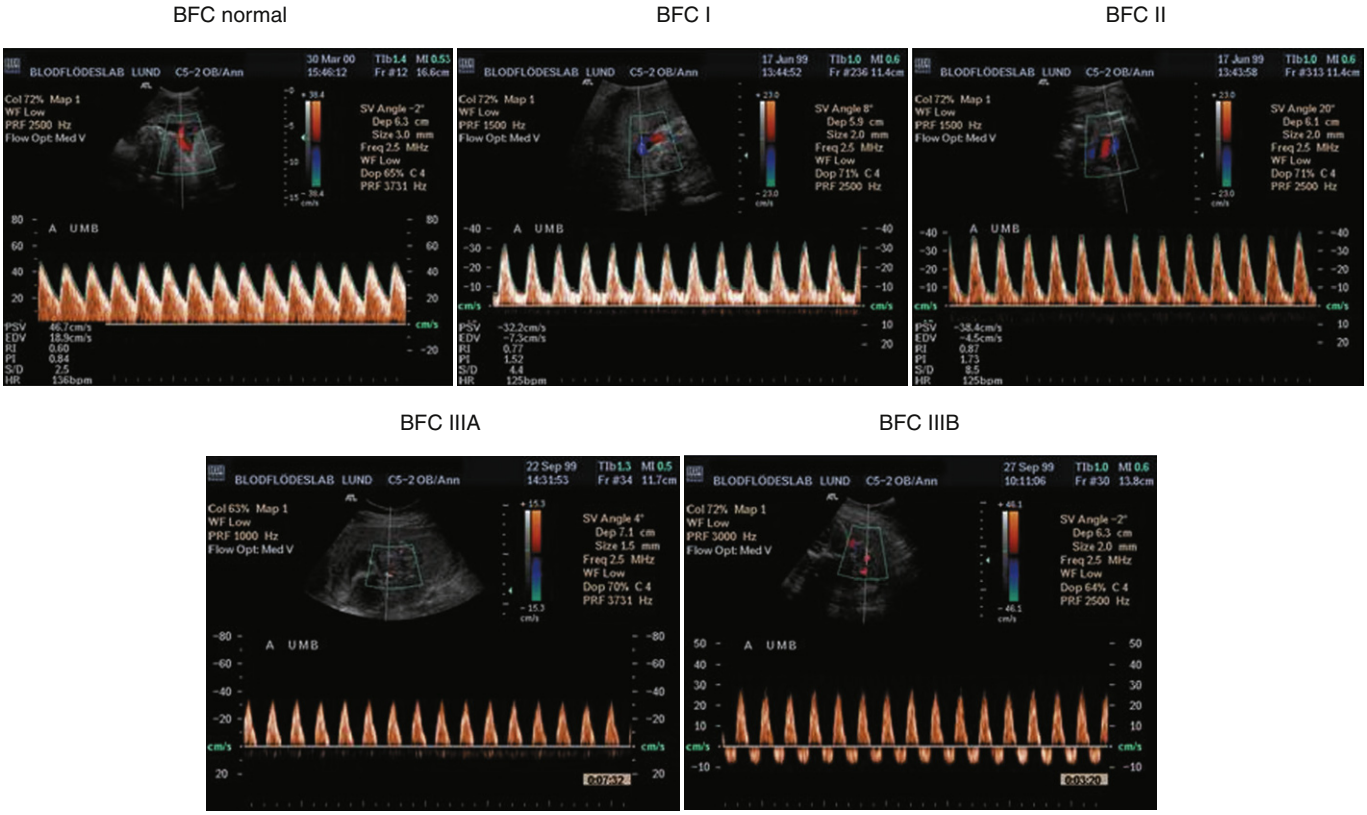


Figure 75-4 Blood flow classes (BFC) of the umbilical artery flow velocity waveforms. *BFC normal,* Positive diastolic flow, PI within normal limits; *BFC I,* positive diastolic flow, PI > mean + 2 SD and ≤ mean + 3 SD of the reference curve[110]; *BFC II,* positive diastolic flow, PI > mean + 3 SD of the reference curve; *BFC IIIA,* absent end-diastolic flow velocity; *BFC IIIB,* reverse end-diastolic velocity.

energy output to be used in pregnancy (720 mW/cm^2); it is the responsibility of the ultrasound operator to control the output on the basis of the output displays in the form of the mechanical index and thermal index.[25] The operator should always follow the ALARA principle, that is, to use ultrasound energy "as low as reasonably achievable."

HUMAN UTEROPLACENTAL BLOOD FLOW DURING LABOR

PW Doppler ultrasound has been used for recording of flow velocity signals from the uteroplacental vessels in labor.[26,27] CW Doppler ultrasound was also used for this purpose.[28,29] All Doppler studies on uteroplacental vessels in labor have shown that, during uterine contractions, both the systolic and diastolic blood velocities diminish, suggesting a decrease in blood flow. At the acme of contraction, the diastolic velocities disappear completely. An inverse linear relationship has been found between the intensity of the contraction, measured as the intrauterine amniotic pressure, and the degree of the end-diastolic flow. When intraamniotic pressure exceeds 60 mm Hg, an elimination of end-diastolic flow velocity occurs in all cases[30] (Figure 75-5).

A similar pattern of blood velocity changes as in spontaneous labor contractions was described for contractions induced by oxytocin challenge test (OCT).[31] At the time of the top of induced contractions the diastolic flow velocities in the uterine artery diminished, were undetectable, or even were reversed. The reversal was significantly more often seen during induced contractions as compared with spontaneous contractions in a control group.[31]

During the third stage of labor, PW Doppler studies of uterine arteries have shown that after a latent phase, during the contraction and detachment phase, a significant increase occurred in the resistance to flow reflected by an increase in PI.[32] After placental separation, a slight uterine relaxation occurred resulting in decreased resistance to flow in the uterine arteries. By using color flow imaging, the changes of blood flow between myometrium and placenta can be followed.[33] In cases with normal placental separation, blood flow between myometrium and placenta ceases immediately after delivery of the infant during the latent phase. In patients in whom manual removal of placenta was necessary, the blood flow continued into the placenta beyond the latent phase. Thus color Doppler examination might be used to diagnose placenta accreta.

HUMAN FETAL BLOOD FLOW DURING LABOR

Umbilical Artery Blood Flow

According to several reports, the waveform indices (PI and A/B ratio) of flow velocities recorded from the umbilical artery of healthy fetuses in uncomplicated labor do not change during contractions or with the progression of labor.[28,30,32-36] This suggests that normal uterine activity in labor does not increase the vascular resistance on the fetal side of the placenta, which helps the fetus to withstand the stress of labor. Fleischer and associates[37] found that the umbilical artery waveform did not change over a wide range of uterine pressures in labor. Conversely, Malcus and co-workers[38] examined 575 fetuses in early labor and found a significant increase in the PI during contraction, as compared with the recording taken before the onset of contraction. Nevertheless, the absolute difference in the PI was small and probably without importance for an undisturbed fetus. In fetuses with already compromised umbilical blood flow, however, such a slight increase in placental resistance might be of pathophysiologic and clinical importance. This is in agreement with the observation that fetuses that exhibit a lack of end-diastolic velocity during pregnancy often have signs of distress in labor and frequently require operative delivery.[22,39,40] In a study that recorded umbilical artery waveform changes continuously through different stages of labor, a significant increase in S/D ratio was observed during contractions in the second stage of labor.[41] The increase in S/D ratio correlated well with a decrease in umbilical artery pH. No significant changes in the umbilical artery velocity waveform were observed during the first stage of labor.

During Braxton Hicks contractions the PI in the umbilical artery did not change, and the authors concluded that Braxton Hicks contractions have no effect on fetal hemodynamics and on fetal oxygenation in the healthy near-term fetus.[42]

In pregnant women with decelerations in the fetal heart rate (FHR) during contractions in labor, a concomitant increase in the PI,[34] or S/D ratio,[43] has been found. Sakai and colleagues[44] have found that the increase in the umbilical artery resistance

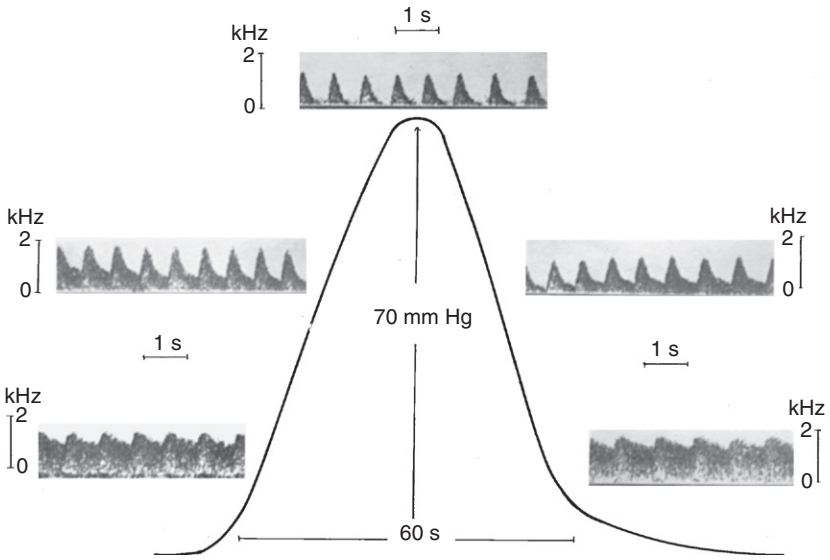


Figure 75-5 Changes of the Doppler velocity waveforms recorded from the uterine artery before, during, and after uterine contraction. *(From Fendel H, Fettweis P, Billet P, et al: Dopplеruntersuchungen des arteriellen utero-feto-plazentaren Blutflusses vor und während der Geburt,* Z Geburtshilfe Perinatol *191:121, 1987.)*

index (RI) was particularly pronounced at downward and bottom stages of variable decelerations. During early decelerations or during contractions not associated with FHR decelerations, no changes were found in the resistance to flow. Tadmor and colleagues,[45] using computerized analysis of umbilical artery velocity waveforms, recorded an increase in S/D ratio and PI in approximately 50% of FHR variable decelerations. They have shown that in one group of pregnancies the increase in the resistance to flow preceded the decrease in FHR. In other cases, the deceleration was not preceded by a measurable increase in umbilical artery resistance. The authors considered this to reflect impaired fetal oxygenation.

Operative delivery is significantly more frequent in patients with abnormal PI values. Brar and associates[46] compared the S/D ratio (see Figure 75-3) in a group of patients with late decelerations with the S/D ratio in matched controls: the mean S/D ratio in the former group was significantly higher than that in the latter group. Among women with late decelerations, a progressive increase also was seen in the incidence of adverse pregnancy outcome with increasing abnormality in the waveform. Patients with late decelerations and a normal umbilical S/D ratio had an incidence of adverse pregnancy outcome similar to that of controls. Murakami and co-workers[47] showed that the blood flow in the intraabdominal part of the umbilical vein decreased in association with late and variable FHR decelerations. The reduction of blood flow was more pronounced with variable than late decelerations, and the blood flow change preceded the deceleration.

Feinkind and colleagues[48] performed a similar analysis of the relationships among FHR tracings and umbilical artery velocity waveform during the first stage of labor and outcome of pregnancy in 273 unselected patients. Both the umbilical A/B ratio and the FHR had similar low positive predictive values (30% and 23%, respectively). If the two methods were combined, the predictive value increased to 56%. In the study by Somerset and colleagues[49] the positive predictive values for prediction of cesarean section for fetal distress were 31% for umbilical artery S/D ratio and 15% for admission FHR recording. The positive predictive value of the umbilical artery PI in early labor was also examined by Malcus and co-workers[38] and was found to be 19% for fetal distress and 15% for intrauterine growth restriction. Similar results have been subsequently reported by others.[50,51]

The rupture of membranes or amniotomy does not seem to have any influence on the umbilical velocity waveform.[36-38,52]

Li and colleagues[53] examined umbilical artery flow velocities during OCT performed in a group of high-risk pregnancies. In cases of positive OCT according to Freeman,[54] the umbilical artery PI increased significantly both during and between contractions induced by oxytocin.[54]

Umbilical artery Doppler velocimetry has been shown to be a clinically useful test when applied for antenatal monitoring of fetal health in high-risk pregnancies.[55] However, the clinical studies on umbilical artery velocimetry in labor reviewed by Farrell and colleagues[56] do not seem to provide similarly convincing results. Therefore this method has not found widespread clinical application in laboring patients.

Fetal Aortic Blood Flow

The fetal aortic volume blood flow, estimated on two occasions during the first stage of uncomplicated labor, has been shown to increase with progression of labor.[57] These measurements were performed between contractions, and the finding of increased fetal flow might be a phenomenon similar to reactive hyperemia in the uteroplacental circulation described in animals.[9] In the study by Lindblad and associates,[57] no change was found in the aortic PI with advancing labor, and no difference was seen between patients with and patients without ruptured membranes. Fendel and colleagues[30] measured the mean fetal aortic velocity in labor and found a drop in the velocity during contractions. The aortic PI and RI remained unchanged.

Fetal Cerebral Blood Flow

The flow velocity in cerebral arteries of fetuses during uncomplicated labor has been examined by using the transcervical access without imaging with either CW[58] or PW[59] Doppler ultrasound. Dougall and co-workers[58] did not find any change in the mean velocity or the RI of the anterior cerebral artery with advancing labor. During contractions, a fall occurred in the diastolic velocities, suggesting that an increase might exist in the cerebral vascular resistance caused by head compression during contractions (assuming that the fetal cardiac output and blood pressure remain unchanged). The mean RI was 0.69 between and 0.81 during contractions ($p < .001$). However, this finding was not confirmed by Maesel and colleagues[59] in the fetal middle cerebral artery.

More recently, several research groups have examined the fetal cerebral circulation in labor, using a combined two-dimensional real-time and PW Doppler ultrasound method. The waveforms of velocities recorded from the fetal internal carotid artery and middle cerebral artery were similar to those recorded before birth.[60,61] Furthermore, no significant difference was found in PI values of velocities recorded between and during uterine contractions.[61] In the late first and second stages of labor, lower values of PI were found in the middle cerebral artery, suggesting a decrease in impedance to flow in fetal cerebral circulation with progress of labor.[35,62] It has been shown that fetal cerebral oxygenation decreases in the second stage of labor,[63] which might lead to vasodilation of the cerebral vascular bed. This has been confirmed by Sütterlin and colleagues,[64] who have measured fetal oxygen saturation simultaneously with Doppler waveforms of the fetal middle cerebral artery. They have shown that, in fetuses with abnormal heart rate patterns and oxygen saturation <30%, the PI and RI of the middle cerebral artery were significantly lower than in control fetuses. More recently, these findings were confirmed.[65]

During uterine contractions in late labor, transient increases in the PI were observed in the fetal internal carotid artery[66] and middle cerebral artery[67] as an expression of higher intrauterine pressure, compared with that in early labor.

During the OCT of fetuses suspected of intrauterine growth restriction, the PI of fetal middle cerebral and anterior cerebral arteries decreased significantly if the PI was primarily within normal limits.[68] No difference was seen between the two vessels in the PI response. No PI decrease occurred in cases where the PI was below the normal lower limit, that is, if signs of so-called brain sparing were already seen before the OCT.

Fetal Venous Blood Flow

Fetal ductus venosus blood flow velocity waveforms during the first stage of labor have been described by Krapp and colleagues.[69] In a group of normal term fetuses, they found that between contractions the mean values of PI for veins and peak velocity index for veins were 0.48 (SD 0.19) and 0.44 (0.18), respectively. During contractions, the mean values increased to 1.66 (0.85) and 1.46 (0.65), respectively. The authors proposed Doppler examinations of ductus venosus in labor to be possibly useful in fetuses at risk. Szunyogh and colleagues,[70] who measured ductus venosus PI between contractions, reported that in term pregnancies with premature rupture of membranes the PI was significantly increased.

The pattern of umbilical venous flow velocities during labor was evaluated by Ghosh and co-workers.[71] They found the occurrence of pulsatile flow to be predictive of fetal hypoxia and operative delivery for fetal distress.

PHARMACOLOGIC EFFECTS ON UTEROPLACENTAL AND FETAL BLOOD FLOW IN LABOR

The possible effects on the fetal and uteroplacental circulation of drugs used in clinical obstetrics for treatment of preterm labor (tocolysis) or for obstetric analgesia in labor have been examined in a number of studies with use of various techniques. Ritodrine given to patients with preterm labor results in a significant decrease in the uterine and umbilical S/D ratio.[72] A concomitant increase in both the maternal heart rate and the FHR occurs, suggesting that the response of the S/D ratio might be secondary to the changes in the heart rate. In a study comparing the effects of nifedipine and ritodrine in preterm labor, no significant effect on umbilical artery Doppler velocimetry was reported for either of the two treatments.[73] On the contrary, Gokay and colleagues[74] reported a decrease in the umbilical artery PI during ritodrine infusion. In addition, they found a selective increase of left cardiac output indicating redistribution of fetal blood flow. Furthermore, an increase occurred in PI of the fetal middle cerebral artery. They commented that these changes might be of importance in preterm fetuses. In the study by Brar and co-workers,[72] magnesium sulfate tocolysis was not associated with any significant changes in the S/D ratio, the maternal heart rate, or the FHR. Keeley and colleagues[75] observed a decrease in the uterine artery PI and an increase in the fetal middle cerebral artery PI during the administration of magnesium sulfate. They interpreted these findings as a physiologic normalization process related to the stressed preterm fetus during labor.

Treatment of preterm labor with indomethacin has been shown to cause a constriction of the fetal ductus arteriosus in approximately 50%.[76] In these cases, Doppler recordings showed very high ductal blood flow velocities. After discontinuation of the indomethacin, normal function of the ductus arteriosus usually returned. No increase in the ductal flow velocity was observed when a selective cyclooxygenase-2 inhibitor (celecoxib) was used in a comparative study by Stika and colleagues.[77]

Tocolysis with an oral dose of 30 mg nifedipine, followed by an additional oral dose of 20 mg after 4 hours, did not influence either fetal or uteroplacental circulation.[78] This is in agreement with other reports on sublingual administration of nifedipine[79] and with the observation from a study using oral nifedipine in women with pregnancy-induced hypertension.[80] Two days after nifedipine maintenance tocolysis (80-120 mg nifedipine daily), a decrease in the uterine artery PI and fetal middle cerebral artery PI was found.[81]

Nitric oxide donors administered for treatment of preterm labor sublingually or transdermally did not influence the RI or PI in the umbilical artery and fetal middle cerebral artery, respectively, but they decrease the resistance in the uteroplacental circulation.[82,83]

After epidural analgesia, a rapid uptake of local anesthetics in the maternal circulation and a rapid transport to the fetus occur. Epidural analgesia for labor uncomplicated by hypotension is, however, not associated with any alterations in placental blood flow as measured by placental scintigraphy.[84,85] Fetal aortic blood flow in women receiving epidural analgesia during labor increases with advancing labor in a fashion similar to that in women without any obstetric analgesia. Furthermore, no signs of negative effects on the fetal circulation are seen.[57] Several Doppler studies have examined umbilical and uterine artery S/D ratios or RI in term parturients before and after establishing epidural block, and in none have any changes been found.[86-89] A similar observation was reported by Mires and colleagues[90] for women with normal pregnancy and uncomplicated labor. However, in women with pregnancy-induced hypertension, epidural analgesia led to a fall in maternal blood pressure and concomitant decrease in umbilical artery S/D ratio.[90] Oláh[91] studied the effects of an epidural top-up in labor and did not find any change in the umbilical artery PI. However, a transient increase in the uterine artery PI was observed, with a maximum at 15 minutes after the top-up. This change in uteroplacental circulation was preceded by an increase in the maternal femoral artery flow, probably causing a loss of circulating volume in the uterine circulation.

Pethidine crosses the placenta rapidly, and maximum concentrations are found in fetal scalp blood and umbilical arterial blood between 1 and 5 hours after an intramuscular injection in the mother.[92] After the intramuscular injection of 75 to 100 mg pethidine, the fetal aortic blood flow has been shown to decrease.[57]

Considerable maternal plasma and fetal scalp concentrations of local anesthetics have been found 20 to 30 minutes after paracervical block.[93] Reduced placental flow has been proposed as the cause of the fetal bradycardia that is sometimes observed after paracervical block.[94] However, no change in the intervillous blood flow[18] or in the uterine artery PI[95] has been observed after paracervical block. As long as no fetal bradycardia occurred, the umbilical artery PI remained unchanged.[95] In the study by Manninen and colleagues[96] paracervical block initiated a significant increase in uterine artery PI, suggesting a vasoconstrictive effect.

FETAL HEART RATE IN LABOR

Rhythmic contractions of the fetal heart are initiated by electric stimuli generated in the pacemaker, the sinus node. The rate of cardiac contractions is subject to autonomic central nervous influences, humoral factors, and the metabolic condition of the myocardium. The main determinants of FHR are the sympathetic and parasympathetic nervous systems, which are in continuous counteraction. This interplay causes changes in beat-to-beat intervals, expressed as heart rate variability. The variability also reflects the function of the fetal central nervous system and shows cyclic changes related to fetal behavioral states. The variability changes due to fetal behavior continue even during labor.[97]

During labor, an increase in fetal blood pressure (e.g., caused by a compression of umbilical cord) leads to bradycardia by initiating a vagal nerve reflex. Fetal hypoxemia can have a direct depressing effect on the function of the central nervous system and fetal myocardium, which can result in a decrease in, or even loss of, FHR variability. The complex effects of hypoxemia and developing acidemia on chemoreceptors of the fetus result in an increase of blood pressure and bradycardia.[98] During labor, signals of fetal heart action can be detected either transabdominally by using a Doppler ultrasound transducer or, after rupture of membranes, transcervically with a fetal scalp electrode. From the measured beat-to-beat intervals, FHR is calculated and recorded simultaneously with the signals of uterine activity as a cardiotocogram. Uterine activity is recorded either with an external tocodynamometer or with an intrauterine pressure catheter. Cardiotocography is widely used today as the preferred method for monitoring fetal health in labor.

FETAL ELECTROCARDIOGRAM IN LABOR

Fetal electrocardiogram (ECG) signals are easily obtained from the fetal scalp during labor. The early interest of researchers in revealing information on fetal myocardial function from the ECG was hampered by difficulties in obtaining signals of good quality and performing accurate waveform analysis.[99] Technologic developments have made it possible to design computerized techniques for improved isolation and analysis of fetal ECG signals.

In studies on animal fetuses, it has been shown that the ECG waveform is changed in a characteristic fashion with fetal hypoxia.[100,101] When the oxygen supply is insufficient to satisfy the energy needs of fetal myocardium, myocardial energy balance becomes negative, metabolism changes to anaerobic, and acidosis occurs. When the fetus compensates for hypoxia by

additional glycogenolysis, an elevation occurs in the ST segment of the waveform and an increase in the height of the T wave. This change can be quantified by calculating a ratio between the amplitude of the QRS complex and the T wave (T/QRS ratio), which in noncompromised fetuses does not exceed 0.25. In normal fetuses, a direct correlation exists between the PR interval and FHR. This relationship is inverted and the PR interval shortens[102] with fetal acidosis, which might enable distinction between FHR decelerations of various origins. The first studies in human fetuses during labor reported promising results, suggesting that changes in the fetal ECG waveform may be an early sign of fetal hypoxia.[103] The clinical usefulness of this new method of fetal surveillance during labor was demonstrated in two large randomized controlled trials.[104,105] In the Swedish trial comprising 4966 parturients, the cardiotocography plus automatic analysis of fetal ST-segment was shown to reduce both the cord artery metabolic acidosis rate and the rate of operative delivery for fetal distress.[105] In addition, it was shown that cardiotocography plus ST analysis provides information on intrapartum hypoxia, thus allowing for intervention in time and prevention of intrapartum asphyxia and neonatal encephalopathy.[106]

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76 Physiology of Resuscitation

Ola Didrik Saugstad

Of the approximately 130 million annual births globally, it has been estimated that 5 to 7 million newborn infants need some kind of intervention immediately after birth; of these, 1.3 million require more extensive resuscitation.[1-5] To perform an optimal resuscitation procedure, it is important to understand the physiologic events that occur as the fetus makes the transition from intrauterine to extrauterine life and the physiologic changes that occur during asphyxia and resuscitation.

It has been estimated that 50% to 70% of infants who require resuscitation at birth come from high-risk pregnancies, and therefore the need for resuscitation often can be predicted.[6] Nonetheless, at least 30% of babies who require resuscitation with endotracheal intubation come from apparently low-risk situations, a finding demonstrating the need for attendance by expert personnel after birth.

BASIC PHYSIOLOGIC CHANGES

Our basic knowledge of the physiologic changes during peripartum asphyxia and resuscitation comes from animal experiments in the early 1960s.[7] However, newer techniques, such as near-infrared spectroscopy and proton spectroscopy, have taught us more about the sequence of biochemical and physiologic changes during and after an asphyxial event.

The classic data from Dawes and associates from experimental studies with rhesus monkeys are presented in Figure 76-1.[4,5,7] This illustration represents the basis for our current understanding of the physiology related to asphyxia and resuscitation. These data also describe changes in pulse, respiration, and blood pressure and emphasize the difference between primary and secondary apnea.

In this model, rhesus monkeys were delivered by cesarean section, the umbilical cord was immediately tied, and the animals were prevented from breathing by immediately sealing their heads in a bag of saline solution. After a few shallow breathing movements, respiratory efforts stopped, and the animals developed primary apnea, which, in some cases, lasted for up to 10 minutes. However, after 1 to 2 minutes of primary apnea, gasping movements often started and progressed with increasing vigor and frequency. These respiratory efforts were accompanied by thrashing movements of the extremities. In this period, spontaneous ventilation could still be induced by appropriate sensory stimuli. The heart rate decreased from 200 beats/minute in the normal fetal rhesus monkeys to 100 beats/minute. After 4 to 5 minutes, a series of spontaneous deep gasps occurred that gradually became weaker until the last gasp after approximately 8 minutes. Cardiac activity continued for 10 minutes or more before cardiac arrest. The period between the last gasp and cardiac arrest is known as secondary or terminal apnea.

Blood pressure initially increased during primary apnea, but it then abruptly decreased and reached a plateau before declining during secondary apnea. The longer the period before the initiation of ventilation, the greater was the delay until the animal took its first spontaneous gasp. For every minute of delay in establishing assisted ventilation, the time to first gasp was delayed by 2 minutes, and the time to spontaneous rhythmic ventilation was delayed by 4 minutes (Figure 76-2).[8]

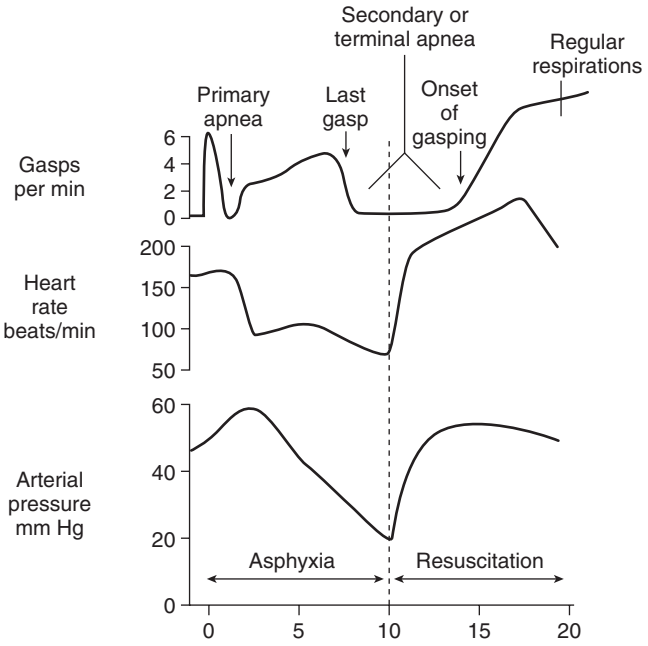


Figure 76-1 The relation between heart rate, blood pressure, and respiratory efforts in rhesus monkeys during asphyxia and resuscitation. *(From Dawes GS, Jacobson HN, Mott JC, et al: The treatment of asphyxiated, mature foetal lambs and rhesus monkeys with intravenous glucose and sodium carbonate,* J Physiol (Lond) *169:167, 1963.)*

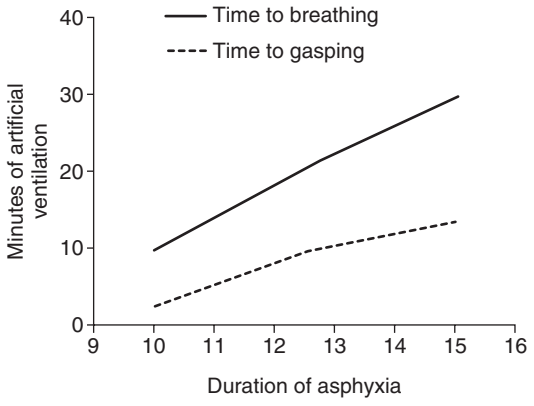


Figure 76-2 The relation between the duration of asphyxia and artificial ventilation until the first gasp and breath are detected. *(Data from Adamsons K Jr, Behrman R, Dawes GS, et al: Resuscitation by positive pressure ventilation and tris-hydroxymethylaminomethane of rhesus monkeys asphyxiated at birth,* J Pediatr *65:807, 1964.)*

TABLE 76-1

Changes in Apgar Scores during the First 10 Minutes of Life

1-min Apgar	5-min Apgar	10-min Apgar
4 (1-7)	7 (3-9)	8 (4-9)
40%*	6%*	3.4%*
94%†	29%†	13%†

Values are median (5th to 95th percentile) Apgar scores in 600 newly born infants needing resuscitation.
*Percentage with Apgar score <4.
†Percentage with Apgar score <7.

During severe asphyxia, the pH drops from 7.30 to 6.80 within 5 minutes, which is primarily the result of an increase in arterial carbon dioxide tension of 10 mm Hg (1.3 kPa) per minute. By the end of secondary apnea, arterial carbon dioxide tension can exceed 100 mm Hg (>13 kPa), and serum potassium level can rise to 15 mmol/L or more.[4]

The physiologic changes that occur with asphyxia and resuscitation were also assessed in a newborn piglet model in which hypoxia was induced by exposure to 8% oxygen (O_2), followed by 20 minutes of active resuscitation with different O_2 concentrations.[9-11] Although this model differs in several ways from human intrauterine asphyxia, it likely describes general principles in the physiologic response to neonatal asphyxia and reoxygenation. In this model, arterial oxygen tension (Pa_{O_2}) typically decreases to 20 to 30 mm Hg (3 to 4 kPa) within 5 minutes, with severe hypotension and lactic acidosis.[9-11]

Another model includes repetitive episodes of intrauterine hypoxia in animals. This causes global neuronal, cortical, midbrain, and cerebellar damage. Damage to the watershed areas between the areas of blood vessels supplying the cerebral cortex is commonly observed.[4] This is an important model because the human fetus may have recurrent episodes of acute, total, or partial asphyxia before the start of labor, with recovery of biochemical abnormalities. Thus severe asphyxial damage may occur without a decrease in fetal pH, and the child may have a normal Apgar score after delivery.[4]

RESPONSE TO RESUSCITATION

A newborn infant who does not breathe may have had birth asphyxia. However, apnea can also be caused by central nervous system depression resulting from maternal drugs, central nervous system injury, septicemia, anemia, primary muscular or neurologic disease, or congenital malformations obstructing the airways. Premature infants may not initiate respirations at birth because of muscular weakness or poorly compliant lungs.[4] These babies are often in need of immediate intervention, and a great need exists for a quick and easy way to assess the response of the resuscitative efforts.

Apgar Scoring

The Apgar scoring system is not the best means to assess the acute response of resuscitation, because it is taken first at 1 minute and then at 5 minutes. Moreover, most babies with an umbilical cord pH lower than 7.10 have normal Apgar scores; only when the pH is less than 7.00 are low Apgar scores commonly observed.[12] The five components of the Apgar scoring system (heart rate, respiration, muscle tone, reflex responsiveness, color) are indicators of different physiologic responses and therefore carry different significance. Heart rate and respiration are obviously more important than reflex responsiveness. Muscle tone represents higher cerebral function, whereas the other components, including heart rate, respiration, and reflex responsiveness, are brain stem responses.[4] Color is dependent on both respiration and heart rate. Rapid achievement of higher Apgar scores has, traditionally, been considered the hallmark of a successful resuscitation, and persistent low Apgar scores may be related to poor prognosis.[13,14]

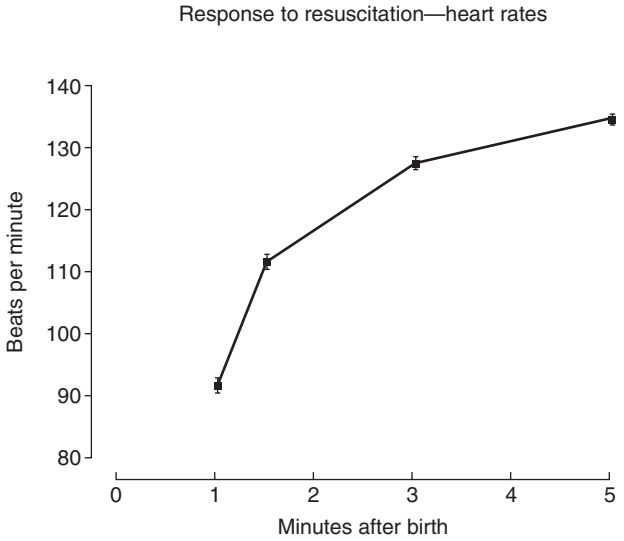


Figure 76-3 Heart rate development in newborn infants requiring resuscitation at birth. *(Data from Saugstad OD, Ramji S, Rootwelt T, Vento M. Response to resuscitation of the newborn: early prognostic variables,* Acta Paediatr *94:890, 2005.)*

In a clinical study in 600 newborn infants who required resuscitation at birth because of bradycardia (heart rate <80 beats/minute) or apnea, the median 1-minute Apgar score increased from 4 to 8 within 10 minutes.[15] Table 76-1 shows the median Apgar scores in this population and the percentage with an Apgar score of less than 4 and less than 7, respectively. However, a controversy has arisen over whether resuscitation with room air is preferable, and in one study the Apgar score was, in fact, higher in those resuscitated with room air compared with pure O_2.[15]

Heart Rate

The best way to assess the response to resuscitation is to count the pulse. This can be done by listening to the heart by stethoscope or by palpating the pulse at the base of the umbilical cord. In infants with a 1-minute Apgar score higher than 6, the median heart rate at 1 minute was more than 130 beats/minute. Figure 76-3 shows heart rate responses, between 1 and 10 minutes of life, in a population of 600 newborn infants who required resuscitation.[15] Median heart rate was restored from approximately 90 to 114 beats/minute at age 60 to 90 seconds. From 3 minutes onward, heart rate remained stable (>130 beats/minute). The lower the Apgar score, the lower was the initial heart rate. When the Apgar score was less than 4 at 1 minute, the median heart rate was 80 beats/minute at 60 seconds and 100 beats/minute after 90 seconds. However, after 3 minutes, the median heart rate returned to a normal range. Table 76-2 shows median heart rates the first 10 minutes of life related to Apgar score at 1 minute.

In the population noted earlier, only 1.7% had a heart rate lower than 60 beats/minute after 3 minutes of resuscitation, and 1% after 5 minutes. In patients with persistent bradycardia, chest compressions are recommended, according to the current resuscitation guidelines of the American Heart Association, the American Academy of Pediatrics, and the International Liaison Committee on Resuscitation.[16,17] In the study population described earlier, chest compressions were performed in nearly 20% of cases, even though this approach was indicated in only

TABLE 76-2

Heart Rate and Arterial Oxygen Saturation in Relation to 1-minute Apgar Score

	Min	1-min Apgar ≥7	1-min Apgar <7	1-min Apgar <4
HR	1	136 (85-156)	86 (40-140)	80 (10-131)
	1.5	140 (110-156)	110 (60-150)	100 (50-158)
	3	142 (120-167)	130 (80-163)	128 (69-160)
	5	142 (136-164)	140 (100-168)	139 (80-168)
	10	142 (120-169)	140 (110-170)	140 (100-174)
Sa_{O_2}	1	75 (57-82)	65 (40-82)	60 (40-81)
		57/79	*67*/60	*65*/58
	3	90 (65-98)	85 (59-92)	80 (41-94)
		65/90	*85*/85	*82*/78
	5	93 (84-98)	90 (68-95)	87 (62-96)
		86/95	*90*/90	*87*/85
	10	94 (88-96)	92 (80-97)	90 (72-97)
		88/95	*92*/90	*90*/90

Values are median and 5th to 95th percentiles for heart rate (HR, beats/minute) and arterial oxygen saturation (Sa_{O_2}, %) according to 1-minute Apgar score the first 10 minutes after birth in 600 newborn infants requiring resuscitation at birth. Median values for Sa_{O_2} are given separately also for *room air*/100% O_2 resuscitated, respectively. (From Saugstad OD, Rootwelt T, Aalen O: Resuscitation of asphyxiated newborn infants with room air or oxygen: an international controlled trial: the Resair 2 study, *Pediatrics* 102:1, 1998.)

1% to 2% of the infants, a finding suggesting an inappropriate and overly aggressive approach in many cases.

Time to First Breath

The time to first breath and first cry (and to a normalization of the breathing pattern) is another simple means to assess the response to resuscitation. It has been reported that time to first breath is approximately 10 seconds in healthy term infants after vaginal delivery and 15 seconds after cesarean section.[18]

In the population of 600 infants noted previously, those with 1-minute Apgar score higher than 6 took their first breath at a median time of 42 seconds. No differences were seen between term infants and preterm infants (>1000 g birth weight). Those resuscitated with ambient air took their first breath and cried significantly earlier than infants given pure O_2.[15] In term babies, median time to first breath increased to 90 seconds when the 1-minute Apgar score was <7 and 120 seconds if the 1-minute Apgar score was <4. Time to first breath was approximately three times longer when the heart rate at 1 minute was <60 versus 80 beats/minute. Table 76-3 shows the median time to first breath and cry related to 1-minute Apgar scores and heart rates at 1 minute. Data for room air, as well as infants resuscitated with 100% O_2, are given.

In another series of normal babies, the mean time to first audible cry was 36 seconds (with a SD of 24 seconds). In asphyxiated babies needing resuscitation, first cry occurred in mean at 72 seconds with room air resuscitation and at 102 seconds for those resuscitated with pure O_2. The mean time to establish a regular, spontaneous respiratory pattern with effective respiratory movements (allowing a maintenance of heart rate and arterial O_2 saturation [Sa_{O_2}] greater than 90%) was less than 1 minute in normal, nonasphyxiated term infants, 4.6 minutes with room air resuscitation, and 6.6 minutes for infants resuscitated with 100% O_2.[19,20] Therefore, as in studies in animals, pure O_2 inhibits respiratory activity in the newborn infant.[21,22]

Oxygen Saturation

Another means to assess the response to resuscitation is to follow Sa_{O_2} by pulse oximetry. Normal Sa_{O_2} values for term infants during the first 10 minutes after birth have been reported.[23]

TABLE 76-3

Time to First Breath and First Cry in Relation to 1-minute Heart Rate and Apgar Score

	1-min HR ≥80	1-min HR <80	1-min HR <60	1-min Apgar >6	1-min Apgar <4
First breath	1.0 (0.25-5.0) *1.0*/1.33	1.5 (0.25-27.4) *1.5*/2.0	3.0 (1.0-44) *2.5*/3.0	0.75 (0.25-2.1) *0.64*/0.75	2.0 (0.6-30) *2.0*/2.5
First cry	2.0 (0.5-14.9) *1.5*/2.0	2.1 (0.5-1032) *1.8*/3.0	3.5 (1.5->) *3.0*/4.3	1.0 (0.5-2.4) *1.0*/1.0	3.5 (1.0->) *3.0*/4.0

Values are median and 5th to 95th percentile time in minutes after birth to first breath and cry according to heart rate (HR, beats/min) at 1 minute after birth in 600 newly born infants requiring resuscitation at birth. Median values are given also for *room air*/100% O_2 resuscitated, respectively, >, An upper limit could not be established based on these data. (From Saugstad OD, Rootwelt T, Aalen O: Resuscitation of asphyxiated newborn infants with room air or oxygen: an international controlled trial: the Resair 2 study, *Pediatrics* 102:1, 1998.)

TABLE 76-4

Changes in Arterial Oxygen Saturation According to 1-minute Heart Rate

Min	1-min HR ≥80	1-min HR <80	1-min HR <60
1	70 (39-82)	60 (40-75)	45 (40-99)
3	85 (41-94)	85 (60-93)	76 (60-94)
5	90 (72-96)	90 (69-95)	80 (60-93)
10	93 (70-97)	90 (80-97)	90 (76-99)

Values are median Sa_{O_2}, % (5th to 95th percentiles) in the first 10 minutes of life according to heart rate (HR, in beats/min) at 1 minute after birth in newly born infants requiring resuscitation at birth. (From Saugstad OD, Rootwelt T, Aalen O: Resuscitation of asphyxiated newborn infants with room air or oxygen: an international controlled trial: the Resair 2 study, *Pediatrics* 102:1, 1998.)

For infants with a 1-minute Apgar score higher than 6, the median Sa_{O_2} value increased from 75% (at 1 minute) to 90% after 3 minutes and to 93% and 94% after 5 and 10 minutes, respectively. When the 1-minute Apgar score was less than 4, Sa_{O_2} values increased from 60% at 1 minute to 80% at 3 minutes and to 87% and 90% at 5 and 10 minutes of life, respectively (see Table 76-2). Surprisingly, the Sa_{O_2} was identical with use of either room air or 100% O_2 for resuscitation—paradoxically, with a tendency to higher Sa_{O_2} in room air–resuscitated infants. However, in children with a 1-minute Apgar score >6, Sa_{O_2} was significantly higher in those given 100% O_2 (90% vs. 75% at 3 minutes and 95% vs. 86% at 5 minutes, with no differences at 10 minutes). Supplemental O_2 therefore does not elevate Sa_{O_2} faster than room air during the first 10 minutes of life in depressed infants, but it does in nondepressed infants. Table 76-4 shows the development in Sa_{O_2} during the first 10 minutes of life according to 1-minute Apgar score. Figure 76-4 depicts the changes in Sa_{O_2} in asphyxiated and nonasphyxiated term infants during the first 10 minutes of life.[24]

METABOLIC CHANGES

The changes in base deficit (BD) during hypoxia and reoxygenation in newborn piglets are shown in Figure 76-5.[11] The increase in BD exhibited a linear relationship with the duration of hypoxia. Hypoxia induced by the breathing of 8% O_2 resulted in a rise in BD of 0.4 mmol/L/minute compared with a rate rise of 0.67 mmol/L/minute when animals breathed 6% O_2. During reoxygenation, BD continued to increase or plateau during the first 5 minutes or so, most likely because of vasodilatation in peripheral tissues with release and washout of lactic acid and

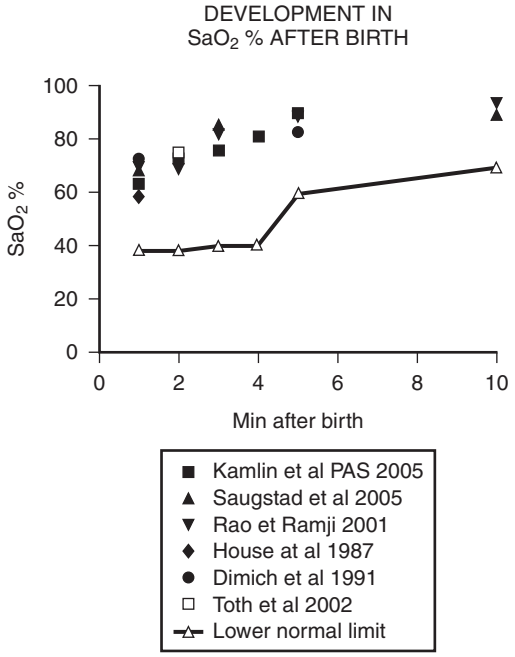


Figure 76-4 Development in arterial oxygen saturation in nonasphyxiated newborn infants. *(From Saugstad OD: Oxygen saturations immediately after birth,* J Pediatr *148:569, 2006.)*

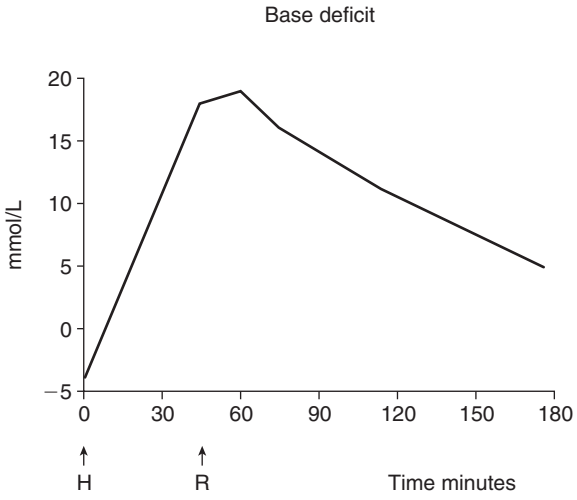


Figure 76-5 Development in base deficit in newborn piglets during hypoxemia *(H)* (inspired oxygen fraction, 0.08) and during reoxygenation *(R). (Data from Medbo S, Yu XQ, Asberg A, Saugstad OD: Pulmonary hemodynamics and plasma endothelin-1 during hypoxemia and reoxygenation with room air or 100% oxygen in a piglet model,* Pediatr Res *44:843, 1998.)*

other metabolites. This phase was followed by a steady decrease of BD with a typical rate of approximately 0.17 mmol/L/minute between 30 and 60 minutes and 0.12 mmol/L/minute between 60 and 90 minutes after onset of reoxygenation.[10-12] These data indicate that, depending on the severity and duration of the hypoxemia, it takes 2.5 to 4 times the duration of hypoxia to normalize BD. A similar pattern and relationship have been observed for arterial hypoxanthine.

Figure 76-6 shows the changes in heart rate compared with BD in newborn infants requiring resuscitation.[25] The decrease of BD

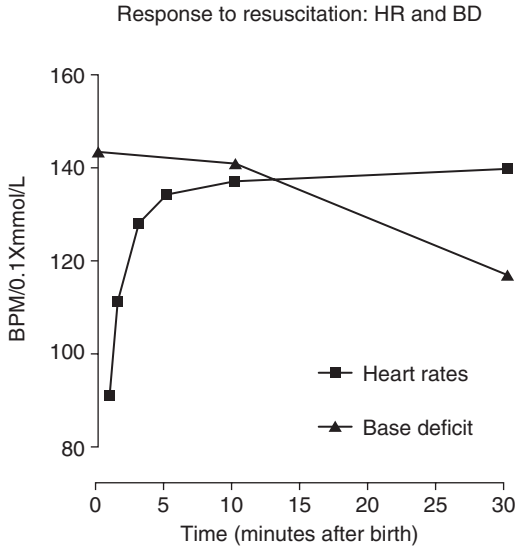


Figure 76-6 The change in heart rate *(HR)* compared with base deficit *(BD)* in newborn infants requiring resuscitation. *(From Saugstad OD: Resuscitation with room-air or oxygen supplementation,* Clin Perinatol *25:741, 1998.)*

in human infants follows the same pattern and rate as in piglets, decreasing 6 to 7 mmol/L/hour. It can be inferred from this figure that heart rate changes represent a rapid response to successful resuscitation, whereas metabolic changes are slower indicators of the success of resuscitation.

In the brain, excitatory amino acids such as glutamate and aspartate increase in the striatum during hypoxemia, and the concentrations double during the first minutes of resuscitation before a rapid decline. Normalization is reached within 15 minutes after start of resuscitation, but a secondary peak in these amino acids may be found several hours after asphyxia.[26]

The concentration of nitric oxide decreases in the brain during hypoxia and increases rapidly during reoxygenation, exceeding basal levels by approximately 40% (more so in infants resuscitated with 100% O_2 than in those resuscitated with room air).[27] Hydrogen peroxide in lymphocytes in the sagittal sinus also increases during reoxygenation, although not when room air resuscitation is used.[28]

REGULATION OF BREATHING

Breathing in fetal life is controlled by several factors. It is inhibited at the brain stem level by humoral factors such as endogenous opioids, prostaglandins, and adenosine, and it is stimulated by carbon dioxide, during active sleep. Hypoxemia completely inhibits breathing. During parturition, breathing movements are completely inhibited; however, severe asphyxia can induce gasping movements, resulting in the aspiration of meconium during delivery.[29]

The first breaths normally are vigorous, a finding suggesting a strong respiratory drive. Breathing is triggered by numerous mechanisms, such as removal of placental humoral inhibitory factors, cooling, catecholamine surge induction of genes encoding for substances important for breathing (e.g., substance P), and rising carbon dioxide concentrations. Because the set point for Pao_2 oxygen tension is relatively low after birth, it seems counterintuitive that a high O_2 concentration during resuscitation would delay the time to first breath. It takes 2 to 3 days until the set point of the peripheral chemoreceptors is increased to adult levels.[29]

EFFECTS OF FIRST INFLATIONS

Insufflation Pressure

The insufflation pressure needed to open the lungs after birth varies, but an opening pressure greater than 10 cm H_2O is rarely needed. In most babies, air enters the lungs as soon as the intrathoracic pressure begins to fall. In more than 80% of newborn infants needing artificial ventilation, this is satisfactorily achieved by bag and mask.[30]

When resuscitation is performed, one should use only the minimal pressure needed to achieve small chest wall excursions. Generally, peak intrathoracic pressures of 20 to 30 cm H_2O are sufficient. However, an occasional infant may require pressures of 40 to 50 cm H_2O if air entry is poor.[31]

When positive pressure ventilation is applied to intubated newborn babies in need of ventilatory support immediately after birth, various reactions have been described. The most common response to lung inflation is the generation of a large positive intrathoracic (intraesophageal) pressure (≤82 mm Hg, 11 kPa). Head's paradoxical reflex is also commonly seen during bag and mask ventilation. This means that the inflation triggers an inspiratory effort, creating a negative esophageal pressure; levels up to 48 cm H_2O have been described. Such inspiratory efforts often produce a fall in inflation pressure. Occasionally, no change in esophageal pressure is observed.[32,33]

Poor Efficacy of Bag and Mask Ventilation

The efficacy of face mask respiration has been questioned. Tidal volume exchange can be less than one third of that noted with endotracheal intubation. Even when very high pressure is used, (≤40 cm H_2O) mean tidal exchange is less than in babies with endotracheal intubation. Face mask ventilation is helpful in establishing a functional residual capacity. Figure 76-7 shows expiratory tidal exchange with the first three breaths in infants receiving ventilation by face mask versus those who had intubation. For the first breath, expiratory tidal exchange by face mask is only one fifth of that measured in infants with endotracheal intubation.[34]

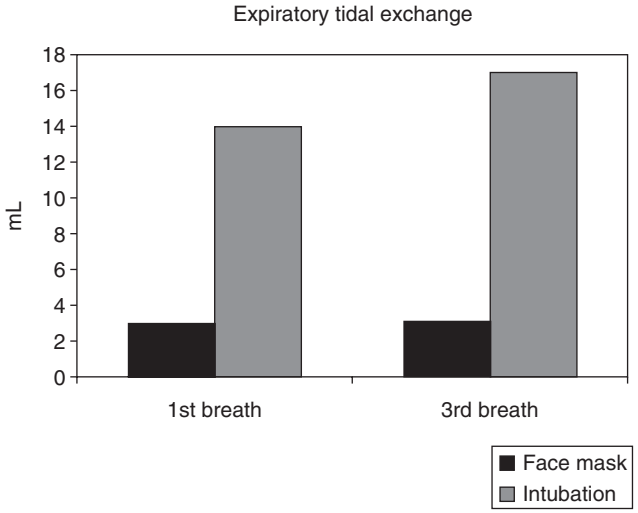


Figure 76-7 Tidal exchange in the first three breaths in term newborn infants receiving ventilation with bag and mask versus intubation. *(Data from Milner AD, Vyas H, Hopkin IE: Efficacy of facemask resuscitation at birth,* BMJ *289:1563, 1984.)*

First Inflations in Premature Infants

The patterns described previously are probably not relevant for premature infants. In the smallest babies, heart rate and blood pressure quickly decline with the onset of hypoxemia. Furthermore, the response to resuscitative efforts in the preterm infant is affected by the state of surfactant sufficiency or deficiency. A few manual breaths with a high tidal volume (30 to 40 mL/kg) in premature, surfactant-deficient newborn lambs can induce lung injury. Large inflations at birth also cause an uneven surfactant distribution and may compromise the effect of surfactant. Therefore it is extremely important to initiate ventilation gently in these babies.[35] In newborn infants less than 29 weeks gestational age who received surfactant before the first breath, the median inspiratory pressure needed was 20 cm H_2O. Fewer than one fifth of these infants needed more than 20 cm H_2O, and none needed more than 30 cm H_2O. The use of high inspiratory pressures may contribute to an increased risk of chronic lung disease.[35-37]

HEMODYNAMIC CHANGES

Systemic Blood Flow and Myocardial Function

Blood pressure follows a typical pattern during hypoxia and resuscitation, as described by Dawes and colleagues.[7] In the newborn piglet model, hypoxia increases mean arterial blood pressure initially from basal levels of approximately 70 mm Hg before it starts to fall[9-11] (Figure 76-8). During resuscitation with either

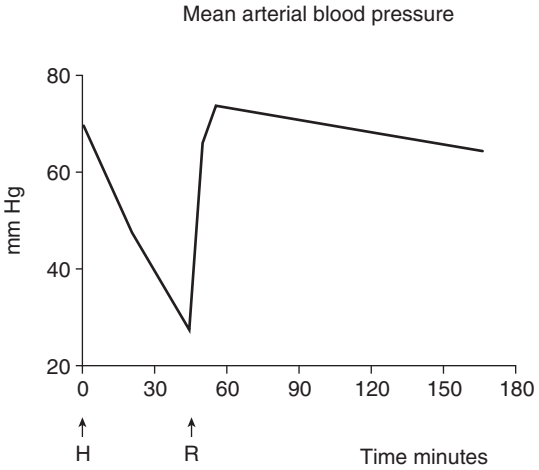


Figure 76-8 Change in blood pressure in newborn piglets exposed to hypoxia *(H)* and reoxygenation *(R). (Data from Medbo S, Yu XQ, Asberg A, Saugstad OD: Pulmonary hemodynamics and plasma endothelin-1 during hypoxemia and reoxygenation with room air or 100% oxygen in a piglet model,* Pediatr Res *44:843, 1998.)*

room air or 100% O_2, a rapid restoration of blood pressure was observed within 5 to 10 minutes; however, a secondary decline in blood pressure often occurred.[11] In this model, 2 hours after reoxygenation was initiated, some piglets needed inotropic support because of myocardial insufficiency, and markers of myocardial damage were found.[38]

Cardiac output follows a pattern similar to that of arterial blood pressure, with a 50% to 60% initial increase followed by a rapid decline, approximately 20 minutes after hypoxemia occurs. At the start of resuscitation, cardiac output was only approximately 50% of basal levels. However, resuscitation restored cardiac output quickly, and it reached levels approximately 30% higher than the basal levels (approximately 180 mL/kg/minute). Subsequently, a relatively rapid decline was observed, to 30% lower than basal levels 2 hours after start of resuscitation. Relatively speaking, the blood pressure is maintained better than cardiac output during

this period.[11] Systemic vascular resistance decreased by 25% to 50% during hypoxemia and increased to 75% to 90% of the basal level within 5 to 10 minutes after start of resuscitation.[11]

Pulmonary Blood Flow

Pulmonary artery pressure and vascular resistance also follow a biphasic pattern during hypoxia and reoxygenation, with a rapid increase in pulmonary vascular resistance during the first few minutes of hypoxemia, followed by a slower increase until a peak is reached at 50% higher than basal levels.[11] Pulmonary arterial pressure declines in parallel with cardiac output until the start of resuscitation. At that point, a rapid restoration of pulmonary arterial pressure is observed, sometimes even to higher than basal values (Figure 76-9). Within 2 to 3 hours, normalization of the pulmonary arterial pressure is found. However, in newborn infants who have inadequate responses to resuscitation, pulmonary arterial pressure may not decrease.

Pulmonary vascular resistance (Figure 76-10) immediately rises (approximately threefold) from basal values during hypoxia, but it returns to basal levels within 5 minutes of resuscitation in piglets. In this model, the pulmonary vascular resistance does not differ whether 21% or 100% O_2 is used during resuscitation.[11]

Cerebral Blood Flow

Cerebral blood flow in newborn piglets, as measured by radiolabeled microspheres, increases initially during hypoxemia, but then it declines with persistent hypoxia. During successful resuscitation, cerebral blood flow is quickly restored.[39] Blood flow in the microcirculation quickly falls to zero both in the cortex and in the striatum of hypoxic newborn piglets.[27,28,40] During resuscitation, blood flow in the cerebral circulation is restored; however, it can take substantial time, and sometimes it never reaches baseline. Resuscitation with 100% O_2 in piglets restored cerebral blood flow more efficiently than 21% O_2 when normocapnia was maintained. However, when resuscitation was performed in moderately hypercapnic animals, this difference was diminished.[40]

Premature infants randomly assigned to receive either 80% or 21% O_2 a few minutes after birth had a 20% reduction in cerebral blood flow in 80% O_2 compared with babies breathing 21% O_2.[41] It is still unknown whether rapid or slower restoration of cerebral blood flow is best for the preterm infant. However, restoration of cerebral blood flow that is too slow may not be beneficial.

BRAIN OXYGENATION

Brain oxygenation can be assessed experimentally by continuous measurements with a Po_2 electrode in the brain region of interest. Clinically, near-infrared spectroscopy can be used. During hypoxia, brain Po_2 quickly falls close to zero and is gradually restored during reoxygenation.

Near-infrared spectroscopy measurements in newborn piglets showed that the so-called *oxygenation index* (change in oxygenated hemoglobin minus change in deoxygenated hemoglobin) rapidly declines to zero; however, 5 minutes after the start of reoxygenation, it is higher than baseline with use of 21% or 100% O_2. The oxygenation index is significantly higher with 100% O_2 than with room air resuscitation.[42]

After the onset of hypoxemia in newborn piglets, the Pao_2 in the sagittal sinus dropped from 40 mm Hg to zero. After reoxygenation, an overshoot was found during the first 15 to 30 minutes, more so in animals reoxygenated with 100% O_2 than in those reoxygenated with 21% O_2. Sao_2 values in the sagittal sinus also dropped to zero during hypoxemia (from an initial level of 50%) and rose to 70% and 90%, respectively, with use of 21% and 100% O_2 for reoxygenation.[42]

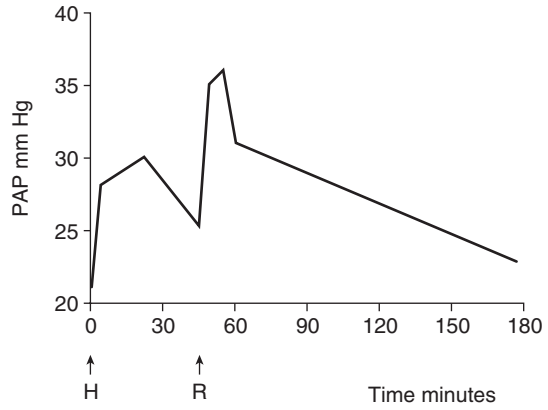


Figure 76-9 Change in pulmonary arterial blood pressure *(PAP)* in newborn piglets exposed to hypoxia *(H)* and reoxygenation *(R). (Data from Medbo S, Yu XQ, Asberg A, Saugstad OD: Pulmonary hemodynamics and plasma endothelin-1 during hypoxemia and reoxygenation with room air or 100% oxygen in a piglet model,* Pediatr Res *44:843, 1998.)*

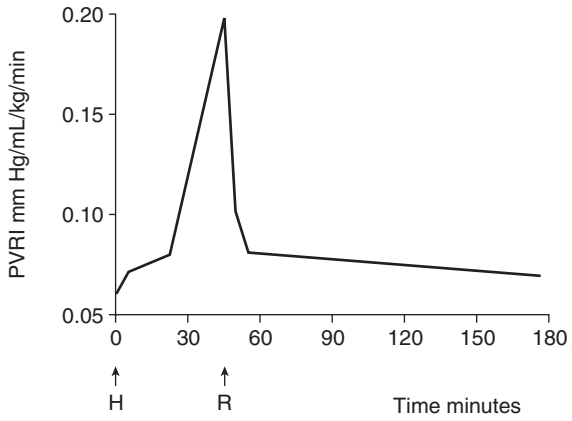


Figure 76-10 Change in pulmonary vascular resistance *(PVRI)* in newborn piglets exposed to hypoxia *(H)* and reoxygenation *(R). (Data from Medbo S, Yu XQ, Asberg A, Saugstad OD: Pulmonary hemodynamics and plasma endothelin-1 during hypoxemia and reoxygenation with room air or 100% oxygen in a piglet model,* Pediatr Res *44:843, 1998.)*

Using near-infrared spectroscopy, several investigations showed that cerebral blood volume increases markedly during hypoxia.[42,43] The blood volume of the brain continues to increase after initiation of resuscitation, and it reaches a maximum at 5 to 10 minutes, returning to baseline values after approximately 1 to 2 hours.

CEREBRAL ELECTROPHYSIOLOGIC CHANGES

The electroencephalogram (EEG) becomes isoelectric within approximately 20 minutes in newborn piglets breathing 8% O_2, but variation is great, with the 25th to the 75th percentile ranging from 7 to 37 minutes. The EEG waves reappear during resuscitation within a median time of 2 minutes in animals resuscitated

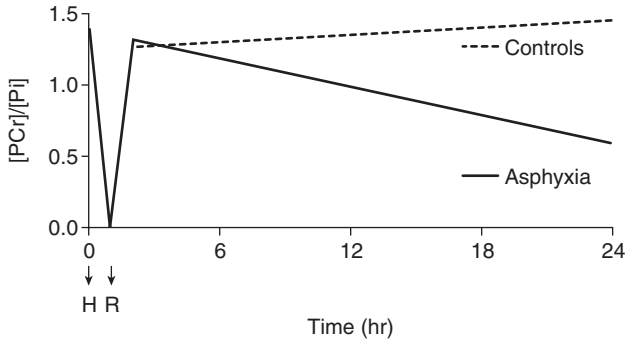


Figure 76-11 The ratio between phosphocreatinine *(Pcr)* and inorganic phosphate *(Pi)* in the newborn piglet brain assessed by proton spectroscopy during asphyxia and the postasphyxic period illustrating primary and secondary energy failure. *H,* Hypoxia; *R,* reoxygenation. *(Data from Lorek A, Takei Y, Cady EB, et al: Delayed ["secondary"] cerebral energy failure after acute hypoxia-ischemia in the newborn piglet: continuous 48-hours studies by phosphorous magnetic resonance spectroscopy,* Pediatr Res *36:699, 1994.)*

with 21% and 12 minutes if pure O_2 is used; however, those values are not significantly different. Within a 2-hour observation period, 75% of the piglets had EEG return to basal levels; of these, one third had an abnormal pattern.[44]

Somatosensory evoked potentials also disappeared during severe hypoxemia, but they reappeared in approximately 90% of the piglets during resuscitation. The amplitude increased steadily from zero at the beginning of resuscitation to approximately 25% after 30 minutes and to 50% to 60% of baseline after 60 minutes, followed by a stable plateau. No differences were found regardless of whether 21% or 100% O_2 was used for reoxygenation.[39,44]

ENERGY STATUS IN THE BRAIN

Proton magnetic resonance spectroscopy of the newborn piglet brain revealed that pH, adenosine triphosphate, and phosphocreatinine rapidly decreased during hypoxemia.[43,45] This is the so-called *primary energy failure*. However, during resuscitation, the energy status is quickly restored to normal levels. Typically, 8 to 36 hours later, a new gradual decline in the energy status of the brain occurs.[45,46] This secondary energy failure is associated with irreversible brain damage (Figure 76-11), a finding suggesting that a window for therapeutic options exists hours after restoration of the primary energy failure and before secondary failure occurs.

With use of spectroscopy techniques, it has also been established that increased intracerebral lactic acid levels are detected weeks after the initial hypoxic insult. In newborn infants with brain injury after a hypoxic ischemic episode, high lactate levels persisted more than a month after birth, in contrast to those who developed normally in spite of a hypoxic insult. Further, this high lactate level has been accompanied by persistent alkalosis. Because alkalosis accelerates lactic acid production, this may be the cause of the elevated lactic acid levels detected.[47] Possible mechanisms leading to persistent cerebral lactic alkalosis are a prolonged change in redox state within neuronal cells, the presence of phagocytic cells, the proliferation of glial cells, or altered buffering mechanisms. An early (<18 hours after birth) elevation of brain lactate seems to indicate a poorer prognosis. It is now clear that brain alkalosis is a negative marker in the newborn infant with birth asphyxia, and the prognosis is best when moderate cerebral acidosis is present.[48]

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Pathophysiology of Shock in the Fetus and Neonate

Cardiovascular compromise in the fetus and neonate often leads to severe organ injury or death, and the success of therapeutic interventions is limited by difficulties with accurate and timely detection of the condition, especially in the fetus, and the sensitivity of the developing organism to alterations in blood and oxygen (O_2) supply. Studies using animal models of fetal and neonatal shock have investigated the cause-specific pathophysiologic changes in the cardiovascular system and, although in much less detail, the cardiovascular response to therapeutic interventions. However, little is known about the cellular effects of shock in the developing animal, and virtually no data exist for the human fetus and neonate. Extrapolation of animal data to humans must be done with caution, and because of the unpredictable impact of interspecies differences on organ development and maturational processes, extrapolation of data obtained in animal models of fetal and neonatal shock to the human fetus and neonate is even more prone to error.

CARDIOVASCULAR COMPROMISE IN THE FETUS

In the fetus, inadequate tissue O_2 delivery is most often caused by decreased blood flow to the uterus, the placenta, or the fetus. In addition, circulatory failure resulting from structural developmental abnormalities, infections, cardiac arrhythmias, and decreased O_2 carrying capacity of the blood also occurs, leading to development of hydrops and, unless intervention is successful, to fetal or neonatal demise. The studies on fetal shock have primarily employed experimental models of decreased blood flow or O_2 delivery, or both, to the fetus, and most of these studies have used the pregnant sheep model.

Developmental Changes in Fetal Gas Parameters

Under normal circumstances, hemoglobin O_2 saturation in the fetal umbilical artery and vein is 50% and 75%, respectively.[1] Thus, the fetus is exposed to lower levels of oxidant stress compared with the newborn. Fetal O_2 tension (P_{O_2}) and pH decrease and carbon dioxide tension (P_{CO_2}) increase with advancing gestational age.[2-4] These changes are primarily driven by the increased O_2 consumption and associated CO_2 production of the growing fetus. The fall in the pH is secondary to the higher CO_2 production as gestational age progresses. Because O_2 carrying capacity also increases, total O_2 content in the fetal blood remains stable throughout gestation. However, although disputed,[4] fetal O_2 saturation has been found to decrease with advancing gestational age.[5] The decrease in fetal O_2 saturation is thought to result from the effect of decreasing pH and increasing P_{CO_2} on the hemoglobin-O_2 dissociation curve and a drop in P_{O_2}.[5]

Oxygen Delivery, Oxygen Consumption, and Oxygen Extraction in the Fetus

Delivery of O_2 depends on blood O_2 content and cardiac output. In the fetus, regional O_2 delivery is unique because the O_2 content of blood flowing to the different organs varies significantly. Under physiologic conditions, despite the low fetal P_{O_2}, O_2 delivery exceeds the metabolic demand of the tissues. The high cardiac index and hematocrit, the increased O_2 affinity of fetal hemoglobin, and the balanced distribution of cardiac output between the placenta and fetus all contribute to ensure appropriate O_2 delivery to the fetal organs.[1] In addition, the tight regulation of uterine and umbilical blood flow plays a major role in ensuring appropriate fetal O_2 uptake. However, reductions in uterine blood flow are relatively well tolerated by the fetus, and, in the sheep, decreasing uterine blood flow by as much as 50% does not adversely affect fetal blood gas parameters.[6] The increase in O_2 extraction is an immediate and effective mechanism compensating for the decrease in O_2 delivery in cases of reduced uterine blood flow.[7] *O_2 extraction,* defined as the ratio between O_2 consumption and O_2 delivery, is about 0.3 in animal fetus.[8,9] Data obtained from umbilical vessels at the time of delivery suggest a higher extraction in human fetus (0.52 to 0.62).[5,10] Because of

lower O_2 consumption in the immature compared with the more mature fetus, O_2 extraction is significantly lower during early gestation.[5,11] When O_2 consumption is increased up to 28% higher than baseline levels by the infusion of norepinephrine or thyroid hormone in the fetus, arterial or venous blood gas values remain unaffected.[12] This finding underscores that, despite the low Pao_2, the fetus is able to adapt to conditions associated with significant increases in O_2 consumption without evidence of disturbances in oxidative metabolism in the tissues.

Fetal Response to Hypoxemia

Lactic Acidosis in Fetal Hypoxemia

Under physiologic conditions, lactate level is higher in the fetal than the maternal circulation. The mild increase in fetal lactate levels is the result of enhanced placental lactate production and decreased fetal gluconeogenic utilization of lactate.[1]

Lactic acidosis develops under pathologic conditions in which O_2 delivery decreases to the point at which compensatory increases in O_2 extraction and cardiac output have reached their limit to satisfy tissue O_2 demand. Critical O_2 delivery in fetal sheep appears to be at 12 mL/kg/minute, and decreases in O_2 delivery to less than this value are associated with impaired oxidative metabolism and the accumulation of lactic acid in the fetal tissues and blood.[13] Indeed, elevated plasma lactate has been shown to be a good indicator of fetal hypoxemia. After 4 to 5 hours of fetal hypoxemia, blood lactate reaches a plateau despite continued anaerobic metabolism. The placental clearance of lactate is responsible for this phenomenon.[7] Thus, despite the ongoing fetal anaerobic metabolism in cases of chronic and severe fetal hypoxemia, the fetal serum lactate level does not rise beyond the point of equilibrium.[7]

The fetus responds differently to various levels of severity of hypoxia. Maternal hypoxia-induced mild fetal hypoxia does not alter fetal acid-base balance when fetal arterial O_2 saturations remain in the 40% range even when the hypoxemia persists for 24 hours.[14] Conversely, when fetal hypoxia results in fetal arterial O_2 saturations of less than 30%, metabolic acidosis develops.[15] Human data are consistent with the results of animal studies indicating that arterial oxygen saturation of 30% is the threshold for developing metabolic acidosis.[16,17] Thus the *critical threshold of arterial O_2 saturation,* defined as the arterial O_2 saturation below which metabolic acidosis develops, is about 30% in the maternal hypoxia-induced fetal hypoxia model in the sheep. It appears that susceptibility of the fetus to hypoxia is developmentally regulated; the preterm fetus is less sensitive to the effects of maternal hypoxia than its term counterpart.[15,18] For instance, when maternal hypoxia results in fetal arterial O_2 saturations of less than 30%, the preterm ovine fetus develops metabolic acidosis at a much slower pace[18] than that described for the fetus near or at term.[18]

The fetus also appears to respond differently to various causes of hypoxia.[19] When fetal hypoxemia is induced by umbilical cord occlusion, the fetal response differs from that seen with maternal hypoxia.[20] When fetal hypoxemia is caused by umbilical cord occlusion, metabolic acidosis develops more rapidly compared with maternal hypoxia-induced fetal hypoxemia.[20] The lack of placental compensatory mechanisms in the cord occlusion model may be responsible for this difference. Finally, the fetal response to hypoxemia is also altered when hypoxia is caused by decreased uterine blood flow. In this model, the critical threshold of arterial O_2 saturation is lower than in the maternal hypoxia or cord occlusion models, and rapid lactate accumulation and fall in pH only occur when fetal O_2 saturation is in the 15% to 20% range.[21] This finding suggests that placental compensatory mechanisms remain effective when blood flow to the uterus is decreased. Because of interspecies differences, these findings obtained in the sheep should be translated to the human fetus with caution.

Endocrine Responses to Fetal Hypoxemia

In the fetus, acute hypoxemia is associated with an increase in the plasma concentration of several hormones, including adrenocorticotropic hormone,[22-24] β-endorphin,[25,26] vasopressin,[27-29] glucocorticoids,[22-24] norepinephrine, and epinephrine.[21,29,30] This stress hormone response facilitates fetal adaptation to hypoxemia in part by ensuring the redistribution of blood flow to vital organs.[31]

However, the effectiveness of the fetal stress hormone response is limited by the developmentally regulated immaturity of certain endocrine organs and the cellular mechanisms of end-organ responses. For instance, although the fetal adrenal glands increase cortisol secretion in response to hypoxemic stress, the increase in the cortisol level is significantly less, whereas the increase in the catecholamine levels is similar to that seen in the mature animal.[21] In addition, expression of the cardiovascular adrenergic receptors and second-messenger systems is developmentally regulated, contributing to the observed differences in the stress response between the fetus and the mature animal.

The exact mechanisms of the 50- to 1000-fold increase in fetal catecholamine release to fetal stress remain to be clarified. One study[32] investigated the effect of metabolic acidosis on adrenal catecholamine secretion. In an effort to avoid the adrenal stimulatory effect of hypoxemia associated with acidosis-induced rightward shift in oxyhemoglobin dissociation curve, 100% O_2 was administered to the pregnant ewe during the infusion of 30% lactic acid to the fetus. Under these experimental circumstances, metabolic acidosis in the absence of hypoxia did not stimulate adrenal catecholamine secretion. Conversely, studies investigating fetal catecholamine release in the presence of hypoxemia have reported increased catecholamine and vasopressin secretion in the presence or absence of associated metabolic acidosis.[27,33]

Cardiovascular Effects of Fetal Hypoxemia

In the fetus, the right and left ventricles work in parallel, and therefore the combined left and right cardiac output is considered the total cardiac output. In the fetal lamb, the combined cardiac output is 450 mL/kg/minute, to which the right and left ventricles contribute 300 and 150 mL/kg/minute, respectively.[34] Similarly, in the human fetus, the contribution of the right ventricle to the combined cardiac output is significantly higher than that of the left ventricle. Data obtained by echocardiography show the combined cardiac output in human fetus to be similar to that of fetal sheep; however, the right ventricle contributes only about 60% of the combined output.[35,36]

In the fetal lamb, the cardiovascular response to acute hypoxemia is characterized by the rapid development of hypertension, bradycardia, increased peripheral vascular resistance, and a 15 to 20% decrease in the cardiac output. The decrease in cardiac output is primarily the result of the increased peripheral vascular resistance (afterload), bradycardia, vagal stimulation, and myocardial depression. However, significant myocardial depression occurs only if the hypoxemia is severe or prolonged or is associated with metabolic acidosis.[31] In the fetus, adenosine may play an important modulatory role in the previously described cardiovascular "maladaptation" by influencing fetal autonomic and glycolytic responses to hypoxia. In the sheep, blockade of the adenosine receptors during fetal hypoxia significantly attenuates the development of hypertension (systemic vascular resistance increase), bradycardia, and metabolic acidosis.[37,38] The fetal cardiovascular response to prolonged hypoxemia is different from that seen in the acute hypoxemia models. In fetal sheep with sustained hypoxemia, cardiac output drops progressively to 38%, and, not unexpectedly, right ventricular output is compromised earlier than the left ventricular output.[39]

In response to fetal hypoxemia, distribution of cardiac output and venous return is altered in an effort to maintain perfusion and O_2 delivery to the vital organs such as the heart, brain, and adrenal glands.[8,40,41] During induced maternofetal hypoxia, the percentage of systemic venous blood recirculated to the fetal body and not sent to the placenta for oxygenation is decreased, whereas the proportion of umbilical venous blood contributing to fetal cardiac output is increased from 27% to 39%.[40] As mentioned earlier, there are differences in the fetal response, including the distribution of cardiac output, venous return, and O_2 delivery among the different models of fetal hypoxia. However, regardless of the cause of hypoxia, the blood flow and O_2 delivery to the heart, brain, and adrenal glands are maintained, and the proportion of umbilical venous blood bypassing the liver through the ductus venosus is increased in all cases.[8,40,41]

Finally, the fetal cardiovascular and endocrine response to acute hypoxemia is altered when repeated hypoxic events occur. This is important because recurrence of hypoxic insults to the fetus may not be infrequent in human pregnancies in which blood flow to the uterus, placenta, or the fetus is repeatedly compromised. It appears that prevailing hypoxemia sensitizes the cardiac and vasoconstrictor chemoreflex responses because enhanced femoral vasoconstriction and marked elevation in plasma norepinephrine and vasopressin have been demonstrated in response to acute hypoxemia in fetal sheep that were previously exposed to sustained hypoxemia.[42]

Renal Responses to Hypoxemia

Although the placentomaternal unit performs most of the effective compensatory functions,[43] the fetal kidney has the ability to contribute to the maintenance of fetal acid-base balance. For instance, ammonium excretion and hence generation of bicarbonate, as well as sodium excretion, increase during the recovery period from hypocapnic hypoxia in the fetal sheep.[44] The possible reason that the foregoing fetal renal compensatory changes only occur during the recovery period from acute hypocapnic hypoxia is the hypocapnia itself. Because fetal P_{CO_2} is low as a result of maternal compensatory hyperventilation to hypoxia in this model and because CO_2 is required for bicarbonate absorption in the proximal tubule, bicarbonate reabsorption and acid excretion in the fetus are delayed until hypoxemia subsides and maternal, and thus fetal, P_{CO_2} returns to normal.

In addition to the effects of hypoxemia on the renal compensatory mechanisms maintaining fetal acid-base balance, there are other changes in renal function induced by hypoxemia. Fractional excretion of sodium is increased by a decrease in proximal tubule sodium reabsorption.[44,45] Urine osmolality also increases, and free water clearance drops secondary to increases in vasopressin release.[44,46] Finally, like the other organs, the immature kidney is also less susceptible to hypoxemia than the mature kidney.[47] The renal response to lactic acidosis induced by acid infusion in the fetal sheep is confined to tubular adaptive responses with decreases in urine pH and increases in ammonium and titratable acid excretion without changes in glomerular filtration rate.[48]

CARDIOVASCULAR COMPROMISE IN THE NEONATE

Definition and Phases of Neonatal Shock

Shock develops when O_2 delivery to the tissues is inadequate to satisfy cellular metabolic demand. Independent of the origin, there are three phases of shock, and each phase is characterized by unique pathophysiologic changes.

In the *compensated phase*, vital organ function is maintained by intrinsic neurohormonal compensatory mechanisms resulting in distribution of organ blood flow primarily to the heart, brain, and adrenal glands and away from other "nonvital" organs. Several hormones and local factors affecting myocardial function, organ blood flow distribution, capillary integrity, systemic and pulmonary vascular resistance, and cellular metabolism are released during this phase. Stroke volume, central venous pressure, and urine output all decrease. However, blood pressure remains within normal limits because the increases in myocardial contractility and heart rate maintain cardiac output close to the normal range. Because blood pressure is the function of blood flow and systemic vascular resistance, blood pressure may not always appropriately reflect the status of organ blood flow in this phase. This observation especially concerns the nonacidotic extremely low-birth-weight preterm neonate with immature myocardium and compensated shock during the first postnatal day.[49,50] In addition, the developing cerebral cortex of the extremely low-birth-weight preterm neonate may function as a "nonvital" organ with low-priority circulation resulting in vasoconstriction and decreased cortical perfusion in cases with low cardiac output,[51] just like the vasoconstriction that occurs to maintain blood pressure in the traditional nonvital organs (e.g., skin, muscle, kidney, liver, mesenterium). Thus, in these patients, blood flow to the brain stem and not to the entire brain may be maintained only during the compensated phase of shock.

If the circulatory compromise is not recognized and treated, neonatal shock will enter the *uncompensated phase,* in which failure of the intrinsic neurohormonal compensatory mechanisms results in decreased microvascular perfusion, myocardial contractility, stroke volume, and blood pressure, with ensuing significant decreases in organ blood flow and tissue perfusion and lactic acidosis. If treatment is delayed or the condition is prone to rapid deterioration, such as in fulminant sepsis, myocarditis, or asphyxia with multiorgan failure, neonatal shock will enter the final and *irreversible phase,* in which cellular damage leading to complete organ failure dominates the clinical picture, and death occurs invariably.

Factors in the Pathophysiology of Shock at the Cellular and Molecular Level

Reactive Oxygen Species

These molecules are involved in initiation and amplification of cellular injury in shock. Under normal conditions, the redox coupling reactions generate water as a result of complete O_2 reduction with only minimal (1%) univalent O_2 reduction ($O^{\bullet}{}_2^-$).[52] However in shock, $O^{\bullet}{}_2^-$ is significantly increased.[53] Ischemia and hypoxia result in accumulation of electron donor compounds such as reduced nicotinamide adenine dinucleotide secondary to impairment of oxidative phosphorylation in the mitochondria.[53] Subsequent reperfusion then leads to enhanced reactive O_2 species production by the enhanced availability of the electron acceptor O_2.

Furthermore, because anaerobic metabolism leads to inadequate production of adenosine triphosphate (ATP), hypoxanthine and xanthine accumulate. Under normal physiologic conditions, hypoxanthine is oxidized to urate by xanthine dehydrogenase and xanthine oxidase, with the latter generating $O^{\bullet}{}_2^-$ and H_2O_2 as byproducts. During ischemia-reperfusion, cellular changes favor hypoxanthine oxidation by xanthine oxidase, with a resultant significant increase in the production of reactive O_2 species. Reactive O_2 species cause cellular injury through their adverse effects on different cellular structures that lead to polysaccharide depolymerization, lipid peroxidation,[54] alterations in the primary structure of amino acids,[55] and nucleic acid oxidation.[56] These effects impair the cellular adhesion and receptor physiology, disrupt cell membrane integrity[54] and the function of many enzymes,[55] and damage DNA,[56] respectively. The fetus and neonate are prone to develop severe cellular injury during the

ischemia-reperfusion cycle because they have immature antioxidant defenses.

Nitric Oxide

Under physiologic conditions, nitric oxide (NO) plays an important role in the regulation of vascular tone. However, under pathologic conditions, inducible NO synthase (iNOS) is upregulated by endotoxin and proinflammatory cytokines, and large amounts of NO are produced.[57,58] Overproduction of NO by iNOS has been implicated in the pathogenesis of shock in pediatric patients, including newborn infants.[59,60] Excessive production of NO then leads to hypotension, decreased vascular response to vasoconstrictor hormones, and myocardial dysfunction.[57] In addition to the adverse cardiovascular effects, NO can cause direct cellular injury through formation of reactive free radicals such as peroxynitrite[61] and by inhibiting mitochondrial respiratory function.[62]

Because excess NO is involved in the pathogenesis of shock, studies have focused on testing therapeutic modalities that inhibit NOS as a possible treatment strategy, especially in septic shock. However, the use of nonselective NOS inhibitors that inhibit both endothelial (e)NOS and iNOS has resulted in deleterious effects in immature[63] and mature animal models of shock and increased mortality in human adults with septic shock.[64,65] In contrast, selective inhibition of iNOS improves hypotension and lactic acidosis in the mature canine endotoxic shock model.[66] However, whether selective iNOS inhibition is helpful in the treatment of neonatal shock remains to be elucidated.[67]

Platelet-Activating Factor

This phospholipid mediator has been implicated in the pathogenesis of shock in both animal models[68,69] and adult subjects.[70] In animal models of septic shock, animals treated with a platelet-activating factor antagonist showed improvement in their cardiovascular status.[71,72] Although there is no study on the role of platelet-activating factor in neonatal shock, its association with necrotizing enterocolitis is well documented.[73,74]

Eicosanoids

In inflammation, arachidonic acid derived from cell membrane phospholipids is metabolized by cyclooxygenase or lipoxygenase to produce inflammatory mediators such as prostaglandins, thromboxanes, and leukotrienes. Although eicosanoids have been implicated in the pathogenesis of organ failure and shock, their exact role remains to be elucidated. Rats deficient in essential fatty acid, and thus unable to produce significant amounts of eicosanoids, are much less susceptible to endotoxic shock and have significantly improved survival rates compared with wild-type rats.

Eicosanoids, such as the predominantly vasodilator prostacyclin and prostaglandin E_2, and the vasoconstrictor thromboxane A_2, play an important role in the regulation of vascular tone. Some eicosanoids (thromboxane A_2) induce platelet and neutrophil aggregation and thus have significant proinflammatory effects, whereas others, such as prostaglandin E_2, also exert an antiinflammatory action by down-regulating cytokine release by macrophages and lymphocytes. Indeed, there are both animal and human data supporting the role of prostaglandins as both proinflammatory and antiinflammatory agents. In animals with hypovolemic shock, administration of prostacyclin and prostaglandin E_1 and E_2 improves cardiovascular status.[75,76] Conversely, in the septic human and animal, inhibition of cyclooxygenase may improve both the cardiovascular status and survival.[77,78]

Studies in animal models and humans have also shown that the levels of thromboxane B_2, a metabolite of thromboxane A_2, are increased in septic shock.[79,80] In addition to its proinflammatory and vasoconstrictor effects, thromboxane A_2 may have a direct myocardial depressant effect, and therefore it may significantly compromise cardiac output.[79]

K_{ATP} Channels

Among the various potassium channels, ATP-dependent potassium channel (K_{ATP}) has emerged as the key channel through which many modulators exert their action on vascular smooth muscle tone.These channels are located in cell membrane of vascular smooth muscle cells. In case of tissue hypoxia, reduction in ATP and increase in lactate level activate K_{ATP} channels, leading to efflux of K^+. The resultant hyperpolarization of the cell membrane decreases vascular tone by decreasing intracellular calcium through inhibition of voltage-gated calcium channels.[81]

K_{ATP} channels have been implicated in the pathogenesis of vasodilatory shock.[82] Some of the mediators of septic shock, such as nitric oxide, are known to activate K_{ATP}.[83] Animal studies have shown an improvement in blood pressure in response to K_{ATP} blockers,[84,85] but a recent small human trial failed to show any benefit of the administration of the K_{ATP} channel inhibitor glibenclamide in adults with septic shock.[86]

Other Factors

Among other factors with potential clinical importance in shock, nuclear factor-κB (NF-κB) has been suggested to have a prognostic value. In patients with septic shock, higher peripheral mononuclear cell NF-κB activity is associated with increased mortality.[87] It is not known whether this is an association or a causative relationship.

Down-Regulation of Adrenergic Receptors

Down-regulation of the adrenergic receptors and second-messenger systems in cases of critical illness and exogenous catecholamine administration,[88] as well as a relative or absolute adrenal insufficiency,[89] have emerged as probable causative factors in adults[90] and neonates[91] for the development of pressor-resistant shock. Because expression of the cardiovascular adrenergic receptors and some components of their second-messenger systems is inducible by glucocorticoids,[92] steroid administration offers a powerful clinical tool to reverse the effects of adrenergic receptor down-regulation.[90,91] These genomic effects of steroids resulting in the synthesis and membrane assembly of new receptor proteins require at least several hours to take place. However, improvement in cardiovascular function occurs within 1 to 2 hours after hydrocortisone administration to neonates.[91] This finding can be explained by the observations that steroids also exert certain nongenomic actions, which affect the cardiovascular system without delay. Glucocorticoids inhibit the catechol-*O*-methyltransferase, the rate-limiting enzyme in catecholamine metabolism, and decrease the reuptake of norepinephrine by the sympathetic nerve endings, leading to increases in the plasma concentration of catecholamines.[93] Physiologic doses of mineralocorticoids and, to a lesser degree, pharmacologic doses of glucocorticoids also instantly increase cytosolic calcium availability in myocardial and vascular smooth muscle cells by acting through putative cell membrane-bound specific steroid receptors.[93] In addition, steroids inhibit prostacyclin production and the induction of iNOS,[94] and they limit the pathologic vasodilation associated with the nonspecific or specific inflammatory response in the critically ill neonate. Finally, by improving capillary integrity, steroid administration may also increase the effective circulating blood volume in neonates with capillary leak.

Physiology of Neonatal Circulation

Cardiac Output

Under physiologic conditions, tissue perfusion is maintained by the provision of uninterrupted blood flow through the microcirculation. Normal microcirculation, in turn, depends on the organ perfusion pressure maintained by the interaction among cardiac output, preload, and afterload. *Cardiac output* is the product of stroke volume and heart rate and is determined by the amount

of blood returning to the heart (preload), the strength of myocardial contractility, and the resistance against which the heart must pump (afterload). When myocardial function is intact, cardiac output depends solely on preload and afterload, according the relationships described by the Starling curve.

Normal ranges for left and right ventricular output for preterm and term neonates have been reported between 150 and 300 mL/kg/minute.[95,96] In the transitional circulation of the newborn infant, in whom ventricular output does not consistently reflect systemic blood flow because of the shunts across the fetal channels,[96] superior vena cava blood flow can be measured and used to estimate systemic blood flow.[50] Normal values for superior vena cava blood flow in well preterm neonates range between 40 and 120 mL/kg/minute, with a median rising from 70 mL/kg/ minute at 5 hours of age to 90 mL/kg/minute at 48 hours.[50]

The strength of myocardial contractility depends on the filling volume and pressure and on the maturity and integrity of the myocardium. Thus, decreases in preload (hypovolemia, cardiac arrhythmia), as well as prematurity and hypoxic and infectious insults, all decrease contractility and lead to decreases in cardiac output.

If the systemic or pulmonary vascular resistance (afterload) is too high, the ability of the myocardium may become compromised to pump against the increased resistance, and cardiac output will fall.[97] In the neonate, significant increases in the afterload may occur with enhanced endogenous catecholamine release during the period of immediate postnatal adaptation as well as in hypovolemia or hypothermia, or when inappropriately high doses of vasopressors are administered to a patient with intact cardiovascular adrenoreceptor responsiveness. Depending on which circulation (systemic or pulmonary) is more severely affected, the high afterload can impair the function of either ventricle. However, by the ensuing decrease in blood return to the initially unaffected ventricle, the reduction in the output of one of the ventricles will influence the function of the other ventricle. In addition, in the immediate postnatal period, shunts through the patent ductus arteriosus or foramen ovale may compromise the circulation.[98] In the extremely low-birth-weight neonate, normal postnatal closure of these fetal channels frequently fails, and as the right-sided pressures fall, pulmonary edema may rapidly develop, further compromising the hemodynamic status of these critically ill patients.[98]

Systemic Blood Pressure

Systemic blood pressure is the product of systemic blood flow and systemic vascular resistance. The gestational and postnatal age-dependent normal ranges for systemic blood pressure in neonates have been described in the literature.[99,100] Although blood pressure only weakly correlates with blood flow in the critically ill extremely low-birth-weight neonate during the period of immediate postnatal adaptation,[50,97] there is an association between low blood pressure and early central nervous system injury in this patient population.[101,102] However, a recent study failed to show any association between hypotension in first 3 postnatal days (as defined by three commonly used thresholds) and brain injury diagnosed by ultrasound obtained before 10 postnatal days in preterm infants.[103] Data indicate that only when mean blood pressures in extremely low-birth-weight neonates are less than or equal to 20 mm Hg and greater than or equal to 40 mm Hg, blood pressure becomes a more accurate indicator of abnormal and normal systemic blood flow.[104] Thus, in extremely preterm neonates with mean blood pressures between 20 and 40 mm Hg in the immediate postnatal period, the state of systemic blood flow is unclear, and the situation can be clarified only with ultrasonographic evaluation of cardiac function and organ blood flow. In more mature preterm and term neonates, blood pressure and organ blood flow appear to have a better correlation. Recent studies have provided some insights into the possible lower blood pressure limit of the cerebral blood flow autoregulatory curve. In extremely low-birth-weight infants during the transitional period (first 2 postnatal days), cerebral blood flow was maintained if mean blood pressure was greater than 29 mm Hg.[105] The cerebral blood flow autoregulation became pressure-passive once mean blood pressure dropped below 29 mm Hg. Even if this cut off value holds true in larger studies, lower blood pressures do not necessarily result in ischemic cerebral injury as compensatory mechanisms provide some degree of protection. Indeed, in one study, cerebral function as assessed by electroencephalogram was affected only when mean blood pressure was below 23 mm Hg.[106]

Organ Blood Flow and Its Autoregulation

Even extremely immature preterm neonates appear to be able to autoregulate their cerebral blood flow.[102,107-109] However, organ blood flow autoregulation is impaired in some preterm neonates, especially in those with birth asphyxia, acidosis, infection, tissue ischemia, and sudden alterations in arterial P_{CO_2}, rendering these patients at higher risk for cerebral injury. As mentioned earlier, because most of the severe cerebral injuries occur in extremely low-birth-weight neonates during the immediate postnatal period, the cortical vessels of these patients may be regulated as low-priority vessels, and thus blood is shifted away from the immature cerebral cortex during periods of circulatory compromise, as it is in other low-priority organ systems such as the kidneys, muscles, skin, and the hepatic and mesenteric circulation.[8,31,40,41] Because afterload abruptly increases with delivery, the immature myocardium struggles to maintain cardiac output in these subjects. Indeed, it is likely that the development of compensated shock and relative cortical ischemia is part of the transition to extrauterine life in most extremely low-birth-weight neonates. The cortical ischemia then sets the stage for reperfusion injury (intracranial hemorrhage or periventricular white matter injury) to occur once myocardial function and organ perfusion improve.[110]

Pathogenesis of Neonatal Shock

The clinical presentation, pathophysiology, and treatment of neonatal shock are significantly affected by the primary cause of the condition.[111] Hypovolemia, myocardial dysfunction, and abnormal regulation of peripheral vascular tone are the primary etiologic factors leading to shock in the neonate. In the critically ill neonate, more than one of these factors may be involved. For instance, in a newborn with septic shock, the capillary leak–induced hypovolemia, direct myocardial injury, and abnormal regulation of vascular tone may all contribute to the development of the circulatory compromise.

Hypovolemia

Hypovolemia is an uncommon primary cause of neonatal shock, especially during the first postnatal days. In preterm newborns, there is no evidence that hypotensive babies as a group are hypovolemic.[112] Hypovolemia causes low cardiac output and hypotension by decreasing the preload. Hypovolemia can result from loss of circulating blood volume after hemorrhage (absolute hypovolemia) or from inappropriate increases in the capacitance of the blood vessels as in vasodilatory shock (relative hypovolemia). In addition, the positive intrathoracic pressure associated with positive pressure mechanical ventilation reduces venous return and hence preload and cardiac output in ventilated preterm and term neonates.[113]

Absolute hypovolemia in the neonate can be caused by intrapartum fetal blood loss resulting from a hemorrhage from the fetal side of the placenta or from acute fetomaternal hemorrhage or acute fetoplacental hemorrhage. The latter may occur in neonates with breech presentation or a tight nuchal cord in whom

the umbilical cord comes under significant pressure.[114] Postnatal hemorrhage may occur from any site and is frequently associated with endothelial damage and disseminated intravascular coagulation induced by perinatal infections or asphyxia. Acute abdominal surgical problems and conditions associated with increased capillary leak with loss of fluid into the interstitium can also lead to significant decreases in the circulating blood volume.

Myocardial Dysfunction and Structural Heart Disease

There are significant differences in myocardial structure and function between the immature myocardium of neonates and the mature myocardium of older children and adults.[111, 115] Of particular interest among the differences are the greater dependence of the immature myocardium on extracellular calcium concentration and its greater sensitivity to an increase in afterload. As for the latter, a neonate is more prone to develop myocardial dysfunction and decreased cardiac output in response to increased afterload compared with a child or an adult. Unfortunately, conditions that can lead to increase in afterload are not uncommon in the immediate postnatal period. With the cessation of low resistance placental flow after delivery, the systemic vascular resistance (SVR) increases. Such a rise in SVR can lead to an increase in left ventricular afterload, with resultant decrease in cardiac output, and to the development of shock, primarily in the more immature preterm neonate during the transitional period. In one study, about half of the preterm infants with hypotension or metabolic acidosis had myocardial dysfunction.[116] In addition, the myocardium in preterm infants with a low superior vena cava (SVC) flow (used as a surrogate of upper body blood flow) appears to be more sensitive to afterload than those with a normal SVC flow.[117] This suggests that myocardial dysfunction due to immaturity plays a potential role in developing poor organ perfusion. On the other hand, even though the myocardium takes months to mature, myocardial dysfunction associated with immaturity does not play a significant role in pathogenesis of neonatal shock beyond the transitional period.

Other than myocardial immaturity, perinatal asphyxia and prolonged septic shock are the major causes of myocardial dysfunction. Preterm infants who undergo ductal ligation may also develop subtle myocardial dysfunction in the immediate postoperative period.[118] However, this transient deterioration in cardiac function is not related to the increase in SVR, as afterload remains unchanged after ligation. The cause of this phenomenon is unknown. It is possible that the acute decrease in myocyte fiber length following sudden decrease in the left ventricular preload after ligation of the ductus arteriosus plays a role and myocardial function rapidly improves as the myocardium resets to the new loading condition.

Finally, cardiomyopathies, structural heart defects that produce duct-dependent systemic circulation (hypoplastic left heart syndrome, critical coarctation of aorta, and critical aortic stenosis), anomalous left coronary artery from pulmonary artery, and tachyarrhythmias can present in early neonatal life with cardiogenic shock.

Abnormal Peripheral Vasoregulation and Neonatal Shock with Complex Pathogenesis

Extreme Prematurity

The transitional circulatory changes in the first 12 to 24 hours after birth represent a period of unique circulatory vulnerability for the extremely preterm infant. During the period of immediate postnatal adaptation, the left ventricle has to double its output. As described in the section on organ blood flow autoregulation, the extremely premature infant has significant difficulties to adjust to extrauterine life and is prone to develop reperfusion injury in the brain.[97,110] Under these conditions, blood pressure in the low-normal to normal range does not necessarily translate into normal organ blood flow and tissue perfusion in the extremely premature neonate.[50,97]

In the transitional circulation of the preterm infant, neither right nor left ventricular output will consistently reflect systemic blood flow because of the shunts across the ductus arteriosus and foramen ovale.[96] Consequently, measurement of either ventricular output can overestimate systemic blood flow by more than 100% in some cases.[96] As mentioned earlier, superior vena cava flow can be used as a marker of total systemic blood flow,[51] and serial measurements of superior vena cava flow have been used to describe the natural history of systemic blood flow changes in preterm neonates in the early postnatal period.[110,119] At least one third of preterm neonates born before 30 weeks gestation have a period of low systemic blood flow, mostly during the first 12 hours of life.[50,110] Gestational age is the dominant predictor of the development of the low-flow state; 70% of babies born before 26 weeks gestation have a period of low systemic flow compared with about 10% at 29 weeks gestation. Thus, the development of a low-flow state with compensated neonatal shock appears to be part of the transitional adaptation process in the extremely low-birth-weight patient population immediately after birth. The low-flow state can persist for up to 24 hours but usually improves thereafter. There is a strong relationship between recovery from the low-flow state and subsequent intraventricular hemorrhage,[110] and the low-flow state itself has been identified as a significant risk factor for poor neurodevelopmental outcome.[120]

Sepsis

Endotoxin or lipopolysaccharide plays a major role in the pathophysiology of septic shock caused by gram-negative organisms. Lipopolysaccharide induces the production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1. In addition to activating the inflammatory response, TNF-α induces apoptosis.[121] NF-κB, a nuclear transcriptional factor, mediates the inflammatory response induced by TNF-α.[122] TNF-α is a potent stimulator of iNOS, and overproduction of NO causes vasodilatation, systemic hypotension, and the generation of reactive free radicals such as peroxynitrite. In gram-negative septic shock, myocardial dysfunction develops, at least in part because of the effect of up-regulated TNF-α production,[123] as evidenced by improvement in cardiac function after administration of anti-TNF-α antibody.[124] Finally, TNF-α, by up-regulating tissue factor production in endothelial cells, also activates the extrinsic coagulation pathway and contributes to the generation of thrombi in the microcirculation.[125]

The triggers of inflammation and the host response in septic shock caused by gram-positive organisms are less well defined. However, cell wall components such as peptidoglycans and lipoteichoic acid, as well as exotoxins, have been implicated in the induction of the cytokine cascade.[126]

Although clinical evidence of circulatory compromise is a feature of many infectious processes in the newborn, the hemodynamics in neonatal septic shock have not been well studied. In older subjects, two distinct hemodynamic patterns occur. *Warm shock* is characterized by loss of vascular tone, increased systemic blood flow, and low blood pressure, and it is difficult to recognize initially unless the blood pressure is closely monitored. *Cold shock,* conversely, is characterized by increased vascular tone, low systemic blood flow, and eventually falling blood pressure, and it has been well described in the newborn.[127]

Pressor-Resistant Systemic Hypotension in Neonates

As discussed in the section on down-regulation of adrenergic receptors, pressor-resistant hypotension in preterm and term infants is now a well-recognized condition.[91,128] The underlying systemic hemodynamic changes, although not well-defined, appear to be similar to those seen in adult vasodilatory shock, with normal to high systemic blood flow and possibly

supranormal cardiac output.[129] Neonates with this condition are more likely to be extremely premature (≤27 weeks) or to have been critically ill or suffered a degree of perinatal asphyxia. Potential mechanisms of the uncontrolled vasodilation include dysregulated cytokine release, excess NO synthesis, vasopressin deficiency, overactivation of the potassium-ATP channels in the vascular smooth muscle cell membrane in response to tissue hypoxia, and down-regulation of the cardiovascular adrenergic receptors.[91] In the neonate, the foregoing mechanisms may be exacerbated by immaturity, relative adrenal insufficiency,[89,130] or preceding asphyxia, or they may be secondary to the transitional circulatory failure of the extremely low-birth-weight neonate.

Treatment of Neonatal Shock

Treatment of neonatal shock must be tailored to the primary cause or pathogenesis and to the level of maturation of the critically ill neonate.[131,132]

Volume Administration

Because hypotensive neonates as a group are not hypovolemic,[112] because myocardial dysfunction frequently contributes to the development of neonatal hypotension,[133] because dopamine is more effective in normalizing blood pressure than is volume administration,[134,135] and because excessive fluid administration increases morbidity, especially in the preterm neonate,[136] fluid resuscitation should be minimized.

Concerning the type of fluid administration, isotonic saline has been shown to be as effective as 5% albumin in increasing the blood pressure.[137] In addition, albumin may cause an impairment of gas exchange, may induce a fluid shift from the intracellular compartment,[138] and may be associated with increased mortality.[139] Therefore, the use of isotonic saline has been advocated.[131,140] However, because of the unbalanced nature of normal saline, its administration in large amounts may worsen metabolic acidosis. If there is an identifiable volume loss, the type of fluid lost should be replaced.

Dopamine and Dobutamine

There is only indirect evidence that the use of these sympathomimetic amines improves neonatal mortality or morbidity. Studies in developing and mature animals showed significant age-dependent differences in the cardiovascular and renal effects of dopamine[141] and the cardiovascular effects of dobutamine.[142] However, owing to maturation-dependent and maturation-independent interspecies differences, interpolation of these findings to the human neonate may be misleading.[107,140] Although a few studies examined the developmentally regulated hemodynamic effects of dopamine[107,109,135,143,144] and dobutamine[97,129,145] in the human neonate, there is still only limited information available on the effects of dopamine and dobutamine on tissue O_2 delivery and consumption[146] and systemic blood flow in the newborn.[97]

Hemodynamic Effects

Dopamine, an endogenous catecholamine, exerts its cardiovascular actions through the dose-dependent stimulation of the cardiovascular dopaminergic receptors and the α- and β-adrenergic receptors. In addition, by stimulating epithelial and peripheral neuronal dopaminergic and adrenergic receptors, the drug exerts significant renal and endocrine effects independent of its cardiovascular actions.[140] Although dopamine affects all three major determinants of cardiovascular function, the drug-induced increases in myocardial contractility[135,140] and peripheral vascular resistance[135,140,145] are the most important factors in increasing systemic blood pressure and improving the cardiovascular status.

Animal studies on the cardiovascular effects of dopamine during development showed that the drug's positive inotropic effect and selective vasodilatory actions in the different regional circulations are maturation dependent.[147-149] However, in the human preterm neonate, clinical studies have demonstrated greater increases in stroke volume[150] and glomerular filtration rate[151,152] in response to dopamine administration than expected from the findings of the developmental animal studies. Thus, functional cardiovascular β-adrenergic and dopaminergic receptors exist, at least beyond 23 weeks gestation, in the heart and the renal circulation of the human neonate.[107,109,135,140,143,144,150] In addition, during the early phases of shock, when the cardiovascular adrenergic receptors are not yet down-regulated, dopamine increases blood pressure in preterm infants at lower doses compared with children and adults.[150-153] The increased sensitivity to the vasoconstrictive (afterload-increasing) effects of dopamine may be related to the enhanced expression of α-adrenergic receptors during early development,[154] as well as to the differences in the maturation of hepatic and renal dopamine-clearance mechanisms.[152]

The original dose range recommendation of 2 to 20 μg/kg/minute of dopamine was based on pharmacodynamic data obtained in healthy adults. However, changes in cardiovascular adrenergic receptor expression by critical illness,[93] relative or absolute adrenal insufficiency and immaturity,[89,130] and the dysregulated production of local vasodilators during severe illness decrease the sensitivity of the cardiovascular system to dopamine and result in the emergence of hypotension resistant to conventional doses of the drug.[91,128] Thus, with the advancing disease process, increased doses of dopamine and other sympathomimetic amines may be needed to exert the same magnitude of cardiovascular response. Indeed, there is no evidence that, when required to normalize blood pressure, high-dose dopamine treatment with or without additional epinephrine administration has detrimental vasoconstrictive effects.[91,128,155] However, there are no data available on changes in cardiac output and organ blood flow in response to high-dose catecholamine treatment in pressor-resistant neonatal shock.

Unlike dopamine, dobutamine is a relatively cardioselective sympathomimetic amine with significant α- and β-adrenoreceptor-mediated direct inotropic effects and limited chronotropic actions.[142,156,157] Dobutamine administration is usually also associated with a variable decrease in total peripheral vascular resistance[157] and, at least in adults, with improved coronary blood flow and myocardial O_2 delivery.[156] Furthermore, unlike dopamine,[140] dobutamine increases myocardial contractility exclusively through the direct stimulation of the myocardial adrenergic receptors. Because myocardial norepinephrine stores are immature and are rapidly depleted in the newborn and because dobutamine may decrease afterload, newborns with primary myocardial dysfunction and elevated peripheral vascular resistance are most likely to benefit from dobutamine treatment.[140,157] Although addition of dobutamine to dopamine in preterm infants with respiratory distress syndrome is effective in increasing blood pressure, this approach is associated with supranormal cardiac output states and lower systemic vascular resistance.[129] Whether the benefits of supranormal cardiac output by providing adequate tissue O_2 delivery throughout the body outweigh the risks of sustained hypercontractility potentially resulting in myocardial injury remains to be investigated. Although dopamine is effective in normalizing the cardiovascular status in most neonates with hypotension,[140] neither dopamine nor dobutamine effectively improves low systemic blood flow in extremely low-birth-weight neonates during the first 24 hours of life.[97] However, in hypotensive very-low-birth-weight (VLBW) infants, stepwise titration of both dopamine and epinephrine improves blood pressure and cerebral perfusion.[158] This finding underscores the earlier-described complexity of the cardiovascular changes during the immediate postnatal transition of the extremely low-birth-weight human neonate.

The vasodilatory dopamine receptors are primarily expressed in the renal, mesenteric, and coronary circulations.[140] Dopamine has been shown to decrease renal vascular resistance selectively[107,109] and to increase glomerular filtration rate[151,152] in preterm infants as early as 23 weeks gestation. However, dopamine decreases mesenteric vascular resistance in preterm infants only beyond the first postnatal day,[107,109,144] and the effects may be variable.[144] Similarly, there are some differences in the reported magnitude of the drug-induced increases in ventricular function, cardiac output, and systemic vascular resistance.[135,144,145] These findings may be best explained by differences in the intravascular volume status, the postnatal age, the developmentally regulated expression of cardiovascular adrenergic and dopaminergic receptors, and the severity of adrenergic receptor down-regulation among the different populations of critically ill preterm infants studied. None of the studies has, however, found evidence of a direct effect of dopamine on cerebral blood flow.[107,109,135,144] Finally, in addition to increasing afterload, dopamine also increases pulmonary vascular resistance in preterm neonates.[159] However, because the drug-induced increases in systemic blood pressure are not associated with impaired oxygenation in most neonates,[140] there is no evidence of consistent increases in extrapulmonary right-to-left shunting during dopamine treatment. In preterm neonates, dopamine also improves right ventricular performance.[160]

There are no data available on the direct renal, cerebral, or pulmonary hemodynamic effects of dobutamine in the human newborn. However, when dobutamine increases blood pressure, it may also increase mesenteric blood flow in preterm infants.[161] Because dobutamine does not stimulate the dopaminergic receptors, β-adrenoreceptor-induced selective vasodilation may be responsible for the observed mesenteric vasodilation.

Epithelial and Neuroendocrine Effects

Independent of the previously described cardiovascular effects, dopamine exerts direct renal[140,152] and endocrine[140] actions in the newborn. Through its direct effects on sodium, phosphorus, and water transport processes and sodium-potassium ATPase activity[162] in the renal tubules, dopamine increases sodium, phosphorus, and free water excretion and may increase the hypoxic threshold of renal tubular cells during episodes of hypoperfusion and hypoxemia.[140] Through its renal vascular and epithelial actions, dopamine also potentiates the diuretic effects of furosemide[163] and theophylline.[164] Among its endocrine actions, the dopamine-induced decreases in plasma prolactin and thyrotropin levels[140] may be of clinical importance. The potential impact on long-term neurodevelopmental outcome and immunologic function of the drug-induced alterations in the neuroendocrine function has not been investigated in the preterm or term neonate. Because dobutamine does not directly stimulate the dopaminergic receptors, its administration is likely to be devoid of neuroendocrine effects.

Other Sympathomimetic Amines and Hormones

Although both epinephrine and norepinephrine have been used in the treatment of hypotension in preterm infants, data in peer-reviewed literature on the cardiovascular actions of epinephrine during development are available only from studies on newborn animals.[165] Studies of the cardiovascular and renal effects of these catecholamines in the human neonate have been published only in abstract form.[166-168] The only published article shows that epinephrine, similar to dopamine, increases blood pressure and cerebral perfusion in VLBW infants.[158] It is not known whether there is a difference in the cardiovascular response or side effects with the combined use of epinephrine and dopamine compared with the use of increasing doses of dopamine beyond 20 μg/kg/minute with or without dobutamine in the human neonate. In addition to sympathomimetic amines, arginine vasopressin has been reported to improve cardiovascular function in newborns with vasodilatory shock.[169]

Steroid Administration

There is emerging evidence that brief steroid treatment stabilizes the cardiovascular status and decreases the need for pressor support in the critically ill newborn with pressor-resistant hypotension.[89,91,128,130,170,171-174] The potential mechanisms of steroid treatment in hypotensive preterm and term neonates on improving the cardiovascular status were described earlier, in the section on pressor-resistant hypotension. As for the effects on hemodynamics, hydrocortisone increases blood pressure by increasing peripheral vascular resistance without adversely affecting the cardiac output in vasopressor-treated preterm and term infants.[174] Finally, there are no data on the potential long-term neurodevelopmental side effects of the attempted physiologic supplementation with hydrocortisone or the use of low-dose dexamethasone to enhance cardiovascular stability and pulmonary function in critically ill preterm and term infants.

Supportive Measures

Maintenance of normal arterial pH and serum ionized calcium concentrations is important for optimizing the cardiovascular response to catecholamines. Because metabolic acidosis with a pH of less than 7.25 compromises myocardial function in preterm infants,[175] maintenance of the arterial pH above this range in infants with acidosis with a significant metabolic component may be warranted.[175] However, the efficacy and potential short- and long-term adverse effects of the administration of sodium bicarbonate or tromethamine have not been studied in the neonatal patient population.

SUMMARY

Treatment of hypotensive, critically ill preterm and term neonates requires the ability to monitor the most important determinants of cardiovascular function, including the blood pressure and systemic and organ blood flow; a thorough understanding of the pathogenesis and pathophysiology of neonatal shock; and knowledge of the developmentally regulated mechanisms of actions, pharmacodynamics, and potential side effects of the sympathomimetic amines and other medications used in the management of neonatal shock.

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SECTION XII

The Lung

78

Susan E. Wert

Normal and Abnormal Structural Development of the Lung

The respiratory system is composed of the lung, its conducting airways, and the respiratory muscles of the thorax. These structures function together to provide oxygen from the external environment to the organism, while removing excess carbon dioxide from the blood. Appropriate anatomic development of these structures is essential for overall respiratory function in the newborn infant, along with histologic, cellular, and biochemical maturation of the lung, which is critical for proper physiologic function at birth. This chapter describes the anatomic development of the lung and its conducting airways, along with a review of associated pulmonary malformations. Selected molecular mechanisms that regulate structural development of the lung and its conducting airways are also discussed briefly. More complete and detailed discussions of these mechanisms can be found in several excellent recent reviews,[1-4] as well as in subsequent chapters of this book.

OVERVIEW OF LUNG DEVELOPMENT

Lung development can be divided into five distinct, but overlapping, chronologic stages of organogenesis, which describe the histologic changes that the lung undergoes during morphogenesis and maturation of its structural elements. These stages include *embryonic, pseudoglandular, canalicular, saccular,* and *alveolar* phases of lung development, which extend throughout gestation and into the postnatal period (Table 78-1). Human lung development is initiated in early gestation as a small, saccular outgrowth, or *diverticulum*, of the ventral wall of the foregut. During subsequent *embryonic* and *pseudoglandular* stages of lung development, formation of the conducting airways, or *tracheobronchial tree,* takes place by a process called *branching morphogenesis.* This process involves rapid growth and repetitive branching of the *primitive respiratory diverticulum* until all of the branches of the tracheobronchial tree are formed. Outgrowth, expansion, cytodifferentiation, and maturation of the alveolar, or gas-exchange, regions of the lung occur later in gestation, during the *canalicular, saccular,* and *alveolar* stages of morphogenesis. The *alveolar* stage of lung development extends into the postnatal period, during which millions of additional alveoli are formed and *maturation of the microvasculature,* or *air-blood barrier,* takes place,[5] greatly increasing the surface area available for gas exchange (Table 78-2). Although definitive alveoli can be found in the human lung by 36 weeks gestation, greater than 85% to 90% of all alveoli are formed within the first 6 months of life.[6] Overall, the number of alveoli increases by approximately six-fold between birth and adulthood, that is, from an average of 50 million (ranging from 20 to 70 million) alveoli in the term lung to 300 million (ranging from 212 to 605 million) alveoli in the adult human lung.[6-8] After the first 6 months of life, alveolar formation continues at a slower pace until approximately 2 years of age, when further growth of the lung becomes proportional to growth of the body. From birth to adulthood, the conducting airways increase in length and diameter (see Table 78-2), and the gas-exchange surface area and its diffusion capacity increase linearly with body weight up to approximately 18 years of age.[9]

Embryonic Stage (3 to 7 Weeks After Conception)

The lung is a derivative of the primitive foregut endoderm and the adjacent mesoderm. The *respiratory primordium* of the lung first appears on postconception (p.c.) day 22 as an enlargement of the caudal end of the *laryngotracheal sulcus*, which is located in the medial pharyngeal groove, an outgrowth of the ventral wall of the primitive foregut endoderm.[5] The *primitive respiratory diverticulum*, or lung bud, appears on day 26 p.c., when the embryo is only approximately 3 mm long, and begins to grow ventrocaudally into the mesoderm surrounding the foregut, that is, in a position that is anterior and parallel to the primitive esophagus. Epithelial cells of the primitive respiratory diverticulum invade the surrounding mesoderm, or *splanchnic mesenchyme,* forming tubules that undergo repetitive lateral and terminal *(dichotomous)* branching to form the proximal structures of the tracheobronchial tree. On day 28 p.c., the respiratory diverticulum bifurcates into right and left primary bronchial buds (main stem bronchi), the left bud being shorter and more horizontal than the right bud. The region proximal (or cephalad) to the first bifurcation becomes the trachea and larynx. Shortly thereafter, the trachea and the esophagus begin to separate into two distinct structures.[10] A second round of asymmetric branching occurs during the fifth week of gestation (day 33-41 p.c.) to yield three secondary bronchial buds on the right and two on the left (lobar bronchi), which become the primary lobes of the right and left lung. During the sixth week (day 41-44 p.c.), a third round of branching yields 10 tertiary bronchi on the right and eight or nine bronchi on the left, which become the bronchopulmonary segments of the mature lung. During this stage extensive proliferation occurs of both epithelial and mesenchymal cells with focal apoptosis in the mesenchyme around branch points and in regions of new bud formation.[11]

At this stage of development, the mesenchyme is composed of a loose arrangement of primitive cells that will later differentiate into progenitor cells of the future blood vessels, lymphatics, smooth muscle, cartilage, and connective tissue components. The extracellular matrix (ECM) is composed primarily of fluid, hyaluronic acid, and proteoglycans. The basement membrane underlying the epithelium contains type IV collagen and other basement membrane glycoproteins, such as laminin and fibronectin, and proteoglycans.[12] The trachea and bronchial tubules lack underlying cartilage, smooth muscle, and nerves; the pulmonary and bronchial blood vessels are not well developed; and the pseudostratified, columnar epithelium is composed of relatively primitive, undifferentiated epithelial cells. At the end of this stage, the lung resembles a small tubuloacinar gland, and separation of the trachea and esophagus is complete.[10]

TABLE 78-1

Human Lung Development and Associated Abnormalities

Stage	Major developmental events	Abnormalities and syndromes
Embryonic, 3-7 wk p.c.	Lung bud arises from ventral foregut endoderm	Laryngeal, esophageal, tracheal atresia
	Branching morphogenesis initiated	Tracheal and bronchial stenosis
	Main stem, lobar, segmental, and subsegmental bronchi form	Bronchogenic cysts
	Trachea and esophagus separate	Tracheoesophageal fistula
	Pulmonary arteries bud off sixth pair of aortic arches	Pulmonary agenesis and aplasia
	Pulmonary veins develop as outgrowths of left atrium	Extralobar pulmonary sequestration
Pseudoglandular, 5-17 wk p.c.	Formation of tracheobronchial tree complete by 16 wk	Renal agenesis and pulmonary hypoplasia
	Pulmonary arterial development parallels airway branching	Intralobar pulmonary sequestration
	Acinar tubules and buds form in peripheral lung	Pulmonary cysts
	Ciliated, goblet, neuroepithelial, and basal cells differentiate	Cystic adenomatoid malformation
	Pulmonary lymphatics appear	Pulmonary lymphangiectasia
	Cartilage, mucous glands, and smooth muscle develop	Tracheomalacia and bronchomalacia
	Pleuroperitoneal cavity closes	Congenital diaphragmatic hernia
Canalicular, 16-26 wk p.c.	Acinar tubules and buds expand	Renal dysplasia and pulmonary hypoplasia
	Air-blood barrier and capillary network forms	Alveolar capillary dysplasia
	Alveolar type I and type II cells differentiate	Respiratory insufficiency
	Lamellar bodies form in type II cells	Surfactant deficiency
Saccular, 24-38 wk p.c.	Distal air spaces continue to branch and grow	Oligohydramnios and pulmonary hypoplasia
	Air spaces expand to form saccules	Alveolar capillary dysplasia
	Mesenchyme thins and condenses	Surfactant deficiency
	Septal walls contain double capillary network	RDS
	Elastin deposited at sites of secondary septal crest formation	HMD
	Surfactant synthesized and secreted by type II cells	Apnea of prematurity
	Fetal lung fluid and fetal breathing	Transient tachypnea
Alveolar, 36 wk p.c. to 2 yr	Secondary septa form, subdividing saccules into alveoli	Lobar emphysema (overinflation)
	Alveolar septal walls thin with loss of connective tissue	Pleural effusions and fetal hydrops
	Alveolar surface area increases	Persistent fetal circulation
	Double capillary network fuses into a single network	Pulmonary hypertension
	Fibroblasts differentiate	Pneumonia
	Collagen, elastin, and fibronectin deposited	Meconium aspiration syndrome
	Surfactant production increases in type II cells	RDS, HMD, surfactant deficiency

HMD, Hyaline membrane disease; *p.c.,* post conception; *RDS,* respiratory distress syndrome.

TABLE 78-2

Postnatal Growth of the Human Lung and Conducting Airways

	Term	Adult	Fold increase
Lung weight (avg)	50 g	800 g	16
Lung volume (avg)	150-200 mL	5000 mL	25-33
Alv SA	3-4 m^2	75-100 m^2	25
SA/kg (avg)	0.4 m^2	1 m^2	2.5
Alv number (avg)	50×10^6	300×10^6	6
Alv number (range)	$17\text{-}71 \times 10^6$	$212\text{-}605 \times 10^6$	8.5-12.5
Alv diameter (avg)	150 μm	300 μm	2
Alv septal wall thickness (avg)	5 μm	2.5 μm	2
Air-blood barrier (avg)	0.6 μm	0.6 μm	0
Tracheal length (avg)	26 mm	184 mm	7
Main bronchial length (avg)	26 mm	254 mm	10

Modified from Hodson WA: Normal and abnormal structural development of the lung. In Polin RA, Fox WW, editors: *Fetal and neonatal physiology,* ed 2, Philadelphia, 1998, Saunders, p 1037.

Alv, Alveoli; *Avg,* average; *SA,* surface area.

Vascular connections with the right and left atria are established at the end of the embryonic stage, creating the primitive pulmonary vascular bed.[13,14] Pulmonary arteries arise from the sixth pair of aortic arches and grow into the surrounding mesoderm, where they accompany the developing airways, segmenting with each bronchial subdivision, and connect to the vascular plexus forming in the pulmonary mesenchyme. During fetal life, the pulmonary artery is connected to the aortic arch by the ductus arteriosus, which enables the right ventricular output from the heart to bypass the pulmonary vascular bed. The pulmonary veins develop as an outgrowth of the left atrium, which divides several times before connecting to the pulmonary vascular bed. Lymphatic vessels originate from the cardinal veins.

Developmental abnormalities that arise during the embryonic stage of lung development are related to lung bud formation, separation of the trachea and esophagus, formation of the proximal conducting airways, and initial lobe formation. These abnormalities include laryngeal, esophageal, and tracheal atresia; tracheal and bronchial stenosis; tracheomalacia and bronchomalacia; tracheoesophageal fistulas; pulmonary agenesis; ectopic lobes; bronchogenic cysts; and arteriovenous malformations (see Table 78-1).

Molecular Mechanisms Regulating Early Lung Development

Experimental studies have demonstrated that dorsal-ventral, lateral, and proximal-distal patterning of the developing lung is regulated by interactions with the surrounding mesenchyme.[1,2,15,16] These interactions involve both positive and negative signaling mechanisms that result in normal growth and organization of the lung. Molecular mechanisms that regulate the initiation of lung bud formation and early branching morphogenesis are (1) expression of nuclear DNA-binding proteins, or *transcription factors,* that are important for specification of the foregut endoderm and its derivatives; (2) endogenous, secreted polypeptides, or *morphogens,* that are important for epithelial-mesenchymal interactions in the lung and pattern formation; (3) *growth factors* that are important for cellular proliferation, migration, and cytodifferentiation; and (4) exogenous factors, such as retinoic acid (RA) (vitamin A), which

functions as a morphogen and is required for early lung bud formation.[1-4,17-20]

Transcription factors that are critical for foregut development and, by extension, early lung development include members of the FOX, GATA, and GLI families of transcription factors. FOXA2 (also known as HNF3β) is a member of a large family of nuclear transcription factors, termed the winged helix/forkhead box (or forkhead DNA-binding domain) family of transcription factors.[21,22] These nuclear proteins are involved in cell commitment, cytodifferentiation, and gene transcription in a variety of organs, including the central nervous system and derivatives of the foregut endoderm.[23,24] FOXA2 is required for formation of the foregut endoderm, as well as organogenesis and cytodifferentiation of the lung. FOXA2 regulates regional specification of cell fate, or cell commitment, in the primitive foregut endoderm and cooperates with another transcription factor, thyroid transcription factor-1 (TTF1, also known as NKX2-1, T/EBP, or TITF1) to determine epithelial cell lineages.[3,4] FOXA2 expression is detected in the pulmonary epithelium throughout lung development, and its expression is maintained in the fully differentiated adult bronchial and alveolar epithelium.[25-27] GATA6 is a zinc finger transcription factor that induces differentiation of the primitive foregut endoderm into respiratory cell lineages via its interactions with TTF1 and FOXA2. GATA6 is expressed in the foregut endoderm and in the pulmonary epithelium during lung development. Genetic ablation of *Foxa2* or *Gata6* in mice disrupts formation of the foregut endoderm and all of its developmental derivatives, including the lung.[28-30] TTF1 is a homeobox protein that is also expressed in the epithelium of the lung throughout development, as well as in the adult lung.[25,31,32] TTF1 is essential for branching morphogenesis, epithelial cell proliferation, development of distal lung structures, and expression of the surfactant proteins.[33] When *Nkx2-1* is inactivated in gene-targeted mice, lung development is perturbed, resulting in the formation of bilateral, sac-like structures that are lined with primitive epithelial cells and originate from a short, common tracheoesophageal tube.[34,35] The absence of TTF1 arrests dorsal-ventral separation of the trachea and esophagus, branching morphogenesis of the lung, and epithelial cell differentiation.

Members of the GLI family of zinc finger transcription factors, GLI1, GLI2, and GLI3, are expressed in the foregut mesoderm and are downstream transducers of sonic hedgehog (SHH) signaling.[36,37] SHH is a secreted polypeptide, or *morphogen,* that is expressed in the foregut endoderm and in the primitive epithelial cells of the lung, as well as in the notochord, the neural plate, and the brain. Its receptor, patched-1 (PTCH1) is expressed in the mesoderm of the lung. When SHH binds to its receptor, another transmembrane protein, called smoothened (SMO), is released and translocated to the nucleus where it activates the GLI2 and GLI3 genes. GLI2 and GLI3, in turn, modulate the expression of other proteins that play a role in lung development, such as GLI1, PTCH1, and FGF10.[1-4,16,17,19] Both SHH and the GLI family members are associated with induction of cellular proliferation, as well as dorsal-lateral patterning of the lung. SHH expression is found at the growing tips of the bronchial tubules, where it is thought to act as a mitogen for the adjacent, primitive, mesenchymal cells. *Shh*-null mutant mice exhibit foregut abnormalities.[38,39] Branching morphogenesis is severely impaired, resulting in extremely hypoplastic, rudimentary lungs that originate from a fused tracheoesophageal tube. Genetic ablation of *Gli2 (Gli2−/−)* in mice also causes foregut defects, including esophageal and tracheal stenosis, pulmonary hypoplasia, and lobation defects (fusion of the four right lobes into one lobe), coincident with diminished expression of PTCH1 and the GLI1 isoform.[40] Genetic inactivation of both *Gli2* and *Gli3* in compound, homozygous, *Gli2−/−;Gli3−/−* mice results in complete pulmonary agenesis, including agenesis of the trachea and the lung, as well as the esophagus. Esophageal atresia and tracheoesophageal fistula with fusion of the lung in the midline is found in compound *Gli2−/−;Gli3+/−* mice.[40] Haploinsufficiency of FOXF1, another SHH target that is expressed in the mesenchyme, also causes esophageal atresia, tracheoesophageal fistula, fusion of the right lung lobes, and pulmonary hypoplasia with altered expression of GLI3, FGF10, and BMP4.[41,42]

Growth factors that are critical for early lung formation include members of the fibroblast growth factor family (FGF) and their receptors, FGFR1, 2, 3, and 4, which induce cellular proliferation, migration, and differentiation in the developing lung.[1-4,16-19,43] One of the most critical events in the induction of early branching morphogenesis is the activation of FGF10 expression in the mesoderm. FGF10 functions as a chemoattractant, inducing growth of the bronchial buds toward regions of FGF10 expression in the mesoderm. FGF10 signaling is mediated by its receptor, FGFR2, which is a tyrosine kinase receptor expressed in the foregut endoderm and primitive respiratory epithelium. Disruption of FGF10 or FGFR2 expression in mutant mice results in the formation of a trachea but no lungs,[44-47] whereas blockade of FGFR2 signaling in transgenic mice, by using a mutant, dominant-negative, FGFR2(IIIb) receptor, completely blocks branching of all the conducting airways distal to the primary bronchi.[48] On the other hand, excision of a single copy of the FGFR(IIIc) isoform, which is expressed in the mesenchyme, resulted in a gain of function mutation with increased expression of the FGFR2(IIIb) isoform in mice.[49] This mutation resulted in kidney, lung, lacrimal gland, and skeletal defects, similar to those found in the human congenital disorders, *Apert* and *Pfeiffer syndromes,* which are caused by missense mutations in the human FGFR2 gene.[50,51] More recently, the hedgehog interacting protein, HIP1, has been shown to play a role in the control of SHH-mediated regulation of FGF10 expression. HIP1 is an integral membrane protein that binds to SHH and is expressed in the distal mesenchyme.[52] Targeted deletion of *Hip1* in mice resulted in defects in lobation and pulmonary hypoplasia, which were attributed to increased SHH signaling with repression of FGF10 expression at sites of secondary bud formation.[53]

RA signaling is required for normal development of many different organs, including the lung. RA signaling appears to be required for lung bud formation, because acute vitamin A deprivation in pregnant animals at the onset of lung development results in tracheal stenosis and pulmonary agenesis.[19] RA activity is mediated by binding to RA receptors, RAR(α, β, γ) and/or RXR(α, β, γ), to form a complex that is translocated to the nucleus, where it modulates gene transcription by binding to RA response elements in noncoding regions of its target genes. These receptors have multiple isoforms that form heterodimers with each other and are widely expressed throughout the embryo, as well as in the pulmonary epithelium and surrounding mesenchyme. Genetic ablation of RA receptors in mice causes abnormalities resembling those found in vitamin A–deficient animals, including malformations of the eyes, skeleton, limbs, aortic arch, genitourinary tract, and the lung. Disruption of RA signaling in compound *RARα1−/−;RARβ2−/−* mice results in tracheoesophageal fistula, agenesis of the left lung, and hypoplasia of the right lung.[54] Subsequent development of the lung during branching morphogenesis is associated with down-regulation of RA signaling, which increases FGF10 expression, limiting proliferation of distal epithelial structures.[55] Conversely, maintenance of RA signaling in embryonic lung cultures disrupts the formation of distal lung structures, while promoting the formation of proximal structures, most likely through up-regulation of the SHH pathway, which inhibits FGF10 signaling.[55]

RA signaling may be further mediated by interactions with the HOX family of homeodomain transcription factors, which determine anterior-posterior patterning of the body axis during development.[56] Expression of the HOX genes is regulated both spatially and temporarily during lung development and is

associated with branching morphogenesis.[57-59] In the mouse, *Hoxb3, b4,* and *b5* are expressed in the foregut endoderm where the lung buds will form. During lung development, *Hoxb3* and *Hoxb4* are expressed in the mesenchyme of the trachea, bronchi, and distal lung, whereas *Hoxa5, Hoxb2,* and *Hoxb5* are restricted to the distal mesenchyme.[59] RA up-regulates *Hoxb5* in embryonic lung cultures, resulting in abnormally elongated bronchial tubes and decreased branching,[60] whereas inhibition of *Hoxb5* expression results in short primary branches with decreased secondary branching.[60] Aberrant expression of HOXB5 has been associated with human *bronchopulmonary sequestration*[61] and *congenital cystic adenomatoid malformations,*[62] suggesting that dysregulation of the *HOXB5* gene may be involved in these pulmonary malformations. Targeted deletion of the *Hoxa5* gene in mice results in laryngotracheal malformation; reduced number of cartilaginous, tracheal, and bronchial rings; tracheal stenosis; impaired branching morphogenesis; and thickened alveolar walls.[63] Loss of HOXA5 was also associated with decreased expression of TTF1 and FOXA2, as well as their target genes, the surfactant proteins, in this experimental model.[63]

Congenital Disorders and Mutations Associated With Early Pulmonary Malformations

Pulmonary agenesis, lung lobation defects, tracheal stenosis (dysplastic tracheal cartilage), and tracheoesophageal fistula have been observed in some patients with *Pallister-Hall syndrome,* which is associated with mutations in the human *GLI3* gene.[50,64,65] Patients with these mutations also exhibit malformations involving the central nervous system (hypothalamic hamartoblastoma, pituitary aplasia or dysplasia), craniofacial features, limbs (postaxial polydactyly), kidneys (renal dysplasia), adrenals (adrenal hypoplasia), and the heart.[50] Esophageal atresia; tracheoesophageal fistula; esophageal and tracheal stenosis (cartilaginous sleeves); laryngomalacia, tracheomalacia, and bronchomalacia; lobar atresia; and pulmonary aplasia have been observed in patients with *Pfeiffer, Apert,* and/or *Crouzon syndromes,* all of which are associated with mutations in the human *FGFR2* gene.[50,51,66-70] Patients with these mutations also exhibit varying degrees of craniosynostosis, or premature closure of the cranial sutures.

Pseudoglandular Stage (5 to 17 Weeks After Conception)

The pseudoglandular stage of lung development is so named because of the distinct glandular appearance of the lung during this period of morphogenesis; that is, the lung is composed of multiple epithelial tubules surrounded by relatively extensive regions of mesenchyme. At the beginning of this stage, the rate of cell proliferation increases, and sequential branching of the airways and vascular structures continues. Formation of the conducting airways, including the terminal bronchioles, is more or less complete by the end of this stage, with 12 to 17 generations of bronchial tubules in the upper lobes, 18 to 23 in the middle lobes, and 14 to 23 in the lower lobes.[71] Numerous *acinar tubules and buds,* which give rise to the adult *pulmonary acinus,* are also formed in the periphery of the lung by the end of this stage, arising as distal branches of the terminal bronchioles.[72,73] The pulmonary acinus includes two to four respiratory bronchioles, each ending in six to seven generations of branched alveolar ducts and sacs.

The bronchial tubules are lined initially by a pseudostratified columnar epithelium. These cells are morphologically undifferentiated and contain large pools of intracellular glycogen, deriving most of their energy needs from anaerobic glycolysis. A prominent basement membrane, rich in laminin and collagen type IV, underlies the epithelium. Mesenchymal cells adjacent to these tubules differentiate into myofibroblasts by 7 weeks p.c., aligning themselves in a circumferential orientation that is perpendicular to the long axis of the tubules. The ECM is composed of various types of glycosaminoglycans and proteoglycans, such as chondroitan, dermatan, and heparan sulfate, as well as laminin, fibronectin, tenascin, entactin, and type I and type III collagen.[12,74] These macromolecules are important for cell proliferation, migration, adhesion, and differentiation during lung development. As branching progresses, the pseudostratified columnar epithelium is reduced to a tall columnar epithelium in the proximal airways and to a cuboidal epithelium in the distal acinar tubules and buds. Cytodifferentiation of the conducting airway epithelium occurs in a centrifugal direction with ciliated, nonciliated (serous), goblet, neuroepithelial, and basal cells appearing first in the more proximal airways.[75-77] Isolated neuroepithelial cells, the first bronchial epithelial cells to differentiate, can be detected in the proximal airways by 8 to 9 weeks p.c.[78] Clusters of neuroepithelial cells, called neuroendocrine bodies, are detected by 9 to 10 weeks p.c. These are located at branch points along the bronchial tree and are innervated by sympathetic and sensory nerve fibers. Ciliated cells appear in the epithelium of the trachea by 10 weeks, in the main stem bronchi by 12 weeks, and in the segmental bronchi by 13 weeks. Mucous cells are present by 13 weeks, and mucous glands appear in the trachea by 11 to 12 weeks and in the bronchi by 13 weeks, with active mucous production by 14 weeks.[79] Cartilage appears in the trachea and bronchi by 10 weeks and in the segmental bronchi by 16 weeks.[80] By the end of this developmental stage, cartilaginous structures extend as far as the segmental bronchi and airway smooth muscle extends as far as the alveolar ducts. Spontaneous contractility of fetal airway smooth muscle can be observed in cultured human fetal lung explants at this stage of development.[81] The smooth muscle layer enveloping the conducting airways is invested by an extensive network, or plexus, of neural ganglia and nerve bundles, which is detected as early as 7.5 to 8 weeks p.c.[82] Elastic fibers are detected in the walls of the trachea and the main stem bronchi, the pleura, and the pulmonary artery.[12] Epithelial cell differentiation is initiated in the distal acinar structures with the onset of surfactant protein B (SP-B) and C (SP-C) expression. These two hydrophobic, lung-specific proteins are expressed selectively in the distal respiratory epithelium by 12 to 14 weeks p.c.[83]

The intrapulmonary arterial system develops along with the bronchial and bronchiolar tubules, branching in parallel with these airways.[13,14,84] The pulmonary veins and lymphatics take a different pathway through the lung, coursing in between the airways in the connective tissue septa that divide and surround each pulmonary segment.[85] All of the preacinar, or distal, arteries and veins are formed by the end of this period.[13,14] Formation of these structures takes place through the process of vasculogenesis, that is, by the coalescence of nascent endothelial cells, or angioblasts (derived from the mesenchyme), into endothelial tubes. These tubes gradually become invested with smooth muscle cells so that, by the end of this developmental stage, smooth muscle actin and myosin can be detected in all of the vasculature.[86-89]

A variety of congenital defects in branching morphogenesis may arise during the pseudoglandular stage of lung development, including bronchopulmonary sequestration, congenital cystic adenomatoid malformation, cyst formation, acinar dysplasia or aplasia, and pulmonary hypoplasia. The *pleuroperitoneal cavity* also closes early in the pseudoglandular period. Failure to close the pleural cavity is often accompanied by herniation of the abdominal contents leading to secondary pulmonary hypoplasia (see later).

Molecular Mechanisms Regulating Branching Morphogenesis

During the pseudoglandular stage of lung development, FGF10 signaling continues to be critical for branching morphogenesis (as discussed earlier). FGF7 (also known as KGF) is also expressed

in the mesenchyme at this time and binds to FGFR2 receptors in the epithelium. FGF7 influences branching morphogenesis by promoting epithelial growth and differentiation. Overexpression of FGF7 in transgenic mice results in the formation of large, fluid-filled, cyst-like structures,[90,91] whereas *Fgf7*-null mutants have no abnormalities, suggesting that FGF7 signaling in the lung overlaps with that of other growth factors.[92] FGF9 is also expressed at high levels during this stage of lung development, but it is synthesized in the pulmonary epithelium, not in the mesenchyme. FGF9 signaling is mediated through FGFR1 receptors, which are expressed in the mesenchyme. Targeted disruption of *Fgf9* in null mutant mice results in pulmonary hypoplasia, because of a reduction in mesenchymal tissue and decreased branching of the airways, although sacculation of the peripheral air spaces and cytodifferentiation of alveolar epithelial cells are not perturbed.[93]

Factors that tend to antagonize or limit FGF signaling, in addition to SHH, include transforming growth factor β (TGF-β), bone morphogenetic protein-4 (BMP4), and the *Sprouty* (*Spry2* and *Spry4*) genes, which may also modulate the EGF and TGF-β pathways.[1-4,16-19,43] TGF-β modulates branching morphogenesis in the lung by limiting epithelial bud formation. TGF-β accumulates in the mesenchyme adjacent to the proximal airways and at sites of cleft formation (between the branching airways), where it inhibits FGF10 expression and promotes the synthesis and deposition of ECM components. Deposition of ECM components stabilizes the proximal airways and prevents further branching at these sites. Induction of SMAD family members, downstream mediators of TGF-β, also inhibits cell proliferation, as well as FGF10 expression.

Members of the TGF-β family are also important for proximal-distal differentiation and left-right patterning in the lung. Weaver and colleagues[94] showed that disruption of BMP4 signaling in mice alters proximal-distal differentiation patterns in the developing pulmonary epithelium. BMP4 is expressed at high levels in the distal epithelium and counteracts FGF10-induced bud formation. Overexpression of the BMP antagonist, *Xnoggin,* or blockade of BMP4 signaling in transgenic mice by using a dominant-negative BMP receptor *(dnAlk6),* resulted in a reduction of distal alveolar cell types with a concomitant increase in bronchiolar cell types (ciliated cells and Clara cells).[94] On the other hand, targeted deletion of *Noggin* in homozygous null mutant mice resulted in esophageal atresia and tracheoesophageal fistula, as well as in defects in branching morphogenesis.[95] Overexpression of Gremlin, a secreted protein that binds to BMP4 and prevents it from interacting with its receptor, resulted in a similar phenotype in transgenic mice,[96] whereas reduction of Gremlin expression in cultured lungs increased branching morphogenesis, epithelial cell proliferation, and cytodifferentiation of type II cells.[97]

Left-right asymmetry is accomplished by a signaling cascade involving four other members of the TGF-β family, LEFTY1, LEFTY2, NODAL, and GDF1, as well as the *Pitx2* gene, which is a transcriptional target of Nodal-signaling.[1-4,16-19] LEFTY1 restricts the expression of LEFTY2 and NODAL to the left side. Inactivation of *Lefty1* in mice leads to bilateral expression of *Lefty2,* resulting in left pulmonary isomerism. In this condition, the lobation pattern normally seen on the right side (four lobes) is replaced by the left-sided pattern (one lobe), resulting in bilateral single, or "left," lobes.[98] In contrast, targeted deletion of *Nodal*, *Gdf1,* or *Pitx2* in mice results in right isomerism of the lung.[98-101] Other factors that may be involved in left-right patterning in the lung include RA signaling and FOXJ1 (also known as HFH4) expression. Targeted disruption of RA signaling in compound *RARα1−/−;RARβ2−/−* null mutant mice results in agenesis of the left pulmonary lobe,[54] and targeted disruption of the nuclear transcription factor *Foxj1* results in random left-right asymmetry, as well as the complete absence of cilia.[102] This phenotype is similar to that seen in the human congenital disorder, *Kartagener syndrome,* in which situs inversus, bronchiectasis, and immotile cilia are found.[50,103]

Additional growth factors that promote branching morphogenesis and growth of the lung via interaction with their respective membrane receptors include (1) FGF1 (also known as acidic FGF) and FGF2 (also known as basic FGF), which up-regulate FGF7; (2) epidermal growth factor (EGF) and TGF-α, which stimulate branching morphogenesis, cell proliferation, and cytodifferentiation; (3) hepatocyte growth factor (HGF), which is a potent mitogen; (4) insulin growth factor (IGF), which facilitates signaling of other growth factors; (5) platelet-derived growth factor (PDGF), which is a potent mitogen and chemoattractant for mesenchymal cells; and (6) vascular endothelial growth factor (VEGF), which regulates vascular growth and patterning.[19,43,104]

Molecular Mechanisms Regulating Vascular Development

Transcriptional factors thought to be involved with specification of vascular progenitor cells include the paired-related homeobox gene, *Prx1,* and the divergent homeobox gene, *Hex,* whereas the homeobox gene *Prox1* is associated with differentiation of venous endothelial cells into the primitive lymphatic vascular network.[105,106] Vascular growth and patterning is regulated primarily by VEGF, which is synthesized in the epithelium and secreted into the mesenchyme, where it binds to its receptors, VEGFR1 (also known as FLT1 in the mouse), VEGFR2 (also known as FLK1 in the mouse, or KDR in humans), and VEGFR3, found on primitive endothelial cells.[13,14,105-107] Targeted inactivation of the *Vegf* gene in mice results in impaired angiogenesis and is lethal in the fetal period,[108] and targeted mutation of *Vegfr1 (Flt1)* is also fetal lethal because of disrupted vascular formation in the embryo.[109] Ruhrberg and colleagues[110] engineered a transgenic mouse expressing an isoform of VEGF-A (Vegf120/120) that lacked heparin-binding or ECM interaction domains. Expression of this isoform in the lung impaired lung microvascular development and delayed air space maturation.[111] Lymphatic endothelial cells undergo proliferation and migration in response to VEGF-C/D signaling through the VEGFR3 receptor, which becomes restricted to lymphatic endothelial cells once lymphatic development is initiated.[105,106] Deletion of VEGF-C in mice leads to failure in migration and proliferation of lymphatic endothelial cells and results in the complete absence of lymphatic vessels,[112] and either VEGF-C haploinsufficiency or insufficient VEGFR3 signaling caused by mutation of the kinase domain results in hypoplastic lymphatic vascular networks.[112,113] Additional molecules involved in both blood and lymphatic vessel formation include members of the ephrin and angiopoietin families of proteins, along with their tyrosine kinase receptors, which are important for assembly, stabilization, remodeling, and maintenance of vessel integrity.[105,106]

Vascular development is also dependent on interactions with surrounding ECM components.[12,74] Fibronectin, which binds to endothelial cells via integrin receptors, is a component of the capillary basement membrane and is important for early capillary tube formation. Fibronectin also promotes cell migration and proliferation and prevents apoptosis, or cell death. Mice that lack the fibronectin gene fail to develop normal vasculature and die in utero.[114] Laminin, a large, noncollagenous glycoprotein, is another important basement membrane component that promotes cell attachment, migration, differentiation, cell growth, and vascular tube formation.[115] Type IV collagen, which is the major structural component of mature basement membranes, also binds to endothelial cells through specific integrin receptors and promotes cell attachment and cytodifferentiation.[12,74] Type IV collagen is expressed later than fibronectin and laminin, and its appearance coincides with a reduction in endothelial cell division, promoting tube formation and stabilizing the structure of newly formed vessels.[12]

Additional growth factors and their receptors that modulate vascular development include the platelet-derived growth factors, PDGFA and PDGFB, which function as mitogens and

chemoattractants for other mesenchymal cells that will be incorporated into the vessel wall. Once these cells contact the endothelium, they differentiate into smooth muscle cells, most likely through the activation and secretion of TGF-β, which in turn inhibits further endothelial cell proliferation and stimulates ECM deposition (elastin and collagen) by the smooth muscle cells. A similar phenomenon occurs with the recruitment of smooth muscle cells by the developing bronchial tubules. In addition to PDGF and TGF-β, endothelial cells secrete a variety of other growth factors and inhibitors that are important for cell proliferation and differentiation. These include EGF/TGF-α, FGF1, FGF2, IGF-I, and IGF-II (and their receptors), all of which stimulate cell proliferation; and TNF-α and heparin sulfate, which inhibit cell proliferation. The deposition of ECM components (collagen and elastin) appears to be stimulated by both PDGF and TGF-β, as well as by IGF-I and IGF-II.[107]

Canalicular Stage (16 to 26 Weeks After Conception)

The canalicular stage is so named because of the appearance of vascular canals, or capillaries, that multiply in the interstitial compartment to form the *air-blood barrier,* or *alveolar-capillary respiratory membrane,* the future gas-exchange surface of the lung.[5] Development of the alveolar-capillary membrane, along with the synthesis and secretion of *pulmonary surfactant,* is critical for extrauterine survival of the immature fetus, if delivered prematurely near the end of this stage. Gas exchange cannot occur in the premature infant unless these capillaries are close enough to the adjacent alveolar epithelium for optimal gas diffusion to take place across their surfaces. Rapid expansion of the capillary bed, with condensation and thinning of the mesenchyme, is the first critical step in the formation of the gas-exchange regions of the lung. During this stage of lung development, the total surface area of the alveolar-capillary membrane increases exponentially with a concomitant decrease in the mean mesenchymal wall thickness, thereby increasing the potential for gas exchange in the immature lung.[5] Disturbances in this stage of lung development result in severe hypoxemia and are not compatible with life after birth.

At the beginning of the canalicular stage, formation of the bronchial tree is complete, and the terminal bronchioles have divided into two or more respiratory bronchioles that have subdivided further into small clusters of short acinar tubules and buds lined by cuboidal epithelium. These structures undergo further differentiation to become the adult respiratory unit, or *pulmonary acinus,* consisting of alveolarized respiratory bronchioles, alveolar ducts, and alveoli. Clusters of acinar tubules and buds grow by further lengthening, subdividing, and widening at the expense of the surrounding mesenchyme. The proportion of dividing epithelial and endothelial cells increases, and the number of dividing interstitial cells (fibroblasts) falls. Apoptosis of cells in the interstitial tissue contributes to mesenchymal involution and thinning of the alveolar septa at this stage.[11] Epithelial growth drops in the larger airways, and cell proliferation occurs predominantly in the peripheral acinar tubules and buds. This peripheral growth is accompanied by the growth and development of intraacinar capillaries (derived from angioblastic cells in the interstitium), which align themselves around the air spaces, establishing contact with the adjacent epithelium to form the primitive alveolar-capillary membrane. Epithelial cell differentiation becomes increasingly complex and is especially apparent in the distal regions of the lung parenchyma, where alveolar type I and type II cells can be detected. Bronchiolar cells begin to express differentiated features and to synthesize cell-specific proteins, such as the Clara cell secretory protein (CCSP).[25,116-118] Cuboidal type II cells lining the distal tubules express increasing amounts of surfactant proteins and phospholipids.[25,83,119-125] Nascent lamellar bodies, the storage form of pulmonary surfactant, are seen in association with rich glycogen stores in cuboidal pre–type II cells lining the acinar tubules and buds.[126-129] Cells of the proximal acinar tubules become flattened and attenuated, acquiring features of typical squamous, type I alveolar epithelial cells. Type I cell differentiation occurs in conjunction with the formation of the alveolar-capillary membrane, that is, wherever endothelial cells of the developing capillary system come into contact with adjacent acinar epithelial cells. Where this occurs, the intercellular junctional complexes, originally localized around the epithelial cell apex, shift to the basolateral aspect of the intercellular cleft. The cells develop thin cytoplasmic attenuations, differentiating into squamous type I cells and losing features previously associated with pre–type II cells. By the end of the canalicular stage of lung development, the potential alveolar-capillary membrane (air-blood barrier) is thin enough to support gas exchange.

Abnormalities of lung development associated with the canalicular stage include acinar dysplasia, alveolar capillary dysplasia, and pulmonary hypoplasia. Pulmonary hypoplasia is often caused by diaphragmatic hernia or compression by thoracic or abdominal masses, by prolonged rupture of membranes causing oligohydramnios, or by renal agenesis (Potter syndrome) in which amniotic fluid production is impaired. Although postnatal gas exchange can be supported late in the canalicular stage, infants born during this period generally have severe complications related to decreased levels of pulmonary surfactant, which cause respiratory distress syndrome (RDS) and hyaline membrane disease (HMD). The administration of exogenous surfactants improves survival in these infants, but bronchopulmonary dysplasia (BPD), a complication caused by ventilatory therapy for RDS, frequently develops later. Surfactant synthesis and mesenchymal thinning can be accelerated by glucocorticoids,[130-133] which are administered to mothers to prevent RDS and HMD when a premature birth is anticipated.[134-136]

Saccular Stage (24 to 38 Weeks After Conception)

During the saccular stage of lung development, the terminal clusters of acinar tubules and buds begin to dilate and expand into thin, smooth-walled, transitory alveolar saccules and ducts, and a marked reduction, or condensation, of the surrounding mesenchymal (interstitial) tissue occurs. The lung continues to grow peripherally by branching and growth of the transitory alveolar ducts so that, by the end of this period, three additional generations of alveolar ducts ending in terminal saccules have formed. These peripheral regions of the lung also increase in size as a result of lengthening and widening of all of the segments distal to the terminal bronchioles. Intersaccular and interductal septa develop that contain delicate collagen fibers and a double capillary network.[5] Overall cell proliferation slows as a result of a sharp drop in division of the epithelial cell population. With the reduction in epithelial cell proliferation comes ultrastructural evidence of cytodifferentiation. Maturation of type II epithelial cells continues and is associated with increased synthesis of surfactant phospholipids[122,137]; the surfactant-associated proteins A, B, C, and D*; and ABCA3, a phospholipid transporter important for lamellar body formation.[141] Glycogen content is reduced, and mitochondrial enzyme activity increases, indicating a shift to aerobic oxidative pathways. Lamellar bodies increase in number and size, and increasing amounts of tubular myelin, the secretory form of pulmonary surfactant, are seen in the terminal air spaces.[126] The concentration of pulmonary surfactant is still low, however, and its phospholipid composition differs significantly from that at term.[122]

Squamous type I cells continue to differentiate and line an increasing proportion of the surface area of the distal lung.

*References 25, 83, 119, 121, 123-125, 128, 138-140.

Enlargement of the potential gas exchange surface is dependent on the development of type I cells with their attenuated, flattened, squamous cell shape. Capillaries become associated more closely with the squamous type I cells, decreasing the diffusion distance between the air spaces and the capillary bed. Basal lamina of the epithelium and endothelium fuse to form the thin-walled alveolar-capillary membrane. In the newborn and adult lung, the mean thickness of the alveolar-capillary membrane is 0.6 μm, which permits passive diffusion of oxygen and carbon dioxide between the alveolar lumen and the capillary bed.[5] Near the end of this stage, the stroma, or interstitial tissue, contains increasing amounts of ECM, and elastin is deposited in areas where future interalveolar septa will form, subdividing the terminal alveolar saccules into true alveoli. The immature lung contains relatively few elastin and collagen fibers, has little elastic recoil, and can be easily ruptured at this stage.[12]

By the beginning of the saccular stage of lung development, the conducting airways have developed both mucous cells and ciliated cells. Cartilage and submucosal glands extend as far down the airway as they do in the adult lung. The epithelial cells are capable of producing fetal lung fluid. Abnormalities associated with the saccular stage of lung development are similar to those associated with the canalicular stage of lung development, including pulmonary hypoplasia, acinar dysplasia or aplasia, alveolar capillary dysplasia, and RDS, HMD, and BPD in the premature infant.

Alveolar Stage (36 Weeks After Conception to 2 Years After Birth)

The alveolar stage is the last stage of lung development and is marked by the formation of secondary alveolar septa, which partition the terminal ducts and saccules into true alveolar ducts and alveoli, and maturation of the alveolar-capillary membrane. This process greatly increases the surface area of the lung available for gas exchange. At the beginning of this stage, the alveolar septa are relatively thick and contain a capillary network on each side of a central core of connective tissue, often referred to as a double capillary network.[5] The secondary interalveolar septa consist of short buds or projections of connective tissue that also contain a double capillary network and interstitial cells that are actively synthesizing collagen and elastin. Restructuring of the terminal saccule into a true alveolus consists of lengthening and thinning of the secondary septa, reduction of septal interstitial tissue, and remodeling of the capillary bed by fusion of the two septal capillary networks into one.[5] Pulmonary vascular resistance decreases as remodeling of the pulmonary vasculature and capillary bed occurs. This stage is accompanied by a phase of rapid cellular proliferation in both the epithelial and mesenchymal cell populations. Interstitial fibroblasts actively proliferate early in this stage but then slow down as increased synthesis and deposition of collagen, elastin, and fibronectin start to occur. Endothelial growth is brisk throughout this stage, and dividing endothelial cells are located primarily in the developing secondary septal crests. Both type II and type I cells increase in number during this stage, but only type II cells are proliferating actively, suggesting that type I cells are derived from type II cells. Type I cells are thought to be terminally differentiated and to lack the capacity for mitosis. Although type II cells account for two thirds of the total number of alveolar epithelial cells in the adult human lung, the larger squamous type I cells actually occupy 93% of the total alveolar surface. Although type I cells form a tight epithelial barrier that is impervious to extracellular fluid and ions, they are easily injured by oxidants, barotrauma, and infection, readily detaching from the alveolar wall when injured. Injury to the lung during this stage of development can result in abnormal remodeling of the lung with a reduction in the number of alveoli and the development of interstitial fibrosis. Additional factors that may cause disturbances in alveolarization include administration of glucocorticoids, which inhibits cellular proliferation, reducing septation and formation of alveoli. Conversely, glucocorticoid administration enhances thinning of the alveolar septa, increases maturation of type II cells, and enhances the production of surfactant in the premature lung and in fetal lung explants.[130-135]

Disorders associated with disturbances in the alveolar stage of lung development, which present at birth, include persistent fetal circulation and pulmonary hypertension of the newborn, lobar emphysema, meconium aspiration syndrome, pneumonia, RDS caused by asphyxia and/or surfactant deficiency caused by mutations in the *SFTPB, SFTPC,* and *ABCA3* genes,[142-145] and defects in cholesterol synthesis.[146]

Molecular Mechanisms Regulating Alveolar Development

Although many studies describing the effects of exogenous substances and/or mechanical ventilation on alveolar development in the premature lung have been published, little is known about the molecular mechanisms that regulate morphogenesis and cytodifferentiation during the canalicular, saccular, and alveolar stages of normal lung development. Recently, factors that modulate elastin formation in the developing lung have been shown to play an important role in the process of alveologenesis. Alveologenesis is characterized by the development of secondary septae that subdivide the terminal air sacs into definitive alveoli. This process appears to be driven by the synthesis and deposition of elastin by alveolar myofibroblasts located at the tips of the developing septa. PDGF, a potent mitogen and chemoattractant for mesenchymal cells, is expressed in the pulmonary epithelium, and its receptor, PDGFRA, is found on presumptive alveolar smooth muscle cells, or myofibroblasts. In mice with a targeted deletion of the *PdfgA* gene, myofibroblasts fail to multiply and migrate into the primary alveolar septa.[147,148] This results in postnatal development of emphysema, or enlarged alveolar saccules, because of a complete failure of alveologenesis, as well as reduced deposition of elastin in the lung parenchyma. Mice with a targeted deletion of the elastin gene *(Eln−/−)* also developed emphysema in the postnatal period because of the absence of elastin synthesis and deposition, which resulted in the complete failure of alveologenesis.[149] The lungs of mice lacking lysyl oxidase, an enzyme responsible for cross-linking both collagen and elastin in vivo, showed impaired development of the distal and proximal airways with enlarged alveoli and disorganization of elastic and collagen fibers in the surrounding mesenchyme.[150] Mice lacking the microfilament fibulin-4, an ECM molecule important for the stabilization and organization of elastic fibers, exhibited severe lung and vascular defects,[151] and fibulin-5–defective mice exhibited severe emphysema.[152,153] RA has been shown to enhance ongoing alveologenesis by up-regulating the transcription of tropoelastin, the soluble precursor of elastin.[154] Mice bearing compound null deletions in the RA receptors, RARγ−/−, RXRα+/−, demonstrate a decrease in elastin gene expression and elastic tissue with a reduction in alveolar formation in the postnatal lung.[155]

FGF signaling has also been implicated in the process of alveologenesis. The lungs of compound null mutant mice, homozygous for targeted deletion of both the *Fgfr3* and *Fgfr4* genes, are completely blocked in alveologenesis and do not form definitive alveoli.[156] In contrast to the *PdfgA*- and *Eln*-null mutant mice, no abnormalities in the alveolar myofibroblast cell population or in elastin synthesis and deposition during alveologenesis were observed, although increased elastin deposition was detected later in the postnatal period (day 21), suggesting that elastin synthesis is increased. Although the double mutant mice were 50% smaller than normal mice, were sickly, and died within the first few months of life, no differences in cell proliferation, cell death, or surfactant protein production were found in their lungs.

Of interest, several nuclear transcription factors important for early lung development are also important for cytodifferentiation

and maturation of the lung. These include FOXA2, TTF1, and GATA6, which are important transcriptional regulators of a variety of target genes during the later stages of lung development, including those important for surfactant production, fluid and electrolyte transport, and vasculogenesis. Mutation of *Titf1*[33] and type II cell–specific deletion of *Foxa2*[157] in transgenic mice impaired lung maturation in the saccular and/or alveolar stage of lung development, resulting in respiratory failure at birth caused by poor gas diffusion. Cytodifferentiation of alveolar type I and type II cells, surfactant synthesis, fluid and electrolyte transport, and vasculogenesis were all disrupted in these models. Lung-specific expression of a GATA6-Engrailed, dominant-negative, fusion protein in transgenic mice also impaired maturation of the alveolar epithelium.[158,159] Transgenic mice lacked detectable alveolar type I cells, with decreased expression of the type I cell markers, aquaporin-5 and T1α. Although the distal airways were dilated, squamous differentiation of the alveolar epithelium was disrupted. Instead, the distal airways were lined with cuboidal alveolar epithelial cells. The alveolar septae were thickened with a relatively undeveloped capillary network. Expression of SP-B, SP-C, CCSP, and FOXJ1 was also decreased.

Metabolic disturbances in pathways associated with cholesterol and lipid synthesis, as well as the generation and packaging of surfactant phospholipids and proteins, also contribute to disturbances in morphogenesis and function in the alveolar stage of lung development. Neonatal mice lacking functional 7-dehydrocholesterol reductase (DHCR7), a model for the human disease Smith-Lemli-Opitz syndrome (SLOS), are deficient in cholesterol and die within 24 hours of birth of respiratory failure caused by pulmonary hypoplasia with impaired sacculation of the lung, defective alveolar type I cell differentiation, and delayed vascular development of the alveolar capillary network.[146] Of interest, pulmonary hypoplasia, abnormal pulmonary lobation, and anomalies of laryngeal and tracheal development have also been reported in patients with SLOS.[160] C/EBPa, a member of the bZip domain–containing family of transcription factors important for the regulation of genes involved in lipid synthesis, is also expressed in epithelial cells of the developing lung.[161] *Cebpa*-null mutant mice die of respiratory failure at birth, exhibiting findings similar to those found in the *Titf1* mutant and *Foxa2*-deleted mice.[162,163] Structural and biochemical maturation of the lung was delayed; cytodifferentiation of alveolar type I and type II cells was arrested, and both surfactant lipid and protein synthesis was decreased. Although null mutant mice lacking the surfactant protein SP-B[164] or the phospholipid transporter ABCA3,[165-167] important for lamellar body formation, do not exhibit structural abnormalities in their lungs, these mice die at birth of acute respiratory distress caused by surfactant deficiency. Likewise, mutations in the human *SFTPB* and *ABCA3* genes are associated with acute respiratory distress and failure in the neonatal period.[168,169]

ABNORMAL DEVELOPMENT OF THE LUNG AND CONDUCTING AIRWAYS

As can be seen from Table 78-1, the majority of pulmonary malformations arise during the *embryonic* and *pseudoglandular* stages of lung development. These malformations represent a spectrum of closely related abnormalities associated with the early stage of lung bud formation, branching morphogenesis, separation of the trachea from the esophagus, and failure of the pleuroperitoneal cavity to close properly. Abnormalities in other organ systems, such as renal agenesis, dysplastic growth of the kidney, or congenital diaphragmatic hernia, may also affect branching morphogenesis of the lung during these early developmental stages. During the canalicular and saccular stages of lung development, abnormalities related to growth and maturation of the respiratory parenchyma and its vasculature predominate, leading to abnormalities in acinar development, alveolar capillary dysplasia, pulmonary hypoplasia, and respiratory insufficiency. Infants born prematurely, during the saccular and early alveolar stages of development, are subject to clinical complications and syndromes that are related primarily to biochemical immaturity of the lung, or surfactant deficiency. Surfactant deficiency causes respiratory distress in the immediate postnatal period and has been associated with HMD in the past. Acute lung injury in the neonatal period may alter subsequent alveolar growth and differentiation, resulting in BPD or chronic interstitial lung disease. Mutations in the *SFTPB* and *ABCA3* genes have been associated with respiratory distress and failure in term infants,[168,169] and mutations in the *SFTPC* gene, and in a subset of mutations in the *ABCA3* gene, have been associated with the onset of chronic interstitial lung disease in the first year of life.[170-173]

Congenital Malformations of the Tracheobronchial Tree

Tracheoesophageal Fistulas

Most congenital malformations of the tracheobronchial tree arise during formation of the respiratory diverticulum and branching morphogenesis of the lung. One of the most critical events in the formation of the respiratory system is the initial separation of the primitive foregut into the respiratory and alimentary tracts. This process begins during the third week of gestation and is complete by the sixth week. Failure of this process to proceed normally results in the formation of a tracheoesophageal fistula, one of the most commonly encountered abnormalities of the trachea. Tracheoesophageal fistulas are usually found in combination with various forms of esophageal atresia.[10,174,175] The most common combination is esophageal atresia with a lower, or distal, tracheoesophageal fistula: the upper segment of the esophagus ends in a blind pouch, and the lower segment originates from the trachea just above the bifurcation. This combination accounts for 80% to 90% of all cases. Other combinations are (1) the upper esophagus ending in the trachea and the upper portion of the lower esophagus forming a blind pouch; (2) a double fistula, where both upper and lower portions of the esophagus join the trachea at separate points along its length; (3) a common fistula without esophageal atresia; (4) esophageal atresia without a fistula, or connection to the trachea; and (5) atresia, or absence, of the trachea, with normal bronchi and lungs. In the latter case, the trachea ends in a blind sac just below the larynx, and the lungs may be connected directly to the esophagus via the bronchi or bronchoesophageal fistulas. Tracheoesophageal fistula in low-birth-weight infants is often associated with a variety of other malformations, which carries a high risk of a poor outcome. In comparison, survival from isolated tracheoesophageal fistula is almost 100% in term infants. Tracheoesophageal fistula is found in VACTERL syndrome, in association with vertebral anomalies, anal atresia, congenital cardiac disease, renal anomalies, and limb abnormalities.[50] From their mutant analysis of the Gli genes, which encode transcription factors mediating SHH signal transduction, Kim and colleagues[176,177] observed that defective SHH signaling leads to a spectrum of developmental anomalies in mice strikingly similar to those of VACTERL. They proposed that VACTERL could be caused by defective SHH signaling during human embryogenesis.

Tracheal Agenesis, Tracheal Stenosis, and Tracheomalacia

Tracheal agenesis is a rare, but fatal, anomaly thought to be caused by displacement of the tracheoesophageal septum. Classification of anatomic variations in this malformation is based on the length of the agenetic segment and the presence or absence of an esophageal fistula.[175,178] The most common variation is complete absence of the trachea below the larynx with a fistula connecting fused main stem bronchi and the esophagus. Other variations are tracheal agenesis with fistulas connecting each main stem bronchus to the esophagus; agenesis of the proximal trachea with main stem bronchi fused to form the distal trachea,

with or without a fistulous connection to the esophagus; or a short segment of tracheal agenesis between the proximal and distal segments of the trachea, linked by an atretic fibrous band of tissue.

Tracheal stenosis is a rare malformation in which the trachea is narrowed, because of either intrinsic abnormalities in cartilage formation or external compression by abnormal vessel formation, or vascular rings.[178] Narrowing of the trachea by compression results in local obstruction to the passage of air, and cartilage deformities may cause obstruction of the airway on both inspiration and expiration. The major underlying causes of intrinsic tracheal stenosis are abnormalities in cartilaginous ring formation, because of either posterior fusion of the normally C-shaped rings or formation of a complete cartilaginous sleeve. The latter malformation has been reported in children with craniosynostosis syndrome, including *Crouzon, Apert,* and *Pfeiffer* syndromes.[50,51,66-70] These syndromes involve abnormal fusion of skeletal or osseous structures and are associated with mutations in the FGF receptor, *FGFR2*.[50,51,69,70] These malformations represent mesenchymal defects in which the cells do not respond normally to FGF signaling. Extrinsic stenosis of the trachea is caused by external compression of the trachea, usually associated with abnormally situated blood vessels, termed vascular rings. These include a double aortic arch, a right aortic arch with a left ligamentum arteriosum, an aberrant (retroesophageal) right subclavian artery, an anomalous left innominate or carotid artery, a sling (retrotracheal) pulmonary artery, or a right aortic arch with aberrant left subclavian artery.

Tracheomalacia occurs when an absence or "softening" of the cartilaginous rings exists, which causes the trachea to collapse on expiration, creating an airway obstruction.[175,178] In the normal trachea, the cartilage–to–soft tissue ratio is 4.5:1, a ratio that remains constant throughout childhood. In tracheomalacia, a reduction in this ratio occurs, in some instances as low as 2:1.[178] This malformation may be segmental or diffuse. In most cases, tracheomalacia is associated with other congenital malformations, such as tracheoesophageal fistula and vascular rings. Tracheomalacia is rare, and no definitive prevalence rates are available.

Bronchial Atresia, Bronchial Stenosis, and Bronchomalacia

Bronchial atresia is a rare anomaly. The most common affected lobe is the left upper lobe, but atresia of the right upper and lower lobes has also been reported. The segmental bronchus is the more common site of atresia, but subsegmental and lobar bronchi can also be affected.[175,178] The distal lung is hypoplastic, emphysematous, or hyperinflated. Often a deficiency of bronchi and vessels exists in the affected lobe, as well as an absence of segmentation and interlobular septae. Like tracheal stenosis, *bronchial stenosis* may be intrinsic or caused by extrinsic compression.[179] Intrinsic bronchial stenosis is rare and is usually associated with anomalous cartilage segmentation. Extrinsic bronchial stenosis caused by compression is usually associated with congenital heart disease. Compression occurs when the pulmonary arteries enlarge in response to pulmonary hypertension, compressing the left upper lobe bronchus. An enlarged left atrium, a bronchogenic cyst, or a teratoma may also compress the left main bronchus. *Bronchomalacia* is caused by abnormalities in the bronchial cartilage, which leads to collapse, or bronchiectasis, of the affected airway during respiration. Bronchomalacia may be associated with other anomalies, various skeletal dysplasias, or widespread, congenital cartilage deficiency.[178]

Bronchopulmonary Sequestration

This malformation develops as a mass of abnormal pulmonary tissue, which is not connected to the tracheobronchial tree and which receives its blood supply from one or more anomalous systemic arteries arising from the aorta.[178-181] Two forms of bronchopulmonary sequestration exist. Intralobar sequestrations occur within the visceral pleural lining of a pulmonary lobe, and extralobar sequestrations are found outside the pleural lining. Both types of sequestration occur more frequently on the left side, sometimes in association with diaphragmatic defects. Rarely, a sequestration may be connected to the esophagus or to the stomach.

Intralobar sequestrations are found most frequently in the posterior basal segment of the left lower lobe. The blood supply is derived from either the thoracic or abdominal aorta, and the venous drainage is to the pulmonary vein of the affected lobe. Embryologically, the pulmonary artery appears to fail to supply a peripheral portion of the lung. Instead, the arterial supply is derived from a persistent ventral branch of the primitive dorsal aorta. Although many cases of intralobar sequestration occur in children, at least half of affected patients are over 20 years old when the diagnosis is made. Clinically, these patients present with recurrent pulmonary infection, cough, or chest pain. The affected tissue is often cystic, lined with either columnar or cuboidal epithelium, and filled with mucus. Again, a continuous accumulation of mucous secretions exists, which gives rise to acute and/or chronic infections and inflammation.

Extralobar sequestration develops as a complete, separate segment of pulmonary tissue, or accessory lobe, which is enclosed within its own pleural sac. Although the embryologic origin of this malformation is unknown, it may develop from the primitive gut as an extra, more distally placed, lung bud, which is then sealed off. Extralobar sequestration usually occurs on the lower left side of the thorax, often between the left lower lobe and the diaphragm. Other extrapulmonary sites of sequestration include paraesophageal, mediastinal, or paracardiac regions, as well as within the muscle of the diaphragm and below the diaphragm in the retroperitoneum. The arterial supply is from the abdominal aorta or one of its branches, while the venous drainage is into the venous system, creating a left-to-right shunt. This malformation is frequently associated with other congenital anomalies, especially congenital diaphragmatic defects, which occur in more than half of the cases. The majority of extralobar sequestrations are found in children under 1 year of age, but the lesion occurs in older children and adults as well. Clinically, extralobar sequestration is usually asymptomatic and is often discovered during repair of a diaphragmatic defect. If the sequestration is located in the wall of the esophagus, however, it may cause dysphasia and hematemesis. Mediastinal or hilar locations may also cause symptoms because of bronchial obstruction and overinflation of the lobe, or, conversely, because of compression of normal pulmonary parenchyma. If the sequestered lobe becomes infected, it may appear to be a chronic pulmonary abscess accompanied by fever, chest pain, cough, and bloody, purulent sputum. Histologically, the lesion may be composed of normal lung or of immature or dysplastic pulmonary parenchyma, with absent or reduced number of cartilaginous bronchi and an irregular pattern of bronchiole-like structures resembling congenital adenomatoid malformation (see later).

Congenital Bronchogenic Cysts

Bronchogenic cysts are caused by abnormal budding and branching of the tracheobronchial tree during its development.[175,179] Most bronchogenic cysts are found in paratracheal, carinal, hilar, or paraesophageal regions (middle mediastinum), but they may also be found in the lungs as pulmonary cysts, which communicate with the tracheobronchial tree. In neonates, communication between a cyst and the tracheobronchial tree may incorporate a check valve mechanism, which results in rapid expansion of the cysts. Sometimes the cyst becomes so large that it compresses the mediastinum, compromising the heart and resulting in death. Mediastinal cysts, on the other hand, rarely communicate with the tracheobronchial tree. They are often lined with ciliated columnar epithelial cells and rarely contain distal lung

parenchyma. These cysts are filled with a clear, serous fluid unless they become infected. The walls of these cysts generally contain smooth muscle, cartilage, and mucous glands.

Congenital Lobar Emphysema (Pulmonary Overinflation)

Congenital lobar emphysema, or pulmonary overinflation, can be divided into two different types: (1) congenital lobar overinflation and (2) regional, or segmental, pulmonary overinflation.[175,180] In congenital lobar overinflation, either an upper or a middle lobe of the lung is hyperinflated and compresses the remaining ipsilateral lung or lobes. If a marked shift occurs of the mediastinum to the opposite side, a ventilatory crisis ensues with dyspnea, cyanosis, and sometimes circulatory failure. Most cases are caused by partial bronchial obstruction. Extrinsic bronchial obstruction may be caused by dilated pulmonary vessels, as in patent ductus arteriosus, total anomalous venous return, and other cardiac defects that cause increased pulmonary blood flow. Intrathoracic masses, such as bronchogenic cysts, extralobar sequestrations, enlarged lymph nodes, and neoplasm may also cause bronchial compression. Intrinsic bronchial obstruction is most often related to defects in the bronchial cartilage with absent or incomplete rings. These abnormal bronchi collapse during expiration, leading to distal air trapping and overinflation of the affected lobe. In regional, or segmental, pulmonary overinflation, only a small segment of the lung is hyperinflated, usually because of bronchial atresia.

Congenital Malformations of the Distal Lung Parenchyma

Pulmonary Agenesis and Aplasia

Pulmonary agenesis and aplasia represent two different forms of arrested lung development that results in the absence of the distal lung parenchyma.[175,178] Pulmonary agenesis is the complete absence of one or both lungs, including bronchi, bronchioles, vasculature, and respiratory parenchyma. In pulmonary aplasia, only rudimentary bronchi are present, each of which ends in a blind pouch, with no pulmonary vessels or respiratory parenchyma. Bronchial hypoplasia with variable reduction in lung tissue (hypoplasia) has also been reported.[178] The incidence of these anomalies is rare and represents failure of one of the primitive foregut branches to develop during the early, embryonic stage of lung development. Developmental arrest at a later stage may result in lobar agenesis or pulmonary dysplasia; some bronchial elements are present but no alveoli exist.

Bilateral agenesis of the lung is very rare and is caused by developmental arrest in the outgrowth of the respiratory primordium during the embryonic stage of lung development. Several cases of bilateral agenesis of the lung have been reported in the literature.[174] In one case, a trachea with 10 cartilaginous rings remained connected to the esophagus throughout its length. No lung buds or pleural cavities existed. In another case, the trachea was separated from the esophagus but ended blindly with only two cartilaginous rings. In other cases, branching morphogenesis was arrested at the bronchial bud stage.

Unilateral pulmonary agenesis is more common than bilateral pulmonary agenesis and may represent an imbalance in the growth of the lung buds. If balanced growth is not established between the left and right lung buds, then one side will develop normally whereas the other will fail completely (aplasia) or undergo limited development (dysplasia or hypoplasia). These defects arise early in lung development, when the respiratory primordium bifurcates into the right and left primitive lung buds at the end of the fourth week of gestation. These malformations suggest an absence or imbalance in the number of progenitor cells located in the two primitive lung buds that results in differential growth or arrest of lung development. Unilateral pulmonary agenesis may affect either lung, although the right side is more commonly affected. Usually no serious clinical consequences occur because of compensatory growth, or hyperplasia, of the contralateral lung. Pulmonary agenesis is frequently associated, however, with other congenital anomalies, such as tracheal stenosis, esophageal atresia, tracheoesophageal fistula, bronchogenic cysts, patent ductus arteriosus, tetralogy of Fallot, and anomalies of the great vessels.

Pulmonary Hypoplasia

Pulmonary hypoplasia is defined as deficient or incomplete development of the lungs and develops as a result of other abnormalities in the developing fetus, such as renal agenesis, dysplastic growth of the kidney, oligohydramnios, urinary outlet obstruction, loss or reduction of amniotic fluid because of premature rupture of membranes, diaphragmatic hernia, pleural effusion, congenital anomalies of the neuromuscular system, and chromosomal abnormalities, including trisomy 13, 18, and 21.[175,178,182] Many of these abnormalities cause a reduction in the volume of the pleural cavity, which physically restricts the growth, or expansion, of the peripheral lung. Disturbances in the production of fetal lung fluid, fetal breathing, loss of amniotic fluid, or external compression of the thoracic cavity can all cause defects in the growth, or expansion, of the distal respiratory parenchyma, resulting in a reduced number of alveoli.

In congenital diaphragmatic hernia, the pleuroperitoneal cavity fails to close.[178] This allows the developing abdominal viscera to bulge into the pleural cavity and stunts the growth of the lung. Usually the left side of the diaphragm is involved more often than the right, probably because the left pericardioperitoneal canal is larger and closes later than the right. The severity of the resulting pulmonary hypoplasia varies depending on the timing of the onset of compression. With early, severe compression of the lung, marked hypoplasia occurs, resulting in the affected lung being less than half the weight of the contralateral lung. This is accompanied by a decrease in alveolar number and size, decreased gas-exchange surface area, and a proportional decrease in the pulmonary vasculature. Infants with diaphragmatic hernia are seen with cyanosis and dyspnea soon after birth and have difficulty nursing properly. Often evidence exists of severe pulmonary hypertension, most likely because of an increased proportion of muscular arteries in the periphery of the lung, which results in increased pulmonary vascular resistance. Diaphragmatic hernia can be surgically corrected at birth. However, if the hernia has resulted in severe pulmonary hypoplasia, the newborn infant may die of pulmonary insufficiency, even if the hernia is repaired.

Oligohydramnios, or an insufficient amount of amniotic fluid, is thought to cause pulmonary hypoplasia by mechanical restriction of the fetal chest wall, that is, by allowing the uterine wall to compress the fetal thorax.[183] Beginning at approximately 16 weeks gestation, a substantial fraction of amniotic fluid is contributed by the fetal kidney. Therefore renal agenesis, or failure of the kidneys to form, results in oligohydramnios and, secondarily, in pulmonary hypoplasia. Reduced amniotic fluid for an extended period of time in the absence of renal anomalies, such as in prolonged rupture of the amniotic membranes, has also been associated with pulmonary hypoplasia.

Congenital Cysts of the Lung

Unlike bronchogenic cysts, bronchiolar cysts are in communication with the more proximal branches of the bronchial tree, as well as with distal alveolar ducts and alveoli, although the connections may be small and tortuous.[175,178] Bronchiolar cysts are lined with ciliated pseudostratified epithelium and may have mucous glands, smooth muscle, and cartilage in their walls. These cysts are usually multiple and restricted to a single lobe. They may be filled with air, fluid, or both. Alveolar cysts are lined with cuboidal or squamous epithelium. No muscle or cartilage appears in the walls, but connective tissue hyperplasia may exist. Both types of

cysts affect the right lung more frequently than the left and lower lobes more frequently than upper lobes. Because congenital pulmonary cysts may be difficult to distinguish from acquired cysts, the pathogenesis of these structures is still obscure.

Congenital Cystic Adenomatoid Malformations

Congenital cystic adenomatoid malformations (CCAM) are most often found in the lungs of infants and have features of both immaturity and malformation of the small airways and distal lung parenchyma.[175,178-180] Five types of CCAM exist, which are classified on the basis of gross appearance and histologic features. Type 0 lesions are rare and are composed of 0.5-cm, bronchial-like, cystic structures with abundant cartilage in their wall. Type 1 lesions are the most common, accounting for more than 50% of the cases and are composed of one or more large cysts ranging from 3 to 10 cm in diameter. These lesions contain fibrous septa lined by pseudostratified, ciliated columnar or cuboidal cells, with clusters of mucus-filled cells resembling goblet cells. Cartilaginous foci are also present in 5% to 10% of these lesions. Type 2 lesions are the second most common and are characterized by evenly spaced, uniform cysts that are usually less than 2 cm in diameter. These bronchiolar-like structures are lined by cuboidal and/or ciliated columnar cells. Striated muscle may be present in the septa or adjacent to the cysts. Type 3 lesions are composed of a solid mass of tissue with multiple, microscopic cysts of less than 0.2 cm that are lined by cuboidal or low columnar epithelial cells. Type 4 lesions are found in the peripheral lung and are characterized by large (up to 7 cm in diameter), thin-walled cysts that are lined by alveolar epithelial cells. Immunohistochemical analysis of cytodifferentiation markers for specific pulmonary epithelial cells revealed that CCAM types 1, 2, and 3 contained clusters of GRP-positive neuroendocrine cells and CCSP-positive Clara cells, demonstrating a bronchiolar origin for these cysts.[184] The epithelial lining of CCAM type 4 cysts was immunopositive for a type I cell–associated surface antigen and the type II cell, surfactant-associated proteins, SP-A, SP-B, and SP-C.[184] These findings suggest that CCAM types 1, 2, and 3 arise during the embryonic and pseudoglandular stage of branching morphogenesis, whereas CCAM type 1 arises during the canalicular and/or saccular stage of acinar development.

Alveolar Capillary Dysplasia

Alveolar capillary dysplasia, with misalignment of the pulmonary veins, is characterized by inadequate vascularization of the alveolar parenchyma resulting in a reduced number of capillaries in the alveolar wall.[178,180] In addition, the pulmonary lobules are malformed, and the pulmonary veins are displaced, or "misaligned," in that they course with the pulmonary arteries in the peribronchiolar connective tissue instead of in the interlobular septa. This malformation causes persistent pulmonary hypertension in the neonate and is fatal.

Congenital Pulmonary Lymphangiectasis

Congenital pulmonary lymphangiectasis is an extremely rare condition, consisting of markedly distended or dilated pulmonary lymphatics, which are found in the bronchovascular connective tissue, along the interlobular septa, and in the pleura.[180] The dilated lymphatic channels are thin walled, lined by endothelial cells, and form a network of communicating channels. This condition has been associated with Noonan, Ulrich-Turner, and Down syndromes. Males are more frequently affected than females (2:1). Pulmonary lymphangiectasis can be divided into three main categories: (1) primary, (2) secondary, and (3) generalized lymphangiectasis.[180] Primary lymphangiectasis is a fatal developmental defect in which the pulmonary lymphatics fail to communicate with the systemic lymphatics. Affected infants present with RDS and pleural effusions and die shortly after birth. Secondary pulmonary lymphangiectasis is associated with cardiovascular malformations, including anomalous pulmonary venous return, atrioventricular valve defects, ostium secundum, pulmonary stenosis, ventricular septal defect, mitral atresia, hypoplastic left heart, cor triatriatum, and atresia of the common pulmonary veins.[178] Generalized pulmonary lymphangiectasis is characterized by proliferation of the lymphatic spaces and occurs in the lung as part of a systemic abnormality in which multiple lymphangiomas are also found in the bones, viscera, and soft tissues.

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79 Regulation of Alveolarization

Jeanne M. Snyder

The alveolus is the primary functional unit of the lung, the site at which oxygen and carbon dioxide are exchanged between inspired air and the blood. Alveolar growth is optimized to provide an enormous surface area throughout which the blood in pulmonary capillaries circulates within a few micrometers of the inspired air. The capillary blood and the inspired air are separated by a barrier that consists of the thin wall of an endothelial cell on the capillary side, a fused basal lamina, and the attenuated cytoplasm of an epithelial cell on the alveolar side (specifically, an alveolar type I epithelial cell).[1] A set of adult human lungs contains approximately 300×10^6 alveoli, most of which are formed by 18 months of age.[2,3] Further growth and development of the distal lung parenchyma involve the thinning of the alveolar wall, an increase in capillary density, and a modest increase in the diameter of the alveoli.[2,3]

The postnatal formation of alveoli is impaired in bronchopulmonary dysplasia (BPD), a disease of prematurity that is thought to be caused by injury resulting from mechanical ventilation, hyperoxia, and inflammation.[4,5] Husain and co-workers[4] have described the "new" BPD as a chronic lung disease, prominent in very low-birth-weight infants who have been treated with surfactant replacement therapy, that is characterized by impaired alveolarization. A similar impairment has been observed in experiments in which premature baboons were subjected to mechanical ventilation with elevated oxygen levels; the lungs of these animals had alveoli that were decreased in number and larger than normal.[6] It is hypothesized that mechanical ventilation or exposure of the lung to high levels of oxygen very early in development leads to impaired alveolarization and eventually chronic lung disease.[5] Although surfactant therapy has lessened the impact of some aspects of the lung injury (fibrosis and inflammation), it has not affected the impairment of alveolarization.[5]

When one lung is removed, new alveoli can form in the remaining lung.[7,8] The number of alveoli in the lung also appears to change in response to physiologic stimuli such as starvation, age, oxygen tension, or hormonal status.[9] In addition, it has been reported that treatment with retinoic acid can increase the number of alveoli in neonatal and adult rats.[10,11] Still not fully understood, however, are the processes that regulate the formation of new alveoli in the developing lung, or how to affect these processes in humans. The ability to program the human lung to form new alveoli is a major and elusive goal in pulmonary biology.

STRUCTURE OF THE ALVEOLAR WALL

The alveolar wall consists of a connective tissue core in which many capillaries are suspended.[1] The capillary bed of the lung alveoli probably is the largest in the body. The cells present within the alveolar wall (i.e., primarily fibroblasts and myofibroblasts) produce an extracellular matrix that is particularly rich in elastin and also contains collagen, proteoglycans, and glycosaminoglycans.[12]

The surface of the alveolar wall is lined by two epithelial cell types, alveolar type I and type II.[13] Type I cells are extremely attenuated squamous cells whose cytoplasm forms part of the

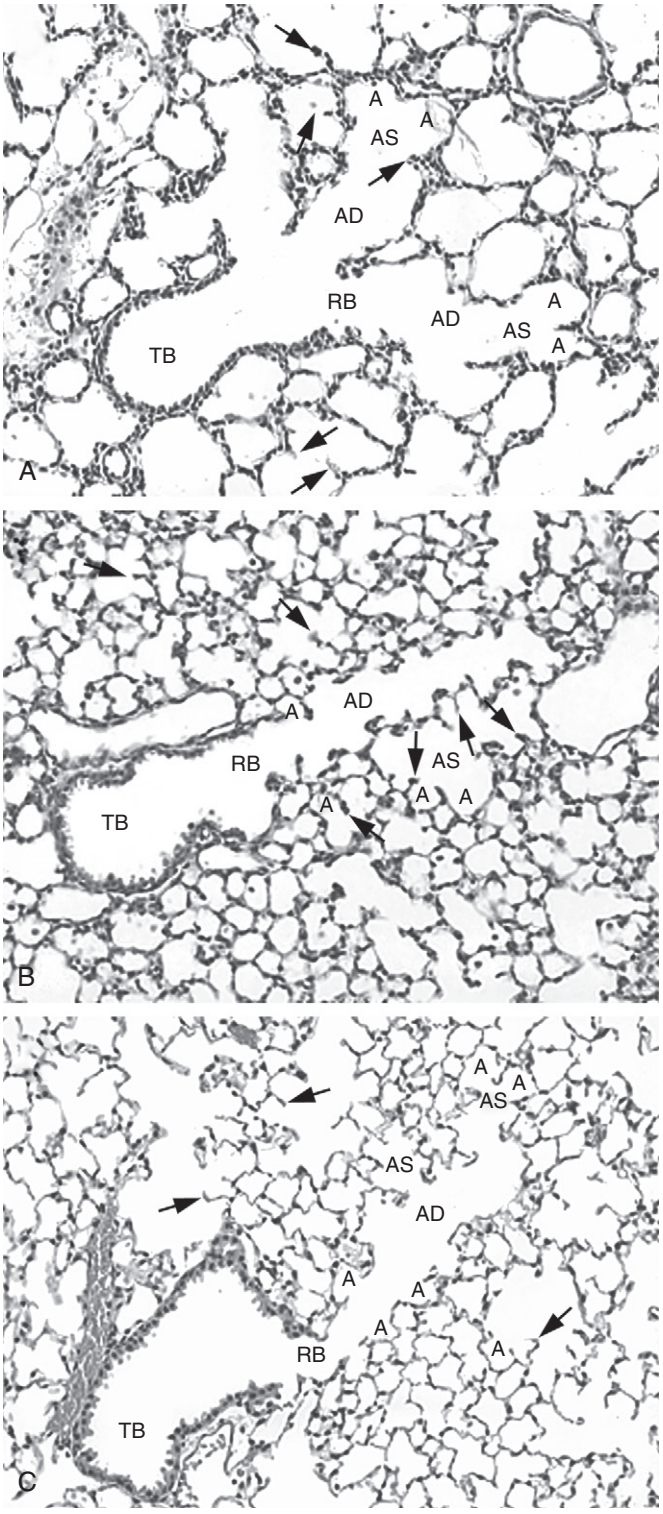


Figure 79-1 Alveolarization in mouse lung tissue. Mouse lung tissue was harvested at postnatal days 0, 12, and 56 (**A, B,** and **C,** respectively) and then inflation-fixed at constant pressure, paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Terminal bronchioles *(TB)* in the lung open into respiratory bronchioles (RB), which in turn open into alveolar ducts *(AD)* ending in alveolar sacs *(AS).* Alveoli *(A)* are found at every level of the respiratory acinus. **A,** At day 0 postnatally, the walls of the alveolar ducts and sacs are the primary septa. Secondary septa *(arrows)* grow into the lumen and begin dividing the primitive alveolar duct and sac air spaces into alveoli. **B,** By 12 days postnatally, the number of secondary septa *(arrows)* has increased dramatically, and many alveoli have been formed, thereby increasing the surface area in the lung available for gas exchange. Note the decrease in the wall thickness of the alveoli from day 0 to day 12. **C,** At 56 days postnatally, the alveolar septa *(arrows)* are much thinner and longer than observed at the earlier postnatal stages of development.

air–blood gas exchange barrier. Approximately 90% of the alveolar surface is lined by alveolar type I cells.[14] Type II cells are cuboidal secretory cells, often located at the corner of an alveolus, that produce pulmonary surfactant, a lipoprotein substance that lowers surface tension in the alveolus and plays an important role in lung host defense mechanisms.[15] Although type II cells account for only a small proportion of the alveolar surface area, approximately equal numbers of alveolar type I and II cells are present in the alveolus.[14]

FORMATION OF ALVEOLI IN THE DEVELOPING LUNG

In the human, some alveoli form prenatally (approximately 20% of the eventual total); however, most alveoli form after birth.[2,3] Studies suggest that most human alveoli are formed within the first 18 months.[16,17] Much of the current understanding of the processes involved in alveolar formation is derived from studies in rodents, primarily the rat and the mouse[9] (Figure 79-1). In these species, alveolarization occurs during the first 2 weeks of postnatal life.[9]

OVERVIEW OF EARLY LUNG DEVELOPMENT

Pseudoglandular Phase

Very early lung development, before the formation of alveoli, is characterized by branching morphogenesis, which occurs during the pseudoglandular phase.[18] The lung buds and trachea originate as diverticula from the endoderm-lined foregut. The lung buds then undergo repeated dichotomous branching to give rise to the conducting airways of the lungs. It has been estimated that approximately 22 orders of branching airways are formed by around 16 weeks of gestation in the human. The branching ducts in the developing lung are lined by tall columnar epithelial cells that begin to differentiate into ciliated and secretory cells proximally but remain undifferentiated at the distal aspects of the branching tree system.[13] Within the epithelium of the conducting airways, at their most distal aspect, are cellular progenitors that will give rise to the alveolar region of the lung.[19]

Canalicular Phase

From 16 to 24 weeks gestation in the human, during the canalicular phase of lung development, the distal aspect of the branching tubes in the developing lung undergoes considerable remodeling.[18] The epithelium that lines the ducts changes from tall columnar to low columnar/cuboidal in shape. The number of blood vessels, primarily capillaries, in the connective tissue between the ducts increases strikingly. Starting at weeks 20 to 22, some of the epithelial cells begin to differentiate into alveolar type II cells, which synthesize and secrete pulmonary surfactant.[13] In addition, the distal lung epithelial cells that are close to the underlying capillaries begin to thin and differentiate into alveolar type I cells.[13]

Saccular Phase

The terminal portion of the branching duct system in the embryonic lung lengthens and will eventually give rise to the alveolar ducts and alveolar sacs, the spaces from which true alveoli will form via septation.[9,18] This phase of lung development, called the saccular phase, extends from approximately 24 weeks to term in the human.[18] Simultaneously with changes in the structure of the epithelium-lined ducts, profound remodeling occurs in the connective tissue surrounding the primitive lung ducts. The relative amount of ductal lumen (the future air space) in the distal lung tissue increases dramatically, while the relative amount of connective tissue (both cells and extracellular matrix components) simultaneously decreases as a component of the distal lung tissue.[18]

FORMATION OF ALVEOLI IN THE DEVELOPING LUNG

The formation of pulmonary alveoli in the human fetus begins prenatally; however, most alveoli are formed after birth (approximately 80%).[16,17] Premature infants who are born when their lungs are quite immature structurally (i.e., in the range of 24 to 28 weeks) are still capable of adequate respiration to maintain viability.[5] In guinea pigs, sheep, pigs, and cows, almost all alveolarization occurs in utero.[9] In rodents such as mice and rats, alveoli are formed during the first few weeks of postnatal life.[20,21] During development, the formation of alveoli increases the pulmonary gas exchange surface area in proportion to the growth of the whole organism.[9] The mechanisms by which the growth of an organism promotes a corresponding increase in the formation of its lung alveoli are not well understood.[9]

Beginning at the end of gestation in the human, and postnatally in rodents such as mice or rats, the terminal sacs in the developing lung begin to undergo septation to form true alveoli.[9,22] The conducting airway system ends at the level of the respiratory bronchiole, which is a structure characterized by occasional alveoli interrupting the airway wall.[1] The epithelium of a respiratory bronchiole is columnar proximally and gradually undergoes a transition to the typical alveolar epithelium that lines the alveolar ducts, alveolar sacs, and alveoli. The entire functional unit (the respiratory acinus) consists, in order, of a respiratory bronchiole (several orders of respiratory bronchioles are possible), alveolar ducts (again, several orders or branches of alveolar ducts can be present), and alveolar sacs.[1] Alveolar ducts can constitute a major portion of the gas exchange tissue in the lung, up to approximately 30% in some species.[23] Alveoli branch from the alveolar ducts, either singly or in clusters from an alveolar sac, which is the terminus of the alveolar duct. Alveoli are present at every level of the respiratory acinus—in the respiratory bronchiole, alveolar duct, and alveolar sac. It is believed that all of these alveoli are equally functional.

In the prenatal human lung and in the postnatal mouse lung, alveoli form by the growth of secondary septa that project from the wall of the terminal saccule.[9] The secondary septa are evenly spaced and extend into the saccule to a similar extent. The growth of secondary septa into the lumen of the terminal saccule is mediated, at least in part, by the proliferation of fibroblasts.[24] A subset of interstitial fibroblasts in the developing lung is characterized by cytoplasmic lipid droplets.[25] The function of these cells is not fully understood, but they probably play an important role in the morphogenesis of the alveolar wall.[25] At the tip of the secondary septum is a myofibroblast, a cell type that produces elastin and other important extracellular matrix components present in the alveolar wall.[26] This particular cell type probably plays a key role in alveolarization, because the absence of myofibroblasts results in a failure to produce alveoli in postnatal life, with an emphysematous lung phenotype.[27,28]

The connective tissue core of the secondary alveolar septa is characterized by abundant elastin, probably laid down by the myofibroblast.[29] The elastin fibers are arranged in an orderly and predictable manner within the alveolar septa.[30-32] Elastin gene expression peaks during the period of postnatal alveolarization.[33,34] The cross-linking of elastin monomers is a critical process in alveolarization, because inhibition of this process leads to a decreased surface area in the lung.[35] Elastin fiber deposition also involves the coordinated expression of fibrillins, emelin, fibulin, and microfibril-associated glycoproteins.[29] In addition, type I, III, and IX collagens are produced by connective tissue cells and are deposited in the alveolar wall during the formation of the secondary septa, where they convey mechanical strength.[36] Glycosaminoglycans, such as hyaluronic acid, chondroitin sulfate, and heparan sulfate, also are present in the alveolar wall and make important contributions to its mechanical properties.[37] Finally, proteoglycans, adhesive glycoproteins such as fibronectin, and components of the basal lamina, including laminins and type IV collagen, also are produced and are laid down during alveolarization in a regulated and integrated manner.[38]

REGULATORS

Vascular Endothelial Growth Factor

The process of alveolarization includes the morphogenesis of an elaborate pulmonary capillary bed in the distal lung, which must form in concert with the differentiation of the alveolar epithelium, and the eruption of secondary septa. The growth and differentiation of pulmonary vasculature begin in the fetal lung coincident with the canalicular and saccular stages of lung development.[39] Initially, the capillaries in the wall of the septa are a double capillary system; that is, one capillary is associated with each surface of the forming alveolar septa.[40] As alveolarization proceeds, however, the alveolar wall thins, and a single capillary system forms in the alveolar wall, with the capillaries coming to lie very close to one of the wall surfaces.[40]

Vascular endothelial growth factor (VEGF) is a growth factor that acts as a mitogen for endothelial cells.[41] VEGF stimulates angiogenesis and vasculogenesis and also regulates endothelial permeability.[41] VEGF binds to at least two receptors: Flk-1 (VEGF receptor 2) and Flt-1 (VEGF receptor 1).[41] VEGF and its receptors are expressed in the developing lung in a coordinated fashion that peaks at the end of gestation and during early neonatal life.[42,43] Several VEGF isoforms of different molecular weights are recognized; however, VEGF 188 predominates in the fetal lung.[43] Transgenic mice that overexpress VEGF in alveolar epithelial cells exhibit disrupted lung development—that is, disrupted branching morphogenesis, hypertrophy of blood vessels, decreased number of terminal saccules, and decreased connective tissue.[44]

VEGF increases cell division in human fetal lung type II cells.[45] In utero administration of anti-VEGF receptor 2 antibodies has been shown to delay fetal mouse lung development.[46] Conversely, lung development in mice, as assessed by the production of surfactant, structural changes, and survival, was accelerated by the prenatal or postnatal administration of VEGF.[46] In a study conducted in adult rats, treatment with a VEGF receptor blocker, SU5416, led to an enlargement of alveoli and a decrease in alveolar surface area—a pattern similar to that observed in emphysema.[47] When the same VEGF inhibitor was administered to newborn rats, alveolar formation was significantly impaired.[48] The inhibitory effects of treatment with SU5416 for 24 hours on the second day after birth persisted until the age of 3 to 4 months.[49] Other inhibitors of angiogenesis, such as thalidomide and fumagillin, administered to neonatal rats during the peak period of alveolarization, also inhibit the formation of alveoli.[49] Taken together, these results suggest that VEGF probably plays a key role in the process of alveolarization.

The growth of pulmonary capillaries and the formation of alveoli during the period of alveolarization are closely linked in the postnatal lung. The functional relationship between these

processes is unknown, but defects in the formation of blood vessels and alveolarization are characteristic of BPD.[4,50] In a primate model of BPD, in the premature baboon, decreased numbers of endothelial cells and a disrupted microvasculature were observed in the distal lung.[6] Bhatt and co-workers[51] observed both decreased numbers and an abnormal distribution of capillaries in the alveolar septa of patients with BPD. The lung tissue of infants with known BPD also was characterized by decreased VEGF and VEGF-2 receptor gene expression.[51] Other animal models in which alveolarization has been impaired also are characterized by decreased numbers of alveolar capillaries (e.g., in neonatal rats exposed to high oxygen and in premature ventilated lambs).[23,52] The role of vascular growth factors in hyperoxia-induced injury in the developing lung has been reviewed by D'Angio and Maniscalco.[53]

Other Regulators of Angiogenesis

In addition to VEGF and its receptors, many other mediators of angiogenesis and vasculogenesis are known whose roles in lung development are still relatively unexplored. For example, angiopoietins, ephrins, and semaphorins, all of which have been shown to play a role in angiogenesis in other systems, are present in the developing lung and may play an important role in alveolarization.[54-56] Angiopoietins 1 to 4 bind to Tie 2 receptors in endothelial cells and act together with VEGF and its receptors to influence angiogenesis.[54] Angiopoietin 1 is expressed in the developing lung but apparently does not change in concentration during development.[57,58] Ephrins are growth factors that bind to tyrosine kinase–linked receptors and are involved in vasculogenesis and angiogenesis.[55] The role of these growth factors and their receptors in lung development is unknown. Semaphorins are a more recently described class of growth factors, initially noted in the central nervous system, that bind to receptors called neuropilins.[56] Neuropilins may act as co-receptors with VEGF receptor 2 and play a role in angiogenesis.[56] Both semaphorins and neuropilins have been detected in the developing lung.[59] Blockade of neuropilins in the newborn rat results in defects in alveolarization.[60]

Platelet-Derived Growth Factors

Platelet-derived growth factors (PDGFs) are dimers of A and B polypeptide chains that bind to two receptor tyrosine kinases, PDGF-Rα and PDGF-Rβ.[61] PDGF is a potent mitogenic factor for connective tissue cells.[61] PDGF-A is expressed by epithelial cells in the developing lung.[62] PDGF-A homozygous gene–deleted mice die early in the postnatal period because of failure of postnatal alveolarization.[27,28] The PDGF-A–knock-out mice lack alveolar myofibroblasts that express PDGF-Rα in their primary septa.[27,28] In addition, these mice do not have a normal elastin deposition pattern in their lungs and do not form secondary septa.[27,28] These data are suggestive that the PDGF-Rα–positive myofibroblasts in the developing postnatal lung play a major role in alveolar septation and that PDGF-A is an important regulator of alveolarization.[63]

Glucocorticoids

Glucocorticoids are steroid hormones produced by the adrenal gland in response to adrenocorticotropic hormone, a peptide produced by the pituitary.[64] Release of adrenocorticotropic hormone from the pituitary is, in turn, regulated by another peptide, corticotropin-releasing hormone (CRH), which is produced in the hypothalamus.[64] Glucocorticoids bind to glucocorticoid receptors (GRs), which are members of the steroid hormone receptor family of transcription factors.[64] Antenatal glucocorticoids, administered to the mother at risk for delivering prematurely, can accelerate lung development and reduce the risk of neonatal respiratory distress syndrome.[65,66] Respiratory distress syndrome is caused by surfactant deficiency; thus glucocorticoids presumably act by increasing the production of surfactant in differentiated alveolar type II cells.[64] However, glucocorticoids have many other effects on the developing lung.[64]

Antenatal glucocorticoids accelerate structural maturation of the lung—in particular, thinning of the alveolar wall.[67,68] In rats, which undergo alveolarization postnatally, glucocorticoids inhibit alveolarization.[67] The formation of secondary alveolar septa is impaired in the lungs of glucocorticoid-treated neonatal rats, resulting in fewer and larger alveoli in the mature lung.[67] Similar findings were obtained when glucocorticoids were administered to the rats antenatally.[69] The glucocorticoid effects on lung structure appear to be permanent, at least in the rat.[69] The effects of glucocorticoids on primate lung development are consistent with observations made in the rat model; that is, glucocorticoids impair alveolarization in the developing lung.[70]

The concept that glucocorticoids play a key role in lung development is supported by the observation that mice that are CRH gene–deleted die of respiratory failure resulting from inadequate lung development.[71] The effect of the CRH gene deletion on lung development probably is structural, because surfactant components are not as strongly affected by the CRH gene deletion.[71] A similar observation was made in GR-deficient mice: The effects of the GR gene deletion include immature structural development of the lung, whereas surfactant components such as the surfactant proteins are not as strongly affected.[72]

BPD is caused by multiple factors; however, mechanical ventilation, oxygen toxicity, and inflammation have key roles in the pathophysiology of this disease.[73] Glucocorticoids are effective in treating BPD, perhaps because of their antiinflammatory effects.[64] The long-term effects of glucocorticoid treatment of premature infants with BPD are still not fully understood and are of great concern. It is known, however, that the lungs of infants with BPD have fewer and larger alveoli, so the use of glucocorticoids, which impair alveolarization, to treat these infants is inherently problematic.[4,74]

Retinoic Acid

Retinoic acid plays a key role in the differentiation and maintenance of differentiation of all respiratory epithelia, from the trachea to the alveolus.[75,76] All-*trans* retinoic acid, which is the best-characterized, most abundant, biologically active retinoid, binds to retinoic acid receptors (RARs) and to retinoid X receptors (RXRs).[77] RARs and RXRs are members of the steroid hormone receptor family of transcription factors.[77] Three RAR genes (RARα, -β, and -γ) and three RXR genes (α, β, and γ), each of which produces several isoforms, have been identified.[77] The RARs form heterodimers with RXRs and bind to *cis*-acting elements in retinoic acid–responsive genes.[77]

All-*trans* retinoic acid has been shown to play a key role in regulating alveolarization in rats and mice.[10,11,78] In the initial report by Massaro and Massaro concerning the effects of retinoic acid, postnatal rats were treated with all-*trans* retinoic acid or dexamethasone.[10] Dexamethasone administration to postnatal rats inhibits alveolarization.[79] Treatment with retinoic acid alone caused an increase in the number of alveoli, but it had no effect on lung gas exchange surface area when compared with alveoli in control animals.[10] In addition, retinoic acid abolished many of the inhibitory effects of glucocorticoids on alveolarization.[10] Specifically, treatment with retinoic acid plus glucocorticoid resulted in greater numbers of alveoli and greater surface area (corrected for body weight) in the lung than in the glucocorticoid-alone condition.[10] In another study by the same investigators, adult rats were treated with intratracheal elastase to create a model of emphysema.[11] When the rats were treated with all-*trans* retinoic acid, the effects of the elastase on the number of alveoli and amount of gas exchange surface area were reversed.[11] It has been

reported that treatment of newborn rats with retinoic acid during exposure to hyperoxia protected their lungs from the deleterious effects of too much oxygen on alveolar formation.[80]

Morphometric studies in transgenic mice revealed that the lungs of RARβ gene-deleted mice have more, but smaller, alveoli.[78] Gas exchange surface area, however, was the same in the RARβ gene–deleted mice and and in wild-type mice.[78] In the same studies, it was shown that treatment of postnatal rats with an RARβ agonist impairs the formation of alveoli.[78] Together, these observations suggest that RARβ may be an endogenous inhibitor of alveolarization.[78] In another transgenic mouse model, McGowan and co-workers[81] showed that RARγ probably mediates some of the effects of retinoic acid on alveolarization. RARγ gene–deleted mice had lungs with fewer and larger alveoli than in lungs of age-matched wild-type mice.[81] The effects of RARγ gene deletion were magnified in mice that were heterozygous for the deletion of RXRα.[81] Taken together, these data support a role for RARγ/RXRα heterodimers in the regulation of alveolarization.[81]

Elastin is an important component of the alveolar wall that is involved in alveolarization and is regulated by all-*trans* retinoic acid.[29,82] Retinyl esters increase in the lung during development and decrease thereafter.[83] The retinyl esters are concentrated in lipid-laden fibroblasts, a prominent cell type in the connective tissue component of the primary septa in the developing lung.[84] McGowan and co-workers[82] showed that endogenous retinoids in the developing lung increase lung elastin gene expression. Retinoids also increase elastin gene expression in vitro in isolated, cultured lipid-laden fibroblasts.[82] In RARγ gene–deleted mice, elastin messenger RNA (mRNA) levels in lipid-laden fibroblasts isolated from postnatal lung were decreased in comparison with those in lung fibroblasts from wild-type, control mice.[81] In addition, the number of elastin fibers was markedly decreased in the alveolar walls of lungs from RARγ gene–deleted mice in comparison with that in the lungs of control animals.[81] Taken together, these data suggest that retinoic acid regulates elastin gene expression and elastin fiber deposition, two important events in alveolarization in the postnatal lung.

The plasma concentration of vitamin A (retinol, the metabolic precursor to all-*trans* retinoic acid) is significantly lower in very low-birth-weight, premature infants.[85] Some of the histopathologic characteristics of lung tissue in vitamin A–deficient models and in BPD are similar.[75,76] Kennedy and co-workers[86] performed metaanalysis of four small clinical trials in which vitamin A supplementation in very low-birth-weight infants decreased mortality and the incidence of BPD. The results of a more recent, large clinical trial suggest that vitamin A supplementation in extremely low-birth-weight infants decreases their risk of subsequent chronic lung disease.[87] These clinical data illustrate the potential usefulness of retinoids in accelerating lung development and in decreasing the incidence of BPD in newborn infants. One of the beneficial effects of the clinical use of retinoids may be in promoting alveolarization; however, this point has yet to be established.

The loss of sufficient numbers of alveoli in the adult lung leads to inadequate gas exchange surface area and the ensuing pathophysiologic changes of emphysema.[88] No clinical treatment that promotes the regeneration of alveoli in the adult lung is available as yet. In light of observations in rats indicating that retinoic acid treatment leads to de novo alveolarization, clinical trials in which all-*trans* retinoic acid is administered to patients with emphysema have been performed.[89] In preliminary findings, retinoid treatment was well tolerated and was associated with relatively few side effects.[89] Unfortunately, no clinical benefits of the retinoid treatment were observed in the feasibility study.[90] The use of a retinoic acid receptor gamma agonist (Palovarotene) is currently being investigated for the treatment of emphysema in clinical trials.[91]

Estrogen and Testosterone

Lung development is influenced by the sex of the fetus: Lung maturation has been shown to be delayed in male fetuses in comparison with that in females.[92] The incidence of respiratory distress syndrome is greater and the infant mortality rate is higher in males than in females.[93] It has been hypothesized that androgens, present primarily in the male, delay lung development in the male fetus.[92] However, the lungs of androgen receptor–negative male mice have been shown to have the same body weight–adjusted surface area as in wild-type male mice.[94]

Some evidence also suggests a sex-specific difference in the formation of alveoli in the neonatal lung.[95] Massaro and co-workers[95] reported that female rats and mice (60 days old) have smaller and more numerous lung alveoli and that their body weight–adjusted lung surface area is significantly greater than in male rats.[95] In another study, these same investigators showed that estrogen mediates the sex-linked effects on lung structure in rats.[94] Treatment of female newborn rats with androgens did not alter their lung structure.[94] Taken together, these data suggest that estrogen regulates alveolarization in the postnatal lung.

During pregnancy, the fetus (male or female) is exposed to very high levels of estrogens, which drop dramatically after birth.[96] Some clinicians have suggested that preterm infants may benefit from estrogen therapy. In fact, a beneficial effect of estrogen therapy in terms of the incidence of chronic lung disease has been reported.[97] It is not known whether this effect reflects differences in alveolus formation.

Nutrition

Caloric restriction reduces the metabolic rate and physiologic requirement for oxygen.[98] Studies performed primarily in rats showed that caloric restriction in adults increases the size of the alveoli and reduces the lung gas exchange surface area available for respiration.[99] Refeeding apparently reverses these changes.[99] These observations were confirmed in a morphometric study in adult mice.[100] Observations in the inhabitants of the Warsaw Ghetto during World War II are consistent with a similar process occurring in humans; that is, an emphysema-like morphology was observed in the lungs of severely malnourished adults.[101]

Massaro and co-workers[9] hypothesized that during periods of low oxygen requirements (e.g., caloric restriction), alveoli are destroyed, whereas during refeeding, which would increase oxygen needs, alveolar regeneration is activated.

Oxygen

The lung alveolar surface area functions as a gas exchange unit, and oxygen has a potent influence on the formation of alveoli in the developing lung.[9] Dimensions of the surface area available for gas exchange are directly related to the oxygen needs of the organism.[102] In general, in newborn rats undergoing alveolarization, hyperoxia tends to decrease the alveolar surface area.[9] Burri and Weibel[103] showed that neonatal rats reared in 40% oxygen had decreased alveolar surface area when compared with air-breathing control animals. Similar observations were made by Randell and co-workers[104] in a study in which neonatal rats were reared in 95% oxygen for the first 7 days after birth. Exposure to high oxygen concentrations for 7 days followed by rearing in room air until the age of 40 days also resulted in impaired alveolarization, that is, a decreased alveolar surface area in the oxygen-exposed animals in comparison with that in control animals reared in room air.[23]

The signaling pathway in the developing lung that senses oxygen levels and regulates alveolar surface area is unclear at present. However, it has been shown that hyperoxia inhibits the expression of VEGF in neonatal rabbit lung.[105] Hyperoxia also increases apoptosis in the distal lung of neonatal mice[106] and is a potent

regulator of alveolar formation.[9,107] Blanco and co-workers[108] and Massaro and associates[109] showed that rearing newborn rats in 13% oxygen results in fewer alveoli and decreased gas exchange surface area.

Inflammation

Alveolarization in premature human newborns is thought to be inhibited by many factors, including mechanical ventilation, oxygen, inadequate nutrition, glucocorticoids, and, possibly, infection. All of these stimuli can result in inflammation. Thus many investigators have hypothesized that inflammatory mediators impair alveolarization.[110-112] Intrauterine infection is a common cause of preterm birth, and it is now apparent that such infection also may influence postnatal lung development.[113] A final common pathway in many inflammatory states is the activation of matrix metalloproteinases, a process that results in the destruction of extracellular matrix components and consequent changes in lung structure and function.[114] An important function of the pulmonary surfactant proteins SP-A and SP-D may be to regulate inflammation in the lung alveolus.[115] In this regard, emphysema develops postnatally in SP-D gene–deleted mice and SP-A/SP-D double gene–deleted mice.[116,117] In addition, inflammation is now closely linked to the destruction of alveoli in adult lung that frequently is observed in chronic obstructive pulmonary disease.[118-120]

Other Mediators of Alveolarization

Treatment of newborn rats with thyroid hormone accelerates the formation of lung alveoli and results in smaller alveoli and an increase in gas exchange surface area.[121] Conversely, a drug that inhibits thyroxine, propylthiouracil, impairs the formation of alveoli and decreases respiratory surface area.[121] Thus thyroid hormone should be added to the list of mediators that influence the formation of lung alveoli in the developing lung.

Fibroblast growth factors (FGF) are a family of peptides that play an important role in many aspects of lung development.[122] These factors bind to one of several tyrosine kinase receptors (i.e., FGFRs).[122] In mice that are deficient in FGFR-3 and FGFR-4, formation of normal alveoli during postnatal life does not occur.[123]

Many other growth factors, growth factor receptors, and transcription factors are known to regulate aspects of lung development.[19,124,125] Many of these mediators affect lung development at multiple sites and at several stages of development. It is therefore difficult to ascribe a specific role to some of these factors in the formation of alveoli. Many of these regulatory factors, however, probably do influence the formation of alveoli in the developing lung.

CONCLUSIONS AND FUTURE DIRECTIONS

Our understanding of the formation of pulmonary alveoli has advanced, but a comprehensive and detailed picture will require additional research. Although careful morphologic and morphometric descriptions of the alveolarization process are available for many species, human data remain incomplete. The processes of formation and destruction of alveoli are known to be responsive to several physiologic stimuli including nutritional status, oxygenation, and removal of one lung or part of a lung (Box 79-1). In addition, strong evidence points to the influence of regulatory factors, including retinoic acid, glucocorticoids, estrogen, and growth factors such as VEGF and PDGF, on the formation of alveoli in the developing lung. Nevertheless, no specific therapies have been clinically proved to increase the formation of alveoli in humans, either in the neonate or in the adult lung.

BOX 79-1
Mediators of Alveolarization

Positive Regulators
- Vascular endothelial growth factor[47]
- Neuropilins[61]
- Platelet-derived growth factor A[28]
- Retinoic acid[11,12]
- Estrogen[92]
- Thyroid hormone[119]
- Fibroblast growth factor[121]
- Nutrition[97]

Negative Regulators
- Glucocorticoids[68]
- Oxygen[102]
- Inflammation[109]

The transgenic mouse model has been extremely fruitful for studying lung development, including the formation of alveoli. However, the study of the formation of alveoli in the lung would greatly benefit from the availability of an in vitro model of alveolarization, as well as from the establishment of biochemical end points that could be used to assess the degree of alveolarization in both in vitro and in vivo model systems. A better understanding of the regulatory link between pulmonary vasculogenesis and alveolarization also is greatly needed. Finally, the hierarchy of genes that influence alveolarization, starting with the transcription factors that may act as master regulators, requires further elucidation. Powerful new tools such as microchip gene array analysis and the availability of mouse genome sequences can be expected to facilitate the search for the regulators of alveolarization.[126,127]

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Richard Harding and Stuart B. Hooper

Physiologic Mechanisms of Normal and Altered Lung Growth Before and After Birth

Lung growth both before and after birth is an important determinant of respiratory health in that appropriate and timely development is essential to produce a system of airways that permit effective exchange of respiratory gases, from infancy through to adult life. Lung growth in the fetus has a major impact on the ability of the neonate to make a smooth transition from fetal to postnatal life. Normal lung growth after birth is necessary to enable the lung to meet increasing metabolic demands during infancy and childhood and into adulthood. During its development, the lung must acquire an intricate system of airways allowing low-resistance airflow to and from the respiratory zone; its internal surface area must be large and closely apposed to a dense vascular network with a high level of blood flow. In terms of its mechanical properties, lung tissue must permit easy expansion during inspiration but retain sufficient recoil to drive expiration, and surfactant is required to prevent collapse at end-expiration. It is now apparent that the physiologic processes controlling lung growth before and after birth are quite different, although the molecular mechanisms are likely to be similar. This chapter presents an overview of the physiologic control of lung growth before and after birth, as well as pathophysiologic processes that perturb normal lung growth.

FETAL LUNG GROWTH

During fetal life, the future air spaces of the lungs are filled with a unique liquid that is produced by the lung as a result of net transepithelial chloride and sodium flux.[1] This fetal lung fluid (FLF) plays a critical role in lung development by maintaining the future air spaces in a distended state; it also limits the entry of amniotic fluid into the lungs, which can have damaging effects. The volume of FLF within the future air spaces, and its flux to and from the lower airways, are influenced by fetal muscular activity as well as by fetal posture and other factors that influence fetal transpulmonary pressure.[2] By maintaining this distended state, FLF serves as an internal "splint" around which the distal air spaces of the lungs develop.[1-3] Without this underlying degree of expansion, the fetal lung is unable to grow and structurally mature.[2] Consequently, much attention has focused on elucidation of how the volume of FLF is controlled and of the mechanisms by which it influences the growth and remodeling of fetal lung tissue. It is now apparent that the fetal metabolic and endocrine environments also play important roles in lung development, especially in lung maturation. The physical factors that are critical for normal lung development and underlie many common disorders of fetal lung growth are the major focus of this chapter.

CONTROL OF FETAL LUNG EXPANSION

Data on the control of fetal lung expansion and its fundamental role in lung growth are derived almost entirely from studies of chronically catheterized fetal sheep.[1,2,4] During the latter half of ovine gestation, the volume of FLF within the fetal lungs progressively increases, reaching 35 to 45 mL/kg during the last weeks of gestation[2]; this is considerably greater than the functional residual capacity (FRC) of the air-filled lung after birth (25 to 30 mL/kg)[5] (Figure 80-1). The high level of fetal lung expansion is actively maintained, involving both fetal breathing movements (FBMs)[6,7] and laryngeal adductor muscles[2] (Figure 80-2). It also is influenced by the transpulmonary pressure gradient, which in turn is influenced by the available intrathoracic and intrauterine space (see further on). Thus values obtained for dead, anesthetized, exteriorized, or paralyzed fetuses will necessarily underestimate lung luminal volumes, because FLF is lost under these conditions[2,6] (see Figure 80-2). Similarly, measurements of FLF volume are questionable unless they were made in the absence of fetal hypoxemia or labor, and in the presence of normal amniotic fluid volume.[8,9] Both labor and reduced amniotic fluid volume reduce FLF volume by compressive forces on the fetus, which increase spinal flexion and, consequently, transpulmonary pressure.[8,9]

In the absence of labor, the volume of FLF in healthy fetuses is determined by fetal muscular activity and the transpulmonary pressure gradient; alterations in the secretion rate of fetal lung liquid have little or no effect.[10] The rate of FLF efflux from the fetal lung by way of the trachea is dependent on the pressure gradient between the lung lumen and the amniotic sac (transpulmonary

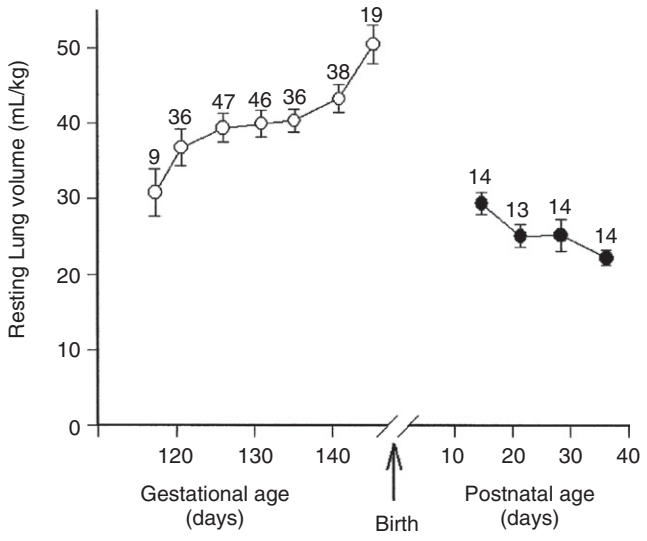


Figure 80-1 Basal lung luminal volumes in fetal and postnatal sheep. The fetal data *(open symbols)* are derived from measurements of the volume of fetal lung fluid within the future airways, made using a dilution technique (numbers of animals are shown above symbols). The postnatal data *(closed symbols)* are derived from measurements of the volume of air remaining within the lungs at end-expiration (functional residual capacity [FRC]), made using a helium dilution technique. *(Data from Harding R, Hooper SB: Regulation of lung expansion and lung growth before birth,* J Appl Physiol *81:209-224,1996.)*

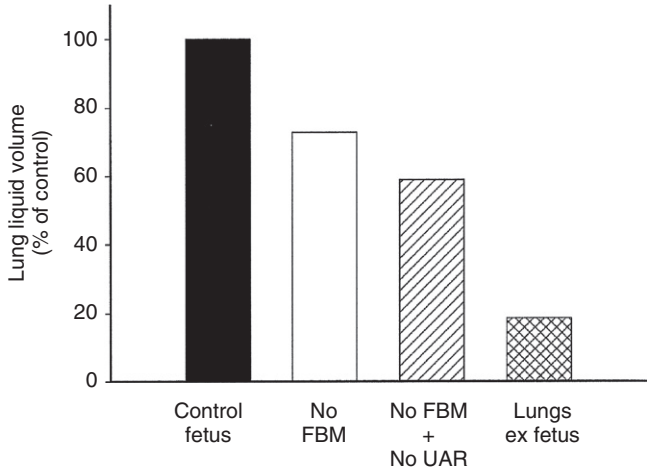


Figure 80-2 The influence of fetal muscular activity on the volume of liquid retained within the future airways. The inhibition of fetal breathing movements (FBMs), by either fetal spinal cord transection[7] or selective blockade of the phrenic nerves,[6] causes an approximately 25% decrease in fetal lung liquid volume from that in control fetuses, demonstrating the importance of FBMs in maintaining the volume of fetal lung liquid. If the fetal upper airway is bypassed, eliminating upper airway resistance *(UAR)* in addition to the abolition of FBMs, the volume of lung liquid is reduced further, demonstrating the independent effect of the fetal upper airway on maintaining fetal lung liquid volumes.The further reduction in lung luminal volume after the removal of the lungs from the fetus demonstrates the contribution of the fetal chest wall to maintaining fetal lung liquid volumes. *(From Harding R, Hooper SB: Regulation of lung expansion and lung growth before birth,* J Appl Physiol *81:209-224, 1996.)*

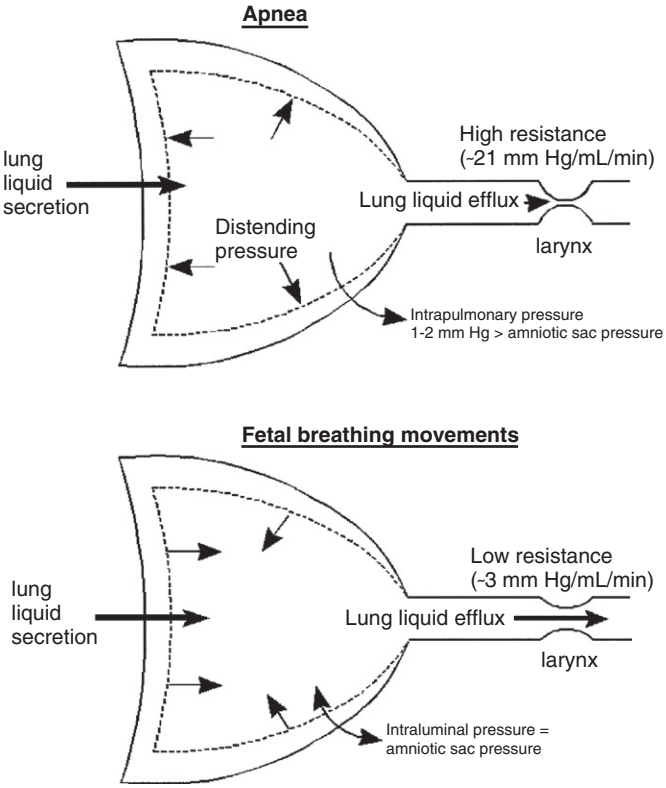


Figure 80-3 Diagram showing the role of the fetal upper airway in regulating the efflux of lung liquid during periods of apnea *(upper panel)* and of fetal breathing movements (FBMs) *(lower panel).* During periods of apnea, the resistance to lung liquid efflux through the upper airway is high (owing to lack of laryngeal abductor activity and presence of tonic adductor activity); as a result, liquid tends to accumulate within the future airways, causing the lungs to expand.This liquid accumulation is responsible for generating a transpulmonary pressure gradient of 1 to 2 mm Hg (intraairway pressure > amniotic sac pressure). During periods of FBM, the resistance to lung liquid efflux through the upper airway decreases (owing to phasic dilator activity of glottic abductor muscles and lack of sustained activity in adductor muscles); therefore liquid leaves the lungs at an increased rate.

pressure) as well as the resistance to efflux in the upper respiratory tract.[11,12] In the absence of FBMs (i.e., with fetal apnea), the pulmonary intraluminal pressure is 1 to 2 mm Hg above amniotic sac pressure,[13] owing to the inherent recoil of lung tissue and the resistance of the upper airway.[12] During apnea, the efflux of FLF throughf the trachea is, on average, lower than its rate of production, and liquid accumulates within the lungs[4] (Figure 80-3). During episodes of FBM, the resistance to efflux is reduced owing to phasic dilation of the glottis,[12] permitting FLF efflux from the lungs.[4]Thus, despite rhythmic contractions of the diaphragm, the average net flow of liquid from the lungs during FBM episodes is two to three times greater than the rate of efflux during intervening apneic periods.[11] Although amniotic fluid can enter the fetal lungs during periods of accentuated FBM, the net flow is out of the lungs.[11] This essentially unidirectional flux helps maintain a constant chemical environment within the future air spaces, limiting the entry of potentially harmful substances in amniotic fluid such as meconium. However, substances dissolved or suspended in amniotic fluid can enter the fetal lungs,[14] which is most likely to occur during episodes of augmented fetal breathing.

The transpulmonary pressure gradient and hence the efflux of FLF also are influenced by factors external to the lungs, such as abdominal pressure.[8] Changes in fetal posture (e.g., trunk flexion) that increase abdominal pressure also increase the transpulmonary pressure gradient, leading to a reduction in FLF volume.[8] Such changes in transpulmonary pressure can be caused by uterine contractions or may develop when intrauterine space is limited as a result of oligohydramnios[8] or presence of multiple fetuses. Lack of amniotic fluid forces the fetus into a more flexed posture.[8,15] Increased flexion increases fetal abdominal pressure and thus transpulmonary pressure, leading to increased FLF efflux and a reduced lung volume.[8] This is the likely cause of lung hypoplasia associated with prolonged oligohydramnios.[8] Because the fetal lungs and chest wall are highly compliant, even small changes in transpulmonary pressure will have marked effects on the degree of lung expansion.

Effects of Reduced Lung Expansion

Sustained reductions in fetal lung expansion are a common cause of hypoplasia and retarded structural development of the lung, including effects on the vascular bed and alveolar epithelial cell (AEC) phenotypes. The hypoplastic effects appear to depend on the degree to which lung expansion is decreased. For example, a 25% reduction in lung expansion causes lung growth to be reduced by approximately 25%,[7] whereas total lung deflation causes lung tissue growth to effectively cease.[3,16,17] Little is known about the underlying mechanisms, although a reduction in the expression of insulin-like growth factor-II (IGF-II) has been observed in two models of reduced lung expansion.[7] It is more likely, however, that the absence of direct mechanical stimuli, which are required to activate or suppress key regulatory gene pathways, is the principal underlying mechanism.[18,19] The

remodeling of lung parenchyma that characterizes normal structural development of the lung also is retarded by reduced lung expansion.[3] In particular, perialveolar tissue volume is increased,[3] leading to thicker septa and a reduced diffusing capacity for respiratory gases. Furthermore, alveolarization is attenuated,[3] which probably is related to impaired elastogenesis.[20] Fetal lung hypoplasia resulting from prolonged underexpansion of the lung also is characterized by reduced growth and maturation of the pulmonary vascular bed, resulting in elevated pulmonary vascular resistance.[21]

The alveolar epithelium of the fetal lung also is affected by prolonged reductions in fetal lung expansion.[3,22] Such reductions promote differentiation of type I into type II cells,[22] whereas prolonged overexpansion reduces the proportion of type II AECs to less than 2% and increases the proportion of type I AECs to greater than 90%.[23] These findings support in vitro evidence that type I cells are not terminally differentiated and can transdifferentiate into type II cells.[24]

Effects of Increased Lung Expansion

Prolonged overexpansion of the fetal lungs, such as that induced by tracheal obstruction, is a potent stimulus for fetal lung growth and tissue remodeling.[3,25] The increase in lung cell proliferation is time-dependent[26] and is restricted to the expanded lung tissue.[16] The growth response to tracheal obstruction differs according to the stage of fetal lung development. During the alveolar stage in fetal sheep, the acceleration in lung growth is completed within 7 days of tracheal obstruction, resulting in an almost doubling of lung DNA content[19,26]; the eventual cessation of accelerated growth probably is due to restraint by the chest wall. By contrast, during the late pseudoglandular–early canalicular stage, the acceleration in lung growth is slower,[27] but eventually results in a greater increase in lung DNA content (an approximately 200% increase)[28]; similar results have been obtained in fetal rabbits.[29] The lower rate of accelerated lung growth in younger fetuses may be due to a lower lung compliance and thus less lung expansion.[27,28] The eventual greater increase in lung DNA content in younger fetuses probably is due to greater compliance of the chest wall, allowing the lungs to expand to a greater degree than in older fetuses.[28]

The mechanisms by which tissue stress affects the rate of lung growth are under active investigation (see further on). It must be recognized, however, that the mechanical stresses imposed on lung tissue by sustained alterations in lung expansion are likely to be very different, depending on the location within the tissue. For example, in response to increased lung expansion, some cells will experience a stretch-like stimulus, whereas other may experience compression.

The characteristics of the lung growth response to increased lung expansion also differ according to the stage of lung development. During the alveolar stage, increased lung expansion causes the proliferation of most major cell types,[25] but during the late pseudoglandular–early canalicular stage, it causes primarily mesenchymal cell proliferation,[28] leading to a large increase in perisaccular tissue volume.[28] By contrast, during the later stages of fetal lung development, prolonged lung expansion accelerates the structural maturation of the lung; that is, the proportion of tissue space is reduced and alveolar number and surface area are increased.[3,25,29,30] The mechanisms by which an increase in fetal lung expansion stimulates septation and alveolarization are unknown but may be linked to the associated increase in tropoelastin expression.[20]

THE ROLE OF FETAL BREATHING MOVEMENTS

FBMs begin early in fetal life and progressively become organized into discrete episodes that are largely associated with a fetal behavioral state resembling rapid eye movement (REM) sleep.[31] During the latter half of gestation, the overall incidence of FBMs is 40% to 50%.[32] The major muscle used in FBMs is the diaphragm; respiratory activity of the intercostal muscles is largely absent, as in postnatal REM sleep. During FBM episodes, the laryngeal abductor (dilator) muscles are active in phase with the diaphragm, whereas during fetal apnea, this dilator activity is absent and the glottis is closed by sustained adductor activity.[12] Typically, individual FBMs lower intrathoracic pressure by up to 5 mm Hg[31] and cause small oscillations of fluid flow within the fetal upper airway.[33,34] Much interest has focused on FBMs because they constitute an important determinant of fetal lung development, although their precise role in vivo has only recently been established.[1,2]

In exploring the functional role of FBMs, numerous techniques have been used to eliminate or blunt their effects, including fetal paralysis,[35] sectioning[36,37] and reversible blockade of the phrenic nerves,[6] sectioning of the fetal spinal cord above the outflow of the phrenic motoneurons,[7,38,39] and replacing sections of the thoracic wall with a compliant membrane.[40] Observations in these studies must be interpreted with caution, owing to confounding factors associated with the experimental procedures. For example, phrenic nerve section causes the diaphragm muscle to atrophy, whereas thoracoplasty may allow lung compression; fetal paralysis abolishes laryngeal adductor activity and may alter fetal posture. To identify the specific role of FBMs in lung development, it is necessary to alter only FBM and then to measure fetal lung luminal volume. With this investigative approach, it is apparent that the reduction in lung growth induced by the abolition of FBM can be explained by the associated decrease in the basal level of fetal lung expansion[6,7]; indeed, the percent decrease in lung expansion is similar to the reduction in lung growth.[7] The decrease in lung expansion after abolition of the thoracic component of FBM can be explained by persistence of laryngeal dilator activity.[7] As a result of this activity, the efflux of FLF is increased during centrally generated "FBM" over that observed in intact fetuses.[6,7] Thus, in intact fetuses, rhythmic activation of the diaphragm in FBM apparently plays a key role in restricting the loss of lung liquid when the resistance to lung liquid efflux is lowered by glottic dilation.[1,2] Collectively, these data show that fetal muscular activity, whether it is active glottic adduction during apnea or activation of the diaphragm during FBM, helps to defend FLF volume and hence the degree of lung expansion (see Figure 80-2). At present, no in vivo evidence has emerged to show that phasic stretch, per se, of the lung during FBMs is an important determinant of fetal lung growth, although in vitro evidence suggests that this is possible.

Numerous in vitro studies have shown that phasic stretch of fetal lung cells in culture, in a manner that attempts to simulate FBMs in vivo, stimulates lung cell proliferation.[41] These experiments have provided important information on the transduction pathways by which phasic mechanical stimuli initiate lung cell proliferation. However, the stimulus used (approximately 5% stretch) probably exceeds that induced by FBMs in vivo. In vivo, individual FBMs are essentially isovolumic, so the percent length change experienced by a lung cell with each FBM will be negligible. This is because the fetal chest wall is very compliant, and fetal lung liquid is very viscous compared with air and, owing to its mass, has a large inertia. Thus, although activation of the diaphragm causes a reduction in intrathoracic pressure, very little liquid is inhaled with each inspiratory effort, because other sections of the chest wall are simultaneously drawn in[42]; free liquid must be present within the pharynx before any liquid can be inhaled.[12] As a result, the tidal volume (V_T) in the fetus is very small; in late-gestation fetal sheep, V_T usually is less than 0.5 mL[31] at FBM rates of up to 3/second. In human fetuses during the last trimester, the mean FBM frequency is approximately 1/second,[33] although respiratory cycle times of up to 1.5 to 2.0 seconds have been reported. Color Doppler ultrasound imaging has been used to measure fluid flow velocity waveforms in the

trachea and nasopharynx of human fetuses, although the contribution of this fluid movement to lung volume changes is currently unclear.[34] Thus, at least in the sheep, fetal V_T is less than 1% of resting lung volume but markedly increases immediately after birth to approximately 20% of resting lung volume.

MECHANOTRANSDUCTION MECHANISMS AFFECTING GROWTH AND DIFFERENTIATION

All cells, tissues, and even whole organs are subjected to physical forces in vivo, including shear stress, stretch, and compression; such forces can result from gravity, osmotic pressures, fluid flow, intracellular tensile forces, body movements, and changes in the internal volume of hollow organs. It has been proposed that cells exist in a state of isometric tension that is generated by the intracellular contractile filaments of the cell.[43] Thus externally applied forces are thought to be imposed on a preexisting equilibrium of forces, causing changes in cell shape and intracellular structural fiber alignment until the force equilibrium is reestablished.[43,44]

Physical forces play an important role in cellular growth and differentiation, are critical regulators of three-dimensional tissue structure,[44] particularly in the lung,[1,45] and constitute an important means by which cells interact with their environment.[43] The transduction pathways by which physical forces are translated into chemical stimuli, leading to changes in cell function, are beginning to be understood. The discovery of cell surface receptors that bind to a variety of extracellular membrane (ECM) proteins has greatly advanced the understanding of mechanotransduction mechanisms. For example, the "integrin" family of transmembrane proteins cluster at focal adhesion sites and bind to a specific sequence, Arg-Gly-Asp (RGD), that is common in many ECM proteins.[46] The intracellular domains of these receptors are mechanically linked to fibrillar-actin bundles via a variety of cytoskeleton-associated proteins (e.g., talin, vinculin, paxillin) and are associated with a number of protein kinases.[46] Because actin bundles are a major component of the intracellular scaffolding, the intra- and extracellular structural components are mechanically coupled, by way of ECM receptors, to form a structural continuum (Figure 80-4). It is through these physical couplings that mechanical forces can be detected and translated into intracellular chemical signals.[44] Although the intracellular signaling pathways are less well defined, they are likely to include stretch-activated ion channels, activation of intracellular second messenger systems, and the direct activation of RNA polymerases and DNA-synthetic enzymes mediated by changes in nuclear shape.

It is possible that increased expansion of the fetal lung stimulates the synthesis and release of specific growth factors that act locally, in a paracrine manner, to stimulate cellular proliferation. The fetal lung produces a number of growth factors including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), IGF-II, keratinocyte growth factor (KGF), and transforming growth factor-β_1 (TGF-β_1). Although IGF-II expression is increased by sustained lung expansion,[19] IGF-II messenger RNA (mRNA) levels do not parallel the increase in DNA synthesis rates[47]; these are maximal at 2 days of tracheal obstruction and then decline to control values by 10 days.[26] Similarly, PDGF-B expression was reduced in response to tracheal obstruction, when DNA synthesis rates were elevated, indicating that the accelerated lung growth is not mediated by increased expression of these growth factors.[47] Although it also is important to understand how expression of specific growth factor receptors changes in response to an increase in fetal lung expansion, the finding that activation of the mitogen-activated protein (MAP) kinase pathway is not increased by this stimulus[47] indicates that growth factor receptor activation is not a major regulatory pathway. However, VEGF expression was transiently increased by tracheal obstruction, indicating that VEGF may regulate endothelial cell proliferation in growth-accelerated lungs.[47]

To identify gene pathways activated or suppressed in response to an increase in fetal lung expansion, differential gene analysis techniques have been used. In an animal model, overexpanded and control lung tissue samples were collected from the same fetus.[18,48] Increased fetal lung expansion induced differential expression of more than 80 genes, many of which have known roles in cell growth and differentiation and ECM remodeling[18]; these genes are likely to play an important role in stimulating and coordinating the growth and development response of the lung. In particular, a number of immediate early response genes including *EGR1, CTGF,* and *CYR61* have been identified, providing

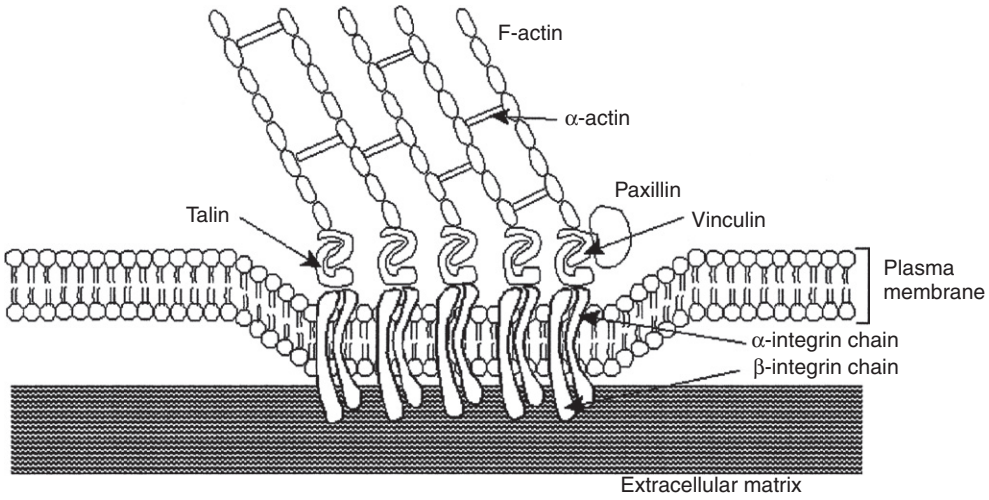


Figure 80-4 Schematic diagram demonstrating how the extracellular matrix (ECM) receptors (integrins) mechanically couple the intracellular microfilaments with the ECM to form a structural continuum. Proteins associated with the intracellular domain are associated with numerous intracellular signaling pathways, which may mediate the response to alterations in fetal lung expansion. *(Data from Ingber D, Karp S, Plopper G, et al: Mechanochemical transduction across extracellular matrix and through the cytoskeleton. In Fransas JA, editor:* Physical Forces and the Mammalian Cell, *San Diego, 1993, Academic Press, p 61; and Rubin K, Gullberg D, Tomasini-Johansson B, et al: Molecular recognition of the extracellular matrix by cell surface receptors. In Comper WD, editor:* Extracellular Matrix, *Amsterdam, 1997, Harwood Academic Publishers, p 262.)*

important clues for identifying factors that initiate the growth response to increased lung expansion.[18]

PHYSICAL CAUSES OF FETAL LUNG HYPOPLASIA

Fetal lung hypoplasia, which can be defined as a reduction in lung tissue weight or cellular content, can result from a diverse group of disorders of pregnancy. Such disorders include oligohydramnios secondary to premature rupture of membranes or fetal anuria; congenital diaphragmatic hernia (CDH); thoracic space-occupying lesions such as pulmonary cysts, tumors, and pleural effusions; and fetal musculoskeletal deformities. The common mechanism for inducing fetal lung hypoplasia in these situations presumably is a prolonged reduction in the degree of fetal lung expansion.[15]

Oligohydramnios

Oligohydramnios occurs in approximately 10% of pregnancies and usually is caused by premature rupture of the fetal membranes. Another cause is inadequate production of fetal urine due to fetal urinary tract disorders, including renal agenesis, renal dysplasia and agenesis or stenosis of the ureters, urethra or urethral valve.[15] The severity of the lung growth deficit depends on the gestational age at onset and the duration and severity of the oligohydramnios.[49] The likely mechanism by which oligohydramnios causes fetal lung hypoplasia involves a prolonged reduction in lung expansion.[8] In the absence of amniotic fluid, the intrauterine space is reduced, so the fetal trunk is increasingly flexed.[8,15] This positioning leads to an increase in abdominal pressure, elevation of the diaphragm, compression of the lungs, and the loss of lung liquid[8]; the efflux of FLF is increased during nonlabor uterine contractions, which further increase the degree of fetal spinal flexion.

Congenital Diaphragmatic Hernia

CDH is less common than oligohydramnios but usually results in more severe, often fatal lung hypoplasia. CDH is a result of failure of the embryonic diaphragm to close (at approximately 12 weeks gestation), allowing abdominal contents to enter the thorax.[50] The hernia can be either unilateral (i.e., left- or right-sided) or bilateral, causing chronic lung collapse on the affected side (s). The perforated thoracic compartment allows unimpeded lung recoil, so the ipsilateral lung is unable to expand, hence lung growth cannot occur. Furthermore, FBMs would not have the effect of opposing lung recoil, as in an intact fetus, because of the diaphragmatic defect; any phasic strain-related effects of FBMs on the lung also would be absent. In addition, the abdominal contents may compress the contralateral lung as a consequence of a shift in the mediastinal ligament. Because the defect occurs early in fetal life, it usually results in severe lung hypoplasia, with major impairments in lung structure and function.

Musculoskeletal Disorders

A variety of fetal musculoskeletal disorders can result in severe lung hypoplasia[15]; although the precise mechanisms depend on the type of disorder, they probably all involve a prolonged reduction in lung expansion. Because the activity of both the diaphragm and glottic adductor muscles plays a crucial role in maintaining fetal lung expansion,[8] interference with such activity would be expected to have an impact on fetal lung growth.

EFFECTS OF CORTICOSTEROIDS ON GROWTH AND MATURATION OF THE FETAL LUNG

During development, the fetal lung is naturally exposed to corticosteroids of maternal and fetal origin, particularly during late gestation and under conditions of fetal distress. Researchers have directed much attention to corticosteroids owing to their therapeutic value in enhancing lung maturity, although their precise effects on lung development in vivo are still unclear. In the fetal lung, elevated circulating corticosteroid levels have important effects on (1) structural development, (2) the surfactant system, and (3) the reabsorption of FLF at birth. Other potential effects of corticosteroids include reduced lung tissue growth and stimulation of type II AEC differentiation. In this section, the focus is on the role of cortisol in fetal lung growth and structural maturation.

It often is assumed that corticosteroids induce fetal lung maturation at the expense of lung growth, but the in vivo data are contradictory, probably reflecting species differences as well as differences in dose, number of doses used, and route of administration. Most studies have used synthetic glucocorticoids (betamethasone or dexamethasone), which have a 30- to 40-fold greater bioactivity than that of cortisol. When administered to the mother, betamethasone[51] causes a decrease in both fetal body and lung growth, effects that become greater with increasing number of doses.[52,53] When administered directly to the fetus, however, betamethasone does not affect fetal body or lung growth.[54] Similarly, physiologic doses of cortisol, infused directly into the fetus to simulate the preparturient increase in fetal plasma cortisol levels, induce structural maturation of the lung without affecting either fetal lung or body growth.[55,56] These data suggest that the effects of maternally administered betamethasone on fetal body and lung growth are dose-dependent, mediated through an effect on the placenta, or related to the duration of exposure (i.e., half-life).

The removal or abolition of the endogenous source of corticosteroids in the fetus also has led to conflicting results in studies examining fetal lung growth. Adrenalectomy[57] and hypophysectomy[58] in fetal sheep significantly reduce lung protein, rather than DNA, content[57]; this observation indicates that endogenous cortisol may contribute to lung protein accumulation. By contrast, knock-out of the corticotropin-releasing hormone (CRH) gene in mice increases fetal lung DNA content and rates of cellular proliferation, particularly in epithelial cells.[59] Similarly, knock-out of the glucocorticoid receptor causes lung hypercellularity (increase in DNA content), indicating that endogenous corticosteroids suppress lung cell proliferation.[60] Other interspecies differences in the pulmonary effects of glucorticoids are evident. For example, homozygous (−/−) CRH knock-out mice from heterozygote (+/−) mothers survive birth, probably as a consequence of the placental transfer of corticosteroids from the +/− mothers.[61] Thus it appears that the survival of newborn mice at birth is not dependent on exposure to the preparturient increase in circulating corticosteroids, as it is in humans and sheep, although some circulating corticosteroids are required.

One of the principal effects of corticosteroids is enhanced structural maturation of the fetal lung, resulting in improved lung mechanics after birth.[62] Both natural and synthetic corticosteroids markedly reduce perialveolar tissue volume, leading to a reduction in cellularity (cell density within the tissue) and a marked increase in potential air space volume; these changes would be expected to increase lung compliance. By contrast, adrenalectomy and hypophysectomy in fetal sheep, hypophysectomy in fetal pigs,[63] and knock-outs of both the GR and CRH genes in mice all have been documented to increase perialveolar tissue volumes, decrease air space volumes, and increase cellularity, resulting in respiratory failure at birth, presumably owing to a reduction in lung compliance. Although their effect on lung structure is arguably the major beneficial effect of corticosteroids on the developing lung, the mechanisms remain largely unknown.[62]

Corticosteroids also can affect alveolarization, although this effect may be dose- or species-dependent. Betamethasone has been shown to arrest alveolarization in postnatal rats,[64] whereas physiologic doses of cortisol administered to fetal sheep increase alveolar number.[65] In addition, the increase in fetal lung

compliance induced by corticosteroids interacts with the relationship between lung expansion and lung growth, causing an enhanced lung growth response subsequent to increased lung expansion.[56]

Antenatal corticosteroids also stimulate the maturation of the pulmonary vascular bed in normal fetal lungs[66] and in hypoplastic fetal lungs.[67] In sheep, antenatal corticosteroids enhance pulmonary vasodilator responsiveness and increase the fall in pulmonary vascular resistance and rise in pulmonary blood flow at the time of birth. These effects may be mediated by acceleration of vascular and parenchymal maturity; it seems unlikely, however, that changes in vascular density or wall structure are involved.[67]

EFFECTS OF METABOLIC FACTORS ON FETAL LUNG GROWTH AND MATURATION

Impaired nutrient and O_2 delivery during fetal life can affect the developing lung. Studies of respiratory function in infants,[68] children,[69] and adults[70] suggest that lung development may be affected by intrauterine conditions that inhibit fetal growth and lead to low birth weight. A common cause of fetal growth restriction (FGR) is chronic placental insufficiency, which reduces the availability of nutrients and oxygen to the fetus. The effects of fetal hypoxemia differ from those of nutrient restriction. Prolonged hypoxemia in the absence of hypoglycemia leads to reduced DNA synthesis rates in ovine fetal lungs.[71] In rats, fetal hypoxia from early in gestation resulted in the lungs being small relative to body weight.[72] Nutrient restriction has been shown to impair alveolus formation. For example, in rats, in which alveolus formation occurs postnatally,[73] intermittent starvation soon after birth was associated with enlarged alveoli with thicker septa and reduced elastin deposition.[74] In sheep, hypoxemia and nutrient restriction during late gestation (coinciding with pulmonary saccular and alveolus formation) also leads to reduced alveolarization.[75] Similar findings have been documented in undernourished postnatal rats[74] and in guinea pigs undernourished during late gestation and in early neonatal life.[76] Impaired alveolarization secondary to FGR could be a result of fetal hypoxemia, hypoglycemia, or elevated corticosteroid levels. Furthermore, glucose transport to the lungs of growth restricted fetal rats is reduced,[77] which could reduce metabolic activity within lung cells.

The alveolar blood-gas barrier also can be affected by restricted fetal growth. In sheep, severe FGR during late gestation increases the blood-air barrier thickness, an effect that persists for at least 2 years after birth.[78] In guinea pigs born after maternal undernutrition during pregnancy, the alveolar surface area was reduced, leading to a reduced diffusing capacity[76]; a smaller surface area was still apparent at 18 weeks, in spite of catch-up growth in body weight and lung volume. Such changes, together with alterations in airway development, may potentially impair lung function later in life.[79,80]

Structural elements of the lung, such as elastic fibers, collagens, proteoglycans, and basement membrane proteins, are laid down during early development and these processes may be affected by nutritional status. Elastin is necessary for airway and alveolar development and affects lung compliance.[81] Tropoelastin is expressed principally by fibroblasts, particularly during alveolus formation.[82,83] Owing to the long half-life of elastin,[84] alterations in elastin deposition during development may exert persistent effects on the mechanical properties of the lung, and it is now apparent that nutritional factors can affect elastin deposition. For example, in growing rats, protein restriction caused a loss of lung desmosine and an increase in alveolar dimensions.[85] In humans, most elastin accumulates in the lungs between 25 weeks gestation and 15 weeks after birth, and this accumulation is not significantly affected by FGR.[86] Similarly, pulmonary tropoelastin expression and elastin content are not altered in fetal sheep subjected to FGR during late gestation.[87]

During fetal life, both hypoxia and fetal undernutrition may be associated with increased levels of corticosteroids[88]; these may affect elastin synthesis as exogenous corticosteroids can both increase[82] and decrease[89] elastin formation in the fetal lungs. Elastin deposition in the lung also may be affected by hypoxia as it down-regulates tropoelastin in pulmonary fibroblasts[90] and in pulmonary artery smooth muscle cells.[91] Paradoxically, hypoxia also increases pulmonary lysyl oxidase activity, the extracellular enzyme responsible for cross-linking elastin and collagen. However, an inhibitory effect of hypoxia on elastin synthesis was not observed in growing rats.[92]

Collagen provides the lung and airways with mechanical strength.[93] In contrast with elastin, collagen is synthesized and degraded throughout life,[93] and it is well established that collagen metabolism is affected by nutritional status.[94] Lung collagen content is reduced in postnatal rats fed a low-protein diet.[85] It is of interest that procollagen gene expression in lung fibroblasts is increased by hypoxia,[95] which is a feature of FGR.

Proteoglycans are a major component of lung ECM, yet no data apparently exist on the influence of impaired nutrition or oxygenation on proteoglycan synthesis in the developing lung. In vascular tissue, hypoxia has been shown to decrease proteoglycan production.[96,97]

Development of pulmonary surfactant is affected by nutritional factors. Undernutrition of pregnant rats results in impaired surface tension–lowering properties of fetal lung extracts[98]; the maturation of type II AECs in these offspring also is delayed, because their glycogen content was increased and lamellar bodies were decreased.[99] Prolonged periods of fetal hypoxemia, induced by placental restriction, also increase expression of surfactant proteins SP-A and SP-B at 0.88 of gestation[100] but not near term.[101] This observation suggests that FGR or nutrient restriction may have beneficial effects on lung maturity in preterm infants but not in those born at term. The effects of undernutrition and hypoxia on type II AECs are likely to differ according to gestational timing and alterations in cortisol levels.

Certain micronutrients are known to be essential for normal lung development. Vitamin A (retinol) and retinoic acid play important roles early in lung development, during embryogenesis and organogenesis. Retinoic acid appears to be the most active of the retinoids[102] and affects many aspects of lung development including early airway branching, alveolus formation, and the synthesis of surfactant and surfactant proteins.[103] For example, vitamin A deficiency during gestation reduces the expression of SP-A, SP-B, and SP-C in fetal rat lungs.[104] Considerable interest has been directed toward understanding the effects of retinoic acid on alveolus formation and surfactant synthesis. These effects must include elastogenesis, because inhibitors of retinoid metabolism decrease tropoelastin expression in fetal rat lung explants,[105] and vitamin A deficiency during pregnancy reduces elastin staining in fetal rat lungs.[106] Some evidence suggests that a reduction in alveolus formation during early development may be reversible, because treatment with retinoic acid is able to increase the number of alveoli, at least in rats and mice.[64,107]

Vitamin D can affect the developing lung, possibly through binding sites on type II AECs.[108] It can advance maturation of type II AECs as indicated by a reduction in glycogen content and increased surfactant synthesis.[109] Selenium also is important, because it is necessary for the activity of glutathione peroxidase, an important antioxidant defense enzyme. Selenium deficiency in rats leads to altered lung development, particularly alveolar septation.[110]

POSTNATAL LUNG GROWTH

In humans, although the formation of significant numbers of alveoli ceases at 1.5 to 3 years after birth,[111] lung growth continues for another 15 to 18 years, until the chest stops growing

in late adolescence. Thus much more lung tissue growth occurs after birth than during fetal life. In the sheep, for example, the weight of the lung tissue increases from approximately 100 g at birth to around 220 g at 6 weeks, and to 600 g in the mature animal. Paradoxically, however, less is known about the physiologic control of lung growth after birth than before birth. Much of what is currently known about the control of postnatal lung growth has been derived from experimental and clinical studies observing lung growth after lung resection (i.e., postpneumonectomy lung growth) and in disorders affecting chest wall development (e.g., kyphoscoliosis).

An understanding of lung growth after birth is of particular interest in the clinical approach to premature infants, infants with hypoplastic lungs (e.g., after preterm premature rupture of membranes [PPROM] or CDH), infants affected by FGR who then experience postnatal catch-up in body growth, and infants and children with feeding problems or nutritional deficiencies. Not only does lung growth increase the surface area for gas exchange, but it is necessary for normal development of the pulmonary vascular bed and, consequently, a normal pulmonary circulation. Children with problems from impaired lung growth before or after birth may benefit from treatments that accelerate lung parenchymal growth and development, some of which are discussed further on.

THE ROLE OF THE PHYSICAL ENVIRONMENT

It generally is assumed that as the thoracic cavity enlarges with skeletal growth, lung growth follows passively to fill the chest; put simply, if the chest wall does not grow, then the lung is unlikely to grow. Thus a full understanding of postnatal lung growth will ultimately require knowledge of how chest wall growth, and its stiffness, are regulated. The lungs exert a chronic traction on the chest wall, so an intimate relation must exist between lung growth and thoracic wall growth, with each affecting the other; it is likely, however, that the lung, which ultimately fills the space available, will stop growing once the chest is no longer enlarging. Immediately after birth, the chest wall is very compliant and can influence lung growth to a significant extent only after the ribs and sternum become ossified; the rate at which this ossification proceeds will therefore have an effect on lung growth. Ossification of the chest wall in preterm infants may be delayed, and this could impair lung growth in these infants, unless they receive CPAP or PEEP.

Lung resection experiments have shown that the postnatal lung will grow to fill the space available, which may be dependent on the presence of a subatmospheric intrapleural pressure. This pressure differential in turn creates a transpulmonary pressure that effectively expands the lung by exerting chronic traction on the lung pleura.[112] The mechanical stress and strain imposed on the lung are presumed to activate intracellular processes that precipitate events that increase cell replication and tissue remodeling. The stimulated lung growth has the effect of reducing stress and strain in the alveolar septa, which limits the stimulus for further growth; it is likely that this process continues until the thoracic cavity reaches its final dimensions.[112]

Available evidence suggests that the basal level of lung expansion is an important determinant of lung growth and structural development after birth, as it is in the fetus, although the mechanical forces exerted on the air-filled lung are considerably more complex. Indeed, in the fetus it is apparent that lung growth may drive chest wall growth, whereas the reverse seems to be true after birth. The relationship between the basal level of lung expansion and lung development is graphically demonstrated by the marked reduction in the rates of lung tissue growth[113] (Figure 80-5) and alveolarization[111,114] (Figure 80-6) that occurs after birth, when the degree of lung expansion is chronically reduced, as shown in Figure 80-1.

An increasing amount of evidence indicates that tissue stretch is a major stimulus to postnatal lung growth. Chronic, localized distention of a region of the lung with perfluorocarbon accelerates lung growth and alveolarization in that region, an effect seen in growing but not adult animals.[115] The use of CPAP and positive end-expiratory pressure (PEEP), both of which would be expected to increase the basal degree of lung expansion by increasing end-expiratory lung volume (i.e., FRC), has been shown to enhance lung growth in growing ferrets[116] and lambs.[117] Similarly, the compensatory lung growth induced by

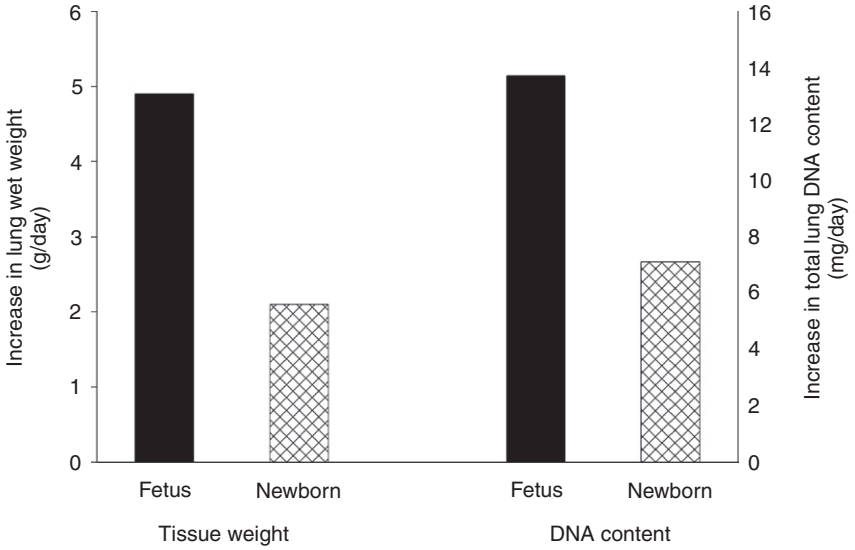


Figure 80-5 Lung growth rates measured in ovine fetuses during late gestation *(closed bars)* and in postnatal lambs *(cross-hatched bars).* Lung growth rates were calculated as the increase in wet tissue weight (g/day) *(left panel)* and total DNA content (mg/day) *(right panel)* using fetuses killed at 114 and 138 days of the 147-day gestation, and postnatal lambs killed at 1 day and 46 days after birth (n = 5 for each age-group). Of note, lung growth rates in postnatal lambs are considerably lower than in the fetus. *(From Hooper SB, Wallace M: Role of the physicochemical environment in lung development,* Clin Exp Pharmacol Physiol *33:273-279, 2006.)*

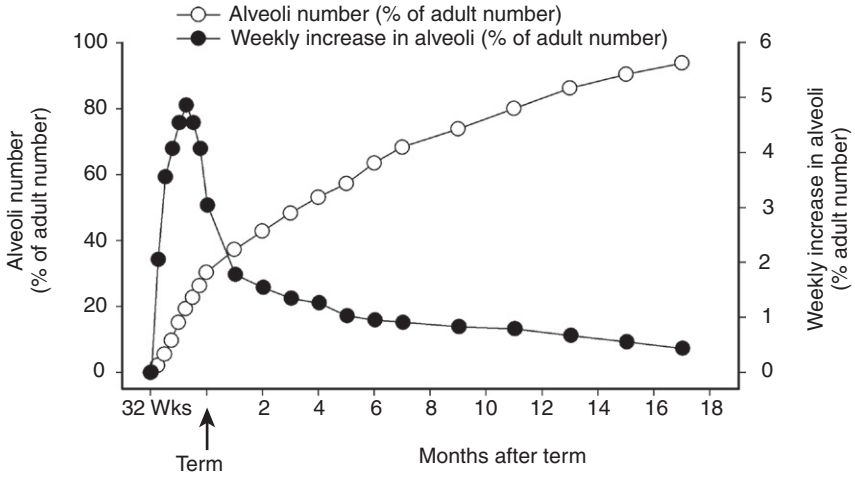


Figure 80-6 Changes with fetal and postnatal age in alveolar number and weekly rate of accumulation of alveoli in humans. The *filled circles* show the weekly rate of alveolus formation expressed as a percentage of the final adult number of alveoli (% alveoli/week); the *open circles* show the percentage of the final adult number of alveoli that have been formed at each time point (% adult number of alveoli). Of note, the rate of alveolus formation is markedly reduced after birth. *(From Jobe A: Lung development and maturation. In Martin RJ, Fanaroff AA, Walsh MC, editors:* Neonatal-Perinatal Medicine, *ed 8, St Louis, 2006, Mosby, pp 1069-1086. Idealized curves data from Langston C, et al: Human lung growth in late gestation and in the neonate,* Am Rev Respir Dis *129:607-613, 1984; and Hislop AA, Wigglesworth JS, Desai R: Alveolar development in the human fetus and infant,* Early Hum Dev *13:1-11, 1986.)*

partial resection of the air-filled lung after birth is thought to be expansion-dependent.[118] It has been suggested that phasic expansion of the lung during breathing acts as a stimulus for lung growth. Of note, however, experimentally increasing the phasic component of postnatal breathing movements does not affect lung growth or lung structure.[119] Combined, these findings indicate that lung growth after birth is regulated primarily by the basal degree of lung expansion, as it is in the fetus.

EFFECTS OF METABOLIC AND ENDOCRINE FACTORS

Although it is now apparent that mechanical tissue strain is the major signaling mechanism underlying postnatal lung growth, nonmechanical factors are likely to play a role in modifying the growth response.[112] These may include metabolic factors such as the availability of nutrients, micronutrients, and oxygen and endocrine factors such as corticosteroids, growth hormone, sex hormones, IGFs, and thyroid hormones.

Nutrition

Numerous studies have shown that as in the fetus, postnatal nutrition can have a profound effect on lung development.[120] In children, severe malnutrition has been shown to adversely affect lung function, but such effects could be related in part to altered musculoskeletal development.[121] Animal studies have shown specific effects of postnatal undernutrition on lung structure, including alveolarization.[74,122] The adverse effects of undernutrition during postnatal development may persist to maturity. For example, a study in sheep has shown that animals that grow slowly after birth have smaller lung volumes, and smaller pulmonary surface areas relative to lung and body size, than those measured in normally growing animals.[122a]

Corticosteroid Exposure

Preterm infants may be administered corticosteroids to accelerate lung maturity and to reduce the time spent on a mechanical ventilator. Although available data on the structural effects of postnatal exposure to exogenous corticosteroids are very limited for human infants, such exposures have been shown in animals to affect lung growth and alveolarization. In postnatal rats, dexamethasone accelerates thinning of interalveolar septa; however, alveolarization is inhibited—an effect that can be permanent or transient, depending on when it is given.[123] Dexamethasone also has been shown to retard rates of both lung cell proliferation and apoptosis[124] and to accelerate microvascular maturation.[125]

POTENTIAL TREATMENTS FOR LUNG GROWTH DISORDERS

Fetal Treatments

It is now clear that lung growth and structural development are a consequence of a complex interaction among mechanical, endocrine and metabolic factors. The challenge is to translate this knowledge into therapeutic treatments that will improve outcomes for preterm infants and infants with hypoplastic lungs. Because increased fetal lung expansion is a potent stimulus for fetal lung growth and structural maturation, it has been tried as an in utero treatment for severe lung hypoplasia. Experimental studies have shown that increases in fetal lung expansion induced by tracheal obstruction can rapidly reverse fetal lung growth deficits in utero[17,126,127] and can reverse postnatal respiratory dysfunction that would otherwise be fatal.[128] However, clinical trials in fetuses with severe pulmonary hypoplasia resulting from CDH have had mixed success, although some improved outcomes have been reported.[129] The major problems relate to preterm labor, failure to adequately stimulate fetal lung growth, and postnatal respiratory insufficiency despite induced lung growth.[129] Thus a significant discrepancy exists between the results of clinical trials and those obtained in animal experiments. These discrepancies may relate to the stage of lung development at which the treatment was applied, the duration of the tracheal obstruction, and the severity of the fetal lung hypoplasia. For example, most tracheal obstruction procedures in humans have been carried out before 28 weeks gestation, when the lungs are at the early to mid-canalicular stage of development.[129,130] When tracheal obstruction is performed at this

stage of lung development in fetal sheep, the induced lung growth is associated with mesenchymal cell proliferation and increased perisaccular tissue volume.[28] Similarly, sustained increases in fetal lung expansion can severely reduce the numbers of type II AECs[23] and limit surfactant protein gene expression.[131-133] Although this reduction in type II AECs[22] and surfactant protein gene expression is not necessarily permanent, the obstruction must be released before birth for normalization to occur.[134] The fetus must then spend sufficient time in utero for the AECs to transdifferentiate and reestablish near-normal proportions. Thus inappropriate growth and reduced type II cell numbers may help explain why infants subjected to prolonged tracheal obstruction in utero can suffer respiratory insufficiency after birth, despite having a normal-sized or larger-than-normal lung.

The failure to stimulate lung growth by tracheal obstruction in human fetuses with CDH also may be explained by the inability of fetal lung liquid secretion to expand the fetal lungs. Because the severely hypoplastic fetal lung is relatively incompliant, it is difficult to expand. Thus, if the internal distending pressure required to expand it is greater than the osmotic pressure driving lung liquid secretion, which is only approximately 4 to 5 mm Hg,[26] the lung will not expand, despite the trachea being occluded. Ongoing lung liquid secretion and increased lung expansion are requirements for accelerated fetal lung growth[26,56]; without continued lung liquid secretion, therefore, the lung growth response will not be activated.[129]

Another complication associated with tracheal obstruction for the correction of CDH-induced lung hypoplasia in human fetuses is the development of fetal hydrops.[129] This phenomenon also has been observed in fetal sheep,[28] but only when the tracheal obstruction procedure is performed during the pseudoglandular-canalicular stages of lung development; it does not occur during the alveolar stage. The hydrops is thought to result from the greater increase in lung expansion and lung growth that occurs in the immature fetus; this is thought to result from an immature chest wall that allows the lungs to expand to a much greater degree.[28] The resultant hydrops probably is due to restricted venous return or to mechanical constraint of the heart by the expanded lungs. Taken together, these data indicate that if tracheal obstruction is to be used successfully to stimulate lung growth in fetal humans, (1) the obstruction procedure is better performed later in gestation, (2) the obstruction needs to be reversible or intermittent, and (3) it may be beneficial to use corticosteroids to improve lung compliance, before the onset of obstruction.

Postnatal Treatments

The stimulation of lung growth in the early postnatal period is of interest in the management of infants born with hypoplastic lungs, such as in CDH. PEEP often is used with preterm neonates to enhance oxygenation and prevent lung collapse and injury. Because PEEP causes a sustained increase in the basal degree of lung expansion (i.e., FRC), it may have the added effect of enhancing postnatal lung growth; this effect has not been demonstrated in human infants but has been observed in normal postnatal ferrets[116] and lambs[117] Although a potential complication of increasing end-expiratory lung volumes in ventilated preterm infants is an alteration in the proportions of AECs, leading to a reduction in type II cells, a study in normal lambs failed to detect an effect of 48 hours of PEEP on AECs.[117]

SUMMARY

Growth and structural maturation of the lung both before and after birth result from a complex interaction among physical, endocrine, and metabolic factors. Sustained expansion of the developing lung appears to be crucial for normal lung development both before and after birth. In the fetus, a high degree of lung expansion is actively maintained and is dependent on diaphragmatic and laryngeal muscle activity. Factors that inhibit these muscular activities or alter the transpulmonary pressure gradient result in a reduction in fetal lung expansion, now recognized as being the common underlying mechanism by which a variety of disorders result in pulmonary hypoplasia. After birth, the major stimulus for lung growth also is likely to be physical lung expansion, which is provided by growth of the rib cage. The mechanisms by which alterations in lung expansion accelerate or retard the growth and development of the lung are largely unknown but represent vital areas of research that may yield substantial clinical benefits.

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81 Molecular Mechanisms of Lung Development and Lung Branching Morphogenesis

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When one examines the development of animals throughout time, the effect of certain influences on the evolution of body plan can be seen. However, one factor in particular emerges as the most influential of all, because its developmental pressure has affected the evolution of nearly every animal. That factor is oxygen, and the ability to extract sufficient amounts of oxygen from the surrounding environment has had an incredible impact on the design of respiratory systems. In particular, land mammals with their high metabolic rates have been forced to evolve a sophisticated organ to meet their oxygen requirements. We explore this process of development and examine a variety of important molecular regulators affecting lung formation.

Lung development can be subdivided into six distinct stages (Table 81-1).[1-3] The early stages of lung formation comprise the embryonic and pseudoglandular periods, after which the prospective conductive airways have been formed and the acinar limits can be recognized. During the pseudoglandular period the primitive airway epithelium starts to differentiate, and neuroendocrine, ciliated, and goblet cells appear while mesenchymal cells have begun to form cartilage and smooth muscle cells. In the subsequent canalicular period, the airway branching pattern is completed, and the prospective gas exchange region starts to develop. During this period respiratory bronchioli appear, interstitial tissue decreases, vascularization of peripheral mesenchyme increases, and distal cuboidal epithelium differentiates into type I and type II cells. In the saccular (terminal sac) period, the growth of the pulmonary parenchyma, the thinning of the connective tissue between the air spaces, and maturation of the surfactant system are the most important steps toward ex utero life. Although already functional, the lung is structurally still in an immature condition at birth. The air spaces present are smooth-walled transitory ducts and saccules with primitive septa that are thick and contain a double capillary network. The final two phases of lung development are alveolarization and microvascular maturation. These two phases occur concurrently and are distinguished by formation of the alveoli through a septation process that greatly increases the gas exchange surface area and by the fusion of the dual-layer capillaries into a single-layer network. One of the important hallmarks occurring throughout lung development is the signaling between the epithelial and mesenchymal tissue layers. The combination, concentration, and spatial-temporal localization of a multitude of molecular signaling factors all working in harmony determine the fate of branching, growth, and cellular differentiation of the developing lung. In this chapter, we summarize studies on lung development from the past two decades and also explore current thoughts on the molecular mechanisms that determine lung pattern formation.

EARLY LUNG DEVELOPMENT

Lung development starts as an endodermal outgrowth of the ventral foregut around the fourth week of human development. This foregut mass rapidly elongates into a single tube dividing into a ventral esophagus and a dorsal trachea, the latter of which bifurcates into the left and right primary lung buds. In a slightly different process, the mouse respiratory system develops from a pair of endodermal buds in the ventral half of the primitive foregut, just anterior to the developing stomach at 9.5 days gestation. The two buds will then elongate in a posterior-ventral direction, while at the same time starting from the primary branch point the single gut tube begins to pinch into two tubes creating the dorsal esophagus and ventral trachea. In humans, the left lung bud will give rise to two main stem bronchi, and the right lung bud gives rise to three main stem bronchi. In the mouse, the right lung bud will form four stem bronchi whereas the left lung consists of one stem bronchus. The secondary bronchi will then branch and rebranch in a dichotomous manner, eventually forming the airway tree in a process termed branching morphogenesis. Endoderm-derived epithelial cells will line the airways, while the surrounding mesenchyme will provide the elastic tissue, smooth muscles, cartilage, vascular system, and other connective tissues. The formation of the bronchial tree is finished at 16 days gestation in the mouse and 16 weeks gestation in humans. At this stage of development, the tracheobronchial tree from the trachea to the terminal bronchioles resembles a system of branching tubules that terminate in exocrine gland–like structures.

MOLECULAR BASIS OF LUNG BUD AND LOBE FORMATION

The outgrowth of the ventral foregut, formation of the trachea, and outgrowth of the main pulmonary bronchi take place during the embryonic period of lung development. The crucial event at this stage is the initiation of lung formation at the right place along the anterior-posterior axis of the foregut. Genetic studies have implicated several transcription factors, morphogens, peptide growth factors, and their cognate receptors in specifying the morphogenetic progenitor field of the lung along the foregut axis (Figure 81-1). One important transcription factor in this process is forkhead box A2 (FOXA2), formerly known as HNF3B.[4,5] FOXA2 is expressed in the ventral foregut endoderm before and immediately at the start of lung bud formation.[6-8] Targeted ablation of FOXA2 in mice led to embryonic death between E6.5 and E9.5, before the onset of lung formation.[9,10] However, chimeras rescued for the embryonic-extraembryonic constriction showed that FOXA2 was essential for foregut and lung formation.[11]

Fibroblast growth factor (FGF) 10 is a member of the large family of FGFs essential to many processes during embryonic development.[12-14] In the murine lung, FGF10 messenger RNA (mRNA) is dynamically expressed in the distal mesenchyme adjacent to the primitive lung buds.[15] The importance of FGF10 for lung development was shown in Fgf10-deficient mice that die at birth because of respiratory failure.[16,17] The Fgf10-deficient mice had complete lung agenesis; that is, lung development had stopped after the formation of the trachea.[16,17] FGFs bind to and signal via FGF tyrosine kinase receptors (FGFR).[12,13,18] The FGF10 receptor (FGFR2IIIB), an FGFR2 splice variant, is expressed in lung bud epithelium.[19] The appositional expression of FGF10 and FGFR2IIIB is in line with lung patterning being dependent on epithelial-mesenchymal interactions.[19] FGFR2b

TABLE 81-1

Stages of Lung Development

	GESTATIONAL AGE			
Stage	**Human (wk)**	**Mouse (days)**	**Main events**	**Epithelial differentiation state**
Embryonic	3.5-7	9.5-14.2	Formation of lung bud, trachea, left and right primary bronchus, and major airways	Undifferentiated columnar epithelium
Pseudoglandular	5-17	14.2-16.6	Establishment of the bronchial tree; all preacinar bronchi are formed	Proximal: columnar epithelium; ciliated, nonciliated, basal, neuroendocrine cells Distal: cuboidal epithelium; precursor type II cells
Canalicular	16-26	16.6-17.4	Formation of the prospective pulmonary acinus, increase of capillary bed	Proximal: columnar epithelium; ciliated, nonciliated, basal, neuroendocrine cells Distal: differentiation of cuboid type II to squamous type I cells
Saccular	24-38	17.4-5 dpn	Formation of saccules, alveolar ducts, and alveolar air sacs	Proximal: ciliated, nonciliated Clara, basal, neuroendocrine cells Distal: type I cells flatten and II cells mature
Alveolar	36-2 ypn	4-30 dpn	Formation of alveoli by septation of alveolar air sacs, thinning of interalveolar septa	Proximal: columnar epithelium; ciliated, nonciliated, basal, neuroendocrine cells Distal: mature type I and II cells mature
Microvascular maturation	Birth-3 ypn	14-21 dpn	Fusion of the capillary bed to a single layered network	

dpn, Postnatal days; *ypn,* postnatal years.

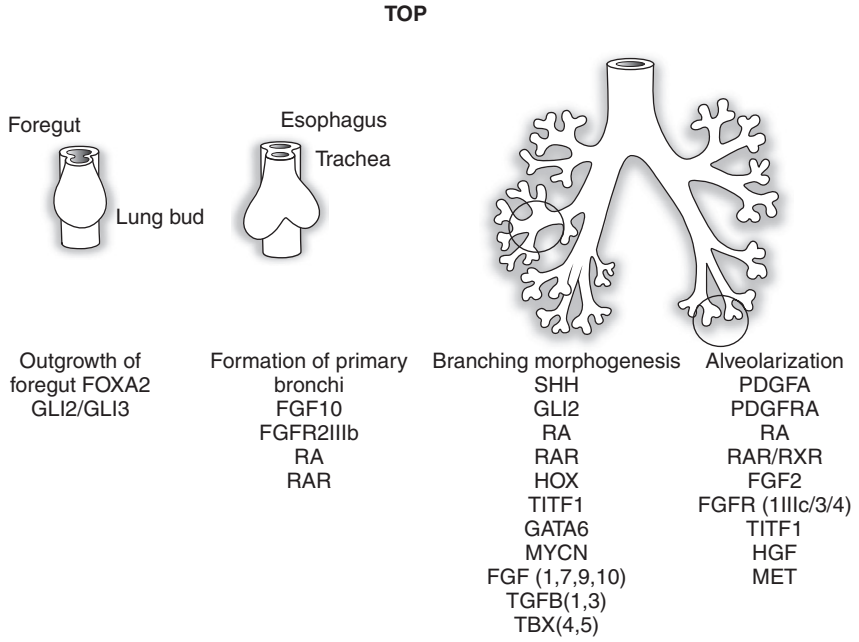


Figure 81-1 Transcription and growth factors regulating lung development (drawings represent a human lung, which in the normal situation consists of two lobes on the left and three lobes on the right side).

is capable of binding FGF1 and FGF7, which have also been implicated in lung development.[13,15,20] Because targeted mutation of FGFR2 resulted in an early lethal phenotype caused by placental insufficiency,[21,22] Fgfr2$^{-/-}$ chimeras were created to overcome this early lethality and allow lung development to be analyzed.[19] As in Fgf10-deficient mice, only the trachea formed without any further pulmonary branching.[19] Similarly, transgenic mice overexpressing a dominant negative FGFR2IIIb splice variant in distal lung epithelium show a severe pulmonary defect, forming only a trachea and two main bronchi, without any lateral branches.[23] Moreover, Cre-mediated excision to generate mice lacking the IIIb form of FGFR2 while retaining expression of the IIIc splice form resulted in mice that had no lungs and that died at birth.[24] Taken together, these data indicate that FGF10 signaling via FGFR2IIIb plays a crucial role in the initiation of lung bud formation (see Figure 81-1). The "no lung" phenotype as a result of inhibited FGF10 signaling shows a striking similarity to a phenotype resulting from the loss of function of either Branchless (BNL) or Breathless (BTL) in *Drosophila*. BNL encodes an FGF homologue that functions as a ligand for BTL, which encodes a *Drosophila* homologue of FGFR. Loss of function of either BNL or BTL prevented tracheal branching in the fly.[25,26]

Sonic hedgehog (SHH) is a secreted signaling molecule and a mammalian homologue of the *Drosophila* Hedgehog (HH) protein, known to be involved in many fundamental processes during *Drosophila* embryonic development.[27] SHH is expressed in the early pulmonary epithelium, with the highest levels of gene expression seen at the tips of developing lung branches.[28] SHH signals via the mesenchymal-located Patched (PTC) receptor, suggesting that the SHH pathway is an important regulator of epithelial-mesenchymal signaling during lung development.[28] The importance of SHH for lung development was shown when the Shh gene was genetically ablated and resulted in a simplistic-looking lung consisting of only one lobe on each side of the trachea (pulmonary left isomerism).[29,30] Thus, in contrast to the Fgf10-null mutant, the initiation of lung formation does occur in SHH-null mutants, but there is an incorrect number of lung lobes and a subsequent failure of branching morphogenesis.[29,30] This was confirmed in work with the creation of a lung-specific Shh-null mouse.[31] In these mice, when SHH expression was removed before E13.5, tracheal, bronchial, and peripheral lung defects were seen similar to those of the Shh-null mouse. The mesenchyme appears to be the primary target of SHH deficiency, showing decreased cell proliferation and increased cell death in the absence of SHH.[29] The effect of SHH on pulmonary mesenchyme was also demonstrated when SHH was overexpressed in distal lung epithelium with use of the surfactant protein C (SFTPC) promoter.[28] Overexpression of SHH resulted in smaller lungs at birth that lacked functional alveoli most likely as the result of hypercellularity of the mesenchyme. Together, these results denote the important function of SHH in epithelial-mesenchymal signaling during early lung formation.

Cubitus interruptus (CI) has been identified as a downstream target in HH signaling in *Drosophila.*[27] Mammalian Gli genes are the putative homologues of *Drosophila* Ci and have also been implicated in mammalian SHH signaling.[27] Three Gli genes have been described in mice: Gli1, Gli2, and Gli3, all of which are expressed in the early pulmonary mesenchyme.[32] Gli3-null mice only show a mild lung defect with a slight reduction in size. However, Gli2-null mice have respiratory failure at birth, presenting with defective airway branching, left pulmonary isomerism, and severe hypoplasia.[33] Concurrent deletion of both Gli2 and Gli3 revealed an even more dramatic phenotype. These $Gli2^{-/-};Gli3^{-/-}$ double-mutant mice have no lung, trachea, or esophagus and die early in gestation. Of interest, other foregut derivatives such as the pancreas, thymus, and stomach do develop in the $Gli2^{-/-};Gli3^{-/-}$-mutant mice, although they are hypoplastic. These data indicate that the combined function of both GLI2 and GLI3 is crucial for the initiation of lung bud formation (see Figure 81-1). The complete absence of trachea and lung formation was ameliorated by the presence of one Gli3 gene, because the $Gli2^{-/-};Gli3^{+/-}$ mutant had a lung consisting of one hypoplastic lobe.[33] The fact that ablation of both Gli2 and Gli3 resulted in a far worse lung phenotype than the deficiency of SHH alone may indicate that SHH is not the only regulator of Gli genes during lung development. The complete absence of a lung in $Gli2^{-/-};Gli3^{-/-}$-mutant mice is similar to the "no lung" phenotype seen in Fgf10-deficient mice. The only difference is that the trachea and esophagus are present in Fgf10-deficient mice but absent in Gli2;Gli3-deficient mice, implicating different signaling pathways. Other single or compound mutants for the three Gli genes show a variable degree of lung hypoplasia with an aberrant number of lung lobes and decreased branching morphogenesis.[33-35] However, another compound mutant recently analyzed revealed an interesting relationship between SHH and Gli3 during lung development. The $Shh^{-/-};Gli3^{-/-}$ mouse shows a phenotype that is less severe than the Shh-null lung alone, with enhanced vasculogenesis and growth potential.[36] Therefore Gli3 must bring about some of the negative effects seen in the Shh-null mouse and clearly denotes the need for SHH to counter the negative function of Gli3 during lung development. For a complete review of SHH-GLI pathway interactions and effect of mutations on lung development, see Rutter and Post.[37]

Retinoic acid (RA) plays a crucial role during gestation and is involved in the developmental processes of almost every organ.[38,39] Both a deficiency and an excess of RA cause congenital defects during human development in a variety of organs.[38,39] RA exerts its effects via the RAR and RXR tyrosine kinase receptors, which function as transcriptional regulators of RA target genes. The RAR family is composed of three genes producing several isoforms: $RARA_{1,2}$, B_{1-4}, and $G_{1,2}$, all activated by both all-trans RA and 9-cis RA, whereas the three isoforms from the RXR family, RXRA, B, and G, are activated only by 9-cis RA.[39] Mice deficient for only one of the isoforms show a less-severe phenotype than expected on the basis of their expression patterns, indicating a high degree of redundancy among the RA receptors.[39] In contrast, compound mutant mice had similar congenital defects as seen with fetal vitamin A deficiency.[38,39] $Rara^{-/-};b2^{-/-}$ double-mutant mice die soon after birth with agenesis of the left lung and hypoplasia of the right lung.[40] Lung hypoplasia was also reported in $Rara1^{-/-};b^{-/-}$ and $Rxra^{-/-};Rara^{-/-}$ double mutants.[41,42] In addition, RA has profound influences on lung development during branching morphogenesis and alveolarization (see later). Furthermore, RA may regulate HOX genes.[43-46] HOX genes form a large family of homeobox-containing transcription factors that are implicated in the specification of cells that form morphologic structures along an anterior-posterior axis. HOX genes are arranged in four chromosomal clusters, and the 3′ to 5′ position of each gene within a cluster corresponds to their expression pattern along the anterior-posterior axis of the developing body.[47] Genes of the 3′ regions of the HOX clusters A and B are expressed in the developing lung, with the HOXB cluster predominantly expressed in the early pulmonary mesenchyme.[43-46,48-53] Within the mesenchyme HOXB genes express in a proximal-distal expression gradient, suggesting a role for HOXB genes in specifying proximal from distal pulmonary mesenchyme.[49] Several mutant models have been created and illustrate the importance of HOX genes during lung development. Single-mutant mice for Hox genes are generally normal, most likely because of redundancy. However, compound $Hoxa1^{-/-};Hoxb1^{-/-}$ mutants have severe lung hypoplasia with a phenotype ranging from five hypoplastic lung lobes to a lung with only two lobes.[54] $Hoxa5^{-/-}$ mice die in the perinatal period and present with laryngotracheal malformations, a reduced tracheal lumen, and lung hypoplasia.[55]

Another homeodomain transcription factor expressed at the onset of lung morphogenesis is thyroid transcription factor 1 (TITF1), previously known as NKX2.1.[56-58] Expression of TITF1 mRNA is localized to epithelial cells of the developing pulmonary tubules and decreases in more proximal conducting airways with advancing gestation.[8,59,60] TITF1 continues to be expressed in adult bronchiolar and alveolar epithelial type II cells, where it plays an important role in the regulation of Clara cell secreted protein (CCSP) and surfactant protein synthesis (see later). Targeted disruption of Titf1 results in severe hypoplasia of the thyroid and lung, with a developmental arrest at the pseudoglandular stage of lung development.[61] In contrast to the different null mutants in the SHH/GLI signaling pathway, Titf1-null mutants lack distal epithelial cell differentiation.[58,61]

SEPARATING ESOPHAGUS AND TRACHEA

The formation of a tracheoesophageal septum will divide the ventral trachea from the dorsal esophagus, and failure to do so will result in a not uncommon congenital defect in humans called tracheoesophageal fistula.[62] Deficiency of all aforementioned growth and transcription factors that are implicated in the etiology of lung agenesis or hypoplasia also results in tracheoesophageal fistula with different gradations of severity

(Table 81-2). Gli2$^{-/-}$;Gli3$^{-/-}$-mutant mice have no trachea, nor do they form an esophagus, and Gli2$^{-/-}$;Gli3$^{+/-}$ mutants have a single tracheoesophageal tube connected to the stomach.[33] In SHH$^{-/-}$-mutant mice, the trachea and esophagus fail to separate, resulting in tracheoesophageal fistula.[29,30] Mice haploinsufficient for the transcription factor forkhead box F1 (FOXF1) also known as HFH8 exhibit foregut abnormalities including narrowing and, sometimes, atresia of the esophagus, as well as frequent fusion of trachea and esophagus.[63] FOXF1 expression was absent in foregut derivates (trachea, esophagus, oral cavity, lungs) of Shh$^{-/-}$ mutants, indicating that SHH signaling is required for activation of FOXF1 in these tissues.[63] Surprisingly, separation of the trachea and esophagus occurs normally in FGF10-deficient mice,[16,17] whereas Titf1-deficient mice have a complete tracheoesophageal defect.[58] Rara$^{-/-}$;B2$^{-/-}$-, Rara1$^{-/-}$;B$^{-/-}$-, and Rxra$^{-/-}$;Rara$^{-/-}$-mutant mice all exhibit a tracheoesophageal septum defect and other tracheal malformations such as disorganized cartilaginous rings and shortening of the trachea.[40-42] The end result of RA signaling in relation to lung development appears to be mediated through FGF10. This became evident in studies using the pan-RAR antagonist BMS493 in foregut explant cultures, which resulted in failure of initial lung bud outgrowth in the prospective lung field because of inhibition of FGF10 expression in the foregut mesenchyme.[64]

LEFT-RIGHT ASYMMETRY

Around 5 weeks gestation, five separate lobes can be identified in the human lung: two on the left and three on the right. In the mouse, the right lung has four major lobes whereas the left lung consists of only one small lobe. Such left-right asymmetries during development are an integral part of the establishment of a body plan, and several highly conserved mechanisms have been found to initiate the vertebrate left-right axis, including the SHH signaling pathway.[65] Molecules such as SHH,[66,67] FGF8,[66,68] N-cadherin,[69] activin B, the activin receptor IIB,[70] and FOXJ1 (HFH4)[71] have all been shown to influence left-right asymmetry during development (Figure 81-2). However, it appears that all these pathways converge to influence the expression patterns of genes in the transforming growth factor B (TGFB) family of cell-cell signaling factors called NODAL, LEFTY1, and LEFTY2.[72-75] SHH and LEFTY1 mutants have left pulmonary isomerisms with only one lobe on each side of the lung.[29,30,67,73] Both SHH and FOXA2 are thought to act upstream of LEFTY1 and are normal in Lefty1$^{-/-}$ mice.[73] Although no data are available on the expression of LEFTY1 in the Shh$^{-/-}$-mutant lung, LEFTY1 is absent in SHH$^{-/-}$ mutant lateral plate mesoderm (LPM).[67] Lefty1$^{-/-}$ mice show bilateral expression of NODAL and LEFTY2 in LPM, which results in ectopic expression of the homeobox transcription regulator paired-like homeodomain transcription factor 2 (PITX2) in the right side of the foregut region.[73] PITX2 is clearly a powerful determinant of left-right asymmetry because ectopic expression of PITX2 in the right LPM alters looping of the heart and gut and reverses body rotation in *Xenopus* embryos.[76] In contrast, the phenotype of Pitx2$^{-/-}$ mice demonstrates right pulmonary isomerism and altered cardiac position.[77-79] Growth differentiation factor 1 (GDF1) encodes another member of the TGFB superfamily, and it is proposed to act upstream of NODAL, LEFTY1 and 2, and PITX2.[80] Mice deficient for this factor, as well as hypomorphic mutants for NODAL, exhibit right pulmonary isomerism.[72,80] It has not been possible to investigate the role of FOXA2 in left-right asymmetry formation of the lung, because Foxa2-null mutant mice die before the onset of lung formation. However, in rescued Foxa2 mutant mice SHH was not detected in the foregut region.[11] Another FOX family member, FOXJ1, has been shown to have a critical role in left-right axis determination because the Foxj1-null mutant shows random left-right asymmetry of the visceral organs (lung included) resulting from defective ciliary development.[71,81]

BRANCHING MORPHOGENESIS

The early branching of the primary bronchi is monopodal; then starting at the level of the secondary bronchus, each bronchus subsequently undergoes dichotomous branching where each branch bifurcates repeatedly into two branches. The enlarging bronchial tree expands through 23 generations of branching (in humans) into the surrounding mesenchyme, which will eventually furrow into the characteristic lobes of the lung: two on the left and three on right in humans and one left and four right in mice. Of the 23 generations of lung branching, the first 16 are genetically hard wired and thus reproducible, but the latter half appear to have a random distribution.[82]

Early studies indicated that branching of the lung buds is controlled by epithelial-mesenchymal tissue interactions,[83,84] where

TABLE 81-2

Separating Trachea and Esophagus

Condition	Trachea	Esophagus	Remarks
FGF10$^{-/-}$	+	+	T-E separation; trachea ends blind
SHH$^{-/-}$	+	+	T-E septum defect
GLI2$^{-/-}$	+	+	T-E separation with stenosis
GLI2$^{-/-}$;GLI3$^{-/+}$	+	–	Single (tracheal) tube connecting to the stomach, esophageal atresia
GLI2$^{-/-}$;GLI3$^{-/-}$	–	–	No esophagus, trachea, or lung
RARA$^{-/-}$;B2$^{-/-}$ RARA1$^{-/-}$;B$^{-/-}$	+	+	T-E septum defect, tracheal cartilage malformations
TITF1$^{-/-}$	+	+	T-E septum defect

T, Trachea; *E,* esophagus.

Transcription and growth factors involved in the separation of esophagus and trachea around gestational week 4 in humans and day 10 in mice. A failure of separation results in a (in)complete trachea-esophagus septum defect, a not uncommon congenital anomaly in humans.

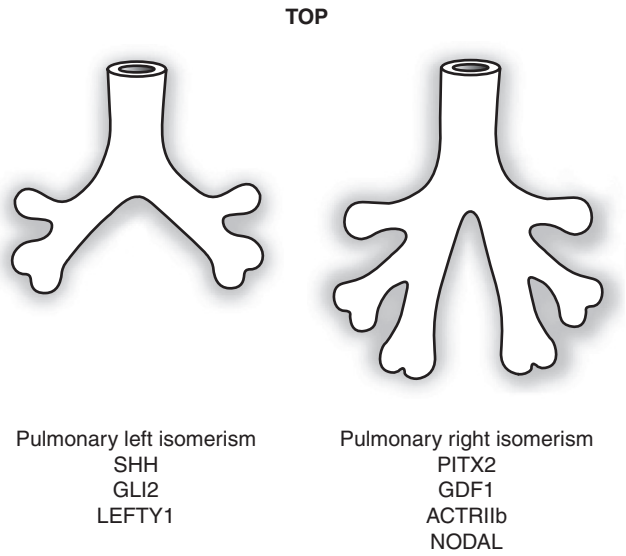


Figure 81-2 Transcription and growth factors involved in left-right determination of the developing mouse lung (drawings represent a human lung, which in the normal situation consists of two lobes on the left and three lobes on the right side).

a soluble factor from the mesenchymal compartment appeared most likely to dictate the branching pattern of the epithelium.[85] The strong inductive capacity of pulmonary mesenchyme was shown when pulmonary mesenchyme was used to induce a lung epithelial phenotype in epithelial ureter buds.[86] Branching appears also to be regulated by positional information along the anterior-posterior axis of the lung because proximal (trachea and main bronchi) and distal (lung bud) mesenchyme differ in their ability to support epithelial branching morphogenesis.[87-89] Some progress has been made in elucidating the complex mixture of transcription factors and morphogens, which guide proper lung branching (see Figure 81-1). Although alterations in cell adhesion and matrix remodeling at the epithelial-mesenchymal interface also contribute to lung branching morphogenesis, they will not be discussed, and the reader is referred to Keijzer and Post.[90]

Although the already mentioned transcription factors belonging to the GLI, HOX, and FOX families are likely involved in this process, other transcription factors that have been implicated in lung branching morphogenesis include GATA binding protein 6 (GATA6) and MYCN. GATA6 belongs to the GATA family of zinc finger–containing transcription factors and is expressed in epithelial and mesenchymal cells of the developing lung bud.[91,92] GATA6 is essential for endoderm formation because targeted deletion of GATA6 resulted in embryonic death before lung formation caused by failure of visceral endoderm formation.[93,94] In the lung, GATA6 appears to be important for branching morphogenesis, because inhibition of GATA6 expression results in decreased branching morphogenesis.[91,94] In addition, expression of a GATA6 engrailed dominant negative fusion protein in the distal lung epithelium resulted in a lack of alveolar type I cells and a perturbation in alveolar type II cells together with a reduction in the number of proximal airway tubules.[95] On the other hand, overexpression of GATA6 using the SFTPC promoter resulted in disrupted branching morphogenesis and a lack of distal epithelial cell differentiation.[96] This suggests that a balanced GATA6 expression level is important for branching morphogenesis during the pseudoglandular period of lung development and also during later stages of lung development when alveolar type I and II cells differentiate and the gas exchange area matures. MYCN is a member of the myc family of proto-oncogenes, which includes MYCN, MYC, and MYCL, and all belong to the basic helix-loop-helix (bHLH) class of transcription factors. In the lung, MYCN is expressed in pulmonary epithelium,[97,98] and mice homozygous for the Mycn-null mutation die at midgestation.[98,99] Leaky null mutants for MYCN survive to the point when lung development starts; however, pulmonary branching morphogenesis is dramatically reduced, resulting in severe lung hypoplasia.[97,100] More recently, mice containing a lung-specific Mycn-null mutation confirmed the critical role of MYCN during lung development, because these mice have abnormal lungs showing a loss of proliferation, increased apoptosis, and a reduction in branching morphogenesis.[101] Additional experiments from this group using transgenic mice expressing MYCN under the SFTPC promoter confirmed the link between MYCN and lung growth because these mice showed increased proliferation, apoptosis, and a reduction in cellular differentiation.

Several growth factors including FGFs and TGFBs have been shown to regulate lung branching morphogenesis.[90,102] FGFs are generally produced by the pulmonary mesenchyme whereas FGFR1 and FGFR2 have been localized to embryonic and fetal lung epithelium.[103,104] Transcripts for FGF7 are detected in lung mesenchyme and are expressed at sites of active branching morphogenesis.[105,106] Exogenous FGF7 inhibits rat lung branching morphogenesis in vitro[107] but stimulates proliferation of adult type II pneumocytes in vitro[108] and in vivo.[109,110] Surprisingly, mice bearing a null mutation of the FGF7 gene had no obvious lung abnormalities,[111] suggesting that the function of FGF7 can be replaced by other factors such as FGF1 and/or FGF10. FGF1 binds to both FGFR2 splice variants, FGFR2IIIb and FGFR2IIIc, and is crucial for branching of embryonic mouse epithelium in mesenchyme-free culture.[112] FGF2, which binds to FGFR2IIIc, but not to FGFR2IIIb, did not affect epithelial branching in these cultures, suggesting that the effects of FGF1 on epithelial branching are mediated via the FGFR2IIIb receptor, which also binds FGF7.[113] As mentioned earlier, FGF10 binds to FGFR2IIIb, and both null mutants for FGFR2IIIb and FGF10 show complete lung agenesis.[16,17,24] Of interest, antisense oligonucleotides directed toward T-box genes 4 (Tbx4) or 5 (Tbx5) to attenuate their transcript levels reduced branching morphogenesis in mouse lung explant cultures.[114] Concurrent knockdown of both TBX4 and TBX5 resulted in complete loss of lung branching in culture. On closer examination it was found that FGF10 transcript levels were completely attenuated in the double knockdown lung cultures and reduced in the case of individual TBX4 or TBX5 targeting. In vitro FGF10 elicits endodermal expansion and bud formation, whereas FGF7 induces expansion of the endoderm but never progresses to bud formation.[115] This suggests that FGF7 and FGF10 signals are transduced in different physiologic responses and may explain why FGF7 cannot compensate for loss of FGF10. Another member of the FGF family, FGF9, is expressed in pulmonary mesothelium and epithelium in early development and later only in the mesothelium.[19,116] However, more recent data indicate that FGF9 is expressed at significant levels in the lung epithelium at E14.5.[117] This expression pattern is different from that of FGF1, FGF7, and FGF10, which are expressed only in the lung mesenchyme. Targeted deletion of FGF9 resulted in severe lung hypoplasia and immediate postnatal death.[116] Analysis of the lungs revealed decreased branching morphogenesis and a lack of alveoli; however, the number of lung lobes and primary bronchi were normal.[116] Recent studies using recombinant FGF9 on lung explant cultures showed potent effects on growth, differentiation, and branching morphogenesis, and furthermore FGF9 exerts its influence on both the epithelium and mesenchyme separately.[117] It also appears that the impact of FGF9 on lung development is effected through changes in expression of FGF10 and FGF7. Additional studies using an inducible system spatially regulated by the SFTPC promoter to express FGF9 during lung development also demonstrate that FGF9 has the ability to induce SHH, PTC, SPRY2, and BMP4 expression, indicating that FGF9 may be a crucial regulator of epithelial-mesenchymal signaling.[118]

TGFB belongs to a superfamily that includes activin, bone morphogenic protein (BMP), and TGFB1, 2, and 3. These peptides can exert a variety of biologic effects including regulation of cell growth, differentiation, and expression of a variety of proteins. In the lung, TGFB1 mRNA and protein are found in the mesenchyme and epithelium, respectively.[119-121] Both addition of exogenous TGFB1 to cultured embryonic mouse lung explants and in vivo overexpression of TGFB1 in distal lung epithelial cells resulted in decreased branching morphogenesis, inhibited distal epithelial cell differentiation, and inhibited formation of the pulmonary vasculature, indicating an inhibitory role for TGFB1 during branching morphogenesis.[122-126] Most, if not all, biologic activities of TGFB are transmitted via transmembrane Ser/Thr kinase receptors, known as type I and type II receptors.[127] Signal transduction requires the formation of a heteromeric complex of type I (TGFBR1) and type II (TGFBR2) receptors. Inhibition of TGFBR2 signaling stimulated lung morphogenesis in whole lung explants in culture, underlining again the negative effects of TGFB signaling on branching morphogenesis.[128] In vivo studies show that Tgfb1-null mutants per se have no gross developmental abnormalities; however, 50% of the null mutants die before E11.5 as a result of defects in yolk sac vascularization.[129,130] Tgfb3-null mutants die after birth with cleft palate and delayed pulmonary development,[131] and Tgfb2-deficient mice die after birth and display a lung phenotype with postnatal collapse of alveoli and terminal airways.[132] SMAD proteins are downstream

effecter proteins in the TGFB signaling pathway.[133] SMAD1-3 proteins are expressed in distal lung epithelium, and SMAD4 is expressed in both distal lung epithelium and mesenchyme.[134,135] Down-regulation of SMAD2/3 and SMAD4 expression increased branching morphogenesis in cultured lung explants. Exogenous TGFB1 did not reverse this inhibitory effect, which is consistent with TGFB1 being upstream of SMAD proteins.[135]

EPITHELIAL DIFFERENTIATION

As branching proceeds, numerous different cell phenotypes are formed along the proximodistal axis of the developing epithelial tubules and associated mesenchymal components, each with different morphologies and patterns of gene expression. This patterning of differentiated lung cells may also be controlled by epithelial-mesenchymal interactions.[85,87] Over the last decade some regulatory molecules involved in epithelial morphogenic patterning in the lung have been identified (Figure 81-3).

Pulmonary neuroendocrine cells (PNECs) are the first cells to differentiate in humans and animals.[136,137] MASH1 is a bHLH transcription factor implicated in neural differentiation.[138] In the lung, MASH1 is expressed in clusters or single-progenitor PNECs.[139] Mice deficient for Mash1 do not develop PNECs, indicating an essential role for this transcription factor in the differentiation of PNECs.[139] HES1 encodes a bHLH protein that is up-regulated in response to NOTCH activation and represses downstream targets such as MASH1, thereby preventing neural differentiation.[140] This finding was supported by recent work using approaches to both knockdown and up-regulate NOTCH1 expression to show effects on HES1 regulation and PNEC differentiation.[141] Indeed, Hes1-deficient embryos have increased MASH1 mRNA expression and PNECs in their lungs.[142] Whether the NOTCH signaling pathway plays a further role in distal epithelial specification remains to be elucidated. Transcription factor 21 (TCF21), formerly POD1, is another bHLH protein that acts as transcriptional regulatory protein that governs cell fate determination and differentiation in a variety of tissues. The Tcf21-null mutant dies at birth with severely hypoplastic lungs that have a reduced number of tertiary branches and a lack of acinar tubules, terminal air sacs, and alveoli.[143] Marker analysis revealed a disturbance in proximal-distal patterning of the lung epithelium with increased CCSP and decreased SFTPC expression.

Epithelial transcription factors such as FOXA2, GATA6, and TITF1 have also been shown to influence lung epithelial specification. In both fetal mouse and human lung, the spatial-temporal distribution of TITF1 follows the pattern of expression of surfactant proteins.[8,59] It has been shown that TITF1 regulates the transcription of SFTPA, B, and C[144-148] and CC10,[144,149] and consequently Titf1-null mutants lack distal epithelial cell differentiation, whereas the proximal epithelial cell marker FOXJ1 was unaffected.[150] Taken together, these data underline the importance of TITF1 for the establishment of the distal epithelial cell phenotype. Also, GATA6 transactivates SFTPA and TITF1,[151,152] and it has recently been shown that GATA6 acts synergistically with TITF1 to influence the activity of the SFTPC promoter.[153] A role for GATA6 in bronchial epithelial specification has been suggested by the observation that Gata6$^{-/-}$ embryonic stem cells fail to contribute to bronchial epithelial cells.[94] In some respiratory epithelial cells, TITF1 is coexpressed with members of the FOX family of transcription factors. Transcripts for FOXA1 and FOXA2 are detected in foregut cells forming the embryonic lung bud and later in the distal epithelium of the developing and mature lung.[8,154] Like TITF1, FOXA1 and FOXA2 appear to modulate the expression of SFTPB and CCSP.[144,154,155] FOXA1 and FOXA2 have been proposed to be upstream regulators of TITF1,[156] and it is possible that both members of the FOX family confer

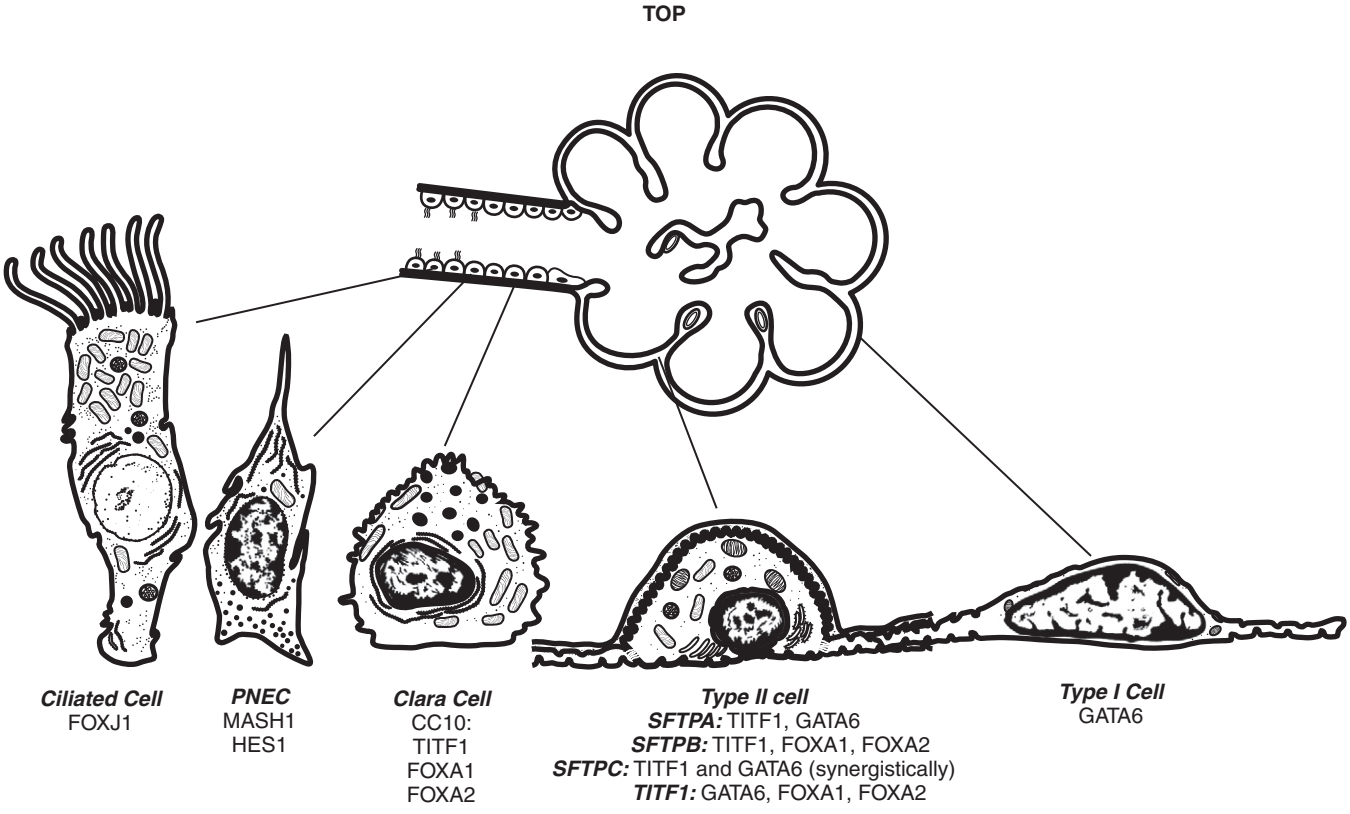


Figure 81-3 Molecular control of epithelial cell differentiation during lung development (drawings represent a human lung, which in the normal situation consist of two lobes on the left and three lobes on the right side).

lung-specific gene expression in the primitive foregut through TITF1 as the intermediate. FOXJ1 is a transcription factor of the winged helix–forkhead family, expressed in various tissues during development. In the developing and adult lung, FOXJ1 expression is restricted to ciliated cells of the bronchial and bronchiolar epithelium.[150,157] The role of FOXJ1 in ciliated cell differentiation was clearly demonstrated when FOXJ1 was overexpressed in distal pulmonary epithelial cells. High levels of FOXJ1 expression inhibited branching morphogenesis and development of distal epithelial cells; however, it enhanced the development of ciliated cells.[158] In contrast, Foxj1-null mutant mice completely lack respiratory ciliated cells.[71,159] These data indicate that FOXJ1 is essential for the development of pulmonary ciliated cells.

The secreted morphogen SHH does not appear to be involved in regulating proximodistal epithelial specification because SFTPC and CCSP are expressed in Shh-deficient mice.[30] In *Drosophila*, HH may regulate the expression of Decapentaplegic (DPP), the *Drosophila* counterpart of bone morphogenetic proteins (BMP).[27] In the murine lung, BMP4 is implicated in lung epithelial specification. BMP4 is expressed in early distal lung tips and at lower levels in the mesenchyme adjacent to the distal lung buds.[160,161] Overexpression of BMP4 in the distal epithelium in vivo resulted in hypoplastic lungs with grossly dilated terminal lung buds separated by abundant mesenchyme.[160] Distal epithelial differentiation was abnormal with decreased SFTPC expression, whereas proximal differentiation (CCSP expression) was normal.[160] In vitro, exogenous BMP4 clearly enhanced peripheral lung epithelial branching morphogenesis and SFTPC expression.[162] The secreted BMP antagonist Xnoggin is expressed in distal mouse lung mesenchyme early on in development.[161] Overexpression of Xnoggin or the dominant negative BMP receptor dnAlk6 in distal pulmonary epithelium resulted in a proximal pulmonary epithelial phenotype.[161] Similar results were obtained when Gremlin, another BMP antagonist, was overexpressed in the distal lung epithelium.[163] These studies clearly indicate a role for BMP4 in proximodistal epithelial differentiation during lung development and as a result probably argue against SHH regulating BMP4 expression.

ALVEOLAR DEVELOPMENT

Alveolarization is the last step of lung development that induces physical changes to the internal surface area of the lung. Alveoli are formed by septation of the pulmonary saccules that form the immature lung. In both humans and mice, alveolarization occurs predominately after birth. One of the key players in the alveolarization process (see Figure 81-1) appears to be one of the three isoforms of the platelet-derived growth factors (PDGF), namely PDGF-A.[164] PDGF-A is normally expressed in early pulmonary epithelium and becomes undetectable at later gestations.[165] Its receptor (PDGFR-α) is expressed in the mesenchyme adjacent to the epithelium that expresses PDGF-A, signifying a paracrine signaling mechanism between the epithelium and the mesenchyme.[165-167] In mice, absence of PDGF-A results in prenatal and postnatal death. Postnatal deaths were characterized by emphysematous lungs with areas of atelectasis, without any formation of septa and/or alveoli, instead, dilated prealveolar saccules were found.[166,167] In normal mice, alveolar septa contain smooth muscle cell alpha actin (α-SMA)–positive myofibroblasts. The postnatal Pdgf-A–null mutant lungs lack alveolar staining for α-SMA, indicating a lack of alveolar myofibroblasts. In addition, they were almost completely devoid of parenchymal elastin fibers, which most likely contributed to the failure of alveolar formation.[166,167] Of interest, myofibroblasts surrounding vessels and bronchioles appeared normal and were tropoelastin positive, suggesting a different developmental lineage.[167] Moreover, PDGFR-α–positive cells were specifically missing from lungs of PDGF-A–null mutants, and it has been proposed that these cells are progenitor cells for alveolar myofibroblasts.[166,167] With these data taken together, PDGF-A is important for the formation of alveolar myofibroblasts that produce elastin, which in turn is important for alveolar formation. Pdgfr-α–deficient mice die in utero with severe skeletal malformations and incomplete cephalic closure. Pdgfr-α–deficient lungs were hypoplastic; however, primary branching and histology were not affected.[168,169] Obviously, postnatal alveolar formation could not be examined in these mice.

Both FGFR3 and FGFR4 are expressed in postnatal pulmonary mesenchyme, whereas their ligands are expressed in pulmonary epithelial cells.[170] Although a null mutation of either FGFR3 or FGFR4 caused no obvious lung defects, concurrent silencing of both receptors resulted in severe overall body growth retardation and a failure of postnatal alveolar formation. Despite the large dilated saccules without any proper alveolar septation, differentiation (including α-SMA–positive myofibroblasts) and proliferation proceeded normally.[170] A recent study examining postnatal alveolar formation in the rat has found a new connection with FGF signaling in postnatal lung development.[171] When signaling of FGF2 (most likely through FGFR1IIIc) was blocked, it inhibited normal postnatal lung development. Histologically these lungs presented with a reduction in secondary septation and a decrease in apoptosis, resulting in increased tissue to air ratio. Besides its role in prenatal lung development, TITF1 also regulates postnatal lung development and homeostasis. After birth, TITF1 expression decreases dramatically; however, expression remains detectable in adult alveolar type II cells. Overexpression of TITF1 in distal lung epithelial cells, using the SFTPC promoter, does not affect prenatal lung development but perturbs postnatal alveolarization, leading to emphysema, severe inflammation, and fibrosis.[172] RA has been shown to increase the number of alveoli in postnatal rats and even abrogates the decreased alveolarization seen after the experimental use of dexamethasone or elastase that decreases alveolar formation. This indicates that RA is a potential controlling factor in "neoalveogenesis" in prematurity or after lung injury.[173-176] Similarly, compound mice homozygous for RARG and heterozygous for RXRA deletion have a reduced number of alveoli and less elastic fiber in their alveolar walls.[177] RARB on the other hand appears to be an endogenous inhibitor of septation, and as a result the Rarb-null mutant shows early onset septation resulting in twice as many alveoli in the null mutant lungs when compared with wild-type lungs.[178] Hepatocyte growth factor (HGF) is a heparin-binding glycoprotein with mitogenic and morphogenic effects on a variety of tissues. Recently it was shown that HGF-neutralizing antibodies injected intraperitoneally into rat pups caused a reduction in the number of alveoli.[179] This was also true when a truncated soluble form of the hepatocyte growth factor receptor (MET) was injected intraperitoneally. These two results combined clearly indicate an important and previously unknown role for HGF signaling in the lung during postnatal alveolarization.

VASCULAR DEVELOPMENT

The lung is composed of a complex network of airways and vessels, and, although a great deal has been discovered regarding the mechanisms controlling lung bud formation and airway branching, the elements guiding vascular formation remain obscure. The lung is unique among most other organs in that it possesses a dual vascular system: the pulmonary system that transports the blood to be oxygenated from and back to the heart and the bronchial system that provides oxygenated blood to the nonrespiratory parts of the lung. Three processes are thought to control pulmonary vascular development: angiogenesis, which is defined as sprouting of new vessels from preexisting ones and gives rise to the central vessels; vasculogenesis, which is de novo synthesis of blood vessels from blood lakes in the periphery of the lung; and the fusion of proximal and peripheral vessels to form the

pulmonary circulation.[180] More recently it was shown that even in the early stages of lung development vascular connections are well established and that pulmonary vascular formation occurs at all developmental stages, with completion of a single capillary network during the period of microvascular maturation.[3,181] A third mechanism of pulmonary vascular formation that relies heavily on vascular remodeling has been proposed and contradicts the other two. This theory uses a distal angiogenesis-driven model in which the vasculature is formed very early on in lung development and surrounds the epithelial bud tips as they grow out into the splanchnic mesenchyme.[182] As the epithelial buds branch and rebranch growing outward, the developing vasculature rapidly expands and remodels with the bud tips. These epithelial-endothelial interactions between the capillary plexus and the epithelial bud tip coordinate growth between the developing airways and vasculature.

Members of the vascular endothelial growth factor (VEGF),[183,184] the angiopoietin,[185,186] and the ephrin families[187] have all been implicated in controlling vascularization of the pulmonary system. VEGF is a potent mitogen for endothelial cells, influencing angiogenesis and vasculogenesis.[188] It is essential for embryonic development, and even haploinsufficiency of VEGF results in embryonic lethality.[189,190] In both the embryonic and adult lung, VEGF mRNA is detected in the lung epithelium[191-193] and signals via two high-affinity receptors: FLT1 and KDR (formerly FLK), which in the embryonic lung are localized to the mesenchyme.[191,192] The complementary expression patterns of VEGF in lung epithelium and the two VEGF receptors in the mesenchyme suggest an epithelial-mesenchymal signaling mechanism and possible influence on lung development and branching morphogenesis.[194] Overexpression of the VEGF 164 isoform in distal airway epithelium results in perinatal death.[195] The lungs appear abnormal, with dilated respiratory tubules and saccules and have a decreased number of terminal buds. KDR expression in the mesenchyme was increased, indicative of a regulatory role of VEGF between pulmonary epithelium and mesenchyme. Mice that lack the VEGF isoforms 164 and 188 and express only the 120 isoform have decreased peripheral vascular development with fewer air-blood barriers and a general delay in lung development.[196] Another factor implicated in pulmonary vascular development is FOXF1. Foxf1-null mutant mice die in utero because of defects in mesodermal differentiation and cell adhesion.[197] In the lung, FOXF1 is expressed in smooth muscle cells surrounding bronchioles and alveolar endothelial cells.[198,199] Heterozygous mutant mice carrying a disruption of the Foxf1 gene, in which FOXF1 levels are reduced by 80%, displayed a 55% postnatal mortality with lung hemorrhaging. Analysis of the lungs revealed abnormalities in alveolar formation and pulmonary vasculature.[198] FOXA2 has also been demonstrated to have effects on vascular development of the lung. Experiments using the SFTPC promoter to express FOXA2 in the distal lung epithelium showed disrupted airway branching, vasculogenesis, and arrested differentiation of epithelial cells.[200] However, this finding was complicated by a more recent study using mice containing a conditional knockout for FOXA2, which showed defects only in alveolarization and differentiation.[201]

From these studies one can reason that a disruption in pulmonary vascular development goes hand in hand with impaired branching morphogenesis and lung hypoplasia. Indeed, some recent evidence suggests that development of the vascular system exerts a strong influence over lung morphogenesis. Blocking VEGF signaling by using a pharmacologic inhibitor has been shown to impair not only vascular growth but alveolarization as well.[202] This was confirmed in other experiments using antisense oligonucleotides targeted toward VEGF that caused a reduction in both airway branching and vascular development.[203] In vivo delivery of VEGF using intratracheal administration of an adenovirus carrying the VEGF expression construct not only results in increased vessel formation but restores alveolar growth in an experimental rat model of BPD.[204] Finally, both loss and gain of function experiments were used in mouse lung cultures to confirm the effect of VEGF on both the lung epithelium and endothelium, in terms of proliferation, differentiation, and branching morphogenesis.[205] However, the question still remains whether the airway branching phenotype is the direct result of physical disruption of blood vessel formation or whether it is the molecular signaling factors of vascular development alone affecting airway branching morphogenesis.

SUMMARY

Molecular studies of lung development have started to unravel the complex series of events that control proper formation of the lung. Observations such as no lung formation are interesting for understanding organogenesis of the lung per se, but the clinical relevance is minimal because having no lung is incompatible with life. However, the finding that factors implicated in foregut specification are also regulating lung morphogenesis is tremendously intriguing for clinical practice (see Figure 81-1). One of the major complications with preterm birth is immaturity of the lung. Despite modern management, many infants exhibit lung dysfunction characterized by arrested lung development and interrupted alveolarization. A better understanding of the molecular basis of pulmonary development may guide clinicians in the design of strategies to mimic normal lung maturation in a premature infant.

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82 Regulation of Liquid Secretion and Absorption by the Fetal and Neonatal Lung

David P. Carlton

The fetal lung is filled with liquid secreted by the developing lung epithelia. The rate and volume of liquid secreted into the fetal lung are calibrated to maintain lung volume at approximately functional residual capacity and are major determinants of normal lung growth. At birth, effective transition from placental to pulmonary gas exchange requires removal of liquid from the lung. The process of emptying the lung begins before birth, is augmented by labor, and is mostly complete after 2 hours of independent breathing. Liquid is removed from the lung lumen by a combination of mechanical drainage and liquid absorption across the lung epithelium (Figure 82-1). The liquid absorption mechanism is switched on by the perinatal epinephrine surge associated with labor and delivery. This epinephrine responsiveness is absent in the immature fetal lung and is induced in the last half of gestation by the rise in active thyroid and steroid hormones in the fetal circulation. The increase in oxygen tension (Po_2) associated with the onset of air breathing at birth consolidates the switch to liquid absorption. Because the absorptive mechanism of the fetal lung develops in the latter part of gestation, infants who are born prematurely may have a restricted ability to clear fluid from their lungs.

DYNAMICS OF LUNG LIQUID FLOW BEFORE AND AFTER BIRTH

Lung Liquid Formation and Flow During Fetal Life

Because it is intuitive that the fetal lung lumen must be filled with liquid rather than air, early debate about the fetal lung revolved around the origins of this liquid. The preferred early hypothesis was that liquid was aspirated by fetal breathing movements into the lung from the amniotic cavity.[1] However, the proposed amniotic origin of lung liquid was disproved in the 1940s by Jost and Policard,[2] who observed progressive lung distention in fetal rabbits whose tracheas had been ligated. Further evidence of local production of fetal lung liquid came from the observation in fetal sheep that the ionic composition of fetal lung liquid differed from that of amniotic fluid—sodium (Na^+) and chloride (Cl^-) concentrations are lower in amniotic fluid, and potassium (K^+) is higher.[3,4] In addition, the observation that the Cl^- concentration of fetal lung liquid was higher that that of plasma or interstitial fluid was the strongest early evidence that lung liquid may be actively secreted by the pulmonary epithelium.[4]

Liquid Secretion

The source of lung liquid was subsequently shown to derive from an active secretory process by the epithelium lining the developing lung.[5] The rate of liquid secretion was measured in fetal guinea pig, goat, and sheep by collection of diverted tracheal fluid, by tracheal flowmeter, and by the indicator dilution method.[5-10] There is an apparent increase in the secretion rate in fetal sheep from approximately 1.5 mL/kg/hour at midgestation to approximately 5 mL/kg/hour in late gestation.[11,12] The secreted liquid is then passively expelled into the trachea and into the oropharynx, where it is either swallowed or expelled into the amniotic cavity. The volume of liquid in the fetal lung lumen is regulated by the distending pressure of approximately 2 mm Hg (relative to amniotic fluid) generated by liquid secretion and resistance to liquid efflux through the fetal larynx.[13-15] The volume of liquid in the fetal lung increases with growth of the fetus and with the increasing alveolarization of the lung during gestation. In the latter part of gestation, the developing fetal sheep lung is distended to a volume (approximately 30 mL/kg) that approximates functional residual capacity of a term newborn infant.[16] The volume to which the fetal lung is distended is apparently crucial for normal lung development. Experimental continuous intrauterine drainage of liquid from fetal sheep lungs resulted in lung hypoplasia, whereas overdistention of the fetal lung by tracheal ligation resulted in hyperplasia.[17-19]

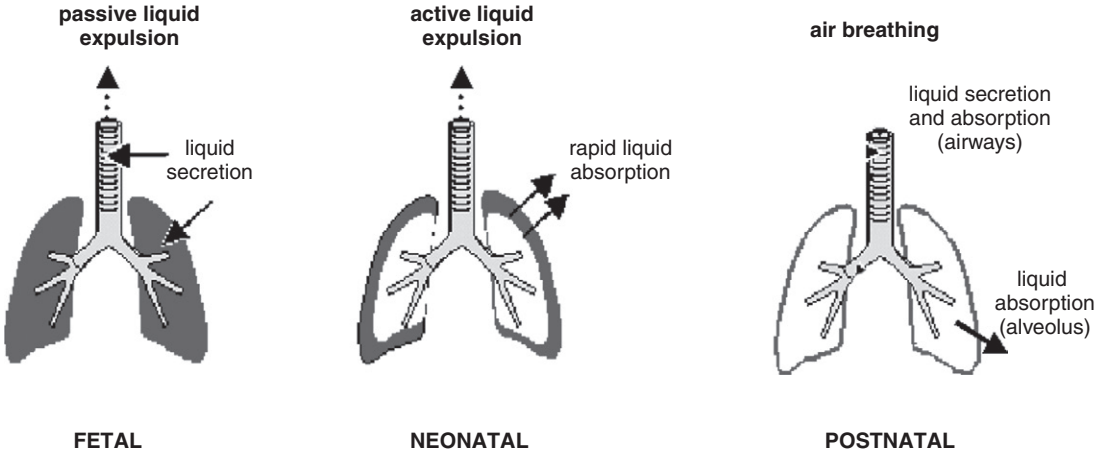


Figure 82-1 Fetal, neonatal, and postnatal phases of liquid flow across lung epithelia.

Fetal Breathing Movements

Fetal breathing movements initiated by diaphragmatic contractions result in small oscillations of liquid flow (~0.5 mL) in the conducting airways.[20-22] Disruption of fetal breathing by phrenic nerve section or cervical cord lesions results in lung hypoplasia through mechanisms that most likely are related to decreased fetal lung volume.[23-25] Although it is likely that these breathing movements are important for normal lung growth, they do not influence the rate of liquid secretion or the net movement of lung liquid out of the lung.

Airway Wall Contractions

Peristaltic contractions of the developing airways cause changes in local lung liquid flow and pressure that may also be important in lung development. Striking contractions of embryonic airways were first observed in chick embryos in the 1920s.[26] Studies on various animal models demonstrate that these contractions cause peristalsis of the airway similar to that seen in the gut.These peristaltic waves propagate distal movement of intraluminal liquid and cause expansion of the endbuds.[27] Smooth muscle contractions were inhibited by the calcium (Ca^{2+}) channel blocker nifedipine.[28] However, it is not clear whether the observed lung hypoplasia after nifedipine administration was the result of inhibition of peristalsis or whether liquid secretion was influenced by this intervention.

Surgical Intervention

Given that ligation of fetal airways promotes lung growth distal to that point, surgical obstruction of the fetal airway has evolved as a technique for prevention or correction of potential lung hypoplasia, particularly with lung anomalies associated with congenital diaphragmatic hernia. However, early enthusiasm for fetal surgery has been tempered by poor results.[29] The traditional hypothesis that lung hypoplasia results from compression by herniating abdominal viscera has been challenged because pulmonary anomalies have been observed before herniation of abdominal contents into the chest.[30,31] The role of ion transport in the pathogenesis lung hypoplasia is not known.

Perinatal Changes in Lung Liquid Flow

At the time of birth, the newborn infant switches from placental to pulmonary gas exchange.This requires an abrupt replacement of the liquid in the lung lumen with air.Within a few hours, approximately 100 mL of fetal lung liquid contained in the human lung lumen must be cleared.This is achieved by the initiation of complex series of mechanical and ion transport events.

Decrease in Lung Liquid Volume Before Labor

Some investigators, working mainly with fetal rabbits and sheep, observed a decrease in both lung liquid volume and secretion rates toward the end of gestation.[6,8,12,32] It is likely that mechanical forces (for example, active fetal exhalation) may participate in some of the expulsion of liquid in the hours leading up to the onset of labor.[33]

Role of Labor

The finding that delivery by cesarean section (CS) slowed lung liquid clearance, particularly when the procedure was undertaken before the onset of labor, indicated that labor was in some way important to the removal of lung liquid.[34,35] Rabbits that were born at term, either vaginally or by CS after the onset of labor, had less water in their lungs than did rabbits that were delivered operatively without prior labor (Figure 82-2). This observation contrasted with the previously held notion that any significant amount of lung liquid is squeezed out of the lungs by thoracic compression during vaginal delivery. These data provided evidence that labor induces other (nonmechanical) mechanisms that must be responsible for lung liquid clearance before birth.

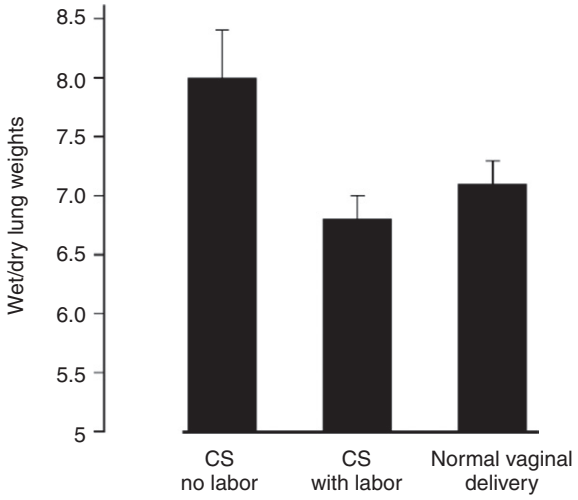


Figure 82-2 Effect of labor on lung water content (wet/dry weight) of newborn rabbit pups born after normal vaginal delivery or by cesarean section *(CS)*. *(From Bland RD, Bressack MA, McMillan DD: Labor decreases the lung water content of newborn rabbits,* Am J Obstet Gynecol *135(3):364–37, 1979.)*

Fate of Lung Liquid During Labor and Delivery

Once labor is initiated, there is rapid removal of liquid from the lung caused by a reversal of the direction of liquid flow across the pulmonary epithelium.[12] The secretory process that dominates fetal life is abruptly switched to allow absorption of liquid from the lung lumen into the fetal circulation.[12] It is now clear that active transcellular Na^+ absorption drives liquid out of the lumen into the interstitial space (see farther on).[36] Most interstitial liquid moves into the fetal-newborn pulmonary circulation and some drains via the lung lymphatics.[37] Starling forces that may assist liquid absorption (for example, osmotic pressure and hydrostatic gradients between the lung interstitium and the lung lumen) do not change during labor, and they are not thought to play a role in removal of fetal lung liquid before birth.

Liquid Movement After Birth

After birth, there is an acceleration of active liquid absorption, and most of the liquid is cleared from the term newborn lung within 2 hours of independent breathing.[12,38] Egan and coworkers[39] noted a transient increase in solute permeability of the alveolar epithelium at the onset of breathing and speculated that this "window" of decreased paracellular resistance may assist with passive liquid absorption at birth.

BASIC MECHANISMS OF TRANSEPITHELIAL ION TRANSPORT

The ion transport mechanisms located in the pulmonary epithelium drive the secretion and absorption of liquid into and out of the lumen of the fetal and newborn lung. Advances in understanding of perinatal lung liquid flow have derived from better characterization of the function, regulation, and molecular identity of these channels and transporters.

Boston, Humphreys, Olver, Strang, and their associates[40,41] launched a series of studies in the 1960s and 1970s that described the flow of liquid from the pulmonary circulation to the lung lumen; characterized the barrier properties of the epithelium and endothelium; and provided the first evidence of secondary active Cl^- transport as the mechanism for lung liquid secretion by the

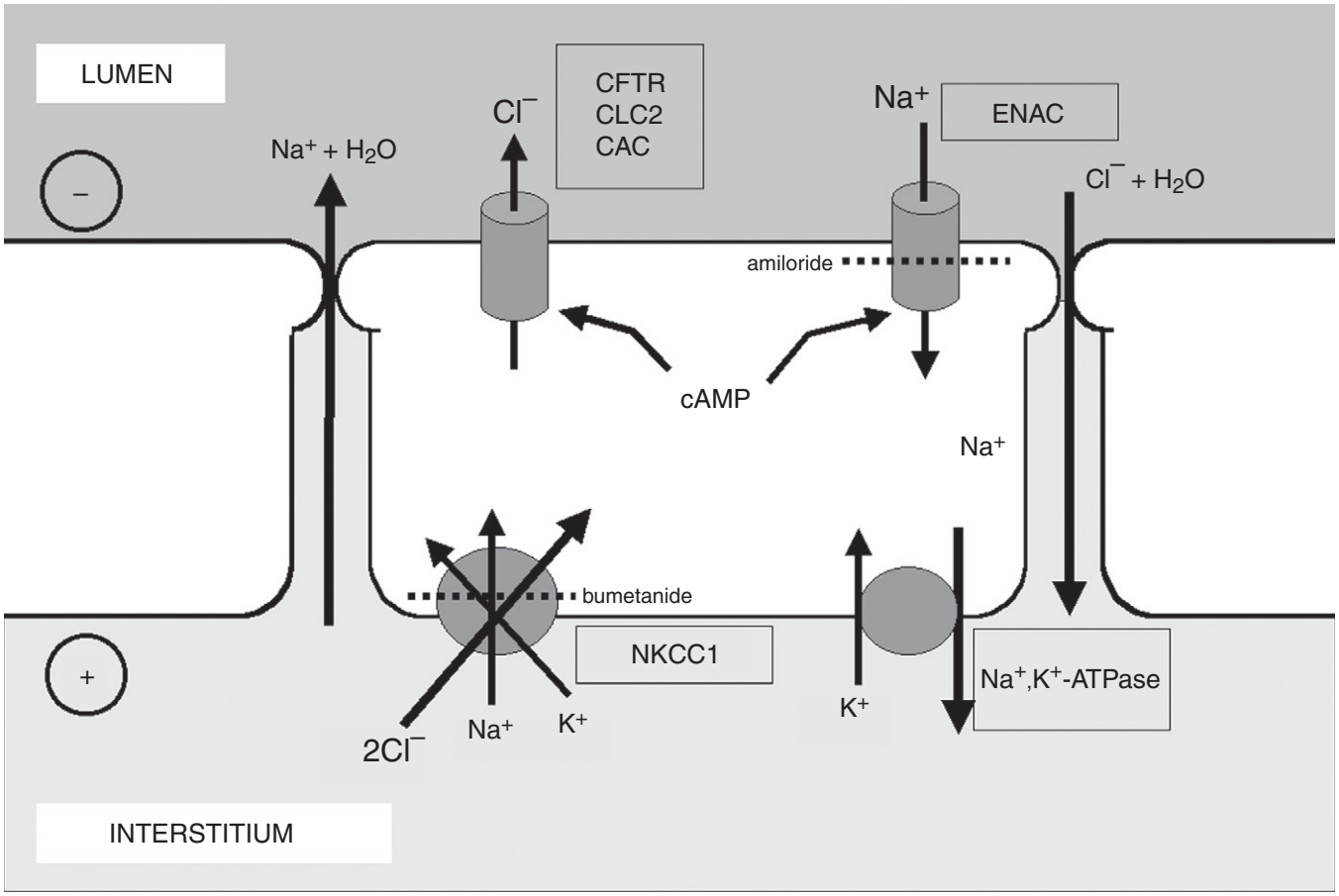


Figure 82-3 Arrangement of ion transporters and pumps in the lung epithelia of fetal lung. Amiloride is a specific inhibitor of the epithelial sodium (Na^+) channel *(ENAC),* and bumetanide blocks Na^+, potassium (K^+), chloride (Cl^-) cotransporter 1 *(NKCC1)*.The "plus" and "minus" signs indicate polarity of the lumen with respect to the interstitium. *CAC*, Calcium-activated channel; *cAMP*, cyclic adenosine monophosphate; *CFTR*, cystic fibrosis transmembrane conductance regulator; *CLC2*, voltage-gated Cl^- channel 2.

fetal lung.[5,11,42]These studies showed that the alveolar epithelium was highly restrictive to macromolecules compared to the endothelium. This barrier property accounts for the virtual absence of protein in the fetal lung lumen, in contrast to the interstitium and circulation. Despite this oncotic gradient across the alveolar epithelium, lung liquid is secreted actively by transcellular Cl^- transport. Researchers from the same laboratory were the first to show that clearance of lung liquid at birth was also driven by an active ion transport process, namely, Na^+ absorption.[36]

The basic configuration of ion transporters and channels follows a pattern that is similar to that found in other mammalian ion-transporting epithelia.The electrochemical gradient for entry and exit of ions to and from the lung epithelial cells is established by Na^+,K^+-adenosine triphosphatase (ATPase) at the basolateral membrane. Activity of this transporter results in the low intracellular [Na^+] that creates a gradient for Na^+ to enter the cell through either the basolateral membrane (entraining Cl^- as part of the secretory process) or through apical membrane (first step in the absorptive process).The main identified transporters and channels that result in liquid secretion and absorption are shown in Figure 82-3.

The molecular identities of the main transporters and pumps located on the interstitial facing membrane (Na^+ pump, Na^+,K^+-ATPase, and Na^+,K^+,2Cl^- cotransporter [NKCC1]) are known (see Figure 82-3). Likewise, the principal Na^+ channel on the luminal membrane (epithelial Na^+ channel [ENaC]) has been cloned.[43] However, the identity of the principal mechanism responsible for fetal Cl^- secretion across the lumen membrane is not known. Candidates for Cl^- channels include the cystic fibrosis transmembrane conductance regulator (CFTR, cloned in 1989) and the voltage-gated Cl^- channels 2 and 3 (CLC2, CLC3, cloned in 1995), all of which are expressed in the fetal lung.[44-47] There is strong functional evidence for a purinoceptor-Ca^{2+}–activated Cl^- channel in fetal lung epithelia, but this channel has yet to be cloned.[48,49]

Chloride Secretion

The "uphill" movement of Cl^- from the interstitium to the lung lumen was shown by Olver and Strang[5] to be the driving force for fetal lung liquid secretion. Since then, the identity of the entry and exit steps for Cl^- transit across the lung epithelium have been sought. From studies of other Cl^--secreting mammalian epithelia, NaK2Cl (NKCC) cotransporter family members were likely candidates for the Cl^- entry mechanism through the basolateral membrane. On the apical membrane, it is assumed that Cl^- channels, exchangers, or cotransporters must be responsible for exit of Cl^- into the lumen.

Sodium, Potassium, Chloride Cotransporter

In fetal sheep and guinea pig lung, addition of the loop diuretic bumetanide or furosemide (specific NKCC inhibitors) to fetal lung liquid caused slowing of lung liquid secretion or liquid absorption.[50,51] Loop diuretics also inhibited liquid secretion in vitro by distal lung explants from fetal rat lung, and they decreased basal short-circuit current by excised fetal canine or rabbit trachea.[52-55] Of the two NKCC isoforms that have been described,

mRNA of NKCC1 but not NKCC2 is abundantly expressed in epithelia from all regions of the late-gestation fetal mouse lung.[56] Postnatally, there is a significant decrease in NKCC1 expression by mouse tracheal epithelia.[57] The likely existence of non-NKCC Cl^- entry mechanisms is suggested by the minimal inhibition by bumetanide of basal and cyclic adenosine monophosphate (cAMP)–induced Cl^- secretion across fetal human and mouse distal lung epithelium and apparently normal survival and lung health in NKCC-knockout ($NKCC^{-/-}$) mice.[48,56-59] Studies of fetal $NKCC1^{-/-}$ did show that maximal (cAMP-mediated) but not basal Cl^- secretion was compromised by NKCC1 deletion. Studies of postnatal $NKCC1^{-/-}$ mice showed that NKCC1-mediated Cl^- secretion by airway epithelium wanes after birth, matching the apparent decrease in NKCC mRNA expression in postnatal airways.[57] The molecular expression of other potential Cl^- entry mechanisms (for example, Cl^--bicarbonate exchanger) in fetal lung epithelia have not been reported.

Cystic Fibrosis Transmembrane Conductance Regulator

Studies of postnatal cystic fibrosis (CF) epithelia indicate that CFTR provides a critical apical membrane conduit for Cl^- secretion and is an important regulator of Na^+ transport in lung epithelia after birth.[60,61] However, non-CFTR mechanisms must exist for liquid secretion by fetal lung epithelia because the lungs of fetuses with CF are normally developed and explants of CF-affected human fetal lung are inflated with secreted liquid.[62,63]

A role for CFTR in fetal lung liquid secretion is suggested by intense expression of CFTR mRNA in all regions of first trimester human fetal lung, with less intense expression in the alveolar and large airway epithelia during the second and third trimesters.[64] In the postnatal lung, CFTR mRNA signal is localized to small airways and submucosal gland epithelium and is not detected in the alveolar epithelium.[65]

Lungs from $CFTR^{-/-}$ mouse fetuses are normally inflated with liquid, and airway explants from these fetuses have basal and cAMP-stimulated anion secretion that is indistinguishable from wild-type littermate controls.[66] There is also evidence of liquid secretion by lung epithelia of human fetuses with CF. However, exposure to cAMP causes no change in liquid volume in CF fetal lung explants, whereas additional liquid secretion was induced by cAMP in normal fetal lung tissue.[63] These studies suggest that CFTR has some role in, but is not required for, basal fetal lung liquid secretion.

Other Fetal Chloride Channels

The identity of non-CFTR anion secretory paths in fetal lung epithelia remains elusive. Purinoceptor-activated Cl^- secretion has been observed in $CFTR^{-/-}$ fetal mouse lung and in wild-type fetal rat and fetal human lung epithelia.[48,49,66] The molecular identity of the purinoceptor-activated Cl^- channel is not known. The developmental expression pattern of CLC2 and CLC3 indicates a possible role for these channels in fetal lung liquid secretion.[47] However, the absence of either functional studies in fetal lung tissues or a knockout model deficient in either CLC2 or CLC3 makes the significance of these channels difficult to determine at present.

Role of Sodium Absorption in Perinatal Lung Liquid Clearance

The observation that spontaneous or epinephrine-induced absorption of fetal lung liquid was associated with a large increase in net Na^+ transport out of the lumen and could be blocked by amiloride, a specific Na^+ channel blocker (Figure 82-4), provided the first conclusive evidence that perinatal liquid absorption is mediated through Na^+ transport.[36] Some Na^+ and cation channels have been identified by electrophysiologic means (patch clamp or Ussing chamber studies) on the apical membrane of fetal distal lung epithelial (FDLE) cells isolated from rodents.[67] However, the observation that all activated and spontaneous liquid absorption could be inhibited by mixing the Na^+ channel blocker amiloride into fetal lung liquid demonstrated a critical role for amiloride-sensitive Na^+ transport in perinatal lung liquid clearance. This finding was strengthened by the observation that amiloride instilled into the trachea of the newborn guinea pig impaired lung water clearance in a dose-dependent manner, and it was later confirmed by ENaC-knockout murine studies (see later).[68,69] The roles of the high-energy Na^+,K^+-ATPase pump on the basolateral membrane and ENaC on the apical membrane have been extensively studied since the molecular identity of these genes became known.[43,70]

Na^+,K^+-Adenosine Triphosphatase

Evidence that labor was also crucial for activation of the lung epithelial Na^+,K^+-ATPase pump came from measurements of the ouabain-sensitive uptake of rubidium-86 ($^{86}Rb^+$, a surrogate for K^+ uptake) in alveolar type II cells freshly isolated from newborn rabbit pups. These studies demonstrated that labor was associated with a threefold to fourfold increase in pump activity in fetal alveolar type II cells.[71,72] In rat and mouse whole lung, expression of $\alpha 1$ and $\beta 1$ subunits increases from low levels in early fetal life to a peak around the time of birth.[73,74] Fetal $\alpha 1$ subunit mRNA is expressed in both epithelium and endothelium, whereas $\beta 1$ subunit expression is localized to epithelium only. By the end of the first week of life, epithelial expression of $\alpha 1$ and $\beta 1$ subunit mRNA is localized to small airways and the basolateral surface of alveolar type II but not type I cells.[74,75]

Epithelial Sodium Channel

ENaC subunit mRNA expression has been studied extensively in developing human and rodent lung epithelia. In both rat and mouse whole lung, an abrupt increase in αENaC mRNA expression in late gestation reaches levels seen in the adult lung shortly after birth.[76,77] In mouse lung, there is a similar increase in expression of γENaC and a more gradual increase in βENaC that peaks in adult lung (Figure 82-5). Expression of γENaC parallels that of αENaC, with intense expression of subunit mRNA in all regions of the fetal lung, whereas βENaC expression in fetal and early postnatal lung is restricted to the airway epithelium.[76] Studies in guinea pig showed that both αENaC mRNA expression and the sensitivity of lung liquid clearance to amiloride and to β-adrenoceptor blockade were highest shortly after birth, and they declined in parallel with endogenous plasma epinephrine concentration, findings providing further support for the key role of β-adrenoceptor activation of Na^+ channels at birth. Immunocytochemical studies of human fetal lung surprisingly showed significant αENaC protein expression in early midtrimester fetal lung, at a time when the fetal lung is not thought to be capable of significant Na^+ transport.[78,79] Whereas the function or location of this protein within the cell is not known, it is possible that there is a discrepancy between developmental ENaC expression in human and rodent lung.

Other Sodium Channels

Other putative amiloride-sensitive Na^+-permeable channels in fetal lung have been described that may contribute to the driving force for lung liquid clearance. These include certain amiloride-sensitive, poorly nonselective cation channels: G-protein–regulated, β-adrenoceptor agonist/Ca^{2+}–activated, and cyclic nucleotide-gated channels.[80-82] According to at least one school of thought, the β-adrenoceptor responsive, nonselective channel may be a multimer of αENaC subunits, and the role of β and γ subunits may be to confer selectivity on the channel.[83] An additional pathway for Na^+ absorption is Na^+–glucose cotransport, which has been reported in fetal sheep lung epithelia.[84]

Knockout Models of Sodium Transport

Certain knockout and other transgenic mouse models were used to explore the role of ion transport in the perinatal regulation of lung liquid flow. Knockout mice for all three ENaC subunits

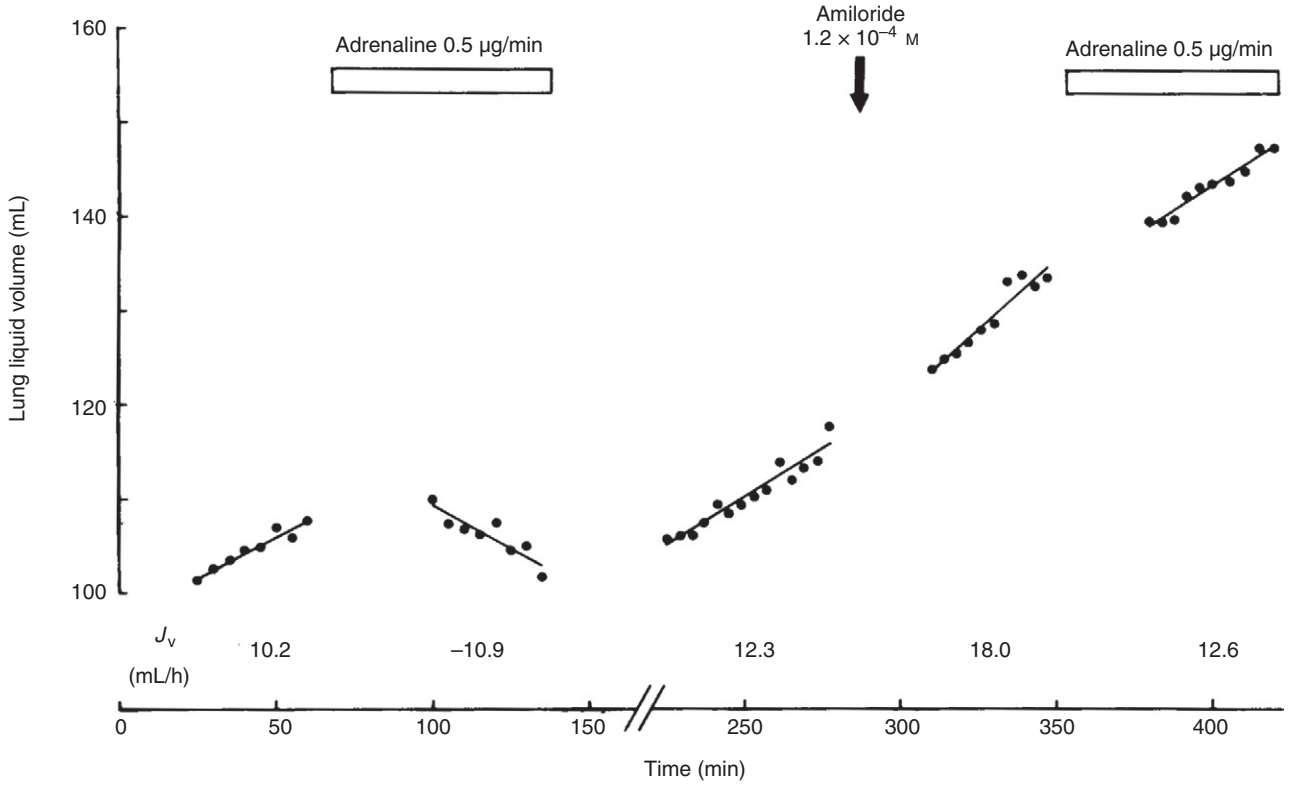


Figure 82-4 Effect of epinephrine (adrenaline) infusion and topical amiloride on cumulative lung volume across the lung epithelium of a near-term fetal sheep. At the start of the study, volume increases because of liquid secretion (J_v 10.2 mL/hour). Epinephrine infusion abruptly causes a decrease in lung volume, reversing the direction of flow (J_v −10.9 mL/hour) from secretion to absorption. Secretion is restored when the epinephrine infusion is discontinued. Addition of the sodium channel blocker amiloride to lung liquid prevents liquid absorption when the epinephrine infusion is restarted. *(From Olver RE, Ramsden CA, Strang LB, Walters DV: The role of amiloride-blockable sodium transport in adrenaline-induced lung liquid reabsorption in the fetal lamb,* J Physiol *376:321–340, 1986.)*

were reported, and additional ENaC mutants were studied.[69,85-88] The most striking finding was that mutant newborns lacking the αENaC gene were unable to clear lung liquid from the alveolar spaces after birth (Figure 82-6). These mice were readily identifiable compared with their unaffected littermates by increased work of breathing, failure to feed, and failure to move around. Mice with γENaC or βENaC null mutations had delayed liquid clearance, but they had near normal lung water content 12 hours after birth. All subunit mutations resulted in severe hyperkalemia, which was the most likely cause of death in the early neonatal period. αENaC null mice that were "rescued" with an αENaC transgene were able to clear lung liquid in the newborn period at near-normal rates with approximately 50% Na^+ channel activity.[88] These studies indicate that ENaC function is a critical requirement for clearance of neonatal lung liquid in the newborn period and suggest that the αENaC subunit is the core, rate-limiting part of the Na^+ channel in lung epithelia. Evidently, neonatal and postnatal lung liquid clearance can be sustained in the murine model with approximately 50% normal Na^+ channel activity.

HORMONAL REGULATION OF ION AND LIQUID TRANSPORT

Cyclic Adenosine Monophosphate–Mediated Liquid Transport

Although cAMP stimulates liquid and Cl^- secretion by both proximal and distal regions of the cultured immature human fetal lung, its net effect in late gestation is the opposite, namely, the induction of liquid absorption.[48,63] Birth is known to be associated with a surge in fetal catecholamine secretion.[89] The link between β-adrenergic stimulation and lung liquid clearance was made in 1978 by Walters and Olver,[90] who discovered that epinephrine and isoproterenol, but not norepinephrine, caused the rapid absorption of lung liquid by late-gestation fetal sheep lungs, and the absorptive response could be inhibited by prior treatment with propranolol. In addition to effects on liquid secretion and lung water, β-adrenoceptor agonists caused release of surfactant, and the two physiologic processes combined to improve early neonatal lung aeration and gas exchange.[91-94]

These observations were subsequently extended to demonstrate that, at any particular stage of gestation, a log-linear relationship exists between fetal plasma epinephrine concentration and the degree of inhibition of secretion or absorption of liquid.[12] The threshold for absorption falls in late gestation to reach an epinephrine level of 0.12 nmol/mL at term. During spontaneous labor, the relationship between endogenous fetal plasma epinephrine and the rate of liquid absorption is similar to that obtained by epinephrine infusion. Classically, β-adrenoreceptor agonists act via the cAMP/PKA (prekallikrein activator) signaling pathway, and, as predicted, the addition of a lipid-soluble analogue of cAMP, dibutyryl cAMP (db-cAMP), can mimic the effect of epinephrine in the mature fetal lamb.[95] The finding that the response to db-cAMP had a similar gestation dependence indicates that the rate-limiting step involved post-cAMP regulation of Na^+ transport rather than changes at the β-receptor.

Because cAMP mediates both liquid secretion and liquid absorption in the fetal lung, the potential exists for an epinephrine surge

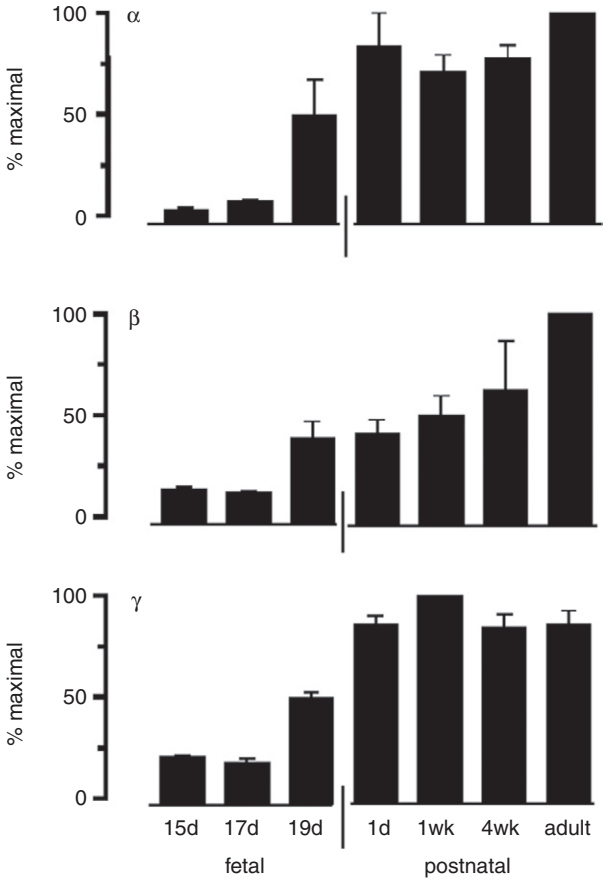


Figure 82-5 α, β, and γ Epithelial sodium channel subunit mRNA expression in fetal (term = 19 days) and postnatal whole mouse lung. *(From Talbot CL, Bosworth DG, Briley EL, et al: Quantitation and localization of ENaC subunit expression in fetal, newborn, and adult mouse lung,* Am J Respir Cell Mol Biol *20:398–406, 1999.)*

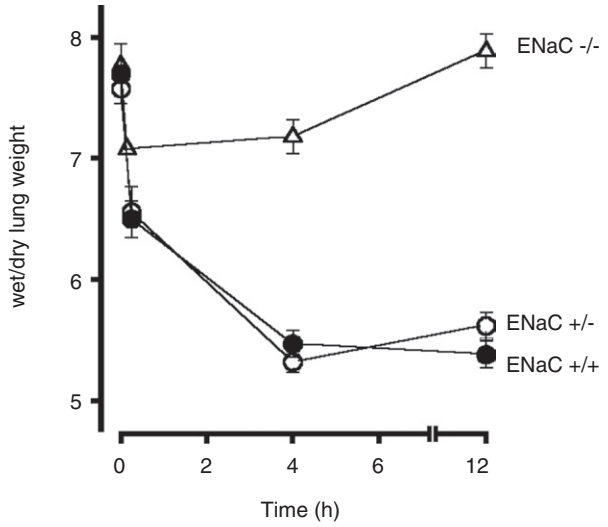


Figure 82-6 Lung water content (wet/dry lung weight) before (0 hour) and after birth (15 minutes, 4 hours, 12 hours) in α-epithelial sodium channel (αENaC) knockout mice (αENaC −/−) and littermate controls (αENaC +/− and +/+). *(From Hummler E, Barker P, Gatzy J, et al: Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice,* Nat Genet *12:325–328, 1996.)*

to cause inappropriate lung liquid secretion in neonates born very prematurely. In explants of human fetal lung, cAMP caused a striking increase in liquid volume (Figure 82-7, *A*). This effect was attenuated by pretreatment of explants with thyroid and steroid hormones (Figure 82-7, *B*). The maturational effect of these hormones on liquid secretion and absorption is discussed later.

Arginine vasopressin, another hormone that increases cellular cAMP, rises during labor. Although it can be shown to inhibit lung liquid secretion, probably acting via the V_1 receptor and protein kinase C, its effect is weaker than that of epinephrine, and its role is uncertain.[96-98] It has been suggested that arginine vasopressin may provide back-up for the β-adrenoceptor–dependent pathway and may explain why β-adrenoceptor blockade fails to prevent lung liquid absorption during labor.[99]

Thyroid and Steroid Hormones

The role of thyroid and glucocorticoid hormones in the maturation of the absorptive response to epinephrine is evident from a series of experiments by Barker and co-workers.[100,101] Their initial studies showed a profound blunting of the response to epinephrine (and db-cAMP) in thyroidectomized fetal lambs that was reversed by infusion of triiodothyronine (T_3) alone. Neither T_3 nor hydrocortisone was capable of advancing maturation of the epinephrine response in normal fetuses with intact thyroid glands when they were given separately, but a powerful synergistic effect was observed when they were given concurrently (Figure 82-8).[102] In thyroidectomized fetal lambs, the response to epinephrine was detectable within 2 hours of the start of infusion of the hormone combination. This induction of epinephrine-sensitive liquid absorption was lost within 24 hours of cessation of hormone administration.[103] These data—together with the observation that T_3 and betamethasone have an additive effect in up-regulating fetal rat lung surfactant synthesis in vivo—emphasize the high degree of coordination of development of the lung liquid clearance and surfactant systems for efficient lung adaptation at birth.[104]

The observation that the rise in αENaC expression parallels plasma cortisol (but not T_3) in the late-gestation and newborn guinea pig, and can be blocked by metyrapone after delivery by CS, underscores the pivotal role of this hormone in the regulation of ENaC near term.[105] Tchepichev and colleagues[77] demonstrated that prenatal steroids, but not thyroid hormones, could advance timing of the increase in αENaC mRNA in fetal rat lung, but neither hormone had any effect on expression of βENaC or γENaC subunit mRNA expression. Champigny and associates[106] also reported no effect of thyroid hormones on ENaC mRNA expression, but they did report a potentiation by thyroid hormones of the steroid effect on Na^+ currents in rat alveolar epithelial cells. Otulakowski and colleagues[107] subsequently identified putative binding sites for thyroid and glucocorticoid receptors in the promoter region of αENaC, and although T_3 alone had no effect on reporter gene activity, it potentiated gene stimulation by steroid hormones.

A similar pattern of regulation has been reported for the subunits of Na^+,K^+-ATPase. Studies of 18-day rat FDLE cells treated with dexamethasone showed a threefold increase in α1 and β1 mRNA expression.[108] Studies of postnatal alveolar cells showed similar effects of steroid hormones on Na^+,K^+-ATPase activity. In addition, there is a large body of evidence in postnatal alveolar cell studies that activity of Na^+,K^+-ATPase can be up-regulated by intracellular cAMP.[109,110] The similarity of hormonal regulation of ENaC and Na^+,K^+-ATPase implies a coordinated increase in activity of the two principal mechanisms that underlie the perinatal absorptive response.

REGULATION OF ION TRANSPORT BY OXYGEN

O_2 appears to be a particularly important factor in maintaining the liquid absorptive response after birth. The onset of breathing results in a sharp increase in alveolar P_{O_2} from the fetal level of

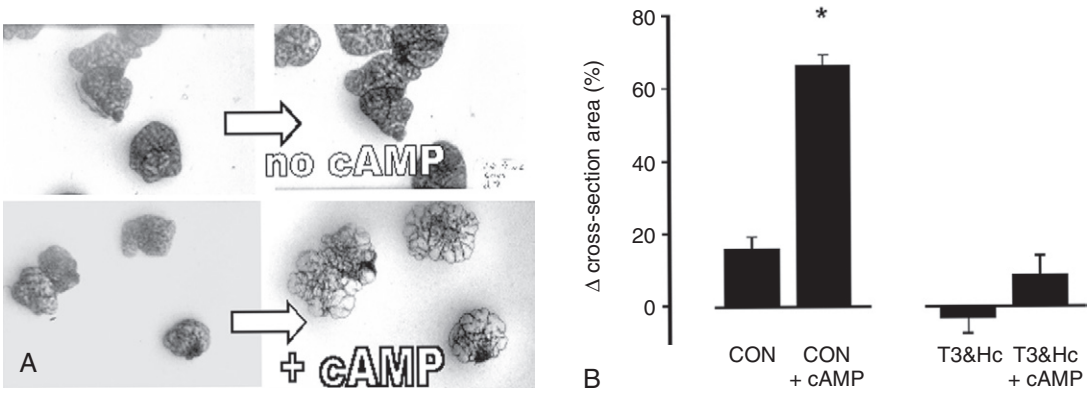


Figure 82-7 **A,** These 20-week human fetal lung explants expanded with liquid when they were exposed for 24 hours to 10^{-4} mol cyclic adenosine monophosphate *(cAMP)*. **B,** This increase in diameter was attenuated in explants preincubated for 48 hours with 10^{-9} mol triiodothyronine (T_3) and 10^{-6} mol hydrocortisone *(Hc)*. *CON,* Control.

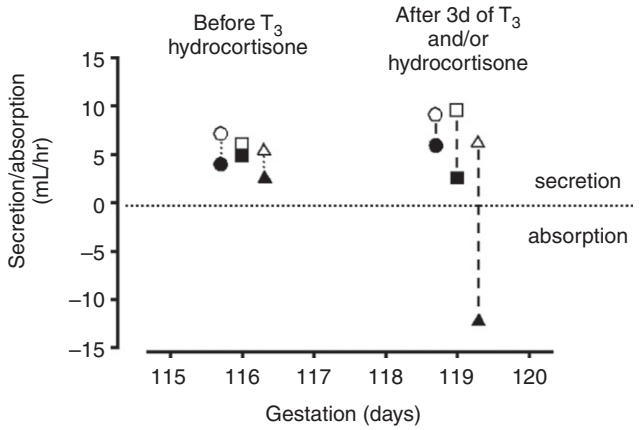


Figure 82-8 Effect of thyroid and steroid hormone on basal secretion *(open symbols)* and the ability of immature fetal sheep (term = 145 days) to respond to epinephrine infusion *(closed symbols)*. Thyroid (triiodothyronine [T_3]) and steroid hormone exposure did not affect basal secretion rates. Only fetuses that had been treated with thyroid *and* steroid hormone for 3 days were able to absorb lung liquid during epinephrine infusion. - -, Effect of IV epinephrine; □, hydrocortisone; ○, T_3; △, T_3 and hydrocortisone. *(From Barker PM, Markiewicz M, Parker KA, et al: Synergistic action of triiodothyronine and hydrocortisone on epinephrine-induced reabsorption of fetal lung liquid,* Pediatr Res *27:588–591, 1990.)*

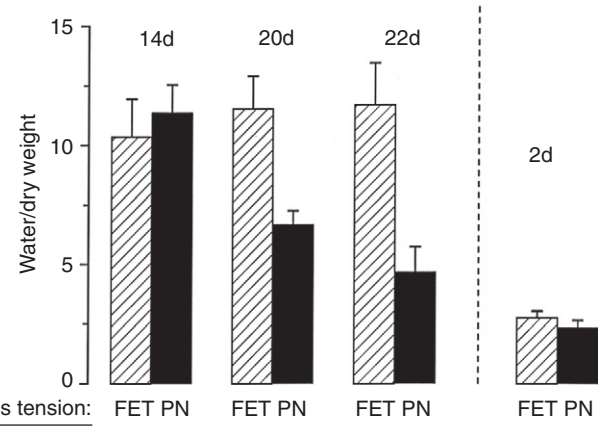


Figure 82-9 Effect of fetal (*FET,* ambient O_2 of 3%) or postnatal (*PN,* ambient O_2 of 21%) conditions on liquid volume of distal lung explants derived from fetal mouse lung. *(From Barker PM, Gatzy JT: Effect of gas composition on liquid secretion by explants of distal lung of fetal rat in submersion culture,* Am J Physiol *265:L512–L517, 1993.)*

23 to 25 mm Hg to about 100 mm Hg postnatally. The potential for this change in ambient O_2 to switch the fetal lung from a secretory to an absorptive state was first shown by Barker and Gatzy[111] in studies of fetal rat distal lung explants. A shift from fetal P_{O_2} (≈25 mm Hg) to room air (≈150 mm Hg) inhibited liquid secretion (reduction in lung water/dry weight ratios and in the number and size of liquid-filled cysts) by fetal lung explants in late-gestation lung explants, whereas there was no inhibition of liquid secretion by high P_{O_2} at very early gestations (Figure 82-9). By contrast, no liquid secretion was induced in postnatal explants with exposure to fetal gas concentrations, a finding suggesting that the switch to liquid absorption may be irreversible 2 days after birth. The effect of O_2 on liquid secretion could be induced in immature explants by coculture in thyroid and steroid hormones. These studies further demonstrated a crucial role of thyroid and steroid hormones in priming the distal lung epithelium to respond to triggers (epinephrine and O_2) that switch transepithelial liquid flow from secretion to absorption at birth.

Subsequently, studies with rat FDLE monolayers showed that the increase in Na^+ transport induced by raising P_{O_2} from 30 to 150 mm Hg was accompanied by an increase in ENaC mRNA expression.[112] This shift in P_{O_2} was accompanied by a temporary fall in epithelial resistance lasting several hours, consistent with the increase in solute permeability noted by Egan and co-workers[39] at the onset of breathing in vivo that may assist passive liquid absorption. Vivona and co-workers[113] showed that hypoxia in adult rats decreased alveolar liquid clearance, an effect that was independent of ENaC and Na^+,K^+-ATPase subunit expression. These studies indicate that the mechanisms of postnatal O_2-induced neonatal alveolar Na^+ and liquid transport include posttranscriptional regulation of ENaC and may be reversible.

Further experiments were undertaken in mature rat FDLE cells to determine how the effects of shifting P_{O_2} from fetal to neonatal levels and the action of hormones are integrated to control lung liquid transport.[114] In these studies, glucocorticoid and thyroid hormones up-regulated Na^+ transport and up-regulated β-agonist–induced apical Na^+ conductance (G_{Na}) independently of ambient P_{O_2}. In contrast, a shift to neonatal P_{O_2} was a prerequisite for hormonal up-regulation of Na^+,K^+-ATPase. These findings contrasted with the reported effects of thyroid hormones

and steroids on immature fetal sheep lung, which were restricted to induction of β-agonist–responsive liquid absorption, with no effect on basal secretion rates.[102] Postnatally, steroids and P_{O_2} also have an additive effect in up-regulating expression of ENaC-like Na^+ channels in type II alveolar cells.[115]

The promoter region of α-rENaC contains a consensus nuclear factor-κB (NF-κB) binding element. Further experiments in rat FDLE cells showed that a rise in P_{O_2} to 150 mm Hg induced this redox-sensitive transcription factor, and blocking NF-κB activation reduced the O_2-evoked rise in G_{Na}.[116,117] These findings could be interpreted as indicating that P_{O_2} activation of NF-κB up-regulated the expression of ENaC, thus increasing Na^+ transport at the onset of breathing. However, subsequent experiments using FDLE monolayers demonstrated that a shift from fetal (23 mm Hg) to postnatal (100 mm Hg) alveolar P_{O_2}, which induces a maximal Na^+ response, resulted in an immediate increase in NF-κB expression and a detectable increase in Na^+ pump capacity within 6 hours, whereas activation of the αENaC promoter was not seen until after 24 hours, reaching a maximum (together with G_{Na}) at 48 hours.[118,119]

It therefore appears that the early increase in fluid absorptive capacity in response to the rise in alveolar P_{O_2} at birth is primarily the result of an increase in Na^+,K^+-ATPase pump capacity, and the increase in G_{Na} may be secondary to this increase in Na^+ transport, not its cause. Both components of the response are enhanced by glucocorticoid and thyroid hormones, which are also required for β-adrenoceptor–mediated control of G_{Na}. This discrepancy between the effects of these hormones in rat versus sheep studies raises the possibility that thyroid and steroid hormones may inhibit basal secretion rates or increase G_{Na} in late, but not early, gestation. The discrepancy in the time course of NF-κB activation and the rise in αENaC expression indicates that activation of the αENaC promoter requires additional transcription factors.

LUNG LIQUID AND ION TRANSPORT IN NEONATAL RESPIRATORY DISEASE

This section reviews the evidence that inefficient lung water clearance can result in neonatal lung disease and examines the contribution of abnormal airway ion transport.

Lung Liquid

Animal studies show a clear correlation between intraluminal liquid volume and respiratory function at birth. Berger and colleagues[120] examined the effect of residual lung liquid volume at birth on gas exchange in lambs delivered by CS near term. Animals that had approximately 45% lung liquid volume removed just before delivery had significantly better respiratory function than those delivered without liquid removal. The first clinical evidence that lung liquid may play a role in neonatal respiratory disease came from an analysis by DeSa[121] of postmortem lung findings after perinatal death. Infants who died of idiopathic respiratory distress syndrome (RDS) had significantly higher lung liquid content (determined as the percentage of wet weight of the lung) compared with infants who died of other, nonrespiratory causes.[122] These findings were supported by other evidence of lung liquid excess on histologic examination after RDS (Figure 82-10).[121]

In an early review, Bland[123] identified certain factors that may predispose the newborn preterm infant to pulmonary edema, as follows:

1. Persistent pulmonary hypertension is more common in this group of infants, particularly if they experience hypoxia or patent ductus arteriosus.
2. A large transpulmonary pressure that drives fluid into the alveolar compartment may develop in areas of the lung with surfactant deficiency. The advent of artificial surfactant therapy has significantly decreased the incidence of RDS, and some of its effect may result from decreased surface tension and improved gas exchange in areas of the preterm lung that are not completely emptied of alveolar liquid at birth.[124]
3. Mechanical ventilation likely disrupts the epithelial barrier and allows entry to the alveolar space of proteins that, in turn, may increase intraalveolar fluid.
4. High inspired O_2 releases toxic metabolites that may interfere with essential cellular functions, including ion transport.

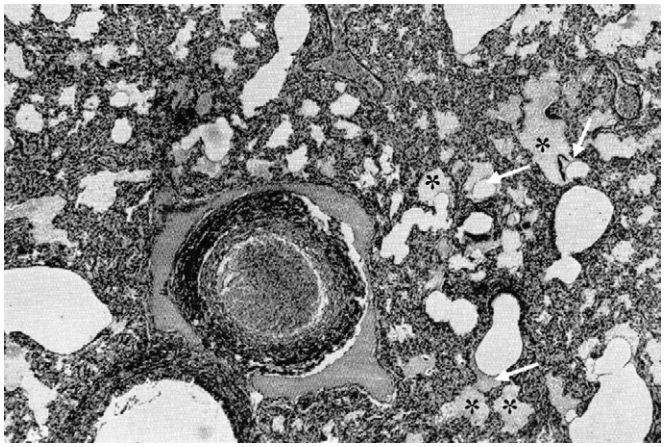


Figure 82-10 Photomicrograph of a lung from a 1300-g infant who died of respiratory distress syndrome at 8 hours without mechanical ventilation. The *arrows* show meniscus of liquid *(asterisk)* contained inside the alveoli. *(From DeSa DJ: Pulmonary fluid content in infants with respiratory distress,* J Pathol *97:469–478, 1969.)*

Against this background of susceptibility to pulmonary edema, ion transport—a major defense against alveolar flooding—is almost certainly incompletely developed in infants born very prematurely.[125]

The correlation between preterm delivery and respiratory distress is clear; however, some clinical studies, both prospective and retrospective, also demonstrate a link between mode of delivery and respiratory condition in the neonate.[126-128] Elective CS without labor is associated with a small but significant increased risk of RDS. This risk is exacerbated by delivery even a couple of weeks before term.[129]

The condition of transient tachypnea of the newborn (TTN) is probably the best-described consequence of inadequate neonatal lung liquid clearance. This self-limiting disease, characterized by an increase in respiratory rate, occurs more frequently in infants delivered by elective CS and is thought to result from delayed activation of liquid clearance in infants not subjected to the stress of labor.[130,131] These clinical data are consistent with animal studies demonstrating the importance of gestation and labor in perinatal lung water clearance and successful adaptation to air breathing.

Airway Ion Transport Studies

Some investigators have used nasal electrical potential difference (PD) to measure ion transport directly across the upper airway in vivo. Nasal PD measures the electrical potential generated by active transport of charged ions (Cl^- and Na^+) across the nasal membrane. The use of nasal PD to infer patterns of ion transport in the lower airways and alveoli has been validated by measurement of similar ion transport patterns in nasal and lower bronchial epithelia for both normal subjects and and those with CF.[132,133] Furthermore, nasal PD can predict who will be susceptible to high-altitude pulmonary edema.[134] Gowen and

colleagues[135] reported that nasal PD could distinguish newborn infants with CF from healthy neonates and those with non-CF respiratory disease. This test also detected ion transport differences that reflected the mode of delivery. Nasal PD was significantly higher in infants born by elective CS compared with those born normally or by CS after labor. Infants with TTN had the highest nasal PD, but they also had decreased amiloride-sensitive Na^+ transport compared with babies who did not have TTN.[136] These preliminary studies support the notion that TTN may result in persistence of a fetal ion transport phenotype after birth.

In a more recent study, these investigators examined the relationship between airway ion transport at birth and development of RDS in infants at or less than 30 weeks gestational age.[137] The RDS group had significantly lower maximal PDs and less inhibition after amiloride perfusion (Figure 82-11), a finding suggesting reduced ENaC-mediated Na^+ absorption. However, this group demonstrated a close correlation between maximal PD and birth weight, and the lower birth weight of the RDS group could represent a significant confounding variable. Although more studies are needed in this area, the available evidence supports an etiologic role for immature ion transport processes in the evolution of acute and possibly chronic lung disease (CLD) in preterm infants.

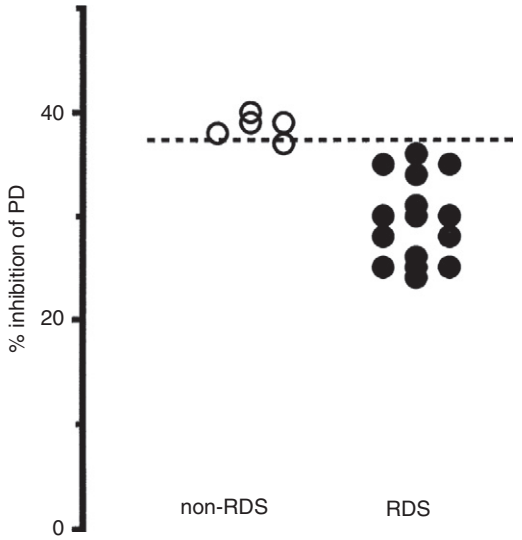


Figure 82-11 The percentage of inhibition of nasal potential difference *(PD)* by the sodium channel blocker amiloride discriminates between infants who subsequently developed respiratory distress syndrome (RDS) *(closed circles)* and those who did not develop RDS *(open circles)*. *(From Barker PM, Markiewicz M, Parker KA, et al: Synergistic action of triiodothyronine and hydrocortisone on epinephrine-induced reabsorption of fetal lung liquid,* Pediatr Res *27:588–591, 1990.)*

Therapeutic Considerations

Our understanding of the role of labor in clearing fetal liquid from the perinatal lung is supported by clinical studies that show a protective effect of labor on subsequent respiratory outcome. Drugs that augment the switch from liquid secretion to liquid absorption at birth are candidates for novel therapies to treat lung disease associated with premature birth. Possible strategies include inhibition of Cl^- secretion and augmenting of Na^+ transport across respiratory epithelia.

Effect of Labor

Respiratory morbidity of both term and preterm infants is related to mode of delivery. In term infants born to mothers undergoing repeat CS, there was a higher incidence of TTN after birth without a trial of labor (6%) compared with those born with a trial of labor (3%). The TTN rate in infants who had a trial of labor was similar to that of those born during routine vaginal births.[138] In both preterm and term infants, the rates of all respiratory problems were significantly higher in infants born by CS without labor compared with those born by either route with labor.[139]

β-Adrenergic Stimulation

In term infants undergoing CS without labor, there was significant improvement in dynamic lung compliance and a decrease in respiratory rate for those whose mothers had received a β-adrenergic agonist during the last 2 hours of labor compared with infants who were not exposed to these drugs.[140] These studies confirm the important role of labor in clearing lung liquid in preparation for air breathing, and the finding supports the hypothesis that β-adrenergic stimulation induced by labor plays a key role in triggering the absorptive process. This beneficial effect of prenatal β-adrenergic stimulation on respiratory morbidity was also observed in preterm infants.[141]

Sodium Transport

Steroid and thyroid hormones exert a major maturational effect on Na^+ transport in the developing fetal lung. Both hormones have been used for prevention and treatment of neonatal lung disease. Meta-analyses of multiple randomized trials suggest that prenatal steroids reduce the risk of RDS by about 50%. But even combined with surfactant therapy, antenatal steroid therapy has not decreased CLD in preterm infants.[142,143] Some clinical trials examining the addition of thyrotropin-releasing hormone to antenatal steroids have been reported. Early trials suggested some reduction in respiratory morbidity in infants of mothers given the hormone; however, subsequent larger studies have not confirmed these findings and have suggested potentially negative outcomes, both short term and long term, after treatment with thyrotropin-releasing hormone.[144-148] Postnatal steroid administration has been used extensively to treat ventilator-dependent lung disease in preterm infants, and studies showed that short courses of dexamethasone given early (in the first 2 weeks of life) may prevent bronchopulmonary dysplasia.[149-152] However, enthusiasm for steroid therapy has been tempered by reports of negative effects of both prenatal and postnatal glucocorticoids on lung development and pathologic findings in multiple organs.[153] These concerns have generated interest in the possibility that lower doses of steroids may be effective and may generate fewer side effects. Pilot studies in very preterm infants suggest that adrenal insufficiency can be reversed and CLD can be prevented with low doses of glucocorticoids.[154-156] The mechanism of low-dose steroid benefit for CLD is not known. We speculate that this treatment may enhance maturity of the ion transport system and may result in more effective lung water clearance in these preterm infants.

Chloride Secretion

Furosemide, a drug that inhibits Cl^- secretion by blocking the basolateral NKCC, has been investigated for the treatment of CLD after preterm delivery. Two systematic reviews for the Cochrane Database examined use of furosemide for CLD after preterm studies. Studies of aerosolized furosemide suggested a short-term benefit in lung mechanics after a single administration; however no data were available on clinical outcome and repeated administration.[157] Similar conclusions were drawn for administration by oral or intravenous routes, although long-term administration by these routes was associated with consistent improvement in lung compliance and oxygenation.[158] Strategies to reduce fetal Cl^- secretion will be assisted by better molecular characterization of fetal Cl^- pathways.

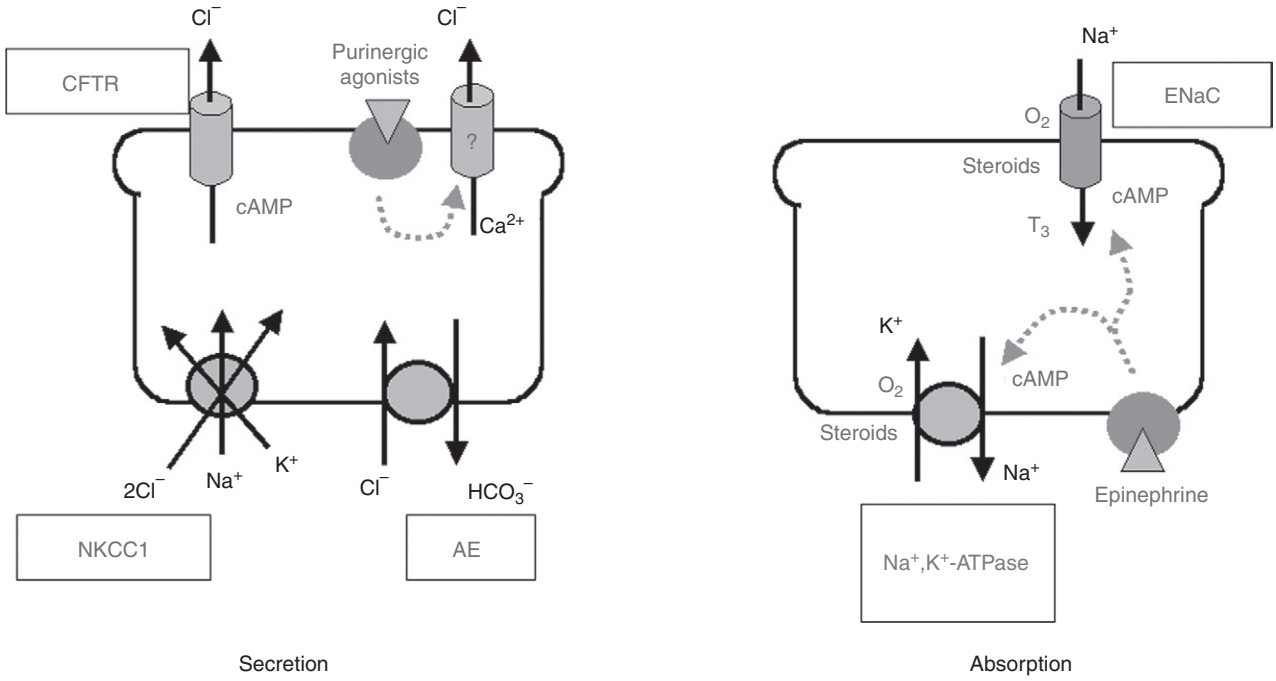


Figure 82-12 Summary of ion transport components and regulations. *AE,* Anion exchanger; *cAMP,* cyclic adenosine monophosphate; *CFTR,* cystic fibrosis transmembrane conductance regulator; *ENaC,* epithelial sodium channel; HCO_3^-, bicarbonate; *NKCC1,* sodium-potassium-chloride co-transporter 1; T_3, triiodothyronine.

SUMMARY

Ion transport has a key role in the switch from liquid-filled to air-filled lungs at birth. Although this physiologic process occurs over hours, the adaptive processes that prepare the fetal lung for birth are initiated at a much earlier stage of pregnancy. There is clear evidence that active Cl^- secretion underpins fetal lung liquid secretion and that this process is critical for normal lung growth in utero. Although there has been progress on elucidating mechanisms for Cl^- entry into the airway cells, the molecular identity of the channel or channels involved in transporting Cl^- into the lung lumen remains elusive. The functional and molecular basis of liquid absorption has been well characterized with detailed animal studies and molecular identification of the key components (ENaC and Na^+,K^+-ATPase) of this process (Figure 82-12). Both Cl^- secretion and Na^+ absorption are activated by a rise in cellular cAMP, and an alternative path for Cl^- secretion is activated by a rise in intracellular Ca^{2+}. The sequence of events that switch liquid secretion to liquid absorption is known. In late gestation, a rise in fetal glucocorticoids and active thyroid hormones readies, but does not activate, the Na^+ absorptive mechanism. In the last few days of gestation, there is some decrease in lung liquid production and intraluminal liquid volume. Labor has an important role in initiating lung liquid clearance in preparation for air breathing. During labor, some liquid is actively exhaled, but the main mechanism for clearing liquid is activation of Na^+ transport by coordinated induction of ENaC and Na^+,K^+-ATPase. A rise in fetal epinephrine levels in response to the stress of contractions and birth appears to trigger the switch from liquid secretion to liquid absorption. After birth, a rise in ambient O_2 augments Na^+ absorption, which completes the transition to the postnatal state. After birth, the lung retains some liquid secretory capability, and a balance of liquid secretion and liquid absorption sustains a thin film of liquid on the airway surface of the air-filled lung. There is strong circumstantial evidence that incomplete maturation of ion transport contributes to respiratory disease in very preterm infants, but future studies will need to confirm this cause-and-effect relationship. Better understanding of the regulation of ion transport across the fetal and newborn lung will open the possibility of novel therapeutic approaches to acute lung disease and CLD associated with preterm birth.

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Thomas H. Shaffer and Marla R. Wolfson

Upper Airway Structure: Function, Regulation, and Development

Although the conducting airways are formed well in advance of fetal viability, they undergo significant maturational changes in late gestation. They are susceptible to damage until they acquire the characteristics of more mature airways. Controversy exists concerning the pathogenesis of bronchopulmonary dysplasia (BPD) in the neonate[1-3]; in any case, prolonged mechanical ventilation and oxygen toxicity appear to be major contributing factors. Serial evaluations of pulmonary function in infants surviving hyaline membrane disease in whom BPD develops subsequently have demonstrated conclusively that the duration and pressure magnitude of mechanical ventilation, rather than increased inspired oxygen tension, damage the airways and lead to interference with their growth.[4,5] Within this context, the greater mechanical ventilation requirements of the very premature infant relative to the older infant precipitate an age-related predisposition to airway damage.

This chapter summarizes the function-structure characteristics and regulation of the developing upper airway and the impact of mechanical ventilation on airway function and reviews the clinical assessment of airway function.

STRUCTURE-FUNCTION CHARACTERISTICS

Airway Embryology

It has been shown that airway development in humans begins during the fourth week of gestation when the respiratory diverticulum, or lung bud, branches from the embryonic foregut.[6] The esophagotracheal septum forms and separates the foregut into the esophagus dorsally and the trachea ventrally. Thus the developing airway is of foregut endodermal origin. Elongation of the respiratory diverticulum forms the trachea, whereas the main bronchi are formed by branching. Growth and elongation continue in the caudal direction under the influence of airway secretions and physical forces.

Maturation of the airway occurs first in the trachea and proceeds distally. Tracheal cartilage formation begins during the seventh week of gestation. Full maturation of the distal airway cartilage is not completed until after birth. Epithelial differentiation begins in the trachea during week 10 of gestation. Together with the phasic contractions of the fetal airway smooth muscle, which are present after gestational week 23, lung fluid secreted from the epithelial cells promotes the growth and development of the respiratory system.[7]

Airway Structure

The airway tree is a branched conducting system whose major functions include the delivery, distribution, and conditioning of gas to the gas exchange units of the lung. The lower airway has three primary components: epithelium, cartilage, and smooth muscle. The epithelium lines the entire length of the airway. From the trachea to the large bronchioles, it is composed of a pseudostratified ciliated columnar epithelium referred to as respiratory epithelium. The designation *pseudostratified* applies because the respiratory epithelium is one cell layer thick and all cells attach to the basement membrane, but not all cells extend out to the airway lumen, giving the histologic appearance of multiple cell layers. Beyond the large bronchioles, the airway epithelium undergoes a gradual transition to a simple ciliated columnar epithelium and, finally, a cuboidal epithelium. The airway epithelium is composed of approximately eight different cell types. The primary cell type in the epithelium is the columnar epithelial cell, which contains a layer of cilia on its apical surface. Other cell types present include mucus-secreting cells, brush cells, small granule cells, and basal cells.

Cartilage is present from the trachea to the bronchioles. In the trachea, cartilage exists as C-shaped rings that are open posteriorly. In the bronchi, the cartilage forms plates that encompass the

entire airway circumference. At more distal airway generations, the cartilage plates become smaller and more discontinuous, gradually disappearing before the bronchioles. As with the airway cartilage, the architectural disposition of airway smooth muscle also varies, with different configurations along progressive airway generations. In the trachea, the airway smooth muscle is confined to the trachealis muscle. The trachealis, along with fibroelastic tissue, bridges the gap between the tips of the C-shaped cartilage rings and forms the posterior wall of the trachea. By contrast, bronchial airway smooth muscle forms a complete circumferential layer that gradually diminishes and becomes discontinuous at lower generations.

A mucosal layer covers the inner surface of the trachea. The mucosa consists of an epithelial layer supported by a basement membrane. This basement membrane is part of a loose connective tissue layer referred to as the lamina propria. The lamina propria is very cellular, containing lymphocytes and lymphatic tissue, plasma and mast cells, eosinophils, and fibroblasts. Also included within the mucosal layer are glands that send ducts to the epithelial surface.

Below the mucosa lies the submucosa. The submucosa is a connective tissue layer containing the distributing blood vessels, lymphatics, and mucus-secreting glands. The submucosa ends when it blends into the perichondrium of the cartilage rings. The outer layer of the trachea consists of adventitia, which binds the trachea to adjacent structures such as the esophagus, neck musculature, and mediastinal structures. The adventitia contains the large blood vessels and nerves supplying the components of the tracheal wall.

The primary innervation of the trachea is cholinergic from the vagus nerve.[8] Vagal stimulation results in contraction of airway smooth muscle. In addition, nonadrenergic, noncholinergic innervation controls both bronchoconstrictive and bronchodilatory actions through mediators such as nitric oxide, vasoactive intestinal peptide, neurokinins, and substance P. Little if any direct sympathetic innervation of the airway is present. Sympathetic control is provided primarily through circulating catecholamines.

More recent studies on structural maturation of airway smooth muscle at the cellular, ultrastructural, and protein expression levels have elucidated structural mechanisms for developmental changes in function.[9-11] Although the individual lengths of isolated tracheal smooth muscle cells increased from prematurity to adulthood, a linear increase in cell length was not demonstrated during the neonatal period, precluding cell length changes as a mechanism for functional development.[9] Similarly, developmental alterations in the electron microscopic ultrastructure of the tracheal smooth muscle could not be identified as potential mechanisms. At the protein expression level, the expression of smooth muscle myosin SM1 and SM2 heavy chain isoforms increased across development. This increase correlates with the developmental increase in tracheal smooth muscle force development, providing a likely mechanism for the functional maturation of this airway smooth muscle.

In developing rabbit trachea, clear patterns of expression or activation of matrix metalloproteinases MMP2 and MMP-9, as well as their (tissue) inhibitors TIMP-1 and TIMP-2, have been demonstrated during the progression of airway development.[12] In the lung, differences in MMP activity have been associated with BPD[13] and other chronic fibrotic lung diseases.[14] Thus it is implied that the progression of lung disease involves the alteration in MMP activity. As noted by Miller and associates, total MMP-2 quantity decreased steadily with increasing age in both the pre- and postgestational periods, whereas the active-to-latent ratio decreased only in the postgestational period. This change in the proteolytic potential in MMP-2 was compounded in the postgestational period by an age-dependent rise in TIMP-2. In that these arbitrary units of protein quantity are relative to a constant total protein load, the rise in TIMP-2 affirms that the significant fall in MMP-2 levels is not likely to be the result of an increase in inert, structural protein diluting the quantity of functional mediators of structural development. Of interest in this study is the lack of MMP-7 activity in developing trachea, in contrast with in the developing lung, where MMP-7 activity is present. These data support the assessment by other researchers that MMP-7 serves as a regulator for passage across the alveolar epithelium[15,16] and thus is not a mediator in development of conducting airways. In this regard, MMP-7 may play a role in the differentiation between airway and lung development.

Mechanics and Regulation of the Developing Airway

It is well accepted that infant airways are more compliant than adult airways. Early studies in necropsied human tracheobronchial segments indicated that airway pressure-volume relationships could be correlated with maturity.[17,18] Accordingly, a reduction in airway compliance with maturity results in decreased collapsibility and increased resistance to deformation during positive-pressure ventilation. Therefore the immature airway is more likely to sustain deformational changes resulting from barotrauma than is the less compliant airway of the older infant or adult.

Animal tracheas have been used extensively as models to study mechanisms that determine maturational changes in airway function. A study of in vitro rabbit tracheal segments,[19] as shown in Figure 83-1, documentedan age-related decrease in compliance in the developing rabbit airway that parallels that seen in neonates.[17] An in vivo study of the innervated and perfused trachea of the lamb demonstrated similar developmental changes in airway compliance and a decrease in the tracheal relaxation time constant.[20] It is noteworthy that in comparison with the results of in vitro studies, the absolute values of specific tracheal compliance in vivo were lower. Contribution of forces of surrounding connective tissue, or neurohumoral influences on airway smooth muscle tone, may affect the elastic properties of the developing airway and account for these observed differences. In addition, the decrease in relaxation time constant with maturation also is suggestive of age-related differences in smooth muscle tone in vivo. Alterations of smooth muscle tone modulate mechanical properties and pressure-flow relationships of the trachea in

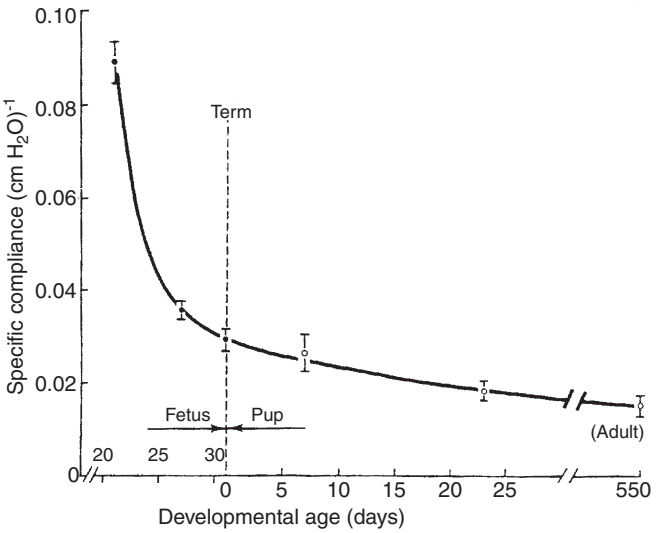


Figure 83-1 Developmental change in specific tracheal compliance in the rabbit. (Term = 31 days.) *(From Bhutani VK, Rubenstein SD, Shaffer TH: Pressure-volume relationships of tracheae in fetal newborn and adult rabbits,* Respir Physiol *43:221-231, 1981.)*

preterm and newborn lambs.[21-23] In general, tracheas stimulated with acetylcholine become stiffer and are less compressible, as reflected by decreased resistance to airflow when they are subjected to compressive forces.[23-25] Specifically, however, comparison of data suggests that the effect of pharmacologic stimulation on airway mechanics is age-dependent, and that the ability of airway smooth muscle to decrease airway compliance and increase flow may be limited in the preterm trachea.

The inability of airway smooth muscle to generate as much force as that produced by its adult counterpart probably contributes to this limitation. The effect of postnatal aging on airway smooth muscle maximal force production and sensitivity to various agonists remains controversial.[26] Some studies show that contractility and sensitivity increase with age,[8,27,28] whereas others suggest that contractility and sensitivity reach their peak early in postnatal life, declining thereafter.[29,30] When the extremes of the developmental spectrum are compared, however, a clear pattern emerges (Figure 83-2). Several investigators have shown that maximal contractility of airway smooth muscle increases between two- and four-fold from preterm or newborn to adult.[8,23,24,27] Furthermore, maximum active stress has been shown to increase significantly during late gestation in both the lamb[27] and pig.[10] This change is not the result of an increase in smooth muscle mass, because normalization for cross-sectional area eliminates that factor.

Developmental changes in force generation of airway and nonairway smooth muscle in various species have been related to an increase in contractile proteins (actin and myosin) per unit area and to age-related differences in amount and type of myosin isoforms.[9,29,31,32] Age-related increases in contractile responses of vessels also have been related to alterations in the orientation of vascular smooth muscle cells, which changes from a circular to oblique relative to the long axis of the vessel wall.[33]

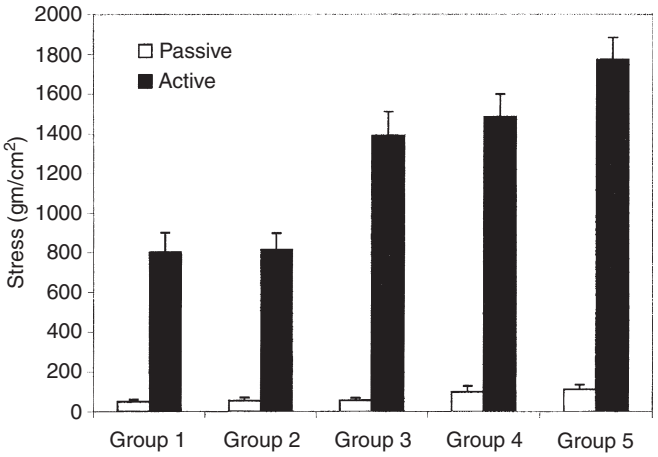


Figure 83-2 Active and passive stress values from muscle bath studies in isolated trachealis muscle strips from preterm, newborn, and adult sheep. Data are normalized for cross-sectional area and for percentage of muscle fibers within the strip. *Group 1:* preterm at 110 days of gestation (n = 8); *group 2:* preterm at 110 to 124 days of gestation (n = 25); *group 3:* preterm at 125 to 140 days of gestation (n = 5); *group 4:* term newborns (n = 10); and *group 5:* adults (n = 16). (Term = 147 ± 3 days of gestation.) A significant increase in both active and passive stress as a function of age was noted ($p < .001$). Post hoc analysis disclosed significant differences in active stress between group 1 and groups 3, 4, and 5, and between group 2 and groups 4 and 5. Similarly, passive stress increased between groups 1 to 3 and group 5. Values are means ± SEM. *(From Panitch HB, et al: Maturational changes in airway smooth muscle structure-function relationships,* Pediatr Res *31:151-156, 1992.)*

Furthermore, age-related shifts in the synthetic activity of vascular smooth muscle cells have been identified in neonatal rats. Initially, synthetic activity is primarily secretory, producing extracellular proteins, collagen, and elastin; after the age of 4 weeks, synthetic activity is primarily contractile, producing the intracellular protein actomyosin.[34] Also along these lines, Driska and co-workers[35] identified age-related differences in morphometry and similar shortening responses in airway smooth muscle cells isolated from preterm and adult sheep. Adult cells were similar in size and appearance to adult arterial smooth muscle cells, but velocity of shortening (0.54 sec^{-1}) was approximately three times faster. Isolated preterm airway smooth muscle cells were approximately half as long and thick as adult cells, but shortening velocities were similar.

Airway epithelium plays an important role in the modulation of smooth muscle function. Studies of adult airways have shown that airway epithelium generates relaxant and contractile factors that modulate the tone of the underlying smooth muscle.[36-40] Furthermore, epithelial damage has been associated with bronchial hyperreactivity.[41] In a similar study of deepithelialized preterm lamb trachea, force generation in response to acetylcholine stimulation was increased over that measured for the intact tracheal smooth muscle strip.[42] These data demonstrate that preterm airway epithelium is able to modulate the responsiveness of smooth muscle. Additionally, the magnitude of the effect was unchanged with maturation (from preterm to adult). Thus, even during late gestation, epithelial integrity may be an important determinant of smooth muscle function, bronchial hyperreactivity, and bronchodilator responsiveness. Not completely understood is whether regional differences in airway epithelium exert differential contractile or relaxant influences on airway smooth muscle in the developing airway.

The fact that the structural arrangement of muscle and cartilage in the adult trachea is different from that in the bronchi suggests that the functional effects of muscle contraction also may be different in these tissues in the adult, and presumably during development as well.[40]

Studies of the adult trachea suggest that the elasticity of the passive trachealis muscle and connective tissue is greater than that of the bronchial wall.[43] Moreover, these studies demonstrated that tracheal smooth muscle is capable of generating larger circumferential tensions than bronchial muscle, presumably because of the circumferential alignment and relatively greater proportion of the smooth muscle cells in the trachealis muscle, compared with helical orientation and fewer smooth muscle cells in the bronchial wall. Regional differences in force generation have been noted in the airway of premature sheep as well.[42] Passive, active, and total stress development decreased significantly as a function of airway generation, from trachea (0 generation 0) to the subsegmental bronchi (generation 4). The receptor-mediated response to acetylcholine was significantly less in generations 0, 1, and 2 than in generations 3 and 4. In addition, the internal radius-to-wall thickness ratio (r/t) decreased from trachea to fourth-generation airway. The law of Laplace predicts that because of this decline in r/t, the trachea would be exposed to the greatest degree of wall stress during positive-pressure ventilation.[42] Taken together, these data help to explain the structural changes as well as physiologic changes in airway reactivity seen in the premature infant after mechanical ventilation.

Although the intact trachea serves as a suitable preparation for studying the mechanical function of the muscle and cartilage, it is important to consider the individual differences in airway smooth muscle function and contribution of cartilage. Along these lines, several investigators have suggested that airway cartilage plays an important role in determining airway compressibility and inflatibility.[44-46] Moreno and co-workers[47] softened rabbit tracheas with papain and demonstrated alterations in unstressed tracheal volume and compliance. In a related study using papain-treated

rabbits, McCormack and associates[42] observed changes in pulmonary function indicative of increased airway collapsibility. The maturational effects of cartilage contribution to airway function was demonstrated by Penn and co-workers,[46] who showed that tracheal cartilage in preterm lambs is extremely compliant relative to that in the adult sheep. In addition, it was suggested that these age-related changes in cartilage paralleled developmental differences in tracheal smooth muscle and tracheal mechanics.[22,27,48] Therefore age-related differences in airway mechanical function may reflect an increase in stiffness that occurs in both airway muscle and cartilage.

Effects of Mechanical Ventilation on Airway Function

Although mechanical ventilation undoubtedly increases the survival of patients in respiratory compromise, it also is associated with increased morbidity.[49] The volumes, pressures, and oxygen concentrations that are incumbent in mechanical ventilation provide a mechanism of injury to a respiratory system that is not suited to such exposure.[50] This dilemma is most obvious in the premature and neonatal patient population.[51,52] Owing to the low compliance of the premature or surfactant-deficient lung, the inspired oxygen concentrations and ventilatory pressure requirements often are extreme.

Even though mechanical ventilation can be associated with systemic morbidity from specific nonpulmonary entities such as intraventricular hemorrhage, the primary pathologic changes consequent to mechanical ventilation involve the respiratory system. Mechanical ventilation is known to result in ventilator-induced lung injury and concomitant respiratory dysfunction. The signs of ventilator-induced lung injury include atelectasis, progressive respiratory failure, and pulmonary edema with hyaline membrane formation. It has been demonstrated in adults and infants that inflammatory cytokines are mediators of respiratory injury and dysfunction.[53,54] Altered production of many cytokines has been implicated, including tumor necrosis factor-α (TNF-α) and the interleukins IL-1β, IL-6, and IL-8. These cytokines not only act on the respiratory system but also exert their effects systemically.

The current thinking regarding minimization of lung injury focuses on limiting ventilatory pressures. Reduction of these pressures, however, comes with a paradox: Although elevated mean airway pressures are implicated in the pathogenesis of lung injury, maintaining adequate positive end-expiratory pressure (PEEP) may attenuate this damage. Including a level of PEEP in the ventilation strategy sufficient to ensure that tidal ventilation occurs at pressures greater than the lower inflection point is thought to minimize lung damage. When end-expiratory pressures fall below the inflection point, some alveoli close. These closed alveoli must then reopen during subsequent inspiratory cycles. This alveolar opening–closing action is thought to be involved in the lung injury and inflammatory process.[55] Additionally, in the absence of PEEP, the closing-reopening of the alveoli has been shown to disrupt the surfactant layer and thereby exacerbate already impaired lung function.

Mechanical ventilation has little effect on adult airways but has been shown to alter the dimensions[55-58] and mechanical properties of preterm and newborn airways.[59,60] The extent of ventilation-induced deformation appears to be directly related to the compliance of the airway and inversely related to age. Our own observations in a preterm lamb model of the effects of mechanical ventilation included an increase in tracheal diameter, thinning of cartilage and muscle, disruption of the muscle-cartilage junction, and focal abrasions of the epithelium.[56] As shown in Figure 83-3, in comparison with unventilated trachea, decreased inflation and increased collapsing compliance of the trachea after ventilation produce a structure analogous to that of a fire hose, which is difficult to expand but easier to collapse.[60]

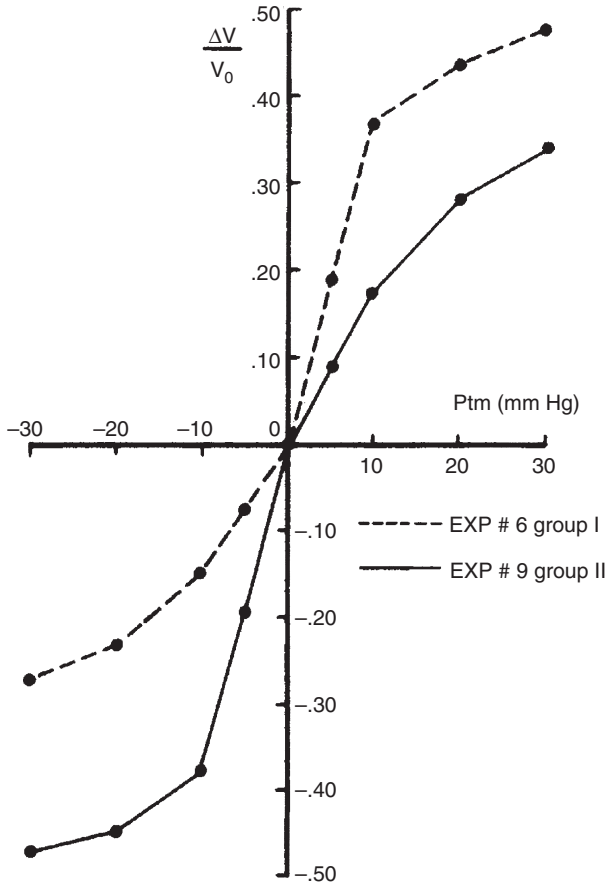


Figure 83-3 Transmural airway pressure *(Ptm)* as a function of normalized volume change ($\Delta V/V_0$) in a typical unventilated *(group I)* and ventilated *(group II)* trachea segment. *(From Penn RB, Wolfson MR, Shaffer TH: Effect of ventilation on mechanical properties and pressure-flow relationships of immature airways,* Pediatr Res *23:519-524, 1988.)*

In addition, ventilated tracheas showed greater resistance to airflow. The clinical implications of these studies include increased dead space, flow limitation, elevated airway resistance and work of breathing, and gas trapping.[61]

The mechanisms that precipitate the alterations in mechanical properties of the ventilated trachea are unclear. Preliminary studies of airway smooth muscle force generation are inconclusive, and further investigation is required. In light of the epithelial disruption demonstrated, it is possible that loss of an epithelial relaxant factor explains the intensive bronchoconstrictor response to inhaled agonists seen in infants with pulmonary dysfunction.[62,63] Focal abrasion and denudation of the airway epithelium after mechanical ventilation have been reported[61] and are illustrated in Figure 83-4. The cells of the airway epithelium play an important role in the modulation of airway smooth muscle. Through the release of both contractile and relaxant paracrine mediators, the epithelium modulates the tone of the underlying smooth muscle. Disruption of this epithelium may have a significant impact on airway function and sensitivity.[64,65]

Disruption of the muscle-cartilage junction may reduce the ability of the airway to resist compressive forces such as excessive supra-atmospheric pressures produced by infants with airflow obstruction. Pressure-induced alterations in the orientation of airway smooth muscle fibers may affect the force-generating capabilities or impair the ability of smooth muscle contraction to stiffen the airway and resist deformation. It also is possible that

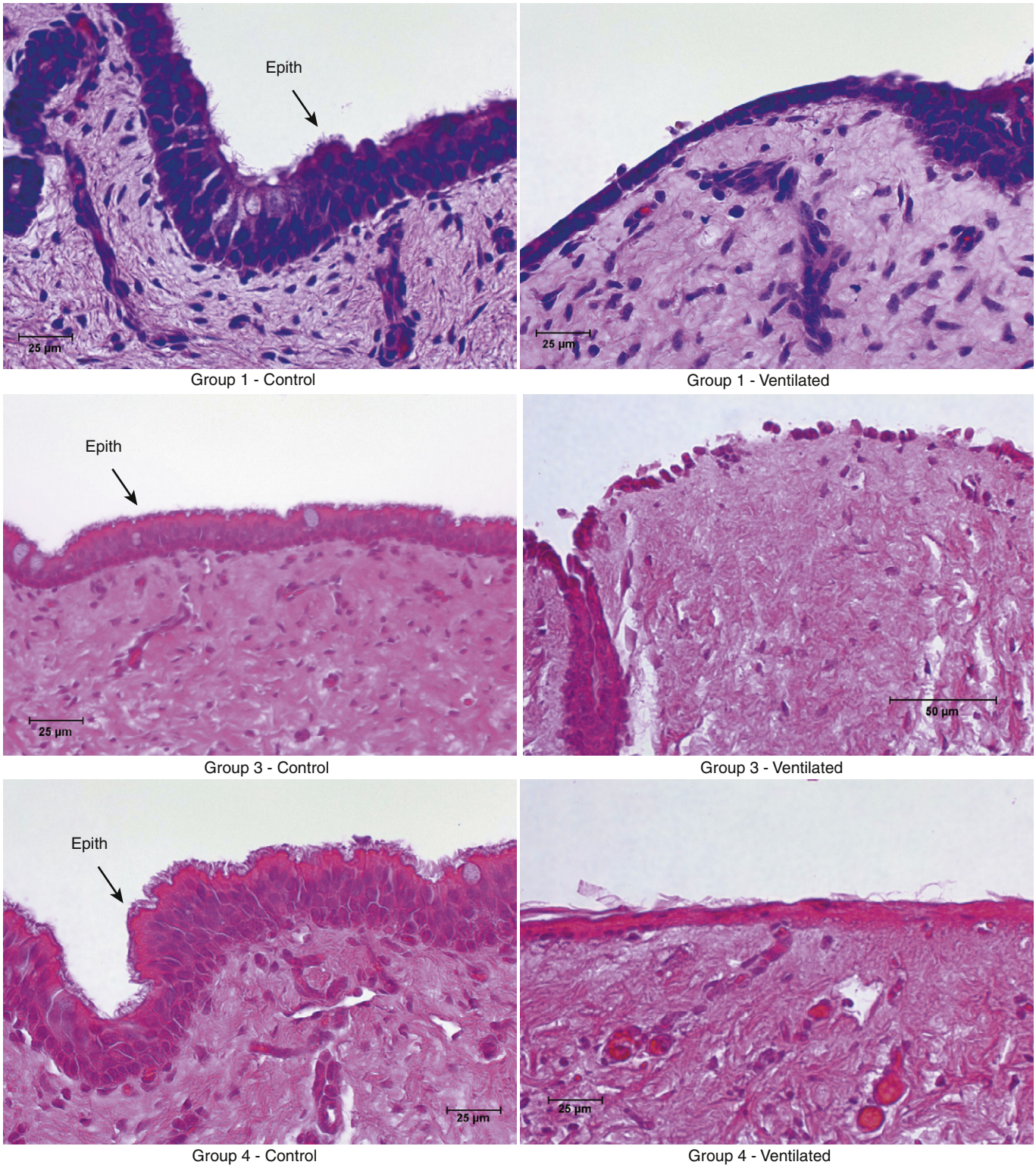


Figure 83-4 Effect of mechanical ventilation on epithelial lining of neonatal lamb trachea in four different age-groups: *group 1:* preterm at 110 to 116 days; *group 3:* preterm at 129 to 132 days; *group 4:* term at 149 to 157 days. (Term = 147 ± 3 days of gestation.) *Left panel:* Nonventilated control animals. *Right panel:* Lambs subjected to mechanical ventilation (mean airway pressure = 15 cm H_2O, duration = 2.8 hours). *(From Cullen AB, et al: The impact of mechanical ventilation on immature airway smooth muscle: functional, structural, histological and molecular correlates,* Biol Neonate *90:17-27, 2006.)*

pressure-induced alterations in the alignment of cartilage components (i.e., proteoglycan-collagen configuration) may attenuate the contribution of cartilage as a structural support for the trachea. In addition to pressure-induced tracheomegaly, histologic studies of ventilated neonatal human and animal lungs describe widening of the peripheral airways.[66,67] Apart from the qualitative assessment of dimensions, the effects of ventilation on the peripheral airways are not known. Presumably age-related and regional differences in the amount of cartilage, orientation, force-generating capabilities, and receptor sensitivity of airway smooth muscle may exacerbate the effect of ventilation on the relatively more compliant distal (as compared with proximal) airways. This effect would be further potentiated by a ventilatory schema wherein an inspiratory hold is produced by long inspiratory times. Long inspiratory times favor equilibration of pressures throughout the tracheobronchial tree, thereby increasing the length of time over which the highly compliant distal airways are subjected to barotrauma. Of note, however, high-frequency jet ventilation (HFJV) evaluated in preterm rabbit airways was associated with significant dimensional and mechanical deformation of tracheal segments, as well as an increased propensity for collapsibility after cessation of the ventilation.[68] Therefore ventilation techniques that attempt to minimize pulmonary barotrauma may potentially still have some deleterious effect on immature proximal airways.

Airway conductance of the adult tracheobronchial tree also may be affected.[69] It previously has been shown that increased muscle tone can increase the rigidity of the trachea in the full-term newborn lamb.[19] This finding suggests a mechanism whereby ventilation-induced alterations of the airway can be ameliorated: If the ability of airway smooth muscle to increase tone in response to pharmacologic stimulation is age- or region-dependent, then prophylactic treatment before ventilation may have differential effects on the proximal versus peripheral airways of the developing animal.

Both mechanical ventilation and chronic lung and airway disease have been associated with the presence of inflammatory mediators in airway aspirates. These mediators attract and activate neutrophils, which in turn generate additional free radicals, cytokines, and proteolytic enzymes such as elastase and collagenase. Previous studies have shown increased levels of TNF-α, endothelin-1 (ET-1), IL-6, IL-8, IL-1β, and IL-1 and decreased fibrinolytic activity in tracheal aspirates from infants in whom BPD and chronic lung disease subsequently develop. Additionally, the infants with BPD exhibit a prolonged neutrophil influx during the first week of life compared with infants with self-limited respiratory distress syndrome (RDS).[54] Thus substantial, chronic airway injury is associated with mechanical ventilation, necessitating elucidation of its pathogenesis to minimize further injury.

In a recent report, it was demonstrated that ultrasound imaging can provide insight into the mechanical properties of the developing airway.[70] Ultrasound imaging of the isolated tracheal segment allows for real-time assessment, with the following specific advantages: (1) airway dimensions can be assessed with the adhesions to surrounding tissue as well as blood supply and innervations intact; (2) static pressure-volume relationship data can be corrected with a more accurate resting volume calculation and without sacrifice of the preparation; and (3) stress-strain relationships can be recorded and analyzed during dynamic pressure pulses, which account for tissue kinetics and the trachea's elastic modulus. More important, an application of this methodology demonstrated that mechanical ventilation has a significant effect on both static and dynamic mechanical properties of the sheep neonatal trachea.[71] Furthermore, mechanical ventilation resulted in breakdown and thinning of the trachealis muscle boundary, as well as increased segment volume and thus anatomic dead space. These data serve as a standard for comparison to evaluate various "protective" alternatives to conventional mechanical ventilation with respect to preservation of airway function. In addition, with an indication of the time dependence of acute alteration in tracheal mechanical properties, future studies can correlate these trends with biomarkers from tracheal tissue samples and segment washings that reflect tissue damage and structural modeling.

CLINICAL ASSESSMENT OF AIRWAY FUNCTION

Modalities for evaluation of airway function in the pediatric population include measurement of lung function during tidal breathing or forced exhalation, radiography, fluoroscopy, and airway endoscopy. In combination, these assessment modalities can be used to identify and quantitate the predicted functional abnormalities found in preterm airways exposed to positive-pressure ventilation, including elevated resistance, decreased forced expiratory flow, airway hyperreactivity, and excessive central airway collapsibility. In addition, these diagnostic procedures have provided important insight into the effects of early injury on future airway growth and function and can be used to test the effectiveness of newer therapies. Evaluation of airway function is especially useful and should be considered in infants whose clinical course is not one of gradual improvement or is marked by frequent severe pulmonary exacerbations, and in infants who demonstrate stridor, chronic wheezing, or focal areas of chronic atelectasis or hyperinflation.

Pulmonary Function Profile

With the simultaneous measurement of respiratory pressures, volumes, and air flow, pulmonary mechanics can be monitored relatively noninvasively.[72] Pulmonary function profiles can be determined to assess initial disease etiology, response to therapy, and disease sequelae during follow-up.[73] For example, the pressure-volume relationship for a premature infant can identify the degree of respiratory function and monitor the response after surfactant administration. In addition, the evaluation of tidal breath pressure-volume relationships serves as a tool to optimize the parameters of mechanical ventilation. Over time, sequential assessments of pulmonary mechanics can be used to monitor progression or improvement in both lung and airway abnormalities.

Measurements of Airway Function During Tidal Breathing

Dynamic pulmonary compliance as measured by the esophageal balloon and pneumotachnometry technique is significantly lower in infants in whom chronic lung disease develops subsequently than in normal infants or those who recover uneventfully from neonatal respiratory distress.[73-75] Although these measurements reflect the elastic properties of the lung to some degree, they also are influenced by maldistribution of ventilation associated with regions having differing time constants (frequency dependence of compliance).[74] Thus dynamic compliance measurements in infants with chronic lung disease probably also reflect some degree of airway obstruction. Quasistatic methods of measuring respiratory system compliance in infants with BPD have verified that alterations in the elastic properties of the lung contribute significantly to the observed decrease in compliance.[76] Studies comparing static and dynamic compliance measurements in this group of infants, however, have not been performed. Thus it is not clear whether the improvement in dynamic compliance seen in infants with moderate to severe BPD studied longitudinally[73,75] represents a change in the characteristics of the lung parenchyma, or if it is reflective of a coexistent decrease in respiratory rate or improvement in airway obstruction. Of interest, both static and dynamic measurements of compliance have been shown to have predictive value regarding the development of BPD in mechanically ventilated preterm infants.[77,78]

Resistance measurements, including airway resistance as determined by plethysmography, pulmonary resistance measured by the esophageal balloon and pneumotachometry technique, and respiratory system resistance determined by the airway occlusion technique, are significantly elevated in infants with BPD.[73,75,79-81] When measurements of resistance or its reciprocal, conductance, have been made serially, values have approached normal over the first 2 to 3 years of life.[73,75] Longitudinal changes in resistance can reflect either resolution of airway obstruction or merely an increase in airway diameter related to growth. To eliminate the influence of growth or changes in lung volume on these measurements, investigators also have reported size-corrected values for resistance or conductance—namely, *specific conductance,* defined as the conductance divided by lung volume at functional residual capacity (FRC). Data reported in this way confirm the presence of airway obstruction in infants with BPD.[73,75] In the relevant study, although pulmonary resistance decreased to only one fourth of the original measurement over the first 3 years of life, specific conductance rose only minimally and remained below normal at the end of the study period. Arad and co-workers[82] found that specific conductance rose from 57% ± 7% of predicted in infancy to 90% ± 8% of predicted by the age of 5 to 7 years, although the children who required mechanical ventilation in infancy demonstrated air trapping and small airway obstruction at the time of the study.

Forced Expiratory Flow Measurements

Measurements of tidal mechanics include significant contributions of the nasopharynx, pharynx, and central airways. In a number of studies, either the rapid thoracic compression technique[79,83,84] or the rapid deflation technique[85,86] has been applied in infants with BPD to obtain information more reflective of small airway status, and to compare data obtained in infancy more easily with spirometric measurements made in routine testing performed later in childhood. Such studies also demonstrate significant airway obstruction in infancy, with evidence of incomplete recovery with growth. When maximum expiratory flow-volume (MEFV) curves were generated by the rapid deflation technique in a group of preterm infants during the acute phase of BPD, severe small airway obstruction as determined by a marked reduction in $Vmax_{25}$ was noted, and the shape of the MEFV curve was concave to the volume axis.[85] When patients with moderate BPD who weaned from mechanical ventilation before 5 months of age were studied longitudinally with the same technique, there was a gradual increase in $Vmax_{25}$ to approximately 40% of predicted by the age of 3 years.[86] By contrast, those patients who required extended periods of mechanical ventilation (>10 months) showed no increase in $Vmax_{25}$ over the same time period.

Similar evidence of airway obstruction has been demonstrated using the rapid compression technique.[79,83] Partial expiratory flow-volume (PEFV) curves were generated over the tidal range of breathing and quantitated by measuring maximal flow at FRC ($Vmax_{FRC}$) in young infants with BPD. Furthermore, the separation between tidal flow and forced flow curves was interpreted as a measure of expiratory flow reserve.[83] The shape of the PEFV curve typically was concave to the volume axis, and expiratory flow reserve was smaller than in infants with normal lungs. Size-corrected forced expiratory flows were only half those of normal controls, and did not increase by the age of 14.5 to 22 months.[83] Furthermore, the slope of the regression equation for $Vmax_{FRC}$ versus age was lower in the BPD group than in normal infants. These data suggest that early exposure to positive-pressure ventilation and high concentrations of oxygen not only caused early airway damage but also interfered with subsequent normal airway growth.

Values of $Vmax_{FRC}$ usually have been considered to be representative of small airway function. More recently, however, studies have shown that central airway collapsibility (i.e., tracheomalacia or bronchomalacia) can cause a marked reduction in values of $Vmax_{FRC}$ and appear graphically similar to small airway obstruction.[84,87] Panitch and co-workers[84] studied five infants with BPD who had bronchoscopic evidence of tracheobronchomalacia using the rapid compression technique. As shown in Figure 83-5, when continuous positive airway pressure (CPAP) was applied to the airway opening, an incremental increase in $Vmax_{FRC}$ was observed. Additionally, in several subjects, the shape of the PEFV curve changed from concave to convex, suggesting that CPAP acted as an intraairway stent to prevent collapse. Furthermore, the ratio of forced-to-tidal flows at midexpiration, a reflection of expiratory flow reserve, increased with application of CPAP. Thus it is possible that reductions in $Vmax_{FRC}$ previously ascribed to small airway damage in infants with BPD may also represent a component of central airway injury.

Assessment of Airway Reactivity

Airway reactivity, as determined by bronchodilator responsiveness, has been well documented in infants with BPD using assessments of both tidal mechanics and forced expiratory flows. Acute decreases in pulmonary, respiratory system, and airway resistance ranging from 23% to 48% have been reported in response to a variety of β-agonist and anticholinergic drugs.[80,81,88-92] The magnitude of this response was even more dramatic when forced flows were tested: Kao and colleagues reported an increase of 86% over baseline values of $Vmax_{FRC}$ in 15 infants with BPD at a mean age of 15.8 weeks after treatment with metaproterenol, and of 45% over baseline after treatment with atropine.[80] Similarly, Motoyama and associates[85] found an increase of 214% over baseline values of $Vmax_{25}$ after isoetharine inhalation in 32 intubated and mechanically ventilated infants. Bronchodilator responsiveness could be demonstrated in infants as young as 26 weeks postconceptual age, and as early as at 12 days postnatal age.

Other investigators have also demonstrated early evidence of bronchodilator responsiveness. Gomez-Del Rio and associates[90] found significant improvement in pulmonary resistance in 30 mechanically ventilated preterm infants younger than 20 days of age after isoetharine inhalation. The youngest was only three days of age, and gestational ages ranged from 27 to 34 weeks. Denjean and co-workers[88] measured significant decreases in respiratory system resistance after salbutamol inhalation in ventilator-dependent preterm infants at 13.3 ± 4.9 days of postnatal age. These observations of early bronchodilator responsiveness suggest that loss of epithelial modulation, as well as airway smooth muscle hypertrophy, probably contribute to airway hyperreactivity seen in chronically ventilated infants.

Radiographic Evaluation of Airway Injury

Acquired tracheomalacia has been evaluated fluoroscopically and by computed tomography (CT). Sotomayor and colleagues[93] used fluoroscopy in anteroposterior, oblique, or lateral views to document central airway collapse in infants with BPD who required mechanical ventilation for periods of 3 weeks to 4 months. These investigators also used fluoroscopy to determine the amount of distending pressure required to maintain airway patency in those infants. McCubbin and co-workers[94] studied central airway collapse in 10 infants (3.3 to 20.5 months of age) with BPD, using cine-CT. A group of seven children of similar age with glottic or supraglottic obstruction but no evidence of lower airway disease was used as a control group. The median percent decrease in airway cross-sectional area during exhalation in the BPD group was 63.5% (range, 23% to 100%), whereas that of the

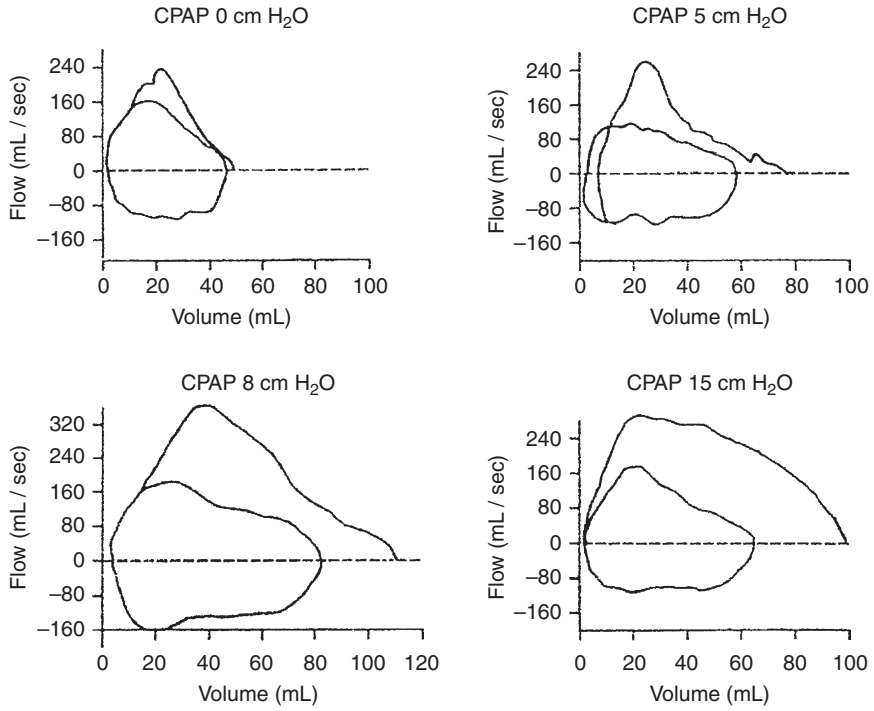


Figure 83-5 Series of partial expiratory flow-volume curves from an infant with bronchopulmonary dysplasia (BPD) and bronchoscopically documented tracheobronchomalacia, showing the effect of increasing levels of continuous positive airway pressure (CPAP). Off CPAP, the curve shape becomes straight, and at higher levels of CPAP, it becomes convex. At each level of CPAP, expiratory flow reserve increases as well. Values for maximal flow at functional residual capacity (V_{maxFRC}) at each level of CPAP are 26 mL/second at 0 cm H_2O; 53 mL/second at 5 cm H_2O; 120 mL/second at 8 cm H_2O; and 204 mL/second at 15 cm H_2O. *(From Panitch HB, et al: Effects of CPAP on lung mechanics in infants with acquired tracheobronchomalacia,* Am J Respir Crit Care Med *150:1341-1346, 1994.)*

control group was only 9% (range, 5% to 13%). This significant difference in collapsibility was present in a short segment of airway in six children and was diffuse in the other four patients. Because narrowing was not always diffuse, these authors speculated that the underlying cause of collapse must include local sites of injury as well as transmural pressure changes.

Bhutani and associates[58] described roentgenographic evidence of acquired tracheomegaly in very preterm neonates (i.e., with birth weight less than 1000 g) who had received mechanical ventilatory support. Increases in tracheal width at the level of the thoracic inlet and carina were present when compared with individually weight-matched nonventilated control infants. These workers speculated that the persistent airway dimensional deformation seen in those infants resulted in increased anatomic dead space and contributed to carbon dioxide retention after extubation.

The ability to evaluate small airway abnormalities with conventional bronchographic agents has been limited by poor resolution of the bronchioles at the secondary lobule level.[87] Because of the high density, low surface tension, and radiopacity of the perfluorochemical liquid perflubron, it has been used as a bronchographic contrast agent for high-resolution computed tomography (HRCT) direct visualization of the airways through the centrilobar bronchioles and their first-order branches, with definition within a millimeter of the lung surface.[96,97]

Radiographic studies of the perflubron-filled lungs of animals and humans with congenital diaphragmatic hernia have proved informative in qualitative delineating the degree of pulmonary hypoplasia and in identifying the distribution and elimination pattern of the perfluorocarbon liquid.[98,99] Regional differences in perfluorocarbon clearing, detected on x-ray studies, were related to localized bronchial obstruction.[98]

Endoscopic Evaluation of Airway Injury

Airway endoscopy provides for both diagnosis and treatment of anatomic lesions of supraglottic, glottic, and subglottic regions, as well as of the trachea and bronchi down to the segmental level.[100-106] Two recent prospective studies of infants who required intubation in the newborn period found that the incidence of moderate to severe subglottic stenosis was 9.8% and 12.8%, respectively,[101,102] but the incidence of other fixed anatomic lesions is unknown. Endoscopy of the airway was selected when infants presented with acquired lobar emphysema, persistent lobar atelectasis, or unexplained medical failure.[104,105,107-110] Typically, with lobar emphysema, the right lower lobe and right middle lobes were more commonly affected.[104,105,107-109,111]

Direct visualization of the airways during spontaneous breathing is the most direct method of identifying central airway collapse. Although both rigid and flexible fiberoptic bronchoscopic techniques are available for the study of pediatric airways, rigid bronchoscopy must be performed while the patient is under general anesthesia. Often the child receives assisted ventilation during the procedure. Consequently, the patient's effort of breathing is decreased, and exhalation may be completely passive. Thus many cases of central airway collapse would be underdiagnosed with use of this approach.

By contrast, flexible bronchoscopy usually is performed using conscious sedation. The patient breathes spontaneously but must breathe around the bronchoscope as well. Variation in expiratory effort can influence the degree of intrathoracic airway collapse. Because of these technical considerations and the lack of universally agreed-on criteria, the frequency of diagnosis of central airway collapse will vary from center to center. In order to circumvent these inconsistencies, some researchers have based the diagnosis on extent of airway narrowing observed during

exhalation.[84,106,112] Mair and Parsons have recommended a grading system based on percent of airway narrowing present at end-expiration during spontaneous respiration, together with an increase in the membrane-to-cartilage ratio.[112] Other workers have defined tracheobronchomalacia as collapse resulting in either less than 50%[84,113] or less than 75%[93] obstruction during spontaneous breathing, with no mention of changes in the proportion of membrane relative to cartilage.Acquired extrathoracic tracheomalacia also has been described in patients with BPD.[102]

To date, most quantitative studies of the inherent characteristics of the developing airway wall have required excision of an airway segment or surgical creation of an isolated segment. A more recent laboratory study demonstrated that airway wall characteristics at various collapsing pressures and attendant changes in stiffness after smooth muscle stimulation could be quantitated bronchoscopically from airway pressure-area relationships.[114] Neonatal lamb tracheal segments were suspended over hollow mounts in a buffer-filled chamber and then subjected to a range (0 to –4.0 kPa) of pressures to determine wall stiffness under collapsing forces before and after stimulation of the trachealis with methacholine. Luminal images were recorded through a 3.6-mm flexible bronchoscope under the same conditions, and the cross-sectional area was calculated from data thus obtained. Both pressure-volume and pressure-area relationships detected significant changes in airway wall stiffness after methacholine administration ($p < .002$), with similar magnitudes of change for both quantities. In a subsequent study, this same method was reported to quantitate airway stiffness in vivo.[115] The speculation is that this technique may be clinically useful to quantitate airway collapsibility and can differentiate whether deformation is secondary to effort or to intrinsic abnormalities of the airway wall.

SUMMARY AND FUTURE DIRECTIONS

Considerable progress has been made in characterizing developmental (preterm, newborn, and adult) differences in airway physiology with respect to pressure-volume and pressure-flow relationships, the effects of mechanical ventilation, and pharmacologic stimulation. In this regard, studies conducted using in vivo, in vitro, and muscle bath preparations have provided valuable insight into how the very premature infant may differ from the later-term and full-term neonate with respect to airway function. Nevertheless, little is known about how positive-pressure ventilation alters the cellular, biochemical, and molecular constituents of the components of the immature airway, and why mechanical ventilation is associated with the development of severe airway dysfunction in some premature infants but not in others. Ultrastructural studies that assess airway damage with greater sensitivity and biochemical and molecular analysis that can detect alterations in collagen muscle and connective tissue composition would improve current understanding of the pathogenesis of pressure- or stretch-related injury. Data from such studies, coupled with clinical results of pulmonary function testing and further characterization of airway anatomy, would be expected to elucidate basic biologic development of the lungs, facilitating continual advancement of neonatal and pediatric clinical respiratory management.

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84 Regulation of Lower Airway Function

Richard J. Martin and Musa A. Haxhiu

Significant progress has been made in elucidating development of lower airway structures and regulation of their function. During early development airway smooth muscle differentiates from the mesenchyme of the primordial lung and envelops the emerging bronchial tree.[1] Airway smooth muscle at this early stage provides phasic rhythmic contractility that is thought to propel lung fluid distally and enhance lung development. Neural structures emerge in parallel to airway muscle, and their functional roles are rapidly integrated such that during postnatal life tonic, rather than phasic, contractile and relaxant functions dominate.[2]

Although it is well accepted that the potential effect of the lower airway on prenatal and postnatal lung function is considerable, our understanding on the link between neurotransmitter specific networks involved in the regulation of airway function is incomplete. Furthermore, when compared with healthy and diseased adult airways, little attention has been paid to the diverse neural mechanisms that regulate airway caliber in the perinatal period. This subject has gained considerable interest because of the injurious effects of increased inspired oxygen and positive pressure ventilation on neonatal airway function. It is therefore important to gain greater understanding of the normal maturational changes exhibited by airway smooth muscle contractile and relaxant mechanisms superimposed on the immature structural elements that compose the airways.[3]

THE HIERARCHIC NEURAL REGULATION OF THE LOWER AIRWAYS

Airway-Related Vagal Preganglionic Neurons: Cholinergic Control of Lower Airways

During development most studies on neural control of the airways have focused on the regulation of smooth muscle, even though many of the same principles apply to control of mucus production, and blood flow in the airways.[4] In general, at any given age neural control of airway functions (Figure 84-1, *level 4*) involves integrated networks along the neural axis that funnel information to tracheobronchopulmonary effector units via airway-related vagal preganglionic neurons (AVPNs) in the medulla oblongata.[5,6] The AVPNs are the final common pathway from the brain to the airways and transmit excitatory signals to the intrinsic tracheobronchial ganglia that are part of the network for feedback control *(level 3)*.

The AVPNs innervating airways, from the extra thoracic trachea[5] to the most distal bronchiole,[7,8] arise mainly within the

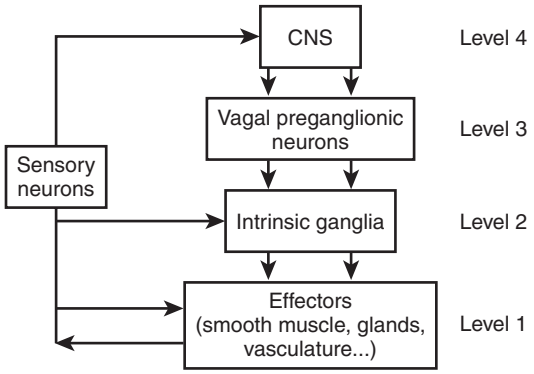


Figure 84-1 General scheme illustrating the organization of autonomic parasympathetic control of airway functions. Central nervous system (CNS) cell groups *(level 4)* regulate the activity of airway-related vagal preganglionic neurons (AVPNs) *(level 3)* that present the final common pathway to the airways. Projections from the AVPNs' synapse on intrinsic ganglionic neurons within the airway walls *(level 2)*. These ganglia give rise to postganglionic fibers that control the function of specific effector targets (i.e., airway smooth muscle, mucus glands, and blood vessels). Sensory feedback for these systems occurs via sensory fibers originating from sensory ganglia (nodose and jugular ganglionic neurons). These fibers innervate sensory receptors and transmit information from the airways to the CNS. They modulate the activity of the AVPNs through the central multisynaptic pathways and may affect function of effector organs via two ill-defined local networks that include axon reflex responses *(level 1)* and sensory innervation of intrinsic airway ganglia *(level 2)*.

brain stem from the rostral nucleus ambiguus (rNA) and to a lesser degree from the rostral portion of the dorsal motor nucleus of the vagus (DMV). These cholinergic cells (see Figure 84-1, *level 3*) that express vasoactive intestinal peptide (VIP)[9] and brain-derived neurotrophic factor,[10] transmit cholinergic signals to intrinsic tracheobronchial ganglia[11] (see Figure 84-1, *level 2*). The individual innervation of postganglionic neurons from the vagus nerve gives rise to postganglionic fibers that regulate functions of specific effector targets (see Figure 84-1, *level 1*). Varicose fibers from these postganglionic neurons are randomly distributed around airway smooth muscle cells, submucosal glands, and arterial blood vessels. In the postnatal period neuronal innervation is already well developed and choline acetyltranferase, a specific marker for cholinergic traits that synthesizes acetylcholine, appears in vagal preganglionic neurons, postganglionic neurons, and postganglionic fibers.

Signals transmitted through preganglionic nerves are relayed, filtered, integrated, and modulated by intrinsic ganglionic neurons before reaching their airway neuroeffector sites through postganglionic axons. This structural organization could explain the strong effects of a relatively small number of vagal efferent fibers on coordinated reflex changes in airway smooth muscle tone, submucosal gland secretion, and blood flow along the tracheobronchial tree. Each ganglion, located in proximity to its effector system, possesses a relatively large number of neurons that can be considered as an expanded parasympathetic, preganglionic efferent motor system. Postnatally, cholinergic innervation is the principal tonic input to airway smooth muscle bundles, and serves to protect the airways from collapsing during expiration-induced compressive narrowing.[12]

The postganglionic cholinergic innervation does not exclude the probability that some of the AVPNs also provide, to a lesser degree, direct innervation of airway smooth muscle bundles,[1] epithelial cells, and the alveolar interstitium of the lung, because ganglia are absent altogether from the bronchial subepithelial space and the most distal gas exchanging units.[7] These observations suggest that AVPNs may be involved in regulating the release of biologically active molecules from epithelial cells, that is, activation of nitric oxide synthase (NOS) and release of nitric oxide (NO), that might oppose excessive cholinergically mediated airway contractile responses at peripheral sites (see later). Furthermore, cholinergic mechanisms are involved in controlling the conductivity of the most distal airways[13] and tissue resistance[14] in early life by influencing smooth muscle tone, lung interstitial pericytes, and alveolar myofibroblasts.[15] However, it is not clear whether individual AVPNs provide parallel simultaneous innervation to airway smooth muscle, submucosal glands, and local blood vessels. Alternatively, it is also possible that groups of functionally selective AVPNs may exert a highly coordinated control over multiple airway functions by central mechanisms that synchronize their output to individual effectors. Presumptive gap junctions between identified AVPNs may serve to synchronize their electrical activity in the rNA of the brain stem. Alterations in central mechanisms that mediate such synchronization could cause regional differences in airway reflex responses, expressed as inhomogeneity in ventilation. For example, in induced bronchoconstriction some branches of the airway may totally close while others remain normal. Future studies should address the question of the central mechanisms and origin of synchronization and asynchrony of bronchoconstrictive reflex responses during development.

Acetylcholine, released from cholinergic nerve endings, may affect lower airway function via signaling through muscarinic and nicotinic acetylcholine receptors (nAChRs) (Figure 84-2).

Muscarinic Receptor Signaling

Muscarinic receptors mediate the responsiveness of airway smooth muscle, submucosal glands, and blood flow to acetylcholine during early development and adult life. Studies in the

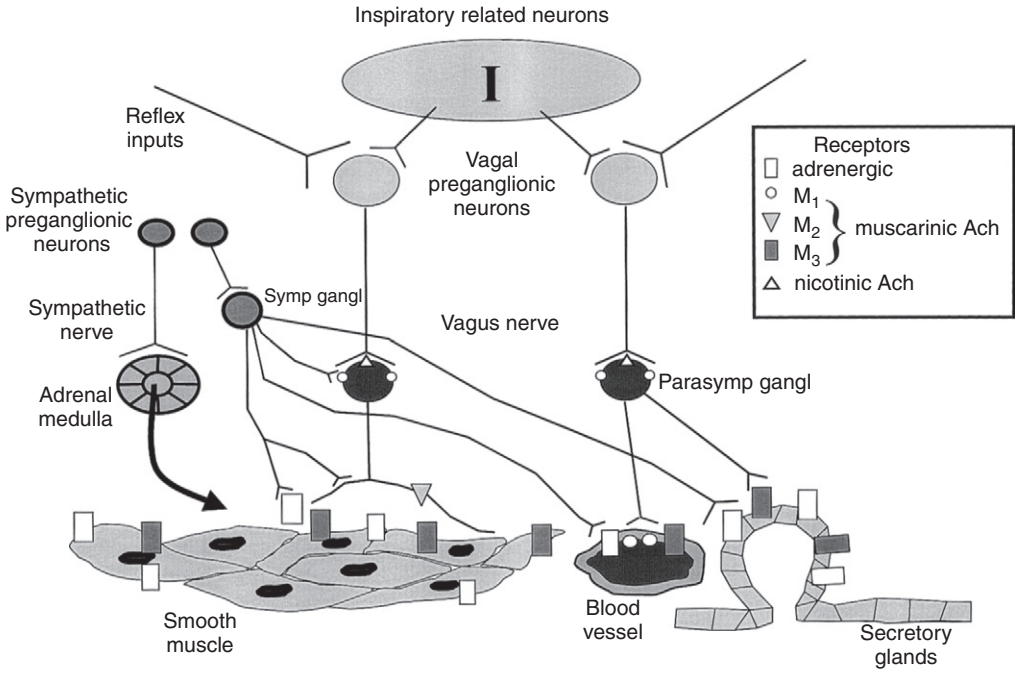


Figure 84-2 Schematic representation of the major parasympathetic, or cholinergic (contractile), and sympathetic, or adrenal adrenergic (relaxant), pathways that innervate (or regulate) airway smooth muscle. Efferent output from vagal preganglionic neurons to airways is influenced by various afferent reflex inputs, and inspiratory-related neurons (I) in the brain stem. Excitation of parasympathetic efferents also increases the output of submucosal secretory glands and results in relaxation of adjacent vascular structures. See text for the functional roles of the various muscarinic (cholinergic) receptor subtypes.

developing airways and porcine lung from birth to adulthood reveal maturational changes in muscarinic receptor subtypes (M_1, M_2, M_3) that may explain pharmacologic changes during development.[16] These muscarinic receptor subtypes, coupled to the family of G proteins, mediate airway contractile responses and their modulation, although there are considerable interspecies differences in their roles.[17] M_1 receptors are largely present on neuronal tissue and ganglia, and the selective M_1 receptor antagonist pirenzepine reduces the contractile response to vagal stimulation in newborn animals.[18,19]

M_2 receptors are located on prejunctional postganglionic cholinergic fibers in airway smooth muscle in some species, and exhibit an autoinhibitory action whereby the quantal release of ACh in response to nerve stimulation is reduced due to feedback inhibition.[20] Sinus node M_2 receptors also mediate the bradycardia seen with vagal stimulation. Selective blockade, or down-regulation, of M_2 receptors may enhance vagally mediated bronchoconstrictor responses and cause a reduction in the bradycardia response. M_2 autoinhibitory actions may be reduced or absent in the newborn because blockade of M_2 receptors does not enhance bronchoconstrictor responses to vagal stimulation.[19] The potential role of lung injury and infection in modulating physiologic responses coupled to M_2 receptors in the newborn remains to be explored.

M_3 receptors are present on smooth muscle and mucus glands and airway epithelial cells, where they initiate the events leading to smooth muscle contraction, airway narrowing, and mucus secretion.[21,22] In the newborn, the density of M_3 receptors has been reported to be similar to that in the adult; however, they do not appear to be tightly coupled to G-protein signal transduction mechanisms that lead to smooth muscle contraction.[18] In vivo studies show that M_3 receptor antagonism decreases bronchoconstrictor responses to vagal stimulation in the newborn that, at low dosages, does not affect the bradycardic response.[19] Among the many unanswered questions in the newborn are the extent of receptor subtype differentiation in human neonates, investigation of G-protein signal transduction, the role of M_2 autoinhibitory receptors in modulating airway tone, and the possible effect of inflammatory lung disease on muscarinic receptor regulation and its pharmacologic manipulation.

Nicotine Receptor Signaling

nAChRs are ligand-gated ion channels formed by five homologous subunits that are involved in processes including signal transduction, proliferation, and apoptosis. The developmental role of these receptors and their role in pathology of lower airways during development, however, is unclear. Nicotine AChRs are composed of 12 subunits that play the important role in mediating ganglionic transmission to their natural ligand acetylcholine, or transmitting effects of nicotine and nicotine-like substances. During ontogeny, nonneuronal and neuronal nAChRs undergo changes in distribution and subunit composition. Nonneuronal expression of nAChR subunits may precede functional innervation and then could undergo changes in distribution on the surface of cells. Airway-related vagal preganglionic neurons express multiple nAChRs subunits.[23] The β4 subunits are also abundantly expressed by ganglionic cells, forming acetylcholine-binding sites with α3 or α4, α5, or α7 subunits as pentameric receptors. In the lungs, nAChRs are found in a variety of nonneuronal locations, such as respiratory epithelial, mesenchymal, and collagen-producing cells. Maternal nicotine exposure up-regulates collagen gene expression that may explain the observed alterations in lung mechanics in the infants of mothers who smoke during pregnancy.[24] In these infants postnatal exposure to environmental smoke may exacerbate pediatric respiratory disease and possibly[24,25] predispose infants to sudden infant death syndrome (SIDS).

Vagal Afferent Sensory Fibers Innervating the Lower Airways

The bronchopulmonary sensory receptors are specialized units for detecting changes in chemical, mechanical, or thermal stimuli. They originate from the bipolar airway vagal afferent neurons that are located in the nodose and jugular ganglia and participate in reflex events. Sensory fibers affect function of lower airway effector units via a local network that includes axon reflex responses. Endogenous substance P released from sensory nerve endings (i.e., C fibers that are mainly nonmyelinated nerve terminals), facilitates synaptic transmission in airway postganglionic neurons,[26] suggesting the presence of control mechanisms within the tracheobronchial system, that also reflexly influence activity of AVPNs. Furthermore, afferent nerve endings (i.e., C fibers) are also believed to be responsible for mediating local axon reflexes and neurogenic inflammation via release of neuropeptides such as substance P,[27] other members of the neurokinin family, and activation of neurokinin receptors (i.e., NK-1, NK-2, NK-3R) or calcitonin gene-related peptide (CGRP).

Vagal Afferent Sensory Fibers Controlling Discharge of AVPNs

The central portion of sensory neural fibers enters the brainstem through the solitary tract and make synapses with the second-order nucleus tractus solitarius (NTS) neurons that project to the AVPNs. Excitatory signals arise from bronchopulmonary rapidly adapting lightly myelinated receptor afferents or nonmyelinated C-fiber terminals via release of glutamate that activates AMPA receptors expressed by these NTS neurons.[28] Processed information is then transmitted to AVPNs via the glutamate-AMPA signaling pathway, and from the AVPNs to the bronchopulmonary effector system through acetylcholine release. The strength of bronchoconstrictive reflex responses can be increased by stimulation of peripheral chemoreceptors, but attenuated by multiple peripheral inputs, such as those from myelinated pulmonary stretch receptors, somatosensory afferents, and baroreceptors. Furthermore, central nervous system (CNS) cell groups that project to the AVPNs, such as GABAergic, serotonergic, and catecholaminergic pathways, inhibit cholinergic outflow to the airway, via hyperpolarization of AVPNs.[28] Catecholaminergic pathways also affect airway smooth muscle tone, mainly via adrenergic receptor subtype expressed by airway effector cells at prejunctinal or postjuctional levels.

Sympathetic Control of Lower Airways

Extrinsic sympathetic innervation of airway smooth muscle is highly species specific, and in airway smooth muscle in humans, direct sympathetic innervation appears to be lacking (Figure 84-3). Nevertheless, circulating catecholamines activate airway adrenoreceptors to exert specific actions that affect smooth muscle contractile function.[29,30] β-Adrenergic responses in airway smooth muscle are composed of two inhibitory actions: first, relaxation of airway smooth muscle mediated by airway β_2-receptors that are coupled to the stimulatory G-protein (Gs) and adenylate cyclase; and second, inhibition of ACh release from postganglionic vagal axons through prejunctional α_2-adrenergic and β_1 receptors in some species.[31] Activation of β-adrenergic receptors is the pharmacologic basis for neonatal bronchodilator therapy. Maturational studies have demonstrated that β-adrenergic receptors in lung tissue increase with advancing gestation and subsequent postnatal development, but this may be more important for their role in surfactant synthesis and release.[32,33] The airway relaxant response to β-adrenoreceptor stimulation actually appears to decrease with advancing maturation, and several mechanisms including greater muscarinic antagonism of

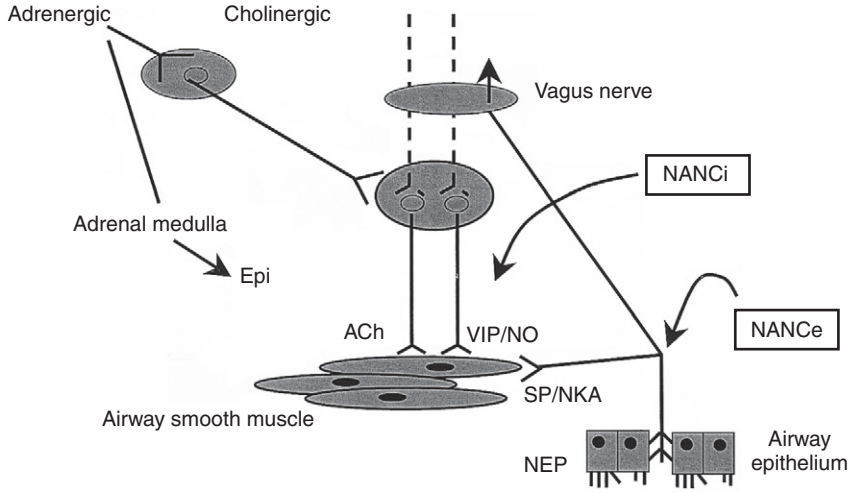


Figure 84-3 Representation of the nonadrenergic noncholinergic (NANC) innervation of airway smooth muscle and related epithelium that complements traditional cholinergic and adrenergic pathways. The inhibitory component of this system (NANCi) appears to be mediated primarily by release of nitric oxide (NO) and vasoactive intestinal peptide (VIP). NANC excitatory mechanisms (NANCe) are initiated by release of neuropeptides such as substance P (SP) and neurokinin A (NKA) from C-fiber afferent nerve endings, and this is modulated by the neuropeptide degrading enzyme, neutral endopeptidase (NEP), in epithelial cells.

β-receptor responses and attenuated expression of M_2 muscarinic receptors have been proposed.[34-36]

The second category of adrenergic responses is attributed to α-adrenoceptors, of which both α_1- and α_2-subtypes play a role. Available data indicate that in adult humans α-adrenergic contractile responses of airway smooth muscle are weak or absent, although this may not hold true for the newborn. A potential role for α-adrenergic receptors in the control of airway smooth muscle in newborn infants with bronchopulmonary dysplasia is supported by observations in preterm infants with chronic lung disease in whom ophthalmic application of the α_1-adrenergic agonist phenylephrine resulted in an increase in total pulmonary resistance and a decrease in compliance.[37] The deterioration in lung mechanics was attributed to α_1-receptor–mediated contraction of airway smooth muscle. Furthermore, adrenergic agonists having mixed α- and β-receptor actions cause airway smooth muscle contraction in newborn puppies,[38,39] and both α_1- and α_2-adrenoreceptors appear to be involved in mediating the response. Neonatal porcine tracheal smooth muscle does not display a contractile response to norepinephrine,[40] a finding similar to that of the adult.[41] Nevertheless, infusion of norepinephrine in newborn pigs increases lung resistance reminiscent of the adult response. In the piglet the contractile effects of norepinephrine are evident in parenchymal tissue, suggesting that circulating catecholamines may exert an action on gas exchange, at least in this species.[40] The implications of the mentioned studies for human infants remain to be seen; however, they may be part of a previously unappreciated component of the pulmonary response to the surge of systemically released catecholamine at parturition.[42] α_2-Adrenergic receptors are present in preganglionic and postganglionic nerve terminals as well as nonmyelinated C-fibers. Their activation inhibits release of ACh and substance P (SP), implicating multiple neurotransmitter systems.[43]

Nonadrenergic Noncholinergic Control of the Lower Airways

Rather than representing a separate pathway for modulation of airway caliber, the nonadrenergic noncholinergic (NANC) system comprises both inhibitory and excitatory mechanisms modulated by several neurotransmitters located in intrinsic ganglia and their fibers (see Figure 84-3). Reference to noncholinergic (i.e., NANC) innervation reflects the neurotransmitters known *not* to mediate its effects. The system is highly species specific, but has been identified in human airways. The NANC inhibitory component of vagal innervation[44] is demonstrated in vivo and in vitro by measuring the response to vagal stimulation or to electrical field stimulation, after muscarinic and β-adrenergic effects on airway smooth muscle are blocked by atropine and propranolol; then airway tone is elevated by an infusion of contractile agonist (e.g., serotonin or histamine).[29] Under these conditions stimulation of vagal preganglionic axons causes bronchodilation. Vasoactive intestinal peptide was initially proposed as the primary neurotransmitter of the NANC system. Mice lacking the VIP gene show airway hyperresponsiveness and airway inflammation, partially reversible by VIP.[45] Subsequently, NO was also shown to act as a NANC neurotransmitter, suggesting involvement of multiple substances and molecules in airway smooth muscle relaxation with considerable interspecies variation[46] (Figure 84-4).

Limited information is available about the ontogeny of this system in the airways. Activation of vagal preganglionic axons results in a NANC-mediated bronchodilation in newborn feline airways, a response that is eliminated by ganglionic blockade with hexamethonium, confirming the efferent nature of the response.[47] Comparison with the mature response is confounded by the necessity of infusing an agonist to measure a response, and the possibility that neonatal and adult airways may possess different sensitivities to the contractile agonist. NANC inhibitory innervation also appears to be functional in young guinea pigs and rat pups.[48] In some species NANC inhibitory responses undergo significant developmental changes; for example, NANC relaxation responses are not present in rabbits until 2 weeks of age.[49] Interestingly, allergen sensitization significantly reduced the NANC response at 2, 4, and 12 weeks of age,[49] suggesting that host or environmental factors may alter the maturation of the inhibitory NANC system and predispose to airway reactivity. The NANC inhibitory system has not been explored in human neonatal airways; data on other airway neurotransmitters suggest that it might be expected to be present.[50]

NO has received considerable attention for its ability to reverse persistent pulmonary hypertension of the newborn, and this has largely overshadowed investigation of its role in control

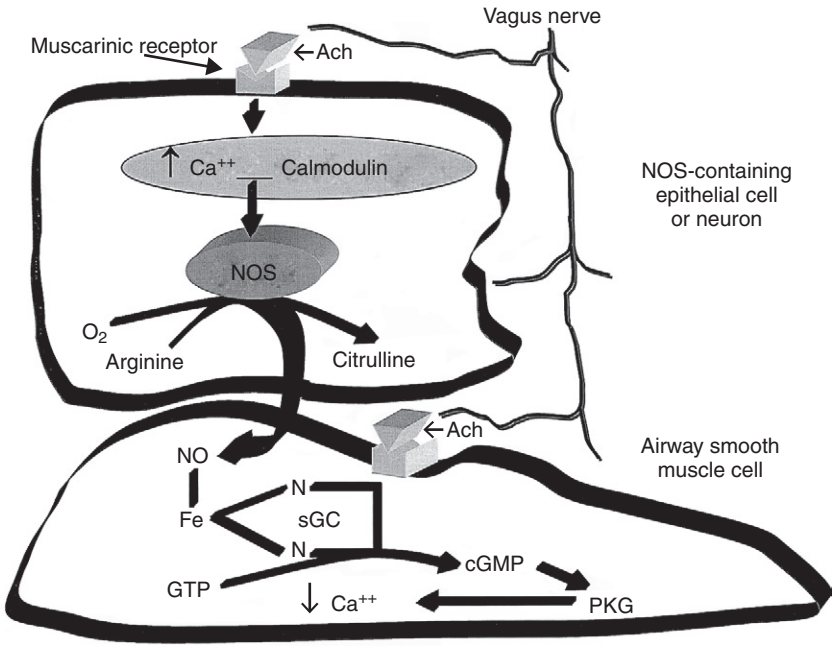


Figure 84-4 Diagrammatic representation of the nitric oxide cyclic guanosine monophosphate (NO-cGMP) pathway that mediates airway smooth muscle relaxation. NO synthase (NOS)–containing epithelial cells or neurons (as well as airway smooth muscle cells) are innervated by cholinergic fibers and express muscarinic receptors. Activation of these NOS-containing cells results in production of NO (from its L-arginine substrate), which diffuses into the smooth muscle layer and interacts with the heme moiety of soluble guanylyl cyclase (sGC), leading to intracellular accumulation of cGMP. This causes activation of protein kinase G (PKG), which in turn causes a decrease in inositol 1,4,5-triphosphate ($InsP_3$)–stimulated elevation of intracellular Ca^{2+} and consequent smooth muscle relaxation.

of airway smooth muscle. Nevertheless, some evidence indicates that expression of neuronal NO synthase is developmentally regulated, declining during late gestation, but subsequently increasing postnatally in the rat.[51] In developing sheep lungs, all three isoforms of NO synthase are expressed in airway epithelium and so might be expected to modulate underlying contractile responses.[52] It has been shown that release of endogenous NO from airway epithelium opposes cholinergically mediated contraction of piglet tracheal smooth muscle.[22] This phenomenon decreases with advancing postnatal age, requires an intact epithelium, and correlates with a relative up-regulation of M_3 muscarinic receptors in airway epithelium of the youngest animals. In the newborn piglet NO inhalation causes a modest decrease in total lung resistance with decreases in both airway and tissue (lung parenchymal) resistance.[53] In the same animal model, inhibition of NO synthase increases tissue more than airway resistance, suggesting a role for endogenous NO in relaxing primarily distal (parenchymal) areas of lung tissue.[54] Thus the NANC inhibitory system and NO may play a significant role in modulating airway and lung function in the neonate, and this may be disturbed in response to inflammatory airway disease.

NANC excitatory mechanisms also play a role in modulating airway smooth muscle. Within this system the tachykinin peptides, such as substance P and neurokinin A, have undergone some study during early postnatal development. As already indicated, these tachykinins are synthesized in sensory neurons and transported to sensory nerve endings from which they are released and have the ability to elicit airway contractile responses by several interrelated mechanisms. Tachykinin release from C-fiber nerve endings may directly or reflexly elicit smooth muscle contraction, modulate cholinergic responses through muscarinic receptors, and induce histamine release from mast cells. In young rabbits, substance P–induced modulation of ACh release increases with advancing postnatal age.[55] In newborn piglets, exogenously administered substance P elicits weak contractile responses of tracheal smooth muscle when compared with older animals.[56] This weak smooth muscle contractile response elicited by the NK_1-receptor agonist substance P in newborn pigs is associated with an adult complement of NK_1 receptors in trachea and lung, but with immature G protein coupling,[57] as observed for muscarinic receptors in the piglet airway.[18]

There is some debate about whether increased expression of substance P or other neuropeptides contributes to lung or airway pathophysiology. In mature animal models, chronic exposure to irritant gas increases substance P content; however, it is controversial whether this serves to aggravate airway hyperreactivity or serves a protective role for airway and lung structures.[58,59] Furthermore, in addition to eliciting airway smooth muscle constriction, substance P may induce relaxation of preconstricted neonatal tracheal tissue by release of NO and relaxant prostaglandins.[60] Newborn and 3-week-old rats exposed to hyperoxia exhibit increased tachykinin precursor expression, increased substance P content in the lung, and increased cholinergic responsiveness, although the relationship of the latter to the increased substance P content is not clear.[61] Ongoing studies focus on the signaling pathways that modulate airway smooth muscle relaxant responses through NO- and prostaglandin-mediated mechanisms in health and disease.

Carbon monoxide (CO), like NO, a gaseous molecule, is formed in neuronal and nonneuronal cells through enzymatic degradation of heme by the constitutive isoform heme oxygenase 2 (HO-2). This molecule mediates its effects by binding to the heme moiety of the soluble guanylate cyclase,[62] inducing formation of the second messenger cyclic guanosine monophosphate (cGMP) and airway smooth muscle relaxation. Further studies are needed to determine the role of interaction between CO and other molecules, such as VIP that may mediate NANC inhibitory influences in the developing lung and during lung injury.[63,64]

MATURATIONAL CHANGES IN AIRWAY PHYSIOLOGIC RESPONSES

Elegant immunohistochemical studies of developing human and porcine fetal airways have been performed from as early as the first trimester.[1] These have revealed the development of an airway smooth layer by the end of the human embryonic period extending from the trachea to terminal lung sacs, as well as an extensive nerve plexus comprising nerve trunks and ganglia investing the airways and innervating smooth muscle. This layer of airway smooth muscle is functional in the first trimester as evidenced by phasic spontaneous narrowing and relaxation of airways with back-and-forth movement of lung fluid.[1,65] Phasic or tonic activity in airway smooth muscle might stimulate lung growth by providing positive intraluminal pressure. These data are consistent with human autopsy findings that airway smooth muscle is present at 23 weeks gestation at all levels of the conducting airways and increased in amount during the earliest signs of developing chronic lung disease, as early as 10 days of birth.[66]

Despite clear evidence of an intact airway smooth muscle layer early in gestation, the effect of postnatal maturation on airway contractile responses is somewhat controversial.[67] Studies using methacholine (cholinergic) challenge have demonstrated greater airway narrowing during bronchoconstriction in immature animals largely attributable to greater airway compliance or distensibility in the walls of distal airways.[68] This was associated with less cartilage and proportionately more muscle in the airway wall of immature versus mature rabbits.[69,70] In contrast, physiologic studies using isolated tracheal smooth muscle strips from several species have demonstrated decreased cholinergic responsiveness in early postnatal life.[18,71,72] These in vitro studies are complicated by the need to carefully normalize the airway contractile response for smooth muscle mass and myosin content.[73] Nonetheless, the weight of evidence appears to point to an anatomically intact, but functionally diminished, airway smooth muscle layer superimposed on highly compliant airway structures in early postnatal life.

AIRWAY FUNCTION IN NEONATAL LUNG INJURY

Positive pressure ventilation and mechanical strain exert structural and functional changes on airway smooth muscle over a wide range of developmental ages. It appears that vagally mediated bronchoconstrictor tone is minimal under baseline conditions in the newborn period.[74] Furthermore, the high deformability or compliance of the trachea in the preterm period appears to be a consequence of decreased airway smooth muscle contractility[75] and diminished cartilaginous support.[69,76] The obvious result is that tracheas, and possibly lower airways, are vulnerable to deformation during positive pressure ventilation. Greater understanding of the detrimental effects of positive pressure ventilation at high inflating pressures has decreased the risk of deformation injury to the immature airway. However, increased airway reactivity and wheezing disorders in infants and children remain a major problem in the preterm survivors of neonatal intensive care.[77,78] The contribution of neonatal assisted ventilation to this problem is unclear. However, recent data in rat pups indicate that enhancement of airway reactivity occurs even after short-term mechanical ventilation.[79] Abnormal mechanical stress and its effect on smooth muscle contractility may serve as a useful model for characterizing one aspect of neonatal lung and airway injury.[80-82] Cultured airway smooth muscle cells exposed to such stress eliminate the confounding effects of deformational strain on surrounding tissues. Such studies have demonstrated strain-induced increases in cell myosin light-chain kinase (MLCK) accompanied by increased phosphorylation of the myosin light chain, all key steps in the smooth muscle contractile response.[83]

Exposure of the immature airway to increased supplemental oxygen is common in neonatal practice and may predispose preterm infants to the development of airway injury, especially in the presence of assisted ventilation. In newborn animal models, most notably the guinea pig and rat pup, hyperoxic exposure has been associated with development of airway hyperreactivity.[61,84,85] Although hyperoxic exposure may increase smooth muscle area, this effect is variable and does not, of itself, explain the development of hyperoxia-induced airway hyperresponsiveness.[86,87]

Epithelial injury with loss of airway relaxant factors may contribute to the hyperoxia-induced increase in airway contractile responses. This is supported by data from tracheal strips in preterm sheep, in which epithelium removal was associated with greater cholinergic responsiveness.[88] A similar phenomenon is observed in rat pups in whom the response of lung resistance induced by vagal stimulation was increased after nonspecific blockade of NOS in normoxic animals. However, after hyperoxic exposure NOS blockade no longer affected the contractile response induced by vagal stimulation.[89] These findings indicate that NO, released by stimulation of vagal preganglionic fibers, modulates bronchopulmonary contractile responses to endogenously released ACh in rat pups. This effect appears to be lost after prolonged hyperoxic exposure and may contribute to airway hyperreactivity under these conditions. Impairment of the prostaglandin/cyclic adenosine monophosphate (cAMP) signaling pathway may also contribute hyperoxia-induced airway hyperreactivity.[90] These findings are somewhat analogous to observations made in young ferrets infected with human respiratory syncytial virus (RSV) in whom nonadrenergic noncholinergic inhibitory responses of tracheal smooth muscle were significantly decreased for a prolonged period.[91] Data suggest that hyperoxic conditioning during early postnatal life impairs relaxation by impairing myosin light-chain (LC20) dephosphorylation.[92]

There is considerable interest in the potential of inhaled NO to decrease neonatal lung injury or bronchopulmonary dysplasia in preterm infants.[93,94] The underlying mechanism for this beneficial effect of prolonged inhaled NO in some clinical trials remains unclear. However, data from animal studies employing lamb and baboon models of neonatal lung injury implicate decreased airway smooth muscle mass and improved lung resistance in NO-treated animals.[95,96] Inhaled NO therapy may therefore reverse airway and lung parenchymal structural changes associated with depletion of endogenous NO in neonatal lung injury. Potential long-term benefit from such therapy on airway function is being explored.[97]

Neurotrophins and Lung Injury

The neurotrophins belong to a protein family essential for vertebrate nervous system development. They are also expressed in nonneuronal tissues that are under neuronal control. The neurotrophin family includes brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3, and neurotrophin 4. They share common structural features and act through their corresponding high-affinity tyrosine kinase (Trk) receptor subtypes.[98] The rapid excitatory activity of BDNF is via activation of the TrkB receptor. BDNF appears to stabilize excitatory (e.g., cholinergic) postsynaptic receptors, and/or enhance quantal excitatory neurotransmitter release probably via postsynaptic Ca^{++} signaling.[10,99]

During development, regulation of the lower airway appears to be influenced by neurotrophins. Recent data show the presence of neurotrophins and their corresponding receptor on airway smooth muscle cells in developing rat pups and up-regulation of the BDNF system after hyperoxic exposure.[100,101] This is consistent with observations from adult human tissue in which BDNF immunoreactivity was intense in bronchial epithelium,

although neurotrophin immunostaining was seen in bronchial smooth muscle, and TrkB (BDNF high affinity receptor) immunoreactivity was strongly expressed in bronchial smooth muscle.[102] Furthermore, BDNF, acting via TrkB receptors, facilitates responses of airway smooth muscle to different contractile stimuli, including ACh. The precise role of BDNF in up-regulating contractile responses of immature airways and respiratory structures remains to be determined.[103] Consistent with these findings, neurotrophins are overexpressed in the lower airways of RSV-infected human infants known to be at great risk for increased airway reactivity.[104]

CONCLUSIONS AND FUTURE STUDIES

The degree and nature of the relationship between different pathways regulating lung homeostasis during development are imperfectly known, a fact that should elevate the enthusiasm for future studies. For example, investigation of the molecular network and signaling pathways, that lead ultimately to hyperresponsiveness of AVPNs, will provide a better understanding of the mechanisms through which lung injury sets the stage for airway hyperreactivity. Another example of our limited knowledge relates to the biologic basis of a link between neurotransmitter specific networks involved in state and arousal and the regulation of airway function during early development. Progress made in our understanding of the control of the airways will contribute to the construction of a more complete conceptual framework that will provide deeper insight into the pathophysiology of airway disorders, and allow us to identify effective new treatment of neonates and infants with airway dysfunctions.

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85 Functional Development of Respiratory Muscles

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This chapter provides a snapshot of fetal and neonatal development of the diaphragm muscle (DIAm), the major inspiratory muscle in mammals located between the thoracic and abdominal cavities. The DIAm appears rather late in evolution, being present only in mammals, whereas other vertebrates use different means of ventilation. The embryologic origin of the DIAm has been elucidated by recent studies using molecular markers of muscle development, reflecting a common origin to the DIAm in the pleuroperitoneal fold. The traditional view of DIAm development called for multiple sites of origin reflecting a complex derivation, consistent with its multiple anatomical origin and insertion sites. The mechanical actions of the DIAm reflect its major function is in inspiration. The DIAm is also involved in several nonventilatory motor behaviors such as coughing, defecation, emesis, micturition, parturition, sneezing, vocalization and weightlifting.

Neural control of the DIAm is similar to neural control of other skeletal muscles and is based on recruitment and frequency coding of motor units. Motor units in the adult DIAm, each comprising a motoneuron and the muscle fibers it innervates, vary considerably in their mechanical, histochemical, and biochemical properties,[1-3] and this heterogeneity provides the range of control of muscle force generation that occurs during different motor behaviors. It is the cumulative contractile and fatigue properties of the motor unit pool that determine the constraints under which the DIAm responds to the various mechanical demands placed on it during different ventilatory and nonventilatory behaviors. Clearly these motor demands can change during development, and the DIAm must adapt or remodel to accommodate these changing demands.

The DIAm becomes rhythmically active during late fetal development (fetal respiratory movements), and at birth it must be ready to sustain ventilation. Postnatally, the DIAm is one of the most active skeletal muscles, with a duty cycle (time active versus relaxed) of approximately 30% to 40%, compared to limb muscles (duty cycles ranging from 2% to 15%). It is not surprising therefore that fetal and neonatal pathology of the DIAm often lead to ventilatory failure and premature death. Despite the vital importance of the DIAm, previous studies have provided mostly descriptive information about its development and growth. However, there is accumulating information about myogenesis and neural development that derives from in vitro model systems, which may be applicable to the mechanisms regulating DIAm development in vivo. Yet, the applicability of these in vitro results regarding the basis of myogenesis and neural development remains to be established, especially in the context of maturation of other systems, for example, the central nervous system, the lung, the thoracic, and abdominal walls. Unfortunately such integrative information is lacking.

Rodent models are increasingly being used to explore the genetic basis for developmental plasticity in neuromotor control. Accordingly, chapter focuses on recent results obtained in rats and mice. Benchmarks for the development of the mouse DIAm are summarized in Figure 85-1. The fetal and neonatal developmental benchmarks in rats are generally comparable to those in mice (offset by approximately 2 days). In mice, developmental benchmarks for the DIAm begin at approximately embryonic day 11 or 12 (E11, E12), when the phrenic nerve makes initial contact with the primordial DIAm. In both rats and mice, the final pattern of adult motor units and muscle fiber types is not achieved until postnatal day 28 (D28). During this 5- to 6-week span, dramatic changes occur in DIAm innervation, contractile protein expression, and function. The timeline for such developmental benchmarks in humans is far more protracted, but it is likely that similar convergence of neural and myogenic events also occurs. Therefore much can be learned by studying the integrated aspects of myogenesis and neural development of the DIAm in rodent models.

Embryologic Derivation and Innervation of the Diaphragm Muscle

Myogenesis

Skeletal muscle fibers, including those in the DIAm, are formed in two stages. The first stage is commitment (also known as determination), when mesodermal progenitor cells are transformed to myoblasts. The second stage is terminal differentiation, when myoblasts fuse to form myotubes and myofibers (Figure 85-2). Myogenesis may proceed via an intrinsic genetic program, or it may require either the presence of positive extrinsic signals or the removal of inhibitory signals.[4] None of these potential mechanisms can be presently excluded. However, there is converging evidence that myogenesis is induced in a variety of nonmuscle cells by the expression of proteins belonging to the basic helix-loop-helix (bHLH) superfamily.[5,6] Collectively, these are known as muscle regulatory factors (MRFs) and include MyoD,[7] myogenin,[8] Myf-5,[9] and MRF4.[10] In human muscle, MRF4 is also known as Myf-6.[11] In addition to providing a molecular mechanism for the regulation of myogenesis in fetal and neonatal muscle, MRFs are also involved in determining the expression of myosin heavy chain (MHC) isoforms in adult muscle.[12]

Genetic determinants of myoblast commitment have been most extensively studied in *Drosophila*, but this information is likely to be directly applicable to myoblast commitment in the mammalian DIAm as well. In *Drosophila* it has been shown that neural-derived proteins (for example, sonic hedgehog, Wnt 1, 3, and 4) promote commitment to myoblasts within a myotome.[4,13] Expression of sonic hedgehog (or other signals) commits mesodermal cells to form myoblasts possibly by releasing suppression of the expression of specific MRFs.[14] The process of myoblast commitment is marked by the expression of the paired-box protein Pax-3, which appears to positively regulate MRF expression. For example, it has been shown that ectopic expression of Pax-3 induces both MyoD and Myf-5 in embryonic tissue.[15] The consequent increase in MRFs then initiates a cascade of signals that continues through differentiation, thus sustaining myogenesis. Indeed, transplantation of Pax-3–expressing embryonic

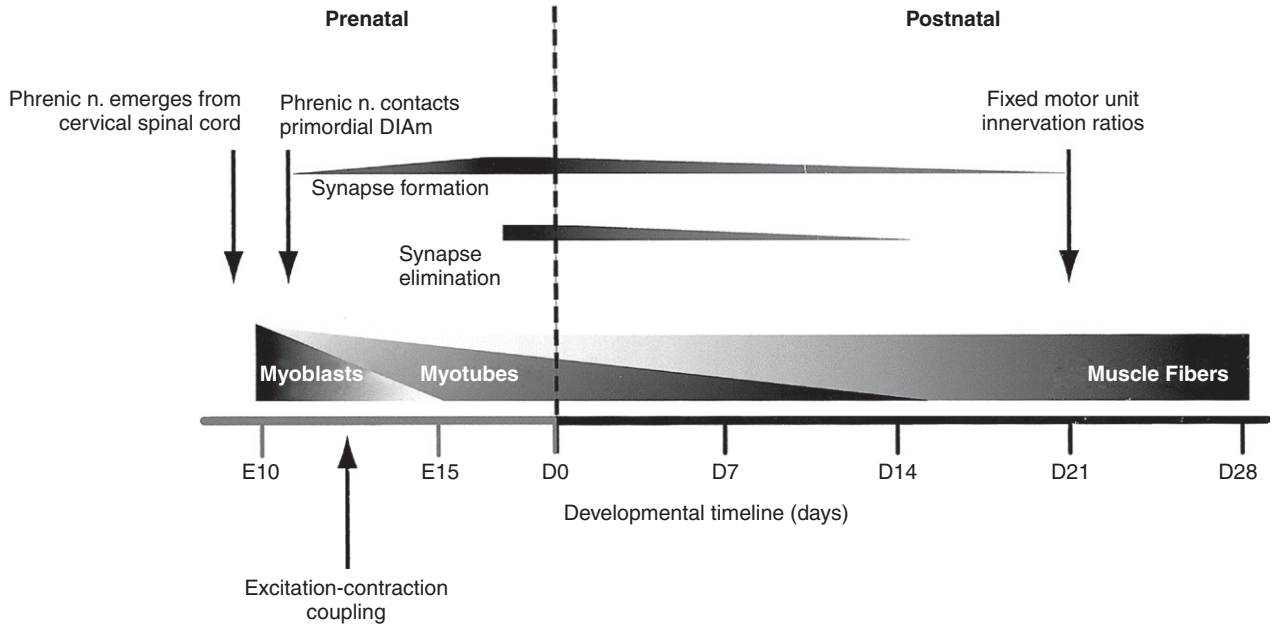


Figure 85-1 Timeline for major events in the development of mouse DIAm. Similar seminal events occur in the embryonic and early postnatal development of the DIAm in other mammals including humans, although temporal characteristics and interrelationships may vary.

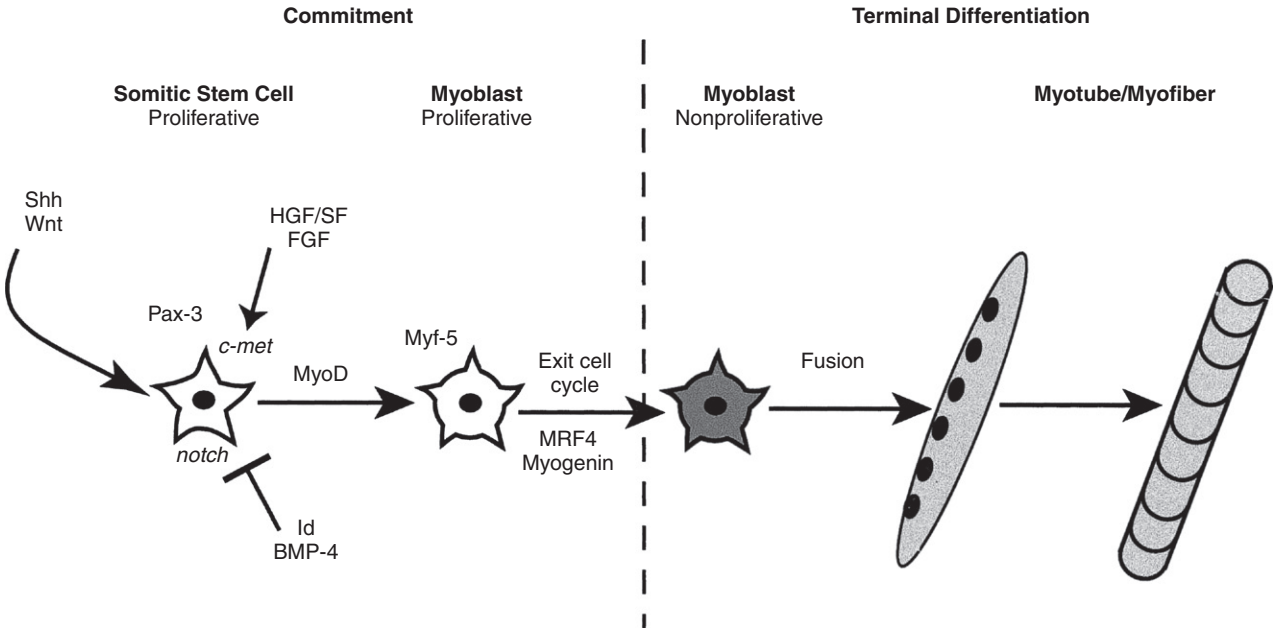


Figure 85-2 The two major stages of myogenesis—commitment and terminal differentiation. During commitment, somitic stem cells proliferate and are committed to myoblasts, a process marked by MyoD and Myf-5 expression. This process ends when proliferating myoblasts exit the cell cycle, influenced by expression of MRF4 and myogenin. The exit of myoblasts from the cell cycle marks the beginning of the differentiation stage. Thereafter, nonproliferative myoblasts fuse to form myotubes, and subsequently, myotubes become myofibers once distinct sarcomeres are present. The differentiation process continues until adult fiber types are established.

stem cells into dystrophic mice resulted in engraftment of stem cell precursors with adult myofibers and enhanced contractile function.[16]

The MRFs function as molecular switches, initiating myogenesis by inducing expression of muscle-specific genes through binding of the bHLH motif to *cis*-acting DNA control elements of muscle-specific genes, known as E-boxes.[17] The E-box is a CANNTG sequence-containing motif present in the promoter regions of many skeletal muscle specific genes.[18] Transcriptional regulation mediated by MRFs may also involve interactions of MRFs with the family of MADS-box proteins, termed myocyte enhancer factor 2 (MEF2A-D), which may determine muscle specificity for genes lacking MRF binding domains. Binding sites for MEF2 factors are located in the promoter regions of many

muscle-specific genes including myogenin. However, MEF2 binding alone is not sufficient to induce myogenesis.

It is also possible that myoblast commitment is determined by release from inhibition by signals that actively suppress MRF expression. For example, a class of HLH proteins termed Id factors (Id1-Id4) inhibit MRF activity by forming heterodimers with E2 products (ubiquitously expressed HLH-containing proteins encoded for by E2 genes). These products prevent dimerization with MRFs and thus block subsequent activation of skeletal muscle specific genes.[19] The Id proteins lack the basic DNA binding domain; therefore heterodimerization of bHLH proteins with Id proteins prevents the strong binding to DNA by either E proteins or MRFs.[20] Twist is another protein that in mice sequesters E-proteins inhibiting MRF activity by preventing the formation of MRF-E protein heterodimers.[21] Yet another inhibitory factor is Mist1, which binds with MyoD to form an inactive heterodimer.[22] The balance between inhibitory and promyogenic signals likely determines the timing and location of muscle precursor development. Recently, calcineurin was shown to activate MyoD indirectly by decreasing the expression of the Id-inhibitory proteins.[23] Myogenic inhibitory signals may also be critical to prevent ectopic formation of skeletal muscle since mesodermal somite cells can follow both myogenic and nonmyogenic programs.

In hypaxial muscles (for example, limb muscles, chest and abdominal wall muscles, and DIAm) MyoD is the first MRF to be expressed. MyoD expression then initiates a cascade resulting in subsequent expression of Myf-5, MRF4, and myogenin.[24] The sequence of MRF expression appears to be critical for the development of normal muscle. MyoD and Myf-5 are highly expressed in proliferating myoblasts, suggesting a primary role for these MRFs during this stage of myogenesis.[7] In mutant mice lacking MyoD or MRF4,[25,26] there is no apparent effect on normal skeletal muscle development, but Myf-5 and myogenin are upregulated, respectively. Therefore it is possible that redundant MRF expression may rescue normal skeletal muscle phenotype in these animals. Similarly, Myf-5–deficient mice appear to have normal skeletal muscle, but they succumb to asphyxiation soon after birth due to rib cage abnormalities.[27] In contrast, myogenin-deficient mice have only myoblasts without the development of myotubes/myofibers,[28,29] suggesting that myogenin is indispensable for differentiation of myotubes/myofibers. In mice lacking both Myf-5 and MyoD, no myoblasts are present suggesting that expression of both of these MRFs is required for myoblast commitment.[30] Mice lacking Myf-5 and MRF4 resemble Myf-5 knockouts, suggesting that MRF4 regulates later aspects of myogenesis.

With terminal differentiation, committed mononucleated myoblasts are nonreversibly transformed into multinucleated myotubes/myofibers with expression of contractile proteins (see Figure 85-2). Thus terminal differentiation represents the irreversible exit of proliferating myoblasts from the cell cycle. Yet, not all myoblasts lose their ability to proliferate because a pool of myoblasts (satellite cells) persists into adulthood, and their proliferative capacity is important in processes of injury and repair as well as other conditions of muscle remodeling. Throughout life, proliferating myoblasts are susceptible to apoptosis (programmed cell death) until they are terminally differentiated and exit the cell cycle. The balance between proliferation and apoptosis may play an important role in controlling the total number of muscle fibers of a given type.[31] Terminal differentiation of myoblasts in hypaxial muscles does not occur in vivo until they have migrated from the myotome to their final location (thoracic or abdominal walls or the limbs). As myoblasts migrate from the lateral dermomyotome, they express Myf-5 and *c-met* (a tyrosine kinase receptor for hepatocyte growth factor/scatter factor), which thus can be used as markers for this process of differentiation. In hypaxial muscle precursors, including those for the DIAm, expression of Myf-5 and *c-met* begins at approximately E10 and continues through E12.[32] Expression of MyoD is also important in the process of terminal differentiation and can be inhibited in vitro by bone morphogenic protein-4 or fibroblast growth factor-5,[14] both of which have been implicated in maintenance of the myoblast proliferative capacity and inhibition of differentiation.

Following terminal differentiation, myoblasts fuse, forming multinucleated myotubes/myofibers. Although the formation of myotubes/myofibers is coincident with the initial appearance of innervation in hypaxial muscles, converging evidence from in vitro and in vivo models indicates that terminal differentiation can be initiated in the absence of neural influence. Myotubes/myofibers can form in vitro in the absence of innervation, but at a much slower pace compared to that observed in vivo. This suggests that neural influence may facilitate the normal process of terminal differentiation and formation of myotubes/myofibers. This potential facilitating influence of innervation needs to be further explored. There is evidence, based on in vitro model systems, that terminal differentiation and myotube/myofiber formation depend on the expression of several nonneural proteins. This includes those present in the extracellular matrix (for example, fibronectins and laminins); basal lamina (for example, muscle cell adhesion molecule [M-CAM], neural cell adhesion molecule [N-CAM], and M-cadherin); cell membrane (for example, β1-integrin); and cytoskeleton (for example, actin and desmin).[33,34] Obviously, terminal differentiation and myotube/myofiber formation involves very complex interactions between differentiating cells and their surrounding environment. Current research is only beginning to unravel these complex interactions. It should be noted that even though the process of myoblast fusion into myotubes/myofibers is called terminal differentiation, there is subsequent differentiation of these nascent muscle fibers into adult muscle fiber types (see later discussion).

Other factors may also influence myogenesis. For example, in vitro passive mechanical strain results in both myotube hyperplasia and hypertrophy.[35,36] Thus both myoblast proliferation and differentiation are affected by passive strain. Passive stretching can also prevent the atrophy of myotubes, which normally occurs in culture media lacking growth factors. The mechanisms responsible for transduction of passive strain into a proliferative and/or trophic influence is unclear but may involve mediators such as $PGF_2\alpha$, IGF_1 or other cytokines.[36] Although these in vitro studies employed passive strain, these results suggest that early mechanical activation of the fetal DIAm (fetal respiratory movements) may be important in promoting further myogenesis.

Myosin Heavy Chain

Myosin is a hexameric protein (MW = 480 kD) comprising two heavy chains and four light chains. At the rod-shaped COOH-terminus end of the myosin molecule, the two heavy chains (MW = 200 kD) dimerize into a 200 nm alpha-helical tail. At the NH3-terminus, the heavy chains separate and form two distinct heads (S-1), which contain both actin and nucleotide-binding domains. The S-1 converts chemical energy into mechanical work through a process that involves stereo-specific docking of S-1 with actin, thereby reversing the intramolecular conformational changes induced by ATP hydrolysis.[37,38] A number of myosin heavy chain (MHC) isoforms exists, all of which are encoded by a highly conserved multigene family located on chromosome 17 (human) or 11 (mouse).[39] The rate of ATP hydrolysis at the S-1 varies among the different MHC isoforms, thereby providing the molecular basis for fiber-type differences in mechanical properties.[40-43] The essential (MLC_{20}) and regulatory (MLC_{17}) myosin light chains provide structural support and possibly modulate mechanical performance of the MHC.[44] Different MLC isoforms may differentially modulate the kinetic properties of MHC.[45] Evidence also exists that both Ca^{2+} binding to MLC_{17} and phosphorylation of MLC_{17} modulate S-1 ATPase activity.[46-48]

Adult skeletal muscle fibers are classified histochemically as types I, IIa, and IIb, based on the pH lability of myofibrillar ATPase staining.[49] Muscle fiber type classification in the adult corresponds with the expression of different MHC isoforms. Fibers classified as type I express MHC_{Slow}. IIa fibers express MHC_{2A}. IIb fibers express MHC_{2B} and/or MHC_{2X}.[39,50-52] Such histochemical classification of muscle fiber types is not possible during fetal and neonatal development. During this period there are dramatic transitions in MHC isoform expression, with a high incidence of co-expression of MHC isoforms that precludes ready distinction of different muscle fiber types.[53-56] In the fetal mouse and rat DIAm, an embryonic MHC isoform (MHC_{Emb}) is predominantly expressed together with MHC_{Slow} and MHC_{2A}. At or close to birth, the predominant isoform expression switches to a neonatal MHC isoform (MHC_{Neo}), together with MHC_{Slow} and MHC_{2A}.Thereafter, MHC_{Neo} expression gradually disappears and is totally absent in the mouse and rat DIAm by D28. Expression of MHC_{2X} and MHC_{2B} isoforms emerges only after D14 in the mouse and rat DIAm, and the proportion of fibers expressing these isoforms increases until about D28, when the adult pattern of MHC isoform expression is fully established.[57] This dramatic postnatal transition in MHC isoform expression in the DIAm represents an important stage of muscle fiber differentiation, especially with respect to the development of mature contractile properties (see section on Association of Contractile Properties with Fiber Type and MHC Isoform Expression, later). Beyond D28, the relative contribution of each MHC isoform changes as a result of the disproportionate growth of DIAm fibers, for example, the growth of fibers expressing MHC_{2X} and MHC_{2B} is approximately twofold to threefold greater than that of fibers expressing MHC_{Slow} and MHC_{2A} isoforms.[58,59] Based on the temporal associations between innervation and the major developmental events in myogenesis (see section on Innervation, later this page), it would seem reasonable that the nervous system can either directly (for example, activity, nerve traffic) or indirectly (for example, secretion of trophic factors) influence DIAm development.[60] However, the precise mechanisms by which activation history and/or trophic factors influence DIAm development remain largely unknown.

The time course for developmental transitions in MHC isoform expression in the DIAm is also influenced by the hormonal milieu (thyroid hormones, growth hormone, insulin-like growth factor, etc.) surrounding the fibers. For example, MHC expression is sensitive to thyroid hormone levels.[61,62] Nutritional status can also affect myogenesis and MHC isoform expression.[58]

The determination of skeletal muscle fiber type has received considerable attention. Expression of class II histone de-acetylases (HDACs) may determine muscle fiber type by inhibiting the transcription factor MEF2.[63] MEF2 factors are required for the transcription of oxidative genes found in slow fibers.[64] HDAC activity is also regulated posttranslationally via ubiquitination and proteasome degradation.[65] Whether these mechanisms play a role in the developmental determination of muscle fiber type is still to be explored. In recent studies we examined MHC mRNA expression during postnatal development of DIAm and found postnatal changes in mRNA and protein expression were not concordant for adult MHC isoforms, suggesting that changes in MHC expression in the developing rat DIAm are not driven solely by changes in mRNA expression.[57] Furthermore, myonuclear domain size (reflecting the volume of myoplasm under transcriptional control by a single myonucleus) increased postnatally as fibers increased in cross-sectional area, indicating that changes in transcriptional activity (although present) do not exclusively determine the postnatal growth of the DIAm.[66]

Embryologic Origin

The DIAm derives from the pleuroperitoneal fold.[67] The pleuroperitoneal fold forms a partition separating the coelomic cavity into thoracic portions (superior, containing the developing heart and pericardium) and abdominal portions (inferior, containing the future peritoneal cavity). Growth of the embryonic axis causes a progressive caudal displacement of the pleuroperitoneal fold, with its anterior edge eventually becoming attached at the midthoracic level while its dorsal edge becomes attached at the lowest thoracic level. Committed myoblasts migrate within the pleuroperitoneal fold and eventually differentiate to form DIAm myotubes/myofibers. Coincident with myoblast migration, the phrenic nerves exit the cervical spinal cord following the septum transversum and migrating myoblasts.[68,69] The exact target of phrenic nerve terminals is not clear, but the site of phrenic nerve contact seems to be consistent (located medially within the pleuroperitoneal fold) with nerve axons being surrounded by migrating myoblasts.[70,71] Phrenic motor axons start branching within the pleuroperitoneal fold as myoblasts radiate into the dorsocostal, sternocostal, and crural regions of the DIAm.[67] The mechanisms by which phrenic nerve axons are guided toward the primordial DIAm remain unclear.

Innervation

All regions of the DIAm are innervated by cervical spinal cord segments.[72] Innervation of the DIAm displays a somatotopic pattern, with the sternal and more ventral aspects of both costal and crural regions being innervated by phrenic motoneurons located in more rostral segments of the cervical spinal cord. It remains unclear whether the somatotopic pattern of DIAm innervation develops before or after initial synapse formation (that is, it could reflect either the pattern of phrenic nerve outgrowth or the location of myotubes/myofibers).

In the mouse, the phrenic nerve arrives at the developing DIAm by E11 (see Figures 85-1 and 85-3) 2 days sooner than in the rat.[71] After arrival at the primordial DIAm, phrenic motor axons branch extensively, in close proximity with Schwann cells (Figures 85-3 and 85-4). Both Schwann cells and nerve terminals at this early stage display coated vesicles suggesting vesicular release (Figure 85-5). A number of regulatory processes may be involved in guiding Schwann cell and phrenic axon terminal outgrowth, both with respect to the initial targeting to the pleuroperitoneal fold and the subsequent contact with myotubes/myofibers. These regulatory processes may involve components of the extracellular matrix, the release of trophic factors or chemotactic substances. Schwann cell migration and branching may be essential to the outgrowth and branching of phrenic nerve terminals. Unfortunately, most of the available literature in this important area is limited by the fact that it is based on measurements of axonal outgrowth and branching using in vitro preparations. Recent studies, however, suggest that perineurial sheaths may serve as a scaffold for Schwann cell growth and alignment, thus targeting motor axons toward muscle fibers.[73,74] Clearly, this is a complex integrated system in which in vitro measurements may not accurately reflect in vivo conditions.

Trophic factors derived from motor axons may be important in determining DIAm development. For instance, nerve-derived agrin is important not only for neuromuscular junction development but also for the development of excitation-contraction coupling mechanisms in cultured human myotubes. Maturation of cultured myotubes including expression of ryanodine receptors and voltage-gated L-type Ca^{2+} channels is promoted by spinal cord explants as well as exogenous administration of neural agrin.[75,76] This suggests that soluble factors (rather than electrical activity) may be responsible for the effects of nerves on muscle development. Neuregulin (a member of the larger epidermal growth factor family)[77-80] is also expressed by motoneurons during embryonic and early postnatal development and may activate receptor tyrosine kinases of the ErbB family present in mature skeletal muscles,[81] including rat DIAm.[82] Neuregulin is known to induce acetylcholine receptor expression at embryonic development via its effects on Schwann cells.[83] Whether neuregulin constitutes a nerve-derived trophic influence for muscle fiber

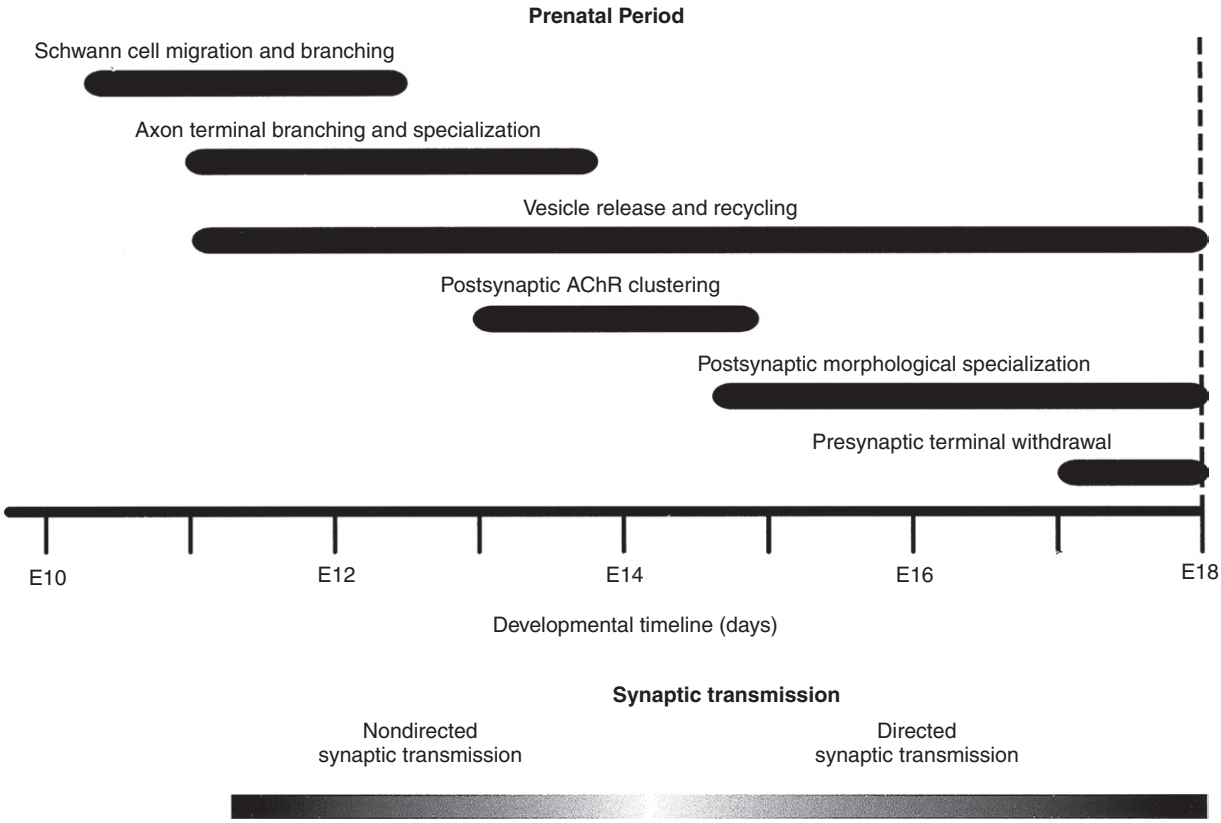


Figure 85-3 The timeline for major events in synapse formation in the mouse DIAm including differentiation of presynaptic and postsynaptic elements of neuromuscular junctions. Experimental evidence suggests that synaptic transmission is initially "nondirected" toward specific postsynaptic targets (that is, cholinergic receptor clusters that define motor end plates). With cholinergic receptor clustering and differentiation of postsynaptic specializations, synaptic transmission becomes "directed" toward these targets.

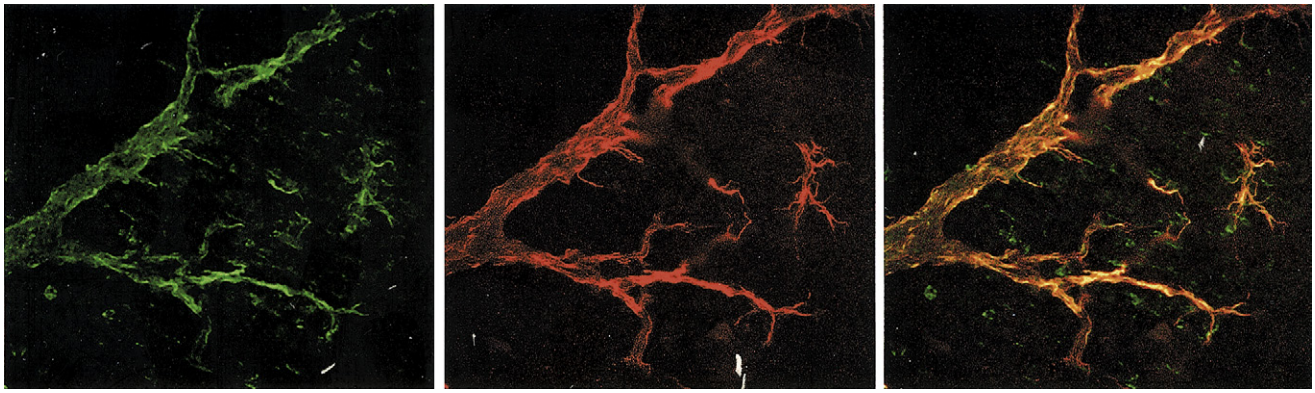


Figure 85-4 Confocal photomicrographs displaying immunoreactivity for neurofilamin (NF, to mark branching nerve fibers) and S-100 (to mark Schwann cells) in the mouse DIAm at E12.5. Note the extensive branching and overlap of both nerve fibers and Schwann cells at this early age.

growth during perinatal development and the relative importance of different trophic factor signaling pathways (for example, agrin versus neuregulin) remain to be determined.

Concurrent with the outgrowth of phrenic motor axons, there is also a progression of events on the postsynaptic side, with the expression and aggregation of cholinergic receptors. In the mouse DIAm, aggregation and clustering of cholinergic receptors is apparent at approximately E13 (see Figures 85-3 and 85-6). The process of aggregation of cholinergic receptors has received considerable attention, and it is known that this process involves agrin secretion by the nerve terminal and incorporation into the basal lamina, with subsequent activation of MuSK receptors at the postsynaptic membrane and finally rapsyn-mediated cholinergic receptor clustering.[84-86] MuSK phosphorylation seems critical to cholinergic receptor clustering, as the clustering is absent in MuSK double mutants and mice treated with inhibitory RNA designed to block MuSK transcription.[87] MuSK receptors show a gradient of expression in muscle fibers, most abundant in their central regions where MuSK can undergo autoactivation,[88] leading to cholinergic receptor clustering in the absence of neural influence. In neighboring sections of the muscle fiber where MuSK is not as richly expressed, agrin released by motor axons

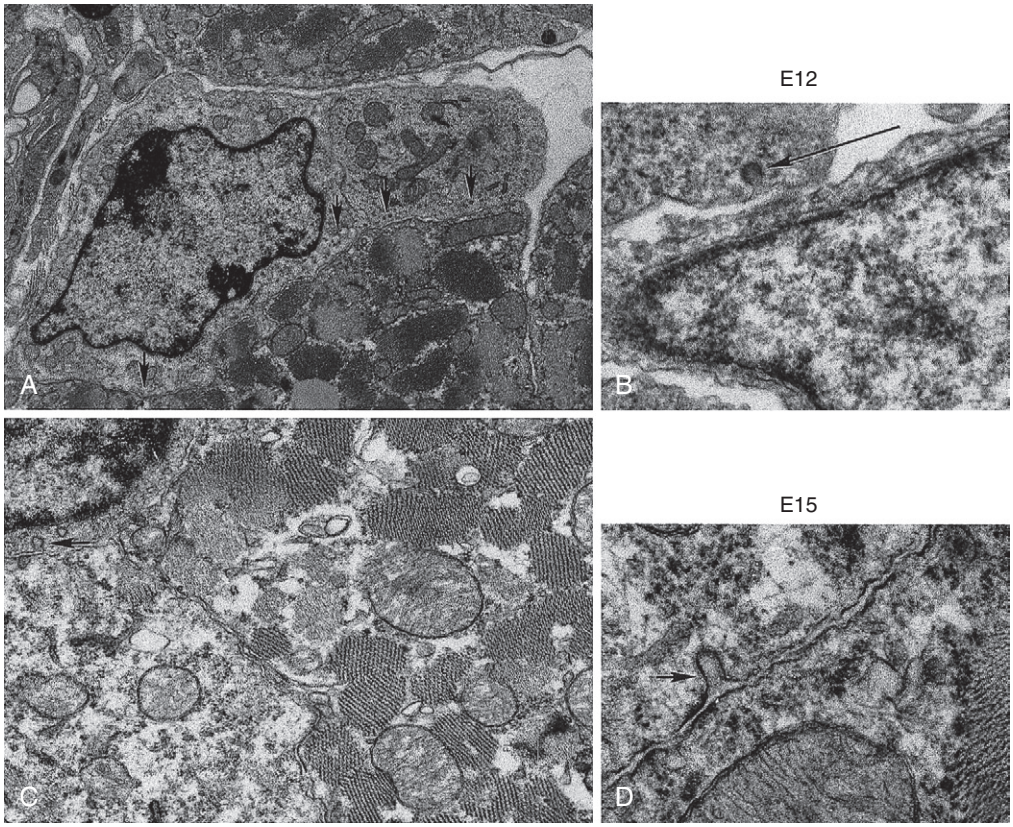


Figure 85-5 Electron photomicrographs showing **A,** the presence of coated vesicles *(arrows)* in both a nerve terminal and Schwann cell (noted by the presence of a distinct nucleus) and **B,** an exocytotic vesicle at a nerve terminal in a mouse DIAm at E12. **C**, Evidence for nondirected vesicular release from both nerve terminals and **D,** Schwann cells continues through E15.

can also lead to cholinergic receptor clustering.[89] This recent model for the formation of postsynaptic components of a neuromuscular junction can account for the apparent "targeting" of motor axons to "preformed" cholinergic clusters,[90,91] as well as the induction of new clusters by nerve-derived influences.[83] Following aggregation of cholinergic receptors, there is further specialization of the postsynaptic membrane with formation of synaptic folds and differentiation of the complex neuromuscular junction structure characteristic of adult DIAm (see Figures 85-3 and 85-7). Previously, it was demonstrated that in the adult rat DIAm, neuromuscular junction morphology varies across different fiber types (Figure 85-8), being far more complex in fibers expressing MHC_{2X} and MHX_{2B} isoforms as compared to fibers expressing MHC_{slow} and MHC_{2A} isoforms.[92,93] Neuromuscular junctions are much smaller and morphologically far less complex in the fetal and neonatal DIAm compared to the adult (Figure 85-9), but there is very little information regarding the mechanisms responsible for development of fiber type-specific differences in neuromuscular junction structure.[94]

Initially, a single myotube/myofiber can be contacted by multiple motoneurons (polyneuronal innervation). Subsequently, polyneuronal innervation disappears through the process of synapse elimination, which in the mouse and rat DIAm is complete by about D14 (see Figures 85-1 and 85-10).[85,95-99] The process of synapse elimination is not fully understood. It has been suggested that there may be competition among motoneurons for target cell innervation depending on activity (Hebbian competition).[98-100] Accordingly, terminal synapses of more active motoneurons would persist at the expense of less active motoneurons. This theory is confounded by the fact that in adult muscle, motor units with the largest innervation ratio (that is, number of muscle fibers innervated by a single motoneuron) are those that are least active (for example, fast-fatigable motor units).[101-103] Thus either the activity patterns of these motoneurons must change dramatically during development (transitioning from most to least active) or these motoneurons must initially innervate a greater number of muscle fibers to account for the subsequent greater loss of synaptic contacts (see Figure 85-10). The pattern of activity (not total activity) and the secretion of so-called synaptotrophins or synaptotoxins may also contribute to maintenance or elimination of synapses.[85] Another possibility is that muscle fibers use intracellular signals called synaptomedins to maintain contact with selected nerve terminals, and this may or may not be dependent on differential activity.[104] None of these possibilities can be excluded at the present time, and it is likely that a combination of mechanisms is responsible for the final pattern of motor unit innervation. However, because differences in innervation ratio exist across motor unit types, it is likely that the mechanisms underlying synapse elimination are linked to muscle fiber lineage or MHC isoform expression. Ultimately, motor unit composition may also be affected by ongoing postnatal myogenesis and formation of new myotubes/myofibers, which occurs until the third postnatal week in rat and mouse DIAm.[58] The final innervation ratio of motor units is not established in the mouse and rat DIAm until about D28.

Fetal respiratory movements indicating intact inspiratory drive and functional synapses have been observed in a number of species, including humans. In the rat, fetal respiratory movements begin at about E17,[105] but the presence and timing of such movements in mice has not been determined. The onset of fetal respiratory movements does not particularly denote the beginning of functional synapses and excitation-contraction coupling in DIAm

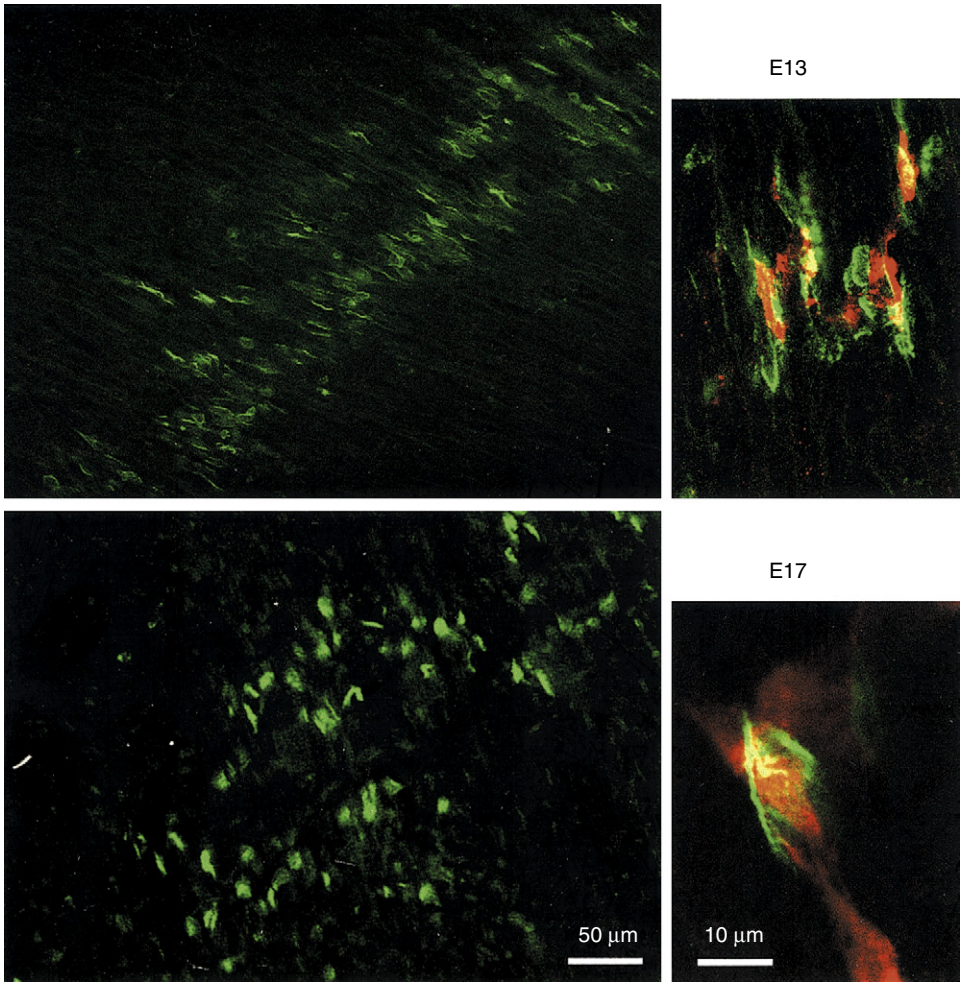


Figure 85-6 Confocal photomicrographs displaying α-bungarotoxin (BTX) labeling of cholinergic receptors and neurofilamin (NF) labeling of phrenic nerve axons and nerve terminals in the mouse DIAm at E13 and E17. Note the more diffuse pattern of BTX staining at E13 compared to E17, indicating a progression toward clustering of cholinergic receptors. Also note the greater overlap between nerve terminals (NF staining) and cholinergic receptors (BTX staining) at E17.

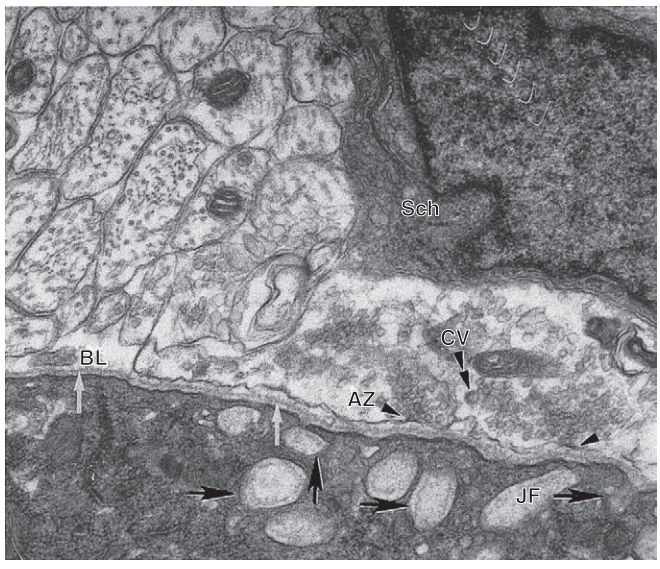


Figure 85-7 Electron photomicrograph illustrating the presence of postsynaptic specializations (for example, basal lamina—*BL, white arrows;* and junctional folds—*JF, black arrows*) at a neuromuscular junction in the mouse DIAm at E16. The electron micrograph also displays nerve terminals containing coated synaptic vesicles *(CV)* aggregating around active zones *(AZ)* and a Schwann cell *(Sch).*

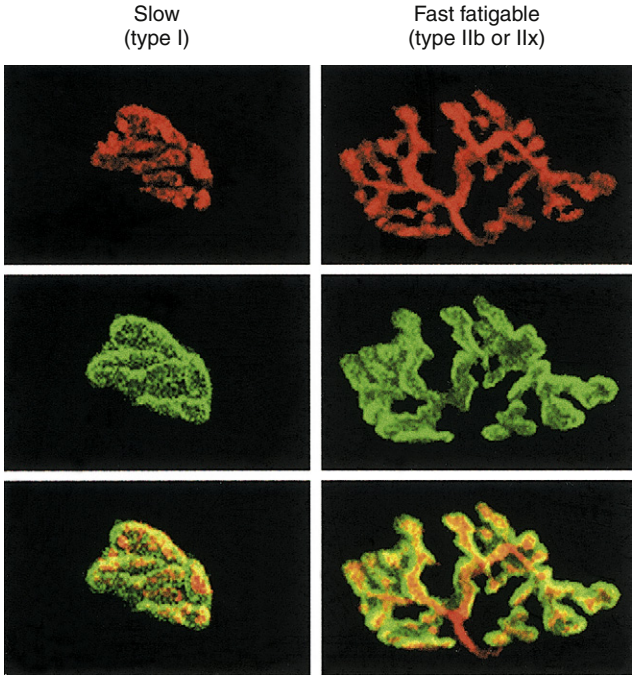


Figure 85-8 Confocal photomicrographs of presynaptic and postsynaptic elements of neuromuscular junctions in the adult rat DIAm. Note differences in complexity and size of neuromuscular junctions that depend on motor unit (muscle fiber) type.

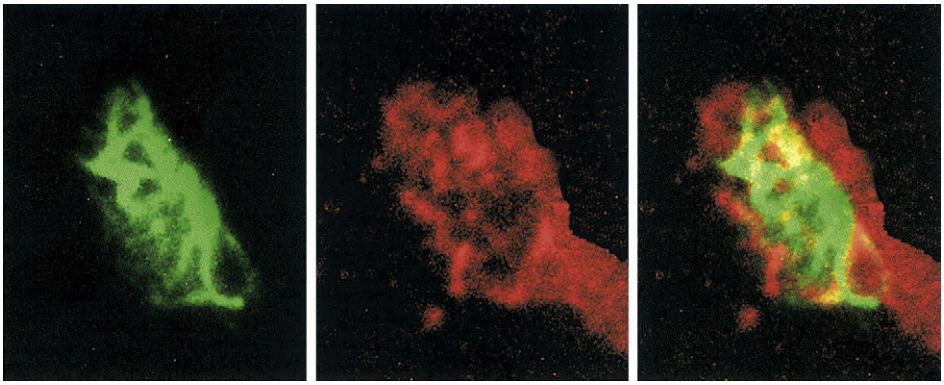


Figure 85-9 Confocal photomicrograph showing the morphology of neuromuscular junctions in the mouse DIAm at E17. Note that in comparison to neuromuscular junctions in the adult DIAm (see Figure 85-8), neuromuscular junctions at this age are substantially smaller and far less complex.

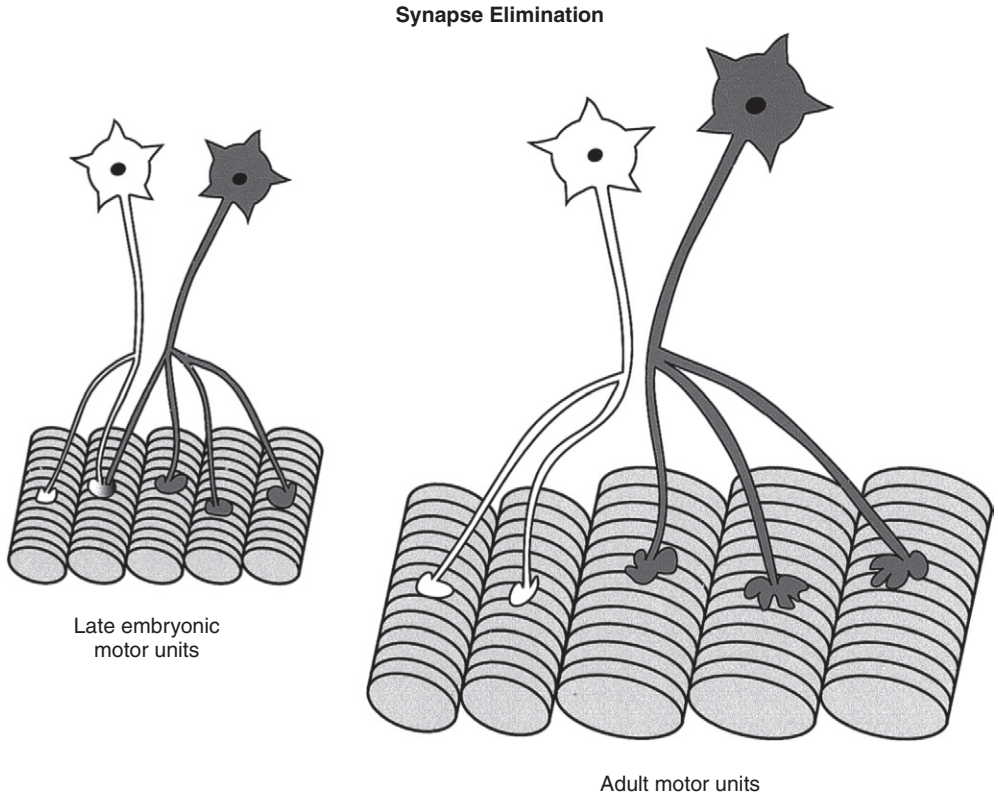


Figure 85-10 Process of synapse elimination. It is thought that differential patterns of motoneuron activity lead to selective withdrawal of motor axon terminals, which may be regulated by the secretion of synaptotrophins or synaptotoxins by the nerve terminal and/or production of synaptomedins within myofibers.

fibers. We found that intracellular Ca^{2+} and contractile responses could be elicited in the mouse DIAm by indirect nerve stimulation as early as E12.5 (Figure 85-11). These intracellular Ca^{2+} and contractile responses were blocked by d-tubocurarine and α-bungarotoxin indicating dependence on acetylcholine release and activation of cholinergic receptors. Yet, at this early age, synapses are only primordial at best, with no postsynaptic specialization and limited clustering of cholinergic receptors. These observations are consistent with the presence of coated vesicles and other indications of vesicular release (see Figure 85-5). Myotubes/myofibers that are forming in the DIAm at this time also display the onset of sarcomeric organization necessary for a mechanical response (Figures 85-12 and 85-13). Thus it appears that the nervous system can induce mechanical responses even before well-defined synapses are present. Such mechanical responses may be important in the subsequent development of myofibers in the DIAm (for example, further sarcomeric organization and alignment of sarcomeres).

Clearly, neuromuscular transmission in the fetal and neonatal DIAm is quite different from that in the adult muscle. In previous studies, it was shown that the neonatal rat DIAm is far more susceptible to neuromuscular transmission failure during repetitive activation than the adult rat DIAm.[106-109] Certainly, the increased susceptibility of the fetal and neonatal DIAm to neuromuscular

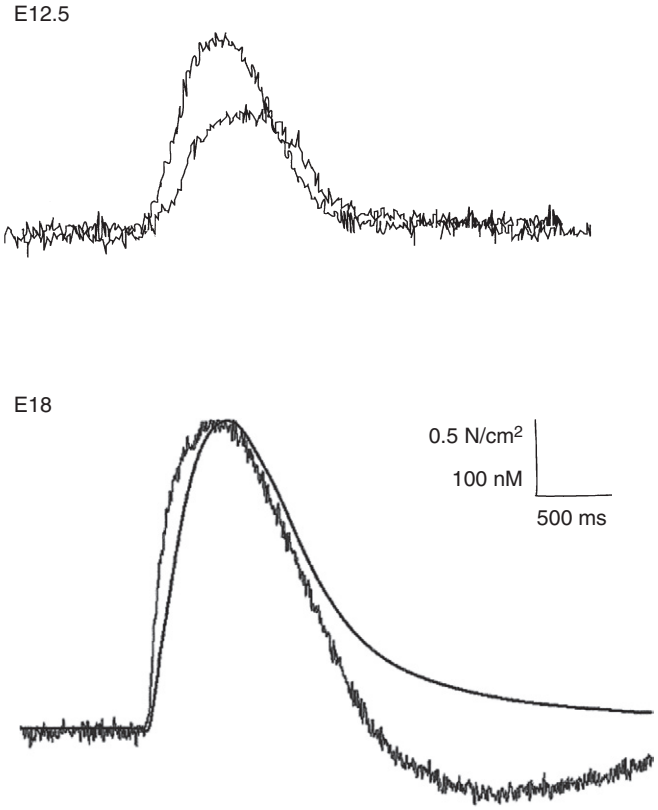


Figure 85-11 Simultaneous measurement of force and intracellular Ca^{2+} responses to phrenic nerve stimulation in the mouse DIAm at E12.5 and E18. Myofibers mounted between force and length transducers in a flow-through chamber were loaded with Fluo-3AM. Phrenic nerve terminals were stimulated using low current short duration (0.1 ms) square wave pulses. Responses were blocked using d-tubocurarine (10 μM).

transmission failure can be attributed, at least in part, to the more extensive branching of phrenic axons (as a result of polyneuronal innervation); thus the greater likelihood of failure of action potential propagation at axonal branch points.[107,109-111] It is also likely that differences in the number of synaptic vesicles, presynaptic neurotransmitter release, or consequent activation of cholinergic receptors all could account for the developmental differences in neuromuscular transmission failure. In the early postnatal DIAm, reduced spontaneous miniature end plate potential frequency and amplitude, as well as evoked end plate potential amplitude, compared to the adult are consistent with reduced neurotransmitter release and/or disproportionately smaller end plate regions (that is, fewer cholinergic receptors).[106-108]

Muscle Fiber Contractile Properties

Excitation-Contraction Coupling

As previously stated, fetal respiratory movements are present in the rat at about E17, but this does not necessarily reflect the onset of excitation-contraction coupling. In fact, intracellular Ca^{2+} and contractile responses are elicited in the mouse DIAm by indirect nerve stimulation as early as E12.5 (see Figure 85-11), indicating excitation-contraction coupling. These early mechanical responses are coincident with the earliest formation of myotubes/myofibers, expression of contractile proteins, and presence of rudimentary sarcomeres (see Figures 85-12 and 85-13). However, the coordination of these events with the emergence of other structures important in excitation-contraction coupling (for example, functional synapses, T-tubules, triads, and sarcoplasmic reticulum) is largely unknown. A number of studies have focused on the embryologic development of the sarcoplasmic reticulum, T-tubules, and triads and their association with myofibrillar organization.[112-122] In recent ultrastructural studies, we observed the presence of T-tubules, triads, and sarcoplasmic reticulum in the mouse DIAm at E14 (Figure 85-14). Takekura and colleagues[123] also presented ultrastructural and confocal evidence for the presence of T-tubules, triads, and sarcoplasmic reticulum in the embryologic mouse DIAm but only as early as E17. These investigators reported the consistent presence of ryanodine receptor clustering at E15 in the mouse DIAm, suggesting the presence of sarcoplasmic reticulum at this early developmental time point. Interestingly, knockout studies indicate that the apposition of sarcoplasmic reticulum to either plasmalemma or T-tubules occurs despite the absence of ryanodine receptors, L-type Ca^{2+} channels (dihydropyridine receptors), or both, despite the lack of functional activation in these mutants.[121,124,125] However, functional Ca^{2+} release is necessary for normal muscle development. Clearly, further studies are needed to elucidate the maturation of the excitation-contraction apparatus and its function during perinatal development.

Association of Contractile Properties with Fiber Type and MHC Isoform Expression

It is well established that mechanical properties of skeletal muscle fibers correlate with fiber type and MHC isoform composition.[39,42,126-133] In rat and mouse DIAm, fibers that express MHC_{2X} and/or MHC_{2B} display faster maximum unloaded shortening velocities (V_o), as seen in Figure 85-15, and greater specific forces (that is, force generated per muscle cross-sectional area), as seen in Figure 85-16, than fibers expressing MHC_{Slow} and MHC_{2A}. In the adult rat DIAm, the greater maximum specific force of fibers expressing MHC_{2X} and/or MHC_{2B} is at least partially explained by differences in MHC protein content per half sarcomere (that is, greater number of cross-bridges in parallel) as well as the force per cross-bridge (see Figure 85-16).[131,132,134,135] Similarly, the faster V_o of adult rat DIAm fibers expressing MHC_{2X} and/or MHC_{2B} is at least partially explained by faster cross-bridge cycling kinetics and a correspondingly higher rate of ATP consumption.[42,129]

During fetal and early postnatal development, DIAm fiber contractile properties change dramatically, corresponding with the transitions in MHC isoform composition that occur. For example, from E15 to approximately D28, specific force generated by rat DIAm increases almost twentyfold (Figure 85-17),[56,58-60,62,136-138] together with an increase in relative expression of fast MHC isoforms and MHC content per half sarcomere.[56,59,132,136] The lower specific force of fetal and neonatal DIAm is due to both a lower MHC content per half sarcomere and reduced force per cross-bridge.[132] During the same developmental period, V_o of the DIAm increases by more than sixfold (Figure 85-18),[59,60,62,138] again corresponding with the increase in relative expression of fast MHC isoforms.[59] The slower V_o of fetal and neonatal DIAm reflects the slower rate of ATP consumption of these fibers, which predominantly express MHC_{Emb} and MHC_{Neo} isoforms (Figure 85-19).[56] With an increase in both force and velocity, there is an accompanying increase in maximum power output and work performance of rat DIAm from birth to D28.[138] However, as power output and work performance of the DIAm increase postnatally, fatigue resistance to repeated activation declines.[137,138]

While it is clear that during early development the contractile properties of DIAm undergo profound shifts, which correspond to the transitions in MHC isoform expression, the signals driving these changes are not yet known. Changes in contractile properties induced by pathophysiologic conditions may not always be reflected by changes in MHC isoform composition alone. For example, with perinatal hypothyroidism there is a dramatic reduction in maximum specific force and a slowing of V_o in the rat DIAm that cannot be fully attributed to the moderate changes in MHC

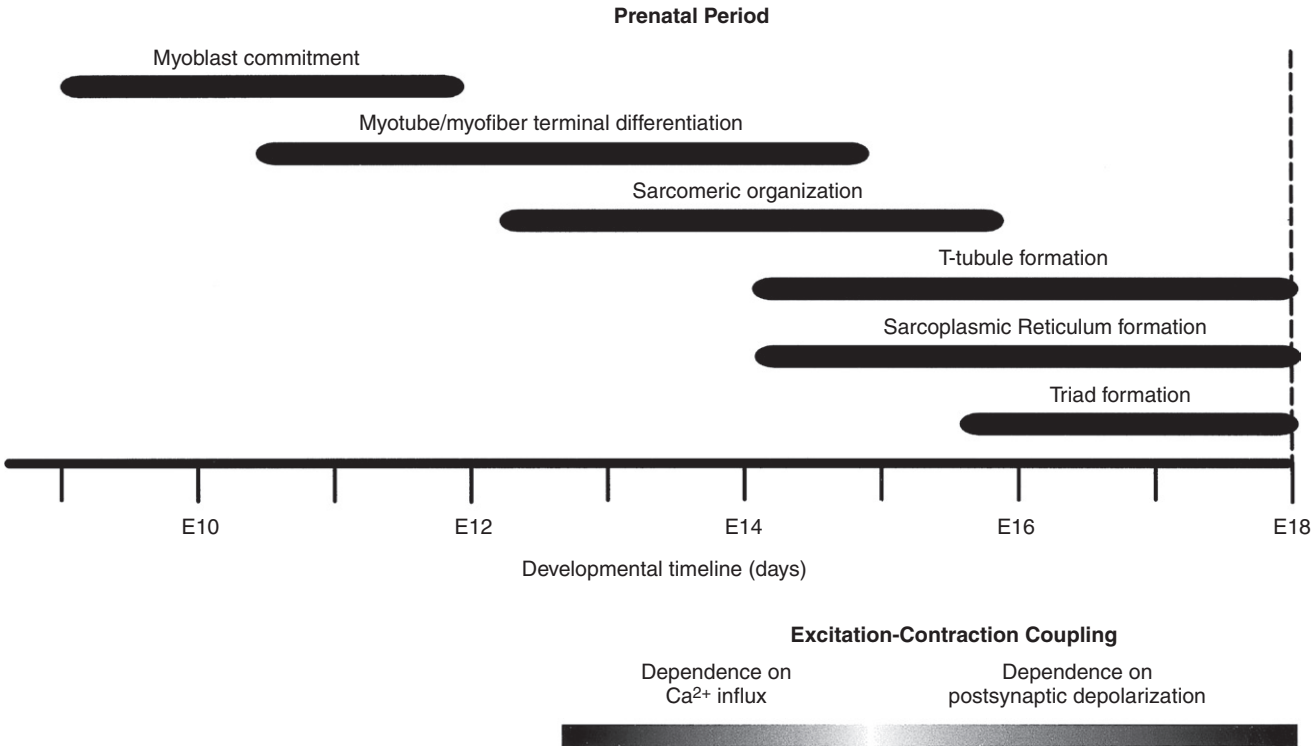


Figure 85-12 Timeline for major embryological events in the formation of myotubes/myofibers in mouse DIAm. It is likely that before the differentiation of distinct T-tubules, triads, and sarcoplasmic reticulum, excitation-contraction coupling is mediated primarily via Ca^{2+} influx, whereas in later embryonic stages mechanisms underlying excitation-contraction coupling resemble those in the adult.

isoform composition.[62] Similarly, following unilateral denervation of the DIAm at D7, there is a marked reduction in maximum specific force and a slowing of V_o that are not predicted from the slight changes in MHC isoform composition. As mentioned above, the changes in specific force may reflect the total MHC content per half sarcomere (that is, number of cross-bridges in parallel) of DIAm fibers rather than MHC isoform expression per se.[131] The slowing of V_o is more difficult to explain. Clearly, these results indicate that cross-bridge cycling kinetics can be slowed independent of the MHC isoform expressed. It is possible that these pathophysiologic conditions affect actomyosin ATPase activity of the myosin S-1 fragment or that there is an increased internal resistance to shortening. In support, we observed that following unilateral denervation of the adult rat DIAm maximum isometric ATP consumption rate was reduced independent of MHC isoform expression.[139]

MHC Isoform Expression and Cross-Bridge Cycling Kinetics in Single Muscle Fibers

In the original model of muscle contraction proposed by Huxley,[140,141] cross-bridges cycle between two functional states: a force-generating state, in which cross-bridges are strongly attached to actin; and a non–force-generating state, in which cross-bridges are detached from actin. The transitions between the two primary functional states of cross-bridges are described by two apparent rate constants: one for cross-bridge attachment (f_{app}) and the second for cross-bridge detachment (g_{app}). An increase in isometric force with increasing myoplasmic Ca^{2+} is explained by the recruitment of cross-bridges into the force-generating state (described by f_{app}). The transition of cross-bridges from force-generating to nonforce-generating states (described by g_{app}) requires the hydrolysis of ATP (actomyosin ATPase). Thus, in the Huxley model, the transduction of chemical to mechanical energy is implicit. Brenner proposed an analytical framework based on Huxley's two-state model of cross-bridge cycling in which this transduction of chemical to mechanical energy is more explicitly described.[142-144] In the Brenner analytical framework, the steady-state fraction of strongly bound cross-bridges in the force-generating state (α_{fs}) is given by:

$$\alpha_{fs} = f_{app} / (f_{app} + g_{app})$$

Isometric force generated by a muscle fiber is then described by following relationship:

$$\text{Isometric Force} = n \cdot F \cdot \alpha_{fs}$$

where n is the number of cross-bridges in parallel per half sarcomere, and F is the mean force per cross-bridge in the force-generating state. Assuming the hydrolysis of one ATP molecule during each cross-bridge cycle, isometric ATP consumption rate (actomyosin ATPase activity) is described by the following relationship:

$$\text{ATPase} = n \cdot b \cdot g_{app} \cdot \alpha_{fs}$$

where b is the number of half sarcomeres within the fiber. At maximal Ca^{2+} activation, where α_{fs} remains constant, actomyosin ATPase activity is directly proportional to g_{app}.

Previously, we employed a quantitative histochemical procedure to determine the maximum velocity of the actomyosin ATPase reaction (V_{max} ATPase) in DIAm fibers expressing different MHC isoforms.[42,129,145] Fibers expressing MHC_{2X} and MHC_{2B} have higher V_{max} ATPase compared to fibers expressing MHC_{Slow} and MHC_{2A}. To determine the ATP consumption rate of single permeabilized rat DIAm fibers during maximum isometric activation (ATP_{iso}), we used an NADH-linked fluorometric procedure.[42,129,146] ATP_{iso} was 30% to 45% lower than V_{max} ATPase, but the dependence on MHC isoform expression was still present, being highest in fibers expressing MHC_{2X} and MHC_{2B} and lowest in fibers expressing MHC_{Slow} and MHC_{2A}. This reflects a reserve capacity for ATP consumption in DIAm fibers, which is consistent with the well-known fact that ATP

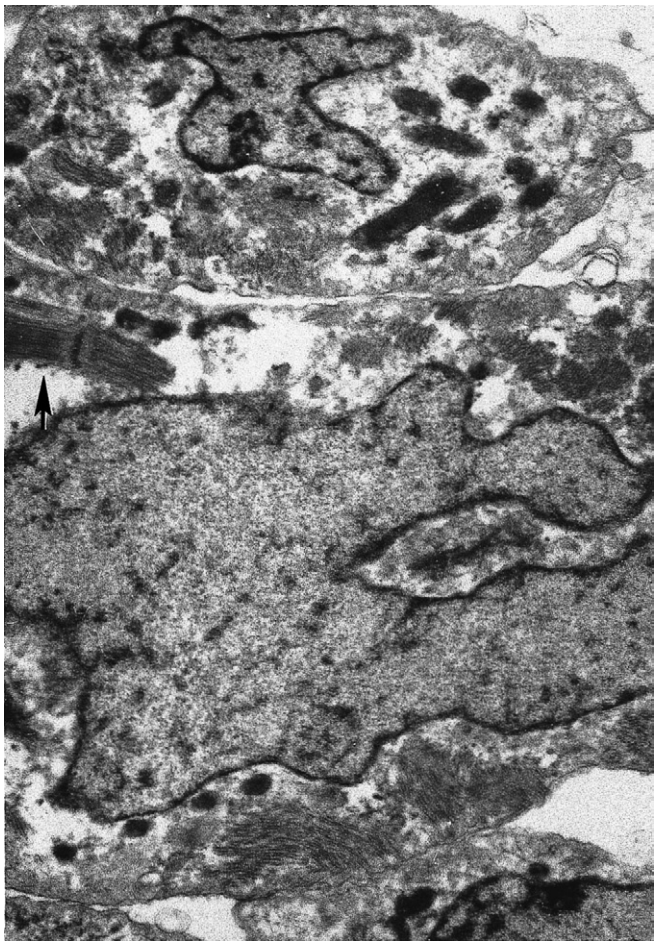


Figure 85-13 Electron photomicrograph displaying rudimentary sarcomeric organization *(arrow)* in the mouse DIAm at E12.5.

consumption rate increases in proportion to work.[147,148] Differences in reserve capacity for ATP consumption may account, at least in part, for differences in fatigability among DIAm fibers.[42,129] In a recent study, we found that the postnatal transition from MHC_{Neo} to adult fast MHC isoform expression in rat DIAm fibers is accompanied by an increase in V_{max} ATPase and ATP_{iso} (Figure 85-19).

MHC Isoform Expression and Ca^{2+} Sensitivity of Muscle Fibers

In skeletal muscle fibers, force generation depends on myoplasmic $[Ca^{2+}]$. The dependency of force generation of myoplasmic $[Ca^{2+}]$ is usually expressed as the force/pCa (-log $[Ca^{2+}]$) relationship. The force/pCa relationship of rat DIAm fibers expressing MHC_{Slow} is shifted leftward compared to fibers expressing adult fast MHC isoforms,[130] consistent with greater Ca^{2+} sensitivity of MHC_{Slow}-expressing DIAm fibers.[149-152]

It is widely recognized that in skeletal muscle, Ca^{2+} binding to the regulatory protein troponin C (TnC) underlies the dependency of force on myoplasmic $[Ca^{2+}]$. Ca^{2+} binding to TnC may simply act to increase the availability of myosin binding sites on actin and thus increase the probability of cross-bridge formation. Different isoforms of TnC from fast (TnC-f) and slow (TnC-s) muscle fibers have different numbers of regulatory binding sites for Ca^{2+} (2 for TnC-f versus 1 for TnC-s) and different binding affinities for Ca^{2+}. These differences in TnC isoforms may underlie the fiber type differences in force/pCa relationships. In support, the force/pCa relationship of rabbit psoas fibers changes when

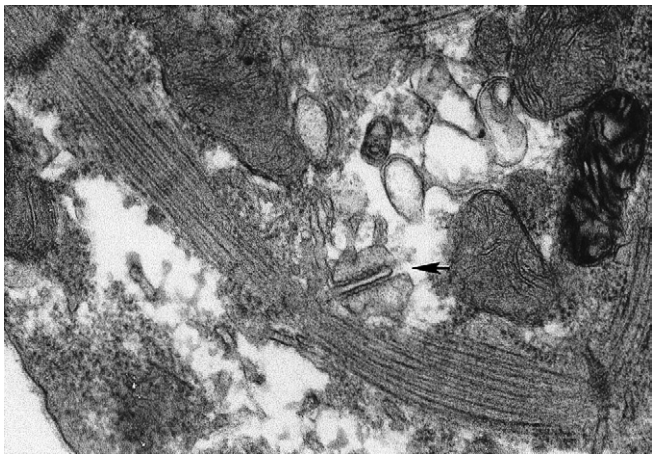


Figure 85-14 Electron photomicrograph showing the presence of a triad *(arrow)* in the mouse DIAm at E14.

the TnC-f present in these fibers is substituted with TnC-s.[153,154] The role of other troponin subunits (TnI or TnT) in regulating muscle contractility is not as well understood, although both slow and fast isoforms exist, and in agreement with TnC findings, fibers containing slow TnI and TnT have higher Ca^{2+} sensitivity than fast Tn-containing fibers.[155]

Functional Implications of Developmental Changes in Mechanical Performance of the DIAm

The DIAm plays a vital role in sustaining ventilation from the time of birth onward, yet its mechanical performance is affected by the concurrent maturation of other structures. For example, with maturation of limb muscles and locomotor function, ventilatory demands placed on the DIAm increase dramatically, and the DIAm must be capable of meeting the increased range of functional demands that coincide with postnatal development.[94] In addition, compliance of the lung and chest wall changes markedly during early development. The neonatal lung is stiffer as a result of greater alveolar recoil, which is offset by expression of surfactant, whereas the chest and abdominal walls are more compliant. As a result, the DIAm must generate greater relative intrathoracic pressures to produce a given level of inspiratory airflow and tidal volume. Yet, the neonatal DIAm generates much lower maximum tetanic force (see Figure 85-17) and has a much slower maximum unloaded shortening velocity (see Figure 85-18) compared to the adult. Thus the functional reserve capacity of the neonatal DIAm is greatly reduced, and a far greater fraction of maximum power output must be recruited to accomplish ventilation. Polyneuronal innervation of DIAm fibers may be particularly important as a neural strategy to accomplish greater fractional recruitment of DIAm motor units. As synapse elimination proceeds, more selective recruitment of DIAm motor units becomes possible, coinciding with the increased range of functions required of the DIAm. In addition, during early postnatal development, expression of MHC_{2X} and MHC_{2B} isoforms in DIAm fibers increases overall functional range, but at the expense of lower energy-efficiency and greater susceptibility to fatigue.[1,3] It is likely that DIAm fibers expressing MHC_{2X} and MHC_{2B} isoforms are recruited only during motor behaviors that require high force output and are of short duration, for example, expulsive behaviors.[1,3] Such motor behaviors of the DIAm may be less necessary in the neonate. Yet, the absence of such functional reserve may cause the neonate be more susceptible to aspiration and asphyxiation. Clearly, greater attention should be focused on the integrative aspects of DIAm development, and it will be important to place the genetic regulation of myogenesis and changing innervation patterns of the DIAm in this more global context.

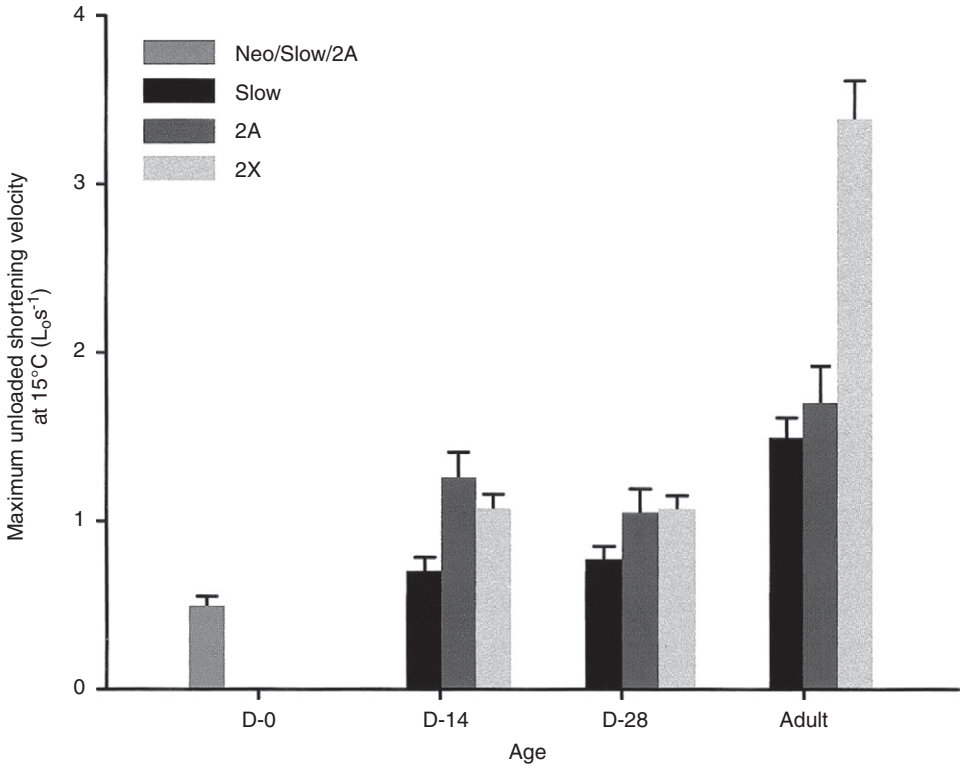


Figure 85-15 Maximum unloaded shortening velocity of single triton-X permeabilized rat DIAm fibers expressing different myosin heavy chain (MHC) isoforms. Maximum unloaded shortening velocity was not solely dependent on MHC isoform expression, but increased with age for fibers expressing the same MHC isoform.

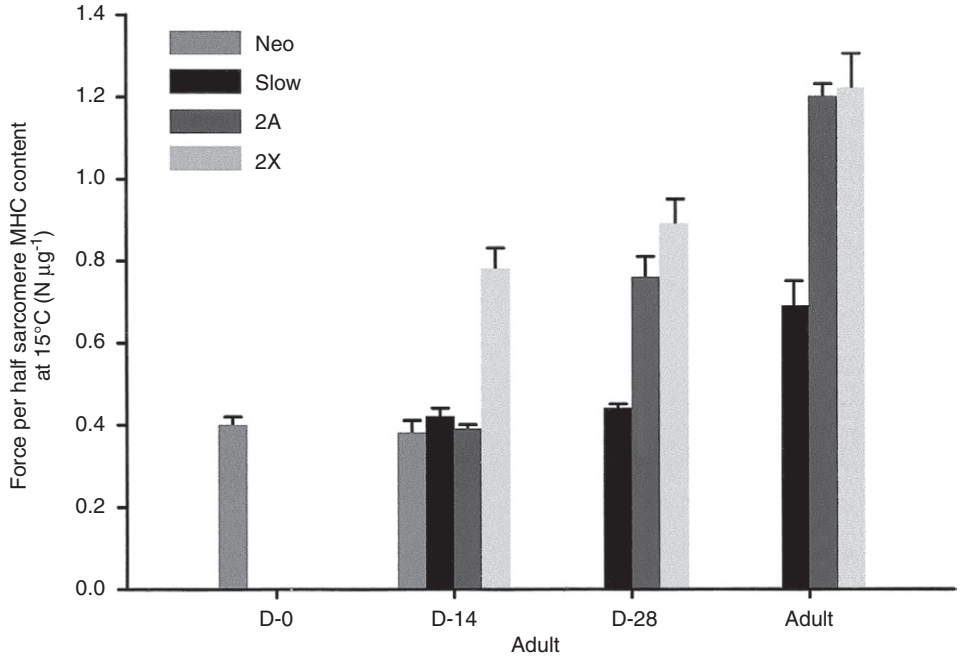


Figure 85-16 Maximum force of single triton-X permeabilized rat DIAm fibers expressing different myosin heavy chain (MHC) isoforms was normalized for MHC content per half sarcomere. Maximum force per half sarcomere MHC content (equivalent to force per cross-bridge) varied with MHC isoform as well as across age-groups for a given isoform.

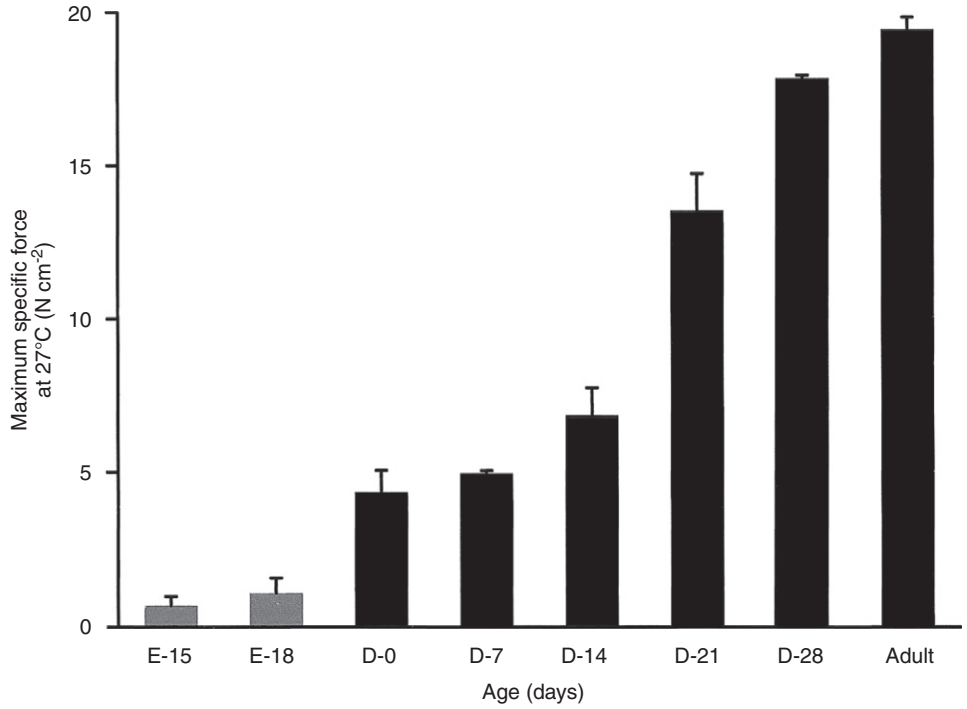


Figure 85-17 Developmental changes in maximum specific force (force per cross-sectional area) of rat DIAm. Maximum specific force increases almost twentyfold from E15 to adulthood.

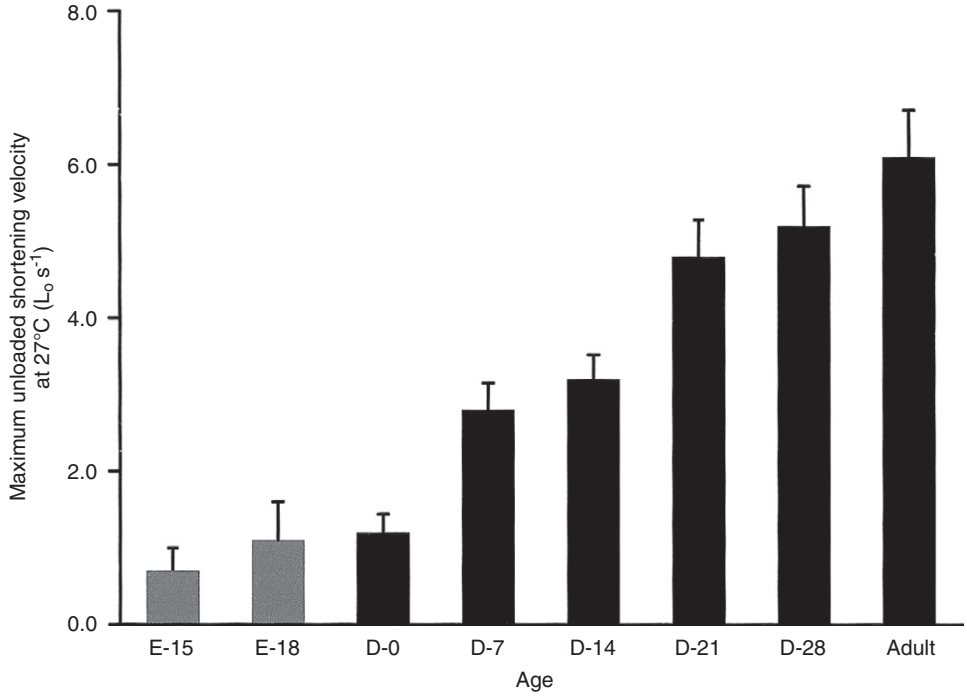


Figure 85-18 Developmental changes in maximum unloaded shortening velocity (expressed as $L_o\ s^{-1}$) of the rat DIAm. Maximum unloaded shortening velocity increases more than sixfold from E15 to adulthood.

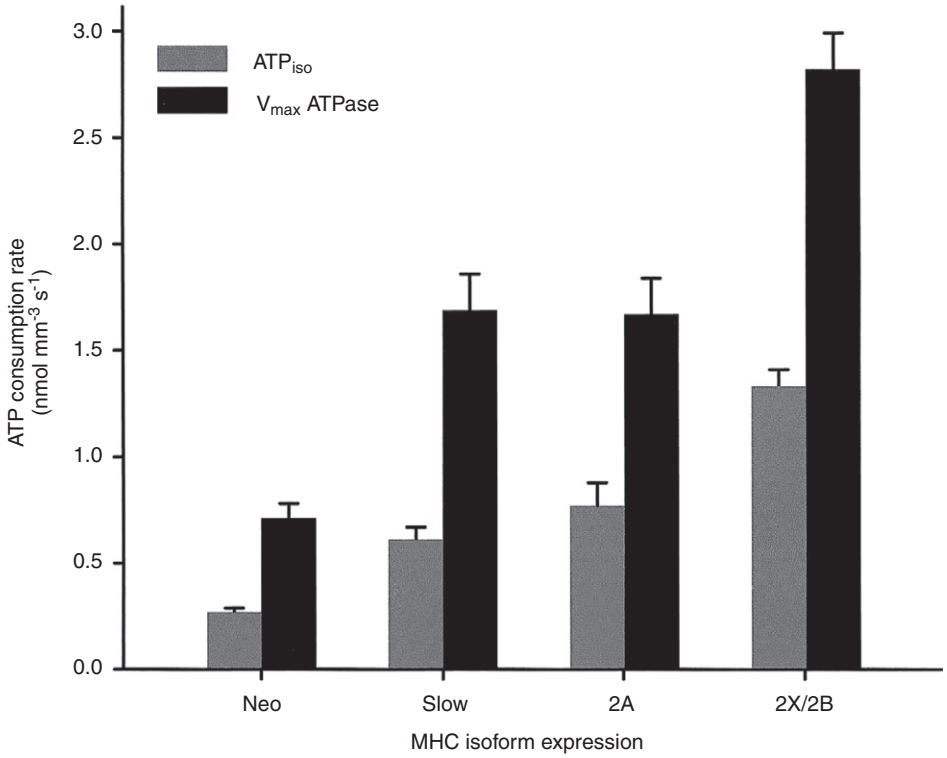


Figure 85-19 Maximum velocity of the actomyosin ATPase reaction (V_{max} ATPase) and maximum isometric ATP consumption rate (ATP_{iso}) of rat DIAm fibers expressing different MHC isoforms. For each fiber type, V_{max} ATPase was greater than ATP_{iso}, indicating substantial reserve capacity for ATP consumption. Although, V_{max} ATPase and ATP_{iso} varied across MHC isoforms and with age, the reserve capacity for ATP consumption remained relatively constant.

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86 Mechanics of Breathing

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In the chain of events that contribute to the translation of the output of the respiratory rhythm generator into pulmonary ventilation, the mechanical properties of the respiratory system represent a critical step. After activation of the inspiratory muscles, the magnitude of force generated depends on the physical properties of the muscles (i.e., their force-length and force-velocity characteristics). The physical translation of force into inspiratory pressure depends on the configuration of the muscle and the mechanical characteristics of the structure to which the force is applied. Finally, of the total muscle pressure generated, part is required to overcome the elastic properties of the respiratory system to change lung volume *(V),* and part is dissipated to overcome the resistive characteristics of the respiratory system to move air, that is, to generate flow $(\dot{V})$. The additional pressure component required for the acceleration of the gas, and dependent on the inertia of the respiratory system, is usually very small and can be neglected. Hence, the total pressure P generated is equal to the sum of elastic *(Pel)* and resistive *(Pres)* components, the former proportional to V and $1/C$, the latter proportional to $\dot{V}$ and R,

$$P = Pel + Pres = (V \cdot 1/C) + (\dot{V} \cdot R) \quad (1)$$

where C and R are proportionality factors determined by, respectively, the elastic and resistive characteristics of the system.

During *spontaneous breathing*, the total P for inflation is generated by the respiratory muscles, hence P = muscle pressure *(Pmus),* and inflow occurs whenever *(Pmus − Pel)* is greater than 0. Normally, during resting breathing, because tidal volume is entirely above the resting volume of the respiratory system *(Vr),* the inspiratory muscles generate *Pmus,* and expiration is passive. However, in some conditions, for example, certain cases of hyperventilation or during breathing against a positive airways pressure, the expiratory muscles may become active, and breathing occurs across *Vr.* In these cases, inspiration is the combined result of the recoil of the respiratory system after expiratory muscle relaxation plus the active contraction of the inspiratory muscles.

During *artificial ventilation*, $Pmus = 0$, and the driving pressure is generated by the ventilator, which opposes *Pel* and *Pres.* Whenever $Pmus = 0$, we say that the system is in a *passive* mode; this is the case during lung expansion by an external ventilator. It also occurs during the last portion of expiration during resting breathing. In fact, during this phase, the elastic pressure stored during inspiration *(Pel)* is the sole driving pressure generating expiratory flow, that is, $Pel = Pres = \dot{V} \cdot Rrs$.

Whenever *Pmus* differs from 0, we say that the system is in an *active* mode; this is of course the case during spontaneous inspiration, but it can also occur when $\dot{V} = 0$, such as during voluntary breath holding (in which case $Pmus = Pel$).

Whenever $\dot{V} = 0$, Equation 1 simplifies to

$$P = Pel = V \cdot 1/C \quad (2)$$

This condition is defined as *static*, irrespective of whether *Pel* is offset by muscle activity (e.g., during breath holding) or by external means with $Pmus = 0$ (e.g., relaxation against an artificial occlusion of the airways). On the contrary, whenever $\dot{V}$ differs from 0, the system is in a *dynamic* condition, again, irrespective of whether the respiratory muscles are active (e.g., inflation during spontaneous inspiration) or not (e.g., inflation by a ventilator). In conclusion, therefore, the operating modes of the respiratory system can be summarized into four combinations, *static* or *dynamic*, depending on the absence or presence of $\dot{V}$, each either *active* or *passive*, depending on the presence or absence of muscle activity (Box 86-1).

A dynamic condition with no changes in V (or changes in V so small that the component $V \cdot 1/C$ of Equation 1 is negligible) can be approached when $\dot{V}$ is very high and V is very small. This can happen during panting or during high-frequency ventilation. During an inspiratory effort against closed airways, because the change in lung volume equals 0 (and therefore also $\dot{V} = 0$), no external work is performed, and the total pressure is entirely dissipated as heat.

PASSIVE MECHANICAL PROPERTIES OF THE RESPIRATORY SYSTEM

As stated earlier, the respiratory system is in a passive mode when $Pmus = 0$. This is the case during paralysis, with the lungs ventilated by external means (e.g., positive pressure ventilation). Similarly, a passive condition occurs after a period of hyperventilation, when the arterial partial pressure of carbon dioxide ($Paco_2$) is lowered below the threshold for muscle activation, which is just a few mm Hg below the resting $Paco_2$ value.[1] In a spontaneously breathing subject, the passive mode can also be achieved by passively maintaining the lung volume above the resting position of the respiratory system, as for example with an external occlusion of the airways at end-inspiration. In fact, maintaining lung inflation activates the airway slowly adapting stretch receptors and triggers the vagally mediated Hering-Breuer inflation reflex, causing relaxation of the inspiratory muscles. This brief period of artificially provoked muscle relaxation is important in the context of pulmonary function testing in human neonates and is sufficient to evaluate the passive mechanical properties of the respiratory system in a spontaneously breathing infant.

Passive Respiratory Mechanics During Artificial Ventilation

To some extent, measurements of passive mechanics of the respiratory system are easier to perform during artificial ventilation than during spontaneous breathing. In fact, because $Pmus = 0$, the passive conditions are guaranteed. In addition, because it is customary to ventilate the lungs through an endotracheal tube, the infant's upper airways are bypassed, eliminating an important source of nonlinear mechanical behavior. Therefore the respiratory system is more likely to perform as a first-order mechanical system, which substantially simplifies the conceptual approach and the analysis of the measurements.

In an infant with intubation and artificial ventilation, $\dot{V}$ can be easily measured by a pneumotachograph placed in series between the endotracheal tube and the ventilator. The signal is amplified and electronically integrated to obtain the changes in lung volume *(V).* During artificial ventilation, the total P needed to inflate the whole respiratory system (P of Equation 1) is

BOX 86-1

Glossary of Terms, Units, and Common Terminology

BTPS	Body temperature, pressure, saturated with water vapor
C (C_L, *Crs, Cw*)	Compliance (of lungs, respiratory system, chest wall), mL/cm H_2O
FRC	Functional residual capacity (end-expiratory volume), mL
P	Pressure, cm H_2O
Pao	Pressure at the airway opening, cm H_2O
Pel	Elastic pressure (proportional to lung volume), cm H_2O
Pes	Esophageal pressure (proportional to pleural pressure), cm H_2O
Pmus	Pressure generated by the respiratory muscles, cm H_2O
Pres	Resistive pressure (proportional to airflow), cm H_2O
R (R_L, *Rrs, Rw*)	Resistance (of the lungs, respiratory system, chest wall), cm $H_2O \cdot mL^{-1} \cdot s$
τrs	Passive time constant of respiratory system, s
$\tau rs(exp)$	Expiratory time constant of respiratory system, s
V	Volume, mL (BTPS)
V_T	Tidal volume, mL (BTPS)
$\dot{V}$	Flow, mL (BTPS)/s
Vr	Static relaxation volume of respiratory system, mL
Active mode:	*Pmus* >0, respiratory muscles are active.
Passive mode:	*Pmus* = 0, respiratory muscles are relaxed.
Static condition:	$\dot{V} = 0$. (1) *Active:* breath holding by muscle contraction above or below *Vr;* (2) *passive:* relaxation against closed airways above *Vr* (*Pao* >0), below *Vr* (*Pao* <0), or at *Vr* (*Pao* = 0).
Dynamic condition:	$\dot{V} > 0$. (1) *Active:* activation of the respiratory muscles, as during spontaneous breathing; (2) *passive*: respiration driven by external means, with respiratory muscles relaxed, as during mechanical ventilation or in the late portion of expiration during resting breathing.

conveniently measured at the airway opening *(Pao),* such as the mouth or the inlet of the endotracheal tube. From these measurements, the values of *C* and *R* of the respiratory system (*Crs* and *Rrs,* respectively) are either separately computed or derived from the equation of motion of the respiratory system (Equation 1). In fact, because three ($\dot{V}$, *V, Pao)* of the five variables are measured, it is possible to mathematically derive the two unknown constants, *Crs* and *Rrs.* With a conceptually similar approach it is possible to compute *C* and *R* of lungs and chest wall.

The possibility of air leaks around the endotracheal tube and the need for accurate estimation of the tube resistance[2] are common problems. Tube resistance can be estimated in vitro, being aware that the fluid dynamics of small tubes can introduce substantial errors in the traditional computation of resistance from delta pressure and flow.[3] Air leaks can introduce enormous errors in the computation of volume of air delivered to the lungs and parameters related to it. Measuring the changes in inflation pressure at the distal end of the tube gives a more accurate estimate of changes in lung volume than the common way of measuring pressure more proximally. Nevertheless, the pressure value includes a component caused by airflow that increases as the leak increases; therefore it cannot be used with confidence to estimate the changes in lung volume. Methods for measuring changes in lung volume in infants on artificial ventilation—other than using a cuffed endotracheal tube—are the head-out body plethysmography and the respiratory inductance plethysmography; however, the former technique is cumbersome and the latter has some approximation.[4]

Compliance

At end-inflation, the compliance of the respiratory system, *Crs,* equals $\Delta V/\Delta Pao$, where ΔV and ΔPao represent the difference in *V* and *Pao* between the onset of inflation and the zero-flow point at end-inflation. It is important to realize that, at the onset of the inflation, *Pao* is not necessarily zero. In fact, *Pao* is above zero whenever the infant's lungs are ventilated with some positive end-expiratory pressure, which is the most frequent condition. Therefore, if one had to compute *Crs* simply as the ratio of *V* and *Pao*, instead of $\Delta V/\Delta Pao$, the resulting value of *Crs* would be incorrectly low.

Lung compliance *(C_L)* is measured as the ratio between the change in *V* (ΔV) and the change in transpulmonary pressure (ΔP), defined as the difference between *Pao* and pleural pressure; from *Crs* and C_L, chest wall compliance *(Cw)* is computed as $Cw = 1/(1/Crs - 1/C_L)$. As in adults, newborn pleural pressure is usually measured as esophageal pressure *(Pes),* with an esophageal balloon or an esophageal liquid-filled catheter. However, especially in premature infants, to what extent the *Pes* is a faithful representation of the mean change in pleural pressure is debatable.[5-10] Problems originate not only from the technical aspects of the measurements (including the frequency response of the esophageal catheter) but also because of the distortion of the chest, a phenomenon much more pronounced in infants than in adults. In spontaneously breathing infants, a test performed to evaluate the accuracy of the *Pes* recording is accomplished by comparing the change in *Pes* with that of *Pao* during an inspiratory effort with occluded airways.[6,10,11] Because during an inspiratory effort with an occluded airway neither lung volume nor transpulmonary pressure changes, the *Pao-Peso* difference should remain nil. However, in infants, particularly in premature infants, the distortion of the chest is often great and not necessarily the same between the occluded inspiratory effort used to test the reliability of the *Pes* measurement and the normal open-airway inspiration. Therefore one is left with the possibility that what may seem a reliable measurement during effort is not equally valid during normal spontaneous breathing.

Compliance measured as described earlier is often labeled *dynamic* compliance, a term that may be confusing because it seems to contradict the requirement for *static* conditions. In effect, the word *dynamic* is used to stress the fact that the measurement is performed during the dynamic state of continuous ventilation, by taking advantage of the very brief end-inflation condition, when, in fact, $\dot{V} = 0$. Truly static conditions can be achieved by stopping the ventilator, and recording *P,* while the lungs are inflated at a constant level. When this is done in healthy adults, the values of *dynamic* compliance are found to be quite close to the *static* measurements. In infants, however, values of *dynamic* compliance are consistently lower than *static* values,[12-14] mainly because of the lung stress relaxation. Stress relaxation is a phenomenon originating from the viscous properties of biologic tissue and substantially more marked in newborns than in adults.[15,16] The asynchronous behavior of peripheral lung units characteristic of newborns contributes to the *dynamic-static*

difference of compliance,[17] and even more so in conditions of marked chest distortion.[18]

Resistance

At any given time during lung *inflation* the total resistance of the respiratory system *(Rrs)* can be calculated as *Pres/$\dot{V}$*, where *Pres* is the difference between the total *P* of the respiratory system and its elastic components (Equation 1).[19] The measurement is more accurate at the time of peak flow, which is usually in the middle third of inflation. Alternative analytic approaches are based on the computation of *Pres* by planimetry or on various solutions of Equation 1.[20] The total pulmonary resistance *(R_L)* is computed in a very similar way; in this case, *Pres* is the difference between the total inflating transpulmonary pressure and its elastic component. Finally, chest wall resistance *(Rw)* is the difference *Rrs* − *R_L*.

During lung *deflation*, to the extent that the respiratory apparatus behaves mechanically as a first-order system, the decrease in volume and flow follows exponential trajectories. Therefore, during deflation, the logarithmic representation of either signal yields a linear relation, and the reciprocal of the slope represents the time constant (*τrs*).[21] Because the time constant of a first-order mechanical system is the product of the system *C* and *R*, *Rrs* is computed as *τrs/Crs*.

Passive Respiratory Mechanics in the Spontaneously Breathing Infant

For these measurements, the infant is breathing through a face mask connected to a $\dot{V}$- recording device, the pneumotachograph. The $\dot{V}$ signal is electronically integrated to obtain the changes in lung volume *(V),* while also measuring the pressure at the airways opening *(Pao).* Although the face mask modifies the breathing pattern, because of the stimulation of the trigeminal area[22] and because of the added dead space, neither of these has an important effect on respiratory mechanics. In its simplest approach, the investigator holds the mask on the infant's face, covering both mouth and nostrils, then intermittently performs manual brief occlusions of the pneumotachograph outlet, with the effect of maintaining the infant's lung volume constant. Alternatively, the use of a solenoid valve with computerized control[23] may be desirable for the accurate timing of the occlusion. This brief airway occlusion is the maneuver at the core of the measurements of *Crs* and *Rrs* to be described.

Compliance

During the occlusion of the airways at end-inspiration or in expiration (Figure 86-1), *Pao* rises to the recoil pressure of the respiratory system at that volume. The time required for *Pao* to reach its new equilibrium depends on the time needed by the inspiratory muscles to reach full relaxation. This relaxation is triggered by the activation of the airway slowly adapting stretch receptors, which respond to transpulmonary pressure. Therefore the larger the lung volume at which the occlusion is performed, the greater the likelihood of complete muscle relaxation. The passive condition required for the measurement lasts not more than a couple of seconds; after this time, the same vagal reflex inhibiting the inspiratory muscles eventually activates the expiratory muscles. However, this short time is sufficient for the measurements. Because *Crs* is the slope of the Δ*Pao*-Δ*V* relationship (x- and y-axis, respectively), the accuracy of the *Crs* computation improves by collecting many δ*Pao*-δ*V* data points over a wide range of *V*.[12,24-27]

Several other approaches have been proposed to compute *Crs,* with small variations from the method just described.[28-30] One of these methods computes the functional residual capacity (FRC)-*Vr* difference by extrapolation of the expiratory $\dot{V}$-*V* loop (see later); then the FRC-*Vr* is added to the Δ*V* for the correct computation of *Crs,* as (*V* + FRC-*Vr*)/*Pao*). With this approach, during one single brief airway occlusion and its immediate release, it is theoretically possible to compute not only *Crs* and FRC-*Vr* but also the time constant and the resistance of the respiratory system (respectively, *τrs* and *Rrs;* see later).[28] Another approach consists of two brief occlusions of the airways.[29] The first occlusion is performed to establish a reference *V* and the corresponding static *Pao,* and the second occlusion is done at a different *V,* either later in expiration or during the following breath. Then *Crs* is computed from the *V* difference between the two occlusions (Δ*V*) and the corresponding difference in *Pao* (Δ*Pao*), as *Crs* = Δ*V*/Δ*Pao*. This method circumvents the potential problems introduced by the breath-by-breath oscillations in FRC. In all these cases, if also *Pes* was monitored, as schematically indicated in Figure 86-1, it would be possible to partition *Crs* into its lung and chest wall components (respectively, *C_L* and *Cw*).

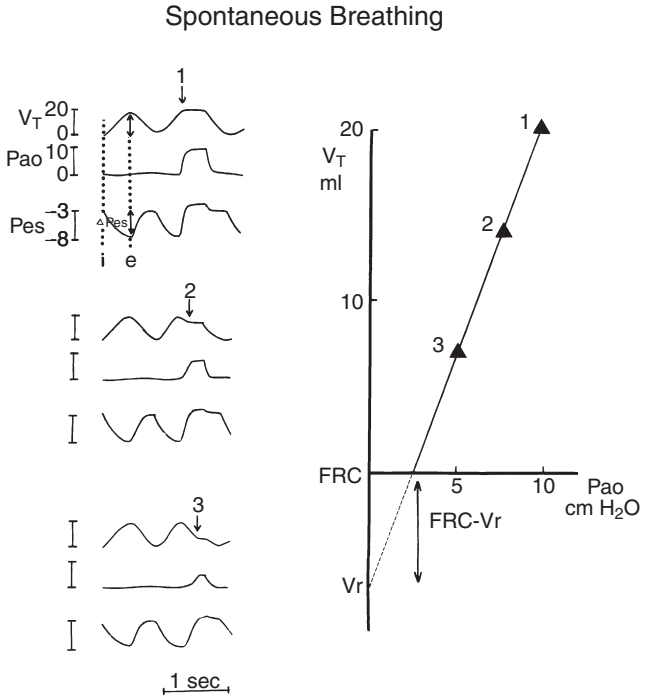


Figure 86-1 Schematic representation of the changes in lung volume (tidal volume, *V_T*), pressure measured at the mouth *(Pao),* and esophageal pressure *(Pes)* in a spontaneously breathing infant. In each of the three examples indicated, from *top* to *bottom,* the recordings start with a breathing cycle followed by an artificial occlusion of the airways. At *1,* the occlusion is at end-inspiration; at *2,* in the first third of expiration; at *3,* in the second half of expiration. The corresponding *Pao-V* points are plotted in the diagram at *right;* the slope of the linear regression represents the compliance of the respiratory system *(Crs),* and the intercept on the y-axis represents the difference between the end-expiratory level and the resting volume (FRC-*Vr* difference). Although only three data points are represented, many more data points through the whole *V_T* range are desirable to improve the accuracy of the linear regression. From *V_T* and *Pes* dynamic lung compliance can be computed as *V_T*/Δ*Pes*, between the two zero-flow points at beginning and end-inspiration *(left panels: i, e)*.

Time Constant and Resistance

Both *C* and *R* vary with large changes in lung volume.[31] Within the *V_T* range, though, their values remain approximately constant. Therefore, during a passive expiration (such as after reopening the airways following a brief airway occlusion at end-inspiration), both *V* and $\dot{V}$ decrease exponentially. The semilog representations

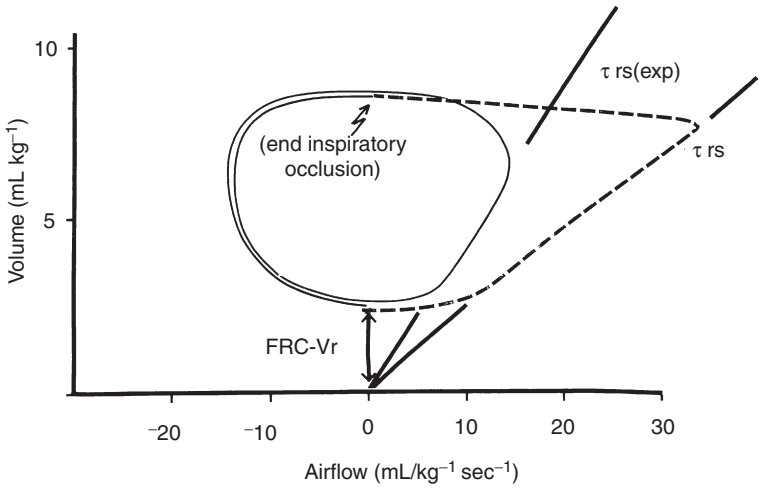


Figure 86-2 Infant, spontaneously breathing. Respiratory airflow and volume are continuously recorded and schematically plotted on the x- and y-axes *(continuous line).* Inspiration is at *left* (negative flows) and expiration is at *right* (positive flows). At end-inspiration of the second breathing cycle, the airways are briefly occluded by the investigator (end-inspiratory occlusion); after release of the occlusion, the slope of the deflation flow-volume curve *(dashed line)* represents the passive time constant of the respiratory system (τrs). Usually, during resting breathing, the expiratory flow-volume curve is at the left of the passive curve, indicating that the expiratory time constant, $\tau rs(exp)$, exceeds τrs. The extrapolation to zero flow of the linear portion of the expiratory flow-volume relationship permits computation of the volume difference between the end-expiratory volume *(FRC)* and the resting volume of the respiratory system *(Vr).*

of such trajectories are linear, and the reciprocal of the slope is the passive time constant of the respiratory system (τrs).[32] More simply, because the ratio of two exponential decays is a constant,[21] the plot of $\dot{V}$ (x-axis) − V (y-axis) during the passive expiration is a linear relationship, the slope of which is τrs[24,28,33] (Figure 86-2). Then Rrs is calculated as $\tau rs/Crs$. Such a simple and very practical approach is not readily applicable whenever the expiratory $\dot{V}$ and V do not follow exponential trajectories. Deviations from the exponential pattern could be due to several reasons, including respiratory muscle activation or nonlinear mechanical behavior of the upper airways. In these cases, the values of τrs and Rrs depend on the $\dot{V}$ at which they are measured.

In infants, during resting breathing, the extrapolation of the linear segment of the $\dot{V} - V$ expiratory curve to zero $\dot{V}$ hits the V-axis below FRC; this difference represents the dynamic elevation of the end-expiratory level (or FRC-Vr difference).[24,25] In fact, in infants, expiration is short with respect to τrs, and inspiration begins at a lung volume higher than Vr.[20] The FRC-Vr difference, sometime referred to as the *dynamic elevation of FRC*, can also be computed from the *Pao-V* curve, as described above (see Figure 86-1); in infants a few days old it averages 7 to 15 mL,[24,25,34,35] or approximately 20% to 30% of tidal volume.

As for other pulmonary function tests, the techniques described previously for measurements of C and R have been the object of numerous discussions, and their technical details can be found in review articles.[27,36-38] Because the transition from the research setting to the bedside is recent, one goal of the discussions is to make the techniques uniform and standardized to facilitate comparison among studies and identification of pathologic situations.

ACTIVE MECHANICAL BEHAVIOR OF THE RESPIRATORY SYSTEM

An important question is whether or not during spontaneous breathing the mechanical properties of the respiratory system are the same as measured during passive conditions. The answer is that the system does not behave as expected on the basis of passive measurements either in inspiration or in expiration. One reason is the difference between *dynamic* and *static* compliance, mentioned earlier. Furthermore, the uneven distribution of forces applied to the chest wall during muscle contraction leads to distortion of the chest; these functional implications are discussed in the next section. During expiration, residual activities of the inspiratory muscles and laryngeal muscles control the expiratory flow and mean lung volume, increasing R and prolonging τrs.[20]

Mechanics of Chest Wall Distortion

During passive inflation of the respiratory system (as during external ventilation provided by a ventilator) both the abdominal and pleural pressures increase. In fact, in the absence of diaphragmatic tension, the changes in pleural and abdominal pressures are the same, and any region of the chest wall (specifically, its two main compartments, rib cage and abdomen) will be driven by the same pressure applied uniformly to all regions.

In active conditions ($Pmus$ >0), when the diaphragm is contracting, the abdominal pressure increases as it does with passive inflation, expanding the abdomen. In contrast, the motion of the rib cage depends on the interplay of several factors.[20] During spontaneous inspiration the pleural pressure is subatmospheric, a situation that tends to collapse both the upper and lower regions of the rib cage, as in quadriplegic patients who have lost the use of the extradiaphragmatic muscles.[39] With respect to the lower rib cage regions, the tendency to collapse is partly offset by the expanding action of the abdominal pressure and also by the direct outward-pulling of the diaphragmatic fibers. In normal infants the inspiratory collapse of the upper rib cage is a common event because their characteristically high Cw is responsible for the poor mechanical coupling between upper and lower rib cage regions. It follows that during spontaneous inspiration, an expansion of the rib cage (to a degree comparable to that observed during passive inflation) can occur only with the intervention of the intercostals or the other extradiaphragmatic muscles, which, indeed, are recruited to compensate the inevitable distortion of the chest wall.[40] The two situations (*absence* of distortion and *compensated* distortion) are quite different when examined in light of the energetics of breathing.

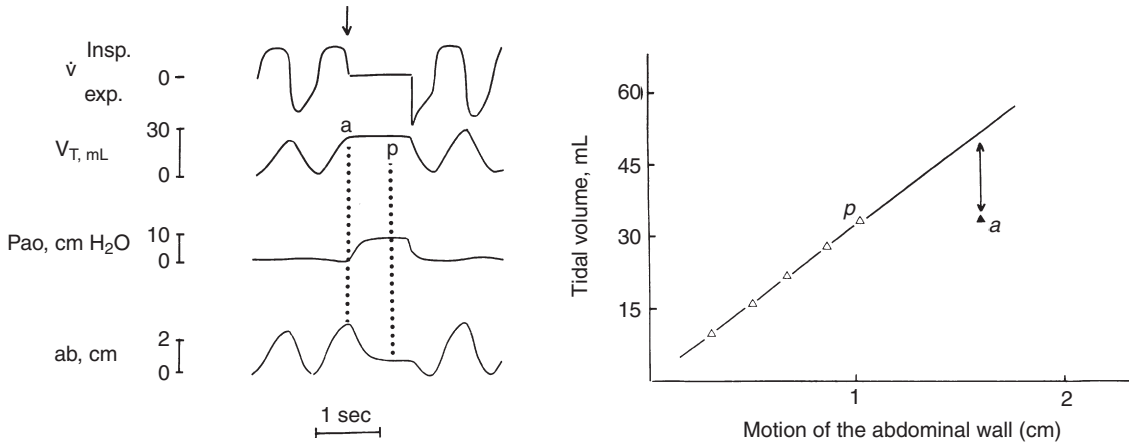


Figure 86-3 Schematic representation (from *top* to *bottom*) of the changes in respiratory airflow *($\dot{V}$),* tidal volume *(V_T),* pressure at the airway opening *(Pao),* and motion of the abdominal wall *(ab)* in a spontaneously breathing infant. At *arrow*, the investigator briefly occludes the airways, and muscle relaxation occurs at the plateau of *Pao.* The active *(a, solid triangle)* and passive *(p) V-ab* points are plotted at *right.* The passive relationship *(open triangles)* is obtained by multiple occlusions at different lung volumes during expiration. The vertical distance between *a* and the passive curve *(double arrow)* indicates the volume loss because of distortion.

A functional evaluation of the net effect of chest distortion can be done by comparing lung volumes between active and passive conditions at the same abdominal pressure (or at the same degree of abdominal expansion, because, in the absence of abdominal muscle activity, changes in abdominal pressure and abdominal displacement are closely related).[40] A schematic example is presented in Figure 86-3, which refers to a newborn infant during sleep. $\dot{V}$, *V,* and *Pao* are recorded, together with the motion of the abdominal wall. At end-inspiration the experimenter briefly occludes the infant's airways, and the plateau in *Pao* during the short time of the occlusion suggests relaxation of the respiratory muscles. Hence, at the same lung volume (end-inspiration), two situations can be recognized; one is *active*, at the end of the spontaneous inspiration, and the other one is *passive*, during the occlusion. By performing many such maneuvers at different lung volumes during expiration, it is possible to construct the passive relationship between abdomen motion and *V,* similar to what was described earlier for the measurement of *Crs* during spontaneous breathing. The difference on the y-axis between the active points (at end-inspiration) and the passive relationship represents the *V* difference between active and passive conditions. Normally (infants during resting breathing, at any given abdominal pressure) V_T is less than the passive *V,* by as much as 50%. This means that breathing in infants involves a substantial *volume loss*.[40] The volume loss is likely to increase in rapid eye movement sleep, when the intercostal muscles are less active and the inspiratory distortion of the rib cage is maximal. In addition, the volume loss probably increases in the supine posture, compared with the prone position,[41] because abdominal compliance is higher in the former case. A very large volume loss could occur in conditions of high *Cw/CL* ratio, as in premature infants, in diseases lowering *CL,* such as bronchopulmonary dysplasia, or in conditions of increased airway resistance. Conversely, a very small volume loss, or a V_T exceeding the passive *V,* could occur in hypoxia or in other situations of elevated motor output to the extradiaphragmatic muscles.

Expiratory Time Constant

Because, in resting conditions, expiration is a passive process, one may expect the expiratory $\dot{V}$ and *V* to follow exponential profiles; hence, the time constant measurable during tidal expiration, $\tau rs(exp)$, should coincide with the passive time constant of the respiratory system (τrs; see Figure 86-2). However, this is often not the case,[24,25,35] and $\tau rs(exp)$ exceeds τrs mainly for two reasons. First, the inspiratory activity does not cease abruptly at end-inspiration but proceeds during part of the expiratory phase, effectively reducing the elastic recoil pressure of the respiratory system. Second, the adduction of the vocal folds during expiration increases airway resistance, prolonging τrs. Graphically, the effects of the activity of the inspiratory and laryngeal muscles in expiration can be appreciated by comparing the tidal expiratory flow-volume loop with the curve obtained after release of an end-inspiratory airway occlusion (see Figure 86-2).[24,25] The finding that the expiratory curve is positioned at the left of the passive curve indicates that $\tau rs(exp)$ is longer than τrs at all volumes during expiration.

The prolongation of $\tau rs(exp)$ causes mean lung volume to remain higher throughout the expiratory phase of the breathing cycle. Also the next inspiration begins at a volume (FRC) higher than *Vr.* Indeed, more than 30 years ago Olinsky and co-workers[42] showed that, during a short period of apnea, FRC decreased in infants, recognizing that during resting breathing, FRC must be maintained actively above *Vr.* Later, it was possible to compute the FRC-*Vr* difference, from the static pressure-volume curve (see Figure 86-1) or from the expiratory flow-volume curve (see Figure 86-2).[24,25,34,35,43] The elevation of FRC above *Vr* results from the combined effects of the breathing rate (which in infants is higher than in adults) and the expiratory action of inspiratory and laryngeal muscles[44] and represents an important mechanism to counteract the infant's predisposition to a low *Vr* caused by the high *Cw-CL* ratio.[20] These mechanisms to control FRC are lost in infants whose lungs are artificially ventilated through an endotracheal tube, because the breathing frequency is controlled by the ventilator, and the laryngeal region is bypassed. In these cases, an end-expiratory *P* of a few centimeters of water[45,46] is added to the expiratory line of the ventilator to avoid the complete deflation of the lungs to *Vr.*

ASPECTS OF THE ENERGETICS OF BREATHING

From the equation of motion of the respiratory system (Equation 1), it is apparent that once *Crs* and *Rrs* are known, it is possible to compute the total pressure required to inflate the lung to a given

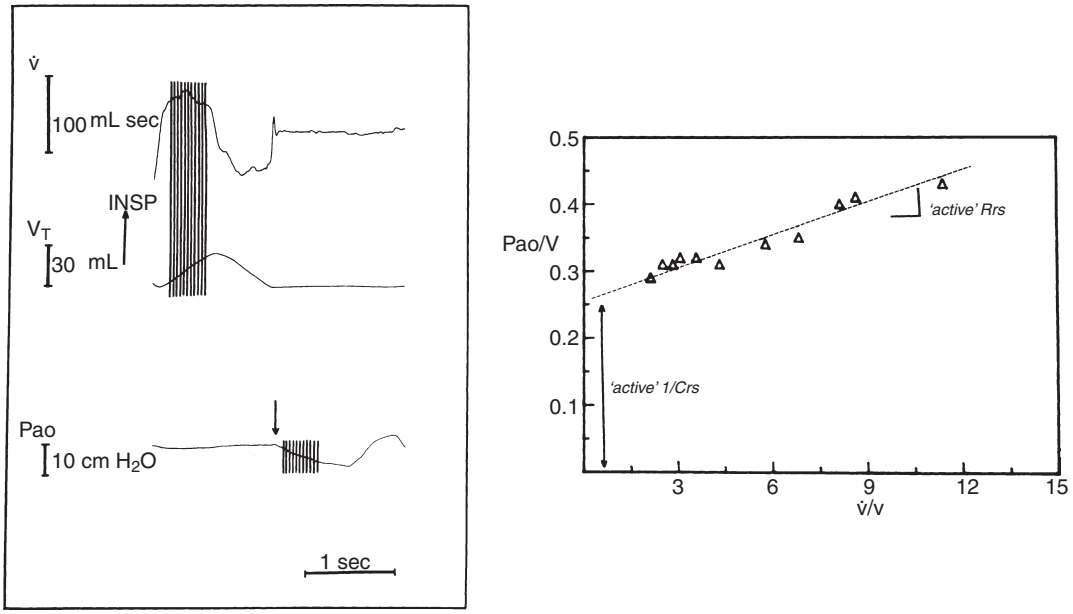


Figure 86-4 *Left*, Newborn infant, spontaneously breathing. From *top* to *bottom*, records of respiratory airflow ($\dot{V}$), tidal volume *(V_T),* and mouth pressure *(Pao).* At the time indicated by the *arrow*, the investigator occludes the infant's airways; then the infant makes an inspiratory effort, as shown by the drop in *Pao* with no changes in V_T. The *vertical parallel lines* indicate isotime measurements of *Pao* (from the onset of the effort), $\dot{V}$, and V_T (from the onset of the preceding breath). *Right*, The isotime *Pao/V-V̇/V* points are plotted; from the linear regression through the data points, the slope and the reciprocal of the intercept represent, respectively, the active resistance and active compliance of the respiratory system. *(Modified from Mortola JP, Saetta M: Measurements of respiratory mechanics in the newborn: a simple approach,* Pediatr Pulmonol *3:123, 1987. With permission from Wiley-Liss, a division of John Wiley & Sons, Inc.)*

volume in a given time. The total work can be calculated on the assumption of a sinusoidal flow pattern, based on the formula originally proposed by Otis and co-workers.[47] These computations have indicated that in newborns, as in adults, resting breathing frequency is controlled to fall within the optimal range of minimal external work although the mechanisms that lead to this optimal performance are still unknown.[24] The respiratory work calculated in this way represents the *passive* work of breathing, because it is based on values of *Crs* and *Rrs* obtained in passive conditions. During spontaneous breathing, though, the *active* work of breathing is likely to exceed the passive value. The main reason for this difference is that the respiratory muscles cannot apply a force to the chest wall as uniformly as it occurs in passive conditions. For example, the main inspiratory muscle, the diaphragm, acts more like a piston expanding the abdomen and simultaneously collapsing the upper rib cage than a uniform chest wall expander. The result of this uneven distribution is that some muscle force is lost in the distortion of the chest wall, instead of being translated into pressure driving the respiratory system. Furthermore, during contraction, a muscle loses force according to its length and velocity of shortening. Therefore, from the point of view of the dynamics and energetics of breathing, one could state that, during active breathing, the respiratory system behaves as if its impedance were higher than in passive conditions, because of a lower *Crs,* higher *Rrs,* or both.

Estimates of the active pressure required to expand the respiratory system have been obtained by measuring the pressure in the airways *(Pao)* generated during an inspiratory effort against an occlusion. In fact, in this condition, the change in lung volume is negligible, and, supposedly, the inspiratory muscles are contracting isometrically. The main assumption underlying this approach is that the *Pao* generated by the muscles is the same pressure required to generate V and $\dot{V}$ during normal breathing. Measurements of *Pao* are made at known time intervals from the onset of the occluded effort and those of flow and volume at the corresponding times from the onset of the preceding open airway breath (Figure 86-4, *left*).[48] From these data, it is possible to construct the *Pao/V* (y-axis) – $\dot{V}/V$ (x-axis) relationship; the slope and intercept represent, respectively, the *active Rrs* and 1/*Crs* (see Figure 86-4, *right*). From several studies that have applied this or similar approaches, it can be concluded that the newborn infant's *Crs* during spontaneous breathing is approximately 65% of the passive value, whereas *Rrs* is approximately the same as in passive conditions. This would mean that the elastic work (and cost) of breathing in a spontaneously breathing infant is approximately 50% (1/0.65) higher than expected from values of respiratory mechanics obtained in passive conditions. Notwithstanding the technical and analytic difficulties, estimates of the active work are useful because they provide a more realistic evaluation of the work imposed on the respiratory muscles than passive measurements would. In addition, the knowledge of the active values of *Crs* and *Rrs* yields more accurate predictions of the ability to maintain ventilation in the face of external elastic or resistive loads.[49]

Several other techniques, in addition to those described, are available to gain insights into the mechanical conditions of the infant's respiratory pump. Among these are methods oriented to measure thoracic gas volume, the mechanical properties of the airways, and their interaction with those of the lung. Lung volumes are usually measured by body plethysmography and the mechanical properties of the airways by forced expiratory maneuvers. These measurements, commonly performed in adults, have some practical difficulties when applied to the infant, mainly because the infant cannot cooperate.[50-52] Forced expiratory maneuvers, for example, require forced suction of air from the airways[53] or forced squeezing on the thorax;[54,55] either approach yields results that depend greatly on how the pressure is applied and how the maneuver is performed.[56,57] Thus these methods are usually confined to specific situations or to the research setting.[58,59] Differently, the measurements of *Crs* and *Rrs*

described in the previous sections are simple techniques readily applicable to a wide range of healthy and sick infants. Because in infants *Cw* is very high by comparison with *CL, Crs* can reflect pathologic changes in *CL* more readily than is the case in adults. Hence, measurements of *Crs* have been found useful in predicting the course and outcome of neonatal respiratory problems, as well as in classifying the severity of some respiratory diseases.

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87 Pulmonary Gas Exchange in the Developing Lung

William E. Truog

Integral to the system of pulmonary gas exchange are mechanisms to maintain matching of pulmonary perfusion and alveolar ventilation. The goal is that atmospheric oxygen and pulmonary capillary blood have intimate contact. Requisite features of the gas-exchange apparatus include sustained effective ventilation to replenish the oxygen stores in alveolar gas and free diffusion of both oxygen and carbon dioxide across the alveolar-capillary barrier. Evolution has produced a situation in which gas exchange must and almost always does switch sites from placenta to lung in a very few minutes. Strictly in terms of O_2 exchange, this switch represents an improvement from the circulatory counter-current pattern of the human placenta. Placental gas exchange is associated with a gradient from maternal uterine artery PO_2 of approximately 85 mm Hg at ambient air at sea level to a fetal umbilical venous PO_2 of approximately 35 mm Hg, a gradient approaching 50 mm Hg. Most neonatal lungs demonstrate improved efficiency (see below).

Mechanisms for maintaining the matching of alveolar ventilation ($\dot{V}A$) and pulmonary perfusion ($\dot{Q}p$) and the factors that influence changes in intrapulmonary distribution of $\dot{V}A$ and $\dot{Q}p$ in neonates are the topics of this chapter. The effects of insufficient respiratory drive, insufficient oxygen-carrying capacity, and diminished cardiac output on pulmonary and tissue gas exchange are discussed elsewhere.

Pathophysiologic mechanisms predisposing to pulmonary gas exchange abnormalities can be divided between extrapulmonary and intrapulmonary (Table 87-1). Hypoventilation can be exacerbated by severe hypoxemia, a factor in neonates that illustrates the importance of lung-brain interaction.

A particular feature of postnatal gas exchange to which the lung must accommodate is the different frequency at which $\dot{V}A$ and $\dot{Q}p$ occur. The pulmonary circulation maintains exclusively unidirectional flow, although at varying velocity, through the pulmonary microvasculature. By contrast, the inhalation of gases is periodic, occurring normally at a rate in neonates that is 20% to 50% of the cardiac rate during health and closer to 75% with pulmonary disorders. These different rates of ventilation and perfusion must be accommodated in achieving or maintaining optimal pulmonary gas exchange, a process which is continuous.

To help sustain sufficient oxygen and carbon dioxide flux between gas and blood, an adequate alveolar gas volume or functional residual capacity (FRC) must be established shortly after birth and sustained thereafter. Development of the FRC occurs in the process of transition from fetal to neonatal life as the fluid-filled lung empties itself of liquid and refills itself with resident gas[1] (Figure 87-1). Once established, this gas volume serves as an intrapulmonary reservoir for oxygen.

TABLE 87-1

Abnormalities in Pulmonary Gas Exchange

Extrapulmonary	Intrapulmonary
Hypoventilation	Ventilation/perfusion mismatching
Alveolar hypoxia	Intrapulmonary or extrapulmonary shunt
Cardiac shunt	Diffusion impairment

The newborn infant is particularly vulnerable to the development of arterial hypoxemia for several reasons. First, because the PaO_2 of normal newborns is low compared with that of adults,[2,3] intravascular oxygen reserve is less during periods of no oxygen movement into the lungs (i.e., during apnea). Second, the neonatal lung has a FRC that is close to airway closing volume. Atelectasis, or airway closure, may develop, especially considering the relative paucity of channels of collateral ventilation in the newborn. Third, the metabolic demand for oxygen in infants is greater on a per kilogram basis than in adults. Therefore the infant more quickly depletes oxygen stores in the blood and in resident alveolar gas in attempting to maintain aerobic metabolism. These factors are accentuated in premature babies, who manifest additional problems of susceptibility to apnea because of immaturity of the respiratory centers and to lung segment collapse at end-expiration because of extremely compliant chest walls.

ASSESSMENT OF VENTILATION-PERFUSION RELATIONSHIPS

Oxygen Gradient

The measurement of the partial pressure of oxygen in arterial blood (PaO_2) provides an excellent approximation about the efficiency of the lung as a gas-exchanging organ. The idealized alveolar to arterial oxygen gradient can be calculated by solving the alveolar air equation:

$$PACO_2 = PIO_2 - PACO_2 / R + PACO_2 \times (1 - R) / R$$

where R = respiratory exchange ratio. $PACO_2$ is assumed to be approximately equal to $PaCO_2$ and PIO_2 is calculated from measured barometric pressure and body temperature and assumes that inspired gas is 100% saturated with water vapor on reaching the acinar space.

The alveolar-arterial oxygen partial pressure calculated difference ($AaPO_2$) in normal adults breathing ambient air at sea level is 10 to 20 mm Hg. This value is increased with increasing FIO_2. Neonates have large $AaPO_2$ values during ambient air breathing (as high as 40 to 50 mm Hg shortly after birth), even allowing for R values close to 1.0 (presumably based on relatively high utilization of carbohydrate). The $AaPO_2$ may remain in the 20 to 40 mm Hg range for days after birth in term nondistressed infants[4,5] even with SpO_2 values of >90%.

The $AaPO_2$ quantifies the degree of venous admixture plus alveolar capillary membrane diffusion disequilibrium. However, neither the $AaPO_2$ nor the calculated ratio of arterial to alveolar oxygen tension (the PaO_2/PAO_2) discriminates between these components and both are very insensitive to situations with intrapulmonary shunt and pulmonary parenchymal diseases coexist. Venous admixture includes both intrapulmonary shunt (perfusion of pulmonary capillary blood past nonventilated lung regions before joining the stream of pulmonary venous blood) and perfusion of low $\dot{V}A/\dot{Q}$ areas in which alveolar ventilation is insufficient to restore PAO_2 to the value predicted from the "idealized" PAO_2 calculated earlier. Pulmonary end-capillary to alveolar space equilibrium can be predicted to occur for oxygen and carbon dioxide in low $\dot{V}A/\dot{Q}$ areas because alveolar PO_2 is low and only a small rise in pulmonary arterial PO_2 occurs as blood traverses

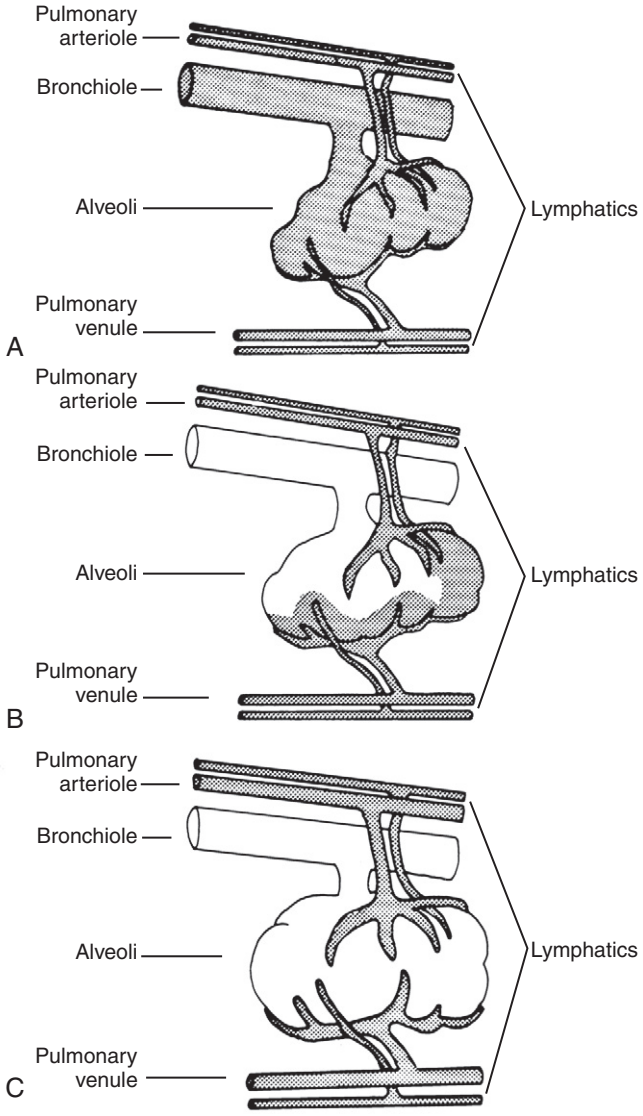


Figure 87-1 Airway fluid is removed from the lungs in several steps during birth. A, Fluid secretion from the lung stops during labor, although the air sacs and airways remain fluid filled. **B** and **C,** Fluid remaining in the airways is squeezed out the mouth and nose and is rapidly reabsorbed into the now expanded pulmonary capillary bed as well as into the lymphatic drainage system of the lung. *(From Carlo WA, Chatburn BL [eds]:* Neonatal Respiratory Care, *ed 2, Chicago, Year Book Medical Publishers, 1988.)*

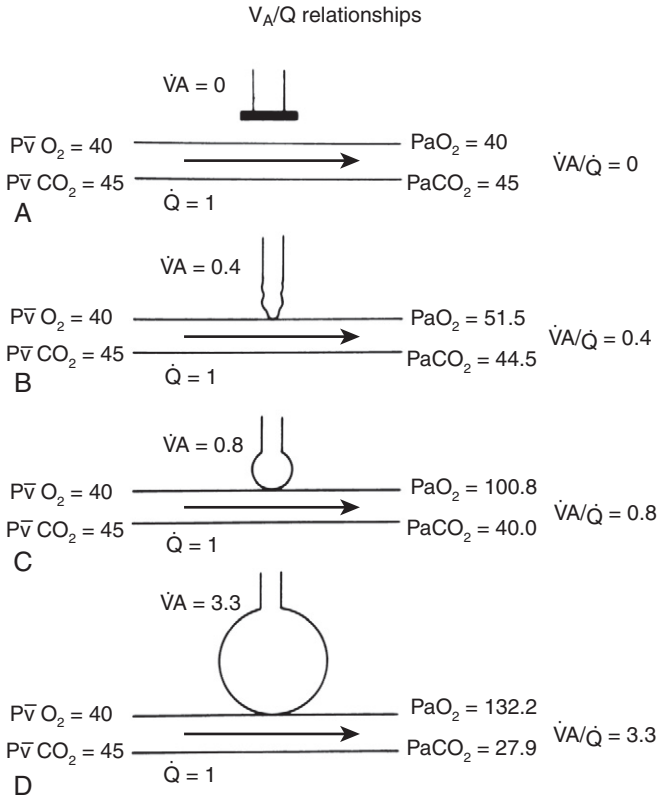


Figure 87-2 Effects of $\dot{V}A/\dot{Q}$ ratios on blood gas tensions, expressed in mm Hg. A, Intrapulmonary shunt leaves mixed venous blood gas tensions unaltered. **B,** Alveolus with low $\dot{V}A/\dot{Q}$ ratio: Only partial oxygenation occurs. **C,** Relatively normal $\dot{V}A/\dot{Q}$ ratio with satisfactory oxygenation of pulmonary capillary blood. **D,** Underperfused alveolus with high $\dot{V}A/\dot{Q}$ ratio. *(From Thibeault DW, Gregory GA [eds]:* Neonatal Pulmonary Care, *ed 2, Norwalk, CT, Appleton & Lange, 1986.)*

the pulmonary microcirculation[6] (Figure 87-2). Depressed pulmonary end-capillary PO_2 is associated with disproportionately depressed oxygen content because of the shape of the oxygen-hemoglobin curve, leading to an exaggerated depression of the mixed arterial PO_2 as measured in the left atrium or systemic arteries (assuming no extrapulmonary right to left shunts). Therefore overall pulmonary gas exchange is the flow-weighted and alveolar ventilation–weighted sum of gas exchange occurring in lung regions or groups of acinar units with a common $\dot{V}A/\dot{Q}$. Note that the rise in PaO_2 in the high $\dot{V}/\dot{Q}$ region depicted (see Figure 87-2, *D*) and the decrease in $PaCO_2$ do not equal levels of alveolar gas partial pressures that would be found if no regional perfusion existed. Lung units with both low $\dot{V}A/\dot{Q}$ and with normal $\dot{V}A/\dot{Q}$ relationships are depicted in Figure 87-3. Also shown is a pulmonary arterial to pulmonary venous connection bypassing any aerated area, which in the neonate could represent shunt through the ductus arteriosus, through the foramen ovale, through connections between pulmonary and bronchial arteries, or through the thebesian circulation.

The $AaPO_2$ measured in ambient air provides an overall summary of pulmonary oxygen exchange efficiency, but tells little about pulmonary reserves or about the cause of an increased difference between alveolar and arterial oxygen partial pressure. The simplified calculation, the oxygen index, combines arterial oxygen tension, FIO_2 and lung distending pressure. This commonly used index in neonatology also does not discriminate among intrapulmonary or extrapulmonary causes of hypoxemia (see Table 87-1).

The presence of a moderate to severe degree of $\dot{V}A/\dot{Q}$ mismatching has an especially depressing effect on PaO_2 when the inspired oxygen fraction is between 0.3 and 0.7 (Figure 87-4).[7] The effect of low $\dot{V}A/\dot{Q}$ areas on the calculation of $AaPO_2$ can be eliminated by breathing 100% oxygen to remove nitrogen from any open but underventilated lung regions. However, absorption atelectasis may then develop, increasing the size of the shunt, which was the subject of the measurement. There is evidence that increased shunt with oxygen breathing occurs in premature infants[8] and neonatal lambs but not in adult dogs.[9] These differences have been attributed to different degrees of development of pathways of collateral ventilation. Hence neither PaO_2 nor the ratio of arterial to alveolar PO_2 provides detailed knowledge of the state of ventilation-perfusion relationships in the lung. The

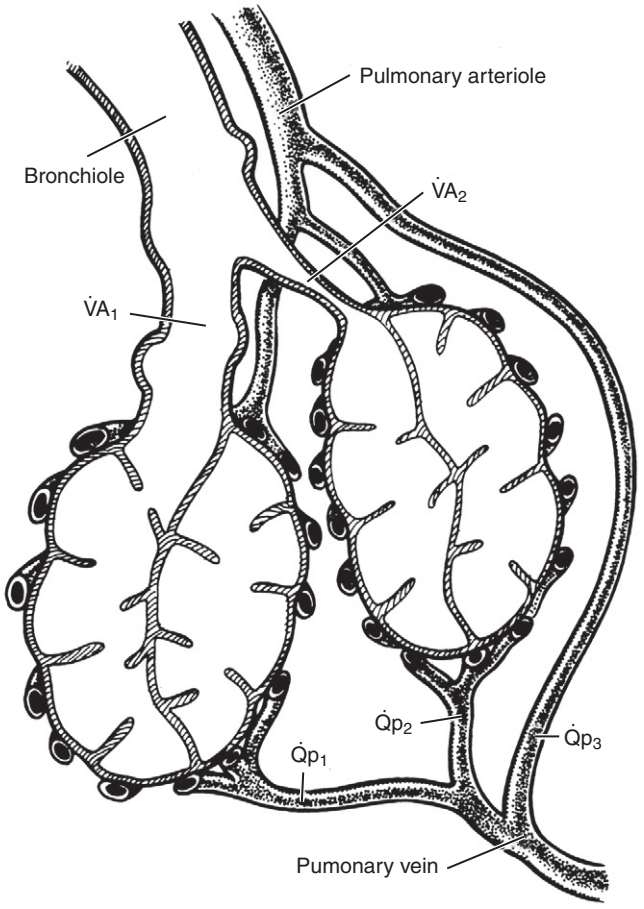


Figure 87-3 Schematic illustration of airway and vascular relationships. The acinar unit, containing many alveolar sacs, is shown with multiple pulmonary capillaries enveloping the saccules, providing maximal gas-exchanging surface area. $\dot{V}A_1/\dot{Q}p_1$ corresponds to an acinar region (containing multiple alveoli) with normal $\dot{V}A/\dot{Q}$; $\dot{V}A_2/\dot{Q}p_2$ represents an acinar region with a low $\dot{V}A/\dot{Q}$ and poor gas exchange. The pulmonary arteriole to pulmonary venous connection, $\dot{Q}p_3$, which bypasses any air-containing spaces, could represent intrapulmonary as well as extrapulmonary shunts.

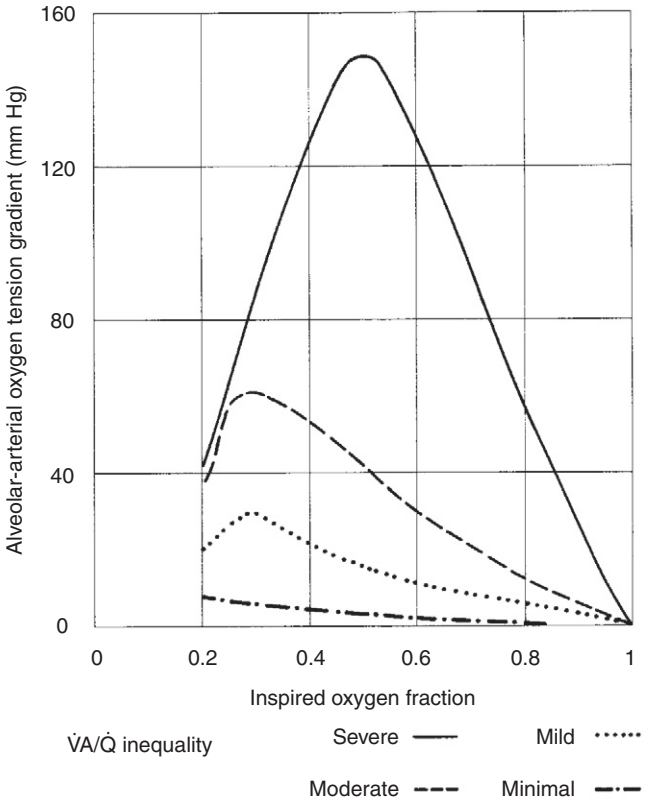


Figure 87-4 The effects of changes in inspired oxygen fraction, shown on the abscissa, on the calculated alveolar-arterial oxygen gradient are plotted for varying degrees of $\dot{V}A/\dot{Q}$ inequality without any coexisting shunt. *(From Dantzker DR: Physiology and pathophysiology of pulmonary gas exchange,* Hosp Pract (Off Ed) *15:135, 1986.)*

unique shape of the oxygen and carbon dioxide hemoglobin dissociation curves make precise quantification of exact patterns of $\dot{V}/\dot{Q}$ heterogeneity difficult to determine. Even the use of multiple inert gases measured together (see later discussion) has not eliminated completely uncertainty in $\dot{V}A/\dot{Q}$ distribution.

Nitrogen Gradient

When PAO_2 is low because of diminished alveolar ventilation (i.e., insufficient replacement of alveolar oxygen from inspiratory gas relative to the rate of removal by pulmonary capillary blood), the alveolar partial pressure of nitrogen (PAN_2) rises because during "no flow" states (end-expiration and end-inspiration) the sum of gas partial pressures in the alveolar spaces must equal atmospheric pressure. PH_2O and $PACO_2$ are relatively constant; therefore when PAO_2 is diminished, the PAN_2 must be increased. If the elevated PAN_2 is sustained, there will be increased absorption of nitrogen into pulmonary capillary blood and development of an arterial to alveolar nitrogen gradient ($aADN_2$), which has been used to estimate the presence of low $\dot{V}A/\dot{Q}$ lung regions. Perfusion of nonventilated lung units produces no increase in PAN_2, because the end-capillary and mixed venous partial pressures are the same for oxygen, carbon dioxide, and nitrogen, as well as any other inert gas solution. An $aADN_2$ gradient can develop because of the presence of either a small area of very low $\dot{V}A/\dot{Q}$ or a larger region of less imbalanced $\dot{V}A/\dot{Q}$, as long as $\dot{V}A/\dot{Q}$ is less than 1.0. Using the $aADN_2$ to indirectly estimate shunt ($\dot{V}A/\dot{Q}=0$) may result in an overestimation of shunt because of the sigmoidal shape of the oxygen-hemoglobin dissociation curve (i.e., small reductions in PAO_2 can disproportionately depress the arterial oxygen content when on the steep part of the curve).

Based on measurements of $AaPO_2$ and $aADN_2$ in healthy newborn infants, Krauss and colleagues[10,11] inferred that a relatively small low $\dot{V}A/\dot{Q}$ region occurred after 1 to 2 days of postnatal life. Furthermore the calculated $aADN_2$ in infants with respiratory distress syndrome showed little contribution from low $\dot{V}A/\dot{Q}$ areas.[12] This observation suggests that the large venous admixture occurring with respiratory distress syndrome results from intrapulmonary or extrapulmonary shunt.

Carbon Dioxide Gradient

Arterial to alveolar differences for carbon dioxide ($aADCO_2$) reflect areas of the lungs that are poorly perfused but have a large dead space because they receive a substantial fraction of minute ventilation ($\dot{V}E$). Mismatching of $\dot{V}A/\dot{Q}$ as a cause of carbon dioxide retention has been emphasized to explain the development of respiratory acidosis when minute ventilation is elevated above normal.[13] A small $aADCO_2$ exists in some newborn infants,[5] but when a normal FRC has been established, the small magnitude of

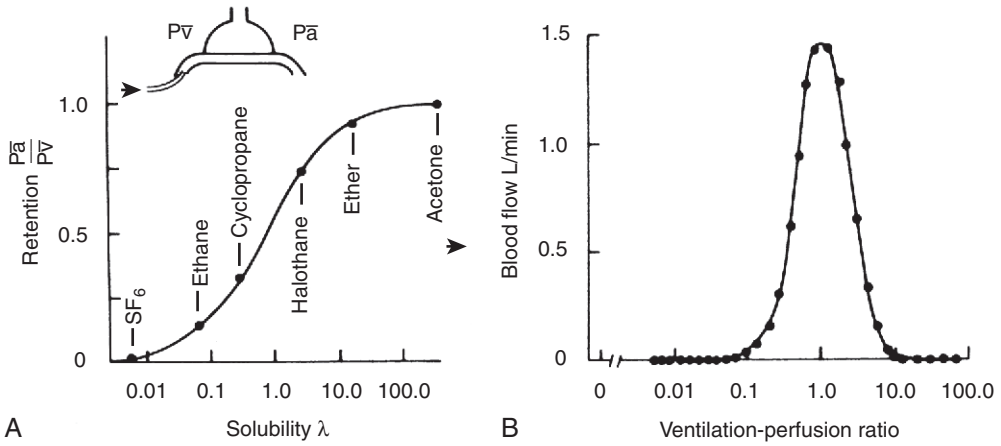


Figure 87-5 A, Retention solubility curve created by the experimental techniques referred to in text. Each of the six gases is plotted against its measured solubility. *Pa,* Partial pressure of each inert gas in arterial blood; *Pv,* partial pressure in mixed venous blood. **B,** The retention solubility curve can be used to derive a distribution of fractional perfusion to each of 50 theoretical lung compartments. *(From West JB: State of the art: ventilation-perfusion relationships,* Am Rev Respir Dis *116:919, 1977.)*

the gradient implies only a modest presence of high $\dot{V}A/\dot{Q}$ areas, except in premature infants.[5] Deficiencies in carbon dioxide exchange during respiratory disorders probably result from excess dead space ventilation with normal or increased tota 1 $\dot{V}E$.[14]

Trace Inert Gases Gradient

Trace quantities of inert gases can be used to quantitate the continuous distribution of ventilation/perfusion ratios from shunt to dead space ($\dot{V}A/\dot{Q}$ of 0 to $\dot{V}A/\dot{Q}$ to infinity).[15] Wagner and colleagues[16,17] described a technique that employs six inert gases (which encompass a wide range of solubility in blood) and in which retention of each gas in blood perfusing the lung can be expressed by the simple relationship:

$$Pa/Pv = Palv/Pv = \lambda/(\lambda + \dot{V}A/\dot{Q})$$

where λ = Ostwald blood-gas solubility coefficient (unique for each gas) and Pa, Pv, and Palv are the partial pressures of the gas measured in arterial blood, mixed venous blood, and alveolar gas (in reality measured in mixed expired gas and corrected for dead space ventilation). The equation assumes that each gas demonstrates a linear dissociation between blood or plasma and air, behavior unlike that demonstrated by the respiratory gases, oxygen ,and carbon dioxide.

Under conditions of presumed steady-state gas exchange, which is always an approximation of in vivo situations, the multiple inert gas elimination technique (MIGET) allows quantification of shunt, dead space, and low and high $\dot{V}A/\dot{Q}$ lung regions during both normal and experimental conditions. A major advantage of the technique is the quantification of shunt, separate from low $\dot{V}A/\dot{Q}$ areas, without the need to resort to 100% oxygen breathing. The experimental data consist of the measured ratios of the mixed arterial to mixed venous and mixed expired to mixed venous partial pressure for each of the gases employed (usually sulfur hexafluoride, ethane, cyclopropane, halothane, diethyl ether, and acetone) and the measured solubility of each gas (Figure 87-5).

Calculation of the standard deviation of pulmonary blood flow (SD $\dot{Q}p$) provides a quantitative index of dispersion of $\dot{Q}p$ about the mean $\dot{V}A/\dot{Q}$ value, separate from shunt and dead space.[18] Under experimental conditions, each of these assessments appears to be consistent with the others in providing semiquantitative estimates of the $\dot{V}A/\dot{Q}$ heterogeneity of the lung (Figure 87-6). Limitations of the inert gas technique include the lack of on-line analysis capacity for assessment of $\dot{V}A/\dot{Q}$ relations and the necessity of the assumption of steady-state gas exchange.[18]

Pulse Doppler Oximetry

The substitution of pulse Doppler oximetry saturation (SpO_2) for arterial PO_2 is seductive but can be misleading. The SpO_2 is precise, reproducible, harmless and painless to obtain. However, change in SpO_2 may not reflect concomitant change in PaO_2 but, rather, may reflect shifting position of the oxyhemoglobin dissociation curve. Acute increases in pH and decreases in PCO_2 can produce leftward shift of the curve, raising SpO_2 without changing PaO_2. Transfusion of adult hemoglobin into term and especially preterm infants will alter the P50, producing a rightward shift and a lower SpO_2 with no change in PaO_2. Lack of awareness of this potential discrepancy may result in inappropriate undertreatment or over-treatment in an effort to maintain SpO_2 in a narrow window. This limitation of SpO_2 predicting PaO_2 also limits the interpretation of the "physiological" diagnosis of bronchopulmonary dysplasia.[19]

Assumptions About Assessment of Ventilation and Perfusion

One important assumption is uniformity of the inspired gas composition. Inspired gas that is altered in composition by rebreathing of expired gas from more slowly emptying adjacent lung areas in the nonhomogeneously ventilated lung will produce series inequality of ventilation, which is difficult to differentiate from the more familiar parallel inequality.[20]

Although the composition of pulmonary arterial blood is relatively uniform, intraregional differences in hematocrit can affect gas exchange.[21] Gas exchange is inhibited when lung units with low $\dot{V}A/\dot{Q}$ are perfused by blood with high hematocrit. If high neonatal hematocrits predispose to greater pulmonary interregional variability, this effect could theoretically contribute to depressed oxygen exchange but the effect is likely minor in infants.

Alveolar end-pulmonary capillary partial pressure disequilibrium, as may occur during a shortened transit time across the pulmonary capillary bed, may limit transport of oxygen (see later discussion). Possibility of alveolar end-capillary disequilibrium for inert gases, with their rapid equilibrium time, is less than with respiratory gases.[22]

All the techniques used for gas exchange analysis have limits because of invasiveness, inaccuracy, reproducibility. They depend on assumptions about steady-state conditions, which in periods of rapid transient change in $\dot{Q}p$ or $\dot{V}A$, or in the presence of pulmonary disease, may not be true. During rapidly evolving disease states, the techniques may not reflect rapidly changing contributions to overall gas exchange inefficiency.[23]

PERINATAL TRANSITION AND GAS EXCHANGE

The decline of Ppa and PVR following birth occurs in conjunction with elimination of some of the intraparenchymal pulmonary fluid. Factors modulating the decline in Ppa include the endogenously produced arachidonate metabolite and vasodilator, prostacyclin,[24] and endogenously produced nitric oxide.[25] Mean Ppa does not approach adult values until days after birth. Wagner[26] has suggested that moderate elevation in Ppa may help maintain $\dot{V}A/\dot{Q}$ matching, given the relatively unstable lung volume of the neonate. However, elevations in PVR also favor passage of vascular fluid into the extravascular alveolar lung spaces, especially if the anatomic site of increased pressure is venular. Such fluid extravasation can have secondary effects on $\dot{V}A/\dot{Q}$ matching by altering bronchiolar dimensions (see Figure 87-3).

The investment of pulmonary arteries with their smooth muscle sheath during the third trimester creates the possibility of an inappropriately vigorous pulmonary vascular response to vasoconstrictive stimuli applied postnatally. Persistent pulmonary hypertension of the newborn (PPHN) may in some instances be a clinical consequence of this phenomenon. In support of this hypothesis, PPHN has been associated with the accumulation of leukotrienes in tracheal lavage fluid; leukotrienes, among many other substances, are potent vasoconstrictors.[27]

Two studies provided data on rate of change in pulse Doppler oxygen saturation in the minutes after delivery of healthy full-term infants.[28,29] Combining a large number of observations in term infants, Kamlin and colleagues[28] demonstrated a rise in SpO_2 from 60% at 1 minute after clamping of the cord (range: 38% to 82%) to a median of 84% by 5 minutes of age (Figure 87-7).[28] The authors note that saturation of greater than 90% was achieved at less than 4 minutes (median time of 2.5 minutes) and a saturation of greater than 90% was achieved in less than 7 minutes in term infants born either vaginally or by cesarean delivery. The previously noted limitations with the use of pulse Doppler oximetry and its correlation with arterial oxygen tension in these infants breathing ambient air also apply to these studies.

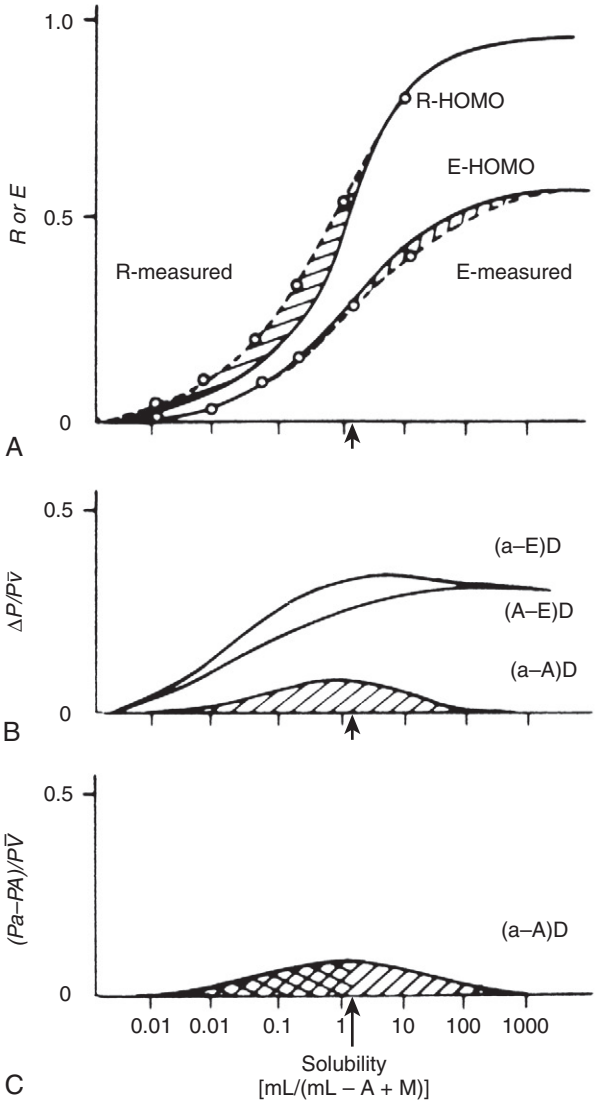


Figure 87-6 **A,** Measured and homogeneous retention *(R)* and excretion *(E)* curves are plotted against solubility, with R and E values shown as open circles. The homogeneous curves would occur if there were no $\dot{V}A/\dot{Q}$ heterogeneity in the lung (i.e., an idealized lung). Hatched areas between measured and homogeneous R and E curves occur because of $\dot{V}A/\dot{Q}$ heterogeneity. **B,** The sum of the areas under the aAD curve is shown. The area under the (a-A) D curve is calculated by subtracting the (A-E) (D) curve from the (a-E) D curve. *Arrow* on the abscissa indicates solubility that is numerically equal to mean $\dot{V}A/\dot{Q}$ in the lung. **C,** The area under the curve to the left of the mean $\dot{V}A/\dot{Q}$ represented by the double-crossed hatch area is an index of low $\dot{V}A/\dot{Q}$ lung areas. *(From Truog WE, Hlastala MP, Standaert TA, et al: Oxygen-induced alteration of ventilation-perfusion relationships in rats,* J Appl Physiol *47:1112, 1979.)*

FACTORS REGULATING VENTILATION-PERFUSION MATCHING IN THE DEVELOPING LUNG

Effects of Increased Pulmonary Arterial Pressure

Pulmonary arterial pressure (Ppa), which equals or exceeds systemic arterial pressure (Psa) during fetal life, declines normally in the hours surrounding birth to a level approximately 50% of Psa.

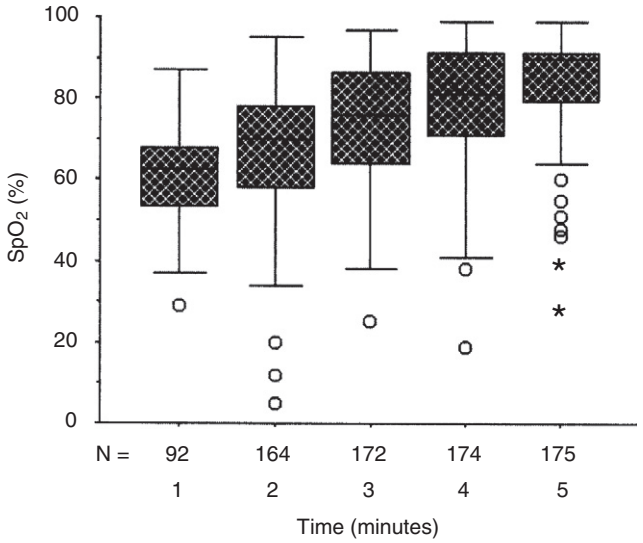


Figure 87-7 Box plots showing the median, quartiles, range (1.5 times the quartile on that side), outliers, and extreme values for SpO_2 at each minute after birth for the first 5 minutes (*N,* Number of patients in whom SpO_2 was obtained). A number less than 175 indicates that SpO_2 was not obtained in all cases. *(From Kamlin CO, O'Donnell CP, Davis PG, Morley CJ: Oxygen saturation in healthy infants immediately after birth,* J Pediatr *148:585, 2006).*

The further decline to adult levels occurs more slowly. Marked (suprasystemic) levels of Ppa can cause hypoxemia due to extrapulmonary shunt, as in PPHN. It is unknown, however, to what extent increased Ppa or persistent elevation of Ppa undermines or contributes to the stability of ventilation-perfusion matching in the neonatal lung during the normal transition to extrauterine life or during pulmonary diseases. Neonatal pulmonary diseases are commonly characterized by elevated Ppa, pulmonary vascular resistance (PVR, and marked vasoreactivity). The diseased neonatal lung may also demonstrate collapsed segments or lobes, which can interfere with $\dot{V}A/\dot{Q}$ matching.

An elevation in Ppa may change the intrapulmonary distribution of $\dot{Q}p$. Elevations in Ppa can occur either because of constriction of smooth muscle (or possibly nonmuscularized endothelial cells) lining the vessels that comprise the pulmonary microvasculature or because of an increase in left atrial pressure. Different vasoactive substances may mediate vasoconstriction at different sites within the microvasculature.[30] If a particular stimulus induces vasoconstriction only in previously dilated small muscular arteries and arterioles (which are conveying blood to well-ventilated areas), and not in already constricted vessels, the ratio in regional resistance of constricted vessels in nonventilated lung areas to resistance in previously dilated vessels in well-ventilated areas would be decreased. One result of this change in the ratio of local vascular resistance could be increased intrapulmonary right to left shunt (perfusion of nonventilated intrapulmonary regions) because of a redistribution of $\dot{Q}p$ favoring flow to nonventilated (hypoxic) regions.

Changes in Ppa are relevant to gas exchange in both the adult and newborn. However, in establishing and maintaining $\dot{V}A/\dot{Q}$ matching, neonates appear to respond differently from adults to certain stimuli affecting Ppa, PVR, and at least theoretically, bronchial and bronchiolar smooth muscle constriction. For instance, lambs have a more vigorous hypoxic pulmonary vasoconstrictive response than do adult sheep, and the onset of the response can be detected at a higher PAO_2.[31] If this phenomenon is also true in humans, it has implications in neonates regarding the effects of even brief apneic episodes on $\dot{V}A/\dot{Q}$ matching. Inferences about effects of the changes in Ppa in neonates with both regional and generalized lung injury cannot be easily extrapolated from results in adults.

Hypoxic Pulmonary Vasoconstriction and $\dot{V}A/\dot{Q}$ Matching

Hypoxic pulmonary vasoconstriction prevents localized mismatching of ventilation and perfusion by diminishing blood flow locally to those areas of the lungs that are underventilated or not ventilated at all. As regional alveolar and acinar PO_2 decline, due to reduced localized ventilation, blood flow through the local pulmonary microvasculature is inhibited by an increase in resistance in muscular small arteries. Blood flow and presumably alveolar ventilation are then redirected into well-ventilated lung regions. The mechanisms by which a decrease in local PO_2 induces constriction of vascular smooth muscle remain incompletely understood. Many modulators, including vasoconstrictive leukotrienes, have been described.[32] However, mediation of this response, as opposed to modulation, may depend on changes in calcium and potassium selective membrane channels in the pulmonary vascular smooth muscle cell membrane.[33]

When regional alveolar hypoxia is created by lobar or segmental atelectasis induced by endobronchial balloon obstruction, a relationship exists between the increase in resistance and increase in overall intrapulmonary shunt (Figure 87-8). Marshall[34] showed that with progressively larger areas of pulmonary parenchymal collapse, up to unilateral atelectasis, the diversion of $\dot{Q}p$ away from atelectatic lung areas and the capacity to maintain satisfactory $\dot{V}A/\dot{Q}$ matching becomes less effective (see Figure 87-8). These studies are particularly relevant to the neonatal condition, in which both lobar and multiregional atelectasis can develop.

In addition to the size of the collapsed and nonventilated segment as a modifier of hypoxic pulmonary vasoconstriction and $\dot{V}A/\dot{Q}$ matching, the presence of inflammatory cells and their products in the affected region has been addressed. Secretions containing bacteria and inflammatory cell products can become trapped in the atelectatic region, possibly inhibiting local hypoxic pulmonary vasoconstriction, resulting in persistent regional blood flow and hypoxemia.

Global or total lung exposure to alveolar hypoxia produces a different challenge for control of pulmonary gas exchange. With these circumstances is the potential for an increase in regional $\dot{Q}p$ to preexisting shunt or low $\dot{V}A/\dot{Q}$ regions. Superimposition of global alveolar hypoxia produces increased resistance to $\dot{Q}p$ in the regions in which resistance beds will become more balanced, with redirection of $\dot{Q}p$ back into low $\dot{V}A/\dot{Q}$ areas. However, in lambs exposed to acute alveolar hypoxia ($FIO_2 = 0.12$), no evidence shows an alteration in $\dot{V}A/\dot{Q}$ matching in spite of a doubling (beyond room air baseline values) of Ppa and PVR.[35] Hansen and colleagues[36] demonstrated redistribution of $\dot{Q}p$ in neonatal lambs during acute alveolar hypoxia, providing indirect evidence of heterogeneity of local vascular resistance during elevated PVR.[36]

One effect of increasing Ppa is the recruitment of pulmonary capillaries, particularly in upper lobes. This process increases gas exchange surface area and represents a "reserve" mechanism to help sustain pulmonary gas exchange during adverse conditions. However, evidence shows that neonatal lambs have an already fully recruited microvasculature, at least as assessed by direct vital microscopy during hypoxia.[37] Indirect evidence for full capillary recruitment present under baseline conditions has also been inferred from work in piglets.[38] If these findings can be generalized to newborn humans, it represents another instance of the decreased reserves available to sustain gas exchange and points to a relative lack of vascular compliance in the neonatal lung.

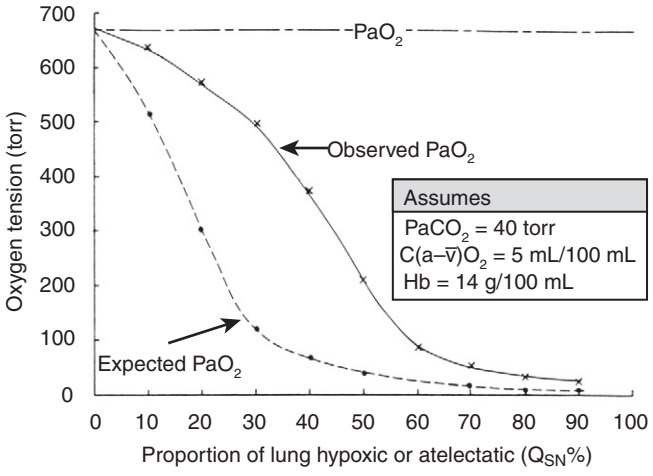


Figure 87-8 The effect of progressively increasing size of atelectatic lung region on arterial oxygen tension is demonstrated by the dashed line, which would occur if no redistribution of blood flow away from atelectatic area occurred. The solid line indicates effects of presumed hypoxic pulmonary vasoconstriction. Note the conversion of the two lines when more than 40% of the lung develops atelectasis. The assumptions regarding O_2 content (CaO_2) are shown in the inset. *(From Marshall BE: Importance of hypoxic pulmonary vasoconstriction with atelectasis,* Adv Shock Res *8:1, 1982.)*

The role of acute hypoxic vasoconstriction in the maintenance of ventilation-perfusion matching has been tested in neonatal animals subjected to 3 days of breathing more than 90% inspired oxygen.[39] This was done to determine if hyperoxia-induced blunting of the vascular response to acute alveolar hypoxia ($FI_O2 = 0.12$) would be associated with worsened $\dot{V}A/\dot{Q}$ matching. Preexposure of animals to hyperoxia did result in diminished hemodynamic response and in deterioration in $\dot{V}A/\dot{Q}$ matching during exposure to acute vasoconstrictive stimuli, such as alveolar hypoxia.

Possible genetic contributions to postnatal regulation of gas exchange in a relatively hypoxic environment due to high altitude have been examined in Tibetan infants.[40] Comparisons of SpO_2 were made in healthy infants born at 3300 meters (~11,800 feet) and normalized for activity state. The infants of the Tibetan (multigenerational) dwellers at altitude demonstrated consistently higher SpO_2 than infants of the Han (recent dwellers) during all relevant activity states (Figure 87-9). As shown, Han infants consistently demonstrated lower O_2 saturation than Tibetan children, suggesting a role for genetics in contributing to hypoxia. Comparisons of SpO_2 were from values obtained during active sleep, the behavioral state associated with irregular breathing effort and reduced FRC because of changes in intercostal muscle tone. Multiple factors contribute to maintenance of SpO_2; the relevant ones that contributed to these findings could not be evaluated. However, the findings suggest that susceptibility to pulmonary hypertension may be genetically determined, even in very young infants.

Vasoactive Mediators Influencing Pulmonary Vascular Resistance and $\dot{V}A/\dot{Q}$ Matching

Stimuli that produce an acute elevation in Ppa may impair $\dot{V}A/\dot{Q}$ matching in neonates. Vasoactive peptides and phospholipid substances are two classes of such agents. These substances are synthesized and released into the circulation in response to bacterial infusion or endotoxin infusion. Their effect on PVR and gas exchange varies with species and with postnatal age. Intravenous infusion of *Escherichia coli* endotoxin into adult goats produced a 200% to 250% rise in Ppa and PVR, yet was associated with a small decrease (of 8 to 9 mm Hg) in PaO_2.[41] Infant or neonatal animals demonstrate a response different from that of adults. In piglets, despite a similar rise in Ppa, as seen in adult animals, infusion of either group B streptococcus or other bacteria resulted in a 30 to 40 mm Hg decline in PO_2.[42,43] This decline was associated with a generalized mismatching of $\dot{V}A/\dot{Q}$, with the development of low $\dot{V}A/\dot{Q}$ areas, but not with an increase in intrapulmonary shunt.[44] The elevation in Ppa induced by bacterial products correlated with increased plasma levels of the vasoconstrictor arachidonic acid metabolite, thromboxane A_2, implying that arachidonate products can alter distribution of perfusion and ventilation.

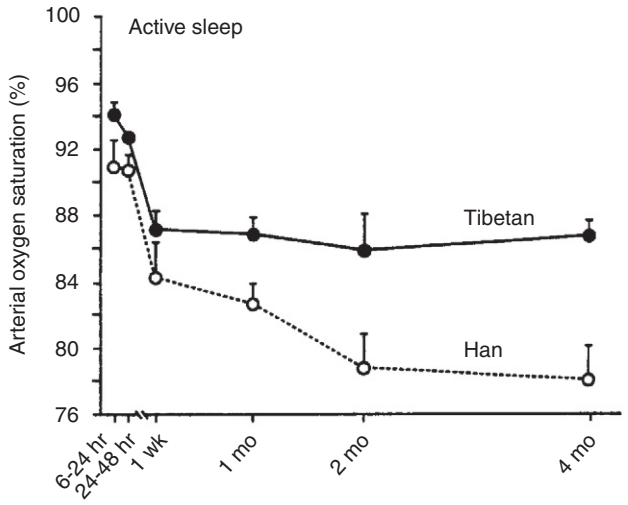


Figure 87-9 Oximetry recorded in multigenerational dwellers' infants at high altitude (Tibetan) versus single generation dwellers' infants (Han). Data were obtained during active sleep in apparently healthy term infants. *(From Figure 1 of Niermeyer S, Yang P, Shanmina, et al:* N Engl J Med *333:1248, 1995.)*

The young piglet has served as a useful model for examining the effects on pulmonary gas exchange of potential antiinflammatory or antiinfective therapies, including antivasoactive agents. After pretreatment with a thromboxane synthase inhibitor, no immediate increase in Ppa, decrease in PaO_2, nor development of $\dot{V}A/\dot{Q}$ mismatching[45] during an infusion of group B streptococcus in piglets was observed. When administered after 2 hours of bacterial infusion, inhibitors of thromboxane synthase decreased Ppa and PVR but did not improve inert or respiratory gas exchange.[46] These findings imply that regulation of hemodynamics and the regulation of $\dot{V}A/\dot{Q}$ matching become increasingly independent phenomena under conditions of ongoing bacterial product stimulation. Gibson and colleagues[47] demonstrated in experimental animals during controlled bacteremia that multiple vasoactive agents participate in the persistence of elevated PVR.

Catecholamines form another class of substances that can alter $\dot{V}A/\dot{Q}$ matching in the neonate by increasing Ppa. Truog and Standaert[35] demonstrated that when dopamine (25 μg/kg/min) is administered to neonatal lambs in which Ppa and PVR are increased due to hypoxic gas ($FIO_2 = 0.12$), a further rise is observed in Ppa, coupled with a deterioration in gas exchange associated with an increase in both intrapulmonary shunt and $\dot{V}A/\dot{Q}$ mismatching, separate from shunt.

Products of the endothelium, endothelin and nitric oxide, participate in regulation of $\dot{Q}p$ by changing local vasoconstriction and hence local vascular resistance.[48] The vasoconstricting effects of endothelin-1 are augmented and the vasodilating effects are attenuated with increasing age in sheep.[49] Vasodilating effects are mediated in part by nitric oxide. Administration of inhaled nitric oxide can result in a simultaneous reduction in PVR and an improvement in PaO_2 in patients with diffuse parenchymal lung diseases, such as ARDS.[50,51] Effects on each (decrease in PRV, V/Q) are dose-related, suggesting different physiologic mechanisms. Other investigators have failed to show significant change in respiratory or inert gas exchange during treatment with inhaled nitric oxide in animals with pulmonary hypertension caused by bacterial infusion.[52]

The effect of elevated Ppa on gas exchange can be difficult to predict in the clinical setting of a neonate with one of many possible causes of respiratory distress. In addition to the disorders discussed earlier, elevation of Ppa may result from a diminished cross-sectional microvascular area secondary to either a primary developmental failure or delayed growth (or inappropriate regression of) the microvasculature. The diminished cross-sectional area increases vascular resistance and results in an extrapulmonary shunt or failure to redistribute $\dot{Q}$ or to recruit lung vessels. Further, presumably a spectrum of local resistances exists across the pulmonary microvasculature shortly after the time of birth, as long as lung fluid is being removed from potential gas-exchange areas. Sudden vasoactive stimulation may result in a redistribution of $\dot{Q}p$, $\dot{V}A/\dot{Q}$ mismatching, and arterial hypoxemia, producing a vicious cycle with diminished pulmonary PVO_2 and further decline in PaO_2. Low mixed venous PO_2 is a secondary stimulus inducing pulmonary arterial hypertension.[53] In situations characterized by low cardiac output and $\dot{V}A/\dot{Q}$ mismatching, the low PVO_2 increases Ppa and PVR. Intrapulmonary shunt or extrapulmonary shunt through the foramen ovale or ductus arteriosus

may result. Measurement of simultaneously obtained pulmonary venous and preductal and postductal arterial PO_2 would be necessary to differentiate among the anatomic sites for the shunt.

Distribution of Ventilation and $\dot{V}A/\dot{Q}$ Matching

Although many studies have examined postnatal changes in lung mechanics, less information is available describing the distribution of ventilation.[54] Analysis of $\dot{V}A$ distribution by nitrogen or xenon washout during 100% oxygen breathing is difficult to perform in sick (or well) neonates because of the need for multiple breath analysis and exposure to radioactivity. However, analysis of $\dot{V}A$ distribution does demonstrate at least two types of acinar spaces, which empty at different rates.[54] The pulmonary function measurement, pulmonary clearance delay, quantitates the presence of these two acinar compartments. It is difficult to draw conclusions about $\dot{V}A/\dot{Q}$ matching from this index because it relates only to ventilation irrespective of relative distribution of $\dot{Q}p$. Engel[55] has reviewed the theoretical and experimental evidence for incomplete alveolar gas mixing with inspiration that develops because of the interaction between diffusion and convection at airway branch points subtending branches of unequal length. It is unknown if this problem is diminished or magnified in the much smaller neonatal lung or to what extent the resulting inhomogeneous alveolar gas mixtures contribute to overall $\dot{V}A/\dot{Q}$ heterogeneity in the neonate. Much would depend on the matching of intraacinar Q distribution and intraacinar V distribution.

The immature lung, with its delayed and variable distal airway and acinar development has not been well studied with regard to the contribution of "regional" $\dot{V}A/\dot{Q}$ inequality to overall gas exchange abnormalities. Similarly, $\dot{V}A/\dot{Q}$ scans that rely on insoluble radioactive tracers injected intravenously may inaccurately portray $\dot{V}A$ or $\dot{Q}p$ distribution because of both shunt and spatial restrictions imposed by the small chest of the neonate or young infant. Factors that influence bronchial and bronchiolar constriction can alter the distribution of each inhaled breath. Some factors relevant in young infants are listed Box 87-1.

Cardiac Function and PVR

An additional factor influencing PVR is the diminished cardiac reserve of neonates, especially premature neonates, who depend largely on chronotropy for raising cardiac output. Older children or adults, by contrast, have effective inotropic mechanisms for increasing cardiac output. The newborn has difficulty doubling the resting cardiac output either by pharmacologic stimulation or by increased sympathetic nervous system activity. Thus efforts to increase PaO_2 (assuming constant tissue oxygen extraction and an unchanging degree of $\dot{V}A/\dot{Q}$ mismatching) by increasing cardiac output (and PVO_2) are not usually successful in the newborn.

NITRIC OXIDE AND VENTILATION PERFUSION MATCHING

Recent investigations have produced a markedly increased appreciation for the complexity of endogenously produced nitric oxide (NO) and its role in regulation of many pulmonary functions, including that of pulmonary gas exchange, specifically ventilation perfusion matching and regulation of pulmonary vascular reactivity.[56,57] Evidence is now available that NO regulates pulmonary function by cGMP-independent mechanisms. Endogenous NO was originally thought to have its primary mode of action by activation of guanylyl cyclase, altering vascular smooth muscle intracellular concentration of cGMP and, by doing so, regulating local calcium concentration and actin-myosin contractility. Evidence is growing that an equally important pathway for NO signaling in the respiratory system is through the S-nitrosothiol (SNO) system. NO synthase activity leads directly to SNO formation and, with the creation of SNO radicals, results in signaling that helps regulate ventilation perfusion matching, pulmonary arterial pressure, and respiratory muscle function. A schematic summary of some of the pathways pertinent to pulmonary gas exchange is shown in Figure 87-10.[56] The contribution of SNOs in bronchial or epithelial lining fluid and in the pulmonary arterioles and erythrocytes in the pulmonary microcirculation provides a exquisitely sensitive level of regulation for small changes in airway muscular tone and lumen size, thus promoting local ventilation perfusion matching. Alterations in SNO metabolism resulting in deficient SNO, especially in the airways, can be associated with bronchoconstriction and overall change in the airway smooth muscle tone and/or change in pulmonary and systemic vascular resistance. Future studies of the therapeutic efficacy to toxicity ratio of NO or NO donor administration must account for SNO and other NO metabolites and changes in endogenous NO production and availability.[57] Few studies in human neonates to date have provided insight into how endogenous NO biology may be altered in important clinical disorders, such as PPHN or bronchopulmonary dysplasia (BPD).

DIFFUSION AND PULMONARY GAS EXCHANGE

Respiratory gas exchange depends on prompt diffusion of respiratory gases between the tissue and plasma, erythrocyte and plasma, plasma and resident alveolar gas, and alveolar gas and gas in the conducting airways. Of these, alveolar-capillary membrane diffusion has been considered the most likely barrier to gas exchange, but it has never been well documented as abnormal in any neonatal condition, including extreme prematurity, when corrected for the size of the gas-exchange surface area available.

Gas movement between alveolar space and pulmonary capillary blood is a passive process, summarized by Fick's law, which asserts that the amount of gas transferred is governed by the partial pressure of that gas in the two compartments, the inverse of the square root of the molecular weight of the gas, and the specific characteristics of the diffusion barrier (thickness and surface area). Thus the transport of gas is expressed by $\dot{V}$ <pi>pt $Dm(PA - P_b)$ where Dm = alveolar-capillary membrane diffusion conductance and $(PA - P_b)$ represents the partial pressure difference across the membrane. To simplify the analysis, it is useful to study a single gas that binds firmly to hemoglobin. Because carbon monoxide combines avidly with hemoglobin, the PCO in pulmonary capillary plasma is virtually zero when small quantities of carbon monoxide are inspired, simplifying the analysis of

Box 87-1

Some Factors Capable of Producing Bronchoconstriction or Bronchiolar Narrowing in the Newborn

Airway inflammation
Mucosal edema
Excess mucous secretion
Sloughing of damaged epithelial cells
Hyperreactive bronchial smooth musculature
Inspiration of cold, dry gas
Increase in parasympathetic nervous system stimulation
Congenital airway stenosis or deficient cartilage development
Bronchiolar narrowing secondary to vascular engorgement
Presence of any foreign body, including partially occluded endotracheal tubes
Trauma from suction catheters
Aspiration of stomach contents into upper airway
Hypoosmolar or hyperosmolar solution in the airway

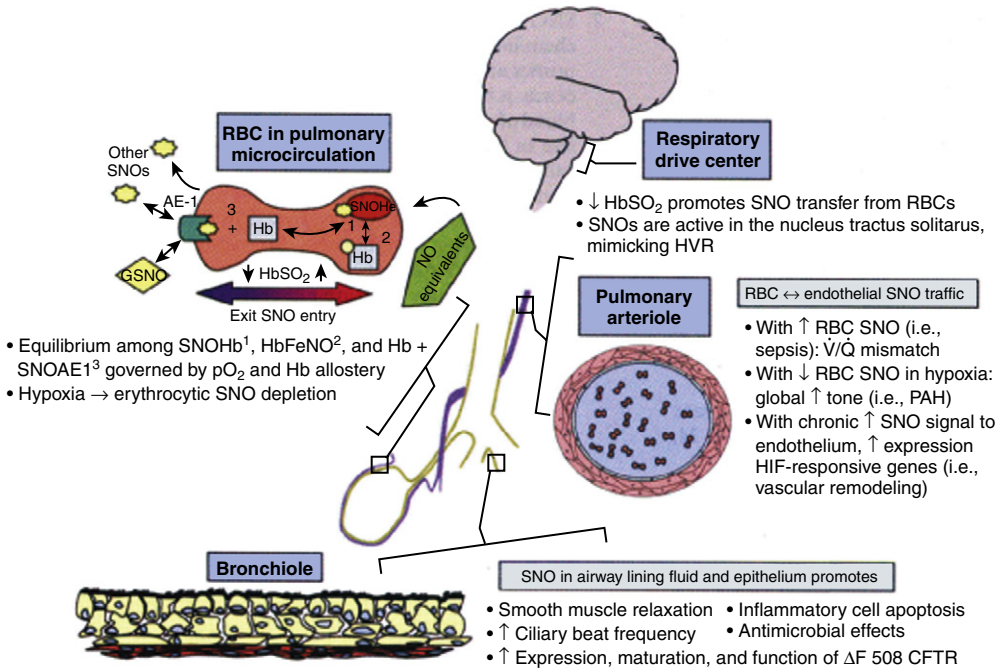


Figure 87-10 Overview of S-nitro-sothiol (SNO) signaling in the respiratory system. SNO signaling affects respiratory control, airway function, and pulmonary vascular tone. *AE1,* Anion exchange protein 1 on the erythrocyte membrane; *CFTR,* cystic fibrosis transmembrane regulatory protein; *GSNO,* S-nitrosoglutathione; *Hb,* hemoglobin; *HbFeNO,* hemoglobin iron nitrosyl; *$HbSO_2$,* oxyhemoglobin saturation; *HIF,* hypoxia inducible factor; *HVR,* hypoxic ventilatory response; *PAH,* pulmonary arterial hypertension; *RBC,* red blood cell. *(From Gaston B, Singel D, Doctor A, Stamler JS: S-nitrosothiol signaling in respiratory biology,* Am J Respir Crit Care Med *173:1186, 2006).*

diffusing capacity. In clinical practice, the diffusing capacity of the lung is expressed by the following:

$$DmCO = \dot{V}CO / PA_{CO}$$

The diffusing capacity of the lung for carbon monoxide is expressed as the volume of carbon monoxide transferred in milliliters of carbon monoxide per minute per millimeter of mercury of alveolar partial pressure of carbon monoxide. Diffusing capacity is usually abbreviated as D_{LCO}. Both a single-breath technique (which is difficult to perform in a neonate) and a steady-state technique can be used for assessing PA_{CO}.

Use of carbon monoxide as the marker gas does not eliminate other variables in interpretation. Ventilation-perfusion heterogeneity, reduced pulmonary capillary transit time, lung volume, and pulmonary capillary blood volume all can affect interpretation of diffusing capacity, as well as intrinsic properties of the alveolar capillary membrane itself—the actual subject of the measurement. Detailed analysis of the effects of these factors on diffusion measurement is available elsewhere.[58] Comparing serial measurements of D_{LCO} assumes that effective $\dot{Q}p$ ($\dot{Q}p_{total} - \dot{Q}p_{shunt}$) is unchanged. This may represent an insupportable assumption in many neonatal pulmonary diseases. Rapid postnatal lung growth and alveolarization, with change in lung volumes, complicates interpretation of serial measurement of diffusion capacity in the neonatal lung. However, Escourrou and colleagues[59] inferred from cross-sectional studies using MIGET in neonatal and infant piglets that diffusion limitation may contribute to early postnatal hypoxemia in animals breathing room air. Either a reduction in surface area or an increase in thickness of the alveolar capillary membrane can reduce D_{LCO}. Although these conditions may occur in neonatal pulmonary diseases, they probably have little practical significance for transport of oxygen because virtually every pulmonary disease is treated with increased PAO_2. Hence, the driving pressure (partial pressure gradient) across the membrane becomes very large for oxygen. Even in these circumstances, there may be lung regions in which diffusion disequilibrium may occur because neonatal pulmonary conditions rarely affect the lung uniformly.

Diffusing capacity for carbon monoxide has been measured acutely in premature infants with and without respiratory distress syndrome and no significant differences have been found.[60] However, D_{LCO} was notably lower than D_{LCO} values measured in earlier studies conducted in full-term healthy infants.[61] The different results may relate to the smaller quantity of both intrapulmonary gas and blood found in the premature newborn. The differences highlight the problems in interpretation of cross-sectional studies of D_{LCO} in the newborn.

Perhaps the most important diffusing capacity measurements are made after recovery and growth. Hakulinen and colleagues[62] made the observation that children born very prematurely and studied at age 7 to 11 years demonstrated modest but significant reduction in D_{LCO} (single breath). This was true for premature infants with or without the diagnosis of BPD. Some "normal" values for D_{LCO} may have overestimated D_{LCO} in some of these children because airflow obstruction, which was common in the preterm infants, may have produced an overestimation of the true D_{LCO}.

IMPAIRED GAS EXCHANGE IN BRONCHOPULMONARY DYSPLASIA

Principles of pulmonary gas exchange discussed in the above sections are supplemented with a brief review of gas exchange abnormalities arising from an important clinical disorder, BPD or

TABLE 87-2

Bronchopulmonary Dysplasia and Impaired Gas Exchange

Abnormality	Effect
Distal airway simplification	Increase in high $\dot{V}/\dot{Q}$ areas and increase in dead space
Bronchial lining thickening	Maldistribution of $\dot{V}$ E
Decrease in capillary network	Increase in high $\dot{V}/\dot{Q}$ areas
Mesenchymal location of pulmonary capillary bed	Decrease in diffusing capacity
Pulmonary to bronchial venous connections	Increased intrapulmonary shunt
Anemia, decreased systemic venous O_2 saturation, decreased Pv O_2	Increased risk of end capillary-alveolar disequilibrium
Altered metabolic function in the lung	Increased Ppa and PVR

chronic lung disease of prematurity. BPD is characterized by multifocal radiographic evidence of lung injury, by postmortem findings of airway and alveolar/acinar simplification with disordered development of the pulmonary microvasculature. A physiological definition of BPD relies on assessment at 36 weeks postmenstrual age. The FIO_2 necessary to maintain SpO_2 of ≥90% is assessed at that age. Although the focus is on impairment in oxygen exchange, alveolar ventilation is also impairment as assessed indirectly by serum bicarbonate measured at the same postmenstrual age. Minute ventilation is normal or increased,[63] so the finding of elevated PCO_2 or serum bicarbonate indicates alveolar hypoventilation and increased high $\dot{V}/\dot{Q}$ areas. Reasons for impairment both in O_2 and CO_2 exchange include the development of intrapulmonary shunt, of both and high low $\dot{V}/\dot{Q}$ areas, possibly diffusion disequilibrium, depending on other circumstances as listed (Table 87-2). Even when infants reach near-term equivalent postmenstrual age, impairment in CO_2 elimination remains. In preterm infants with moderate BPD (FIO_2 ≥0.3) compared with age-matched infants with no BPD and using serum bicarbonate as a proxy for PCO_2, impairment in CO_2 elimination remains.

Infants requiring FIO_2 ≥0.3 at near-term equivalent age are at increased risk for recurrent hospitalization for pneumonia and for reactive airway disease, and the long-term consequences of their pulmonary insufficiency as measured at 36 weeks remain yet to be well-defined. The very profound changes in small airway function, documented in children years after "recovering" from BPD provide a warning to work to reduce the gas exchange inefficiencies accompanying profoundly preterm births.[64]

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Maria Delivoria-Papadopoulos and Jane E. McGowan

Oxygen Transport and Delivery

OXYGEN TRANSPORT SYSTEM

The oxygen transport system in humans depends on many interrelated factors, including the fraction of oxygen in inspired air, partial pressure of oxygen in inspired air, alveolar ventilation, relation of ventilation to perfusion of the lungs, arterial blood pH and temperature, cardiac output, blood volume, hemoglobin concentration, and affinity of hemoglobin for oxygen. In normal persons, this complex system, which adjusts to tissue requirements to maintain an adequate end-capillary oxygen tension, has a reasonable reserve capacity and the ability to respond rapidly to changes in oxygen need. Gestation and the immediate postnatal period tax the oxygen transport system more than does any other time period in the human life cycle. In addition to the factors just listed, fetal oxygenation also varies with transplacental oxygen transport—which in turn depends on placental perfusion and Pvo_2 in the uterine vein, thus indirectly reflecting the state of the maternal cardiorespiratory system. Oxygenation of the infant at birth requires a successful transition from fetal to newborn circulatory patterns and establishment of adequate alveolar respiration.

Aerobic metabolism is critically dependent on a constant and adequate supply of oxygen. Although molecular oxygen participates in numerous types of oxidative reactions necessary for cellular metabolism (e.g., production of prostaglandins mediated by cyclooxygenase), its primary role is as the final electron acceptor in the mitochondrial respiratory chain—the process by which energy produced by glycolysis and the citric acid cycle is stored in high-energy phosphate bonds in the form of adenosine triphosphate (ATP). Although ATP is produced during anaerobic glycolysis, the delivery of oxygen to allow aerobic metabolism results in a more than 15-fold increase in the quantity of ATP produced from glucose metabolism. The diffusion of oxygen from midcapillary to the cell is the last step in oxygen transport and depends on several factors, including the oxygen pressure gradient between capillary and cell, the distance between the closest perfusing capillary and the cell, and the impedance to diffusion of the tissue, as reflected by the *diffusion coefficient.* Mitochondrial oxygen supply in vivo ultimately depends on a number of factors, including the distance between the closest perfusing capillary and the cell, the tissue impedance to oxygen diffusion, and the oxygen pressure gradient between capillary and mitochondrion. The pressure gradient, which directly affects mitochondrial oxygen uptake, varies with regional oxygen delivery, tissue oxygen consumption, and the hemoglobin-oxygen affinity. In vitro, mitochondrial function remains at maximal levels at oxygen pressure (Po_2) as low as 0.5 mm Hg; however, a "critical" Po_2 in vivo, below which mitochondrial respiration is compromised, is likely.[1]

Under basal conditions, the lungs load approximately 4 mL of oxygen/minute/kg of body mass onto hemoglobin but can increase this rate 15-fold through input from the carotid and aortic bodies (which sense arterial oxygen content) and brain stem chemoreceptors. Arterial blood transports oxygen from the pulmonary capillaries to the tissues. The oxygen content of the arterial blood usually is high enough to meet cellular oxygen demand. When the oxygen content is decreased, however, local perfusion or hemoglobin oxygen affinity may change to compensate for the lower oxygen content.

The cardiovascular system regulates oxygen supply through variation in cardiac output and distribution of blood flow. Alterations in the metabolic rate of peripheral tissues activate local regulatory mechanisms that modulate arterial blood flow and venous return and, consequently, cardiac output. The distribution of blood flow to specific tissues and organs also is set by local metabolic activity. Accordingly, different controls exist for different tissues.[2] Coronary blood flow, for example, reflects the metabolic activity of heart muscle; because the oxygen extraction of cardiac muscle normally is high, changes in cardiac work

must be matched closely by concomitant changes in coronary blood flow. When oxygen supply is limited, flow is reduced to tissues with low oxygen extraction (such as kidney and gut) in favor of tissues with high extraction (such as heart and brain). The high flow–low extraction areas of the circulation constitute an oxygen reserve system that may be deployed in times of oxygen deprivation.

By contrast, cardiac output does not appear to be directly responsive to moderate changes in either Pa_{O_2} or blood oxygen content[3] (presumably because other mechanisms provide an adequate adjustment) and is virtually unaffected by an increase in Pa_{CO_2} to 50 mm Hg. Blood viscosity and volume are additional determinants of cardiac output. If volume is kept constant and viscosity is altered (e.g., by a change in hematocrit), a reciprocal change in cardiac output occurs. Conversely, cardiac output varies directly with blood volume, presumably through the effect of volume on venous return. The induced alterations in cardiac output that follow changes in viscosity and blood volume are corrected toward normal by subsequent compensatory changes in peripheral vascular resistance. A number of neurohumoral factors also affect cardiac output by direct action on the heart, as reflected in the strength and especially the rate of cardiac contraction.

Hemoglobin concentration is regulated by a renal sensing mechanism that operates to maintain a balance between oxygen supply and oxygen requirement of renal tissues. A decrease in concentration or arterial oxygen saturation of hemoglobin or any increase in hemoglobin affinity for oxygen causes increased erythropoietin production through increased expression of hypoxia-inducible factor (HIF).[4,5] The effect of erythropoietin on bone marrow usually is limited by available iron, so that red blood cell production is stimulated to approximately twice its basal value of 1% of the total red blood cell mass per day. Consequently, red blood cell mass increases slowly in response to hypoxia.[6] Because it increases blood viscosity, a higher hemoglobin concentration at the same total blood volume reduces blood flow and hence oxygen delivery. Normal cardiac output is reestablished by increasing plasma volume proportionately (i.e., by increasing total blood volume).[7]

The affinity of hemoglobin for oxygen, in association with flow distribution, translates oxygen flow into oxygen availability. This characteristic of hemoglobin is classically depicted in the oxygen dissociation curve (oxygen equilibrium curve). Because of its remarkable ability to combine reversibly with large quantities of oxygen, hemoglobin increases the oxygen transport capacity of blood approximately 70-fold over that of oxygen transported dissolved in plasma. For example, if the entire oxygen requirement of the maternal organism had to be met by physically dissolved oxygen, the required cardiac output would be 100 L/minute.

STRUCTURE OF THE HEMOGLOBIN MOLECULE

The hemoglobin molecule contains four heme groups bound to the protein globin. The heme groups, located in crevices near the exterior of the molecule, consist of an organic moiety, protoporphyrin, and an iron atom. The iron in heme binds to the four nitrogens in the center of the protoporphyrin ring (Figure 88-1). The four oxygen-binding sites of hemoglobin are relatively far apart, the distance between the two closest sites being 2.5 nm.

The primary structure of the hemoglobin molecule is genetically determined by the amino acid sequence of the globin chains. The three basic chain structures most important in humans are the α-chain (with 141 amino acids), β-chain (with 146 amino acids), and γ-chain (with 146 amino acids), which together form hemoglobin A (Hb A), with a makeup of $\alpha_2\beta_2$, and hemoglobin F (Hb F), which is $\alpha_2\gamma_2$. The β- and γ-chains differ from each other by only a few amino acid residues. Nine amino acid positions in the sequence are recognized to be the same in all or nearly all species studied thus far. These conserved positions are involved either in the oxygen-binding site directly or in forming hydrogen bonds between helices. The positions of the nonpolar residues in the interior of hemoglobin vary considerably, but substitutions always involve one nonpolar residue for another, thereby conserving the striking nonpolar character of the molecule's interior. Eight α-helices, by convention designated A to H, constitute the secondary structure of the hemoglobin molecule. Helices E and F form a hydrophobic cleft at the insertion of the heme moiety. The position in space of the α-helices with respect to one another provides tertiary structure, whereas the spatial relationship of the subunit globin chains provides the quaternary structure of the hemoglobin molecule.

The quaternary structure of deoxyhemoglobin is termed the *T* or *tense form,* whereas that of oxyhemoglobin is the *R* or *relaxed form.* X-ray crystallographic studies have confirmed that oxygenated and deoxygenated hemoglobin differ in their conformation, with the oxygenated form being more compact.[8] In addition, these conformational changes were found to be crucial for the interactions of hemoglobin with organic phosphates[9] (see later discussion).

In the T form (deoxyhemoglobin), the iron atom is pushed out approximately 0.6 nm from the heme plane because of steric repulsion between the proximal histidine and nitrogen atoms of the porphyrin. On oxygenation, the iron atom moves into the plane of the protoporphyrin ring, forming a strong bond with oxygen. Oxygenation of the first heme group then favors a switch in the quaternary structure from T to R through the proximal histidine group. The heme group and proximal histidine residue make intimate contact with 15 side chains; oxygenation causes

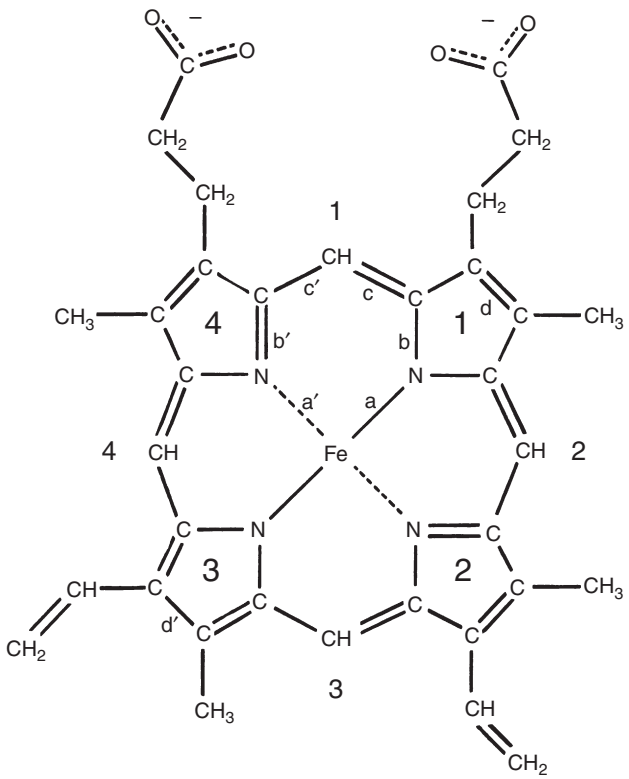


Figure 88-1 The heme group is an essential component of hemoglobins, cytochromes, and enzymes such as catalase and peroxidase. The central porphyrin ring has various side chains: methyl, —CH_3; vinyl, —CH—CH_2; and propionic acid, —CH—CH_2—COOH.

structural changes in the F helix, the EF corner, and the FG corner. These changes are then transmitted to the subunit interfaces (Figures 88-2 and 88-3). The expulsion of a tyrosine residue from the pocket between the F and H helices leads to the rupture of interchain salt links. Consequently, on oxygenation of the first heme group, the equilibrium is shifted in favor of the R form. Each subsequent molecule of oxygen is bound more strongly because fewer salt links remain to be broken. Because each hemoglobin molecule (tetramer) contains four iron atoms, it can combine with four oxygen molecules. Each mole of hemoglobin thus can combine with four moles of oxygen, for a normal blood oxygen capacity of 7.76 mM/L (i.e., 1.94 mM of hemoglobin/L × 4 = 7.76). Expressed alternatively, 1 g of hemoglobin can combine with 1.368 mL of oxygen for an oxygen-carrying capacity of approximately 20 mL/dL when the hemoglobin concentration is 15 g/dL[10] (as detailed in the following section).

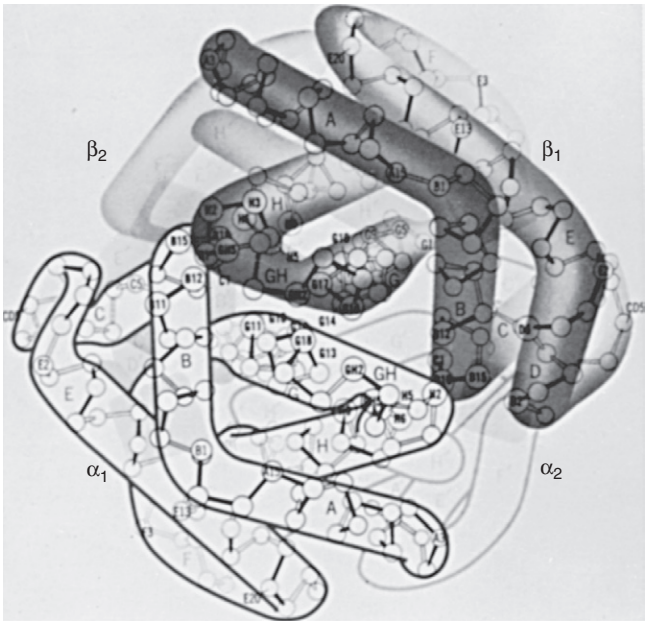


Figure 88-2 Hemoglobin tetramer, $\alpha_1\beta_1$ contacts ($\alpha_2\beta_2$ contacts are identical). The eight α-helices, which constitute the secondary structure, are labeled *A* to *H*. The packing of chains in the hemoglobin molecule is such that close interlocking contact of side chains exists between unlike subunits, but with little contact between α and α or β and β. The $\alpha_1\beta_1$ or $\alpha_2\beta_2$ contacts involving *BGH* helices and the *GH* corner are called *packing contacts* because they represent subunit packing that is unchanged when the hemoglobin molecule goes from its deoxy- to its oxy- configuration.

HEMOGLOBIN-OXYGEN INTERACTIONS

Hemoglobin oxygen affinity is the continuous relationship between hemoglobin oxygen saturation and oxygen tension. It customarily is plotted as the sigmoid-shaped oxygen equilibrium curve, and it can be summarily expressed as the P_{50}—that is, the oxygen tension at which 50% of hemoglobin is saturated with oxygen at standard temperature and pH (Figure 88-4). The sigmoidal shape of the oxygen-hemoglobin equilibrium curve can be explained only if two assumptions are made: (1) that the heme groups react with oxygen in a fixed sequence, and (2) that the oxygenation and deoxygenation of one heme group profoundly affect the oxygenation and deoxygenation of the others. This latter phenomenon has been termed *heme-heme interaction.* As each heme group accepts oxygen, it becomes progressively easier for the next heme group of the molecule to pick up oxygen. This concept is implicit in the Hill equation for percent saturation[11]:

$$y / 100 = k \times Po_2^n / 1 + (k \times Po_2^n)$$

where y is percent saturation with oxygen, k is an equilibrium constant, Po_2 is oxygen partial pressure, and the exponent n is the average number of iron atoms per hemoglobin molecule. The value of n for normal hemoglobin is approximately 2.9.

As blood circulates through the normal lung, Po_2 increases from approximately 40 mm Hg to 110 mm Hg, a pressure sufficient to ensure at least 95% saturation of hemoglobin with oxygen. The oxygen-hemoglobin equilibrium relationship is such that any further increase of oxygen tension in the lung results in only a small increase in saturation. In the normal adult, when oxygen tension has fallen to approximately 27 mm Hg, at a pH of 7.40 and a temperature of 37° C, 50% of hemoglobin is saturated with oxygen (i.e., the P_{50} for whole blood is 27 mm Hg).

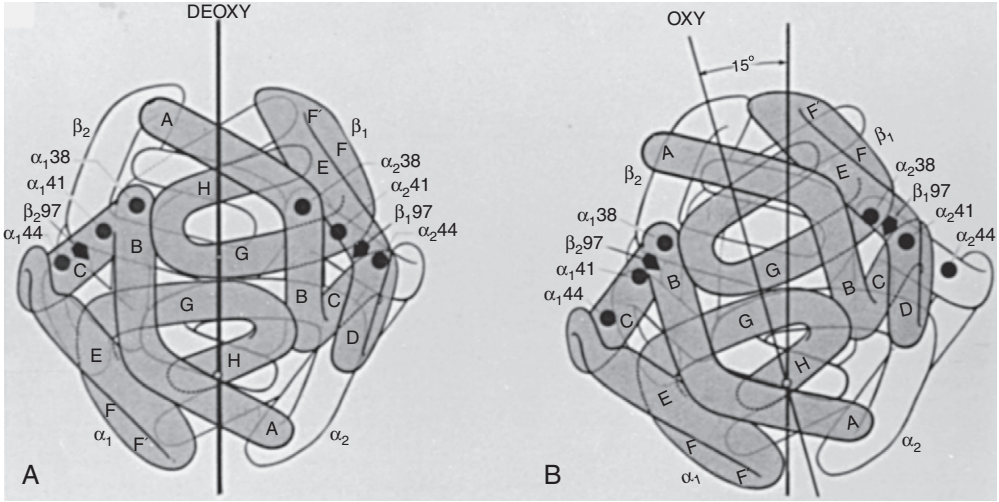


Figure 88-3 Subunit motion in hemoglobin: Combination into deoxyhemoglobin and oxyhemoglobin. Two dimers are superimposed in **A** and **B. A,** The complete tetramer is shown in its deoxyhemoglobin conformation. Note the position of β97 relative to α41 and α44. **B,** In oxyhemoglobin, the $\alpha_1\beta_1$ is rotated 15 degrees relative to $\alpha_2\beta_2$. Note the new positions of β97 between α41 and α38.

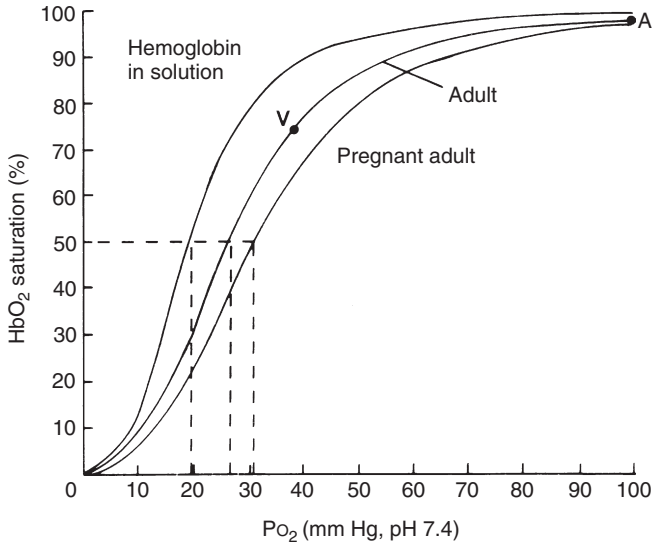


Figure 88-4 Oxyhemoglobin saturation curve under standard conditions for normal blood of pregnant and nonpregnant women. Also shown is the dissociation curve for hemoglobin in solution.

The steep and flat parts of the curve reflect definitive processes in oxygen unloading. As oxygen diffuses from capillary to tissue, at first a rapid fall in Po_2 (represented by the flat part of the curve) occurs until the steep part is reached, after which capillary Po_2 decreases little even though large amounts of oxygen are released. Because oxygen tension at the mitochondrial surface, the point of oxygen utilization, is always approximately 0.5 to 1.0 mm Hg,[1,12] the driving pressure for, and consequently the rate of, oxygen delivery is determined solely by the mean Po_2 in capillary blood. This is in turn set by the position of the dissociation curve on the Po_2 axis and by its steepness, such that relatively little change in driving pressure occurs as the red blood cell moves through the capillary. As the partial pressure of oxygen decreases, tissue oxygenation may become impaired. The term *critical* Po_2 was introduced to indicate the oxygen tension of blood below which diffusion is impaired and organ function is disturbed.[13] A critical Po_2 cannot be a well-defined value that applies to all tissues under all conditions. The oxygen requirements of tissues vary, and in some tissues, such as striated muscle, oxygen requirements are determined by the level of activity. For the brain, an organ in which an adequate oxygen supply is essential for maintaining energy metabolism, the critical Po_2 appears to be approximately 20 mm Hg.

When hemoglobin oxygen affinity is increased (i.e., with lower P_{50}), the curve is shifted to the left, and oxygen (which is bound more tightly to hemoglobin) is released only at lower partial pressures. For example, whereas a Po_2 of 40 mm Hg results in an oxygen saturation of 75% at 37° C and pH 7.40, a leftward shift of the curve results in a higher saturation at the same Po_2. The resulting change in oxygen unloading ultimately could result in impaired diffusion. When affinity is decreased (i.e., with higher P_{50}), the curve is shifted to the right. Consequently, oxygen is bound less tightly to hemoglobin and is released at higher partial pressures, thereby enhancing oxygen unloading at the tissue level. Therefore the release of oxygen from the blood at the tissue level depends on the position of the oxygen equilibrium curve, which in turn is modified by intraerythrocytic pH, Pco_2, temperature, and other factors, including electrolyte concentration, organic phosphate levels, and hemoglobin type.

FACTORS ALTERING BLOOD OXYGEN AFFINITY OF HEMOGLOBIN

The effect of temperature on the oxygen equilibrium curve was first noted by Barcroft and King in 1909.[14] Increased temperature shifts the curve toward the right, thereby facilitating the release of oxygen. In addition, changes in temperature alter both the Bohr factor and the 2,3-diphosphoglycerate (DPG) effect[15] (discussed in the following section).

The *Bohr effect* is the shift to the right of the oxygen equilibrium curve of both adult and fetal blood in response to an increase in Po_2 or a decrease in pH, or both. Oxygen unloading is determined by the Po_2 gradient between blood and tissues. The shift of the oxyhemoglobin dissociation curve to the right as carbon dioxide enters the blood from the tissues tends to raise the oxygen tension, increasing the gradient for any given oxyhemoglobin saturation and facilitating transfer of oxygen to the tissues. The change in log P_{50} per unit change in pH (i.e., $-\Delta \log P_{50}/-\Delta$ pH) is known as the *Bohr factor.* Its value for adult human blood is –0.48, and for the newborn infant it is –0.44. The effective pH is the intracellular pH of the red blood cell, which usually is 0.2 unit less than plasma pH at physiologic levels, although the pH gradient across the red blood cell may vary in disease states. Thus an acute change in pH of 0.1 unit changes P_{50} by approximately 3 mm Hg. The effect of pH changes lasting longer than 2 to 3 hours depends largely on the compensatory change in organic phosphate synthesis (see later discussion). The Bohr effect is more pronounced, at least experimentally, as oxygen saturation decreases and is diminished in 2,3-DPG–depleted blood.[16]

The Bohr effect produced by varying Pco_2 at constant fixed acid is larger (–0.48) than that induced by alterations in metabolic acids at constant Pco_2 (–0.40).[16] The molecular basis of the carbon dioxide effect is two-fold: It follows both carbon dioxide–induced changes in pH and the action of carbon dioxide as a ligand, binding reversibly to uncharged amino groups in the hemoglobin molecule to form carbamates.[17-19] Bound carbamates form salt bridges, stabilizing the T (deoxy-) conformation of hemoglobin and decreasing hemoglobin oxygen affinity. In addition, the affinity of other sites on the hemoglobin molecule for the hydrogen ion (H^+) is enhanced by transition from oxyhemoglobin to deoxyhemoglobin, allowing deoxyhemoglobin to take up much of the H^+ generated from spontaneous decomposition of carbonic acid.

By modifying hemoglobin oxygen affinity, carbon dioxide also facilitates respiratory gas exchange in the lungs. At the lungs, carbon dioxide is given up by red blood cells into the alveoli. Carbon dioxide concentration falls, thereby shifting the oxygen equilibrium curve to the left; the increase in hemoglobin oxygen affinity enhances uptake of oxygen from the alveoli.[20] The combined effects of pH, Pco_2, and temperature on the oxygen equilibrium curve can be viewed, in theory, as advantageous for species survival. An increase in tissue metabolism causes increases in local temperature, H^+ concentration, and Pco_2, all of which raise P_{50}. The result is a higher gradient of oxygen tension between capillary and mitochondrion at the site (tissue) where oxygen consumption is highest. For example, the in vivo oxygen equilibrium curve has been shown to shift markedly rightward during acute exercise as a result of combined Bohr and temperature effects.[21]

EFFECT OF ERYTHROCYTE 2, 3-DIPHOSPHOGLYCERATE ON BLOOD OXYGEN AFFINITY OF HEMOGLOBIN

It has long been recognized that the oxygen affinity of Hb A in free solution is considerably greater than that of the intact fresh erythrocyte. This difference suggested to many investigators that the red blood cell contained a substance or substances capable of interacting with hemoglobin and reducing its affinity for oxygen.

In 1967, it was demonstrated that interaction with a number of organic phosphates decreased the affinity of a hemoglobin solution for oxygen, with 2,3-DPG being the most effective.[22,23] Of the organic phosphates normally found in the human erythrocyte, 2,3-DPG is present in the largest concentrations and thus is both qualitatively and quantitatively the most important phosphate with respect to modulation of hemoglobin oxygen affinity. The content of 2,3-DPG in the human red blood cell averages 4.5 μmol/mL of red blood cells (range, 3.4 to 5.2 μmol/mL), whereas the ATP concentration averages 1.0 μmol/mL of red blood cells (range, 0.8 to 1.4 μmol/mL). The remainder of the organic phosphates generally totals less than 0.4 μmol/mL of red blood cells. Hemoglobin oxygen affinity as indicated by P_{50} is linear with respect to 2,3-DPG concentration over a wide range; a change of 0.43 mmol of 2,3-DPG/mL of red blood cells results in a 1-mm Hg change in P_{50}.[24]

The highly negatively charged anion 2,3-DPG binds preferentially to deoxyhemoglobin in a 1:1 molar ratio under physiologic conditions of solute concentration and pH. On a molecular basis, 2,3-DPG decreases oxygen affinity by stabilizing the quaternary structure of deoxyhemoglobin through cross-linking of β-chains. 2,3-DPG is stereochemically complementary to a constellation of six positively charged groups located on the β-chains and facing the central cavity of the hemoglobin molecule.[25] A single molecule of 2,3-DPG is anchored by six salt bridges to cations at the entrance of the central cavity. On oxygenation, 2,3-DPG is extruded because the central cavity becomes too small. A second mechanism by which 2,3-DPG reduces oxygen affinity is by altering the intraerythrocytic pH relative to plasma pH. The reduction in pH consequently decreases oxygen affinity by the Bohr effect. It has been shown that both mechanisms apply in the intact erythrocyte, and that at concentrations of 2,3-DPG above normal, the latter mechanism predominates.[26] The concentration of 2,3-DPG in human red blood cells varies with gestational and postnatal age and can be influenced by hemoglobin level, pH, oxygenation, and activity of red blood cell enzymes such as hexokinase and pyruvate kinase.[27]

FETAL OXYGEN TRANSPORT

Compared with adult blood, fetal blood has a higher affinity for oxygen and lower P_{50}—an observation first made in 1930.[28] Figure 88-5 shows this relationship for the human fetus near term, when P_{50} is approximately 20 mm Hg under standard conditions. Barcroft working with Elsden[29] and other associates[30] and Hall[31] reported a gradual decrease in the blood oxygen affinity (increase in P_{50}) during the course of gestation, such that the fetal curve progressively approximates the maternal curve. In the placenta, because of the low P_{O_2} at which transfer of oxygen is achieved, the high affinity of fetal hemoglobin favors oxygen uptake in the fetus. The highest P_{O_2} in the fetus is in umbilical venous blood, in which P_{O_2} usually does not go much above 30 mm Hg. At that oxygen tension, the saturation of human fetal blood is 6% to 8% higher than the saturation of maternal blood.[32]

These findings raised questions about the mechanism or mechanisms responsible for the increased oxygen affinity of fetal blood. Hall[31] suggested that this difference might be associated with specific hemoglobin characteristics. Korber,[33] in 1866, had first noted that blood from human newborn umbilical vessels denatured less readily in alkaline or acid solutions than did blood from adults, the difference being most pronounced in alkali. In 1930, Haurowitz[34] demonstrated that the difference in alkaline denaturation resides in the globin chains of hemoglobin and proposed the presence of two hemoglobins in newborn blood: an alkali-susceptible adult fraction and a more resistant fetal fraction. In 1963, the human fetal α-chain was shown to have the same amino acid sequence as for the α-chain of human adult hemoglobin.[35] In addition to the two α-chains, fetal hemoglobin were found to contain two non–α-chains (labeled γ-chains), which resemble the β-chain and contain the same number of amino acids (146) but differ in sequence by a total of 39 amino acids. Each γ-chain contains four isoleucine residues not found in either the α- or the β-chain. In addition, three proline residues present in the β-chain have been substituted by other amino acids.

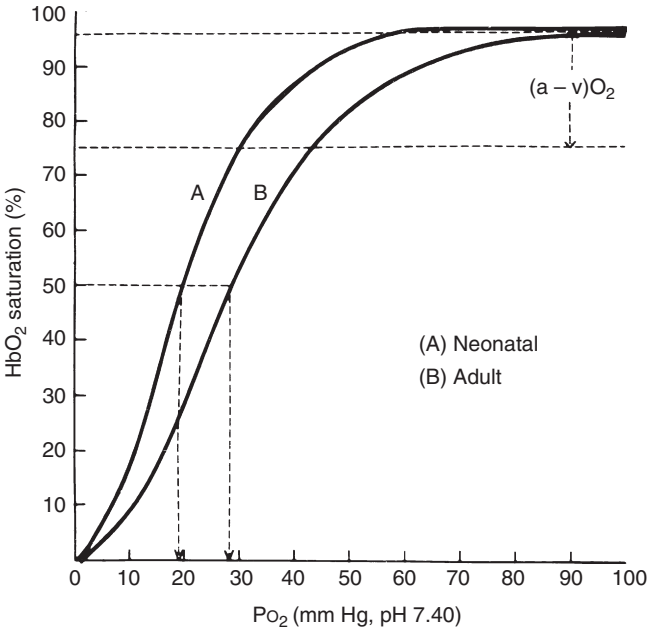


Figure 88-5 Oxyhemoglobin equilibrium curves for blood from term infants at birth and from adults.

A linear correlation between the proportional composition of fetal hemoglobin and the P_{50} at the time of delivery has been noted in humans. At term, Hb A accounts for approximately 25% of total hemoglobin, and the fetal P_{50} is approximately 19 mm Hg.[26,36] Birth, intrauterine hypoxia,[37] and hemolytic disease of the newborn do not cause a change in the proportions of Hb A and Hb F at any given gestational age. During the period of accelerated erythropoiesis near term, however, Hb A synthesis predominates. The subcellular events—transcription and translation—responsible for the gradual, orderly change in synthesis from γ- to β-chains are not completely understood.[38,39] More recent studies, however, suggest that the interaction of multiple transcription factors with the locus control region (LCR) of the globin gene sequence on chromosome 11 regulates the forms of the globin gene (ε, γ, δ, or β) expressed at each stage of development.[40] The erythroid Kruppel-like factor (EKLF) and the stage selector protein (SSP) seem to be particularly important. Although the specific developmental signals that regulate developmental changes in transcription factor expression have not been identified, changes in expression of hypoxia-inducible factor 1 (HIF-1) in response to changes in fetal blood oxygen tension may play a role in this process.[41]

Fetal Blood Oxygen Delivery

Despite an oxygen tension in fetal blood that is only one-fifth to one-fourth that of the adult, fetal arterial blood oxygen content and oxyhemoglobin saturation at term are not much lower than those in the adult (Figure 88-6). This similarity, of course, results from a combination of the high oxygen-carrying capacity and increased oxygen affinity of fetal blood. The greater oxygen affinity of fetal blood generally is considered an advantage in that it allows incorporation of oxygen to nearly saturate fetal hemoglobin at relatively low oxygen tensions. The possible disadvantage

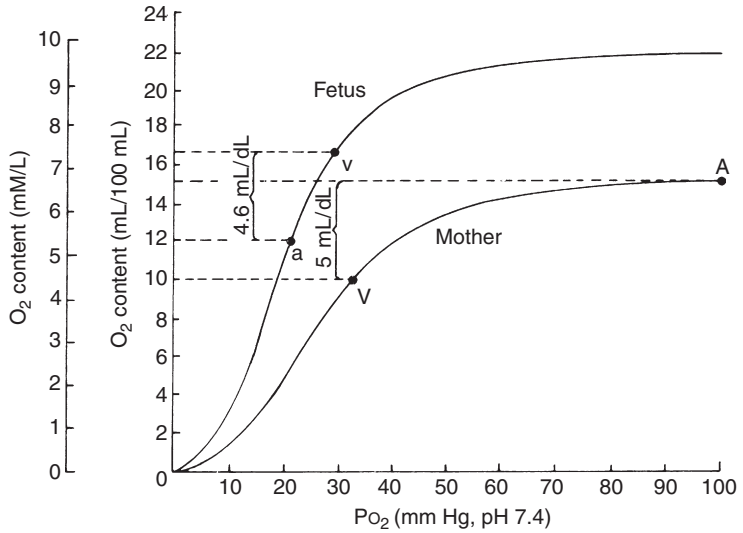


Figure 88-6 Blood oxygen content as a function of oxygen tension in human maternal and near-term fetal blood.

in oxygen delivery to fetal tissues is offset by the fact that the fetal oxyhemoglobin saturation curve is rather steep, so that a small decrease in oxygen tension results in a major decrement in oxyhemoglobin saturation and unloading of oxygen to the tissues.

The actual oxyhemoglobin saturation curve in vivo as blood flows through the placental exchange capillaries is an additional important consideration. Compared with standard conditions, maternal arterial blood is slightly hypocarbic (P_{CO_2} of 32 mm Hg) and alkalotic (pH 7.42), whereas fetal blood is slightly hypercarbic (P_{CO_2} of 45 mm Hg) and acidotic (pH of 7.34). As fetal blood courses through the exchange vessels, it gives up H^+ and carbon dioxide, leading to a rise in pH and a fall in P_{CO_2}. The opposite changes occur in the maternal exchange vessels. Thus, in vivo, the maternal and fetal oxyhemoglobin saturation curves may be almost superimposed (see Figure 88-6). Although the double Bohr effect in the placenta has been credited with significantly augmenting oxygen exchange, theoretical studies suggest that this accounts for only 2% of the total oxygen transferred.[42] As noted earlier, the Bohr effect is expressed by the factor $-\Delta\log P_{50}/-\Delta pH$, which has a value of approximately −0.48 for both fetal and maternal blood at pH values between 7.2 and 7.8. Below a pH of 7.2, however, the Bohr effect is greater for fetal than for maternal blood. An additional factor tending to shift the in vivo fetal dissociation curve to the right is the temperature of the fetus, which exceeds that of the mother by 0.5° to 1.0° C. In human neonates, the dissociation curve also shifts toward that of the mother as the concentration of Hb F decreases.[32,43]

Effect of 2,3-Diphosphoglycerate on Fetal Hemoglobin Oxygen Affinity

In 1971, investigation of the deoxygenation kinetics of isolated fetal and adult hemoglobin revealed significant functional differences.[44] Although 2,3-DPG bound to fetal hemoglobin, the binding constant was lower than for adult hemoglobin.[36] Other experiments performed on whole blood demonstrated a significant fall in P_{50} when erythrocytes were depleted of 2,3-DPG, even in samples of blood with a high percentage of fetal hemoglobin.[45] With the use of pure solutions of fetal and adult hemoglobin, it subsequently has been shown that the effect of 2,3-DPG on the P_{50} of fetal hemoglobin is approximately 40% of its effect on the P_{50} of adult hemoglobin.[46]

On the basis of these findings, the P_{50} of whole blood can be expected to be related to both the 2,3-DPG level and the relative concentrations of adult and fetal hemoglobin. Because the concentrations of adult hemoglobin and 2,3-DPG and, correspondingly, the functioning fraction of DPG (total red blood cell DPG content in nmol/mL of red blood cells times the percentage of adult hemoglobin) increase during gestation, it is not surprising that the P_{50} of whole blood correlates with gestational age (Figure 88-7). (The term *functioning fraction* is not intended to imply that a compartmentalized portion of the red blood cell's 2,3-DPG is in combination with the hemoglobin but instead is used to underscore the fact that both 2,3-DPG and adult hemoglobin act in concert to determine the oxygen affinity of intracellular hemoglobin.) The correlation of P_{50} with gestational age is even closer than with the functioning fraction of DPG, suggesting that other factors contribute to the rise of P_{50} during gestation.

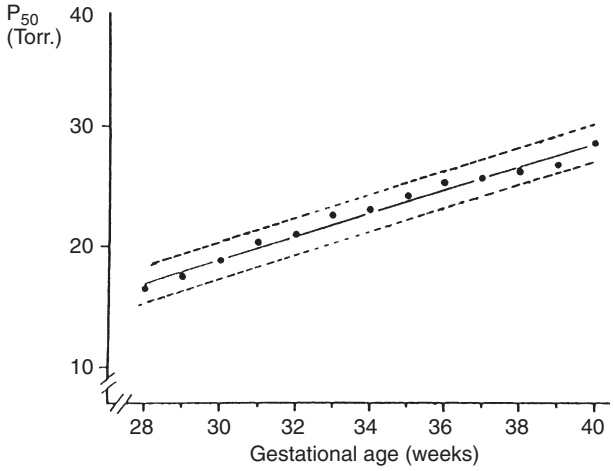


Figure 88-7 Relationship between gestational age and hemoglobin P_{50}. Each *plotted point* represents the mean value for age; *dashed lines* indicate ± 2 SD.

No satisfactory explanation has emerged for the rise in 2,3-DPG levels during gestation. It has been speculated, however, that the increase is the result of increased synthesis of adult hemoglobin, because in vitro studies have shown that the reduced hemoglobin of adults is more effective than the reduced hemoglobin of fetuses in stimulating 2,3-DPG synthesis.[47] This hypothesis also is supported by the significant correlation

TABLE 88-1

Oxygen Transport in Term Infants: Selected Clinical Series

No. of Infants	Age	Total Hb (g/dL blood)	Hct (%)	MCHC (%)	O_2 Capacity (mL/dL blood)	P_{50}* (mm Hg)	2,3-DPG (nmol/mL RBCs)	Fetal Hb (% of Total)	FFDPG (nmol/mL RBCs)	Reticulocyte Count (%)
19	1 day	17.8	52.7	34.2	24.7	19.4	5433	77.0	1246	4.70
		± 2.0	± 7.1	± 1.9	± 2.8	± 1.8	± 1041	± 7.3	± 570	± 1.74
18	5 days	16.2	46.9	34.1	22.6	20.6	6580	76.8	1516	2.15
		± 1.2	± 6.0	± 0.8	± 2.2	± 1.7	± 996	± 5.8	± 495	± 1.64
14	3 wk	12.0	33.5	35.9	16.7	22.7	5378	70.0	1614	0.88
		± 1.3	± 4.3	± 1.2	± 1.9	± 1.0	± 732	± 7.33	± 252	± 0.71
10	6-9 wk	10.5	30.2	34.9	14.7	24.4	5560	52.1	2670	1.63
		± 1.2	± 3.9	± 0.6	± 1.6	± 1.4	± 747	± 11.0	± 550	± 0.65
14	3-4 mo	10.2	30.3	33.8	14.3	26.5	5819	23.2	4470	1.36
		± 0.8	± 2.4	± 1.7	± 1.2	± 2.0	± 1240	± 16.0	± 1380	± 0.45
8	6 mo	11.3	34.0	33.4	14.7	27.8	5086	4.7	4840	1.42
		± 0.9	± 3.6	± 0.7	± 0.6	± 1.0	± 1570	± 2.2	± 1500	± 1.15
8	8-11 mo	11.4	34.8	32.8	15.9	30.3	7381	1.6	7260	0.82
		± 0.6	± 1.9	± 0.9	± 0.8	± 0.7	± 485	± 1.0	± 544	± 0.27

*At pH 7.40.

2,3-DPG, 2,3-Diphosphoglycerate; *FFDPG,* functioning fraction of 2,3-DPG; *Hb,* hemoglobin; *Hct,* hematocrit; *MCHC,* mean corpuscular hemoglobin concentration; *RBCs,* red blood cells.

All values are given as mean ± 1 SD.

Data from Delivoria-Papadopoulos M, Oski FA, Miller LD, Forster RE: The pathophysiology of exchange transfusion of the newborn infant with regard to oxygen transport. In *Preservation of Red Blood Cells,* Washington DC, 1973, National Academy of Sciences, pp 137-147.

between the percentage of adult hemoglobin and 2,3-DPG content in the neonatal period (Table 88-1).

Postnatal Changes in Oxygen Transport

The high oxygen affinity of fetal blood, which is well adapted to oxygen uptake in the placenta, has disadvantages in postnatal life. With adequate postnatal lung function, the pulmonary circulation is exposed to an oxygen tension of 80 mm Hg or more, so that high affinity at low oxygen tensions has no advantage for oxygen uptake during the newborn period. At the tissue level, the low P_{50} decreases the driving potential for oxygen diffusion, limiting the rate at which oxygen can be unloaded. The newborn infant needs more oxygen than the fetus, for even in a neutral thermal environment and at minimal activity, the oxygen consumption of most species increases by 100% to 150% in the first few days of life.[48] Colder environments and muscle activity further increase the metabolic demand for oxygen. Hence a P_{50} adequate for tissue supply in the fetus does not provide a sufficient rate of net oxygen diffusion in the neonate.

To meet the increased oxygen demands after birth, oxygen-carrying parameters of blood change drastically. In most species, postnatal changes take place in both oxygen affinity and oxygen-carrying capacity, but at different rates and by different amounts. Beginning at birth, the infant's blood oxygen affinity decreases rapidly.[26] On the first day of life, the P_{50} in normal infants is 19.4 ± 1.8 mm Hg, in contrast with a value of 27.0 ± 1.1 mm Hg for the normal adult (all values are mean ± SD). In the term infant, P_{50} increases gradually and reaches normal adult values by 4 to 6 months of life (Figure 88-8), corresponding to the time course of the replacement of fetal hemoglobin by adult hemoglobin. The red blood cell 2,3-DPG concentration on day 1 averages 5.43 ± 1.04 μmol/mL of red blood cells and thus does not differ significantly from the adult value of 5.11 ± 0.42 μmol/mL. By the fifth day, 2,3-DPG increases to 6.58 ± 1.00 μmol/mL of red blood cells.[35] Although this increment has only a modest direct effect on fetal hemoglobin oxygen affinity, it lowers intracellular pH, thereby decreasing blood oxygen affinity.

For most of the first year of life, Hb F decreases from around 75% to approximately 2% of the total, whereas 2,3-DPG concentrations in general remain fairly constant at 5 to 6 μmol/mL of red blood cells. At 8 to 11 months of age, the concentration of 2,3-DPG rises to pproximately 7 μmol/mL. The P_{50} at this age averages 30.3 mm Hg, which exceeds the value for the normal adult, whereas the fetal hemoglobin concentration is decreased to normal adult levels. Average values for the percentage of fetal

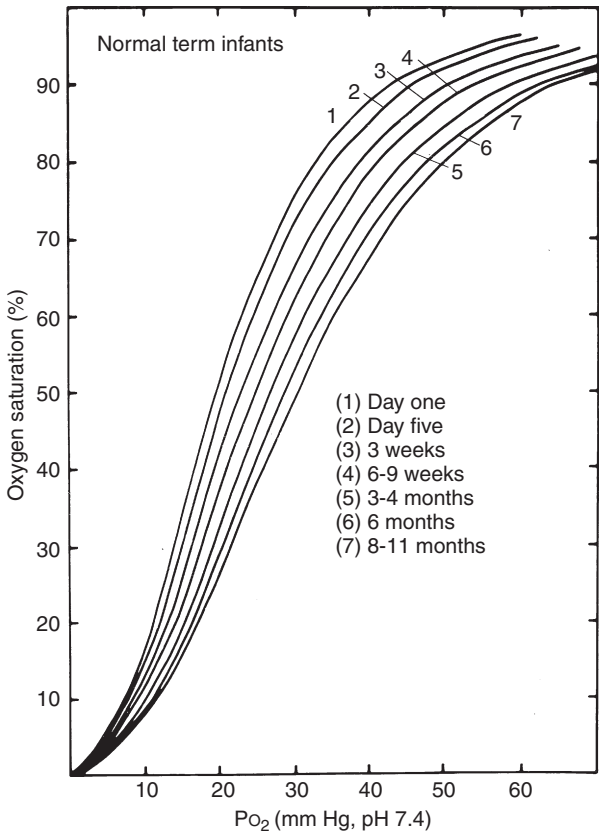


Figure 88-8 Oxygen equilibrium curve for blood from term infants at different postnatal ages.

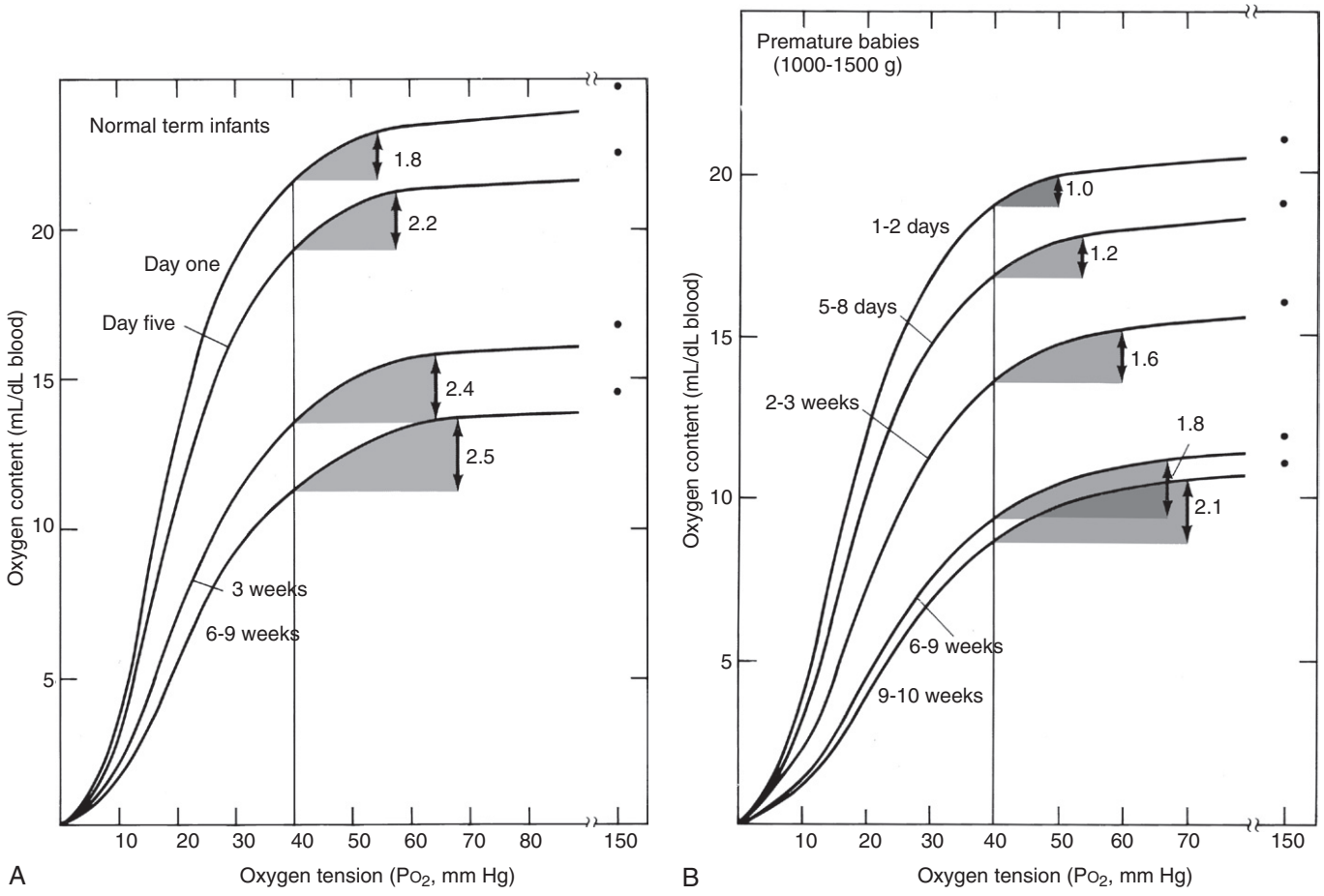


Figure 88-9 Oxygen equilibrium curves for blood from term infants (**A**) and from preterm infants of birth weight 1000 to 1500 g (**B**) at different postnatal ages. *Double arrows* represent the oxygen-unloading capacity between given "arterial" and "venous" Po_2 values. *Points* corresponding to 150 mm Hg on the abscissa are the O_2 capacities; each *curve* represents the mean value for the infants studied in each age group.

hemoglobin, P_{50}, and 2,3-DPG levels for term infants of various postnatal ages are shown in Table 88-1. Thus the postnatal change in P_{50} correlates neither with the change in red blood cell DPG content alone nor with the decline in fetal hemoglobin alone. Instead, the progressive decrease in oxygen affinity during the first 6 months of life correlates significantly with the functioning DPG fraction. The changes in oxygen affinity during postnatal life should be taken into account when the oxygen saturation or content of arterial blood in the neonate is derived from measurements of Pa_{O_2} and pH. In the absence of other measurements, gestational age can be used for a reasonably accurate estimation of the oxygen affinity.

In general, compared with their larger term counterparts, preterm, low-birth-weight infants have a lower erythrocyte 2,3-DPG content, lower P_{50}, and higher fetal hemoglobin concentration. During the first several weeks of life, these small infants have functioning fractions of DPG that are significantly lower than those in term infants. Premature infants have a smaller oxygen-unloading capacity initially than that measured in term infants and do not catch up during the first 3 months of life[3] (Figure 88-9).

Effect of Altered Physiologic States on Oxygen Delivery to Tissues

Various alterations in physiology may have a significant effect on oxygen delivery to the tissues. Under such conditions, variations in oxygen-carrying capacity, blood flow, or hemoglobin oxygen affinity have the potential to improve or impair tissue oxygenation.

Hypoxemia

The response to hypoxemia changes significantly during maturation. Oxidation of the cytochromes of the electron transport chain during hypoxemia is decreased in the newborn infant compared with the adult at any given degree of hemoglobin saturation, presumably because of the presence of fetal hemoglobin in the circulation.[49] The lower level of oxidation preserves mitochondrial metabolism and helps maintain high-energy phosphate production during hypoxemia. The left-shifted hemoglobin-oxygen curve associated with fetal hemoglobin might be expected to be a disadvantage during hypoxemia because release of oxygen to tissues is decreased. On the contrary, tissue oxygen extraction in the fetus and neonate appears to be increased during acute hypoxemia, thereby maintaining normal levels of oxygen consumption.[50] By contrast, animal studies suggest that fetal oxygen extraction is not increased during chronic fetal hypoxemia; instead, fetal oxygen consumption is decreased, presumably reflecting a decrease in metabolic rate in response to reduced oxygen delivery.[51]

The fetal response to hypoxemia also appears to be organ-specific. For example, studies in animal models have shown that cerebral blood flow increases proportionately more during acute hypoxemia when fetal hemoglobin concentration is high. This differential response preserves or even increases oxygen delivery to brain, although the effect is blunted during chronic

hypoxemia.[51,52] Changes in blood flow in response to fetal hypoxemia have not been well-characterized in other organs.

Sepsis

Bacterial sepsis also may affect tissue oxygen transport. Sepsis caused by group B streptococci, for example, is associated with a higher critical venous oxygen saturation (the levels below which tissue acidosis develops) than that typical for hypoxemia.[53] This higher critical point seems to be a specific effect of sepsis, rather than a result of the alterations in circulatory status (e.g., increased catecholamine levels) that occur in response to infection. Oxygen consumption is increased during sepsis, while at the same time oxygen delivery may be decreased as a result of impairment of myocardial function by endotoxin.[54] Additional evidence suggests that sepsis may decrease oxygen extraction at the tissue level, further reducing tissue oxygen delivery.

Anemia

Anemia is a common clinical problem in the premature infant. In otherwise normal infants with no evidence of cardiac disease, pulmonary disease, or increased metabolic needs, a decrease in the oxygen-carrying capacity is a source of concern only if oxygen delivery to the tissues approaches the limit of oxygen stores. As seen in Figure 88-10, infants who at birth exhibit significant differences in total oxygen-carrying capacity but similar oxygen affinities will exhibit the same arteriovenous oxygen content differences. It is worth noting that when oxygen-carrying capacity decreases significantly as a result of anemia, sufficient oxygen unloading occurs without lowering the venous oxygen tension below 30 mm Hg.

A reduction in oxygen-carrying capacity may be more critical under conditions of impaired oxygen uptake, such as in the presence of lung disease. With reduced oxygen uptake, tissue oxygen delivery can be improved by optimizing preload, afterload, Sao_2, and oxygen-carrying capacity (i.e., hemoglobin concentration). The effect of a decrease in Sao_2 on oxygen delivery is significantly less than the effect of anemia.[55,56] For example, Sao_2 would have to decrease from 95% to approximately 80% to have an effect on oxygen content comparable to a 2-g decrease in hemoglobin concentration. Thus, in situations of marginal oxygen delivery, maintenance of hemoglobin concentrations high enough to optimize oxygen-carrying capacity is essential.

Exchange Transfusions

An acute rightward shift of the oxyhemoglobin curve of the newborn infant can be produced by exchange transfusion with adult blood, with its lower oxygen affinity[57,58] (Figure 88-11). The consequences of exchange transfusions with fresh versus stored blood were first demonstrated in 1954 and further delineated in later studies. Infants who underwent exchange transfusion with blood that had been stored for 7 days or longer had a decreased P_{50} compared with that in infants receiving fresh blood (i.e., obtained up to 24 hours previously).[59] The increase in oxygen affinity occurring with blood storage correlated with a simultaneous fall in 2,3-DPG levels.[60] The calculated oxygen-unloading capacity was decreased in infants receiving transfusions of 5-day-old blood, but in those receiving fresh blood it was increased.[36] These studies demonstrated that during the immediate posttransfusion period, the infant's blood reflects the storage characteristics of the blood received.

Further investigations revealed that these findings are specific for blood stored in acid-citrate-dextrose; use of this storage medium results in a prompt fall in 2,3-DPG levels and

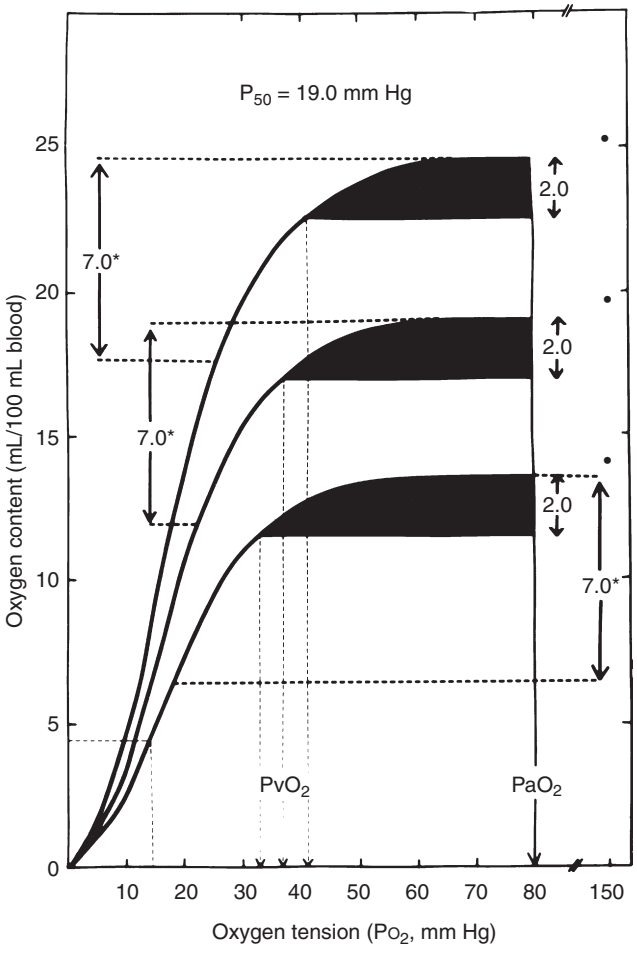


Figure 88-10 The effect of altered hematocrit at a constant P_{50} of 19.0 mm Hg on tissue oxygen unloading in newborn infants. The *asterisk* represents the calculated myocardial extraction of oxygen. In this study, P_{50} = 19.0 mm Hg at pH 7.40 and 37° C.

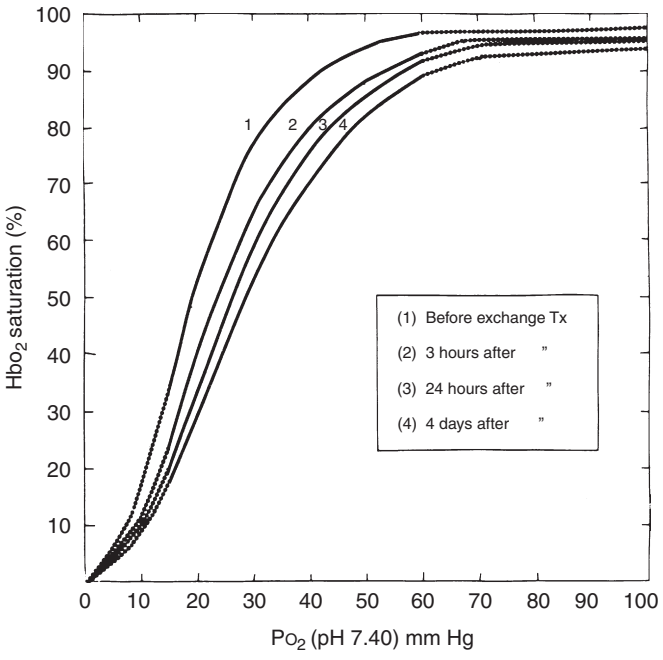


Figure 88-11 The effect of exchange transfusion with fresh adult blood of low oxygen affinity on the oxyhemoglobin dissociation curve for the newborn infant. An acute shift of the curve to the *right* is seen after such a transfusion.

a concomitant rapid increase in oxygen affinity. By contrast, the use of citrate-phosphate-dextrose preserves the oxygen affinity and 2,3-DPG content of the stored blood essentially unchanged throughout normal storage periods.[36] These differences have important physiologic consequences. If blood with a lower P_{50} than that of normal adult hemoglobin is used for exchange transfusion, the amount of oxygen unloaded is decreased. To compensate for this effect, the infant must increase either cardiac output or the arteriovenous oxygen difference.[61] The latter response results in decreased venous oxygen tension, potentially impairing oxygen diffusion from the capillaries to the tissues. Thus knowledge of the storage conditions of the blood products to be used for exchange transfusion may be of critical importance in determining the physiologic impact of the procedure, particularly in the infant with compromised cardiorespiratory function.

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89 Control of Breathing in Fetal Life and Onset and Control of Breathing in the Neonate

Ruben E. Alvaro and Henrique Rigatto

In the study of control of breathing during the fetal and neonatal periods, three considerations are of special importance: First, the fetus sleeps all of the time and the neonate most of the time.[1-4] Accordingly, their control of breathing must be studied during sleep, and the findings need to be compared with data for adult subjects obtained during sleep and not during wakefulness.[5-8] In this respect, accurate comparison has not been the norm in the past, leading to some notable misconceptions. Second, the fetus and the neonate are not cooperative subjects. Study of their respiratory control must therefore proceed without benefit of conscious participation as in the adult; consequently, determining whether the measurements obtained reflect corresponding physiologic processes in adult subjects under similar conditions is difficult. Third, measurements in the neonate usually are made, by necessity, in the decubitus position, whereas those in the adult usually are made with the subject sitting or standing.[6,9] The implications of the effect of different positions on the control of breathing also have not been dealt with in previous investigations. Unless some consistency in methodology can be maintained, defining what is actually distinct or unique about the control of breathing in the neonate will prove challenging.

This chapter reviews some of the concepts and progress made in the area of control of breathing in the fetus and newborn. Major developments are summarized, and a critical analysis of the scientific foundations of current knowledge in this area also is presented.

CONCEPTUAL AND HISTORICAL PERSPECTIVES

Although the major advances in current understanding of the control of breathing generally apply to the fetus and newborn, two important aspects that underscore the unique nature of breathing during this period merit special attention: (1) the presence and purpose of fetal breathing and (2) the physiologic mechanism responsible for the establishment of continuous breathing at birth.

In 1970, Dawes and co-workers[10] presented evidence that the fetal sheep makes regular breathing movements during rapid eye movement (REM) sleep. In their work, tracheal pressure changes were measured as an index of fetal breathing. Simultaneously, Merlet and colleagues,[11] using changes in intraesophageal pressure as the index of fetal breathing, demonstrated respiratory activity in fetal sheep. Subsequent work confirmed and expanded these findings by recording the electrical activity of the diaphragm and clearly showing the central origin of the respiratory output in utero.[2,12-16] It is now universally accepted that the fetus performs breathing "exercises" in utero beginning in early pregnancy.[14,15,17-23] Because no gas exchange is associated with this activity, its purpose is unknown. Dawes previously suggested, in a teleologic vein, that the breathing in utero is important to exercise the respiratory muscles for their vital function after birth.[24]

The discovery of fetal breathing brought a new dimension to the events occurring at birth. What had traditionally been called "the initiation of breathing at birth" must now be called "the establishment of continuous breathing at birth." Breathing begins long before birth. The question now is not what determines the appearance of breathing at birth but what makes it continuous. Alternatively, from another perspective, the issue is what makes fetal breathing episodic, present only during low-voltage electrocortical activity. The answers to these questions remain essentially unknown.

The Fetus

Breathing Pattern at Rest

Fetal breathing occurs primarily during periods of low-voltage electrocortical activity, which accounts for 40% of fetal life during the last trimester of gestation in sheep.[2,10,24-26] In the human fetus, the percentage is similar.[17,21,22] During high-voltage electrocortical activity, no established breathing is present, but occasional breaths may surface after episodic, generalized, tonic muscular discharges associated with body movements[2] (Figure 89-1). During low-voltage electrocortical activity, breathing is irregular, with abrupt onset and ending of activity seen on diaphragmatic electromyography (EMG) tracing. Less frequently observed is a progressive increase in envelope amplitude, comparable to the inspiratory slope observed in the anesthetized newborn lamb (Figure 89-2). A gradual decrease in diaphragmatic electromyographic signal at the end of a breath, reflecting postinspiratory activity (as observed in the newborn infant), is rarely seen in the fetus.[2,19,23,27,28] This irregular diaphragmatic activity generates a negative tracheal pressure of approximately 2 to 5 mm Hg. The corresponding changes in tracheal flow are less than those seen postnatally, probably owing to the higher viscosity of lung fluid in the system. The irregular breathing activity observed during this period probably reflects the influence of the reticular formation on breathing characteristic of REM sleep. The average breath has an inspiratory time of 0.45 second, expiratory time of 0.74 second, and a total duration of 1.12 seconds.[27,29] The physiologic mechanism responsible for the occurrence of fetal breathing only during low-voltage electrocortical activity is unknown.

Fetal State

The occurrence of fetal breathing during low-voltage electrocortical activity has led some investigators to believe that the fetus might be awake during part of this period.[30-32] In fact, on the basis of electrophysiologic criteria, it was postulated that during the last part of gestation in sheep the fetus was awake about 5% of the time.[12,13,32,33]

It was further postulated that a number of chemical and pharmacologic agents could alter fetal breathing by "arousing" the fetus.[12,32,33] In the late 1970s, Rigatto and colleagues[16] became interested in determining whether the fetus was at times awake in utero, and whether arousal could be induced by chemical or pharmacologic agents. As measured in this study using electrocorticography (ECoG) during both low-voltage and high-voltage activity (i.e., LVECoG and HVECoG, respectively), the polysynaptic reflexes for the hindlimbs in the chronic fetal sheep preparation were found to be unusually intense during

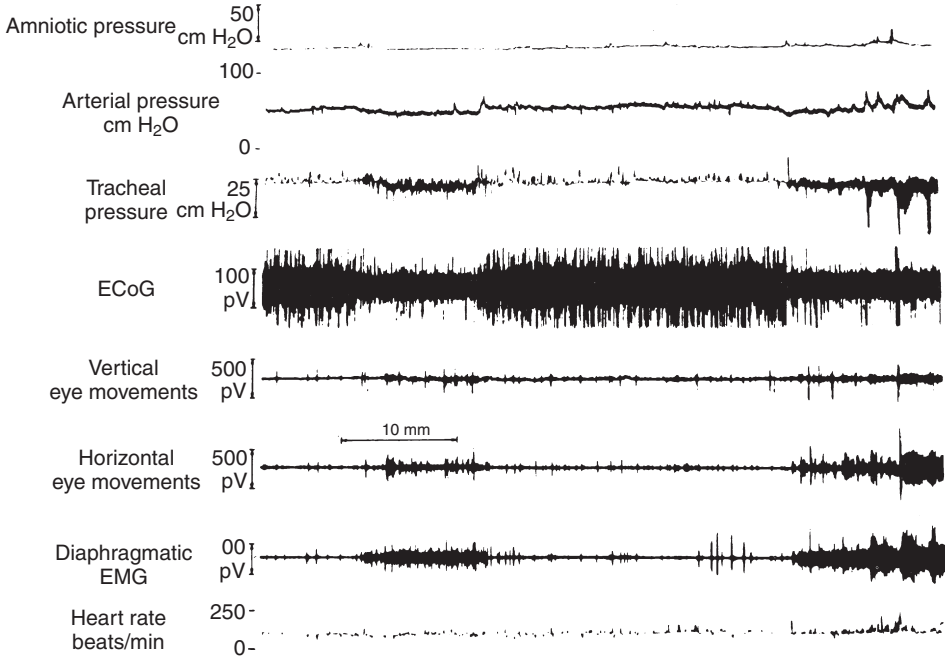


Figure 89-1 Fetal breathing in a fetal lamb at 134 days gestation. Note that the deflections in tracheal pressure and diaphragmatic activity occur during periods of rapid eye movement *(REM)* sleep in low-voltage electrocortical activity *(ECoG)* only. In high-voltage electrocortical activity (quiet sleep), breathing is absent.

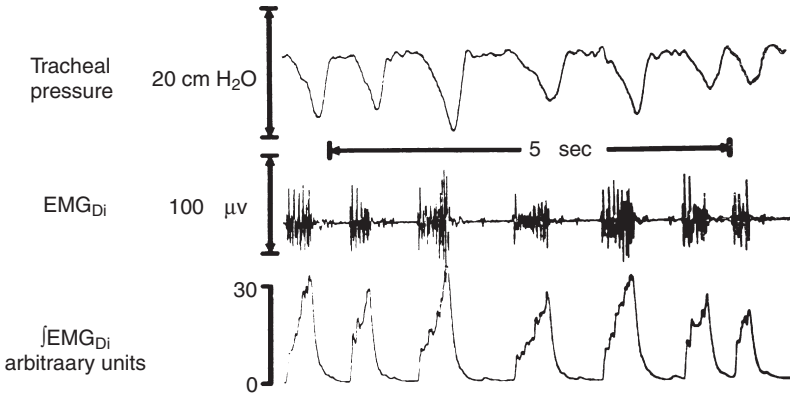


Figure 89-2 Tracheal pressure and diaphragmatic electromyographic activity *(EMG_{Di})* in a fetal lamb at 129 days gestation. The abrupt beginning and ending of diaphragmatic activity in some of the breaths contrast with the progressive increase in activity in others. A gradual decrease in diaphragmatic activity, reflecting postinspiratory activity, as seen in the newborn infant, is rarely seen in the fetus.

transition from low-voltage to high-voltage electrocortical activity.[16] This heightened intensity was speculated to represent fetal wakefulness.

In subsequent studies, a window was implanted on the left flank of the ewe to permit direct observation of the fetus in utero[2,34] (Figure 89-3). The technique proved to be highly successful and has generated substantial new information. Wakefulness, defined by open eyes and purposeful movement of the head, was never observed in the fetus under resting conditions. Analysis of videotapes amounting to more than 5000 hours of observation over 8 years has shown clearly that the fetus alternates between two basic behavioral states: REM (mostly LVECoG) and quiet (mostly HVECoG) sleep.[2,3] Activities such as movement, swallowing, licking, and breathing occur during REM sleep (Figure 89-4). During quiet sleep, the fetus is still and occasionally shows generalized movements associated with tonic discharges. The enhancement of the polysynaptic reflexes observed previously was associated with generalized tonic discharges and rotation of the body and head during the transition from low- to high-voltage ECoG activity. It was not associated with wakefulness.[2,34] This generalized discharge is typical of the transition from low- to high-voltage ECoG. Explication of the neurophysiologic basis for this intense discharge may provide the explanation of how the change from low to high voltage occurs.

Besides the normal irregularity of the respiratory pattern seen in REM sleep, licking and swallowing clearly disturb breathing activity[2,19,28]; breathing becomes slower and irregular, and diaphragmatic activity becomes interspersed with clusters of esophageal electromyographic activity. This digestive tract activity occurs primarily during REM sleep and is translated behaviorally and electrophysiologically as a general increase in electromyographic activity, blood pressure, and heart rate.

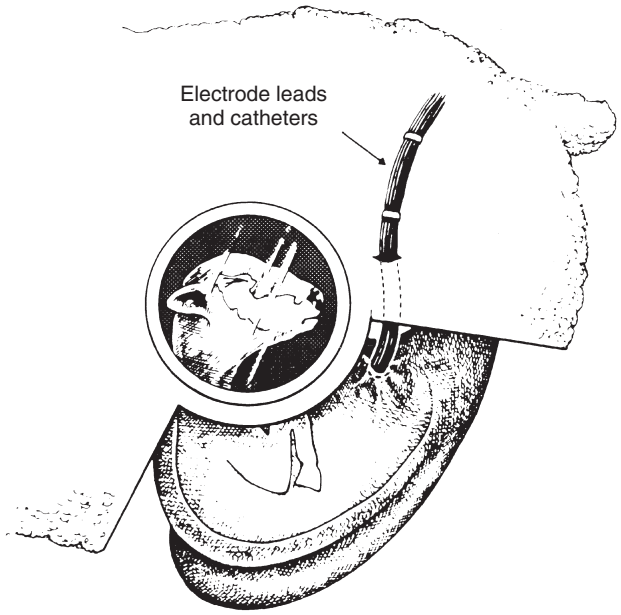


Figure 89-3 View of the head of the lamb fetus through newly created surgical window in the flank of the ewe. The bundle with catheters and electrical leads crosses the abdominal and uterine walls at some distance from the window.

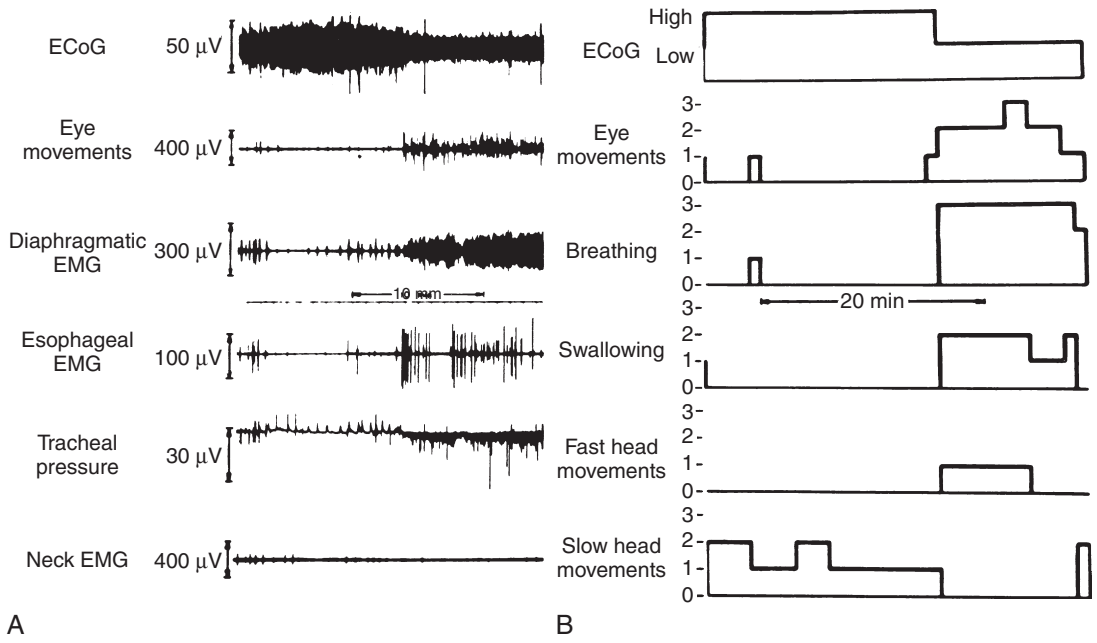


Figure 89-4 A comparative representation of observations made on a polygraph **(A)** and through a double-wall Plexiglas window **(B).** Fetal breathing, eye movements, and swallowing are present predominantly with low-voltage electrocortical activity *(ECoG)* (0 = absent; 1 = low activity; 2 = medium; 3 = high).

Modulation of Fetal Breathing by Carbon Dioxide, Oxygen, Pulmonary Reflexes, and Pharmacologic Agents

Fetal breathing initially was thought by some investigators to depend on behavioral influences, because it was observed only during REM sleep and seemed somewhat refractory to chemical stimuli.[35] Subsequent studies, however, clearly showed that the fetal breathing apparatus is capable of responding well to chemical stimuli and other agents known to modify breathing postnatally. Thus it became clear that the fetus responds to an increase in $Paco_2$ with an increase in breathing.* This increase is associated with increases in tracheal pressure, integrated diaphragmatic activity, and respiratory frequency (Figure 89-5). Both inspiratory and expiratory times decrease, as would be expected from postnatal studies.[29] The increased breathing activity is prolonged into the transitional HVECoG activity but does not continue into the established HVECoG.[29] A specific target of investigation was

*See references 17, 18, 27, 29, 33, 34, 36.

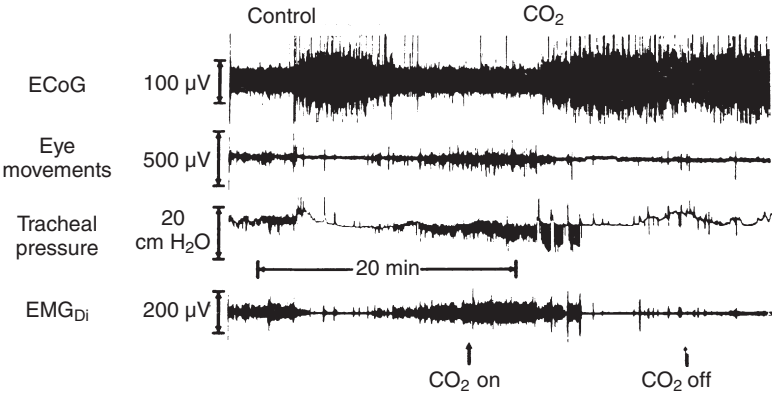


Figure 89-5 Fetal breathing under control conditions and during CO_2 rebreathing. Both tracheal pressure and diaphragmatic activity increase during CO_2 rebreathing. Fetal breathing was prolonged into the transitional low-voltage to high-voltage electrocortical activity *(ECoG)* but stopped in established high-voltage activity.

whether $Paco_2$ could be increased during rebreathing or during direct administration of carbon dioxide to the fetus through an endotracheal tube.[29] Breathing activity was always abolished in established HVECoG, and only when $Paco_2$ was unphysiologically high (greater than 100 mm Hg) and pH was low (less than 7.0 mm Hg) could breathing be initiated in HVECoG. At this level of acidosis, low pH could have been the primary stimulus to initiate breathing, because acidosis has been shown to induce continuous breathing.[37] This increased breathing activity was not associated with wakefulness.[29] Conversely, reducing $Paco_2$ below the "apneic threshold level" abolishes breathing activity,[38] as has been shown in postnatal life.[39-45]

Administration of low oxygen to the fetus by having the ewe breathe hypoxic mixtures abolished fetal breathing. This effect was associated with a decrease in body movements and in the amplitude of the ECoG signal.[18,46] This respiratory inhibition results mainly from direct effects of low O_2 tensions on the fetal brain through activation of adenosine A_{2A} receptors.[47-49] Koos and co-workers[50] found that neuronal lesions of the diencephalon that included the posteromedial group of thalamic nuclei abolished the depressant effects of hypoxia. More recently these investigators observed that neurons within, or proximate or connected to, the thalamic parafascicular nucli were crucially involved in the hypoxic arrest of fetal breathing.[51] Conversely, an increase in arterial Po_2 to levels above 200 mm Hg after the administration of 100% oxygen through an endotracheal tube induced continuous fetal breathing in some experiments in sheep.[52] These findings suggest that the low partial pressure of oxygen in the fetus at rest may be a normal mechanism contributing to inhibition of breathing in utero.

Of the pulmonary reflexes, the inflation reflex of Hering-Breuer is present in fetal life. Lung distention with saline infusion produced decreased frequency of breathing.[14,15] However, fetoplacental gas exchange, the incidence of fetal breathing, and sleep states were not affected by bilateral vagotomy performed during fetal life.[23,30] Although bilateral vagotomy did not prevent the establishment of continuous breathing at birth, vagal innervation was necessary to establish adequate alveolar ventilation in newborn lambs.[53]

A comprehensive review of the effects of various neurochemical agents on fetal breathing has been published.[54] In general, fetal breathing is inhibited by the γ-aminobutyric acid (GABA) agonist muscinol,[55] pentobarbitone,[56] and diazepam[57] acting at the $GABA_A$ receptor complex.[58] Other endogenous inhibitors include adenosine, the prostaglandin PGE_2 (see further on), and endorphins, which may be present at high levels in the fetal circulation. Agents that stimulate fetal breathing movements include prostaglandin inhibitors such as indomethacin, meclofanate, morphine, caffeine, pilocarpine, and 5-hydroxytryptamine (5-HTP). Fetal breathing is inhibited by maternal hypoglycemia, whereas it is stimulated after maternal meals and by hyperglycemia.

Plentiful evidence suggests that the actions of carbon dioxide and those of the pharmacologic agents are central. The effect of hypoxia is poorly understood, but it too seems to act centrally. The peripheral chemoreceptors originally were thought to be inactive in utero; however, the idea that they are completely silent probably was derived from incorrect experimental evidence.[59] Blanco and co-workers[60] have clearly documented activity of the peripheral chemoreceptors in the fetal lamb showing that they are reset at the time of delivery. Johnston and associates[61] found that the response to hypoxia in the fetal lamb with a pontine lesion is mediated through peripheral chemoreceptors. Resection of the carotid bodies does not alter fetal breathing or fetal state substantially and apparently does not alter the establishment of breathing at birth.[62,63] The exact relevance of peripheral chemoreceptors to intrauterine breathing remains undetermined.

Establishment of Continuous Breathing at Birth

The traditional view of the onset of continuous breathing at birth is that labor and delivery produce a transient fetal asphyxia that stimulates the peripheral chemoreceptors to induce the first extrauterine breath. Breathing is then maintained through the input of other stimuli such as cold, touch, and other sensory stimuli.[10,64-67] This overall view was generated through experiments done some years ago in the acute exteriorized fetal preparation at a time when the general consensus was that the fetus did not breathe in utero.

More recent observations have made this general view open to question. First of all, denervation of the carotid and aortic chemoreceptors does not alter fetal breathing or the initiation of continuous breathing at birth[62,63] (Figures 89-6 and 89-7). Moreover, continuous breathing can be initiated in utero, with manifestations of arousal, by raising arterial PO_2 with administration of 100% oxygen to the fetus through an endotracheal tube[52] (Figure 89-8). Continuous breathing does not occur if the arterial Po_2 does not rise. In fetuses in which Pao_2 does not rise, however, continuous breathing will subsequently initiate on cord occlusion.[52] This continuous breathing in response to high arterial Po_2 or cord occlusion is independent of an increase in arterial Pco_2, because the response remains when Pco_2 is kept constant by ventilating the fetus with high-frequency oscillation. These observations during administration of 100% oxygen and cord occlusion suggest that the fetus can resemble a newborn in utero. Breathing can occur in the fetus in the absence

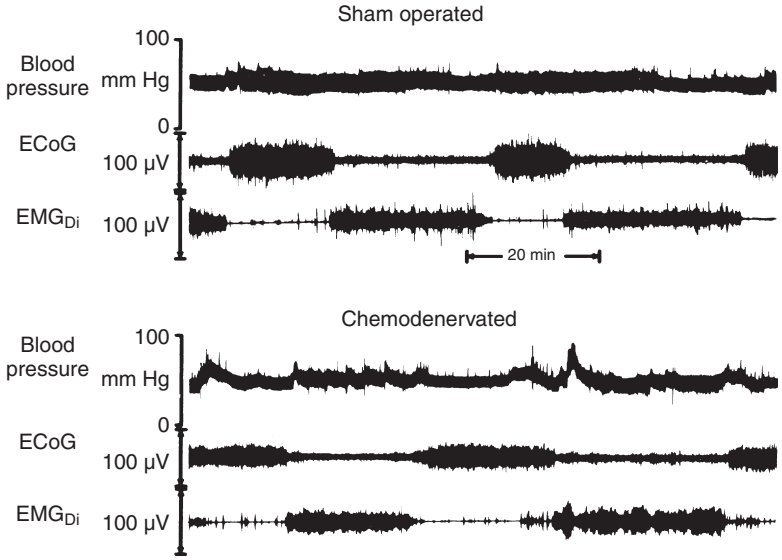


Figure 89-6 Breathing activity in a sham-operated fetal sheep (at 132 days gestation) and in a chemodenervated fetal sheep (at 130 days gestation). Breathing activity remains intact in the chemodenervated fetus. Blood gases were comparable in the two fetuses.

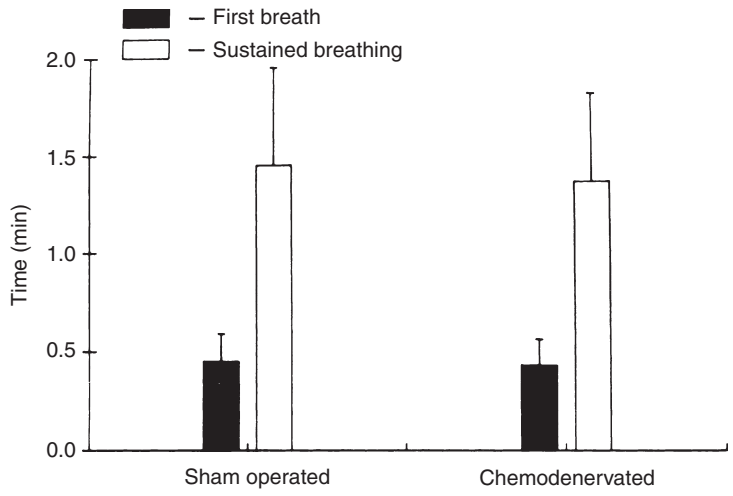


Figure 89-7 Delay in minutes from opening of the window to the appearance of the first breath and to sustained breathing. No significant differences between the two groups are seen.

of transient hypoxemia to stimulate the peripheral chemoreceptors and without any of the sensory stimuli, such as cold, once thought to be important for the establishment of continuous breathing at birth.

Thus the physiologic mechanisms responsible for the inhibition of fetal breathing and the establishment of continuous breathing at birth remain unknown. It has been debated whether the key factors in inducing these changes are intrinsic to the fetal brain or are localized to the placenta. Because placental separation at birth is associated with the onset of continuous breathing, various investigators, ourselves included, have hypothesized that placental factors may be responsible for the inhibition of fetal breathing.[68-72] This line of thinking is based on the assumption that the release of a factor by the placenta into the fetal circulation prevents fetal breathing from being continuous, with inhibition observed during HVECoG, and present only during periods of reticular activation, as observed during LVECoG. In the absence of this factor from the placenta at birth, after cord clamping, the sleep state–related inhibition observed during HVECoG is insufficient to disrupt continuous breathing. An teleologically interesting possibility is that nature may have delegated to the placenta the important role of providing the fetus with gas exchange and nutrients—and it also may have endowed the placenta with some form of chemoreceptor activity regulating fetal breathing and behavior by the secretion of chemical substances into the fetal circulation.

More direct evidence for a placental role has existed since Dawes[65] and Harned and Ferreiro[67] showed that only after the umbilical cord is clamped does the newborn lamb start breathing and behaving like a neonate. Subsequently, Adamson and colleagues[70] induced breathing in the fetus with umbilical cord occlusion and supply of O_2 delivered through an endotracheal catheter. On release of the cord, breathing ceased immediately, before any change in blood gases or pH, suggesting that a factor from the placenta might be involved. Workers in our laboratory were able to induce continuous breathing and wakefulness in fetal sheep by occluding the umbilical cord, so long as a gas exchange area was provided for the fetus in the form of an

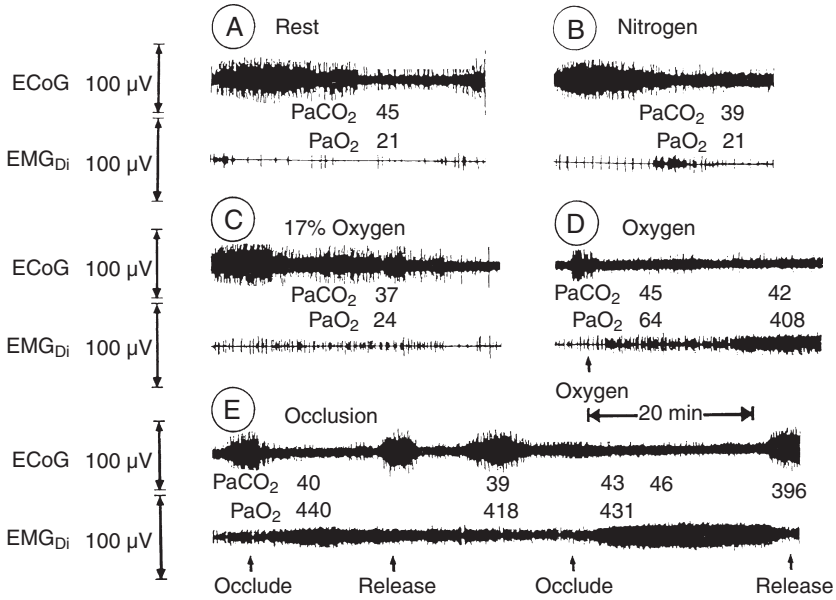


Figure 89-8 Representative tracings showing the effect of various concentrations of O_2 on fetal breathing and electrocortical activity. **A,** Control cycle showing little breathing in fetus in early labor at 143 days gestation. **B,** Lung distention (mean airway pressure 30 cm H_2O) and inspired N_2 do not affect baseline tracing. **C,** 17% O_2 also does not alter breathing. **D,** 100% O_2 induces continuous breathing. **E,** Occlusion on two occasions induces more forceful breathing than that observed with O_2 alone. Of note, continuous breathing was elicited despite preventing the rise of Pa_{CO_2} by subjecting the fetus to high-frequency ventilation (at 15 Hz, with stroke set at 7 cm H_2O).

endotracheal tube.[69,73-75] These experiments suggest the origin in the placenta of a compound that inhibits fetal breathing and fetal activity.

In trying to prove the hypothesis that a factor is released by the placenta, investigators in our laboratory injected the fetal sheep with a placental extract (obtained from cotyledons acutely dissected, sliced, and immersed in Krebs solution) after continuous breathing was induced by cord occlusion.[69] In all experiments, the placental extract decreased or abolished breathing. The infusion of the placental extract into the fetal circulation also inhibited spontaneous fetal breathing present during low-voltage electrocortical activity without inducing significant changes in blood gas tensions, pH, heart rate and blood pressure.[76] This factor appeared specific to the placenta, since the breathing response was absent with extracts from other tissues such as liver, muscle, or blood. Other recent work from our laboratory demonstrated that this factor in the placental extract probably is a prostaglandin, because treatment of the extract with indomethacin–acetylsalicylic acid (ASA) (which significantly reduced the concentration of prostaglandins) eliminated the activity of the extract[77] (Figure 89-9).

Indirect evidence that placental prostaglandins, especially PGE_2, are the mediators responsible for the inhibition of breathing in fetal life has been demonstrated by Kitterman[78] and Wallen[79] and their co-workers, who have shown that infusion of PGE_2 into the circulation of the fetal sheep induced a prompt and complete cessation of breathing movements. In addition, the incidence of fetal breathing movements was inversely correlated with both the PGE_2 dose and the mean PGE_2 concentration. Conversely, intravenous infusion of prostaglandin synthetase inhibitors, such as indomethacin or meclofenamate, induces continuous breathing for many hours in the fetus.[78,80,81] Thus the rate of placental prostaglandin production plays a significant role in setting the level of fetal breathing activity by producing a sleep-related inhibition in the fetal brain stem.

It is unlikely, however, that prostaglandins are involved in the inhibition of fetal breathing observed during hypoxia, because this inhibition persists after the administration of prostaglandin inhibitors. As mentioned previously, several studies have shown that adenosine is the likely mediator of the respiratory depression observed during hypoxia, because intravascular administration of adenosine inhibits fetal breathing and eye movements and the infusion of adenosine receptor antagonists blunts this inhibition.[82,83] In addition, brain disruptions that eliminate hypoxic inhibition of breathing abolish the depressant effects of adenosine.[84]

The Neonate

Breathing Pattern at Rest

The neonate, and particularly the premature infant, breathes irregularly. Great breath-to-breath variability and long stretches of periodic breathing in which breathing and apnea alternate are characteristic.[9,85-89] Haldane's statement that "the surprising fact is not that we breathe regularly, but that we do not breathe periodically most of the time" applies more at this age than at any other.[90] The resting breathing pattern of the neonate is not sleep state–dependent, although sleep greatly modulates it.[3,91]

Sleep traditionally has been divided into quiet, REM, transitional, and indeterminate states. Approximately 29% of sleep time in neonates is spent in quiet sleep, 33% in REM, 7% in transitional sleep, and 31% in indeterminate sleep. The proportion of quiet sleep increases with age, whereas the amount of REM decreases with age. The proportion of wakefulness decreases with decreasing gestational age, and in very immature infants, it becomes difficult to define wakefulness or arousal.[92-94] Studies have shown that periodic breathing, a common breathing pattern in premature infants in which they alternate between breathing intervals and apneic episodes lasting 5 to 10 seconds, occurs in the three states of wakefulness, REM, and quiet, but its prevalence is increased in REM sleep.[3] It frequently is stated in textbooks that in quiet sleep, in analogy with criteria used for adult subjects, breathing is regular. Studies from our laboratory and others, however, have clearly shown that periodic breathing is common

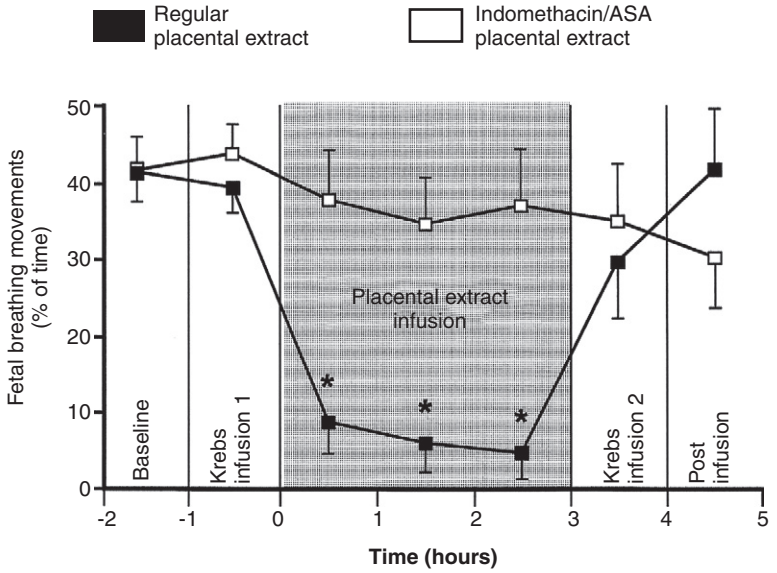


Figure 89-9 The effects of eliminating the prostaglandins from the placental extract on fetal breathing. Of note, fetal breathing remains essentially unchanged with the infusion of a regular placental extract (not treated with indomethacin-ASA), whereas it decreased significantly with the infusion of an indomethacin-ASA–treated extract. *ASA,* Acetylsalicylic acid.

in quiet sleep.[9,95,96] The difference is that periodic breathing is regular in quiet sleep—that is, the breathing and apneic intervals are of similar duration—and very irregular in REM sleep. The most well-defined periodic breathing observable in small babies is in quiet sleep during discontinuous activity in the classic tracé alternant pattern on the electroencephalogram (EEG) (Figure 89-10). Therefore two major differences are recognized between neonates and adults regarding staging of sleep state. One difference relates to the patterns of breathing observed in quiet and REM sleep states and the other to the presence of the tracé alternant EEG pattern during quiet sleep in the neonate. Because this pattern subsides after 44 weeks postconceptual age, it is not used in adults to characterize quiet sleep. Finally, the overall minute ventilation is increased in REM sleep as compared with quiet sleep; this difference is due to a primary increase in respiratory frequency with little change in tidal volume.[3,6,9]

Periodic Breathing and Apnea

Periodic breathing, defined as pauses in respiratory movements that last for up to 20 seconds alternating with breathing, is common in preterm infants. When the respiratory pause is longer than 20 seconds, it is called *apnea.*[97] Although the duration used to distinguish periodic breathing and apnea is arbitrary, it has proved useful and has been widely adopted. Periodic breathing is not as harmful as apnea, because the respiratory pause is short and the decrease in heart rate is minor. By contrast, apnea is a more serious condition, the respiratory pause being longer and frequently associated with decreases in heart rate below 80 beats/minute.[86,97] In very small preterm infants, significant bradycardia can occur with very short apneic pauses. In this instance, therefore, the length of the respiratory pause is not a very useful indicator of severity of the disruption in breathing. For this reason, many centers, including ours, have decided to rely on heart rate as the primary indicator of severity.

Apneic episodes in the neonate are classified according to the absence or presence of breathing efforts during the period of no airflow.[98] Central apneas are those with no flow and no observable breathing efforts. Obstructive apneas are those with no flow despite breathing efforts. Mixed apneas begin as central and end as obstructive apnea. Breath-holding apneas are those in which flow stops at mid-expiration and the remaining expiration occurs just before breathing starts again. A newer method of classifying apneas, devised in our own clinical laboratory, is based on a magnified cardiac stroke–induced pulse observed on the respiratory flow tracing. This method permits detection of presence and timing of airway obstructions with great precision. With use of this method, it has become obvious that some apneas previously classified as central on the basis of absence of respiratory efforts are in fact obstructive.[99] We have been using this method extensively

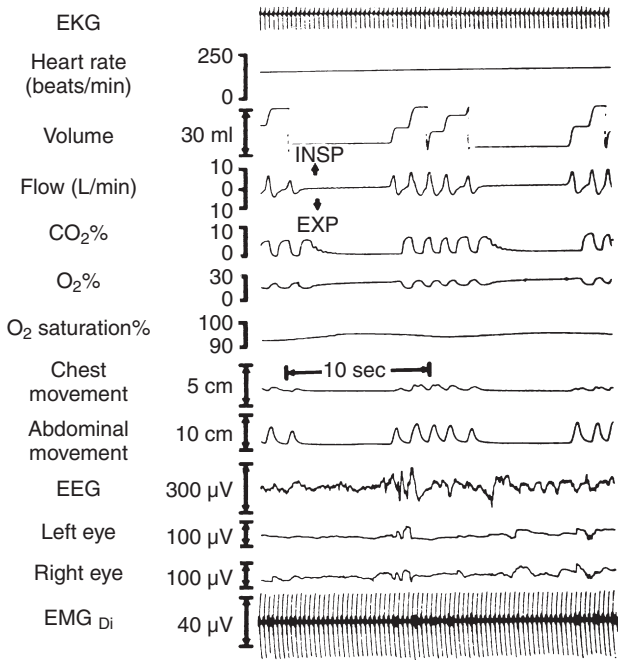


Figure 89-10 Periodic breathing during quiet sleep in an 8-day-old preterm infant born at 32 weeks. The regular periodicity of breathing, with both apneic and breathing intervals of constant length, is characteristic. The classic tracé alternant pattern is evident on the electroencephalogram (EEG).

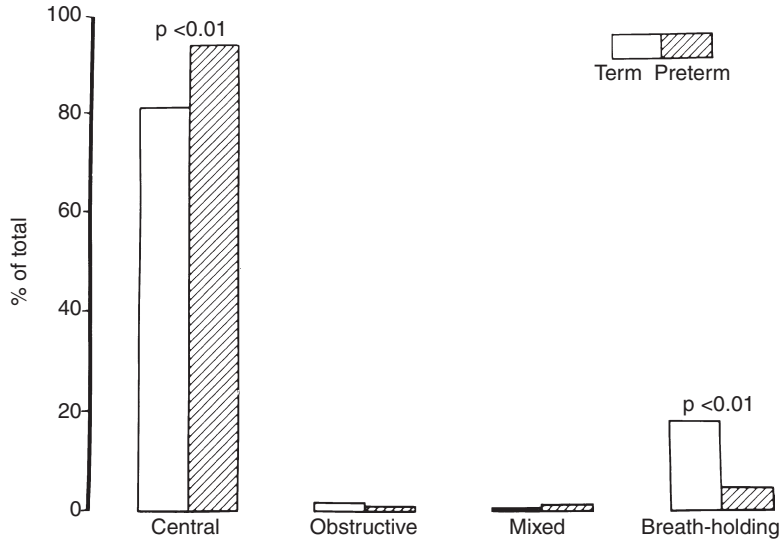


Figure 89-11 Frequency distribution of the various types of apneas in healthy preterm and term infants. Central apneas are predominant. The frequency of breath-holding apneas is greater in term than in preterm infants. Obstructive and mixed types of apneas are rare.

in our experimental studies, but its use in routine clinical care is not yet established.

As determined in preterm infants without underlying disease studied over a period of 3 months, central apneas predominate, and purely obstructive apneas are rare[98] (Figure 89-11). Obstructive apneas more commonly are noted as part of a mixed apneic event. In preterm infants recovering from respiratory support, with some degree of residual lung disease (bronchopulmonary dysplasia), the prevalence of obstructive apneas appears to be increased, accounting for up to 48% of the apneic episodes in some studies. No clear explanation for the obstruction has emerged, but it seems to be at the level of the larynx.[100] Our initial observations suggest that borderline hypoxemia of these infants may be a predisposing factor. One report has described obstruction in 80% of the pauses in preterm infants with periodic breathing.[101] In data from unpublished work in our laboratory, this high prevalence has not been corroborated, but our findings indicate that small preterm infants can exhibit short-duration mixed apneas associated with a significant decrease in heart rate.

Periodic breathing and apnea clearly are a consequence of a disturbance of the respiratory control system, but the precise mechanisms are unclear. Investigators tend to believe that the negative feedback loop controlling respiration is affected by multiple factors related primarily to anatomic and physiologic immaturity. For example, arborization of dendrites at 30 weeks gestation is meager, and neuroconduction and synaptic relay are impaired.[102] Delays in trafficking of neuromessages may then make the system oscillate. Unfortunately, how much immaturity is needed for a given impairment in neurophysiologic traffic has not yet been established. Oscillation in arterial gas tensions, changes in circulation time, incoordination of the respiratory pump secondary to a compliant chest wall, and changes in sleep state all may contribute to this instability of the respiratory control system.[97]

A relevant controversy in the literature has centered on whether periodic breathing and apnea are mechanistically different or whether long apneas constitute just a step further in the basic respiratory disturbance that induces the short apneas of periodic breathing. A study carried out in our laboratory showed that (1) a prolonged apnea almost never occurred in the absence of preceding short apneas, and (2) the risk for occurrence of a prolonged apnea increased significantly when the preceding period contained an increased number of apneic episodes, increased duration of the longest apneic interval, or increased duration of the apneic time.[103] More recent studies have shown that the periodic breathing cycles in REM but not in quiet sleep were associated with progressive decreases in minute ventilation and oxygenation, probably related to the mechanical and chemoreceptor limitations known to be present in this sleep state.[104] In all likelihood, periodic breathing, especially during REM sleep, is a marker for apnea, because apneas almost never arise abruptly in infants breathing regularly but occur only in infants whose respiratory pattern is characterized by significant periodicity.

Compared with infants who are breathing continuously, neonates exhibiting periodic breathing have lower P_{O_2} values, and their peripheral chemoreceptors are more hyperactive, as reflected by the longer apneic period and more pronounced immediate decrease in ventilation in response to inhalation of high-concentration oxygen mixtures.[86,87,105] Thus the major reason for background instability appears to be the significant contribution of the peripheral chemoreceptor drive to normal breathing at this age. Indeed, the arterial P_{O_2} tension in these infants sits on the steep portion of the minute ventilation–arterial P_{O_2} regression curve for human adults. This means that small changes in baseline arterial P_{O_2} produce large changes in baseline ventilation. Hypoxia may be a contributing factor, because inhalation of a low-concentration oxygen mixture easily induces periodic breathing and apnea in these infants.

As reported in a study from our laboratory, the average CO_2 apneic threshold in preterm infants is only 1.5 mm Hg lower than the eupneic P_{CO_2}, whereas in adults it is approximately 5 mm Hg lower.[43] The closeness of eupneic and threshold P_{ACO_2} likely confers a great vulnerability to respiratory stability in these infants. It is not surprising, therefore, that brief startles, movements, or changes in sleep state could allow eupneic P_{CO_2} to dive below the P_{CO_2} apneic threshold, inducing periodic breathing and apnea in these infants (Figure 89-12). We think that the key element responsible for this narrow difference is the well-known hypoxemic status of these infants. Xie and colleagues recently showed that in adults, the time course of the occurrence of apnea after transient hyperpnea was consistent with a peripheral chemoreceptor mechanism. In this study, hypoxia shortened the apnea latency and narrowed the eupneic-apneic alveolar P_{CO_2} (P_{ACO_2}) threshold, whereas hyperoxia delayed the onset of apnea and widened the eupneic-apneic P_{ACO_2} threshold.[45] The same

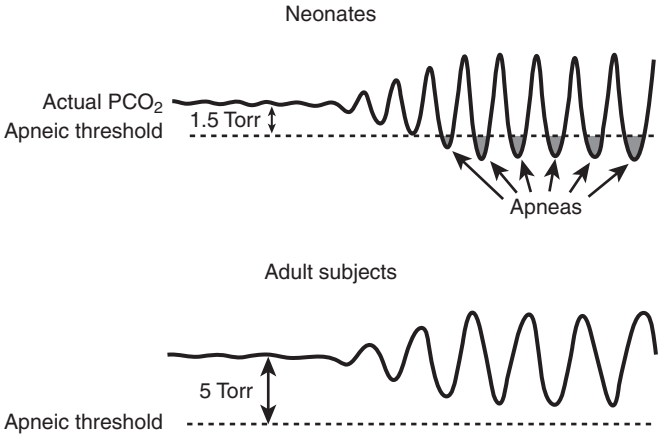


Figure 89-12 Diagrammatic representation of the relationship between the CO_2 apneic threshold and the baseline or actual P_{CO_2} levels in neonates and adults. Because of the proximity of these two levels in the neonate, P_{CO_2} is much more likely to dive below the apneic threshold than in the adult.

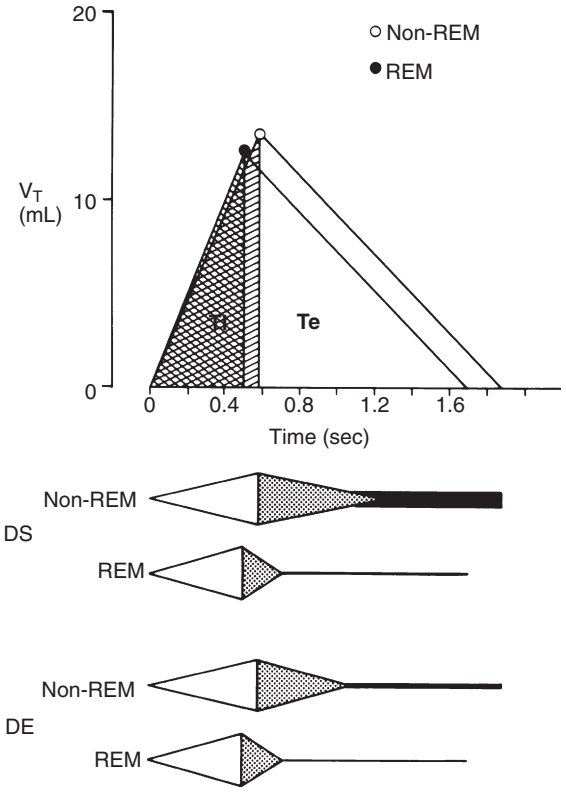


Figure 89-13 Schematic diagram showing changes in tidal volume, timing, and diaphragmatic electromyography (EMG) signal in non–rapid eye movement (non-REM) (quiet) and REM (active) sleep. Of note, total phasic activity diminishes from non-REM to REM sleep. Also, in both sleep states, it is shorter in esophageal *(DE)* than in surface *(DS)* EMG tracings. Expiratory phase activity as a proportion of total phasic activity decreases significantly from non-REM to REM sleep.

investigators previously had shown that the smaller difference between eupneic and apneic P_{ACO_2} during hypoxia was due to a disproportionate reduction in the eupneic P_{ACO_2} rather than a higher apneic threshold.[106] The low arterial P_{O_2} in newborn infants may keep the eupneic P_{CO_2} relatively low, not by hyperventilation as in adult subjects but by a decrease in metabolism that parallels the decrease in arterial P_{O_2}.

The sleep state appears also to be a contributing factor, because periodic breathing and apnea are more frequent in REM sleep than in quiet sleep. The neonate sleeps almost uninterruptedly, continuously alternating between REM sleep and quiet sleep. This pattern increases instability in the respiratory control system. Indeed, minor alterations during sleep, such as a startle or a sigh, produce apnea in these infants. The almost continuous change in baseline ventilation during sleep is what Haldane called "the hunting of the respiratory centre."[90]

Although sleep modulates breathing, it does not cause apnea; apnea also occurs during wakefulness. The high prevalence of apnea during REM sleep may be related to muscle activity in this stage. During REM sleep, the tone of the intercostal muscles is abolished in conjunction with a decrease in diaphragmatic activity and in the tone of the adductor muscles of the upper airway—a combination of factors likely to induce chest distortion, impairment of the braking mechanism during expiration, pulmonary collapse, and apnea[97] (Figure 89-13). When chest distortion occurs, diaphragmatic work increases by approximately 40%, adding to the mechanical impairment. This observation is compatible with the finding that the application of continuous negative pressure around the chest tends to abolish apnea.[107]

Chemical Regulation

Inhalation of carbon dioxide increases ventilation during REM and quiet sleep in newborn infants. The response to steady-state inhalation of carbon dioxide is the same in these two sleep states, but the response during rebreathing of carbon dioxide is less pronounced in REM than in quiet sleep[3,6,8,95] (Figure 89-14). Workers in our laboratory postulated that the differences in response with these two techniques relate to the fact that using rebreathing, it is possible to measure the response in "phasic" REM only, whereas using the steady-state technique the response always covers both "phasic" and "tonic" REM sleep.[95] Because the carbon dioxide response in "tonic" REM is the same as that during quiet sleep, the results with use of the steady-state method tend to resemble those in quiet sleep.[7,108]

The pattern of breathing observed with inhalation of carbon dioxide differs according to the percentage of carbon dioxide inhaled. If the percentage of inhaled carbon dioxide is low (less than 2%) during steady-state inhalation, the response consists primarily of an increase in tidal volume.[9] If the percentage of inhaled carbon dioxide is high (greater than 2%), the response in both sleep states consists of an increase in respiratory frequency and in tidal volume.[87,95] Periodic breathing is abolished with a small increase in inhaled carbon dioxide of approximately 1% to 2%.[9,87] This response has been attributed to the increased central drive and increased stores of carbon dioxide, with better buffering capacity for the oscillations in P_{ACO_2}.

Inhalation of oxygen at low concentrations produces an immediate increase in ventilation (at 1 minute) followed by a later decrease (at 5 minutes).[85,88,109,110] Responses are similar in wakefulness, REM, and quiet sleep, although hyperventilation seems slightly more sustained during late hypoxia in quiet sleep.[3] The more sustained hyperventilation in these infants during quiet sleep reflects the more autonomic control during this sleep state, the system being more responsive to chemical stimuli.[7,108] The immediate increase in ventilation reflects peripheral chemoreceptor stimulation and is associated with an increase in frequency and in tidal volume. The late response is manifested primarily by a decrease in frequency.[86,88]

The mechanism responsible for this response is still unclear and may vary according to species. In humans it is likely to be related to central release of inhibitory neuromodulators.[111] Experiments in kittens and in newborn monkeys, however, suggest that the late

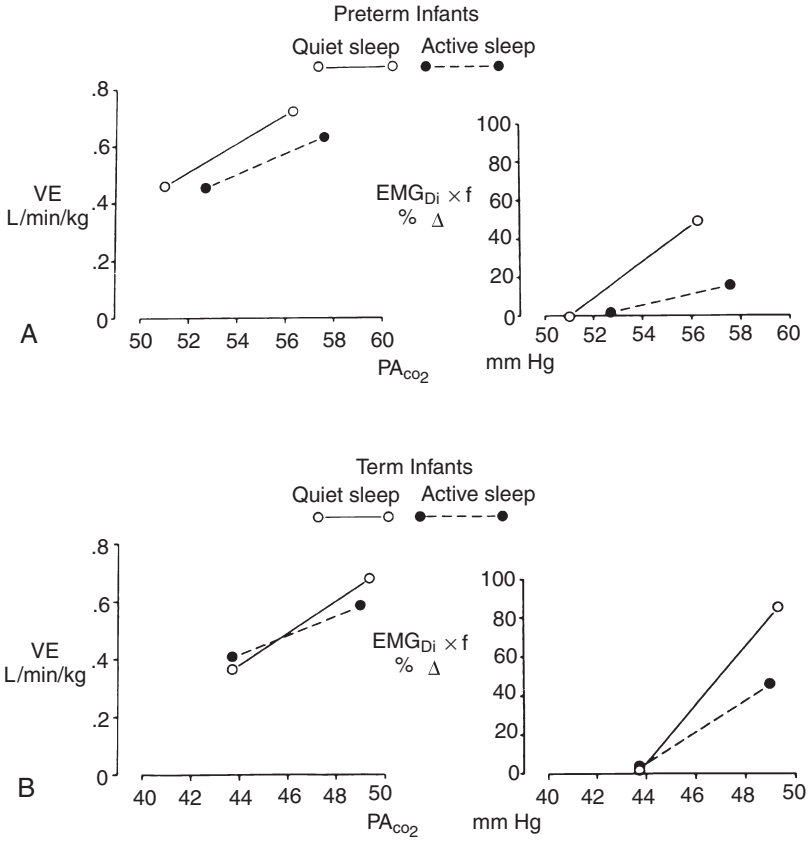


Figure 89-14 The ventilatory response to CO_2 rebreathing in neonates. Both preterm **(A)** and term **(B)** infants showed a decreased response to CO_2 in phasic rapid eye movement sleep as compared with quiet sleep.

decrease in ventilation may be a mechanical effect, rather than a depression of the central respiratory neurons.[112,113] In these experiments, diaphragmatic activity and respiratory frequency remained elevated during hypoxia, but tidal volume decreased below control values during late hypoxia. These experiments were carried out during quiet sleep. The peculiar response of the neonate to low concentrations of inhaled oxygen is of great clinical significance. Infants who are borderline hypoxic tend to breathe periodically or to experience apneic spells. Hypoxia can induce periodic breathing in these infants, as shown previously.[67] Relief of these apneic spells, which frequently are associated with bradycardias, can be obtained by increasing the inspired oxygen concentration.[86,87]

Administration of high concentrations of oxygen, on the other hand, produces an immediate decrease in ventilation followed by hyperventilation—a response that occurs during wakefulness, REM, and quiet sleep alike. These findings suggest a lack of major differences in the activity of the peripheral chemoreceptors during these sleep states.[97,114] The immediate decrease in ventilation after the administration of 100% oxygen is related to a decrease in respiratory frequency (apnea being common in preterm infants) and a decrease in tidal volume. The late increase in ventilation with oxygen probably is related to cerebral vasoconstriction with increased hydrogen ion (H^+) concentration at the chemoreceptor site.[115]

Pulmonary Reflexes

The inflation reflex of Hering-Breuer is much more active in the newborn period than in adult life.[116,117] Small increases in lung volume cause apnea. This response is so powerful in the newborn that many investigators have used this inflation to produce apnea and then study the mechanical properties of the respiratory system during the passive expiratory phase that follows apnea. The action of the stretch receptors is much influenced by sleep, being abolished during REM sleep. The irritant receptors also are poorly developed in preterm infants, and the mediated reflexes are abolished during REM sleep as well.[118] Therefore airway mechanisms responsible for clearing, such as cough, are impaired during REM sleep.

The paradoxical reflex of Head commonly is observed in the neonate in the form of a sigh.[119] Many investigators attribute the high prevalence of sighs to the greater need for lung recruitment at this age.[120] Sighs are more frequent in REM than in quiet sleep and also during periodic breathing than during regular breathing. During periodic breathing, a sigh usually appears during the first or second breath after the apneic episode. When it occurs during regular breathing, it tends to be followed by short apneas.[121] Efforts to discover the mechanisms triggering sighs have been fruitless. Thach and Tauesch[120] showed that asphyxia does not seem to be a stimulus. Alvarez and colleagues,[122] however, have observed that airway occlusion in the presence of hypoxia predisposes to sighs.

Studies of the morphology of sighs in infants and adult subjects found that the sighs in infants were found to be relatively larger than those in adults, and that whereas post-sigh ventilation usually is increased in adults, it is decreased in infants.[123] Because the drive to breathe early in life is dependent on increased peripheral chemoreceptor activity, it is conceivable that the sudden increase in arterial P_{O_2} with sighing could produce a rapid decline in carotid body afferent discharge leading to hypoventilation and apnea. The other almost instantaneous change that occurs with sighing includes a decrease in P_{CO_2}. Because the CO_2 apneic threshold is much closer to the baseline CO_2 in neonates than in adults, the decline in CO_2 below the threshold during a sigh

could trigger an apnea or initiate an epoch of periodic breathing in infants with immature respiratory feedback loop.[43,124] These findings suggest that although the ability to sigh may be an important mechanism to restore lung volume, sighing has the potential to destabilize breathing, leading to hypoventilation and apnea in infants at risk for inadequate control of breathing.[123]

Respiratory Muscles

The activity of the respiratory muscles is much altered by sleep state. Tonic activity of most respiratory muscles is abolished during REM sleep.[19,27,125,126] The disappearance of tone in the intercostal muscles has been suggested as a major factor responsible for the increased chest distortion seen during REM sleep in infants with this condition. Lack of tone leads to chest wall collapse during inspiration, and caudal displacement of the diaphragm has to be twice that normally required to produce the same lung volume displacement.[125,127] Because of chest wall collapse, functional residual capacity is decreased in these infants during REM sleep.[125] Investigators in our laboratory have found that distorted and nondistorted breaths produce the same instantaneous ventilation so long as they are of the same duration, although the work of the diaphragm is 40% greater when distortion is present.[127]

Postinspiratory activity of the diaphragm also is affected by sleep. This activity controls, in part, the duration of expiratory time.[128,129] In neonates, this activity is more pronounced in the lateral than in the crural part of the diaphragm, longer in quiet than in REM sleep, and more prolonged in preterm than in term infants.[130] The duration and variability of this activity in preterm infants suggest that because of their highly compliant chest wall, the postinspiratory diaphragmatic activity can be used as a braking mechanism to maintain lung volume. In addition, controlling expiratory time probably is much more important in preterm infants than in older children and adult subjects. Similarly, sleep state profoundly affects the muscular control of upper airway resistance. Studies in fetal and neonatal lambs suggest that the abductor muscles of the larynx—the posterior cricoarytenoid and cricothyroid—have inspiratory activity in parallel with that of the diaphragm, during both quiet and REM sleep. On the other hand, the adductor muscles of the larynx—the thyroarytenoid, lateral cricoarytenoid, and intraarytenoid—have a phasic expiratory activity during quiet sleep. This activity is lost during REM sleep in the fetus and in the newborn lamb.[19,27] A reduction in adductor activity of the larynx, in conjunction with decreased intercostal and decreased postinspiratory diaphragmatic activity during REM sleep, may cause the decrease in lung volume observed during this sleep state.

CONCLUSION

In summary, although the basic mechanisms involved in the control of breathing during fetal and neonatal life are similar to those investigated more extensively in adult subjects, some relatively recent findings point to the unique nature of control of breathing at this age. First, sleep seems to have a very profound effect during this period of life, particularly in the fetus, in which breathing is allowed to surface only during REM sleep. Second, breathing activity is present in utero since early gestation and without apparent reason, because it is not responsible for gas exchange. The placenta is responsible for gas exchange, being the "lung" of the fetus. It is therefore imperative to discover the purpose of fetal breathing. Third, the discovery of fetal breathing probably is the most exciting contribution made in this area during the past 50-some years. To explain why this episodic breathing in utero becomes continuous after birth is the major challenge of the moment. Elucidation of specific aspects of this change may result in the discovery of key mediators that are at the heart of the mechanism controlling breathing in general.

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Basic Mechanisms of Oxygen Sensing and Response to Hypoxia

A large body of experimental work has been performed on oxygen sensing and on the cellular events that result from oxygen deprivation. This work has been performed in a variety of cell types, tissues, and organs, and these comparative studies have contributed to our understanding of O_2 sensing. As a result of these investigations, a number of new concepts regarding O_2 sensing have emerged. Neurons, renal and respiratory epithelial cells, hepatocytes, myocardial cells, vascular smooth muscle, or endothelial cells—virtually every cell type studied has been shown to sense O_2 one way or another. This type of investigation has also been done at various ages, and age has been very important in determining both sensing and responsiveness to lack of O_2. Because we have generally focused in the past on the study of nerve and glial cells during O_2 deprivation, this chapter focuses on excitable tissues. However, an extensive body of new knowledge has demonstrated that much of what is known about the heart's sensing mechanisms can be applied to the central nervous system, for example, and much of what we know about the renal epithelium can be applied to the respiratory cells. It should be recognized, however, that, although there are similarities among tissues, there are often major differences. These differences reflect either differing environments or the function of that particular cell type of tissue. In certain instances, when the duration and severity of the stimulus are not too overwhelming, nerve cells may adapt and possibly survive hypoxia. Often, however, when the stress is too severe, the response of the cells, from sensing to death, is considerably shortened, and it is often difficult to tease apart the processes that control the various stages of response.

The aim of this chapter is to highlight observations that will demonstrate that a number of potential O_2 sensors exist in nerve cells. We detail results and data regarding ionic flux and controlling ionic flux mechanisms. We also detail some newer observations regarding expressional regulation of genes during O_2 deprivation that occurs over much longer periods of time.

OXYGEN SENSING VIA MEMBRANE PROTEINS: IONIC ALTERATIONS

Potassium Ionic Flux and Its Regulation: Short Versus Long Exposures

Potassium ion (K^+) channel modulation has been shown to be an integral and important cellular response to O_2 deprivation in nerve and cardiac cells. It is not clear, however, what signals precede the modulation of K^+ channels. This modulation could be direct or indirect.[1,2] Part of this modulation occurs as a result of changes in the concentrations of cytosolic factors (e.g., adenosine triphosphate and calcium ion [Ca^{2+}]) that are altered during O_2 deprivation. For example, a number of cytosolic factors change during hypoxia: Ca^{2+}, pH, sodium ion (Na^+), adenosine triphosphate–adenosine diphosphate–adenosine monophosphate ratios, and redox.[3-8] These, in turn, modulate a number of ion channels, including K^+ channels.

If K^+ channels can be modulated by cytosolic changes during hypoxia, one should question whether there are mechanisms originating from changes other than in the cytosol. For example, does the partial pressure of O_2 itself affect plasma membrane channels? To test the hypothesis that membrane-delimited mechanisms participate in the O_2-sensing process and are involved in the modulation of K^+ channel activity in central neurons, experiments were performed with use of patch-clamp techniques and dissociated cells from the rat neocortex and substantia nigra.[1,2] Oxygen deprivation produced a biphasic response in current amplitude: an initial transient increase was followed by a pronounced decrease in outward K^+ currents. The reduction in outward currents was a reversible process because perfusion with a normoxic medium with a Po_2 greater than 100 mm Hg (1 mm Hg = 133 Pa) resulted in complete recovery. In cell-free excised membrane patches, we have demonstrated that a specific K^+ current (large conductance, inhibited by micromolar concentrations of adenosine triphosphate and activated by Ca^{2+}) was reversibly inhibited by lack of O_2. This was characterized by a marked decrease in channel open-state probability and a slight reduction in unitary conductance. The magnitude of channel inhibition by O_2 deprivation was closely dependent on O_2 tension. These studies demonstrated the selective nature of the hypoxia-induced inhibition of some K^+ channels[1,2,8] (Figures 90-1 and 90-2) and also provided the first evidence for the regulation of K^+ channel activity by O_2 deprivation in cell-free excised patches of central neurons.[9,10]

It is not well understood how this inhibition occurs. With the use of specific agents that chelate metal, including heme and nonheme iron and copper, our laboratory has demonstrated that iron-center blockers inhibited the channel in excised patches in a fashion similar to that of low Po_2. These results suggested that K^+ channel activity is modulated during hypoxia by iron-containing proteins, thus providing evidence for an O_2-sensing mechanism in neuronal membranes.

Although most ionic fluxes that have been studied during hypoxia are plasma-membrane related, a number of reports on ion channels in mitochondrial membranes have been published.[11,12] However, it is unclear how these are involved in O_2 sensing or in hypoxic injury. We have obtained evidence that the maxi-K channels can be found in mitochondrial membranes and that they play a role in apoptotic cell death induced by serum deprivation. Furthermore, data demonstrate that mitochondrial channels (adenosine triphosphate–dependent K^+ channels) are present in myocardial cells and may be important for hypoxic injury.[13]

The observation of the relative "insensitivity" or the lack of response of the immature cells or tissues to low O_2 tensions compared with the more mature response of differentiated cells is of particular interest. On the basis of a large number of studies investigating ion fluxes and activities, release of neurotransmitters, or changes in membrane electrophysiologic properties, it is clear that the newborn exhibits a blunted response to hypoxia.[3,4,14-17] Although there is a paucity of cellular and molecular work on this subject in the fetus, it is clear from whole-body studies in the fetus exposed to hypoxia that its response is very blunted, if not totally eliminated. In other types of studies, however, investigating the response of the neonatal lung to hypoxia (compared with that of the adult), the response in terms of septation and alveolar formation was more severe in the neonate than in the adult.[18] For example, hypoxia in the first couple of weeks of life will inhibit septation, whereas in the adult there is very little effect on alveolar size.[18] Therefore, depending on the measured variable, the newborn infant may or may not be as responsive as the adult. Because most systems exhibit plasticity at an early stage in life, strong stimuli may affect function and structure much more in the neonate, especially when these stimuli occur over prolonged periods of time. Acute short stresses, however, especially those in the central nervous system, may not be so significant for the newborn infant.

Sodium Ionic Flux and Its Regulation: Short Versus Long Exposures

The concentrations of Na^+ and chloride ion (Cl^-) change markedly during hypoxia. Anoxia induces a drop in extracellular Na^+ in brain slice, and removal of extracellular Na^+ prevents the anoxia-induced morphologic changes in dissociated hippocampal neurons.[6,19,20] For an understanding of the mechanisms that sense O_2 and lead to acute neuronal swelling during anoxia, the ionic movements of Cl^- and Na^+ during O_2 deprivation in the hypoglossal (XII) neurons of rat brain slices have been studied. Baseline extracellular Cl^- and Na^+ activities ($[Cl^-]_o$ and $[Na^+]_o$) were measured in both adult and neonatal brain slices.[6,19,20] During a period of anoxia (4 minutes), $[Na^+]_o$ decreased markedly in adult slices whereas $[Na^+]_o$ did not show any significant change in the neonatal slice. Anoxia induced a significant decrease of $[Cl^-]_o$ in both the adult and neonate; however, $[Cl^-]_o$ dropped seven times more in the adult than in the neonate. Intracellular Cl^- activity ($[Cl^-]_i$) has been studied in adult hypoglossal cells. It is not surprising that an increase in $[Cl^-]_i$ with O_2 deprivation was seen. To also study intracellular Na^+ ($[Na^+]_i$) in isolated neurons, we used the fluorophore Sodium Green in freshly dissociated rat neurons, and sodium-binding benzofuran isophthalate (SBFI), a fluorescent indicator for sodium, in cultured cortical neurons. Ten minutes of anoxia caused an increase in Na^+_i with a latency of approximately 2 minutes. In these neurons, fluorescence increased by an average of approximately 20%. We conclude that during anoxia (1) intracellular $[Cl^-]$ and $[Na^+]$ increase in the adult, most likely because of entry of extracellular ions into the cytosol, and (2) a major maturational difference exists in mechanisms regulating Cl^- and Na^+ homeostasis between neonate and adult brain tissue.

Although it is well known that Na^+_i increases during hypoxia, the sensors for this ionic alteration are not well documented. It is possible that exchangers or transporters and channels are somehow located within the cascade of events that lead to this increase. The regulation of the voltage-sensitive Na^+ channels during hypoxia has been investigated with use of isolated hippocampal neurons.[5,20] Given the prior data demonstrating an

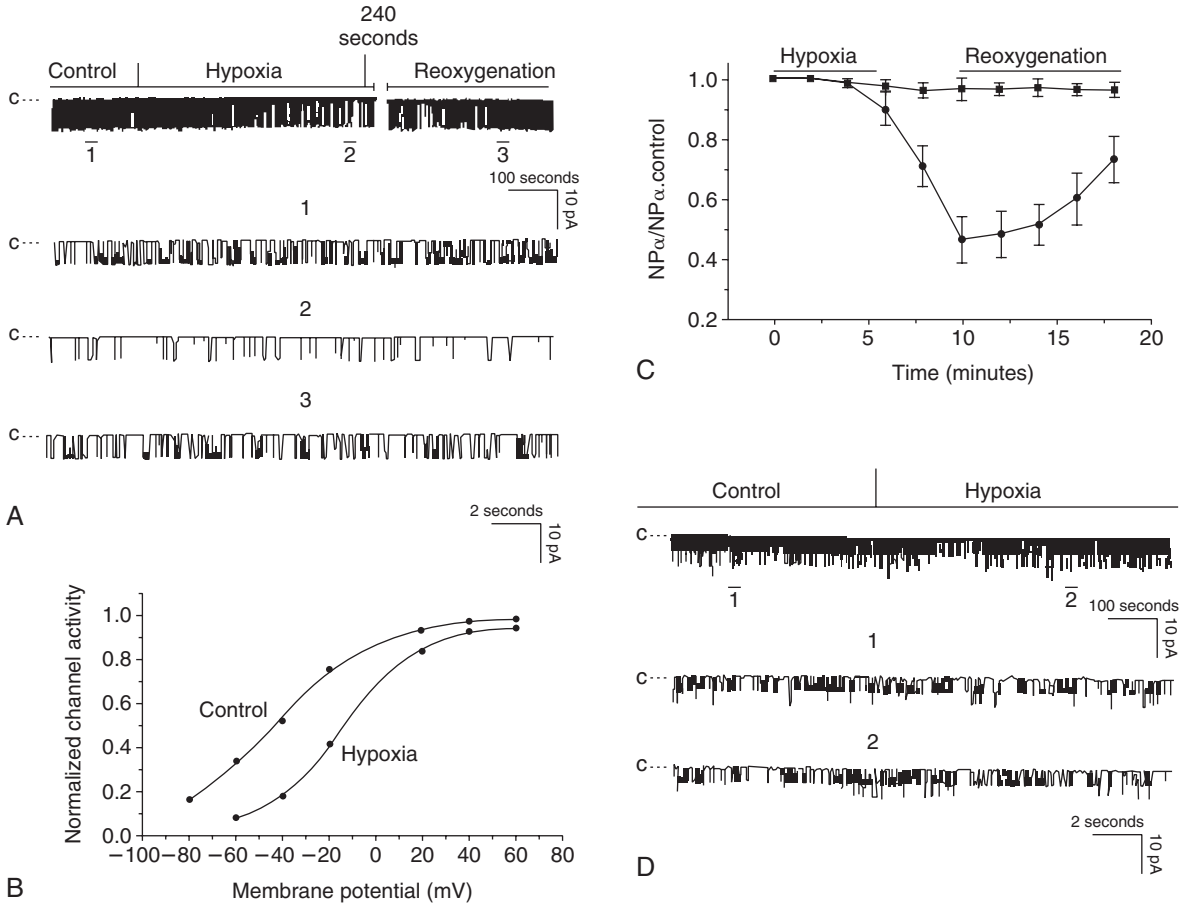


Figure 90-1 Effect of hypoxia on single-channel BK_{Ca} current. A, Effect of hypoxia on single BK_{Ca} channel in a cell-attached patch from a neocortical neuron. Current was recorded with high-KCl (140 mmol/L) solution in the pipette and physiologic solution in the bath at a V_m of −30 mV. The channel closed level is indicated by *C.* Three parts of the compressed trace are shown, as indicated by numbers *1* through *3* (fast-time resolution). **B,** Effect of hypoxia on the voltage activation of BK_{Ca} channel under the ionic condition just described. The line is fitted to a Boltzmann distribution. $V_{0.5}$ shifted from −42.4 ± 4.8 mV to −18.6 ± 3.5 mV after exposure of hypoxia for 10 minutes. **C,** Time course of the hypoxia-induced effect on NP_o. In cell-attached recordings *(filled squares),* channel inhibition started about 5 minutes after the onset of hypoxia, and a maximum inhibition was reached in approximately 10 minutes. After that time, NP_o was markedly reduced to approximately 43% of control level. Reoxygenation led to partial recovery. In inside-out recordings *(filled circles),* NP_o was not significantly affected during hypoxia. **D,** Continuous recording of a single BK_{Ca} channel current from an inside-out patch of a neocortical neuron during hypoxia, with use of a symmetric 140-mmol/L KCl on both sides of the membrane, with a V_m of −30 mV. The channel closed level is indicated by *C.* Two parts of the compressed trace (indicated by the numbers *1* and *2*) are shown at fast-time resolution. *BKCa,* Large-conductance calcium channel; *NPo,* product of number of channels open multiplied by the open probability. *(From Liu H, Moczydlowski E, Haddad GG: O_2 deprivation inhibits Ca^{2+}-activated K^+ channels via cytosolic factors in mice neocortical neurons,* J Clin Invest *104:577, 1999.)*

increase in intracellular Na^+, it is somewhat surprising that hippocampal neurons respond to acute oxygen deprivation with an inhibition of whole-cell Na^+ current (I_{Na}).[10,21] Because kinases can modulate I_{Na} and are activated during hypoxia, we hypothesized that kinase activation may play a role in the hypoxia-induced inhibition of I_{Na}. I_{Na} was recorded at baseline, during exposure to kinase activators (with and without kinase inhibitors), and during acute hypoxia (with and without kinase inhibitors). Hypoxia reduced I_{Na} to approximately 40% of initial values and shifted the steady-state inactivation in the negative direction.[22] Hypoxia produced no effect on activation or fast inactivation. Protein kinase A (PKA) activation with 3′,5′-cyclic adenosine monophosphate, N^6,O_2-dibutyryl, sodium salt (db-cAMP) resulted in a reduction of I_{Na} to 63% without an effect on activation or steady-state inactivation. I_{Na} was also reduced by activation of protein kinase C (PKC) with phorbol 12-myristate 13-acetate (PMA) to 40% or with 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) to 46%. In addition, steady-state inactivation was shifted in the negative direction by PKC activation.

Neither the activation curve nor the kinetics of fast inactivation was altered by PKC activation. The response to PKA activation was blocked by the PKA inhibitor (H-89) and by PKA inhibitory peptide PKA_{5-24} (PKA_i). PKC activation was blocked by the kinase inhibitor (H-7), by the PKC inhibitor calphostin C, and by the inhibitory peptide PKC_{19-31} (PKC_i). The hypoxia-induced inhibition of I_{Na} and shift in steady-state inactivation were greatly attenuated by H-7, calphostin C, or PKC_i but not by H-89 or PKA_i. We conclude that hypoxia activates PKC in rat CA1 neurons and that PKC activation leads to the hypoxia-induced inhibition of I_{Na}. These data indicate that kinases can inhibit whole cell Na^+

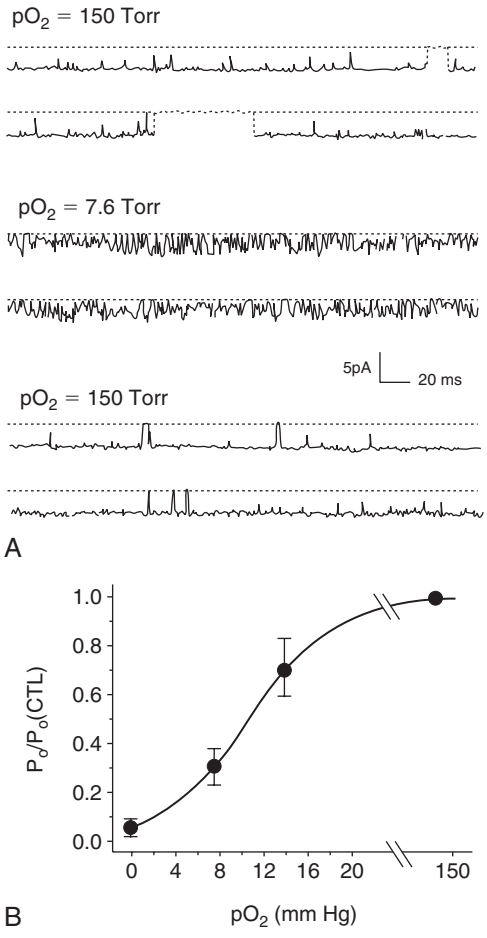


Figure 90-2 A large conductance K^+ current is inhibited during O_2 deprivation. A, Continuous recordings of a single K^+ current from an inside-out patch with the same solution (150 mmol/L K^+) in both internal and external sides, when the membrane potential was held at 20 mV. During baseline *(top two traces),* this channel had a P_{open} of 0.92 and a unitary conductance of 188 picosiemens. *Straight lines* indicate the channel closed level. Hypoxia ($Po_2 \approx 8$ mm Hg) induced a decrease in P_{open} to 0.24 *(middle two traces).* Recovery of P_{open} (0.96) is seen after reperfusion *(lower two traces).* **B,** Dose-dependent inhibition of P_{open} by graded hypoxia. Note that the P_{open} was normalized to its control level. Data presented as means ± SEM (n = 3) are fitted with an equation of $y = 1/\{1 + \exp[(K_d - x)/h]\}$, where $y = P_{open}/P_{open}$ (control), $x = Po_2$, $K_d = 11$, a Po_2 level for 50% inhibition of y, and $h = 4$. $P_{open,}$ Probability of having a channel in the open state. *(From Jiang C, Haddad GG:A direct mechanism for sensing low oxygen levels by central neurons,* Proc Natl Acad Sci U S A *91:7198, 1994.).*

current very much like that observed during hypoxia. However, it is unclear how kinases are activated during hypoxia and what events occur upstream.

From this analysis, we should highlight a few issues pertaining to O_2 sensing: (1) In the studies, sensing is very rapid; whatever the sensor is, the reactions that lead to the electrophysiologic responses observed by using our techniques and approaches must be very quick, on the order of seconds. (2) Most of the alterations are not genetically mediated, and no gene expression is presumably altered in this short period of time.

Chronic hypoxia has also been studied in our laboratory, as well as in others.[23-25] With respect to Na^+ flux, it seems that Na^+ influx increases when neurons are subjected to hypoxia for days.[23,26] It is very important to note that Na^+ influx through voltage-sensitive Na^+ channels can lead to cell death, and this form of cell death is most likely a result of the activation of cell death genes.[25]

OXYGEN SENSING VIA GENE REGULATION: LONGER PERIODS

Neurons vary widely in their capacity to adapt to a limited oxygen supply to the brain, reflecting the diversity of neuronal function and their adaptive mechanisms to oxygen deprivation. The hypoxia-tolerant neurons can be found in every order of vertebrates, such as crucian carp,[27] tadpoles,[28] turtle,[29] and the naked Kenyan mole rat. Neurons from oxygen-sensitive species such as *Rattus norvegicus* are hypoxia tolerant during the embryonic and neonatal periods.[30,31] The discovery of the hypoxia-tolerant property of an invertebrate species, *Drosophila melanogaster*, provided researchers with a new model system to study the mechanism of hypoxia tolerance in neuronal systems.[32,33] Those studies have prompted a number of questions. How do neurons "sense" the lack of microenvironmental O_2? How do they respond? How does sensing O_2 deprivation affect the cascade that follows the initial steps?

Multiple O_2 sensing systems most likely exist, and, although much remains to be learned about how cells sense and adapt to conditions of oxygen scarcity, it is clear that O_2 sensing (and the cascade of events that follow O_2 deprivation) largely depends on the regulation of gene transcription. Such mechanisms are fairly quick, and targets can be multiple. Several transcription factors, such as activator protein 1 (AP-1), early growth response protein-1 (EGR-1),[34] nuclear factor κB (NF-κB),[35] CCAAT enhancer-binding protein beta (C/EBPβ/NF-IL-6),[36] and the hypoxia-inducible factor (HIF), were found to be involved in the modulation of gene expression by oxygen. Among these O_2-sensitive transcriptional systems, perhaps the best described O_2-sensing pathway is the oxygen-sensitive transcription factor HIF, which activates the gene transcription machinery in an oxygen deprivation–dependent manner.[37]

HIFs are heterodimeric transcription factors containing an α- and a β-subunit that belong to the basic-helix-loop-helix (bHLH)-PAS protein superfamily. HIF-1α was first cloned from the human Hep3B cell line[38] and is a member of the PAS superfamily 1 (MOP1). The mouse and rat isoforms also have been cloned.[39,40] In addition, two other α-subunits were cloned, from humans and rats and mice (HIF-2α and HIF-3α). HIF-2α is known as endothelial PAS domain protein 1 (EPAS1), HIF-1α–like factor (HLF), or HIF-related factor (HRF) and is a member of the PAS superfamily 2 (MOP2).[41] HIF-3α is also referred to as MOP7.[41] The β-subunits of HIF, HIF-1β, HIF-2β, and HIF-3β, are members of the arylhydrocarbon receptor nuclear translocator, ARNT family, also named as ARNT1, ARNT2, or ARNT3.[42] HIF-1α and β homologues also exist in *Drosophila* tissues, and studies in this species have provided new data on the cellular adaptive responses to chronic hypoxic stress.[15,43] HIF-1 has been the most extensively studied (Figure 90-3).

Previous investigations have shown that HIF-1 messenger RNA (mRNA) was expressed in the mouse, rat, and human brain.[44] Because the brain is extremely sensitive to hypoxia and ischemia, the regulation of HIF-1 expression is highly relevant. Two of its target gene products in the brain are erythropoietin (EPO) and vascular endothelial growth factor (VEGF).[45,46] In the adult rat brain, HIF-1 mRNA was found in neuronal cells.[47] In hypoxia-treated rats or mice, an increase of HIF-1 mRNA was found in the brain, kidney, and lung. The HIF-1–regulated gene VEGF increased accordingly with a similar expression pattern as HIF-1.

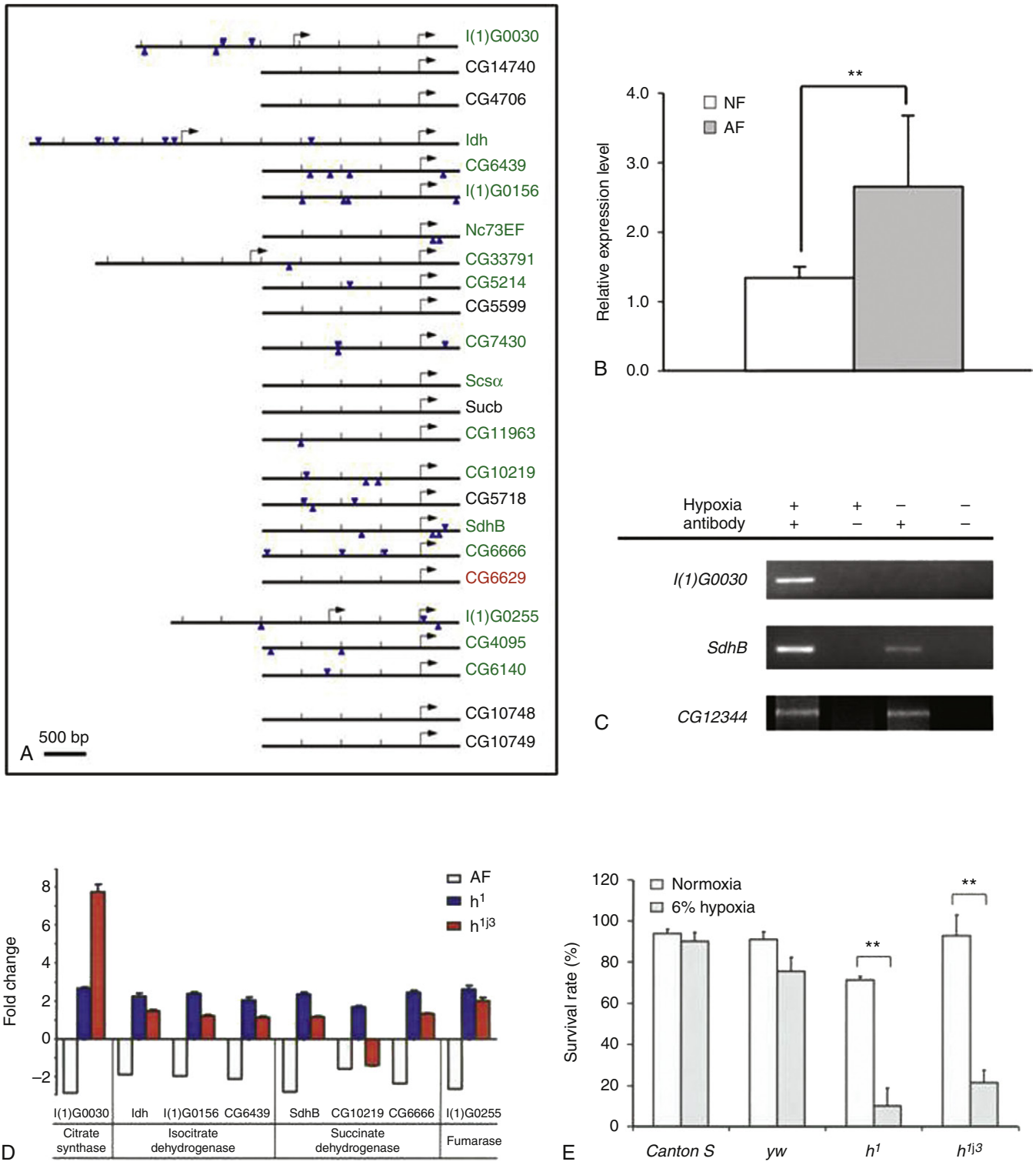


Figure 90-3 Transcriptional suppressor hairy regulates TCA cycle gene expression and hypoxia tolerance. A, Genomic localization of hairy binding elements in the *cis*-regulatory region of genes encoding TCA cycle enzymes. *Arrowheads* indicate consensus hairy binding sites, and *arrows* indicate transcriptional start sites. **B,** Significant up-regulation of hairy expression in hypoxia-selected AF populations ($p < .01$). **C,** Chromatin immunoprecipitation assay of gene l(1)G0030, SdhB, and CG12344 under normoxia or hypoxia. **D,** Abolished suppression of genes encoding TCA cycle enzymes in hairy loss-of-function mutants. **E,** Significant reduction in hypoxia survival in hairy loss-of-function mutants. Embryos from loss-of-function hairy mutants (h^1 and h^{1j3}) and controls (Canton-S and yw) were cultured at normoxic or 6% O_2 hypoxic condition. The ratio of adult flies to pupae was determined as survival rate of each stock. The loss-of-function hairy mutants exhibited a significantly reduced rate of hypoxia survival. Data are presented as means ± SD. $^{**}p < .01$. *AF,* Hypoxia-adapted flies; *TCA,* tricarboxylic acid. *(Modified from Zhou D, Xue J, Lai JC, Schork NJ, et al: Mechanisms underlying hypoxia tolerance in* Drosophila melanogaster: *hairy as a metabolic switch,* PLoS Genet *4(10):e1000221, 2008.)*

This provides evidence for a neuroprotective role of HIF-1 in the central nervous system. HIF-1 has been shown to mediate adaptive responses to reduced O_2 availability, including angiogenesis, glycolysis, and ischemia tolerance in the brain.

Control of angiogenesis through HIF-1 and HIF-2 is regulated by growth factors, including VEGF.[48] Numerous studies have demonstrated that chronic hypoxia induces angiogenesis in the adult brain with an increase in VEGF. A time course study, however, has shown that, after exposure to 10% O_2, HIF-1 rapidly accumulated, remained at high levels for only 14 days, and then decreased by 21 days, despite continuous low arterial oxygen tension. This would indicate either that HIF has a long-lasting effect on target genes or that other HIF targets or long-lasting hypoxia can maintain the induction of genes such as VEGF, because angiogenesis does not regress at a time when HIF has gone back to normal levels.[49] When 21-day adapted rats were exposed to a more severe hypoxic challenge (8% oxygen), HIF-1 increased again indicating that the HIF gene can still be up-regulated with more severe stresses.[49] Thus HIF-1 and its regulated genes appear to have a role in vascular remodeling and metabolic changes that contribute to adaptation of the chronic hypoxia condition.

It has been found that a noninjurious hypoxic stimulation can protect cells from an otherwise lethal hypoxic-ischemic attack several hours or days later. This noninjurious hypoxic stimulation has been termed *preconditioning*. Several studies have suggested that HIF-1 could be an important mediator of hypoxia-induced tolerance to ischemia by preconditioning.[50-52] Indeed, hypoxic preconditioning-induced expression of HIF-1 and its target genes has been found in the neonatal and adult brain. With use of DNA microarray methods combined with real-time reverse transcriptase polymerase chain reaction technologies, a set of HIF-1– but not HIF-2–mediated gene expression has been identified in the neonatal rat brain.[53] In this study, 12 genes were reported to be induced; they are *VEGF*, *EPO*, *GLUT-1*, *adrenomedullin*, *propyl 4-hydroxylase*, *MT-1*, *MKP-1*, *CELF*, *12-lipoxygenase*, *t-PA*, *CAR-1*, and an expressed sequence tag. Some of these genes, such as *GLUT-1*, *MT-1*, *CELF*, *MKP-1*, and *t-PA*, did not show a hypoxic regulation in either neurons or astrocytes, suggesting that other cells are responsible for the up-regulation of these genes in the hypoxic brain. These results also demonstrate that, besides HIF-1 pathway, a number of other endogenous molecular mechanisms might also be involved in the hypoxic preconditioning-induced tolerance.

If the responses to hypoxia over a longer term, such as hours and days, involve HIF-1 and HIF-2 and their target downstream genes, how about hypoxia over even longer periods of time, such as over generations? These types of studies have been relatively scarce. Recently, we have generated a hypoxia-tolerant *Drosophila* strain to study the genetic and molecular basis of adaptation to long-term (i.e., over generations) hypoxic environments.[54] Microarray and genetic analyses have shown that the survival of both larvae and adult flies was accompanied by alterations in a number of major signaling pathways, including EGF, Insulin, Notch, and Toll/Imd pathways. Furthermore, previous studies have demonstrated an enhancement of glycolytic activity as a major metabolic response in cells and tissues after short-term (in minutes or hours) hypoxia. In contrast, our present data showed that many genes encoding glycolytic enzymes were down-regulated in the hypoxia-tolerant flies.[55] Moreover, no significant lactate accumulation was found in the hypoxia-adapted flies (AF).[56] For more than three decades, physiologists have debated the importance of down-regulation of metabolism in hypoxia tolerance.[57,58] Although this idea, based on metabolic data, is intuitively appealing, no information has been obtained about how various metabolic enzymes could be coordinated to survive severe long-lasting hypoxia. Our current work provides the first evidence showing that such coordination can be achieved at a transcriptional level by a seemingly metabolic switch (i.e., *hairy*, a transcriptional suppressor). This notion is supported by a number of observations: (1) expression of *hairy* was up-regulated in hypoxia-tolerant flies; (2) the *hairy*-binding region was present in the *cis*-regulatory regions of the down-regulated genes but not others; (3) functional chromatin immunoprecipitation analysis demonstrated that the down-regulation of these metabolic genes is based on the changes of *hairy*; it is noteworthy that its binding was not increased for other, nonmetabolic target genes, for example, CG12344; and (4) *hairy* loss-of-function mutations abolished the suppression on the expression of tricarboxylic acid (TCA) cycle genes and significantly reduced hypoxia survival in *Drosophila*[55] (see Figure 90-3). The adaptive mechanisms identified in this hypoxia-tolerant *Drosophila* model may also play a crucial role in protecting mammals from hypoxia injury.

Although a great deal of work has been done to understand the biologic role of hypoxia-induced gene expression in the brain, many important questions remain largely unanswered. For example, which genes are expressed and which are inactivated during hypoxia in different brain regions and cell types are still unknown. Further research is needed to describe the diversity of oxygen sensors and the mechanisms by which these sensors regulate the gene transcription machinery.

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91

Martha J. Miller and Richard J. Martin

Pathophysiology of Apnea of Prematurity

EPIDEMIOLOGY AND DEFINITION OF APNEA

Definition

Apnea is a common disorder of respiratory control in premature infants. Such infants may experience respiratory pauses of varying duration, at rest or in conjunction with startles, movement, defecation, or swallowing during feeding. These short respiratory pauses may be self-limiting and are not associated with clinically significant bradycardia (less than 100 beats/minute). *Apnea* has been defined by the American Academy of Pediatrics Task Force as a respiratory pause lasting at least 20 seconds, or a pause accompanied by bradycardia, cyanosis, or pallor.[1] Shorter apnea (e.g., 15 seconds in duration) may, on occasion, require clinical intervention.

Apnea should be distinguished from *periodic breathing,* in which the infant exhibits regular cycles of respiration approximately 10 to 18 seconds in length, interrupted by pauses at least 3 seconds in duration, with this pattern recurring for at least 2 minutes.[2] Periodic breathing has been considered to represent a benign respiratory pattern in the premature or young term

infant. The respiratory pause of apnea, unlike that of periodic breathing, may not be self-limiting and may produce significant physiologic changes. Such changes are considered in detail in the following discussion.

Classification

Apneic events are distinguished not only by their duration but also by the presence or absence of airway obstruction during the episode of apnea. Thach and Stark[3] initially described an increase in the frequency of apnea when the premature infant's neck was flexed. Subsequently, upper airway obstruction was found to accompany apnea in preterm babies, even though neck flexion was not present.[4] The location within the upper airway at which obstruction occurs usually is within the pharynx but may vary. Mathew and co-workers[5] noted that obstruction occurred within the pharynx in 93% of cases of apnea, at the level of the larynx in 1%, and at both the larynx and pharynx in 6% of cases.

The presence or absence of upper airway obstruction forms the basis of the classification of apnea into three types (Figure 91-1). *Mixed apnea* is the most commonly observed clinically significant event in small premature infants and consists of obstructed inspiratory efforts as well as a central pause 2 seconds or longer in duration (see Figure 91-1, *A*). In *obstructive apnea*, obstructed breaths characterized by chest wall motion without nasal airflow continue throughout the entire apnea (see Figure 91-1, *B*). In *central apnea*, inspiratory efforts cease entirely, and obstructed breaths are not observed (see Figure 91-1, *C*). Mixed apnea accounts for approximately 50% to 75% of all instances of apnea in premature infants; obstructive apnea, 10% to 20%; and central apnea, 10% to 25%[6] The proportion of mixed apnea–type events progressively increases (and that of purely central apneic events decreases) the longer the apneic episode lasts.

Apnea duration and classification also may correlate with the neurologic status of the infant. Butcher-Puech and co-workers[7] found that infants who experienced spells of obstructive apnea longer than 20 seconds in duration had a higher incidence of intraventricular hemorrhage, hydrocephalus, prolonged mechanical ventilation, and abnormal neurologic development after the first year of life. It is possible that apnea in a subset of infants is a sign of a diffuse neurologic insult in prenatal or postnatal life that leads to disordered control of breathing. In most infants, however, an underlying neuropathologic process is unlikely, because apnea frequency decreases as the infant matures. Hypothetically, apnea may resolve when central and peripheral chemoreceptors develop to the point at which appropriate responses to change in blood gas status can occur. A further important developmental contribution to resolution of apnea may be the increasing ability of medullary respiratory control centers to activate upper airway–dilating musculature in synchrony with increasing ventilatory drive.

Hypoxemic events resembling apneic spells also have been detected in intubated, mechanically ventilated preterm infants. Bolivar and co-workers[8] described episodes of hypoxemia preceded by an increase in total pulmonary resistance and a decrease in compliance, analogous to changes described by Miller and associates[9] occurring before apnea in unintubated infants. Furthermore, Dimaguila and colleagues[10] reported that such episodes may be preceded by subtle spontaneous movement and are characterized by both central respiratory depression and obstruction to airflow (the latter features being analogous to mixed apnea). These hypoxemic episodes in intubated infants are a consequence of hypoventilation, frequently associated with arousal,[11] and they further illustrate the vulnerability of premature infants to imbalance of central respiratory control and altered pulmonary function.

Physiologic Effects

Cessation of respiration during apnea has significant ventilatory and reflex cardiovascular consequences for the preterm infant. Both hypoxemia and hypercarbia accompany prolonged apnea. The decrease in oxygenation in term infants with apnea has been observed to be directly related to the duration of the apnea and and baseline respiratory status.[12] In most premature infants, apnea, if appropriately treated, is not thought to produce significant long-term complications. In a retrospective analysis, however, Kitchen and associates[13] noted a significant correlation between apnea of infancy requiring treatment with theophylline and later development of manifestations of cerebral palsy. Furthermore, Janvier and co-workers[14] found that neurodevelopmental impairment was significantly associated with the number of days of apnea after extubation. This correlation could reflect early (apnea) and late (cerebral palsy) manifestations of diffuse cerebral hypoxic-ischemic injury in a small subset of premature infants, as discussed later. Whether apnea itself can exacerbate underlying brain injury is as yet unknown.

The reflex effects of apnea include characteristic changes in heart rate, blood pressure, and pulse pressure. Bradycardia may begin within 5 seconds after the onset of the apneic episode[15]

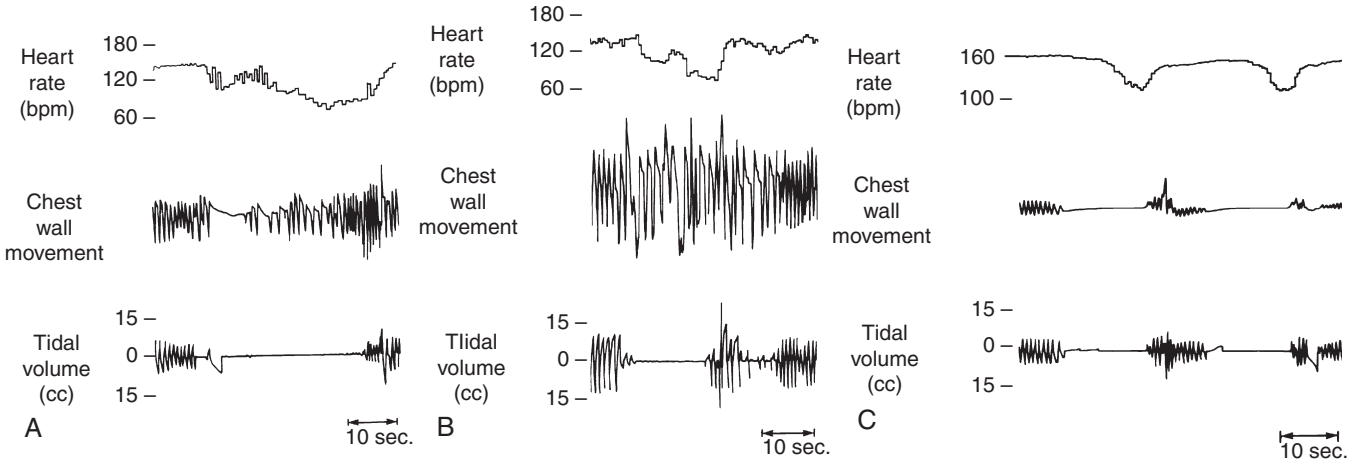


Figure 91-1 **A,** Mixed apnea. Obstructed breaths precede and follow a central respiratory pause. **B,** Obstructive apnea. Breathing efforts continue, although no nasal airflow occurs. **C,** Central apnea. Both nasal airflow and breathing efforts cease simultaneously. *(From Miller MJ: In Edelman N, Santiago T, editors:* Breathing disorders of sleep, *New York, 1986, Churchill Livingstone.)*

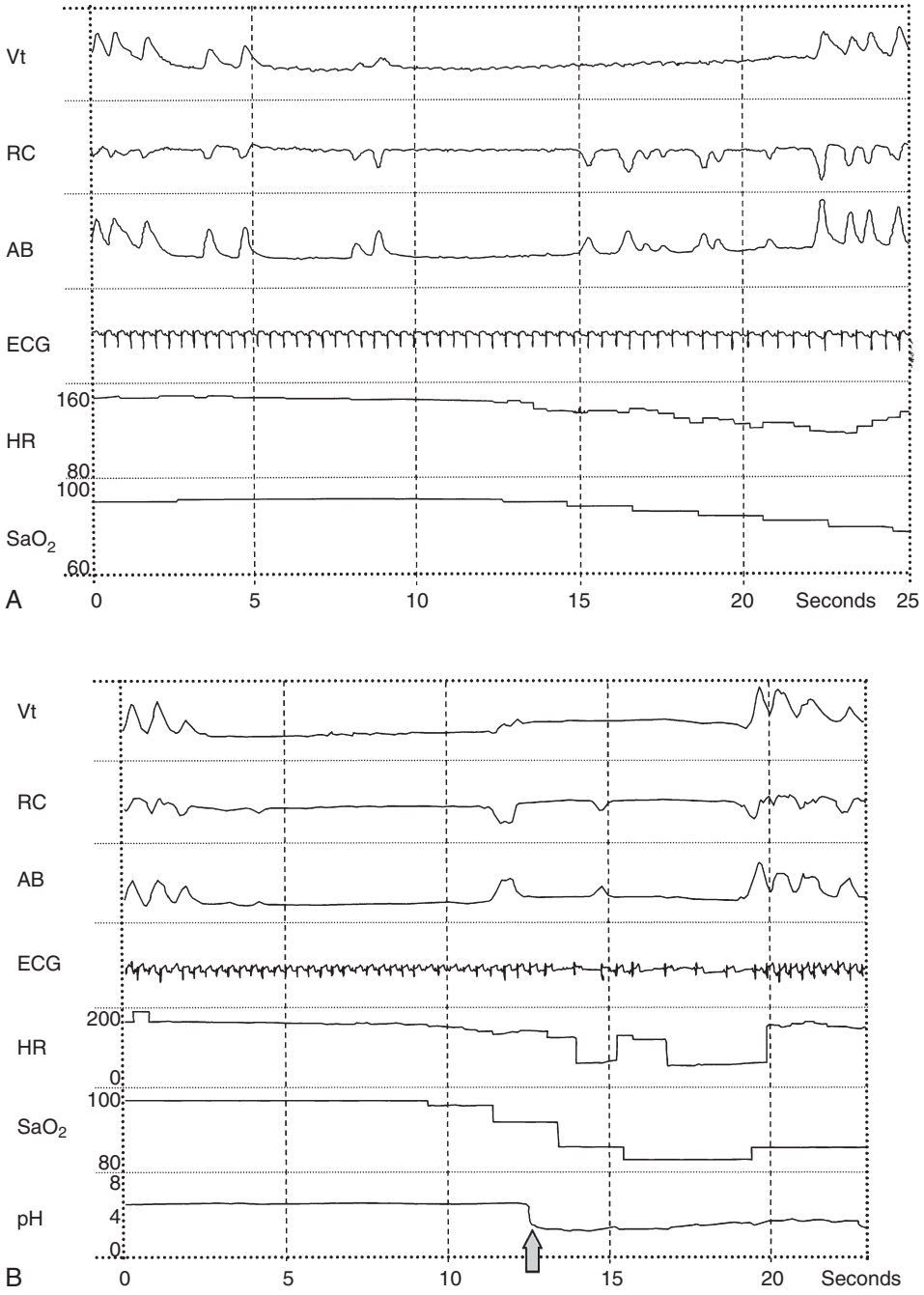


Figure 91-2 A, Relationship between apnea and bradycardia. During this mixed apnea, the heart rate *(HR)* begins to decrease approximately 5 seconds after the apnea begins. **B,** Relationship between apnea and gastroesophageal reflux. Reflux occurs after the onset of apnea, as indicated by the decrease in lower esophageal pH *(arrow). AB,* Abdomen motion; *ECG,* electrocardiogram; *RC,* rib cage motion; Sa_{O_2}, arterial oxygen saturation; *Vt,* tidal volume (estimated).

(Figure 91-2). The bradyarrhythmia most often is sinus in character, with an occasional infant showing a nodal escape. Dorostkar and associates[16] have observed that 1.8% of preterm infants may even have asystolic events during apnea, defined as absence of QRS complex for more than 3 seconds during apnea. This subgroup of infants appears to have a benign clinical outcome. Henderson-Smart and co-workers[17] noted a significant correlation between the decrease in oxygen saturation and heart rate and postulated that bradycardia during apnea could result from hypoxic stimulation of the carotid body chemoreceptors.

The complex relationship between reflex control of heart rate and breathing was studied by Daly and Scott,[18] who determined the effect of hypoxic perfusion of the isolated carotid body on heart rate in the dog. When ventilation was allowed to increase in response to hypoxia, tachycardia occurred. When this reflex increase in ventilation was prevented, however, bradycardia resulted. In the spontaneously breathing subject, the decrease in heart rate secondary to hypoxic stimulation of the carotid body may be masked by a cardiac accelerator response that may derive from stretch receptors in the lung as well as cycling of the respiratory control center in the medulla.[19] At the onset of apnea, at which time cessation of

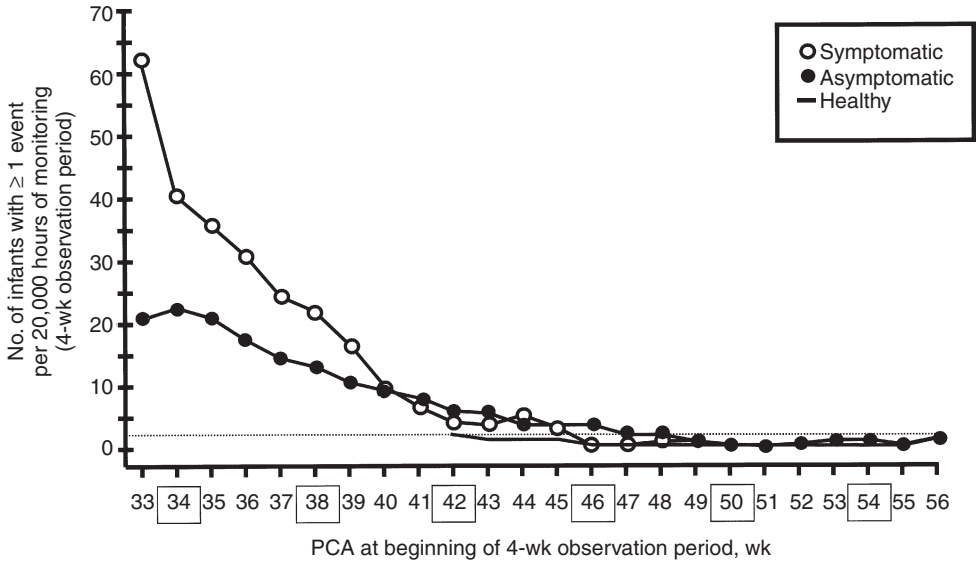


Figure 91-3 The number of infants who experienced at least one apneic episode lasting 30 seconds accompanied by bradycardia decreased with advancing postconceptional age *(PCA).* Data are presented for symptomatic and asymptomatic preterm infants, depending on the persistence of cardiorespiratory events before discharge, and for healthy term infants. *(From Ramanathan R, et al: Cardiorespiratory events recorded on home monitors: comparison of healthy infants with those at increased risk for SIDS,* JAMA *285:2199, 2001.)*

ventilation and onset of hypoxemia occur almost simultaneously, hypoxemia would be expected to produce bradycardia.

Other reflex input may accentuate the bradycardia during hypoxemia. For example, the reflex effects of apnea in infants also have been likened to the diving response of the seal.[20] During reflex apnea in these animals, upper airway afferent input from superior laryngeal and trigeminal nerve stimulation may produce greatly enhanced bradycardia. The contribution of upper airway reflexes to the bradycardia that occurs during apnea has not been studied in human infants. In summary, the rapid onset of bradycardia during apnea may be a complex reflex deriving from multiple sources, including trigeminal receptors and carotid chemoreceptors.

A change in blood pressure also accompanies apnea in newborn infants. Girling[21] noted that the decrease in heart rate during apnea was accompanied by a concomitant rise in pulse pressure, usually owing to an increase in systolic pressure, occasionally accompanied by a fall in diastolic pressure. During bradycardia, filling volume of the heart may increase, leading to a rise in stroke volume and pulse pressure in accordance with Starling's law. With more severe apnea and bradycardia (less than 80 beats/minute), a decrease in systemic blood pressure may occur, accompanied by a fall in cerebral diastolic and systolic blood flow velocity.[22] In premature infants with poor cerebrovascular autoregulation, cerebral blood flow may mirror systemic blood flow, and cerebral perfusion may decrease to very low levels during prolonged apnea. Resolution of apnea then may be accompanied by cerebral hyperperfusion.[23] Thus very prolonged apnea could be analogous to a hypoperfusion-hyperperfusion type of brain insult and theoretically could lead to a form of hypoxic-ischemic brain injury in susceptible premature infants.

Epidemiology

In defining the epidemiology and developmental correlates of apnea, part of the difficulty lies in the numerous definitions of apnea that have been used by various investigators. However, some trends do emerge. Apnea is more frequent in more immature infants, and almost all who weigh less than 1000 g may experience apnea during the neonatal period.[24] Carlo and associates[25] showed that the onset of apnea may be as early as day 1 of life in infants without respiratory distress syndrome. By contrast, spontaneously breathing infants with respiratory distress syndrome may show a delay in the peak frequency of apnea until day 7 of life. Thereafter, both the frequency and duration of apnea decrease with advancing postnatal age,[26] as discussed further on (Figure 91-3). These observations serve to emphasize the developmental immaturity of respiratory control that underlies infantile apnea, as well as the resolution of this disorder over time.

Idiopathic apnea is thought to be related to prematurity. In rare infants, however, underlying specific familial neuropathology may be identified. Adickes and co-workers[27] described a family in which three of six siblings presented between 18 and 26 months of age with sleep apnea and subsequently died of this disorder. Neuropathologic evaluation revealed that the affected children suffered from Leigh disease (subacute necrotizing encephalomyelopathy). In this disease, spongiform degeneration may be present in the areas of the brain stem that regulate ventilation, including the nucleus reticularis and nucleus gigantocellularis. Other disorders affecting the brain stem that may manifest with apnea include olivopontocerebellar atrophy, myotonic dystrophy, and syringobulbia, as well as brain stem infarction resulting from asphyxia.[28]

Apnea can recur in premature infants after the neonatal period in response to specific clinical situations in which respiratory drive is altered. Respiratory syncytial viral infection is well known to elicit apnea, which may be of the typical mixed or obstructive type or may occur in a pattern more reminiscent of periodic breathing.[29] These spells may be severe, necessitating endotracheal intubation. The cause of respiratory depression in respiratory syncytial viral infection is unknown. However, the apnea resolves with recovery from this infection.

Former premature infants also may experience apnea during recovery from general anesthesia.[30] This form of apnea most commonly occurs in the first few months of life, particularly when either ketamine sedation or general anesthesia is used during surgery. For this reason, in former preterm infants, cardiorespiratory monitoring during the acute postoperative period is an important part of their care.

Routine care of the premature infant before discharge includes several practices that may cause temporary recurrence of apnea.

For example, eye examination for retinopathy of prematurity may be associated with cyanosis, apnea, and gastrointestinal side effects.[31] Immunization with diphtheria-tetanus-pertussis vaccine, inactivated poliovaccine, and *Haemophilus influenzae* type b vaccine (DTP-IPV-HIB vaccines) also has been found to be associated with apnea, bradycardia and desaturation. With such practices, therefore, infants require continued monitoring until they are stable, but evidence of a subsequent detrimental effect is lacking.[32]

PHYSIOLOGIC FACTORS

Alteration in Central Drive

Immaturity or depression of central inspiratory drive of the muscles of respiration traditionally has been accepted as a key factor in the pathogenesis of apnea of prematurity. Vulnerability of the bulbopontine respiratory centers in the brain stem to inhibitory mechanisms could explain why apneic episodes are precipitated in preterm infants by such a wide diversity of specific clinicopathologic events, as outlined in Figure 91-4.[33] In other words, apnea may represent the final common response of incompletely organized and interconnected respiratory neurons to a multitude of afferent stimuli. It has been proposed that immature circuits within neuronal networks may be highly susceptible to inhibitory neurotransmitters and neuroregulators such as adenosine and γ-aminobutyric acid (GABA). Unfortunately, the maturation of central respiratory integrative mechanisms and of their biochemical neurotransmitters is inaccessible to study in human infants, and no ideal animal model of spontaneous apnea has been identified for study in the nonanesthetized state.

Using noninvasive techniques, Henderson-Smart and coworkers[34] documented that brain stem conduction times of auditory evoked responses are longer in infants with apnea than in matched premature infants without apnea. This observation provides indirect evidence that infants with apnea exhibit greater-than-expected immaturity of brain stem function on the basis of postconceptual age and supports the concept that stability of central respiratory drive improves as dendritic and other synaptic interconnections multiply in the maturing brain.

The absence of respiratory muscle activity during central apnea unequivocally points to the depression of respiratory center output. In support of this concept, Gauda[35] documented a decrease in diaphragmatic activity on electromyography (EMG) during spontaneously obstructed inspiratory efforts that characterize mixed apnea, as discussed later. Thus both central and mixed apneic episodes share an element of decreased respiratory center output to the respiratory muscles. The role played by the balance of neurotransmitter substances in modulating this inhibition is not yet known. GABA is considered a ubiquitous major inhibitory neurotransmitter within the brain. Physiologic studies in neonatal animal models have implicated GABA in inhibition of respiratory frequency and decreased ventilatory responses during hypercapnia, hypoxia, and superior laryngeal nerve stimulation.[36-38] GABA thus has the potential to play a key role in the vulnerability of preterm infants to apnea.

Other transmitters and neuroeffectors are also involved in control of breathing at the peripheral and central levels. Dopaminergic receptors have been implicated as playing an inhibitory role in both peripheral chemoreceptor responses and central neural mechanisms elicited by hypoxia.[39] Evidence from neonatal animal studies suggests that endogenous endorphins may depress central respiratory drive.[40] Endogenous opiates may modulate the ventilatory response to hypoxia in newborn primate animals[41]; however, a competitive opiate receptor antagonist, naloxone, is of no benefit in resuscitation of the asphyxiated human neonate,[42] nor does naloxone appear to play a therapeutic role in apnea of prematurity.

Infants with sepsis are prone to respiratory compromise, including apnea. The proinflammatory cytokine IL-1β may be released in response to infection, and animal studies have shown

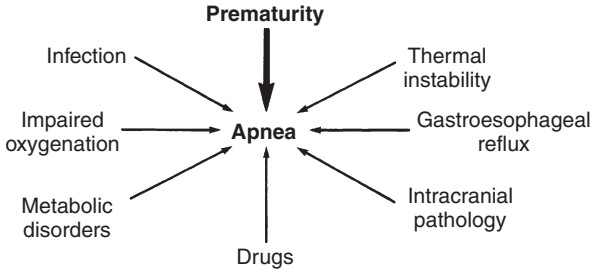


Figure 91-4 Some diverse factors known to precipitate development of apneic episodes in predisposed preterm infants. *(From Martin RJ, Miller MJ, Carlo WA: Pathogenesis of apnea in preterm infants,* J Pediatr *109:733, 1986.)*

that IL-1β can depress respiration by way of a prostaglandin E_2–related mechanism. Thus activation of this pathway may trigger apnea in the infant with infection.[43] The final output of the respiratory control nuclei in the medulla may be a complex function of many inhibitory and stimulatory inputs, both humoral and neural. The exact manner in which these pathways are altered during apnea of prematurity remains to be elucidated.

Role of Sleep State

It became apparent in the mid-1970s that respiratory control is influenced by sleep state in infants. Apnea was observed to occur more commonly during active (or rapid eye movement) and indeterminate (or transitional) sleep, when respiratory patterns are irregular in both timing and amplitude.[44] Apnea is less commonly observed during quiet sleep, when respiration is characteristically regular with little breath-by-breath change in tidal volume or respiratory frequency, although periodic breathing may actually occur predominantly in quiet sleep. In term neonates, respiratory variability alone can be used to stage sleep with a high degree of accuracy.[45] However, sleep state is not easily definable by any criteria before 32 weeks gestation, when apnea occurs most frequently.

The higher incidence of apnea during active sleep probably is the result of the variability of respiratory rhythmicity characterizing that state. Other factors may enhance the infant's vulnerability to apnea during active sleep. Chest wall movements are predominantly asynchronous (or paradoxical) during active sleep, in contrast with quiet sleep.[46] Specifically, abdominal expansion during inspiration is almost always accompanied by inward movement of the rib cage during active sleep, whereas during quiet sleep, rib cage and abdomen expand together. These paradoxical chest wall movements during active sleep appear to be the result of decreased intercostal muscle activity, secondary to spinal motoneuron inhibition.[47] In very preterm infants, however, the paucity of quiet sleep, together with an extremely compliant rib cage, makes paradoxical chest wall movements almost a constant feature. Asynchronous chest wall movements may predispose the infant to apnea by decreasing functional residual capacity and impairing oxygenation.[48] The compensatory increase in diaphragm activity that results may increase diaphragmatic work, potentially leading to diaphragmatic fatigue[49] and collapse of the pharyngeal airway.[50] Thus the enhanced vulnerability to apnea during active sleep may involve simultaneous mechanisms operating at several levels of respiratory function.

Influence of Chemoreceptor and Mechanoreceptor Responses

The ventilatory and respiratory muscle responses to increases in inspired carbon dioxide reflect predominantly central chemoreceptor activity and are less well developed in the immature infant before 33 weeks of postconceptional age.[51]

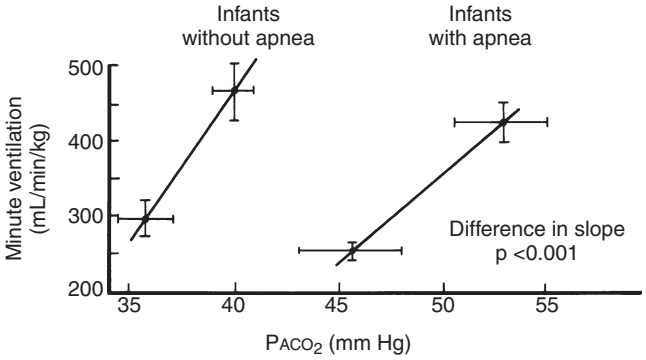


Figure 91-5 Comparison of carbon dioxide sensitivity obtained from ventilatory responses to changing alveolar partial pressure of carbon dioxide (P_{ACO_2}) in preterm infants with and without apnea. Note the less steep ventilatory response in the apneic group. *(From Gerhardt T, Bancalari E: Apnea of prematurity. I. Lung function and regulation of breathing,* Pediatrics *74:58, 1984. Reproduced by permission of* Pediatrics.*)*

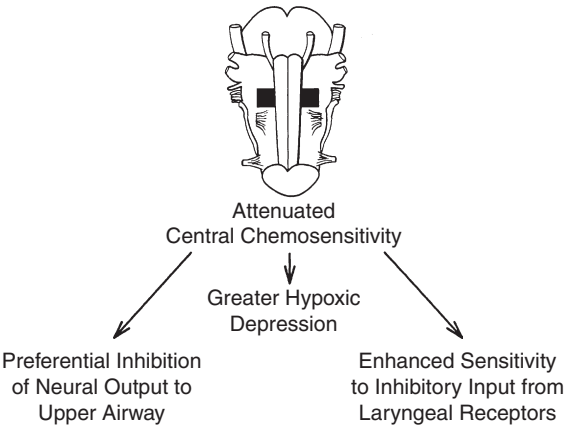


Figure 91-6 Hypothetical framework for mechanisms whereby attenuated central (carbon dioxide) chemosensitivity enhances vulnerability to neonatal apnea. The *black squares* indicate the intermediate area of the ventral medullary surface of the brain stem.

The reduced ventilatory response to carbon dioxide in small preterm infants is primarily the result of decreased central chemosensitivity; however, mechanical factors preventing an appropriate increase in ventilation may contribute. Unlike adults, preterm infants do not increase frequency of ventilation during hypercapnia. In these infants, hypercapnia may be accompanied by prolongation of expiratory duration.[52] It has been shown that the carbon dioxide response curve has a decreased slope (indicating a less steep ventilatory response to increasing carbon dioxide concentrations) in preterm infants who exhibit apnea[53] (Figure 91-5) However, a cause-and-effect relationship between decreased carbon dioxide responsiveness and apnea of prematurity has not been clearly established. Both entities may simply reflect decreased respiratory drive. Administration of carbon dioxide at low inspiratory concentrations would be expected to relieve apnea, as it does periodic breathing; this approach has been investigated but may not be therapeutically feasible in human infants.

A newborn piglet model has been used to identify the physiologic consequences of decreased central (carbon dioxide) chemosensitivity by cooling a discrete area (the intermediate area) of the ventral medullary surface.[54,55] Cooling at this site (or microinjection of inhibitory neurotransmitters) inhibits central chemosensitivity, possibly by directly affecting chemosensitive cells but also by inhibiting neural transmission between central chemosensitive cells and respiratory rhythm generators situated in the brain stem. Obvious limitations arise in extrapolating data from anesthetized newborn piglets to apneic human infants. Nonetheless, as seen in Figure 91-6, attenuated central chemosensitivity may underlie some of the physiologic characteristics of neonatal respiratory control, including preferential inhibition of neural output to the upper airway muscles as compared with the diaphragm, enhanced sensitivity to inhibitory afferents from upper airway (e.g., laryngeal) receptors, and greater hypoxic depression of breathing (as discussed later on).

It has been known for many years that preterm infants respond to a fall in inspired oxygen concentration with a transient increase in ventilation over approximately 1 minute, followed by a return to baseline or even depression of ventilation.[56] The characteristic response to low oxygen in infants appears to result from initial peripheral chemoreceptor stimulation, followed by overriding depression of the respiratory center as a result of hypoxemia. Consistent with these findings is the observation that a progressive decrease in inspired oxygen concentration causes a significant flattening of carbon dioxide responsiveness in preterm infants.[57] This unstable response to low inspired oxygen concentration may play an important role in the origin of neonatal apnea. It offers a physiologic rationale for the decrease in incidence of apnea observed when a slightly increased concentration of inspired oxygen is administered to apneic infants. The biphasic ventilatory response to low inspired oxygen, however, is not unique to apneic infants and probably serves to increase their vulnerability to apnea, rather than causing the problem. Consistent with the prolonged vulnerability of respiratory control in preterm infants is the observation that the characteristic biphasic ventilatory response to hypoxia persists into the second month of postnatal life.[58]

Afferent neural input from pulmonary stretch receptors is capable of substantially modulating respiratory timing in human neonates. This vagally mediated response, called the *Hering-Breuer reflex,* acts to inhibit inspiration, prolong expiration, or both, with increasing lung volume, thereby limiting lung overinflation.[59] Active shortening of expiratory duration with decrease in lung volume may similarly provide a neonatal breathing strategy to preserve functional residual capacity in the presence of a highly compliant chest wall. Another manifestation of this reflex response to lung inflation is that inspiratory duration typically is prolonged after end-expiratory airway obstruction when lung inflation is prevented. This ability of neonates to increase the duration of an obstructed inspiratory effort appears to be an appropriate compensatory mechanism during airway occlusion. More important, upper airway obstruction contributes substantially to the initiation of apneic episodes in preterm infants, and upper airway muscles show preferential reflex activation in response to airway obstruction in infants.[60] Gerhardt and Bancalari[61] compared preterm infants with and those without apnea in their ability to respond to end-expiratory airway occlusion. Prolongation of the occluded inspiratory effort was significantly greater in the nonapneic group, suggesting a more mature respiratory reflex response with stronger compensatory activity to relieve airway obstruction.

Apnea in premature infants is accompanied by complex changes in pulmonary mechanics and ventilatory timing.[9] Before apnea occurs, an increase in total pulmonary resistance has been observed in association with a decrease in tidal volume and a prolongation in expiratory time. Such changes were documented before onset of mixed, obstructive, and central apneic episodes. These observations suggest that a diminution in respiratory drive

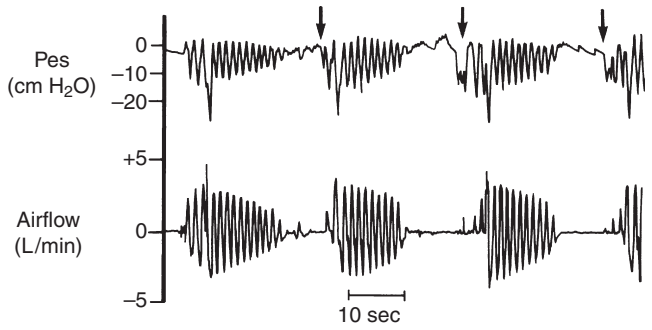


Figure 91-7 During this epoch of periodic breathing, obstructed breaths occur at the onset of each ventilatory cycle, as indicated by esophageal pressure *(Pes)* swings in the absence of nasal airflow. *(From Miller MJ, et al: Airway obstruction during periodic breathing in premature infants,* J Appl Physiol *64:2496, 1988.)*

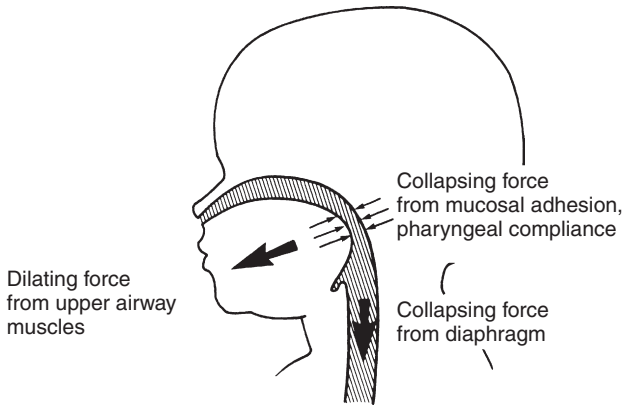


Figure 91-8 Sagittal section of the upper airway, demonstrating various forces operating either to collapse the pharynx or to maintain its patency during normal respiration. *(Modified from Thach BT: The role of pharyngeal airway obstruction in prolonging infantile apneic spells. In Tilden JT, et al, editors:* Sudden infant death syndrome, *New York, 1983, Academic Press.)*

precedes apnea, reminiscent of the cyclic alterations in drive observed by Waggener and co-workers.[62] After the apnea resolves and respiration is resumed, the respiratory drive in premature infants initially is increased (reflected in a higher ratio of tidal volume to inspiratory time), which may represent a response to cumulative hypoxia and hypercapnia. During recovery from apnea, total pulmonary resistance is increased and supraglottic resistance also is elevated, possibly in response to a fall in lung volume and collapse of the upper airway when respiratory drive declines after onset of apnea. Remarkably, within two to three breaths after apnea, the premature infant can restore pulmonary resistance and respiratory drive to normal preapnea values. Thus the neural systems that restore respiratory homeostasis are intact, even in premature infants with apnea.

Relation of Apnea to Periodic Breathing

Periodic breathing, defined earlier as recurrent sequences of pauses in respiration, is so common as to be considered normal in preterm infants.[63] It can be eliminated by increasing environmental oxygen,[63] but because periodic breathing is considered harmless, this intervention is not recommended. Apnea and periodic breathing share many characteristics, but some specific differences have been noted. Both forms of respiratory instability decline in frequency over a 5- to 10-week period after birth. The respiratory pause in both apnea and periodic breathing is preceded by a decline in tidal volume and by a prolongation of respiratory time reminiscent of the cyclic changes in ventilation described by Waggener and co-workers.[62] Both apnea and periodic breathing may be accompanied by obstructed breaths before the onset of respiratory cycles[9,64] (Figure 91-7).

Apnea and periodic breathing do differ in the following respects: The respiratory pauses of apnea are not limited in duration, and they are accompanied by frequent swallows, which are not observed during periodic breathing. Thus the physiologic correlates of these forms of respiratory instability differ in several ways. The specific central neuronal pathways that lead to each have yet to be determined.

Differential Responses of Upper Airway and Chest Wall Muscles

Because premature infants exhibit pharyngeal or laryngeal obstruction during spontaneous apnea, much interest has focused on the interactions among the various respiratory muscle groups in maintaining airway patency. A model was proposed for the pathogenesis of neonatal apnea by Thach,[50] a modification of which is shown in Figure 91-8. According to this model, negative luminal pressures generated in the upper airway during inspiration predispose a compliant pharynx to collapse. Patency can be maintained by activation of upper airway muscles, which may increase tone within the extrathoracic airway through tonic or phasic contraction in synchrony with the chest wall muscles. The relative roles played by active upper airway muscle contraction and passive rigidity of the anatomic framework of the upper airway in maintaining pharyngeal patency in preterm infants are unclear.

Whereas many upper airway muscles, including the alae nasi and laryngeal abductor and adductor muscles, modulate patency of the extrathoracic airway, failure of genioglossus activation has been most widely implicated in mixed and obstructive apnea in both adults and infants. Carlo and co-workers[65] compared activity of the genioglossus muscle with that of the diaphragm in response to hypercapnic stimulation. Consistent with data obtained in animal models,[66] genioglossus activation in preterm infants was delayed for approximately 1 minute after initiation of carbon dioxide rebreathing and occurred only after a P_{CO_2} threshold of approximately 45 mm Hg had been reached.[65] By contrast, diaphragm EMG activity increased linearly with progressive hypercapnia. Thus it is possible that an absent, small, or delayed upper airway muscle response to hypercapnia may result in upper airway instability when accompanied by a linear increase in chest wall activity. This instability may predispose affected infants to obstruction of inspiratory efforts after a period of central apnea. Consistent with this hypothesis is the observation that short apneic episodes are more likely to be central, and longer episodes (lasting longer than 15 seconds) are more likely to be accompanied by obstructed breaths. Furthermore, airway obstruction often occurs toward the end of the longer episodes of mixed apnea, when diaphragmatic activity may be enhanced before that of the upper airway muscles.

Neonates typically exhibit a modest prolongation of inspiratory time when lung inflation is prevented by end-expiratory airway occlusion; as indicated earlier, this response is a manifestation of the Hering-Breuer reflex. Studies in animals demonstrated that this vagally mediated inhibition of normal lung inflation has a greater influence on the upper airway muscles than on the diaphragm.[67] This vagal inhibition is released when the airway is occluded. Consequently, airway occlusion results in a greater increase of upper airway muscle activity, as compared with the diaphragm, for an obstructed inspiratory effort versus the corresponding preocclusion breath. A similar preferential increase in amplitude of the submental (genioglossus) versus diaphragm

EMG tracing has been noted in healthy preterm infants during end-expiratory airway occlusion.[60] It is theoretically possible that exaggerated vagal inhibition of the dilator muscles of the upper airway may contribute to the origin of obstructive apnea.

Gauda and co-workers[68] used sublingual surface electrodes (placed over the insertion of the genioglossus within the mandible) to compare the genioglossus responses with end-expiratory airway occlusion in preterm infants with mixed and obstructive apnea and in nonapneic control infants. In both groups of infants, genioglossus EMG activity typically was absent during unobstructed breathing. Occlusion resulted in immediate release of this inhibition, with resultant augmentation of the genioglossus in the nonapneic infants. In infants with apnea, however, activation of the genioglossus in response to occlusion was significantly delayed. The cause of this delay in genioglossus activation after airway obstruction is not currently understood. This observation suggests that vagal inhibition of the dilator muscles of the upper airway does not contribute to the initiation of apnea.

Subsequently, Gauda and co-workers[35] evaluated the activity of the genioglossus and diaphragm during spontaneously occurring mixed and obstructive apneic episodes. During mixed apnea, the amplitude of the diaphragm EMG activity decreased on the initial obstructed inspiratory effort and did not exceed that for the breath preceding apnea until flow was reestablished (Figure 91-9). Genioglossus activity accompanied approximately 20% of breaths immediately preceding spontaneous apnea; this frequency did not increase significantly until resolution of the apnea, with genioglossus activity present during 40% of breaths associated with reestablishment of airflow. Thus decreased diaphragmatic activity is a major component of spontaneous apnea associated with airway obstruction, and neither diaphragm nor genioglossus activity is increased until resolution of apnea. These findings suggest that central, mixed, and obstructive apneas are caused by a common mechanism—a reduction in central drive affecting the diaphragm and dilating muscles of the upper airway. However, the finding that only 40% of spontaneous apneic episodes were terminated with genioglossus activation indicates that this is not the sole mechanism by which upper airway obstruction is relieved in premature infants.

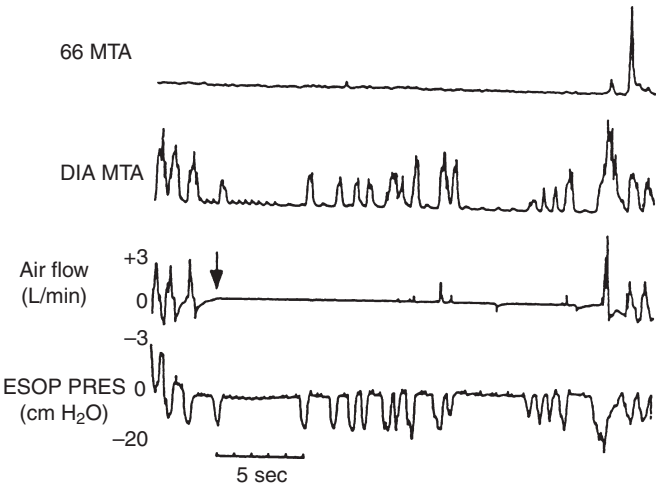


Figure 91-9 A representative tracing of the genioglossus muscle *(GG)* and diaphragm *(DIA)* electromyographic activity during spontaneous apnea. The *arrow* denotes the onset of mixed apnea. The diaphragmatic amplitude of the initial obstructed inspiratory effort is less than the diaphragmatic amplitude of the breath preceding apnea, whereas the diaphragmatic amplitude of the breath at resolution of the apnea exceeds that of the preapneic breath. Electromyographic activity of the genioglossus muscle appears only at resolution of the apnea. The moving time averaged *(MTA)* signals are depicted for both the genioglossus and diaphragm. *ESOP PRES,* Esophageal pressure. *(From Gauda EB, et al: Genioglossus and diaphragm activity during obstructive apnea and airway occlusion in infants,* Pediatr Res *26:583, 1989.)*

Upper Airway Reflexes

Reflexes originating from the upper airway may directly alter the pattern of respiration in infants and may play a crucial role in both the initiation and termination of apnea. The walls of the nasal cavity, nasopharynx and oropharynx, and larynx contain many sensory nerve endings that may respond to a variety of chemical and mechanical stimuli. Sensory input from these upper airway receptors travels to the central nervous system through cranial nerves V, VI, IX, X, XI, and XII and may have powerful effects on respiratory rate and rhythm, heart rate, and vascular resistance.[69] In human infants, interest has focused on two aspects of control of respiration by sensory input from the upper airway—that is, the response elicited from the nasopharynx to changes in luminal pressure and apnea produced by liquid stimulation of chemoreceptors in and around the larynx and hypopharynx.

Abu-Osba and co-workers[70] observed that sensory deprivation of the entire upper airway, including the larynx in rabbits, resulted in pharyngeal collapse. Similarly, topical anesthesia of the upper airway mucous membranes eliminated airway reflex response to pressure and was associated with pharyngeal obstruction, as well as death of the animal. These observations suggest that afferent sensory input from the upper airway is necessary for airway patency. By contrast, in kittens and puppies, steady airflow through the entire upper airway actually has been found to depress respiration.[71] Thus, depending on the species and experimental conditions, inhibitory or excitatory stimuli may originate from the upper airway.

In human infants who have undergone tracheostomy, Thach and colleagues[72] showed that negative pressure within the isolated upper airway depressed ventilation. In adult rabbits, Mathew and Farber[73] examined the effect of upper airway negative pressure on respiratory timing. Application of negative pressure to the isolated upper airway resulted in prolonged diaphragmatic inhibition; however, phasic upper airway muscle activity continued. The ability of the upper airway muscles to respond to increasing negative pressure may be augmented by chemoreceptor drive and inhibited by input from pulmonary stretch receptors.[74] The net balance of these reflex inputs may ultimately determine the patency of the upper airway at any given time. These observations may be relevant to the processes that initiate and resolve obstructive apnea. For example, during apnea, upper airway occlusion would result in increased negative airway pressure below the site of the obstruction. Reflex inhibition of diaphragmatic contraction resulting from increasing negative pressure in the airway lumen could produce the central pause characteristic of mixed apnea (see Figure 91-1), which in turn could result in a decrease in luminal pressure, allowing the airway to reopen.

A second important area of sensory reception in the upper airway lies in and around the larynx. Menon and co-workers[75] described apnea associated with overt regurgitation of gastric contents into the upper airway of human infants. Presumably, acidic stomach contents that enter the larynx excite chemoreceptors that cause both apnea and swallowing. These receptors have been studied in the lamb. Chemoreceptors in the region of the larynx send afferent neural output to the medulla; when these receptors are stimulated by water or ammonium chloride, apnea can be elicited.[76] Menon and co-workers[77] and, more recently, Miller and DiFiore[78] reported an observation that may link idiopathic apnea of prematurity with inhibitory reflexes arising within the upper airway: Swallows were found to be much more common during apnea than during comparable periods of uninterrupted sleep (Figure 91-10). Furthermore, swallowing during the respiratory

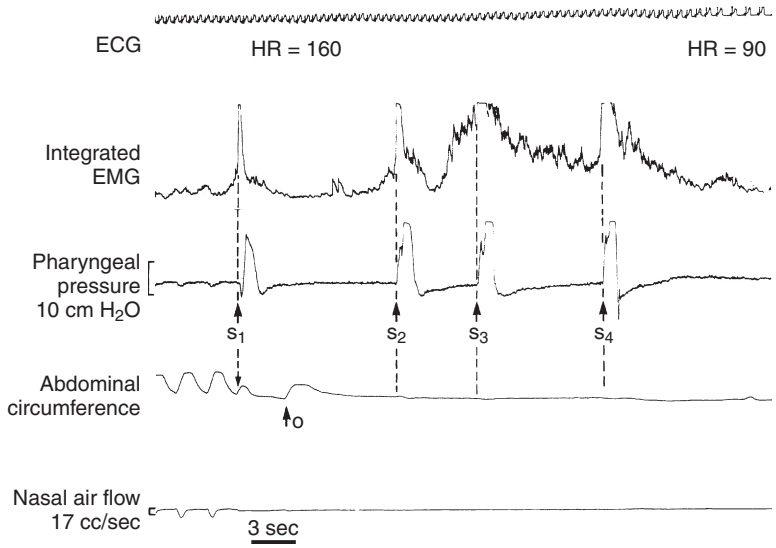


Figure 91-10 A typical prolonged apneic spell with swallowing *(S1)* occurs at apnea onset as well as during the apneic episode *(S2, S3, S4)*. An obstructed breath *(o)* also is seen at apnea onset. *ECG,* Electrocardiogram; *EMG,* electromyogram; *HR,* heart rate. *(From Menon AP, Schefft GL, Thach BT: Frequency and significance of swallowing during prolonged apnea in infants,* Am Rev Respir Dis *130:969, 1984.)*

pause is unique to apnea and does not occur during periodic breathing.[79] The etiology of these swallows is as yet unknown. Apneic episodes of variable duration have been elicited, resembling central or obstructive apnea, by introducing saline boluses into the oropharynx during sleep.[79] Hypothetically, accumulation of saliva in the pharynx could prolong apnea through a chemoreflex mechanism and also elicit swallowing movements.

Gastroesophageal Reflux

Although *gastroesophageal reflux* often is incriminated in causing neonatal apnea, caution should be exercised before the apnea is attributed specifically to reflux. Despite the frequent coexistence of apnea and gastroesophageal reflux in preterm infants, investigations into the timing of reflux in relation to apneic events indicate that they are not commonly temporally related.[80] Monitoring studies demonstrate that when a relationship between reflux and apnea is observed, apnea may precede rather than follow reflux, as seen in Figure 91-2.[81,82] This finding suggests that loss of respiratory neural output during apnea may be accompanied by a decrease in lower esophageal tone and gastroesophageal reflux. Such a phenomenon is supported by data from a newborn piglet model, in which apnea was accompanied by a fall in lower esophageal sphincter pressure.[83] Although physiologic experiments in animals reveal that reflux of gastric contents to the larynx induces reflex apnea, no clear evidence is available showing that treatment of reflux will affect frequency of apnea in most preterm infants.[84] Therefore pharmacologic management of reflux with agents that decrease gastric acidity or enhance gastrointestinal motility generally should be reserved for those preterm infants who exhibit signs of emesis or regurgitation of feedings, regardless of whether apnea is present. Greater understanding of the clinical consequences of gastroesophageal reflux may emerge from studies using transducers embedded in esophageal catheters that also measure impedance to characterize nonacid reflux.

PHYSIOLOGIC RATIONALE FOR THERAPEUTIC INTERVENTIONS

Continuous Positive Airway Pressure

Continuous positive airway pressure (CPAP) delivered by nasal prongs, nasal mask, or face mask at 2 to 5 cm H_2O pressure has proved effective in the treatment of apnea in preterm infants. Initial studies suggested that the beneficial effects of CPAP were mediated by means of an alteration of the Hering-Breuer reflex, stabilization of the chest wall with consequent reduction of the

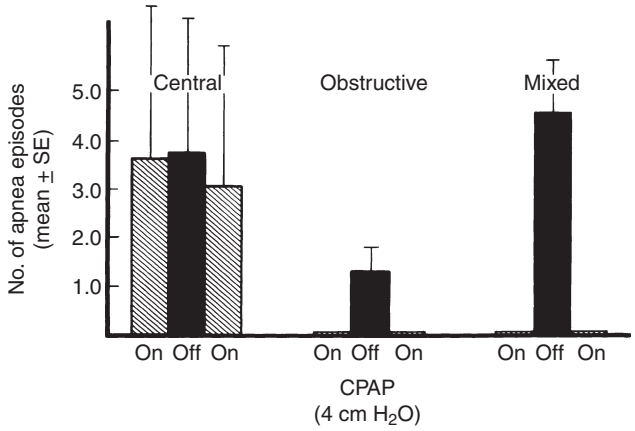


Figure 91-11 Effect of continuous positive airway pressure (CPAP) on the number of apneic episodes lasting 10 seconds or longer in infants of various ages (n = 10). Episodes of mixed and obstructive apnea decreased significantly during both periods of CPAP; however, no effect on central apnea was noted. *(From Miller MJ, Carlo WA, Martin RJ: Continuous positive airway pressure selectively reduces obstructive apnea in preterm infants,* J Pediatr *106:91, 1985.)*

intercostal-phrenic inhibitory reflex, or increase in oxygenation.[85] Although such mechanisms may play a role, nasal CPAP reduces the frequency of only mixed and obstructive apneas, with little or no effect on central apnea in infants[6] (Figure 91-11). It appears, therefore, that CPAP exerts its beneficial effect in infants by splinting the upper airway with positive pressure throughout the respiratory cycle, and nasal cannula flow at 1 to 2.5 L/minute may serve the same purpose.[86] Animal studies have demonstrated that genioglossus activity decreases with CPAP administration[87]; therefore reflex activation of upper airway dilating muscles is unlikely to be involved in the relief of apnea by CPAP.

Pharmacologic Agents

The methylxanthines—theophylline and caffeine—have proved effective in treatment of apnea in the newborn infant.[88,89] Several mechanisms, summarized in Box 91-1, have been proposed whereby methylxanthines decrease the incidence of apnea. Available evidence points to central respiratory stimulation, rather

BOX 91-1
Effects of Xanthines

Physiologic
- Increased minute ventilation
- Shift of CO_2 response curve to left ± increased slope
- Greater efficiency of diaphragmatic contraction
- Improved pulmonary mechanics
- Decreased hypoxic ventilatory depression

Biochemical
- Adenosine receptor antagonism

than bronchodilation, as the major mode of action. Gerhardt and associates[90] observed an increase in respiratory center output in premature infants receiving aminophylline, as measured by an increase in esophageal pressure change per breath, as well as a lowering of the threshold of the respiratory center to carbon dioxide. At the dose of aminophylline used in this study, no increase in carbon dioxide sensitivity or improvement in pulmonary mechanics was observed. A concurrent increase (approximately 20%) in metabolic rate was noted, however, and was subsequently confirmed with similar experiments using caffeine.[91] This metabolic step-up constitutes an adverse effect in preterm infants, who frequently are nutritionally compromised. These findings are in agreement with the central stimulatory effect of theophylline, which has been reported in animals and human adults. Animal data suggest that systemically administered theophylline acts directly on brain stem neurons, because respiratory stimulation occurs in the absence of peripheral (carotid body or vagus) reflex mechanisms, medullary chemoreceptor responses, or suprapontine brain structures.[92] Additional stimulatory effects of aminophylline on peripheral chemoreceptor activity were demonstrated by Cattarossi and associates[93] in the newborn human. Thus, in the infant, aminophylline may exert its effects at both the peripheral and central chemoreceptors.

The xanthines inhibit phosphodiesterase, which normally breaks down cyclic adenosine monophosphate (cAMP), although the relationship of cAMP accumulation with relief of apnea in infants is unproved. Adenosine and its analogues cause respiratory depression, and adenosine antagonism also has been proposed as the predominant mechanism to explain the therapeutic effect of theophylline.[37] In support of this concept, Darnall[94] documented that aminophylline partially reduces the ventilatory depression induced by hypoxia in newborn piglets. Adenosine is formed from nucleotides such as ATP and binds to multiple adenosine receptor subtypes. Whereas activation of adenosine A_1 receptors is primarily inhibitory of neuronal activity, adenosine A_{2A} receptor activation is predominantly excitatory. The latter has been shown to excite GABA-containing neurons, resulting in release of GABA—probably the major inhibitory neurotransmitter.[95] Xanthine therapy thus may excite breathing by blocking either adenosine or GABA effects on respiratory neural output. Xanthines may, however, have additional effects on the respiratory system. In adult patients with emphysema, theophylline appears to enhance diaphragmatic strength, as measured by transdiaphragmatic pressure generated against an occluded airway, while reducing EMG evidence of diaphragmatic fatigue.[96] Enhancement of calcium transport across the sarcolemma is a proposed mechanism by which theophylline increases diaphragm contractility, although the relevance of this action in human infants with apnea is not known.[97] A multicenter clinical trial has demonstrated that caffeine (versus placebo) therapy is associated with decreased duration of necessary ventilatory support in preterm infants.[98] This benefit was accompanied by a temporary reduction in weight gain. Longer-term effects on neurodevelopmental and respiratory outcomes are encouraging.

Doxapram, known to be a potent respiratory stimulant with predominantly peripheral chemoreceptor effects, has been used in neonates with idiopathic apnea of prematurity.[99] This drug may have a limited therapeutic role in infants with apnea refractory to xanthine derivatives, although potential side effects appear to limit its usefulness.[100]

Relation of Apnea to Sudden Infant Death Syndrome

One of the controversial aspects of apnea has been its proposed relation to the *sudden infant death syndrome* (SIDS). Many observations on the epidemiology and physiology of SIDS point to an abnormality of control of ventilation as one underlying cause of this multifactorial disorder. Early reports on the neuropathologic features of SIDS suggested the presence of tissue changes consistent with chronic hypoxia within the areas of the brain stem that regulate breathing and within the carotid body.[101,102] Short and prolonged apnea has been reported in infants with *near-miss SIDS* (now referred to as an *apparent life-threatening event*), a proportion of whom subsequently died of SIDS.[103] Short apneic episodes, periodic breathing, mixed and obstructive apnea, and abnormal ventilatory responses all have been variably identified in infants thought to be at risk for SIDS.[104,105] All of these observations suggested that an abnormality of ventilatory control could contribute to death from SIDS. Such considerations prompted the development of polygraphic monitoring in which variables such as heart rate, nasal airflow, chest and abdominal movement, and transcutaneous oxygen tension or oxygen saturation were measured in an attempt to predict the risk of SIDS in vulnerable infants. However, studies evaluating large cohorts of infants have failed to demonstrate that monitoring of cardiorespiratory variables can prospectively identify SIDS victims.[106] Although evidence implicates an abnormality of ventilatory control in SIDS, the nature of this abnormality is unknown. Indeed, more than one underlying cause of cardiorespiratory instability eventually may be implicated in this disorder. For example, it has been proposed that approximately 2% of infants who die of SIDS may have a sodium channel mutation with resultant predisposition to the long QT syndrome and potentially fatal arrhythmia.[107,108]

More recent research is providing new insights into the genetics of respiratory control development. Congenital central hypoventilation syndrome has been associated with weak or absent hypercapnic ventilatory responses and developmental abnormality of *PHOX2B* gene expression, which is crucial for autonomic nervous system development. Application of knowledge acquired from study of this extremely rare syndrome may solve some of the mysteries of respiratory control development.[109] Similarly, a possible relationship between delayed maturation of serotoninergic chemosensitive medullary neurons and predisposition to SIDS has generated interest in a role for the serotonin transporter gene in disordered respiratory control.[109]

Body Position and Exposure to Smoking

A reduction in postneonatal mortality and in the rate of sudden infant death has been associated with sleeping in the supine position. This finding has been substantiated in many countries and has generated public education campaigns designed to promote supine and avoid prone sleeping.[110] The physiologic mechanisms that underlie this relationship between SIDS and prone positioning are under investigation. Relevant factors include softness and porosity of bedding, which may encourage carbon dioxide rebreathing,[111] and thermal stress, which may destabilize respiratory control by as-yet undetermined mechanisms.[112]

Several studies have documented physiologic benefits for prone versus supine positioning in preterm infants, especially

those with residual lung disease. Such benefits include modest improvement in transcutaneously measured partial pressure of oxygen,[113] more time in quiet sleep,[114] decreased energy expenditure,[115] and greater ventilatory responses to inspired carbon dioxide[116] in the prone as compared with the supine position. The effect of sleep position on apnea is controversial. Heimler and co-workers[117] observed that infants had less apnea in the prone position. However, Bhat and associates noted more central apnea during prone sleeping.[118] Nonetheless, supine positioning also should be recommended for such infants in the home and should be initiated before hospital discharge. Furthermore, it has been proposed that greater sleep efficiency with fewer arousals associated with the prone position may actually enhance SIDS vulnerability. Prenatal and postnatal exposure to smoking also predisposes infants to SIDS. Nicotine affects a multitude of autonomic and sensory functions; however, no clear consensus has emerged regarding how nicotine targets developmental cardiorespiratory control, arousal, and ability to overcome apnea, bradycardia, and hypoxia.[119]

Apnea on a Continuum From Infancy to Childhood and Adulthood and Neurodevelopmental Outcome

Apnea of prematurity generally resolves by approximately 36 to 40 weeks of postconceptional age. In the most premature infants (born at 24 to 28 weeks of gestation), however, apnea frequently persists beyond 36 weeks of postconceptional age, and in occasional infants it may persist beyond 40 weeks. Cardiorespiratory events in such infants return to the baseline "normal" level at approximately 43 to 44 weeks of postconceptional age. In other words, beyond 43 to 44 weeks of postconceptional age, the incidence of cardiorespiratory events in preterm infants does not significantly exceed that in term babies (see Figure 91-3).[26]

Apnea and bradycardia in many preterm infants so affected have resolved by the time they are ready for hospital discharge as determined by maturation of temperature control and feeding pattern. An apnea-free observation period, usually ranging from 3 to 7 days, is used as a criterion for determining discharge date. For a subset of infants, however, the persistence of cardiorespiratory events may delay discharge from the hospital. In these infants, apnea lasting longer than 20 seconds is rare; rather, they exhibit frequent episodes of desaturation to less than 80% or bradycardia at less than 70 to 80 beats/minute with short respiratory pauses. The reason why some infants exhibit marked desaturation and bradycardia with short pauses is unclear.[120-122] For such infants, home cardiorespiratory monitoring until they attain 43 to 44 weeks of postconceptional age may offer an alternative to a prolonged hospital stay.

Because idiopathic apnea most often is seen in high-risk preterm infants, separating the consequences of premature birth from the effects of apnea of prematurity has proved to be difficult. Infants born prematurely often have multiple problems, and many of these conditions, particularly periventricular leukomalacia and intraventricular hemorrhage, may contribute to poor neurodevelopmental outcome. In one study that tracked the progress of preterm infants up to early school age, factors that predicted poor neurodevelopmental outcomes included apnea of prematurity.[123] In another series of very-low-birth-weight infants followed up to 24 months of age, predischarge apnea and desaturation during apnea correlated with lower mental and motor neurodevelopmental scores. The presence of excessive cardiorespiratory events on home monitoring after discharge has been associated with a lower mental developmental index on follow-up.[124] It is possible that recurrent hypoxia is the detrimental feature of the breathing abnormalities exhibited by preterm infants.[125]

A respiratory disorder similar to apnea of infancy, termed *sleep apnea syndrome,* also occurs in adults. Both obese and nonobese adults may present with mixed, obstructive, or central sleep apnea.[126,127] Just as in infantile apnea, adults with sleep apnea syndrome experience upper airway obstruction within the pharynx. Sleep apnea syndrome may be accompanied by daytime somnolence, hypoxemia, cardiac arrhythmias, polycythemia, hypertension, morning headaches, and intellectual deterioration. CPAP has been used effectively to relieve mixed, obstructive, and central apnea in adults.[128] Thus it would seem that the pathophysiology of sleep apnea in adults and infants may share some common elements, including failure of maintenance of patency of the upper airway. Adults and infants do differ in two respects in their respective disorders: In adults, obstructive apnea fails to respond to theophylline therapy.[129] This lack of response may reflect an underlying dissimilarity in the balance of inhibitory and excitatory neurotransmitters that regulate respiration in humans at different ages. Alternatively, the degree of narrowing of the upper airway may be more critical for development of obstructive apnea in adults than in infants. In addition, apnea in adults is not accompanied by an increase in spontaneous swallows. In childhood, sleep-disordered breathing is receiving greater attention, and its potential relation to apnea of prematurity is under study.

The long-term outcome for infants with persistent sleep apnea is not well understood. Guilleminault and co-workers[130] published the first report of data for a series of children 6 months to 17 years of age who had obstructive sleep apnea syndrome. Subsequent studies confirmed an association with pharyngeal narrowing and behavioral problems with poor school performance.[131,132] Thus apnea as a manifestation of instability of respiratory control of the diaphragm and upper airway during sleep has been described in infants, young children, and adults. The underlying complex of factors that produce apnea, including cyclic fluctuation in respiratory center output, imbalance of drive to the upper airway musculature, and anatomic narrowing of the upper airway, may differ in each age-group, but final outcomes, in terms of apnea, are remarkably similar. Sleep apnea thus emerges as a not-so-silent disorder that accompanies humanity from the cradle to the grave.

FUTURE DIRECTIONS

Initiation of Apnea

Two questions may be fundamental both to the cause of apnea and to the nature of the respiratory dysfunction in SIDS:

1. How is apnea initiated?
2. By what mechanisms is apnea terminated?

Cyclic instability of ventilation has been observed in both infants and adults with sleep apnea. Cherniack and Longobardo[133] proposed that apnea may derive from instability in the automatic feedback control of breathing. In this model, cyclic variation in ventilation, such as that observed in periodic breathing, could derive from increased circulation time, increased chemoreceptor sensitivity, or the inherently low stores of oxygen in the circulation. For example, apnea could occur when the respiratory control center in the medulla overcompensates in response to a brief increase in ventilation, resulting in a *central* respiratory pause (central apnea). In addition, obstructive apnea could result from an imbalance of central drive to the upper airway–dilating muscles and diaphragm.

Initiation of apnea also could involve a global alteration in the central regulation of ventilation and muscular function. During all stages of sleep (particularly active sleep), a generalized decrease in skeletal muscle tone is characteristic. A more profound depression of muscle tone may occur during apnea. Schulte and associates[134] reported that monosynaptic reflex excitability decreases during prolonged apnea and speculated that these changes may result from a decrease in output from the reticular activating system to the brain stem as well as higher brain structures. Thus unstable feedback control of ventilation, an imbalance in activity of upper airway muscles and the diaphragm, and a decrease in

output from the reticular activating system all have been proposed as mechanisms that may initiate apnea, but which factor is most crucial has yet to be demonstrated. Sophisticated new anatomic tracer techniques, together with localization of neurotransmitters at key locations in the developing brain stem, should provide an important link to available physiologic data.

Termination of Apnea

Increasing hypoxia and hypercarbia during apnea would be expected to lead to increased ventilatory drive to the diaphragm and upper airway, resulting in eventual resolution of apnea. Why then do some infants apparently fail to respond appropriately to these stimuli? As previously discussed, auditory brain stem conduction times are prolonged in infants with apnea, possibly reflecting decreased synaptic efficiency and myelination.[34] The delayed response of the apneic infant to hypercapnia and hypoxia may simply be the result of immaturity in processing afferent chemosensory information at the brain stem level. The decreased carbon dioxide response observed in apneic infants is consistent with this hypothesis.

Arousal from sleep also may play a role in the ability of the infant to terminate apnea quickly. The nature of the infant's sleep state may determine whether arousal occurs in response to apnea; this issue was investigated by Baker and Fewell[135] in the lamb. In active sleep, arousal was dependent on the rate of change in arterial oxygen concentration. A synergy appeared to exist between arterial carbon dioxide and oxygen levels, with the most rapid arousal occurring when both were changing.[136] Repeated exposure to hypoxemia during sleep resulted in evidence of adaptation, that is, increased time to arousal and decreased saturation at arousal. Such habituation to repeated hypoxic exposure has not been studied in human infants. Depression of reflex response to hypoxia, however, is a potential mechanism by which multiple sequential apneas could occur. Quantification of the frequency and magnitude of episodic hypoxemic episodes associated with apnea is now possible with the latest pulse oximetry technology. Thus several important variables, including peripheral and central chemoreceptor responses, sleep state, and previous exposure to hypoxia, could affect the infant's ability to terminate apnea. Exactly which of these influences is most crucial in resolution of apnea in the human infant remains an important question for future research.

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92

Emidio M. Sivieri and Soraya Abbasi

Evaluation of Pulmonary Function in the Neonate

The ability to measure lung function is essential for understanding its physiologic function, diagnosis of pathologic conditions, and evaluating the effect of therapeutic interventions. Early efforts at evaluating lung function were made possible in the 19th century with the invention of a simple spirometer to measure vital capacity.[1,2] Modern pulmonary function testing in adults and children has been used effectively as a research and clinical tool since the 1950s.[3,4] Although testing of infant pulmonary function was introduced around the same time, its practical use has lagged behind, owing to technical limitations, lack of patient cooperation, difficulty with the long duration of testing in this age-group, and time-consuming manual analysis methods.[5-8] In the 1980s, improvements in technology, including sensor miniaturization and computerization, led to mobile equipment systems enabling the clinician to perform routine pulmonary function testing at the bedside. More recently, a new generation of infant ventilators incorporating continuous display of pulmonary function data, as well as on-line graphic display of respiratory waveforms, have become available. Infant pulmonary function testing has thus moved from the research laboratory to the patient bedside, while efforts to reduce invasiveness and improve accuracy continue.

PHYSIOLOGIC BACKGROUND

Each respiratory cycle is governed by a driving pressure that moves a volume of air into and out of the respiratory tract, resulting in measurable airflow and volume changes. During unaided breathing, the driving force required for initiation of inspiratory airflow is generated by contraction of the respiratory muscles and outward movement of the thorax, resulting in a transient decrease in alveolar pressure from atmospheric pressure at end-expiration to a subatmospheric level and peak airflow at mid-inspiration (Figure 92-1). The expiratory driving force is generated by relaxation of the respiratory muscles and inward recoil of the lung. These actions result in an increase in alveolar pressure, causing an expiratory airflow that reaches a peak at mid-expiration and returns to zero at the end of expiration. At this point, alveolar pressure also returns to zero, and another respiratory cycle commences. Thus alveolar pressure cycles from a negative to a positive value relative to atmospheric pressure, and the magnitude of this change is determined chiefly by airway resistance. Pleural pressure, however, remains subatmospheric throughout the breathing cycle owing to a balance of the opposing forces of lung elastic recoil and chest wall outward recoil.

During tidal spontaneous inspiration and expiration in normal healthy lungs, pleural pressure decreases and increases, respectively. The magnitude of these changes is determined by airflow and tissue resistances and by elastic forces in the lungs and chest wall, reaching a maximum at inspiratory and expiratory midpoints, as do the inspiratory and expiratory airflows. Because no airflow occurs at the end-inspiratory and end-expiratory points of the breathing cycle, no flow-resistive losses result at these points, and the pleural pressure is dependent only on elastic forces. This fact aids in the determination of elastic and resistive pulmonary function components as follows.

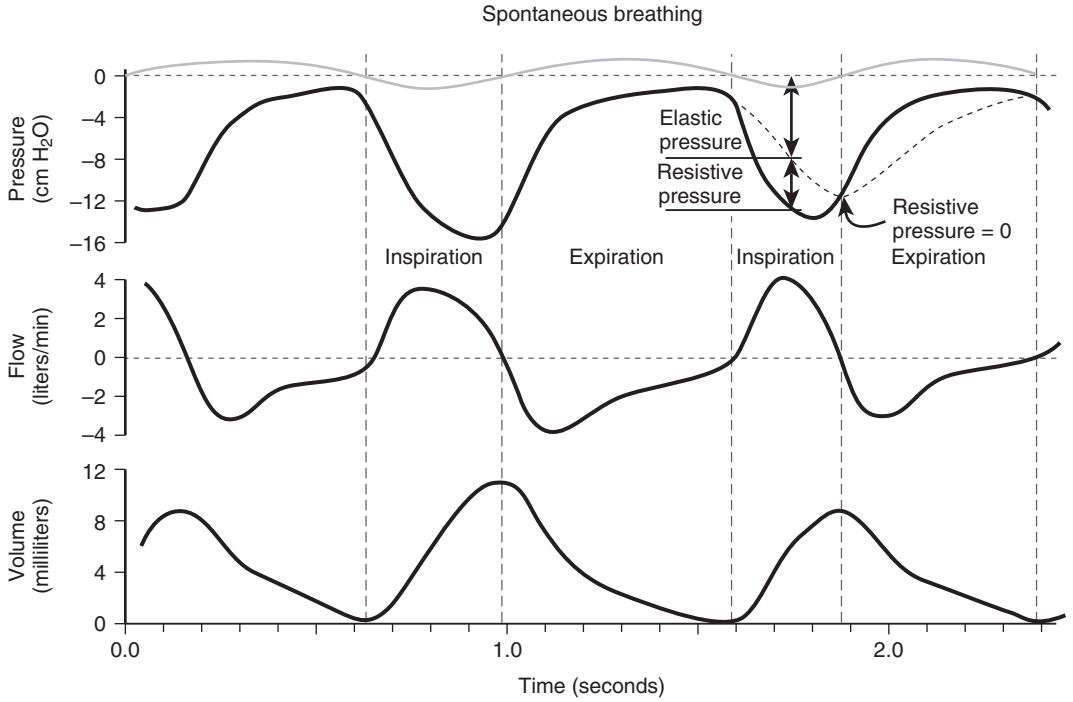


Figure 92-1 Pressure, flow, and volume signals for a respiratory cycle during spontaneous breathing.

Basic evaluation of pulmonary function is based on measurements of the interaction of driving pressure (P), volume (V), and airflow (V').[9,10] The equation of motion relating the motion of gas, chest wall, and lungs is expressed as a force balance equating the total driving force for breathing to a sum of elastic, resistive, and inertial forces:

$$P = EV + RV' + IV''$$

where

E = elastance of the respiratory tract (equivalent to the reciprocal of compliance, or $1/C$)

R = frictional resistance of respiratory tract comprising both airflow and tissue resistances

I = inertance of the respiratory tract, due to gas and tissue acceleration (V'')

This one-compartment linear model relating driving pressure to volume and flow has been considered an ideal elemental equation and constitutes a basic principle of respiratory physiology.[11] However, it is well appreciated that the respiratory system is not a linear system, resistance and compliance are not constants, both being dependent on volume, volume history, flow, and frequency. In addition, the respiratory system is a complex system consisting of multiple components with differing mechanical properties. This consideration is particularly pertinent in the preterm neonate. Despite its inexactness, however, and because of the complexities of dealing with nonlinear modeling, this simple linear model provides a useful framework for examining the dynamic behavior of the normal respiratory system.

INSTRUMENTATION

Instrumentation to measure the pressures, gas flows, and volumes in neonatal pulmonary function testing must meet basic physical capabilities for accuracy, precision, range, frequency response, and calibration integrity.[12,13] Computerized data collection systems also need to conform to signal processing standards such as adequate sampling rates to minimize aliasing in digitized signals, and to permit the use of appropriate automated breath detection algorithms.[14,15] In addition, devices to be used inline with infant breathing circuitry also must meet minimum standards for safety, hygiene, dead space, and resistive load. Equipment specifications and methodologies have been studied extensively in an effort to achieve uniformity among research laboratories and also among the various commercially available infant pulmonary function assessment systems. To this end, comprehensive testing and measurement standards have been established and published.[16,17]

Airflow Measurement

Decades of pulmonary function research have advanced several flow measurement technologies for use in both term and preterm neonates. A consistent objective has been to produce a smaller and lighter flow sensor having minimal dead space and airflow resistance. Some of the most commonly used devices are described next.

Pneumotachometers

Pneumotachometers are flow-resistive–type devices in which gas flows through a tube containing a fixed laminar flow element. Gas flow across the resistive element creates a pressure differential which is easily measured with a sensitive differential pressure transducer connected to each end of the tube. The resistive element can be either a fine-mesh screen or a bundle of small capillaries (Fleish type),[18,19] both of which produce a pressure drop that is linearly proportional to airflow so long as the flow is within a specified laminar flow range.[20,21] Higher flows give rise to turbulence, resulting in a nonlinear output. Accordingly, pneumotachometers should be selected that have linear flow ranges appropriate to the maximum expected normal flow for a given subject. Condensation of water vapor on the resistive element can easily alter its resistive properties, so most pneumotachometers incorporate a heating element to prevent accumulation of moisture. The flow characteristics also may be altered by accumulation of secretions, varying gas viscosity, gas composition, and temperature.[22-25] The pneumotachometer should be calibrated using the

same connectors, adapters, and gas composition as those to be used for actual measurements.[26,27] The differential pressure transducer used in conjunction with the pneumotachometer also must have a linear response over the pressure range being measured and have sufficient frequency response and phase characteristics.[28,29]

Hot Wire Anemometers

Hot wire anemometers are flow sensors that operate on the principle of measuring the amount of electrical current needed to maintain a constant temperature in a fine heated wire suspended across an air stream; the added current increases as the airflow increases and more heat is dissipated.[30-32] The response of the anemometer is inherently nonlinear, requiring linearization either in the signal conditioning circuitry or through computer signal processing algorithms. Another disadvantage is directional insensitivity, usually circumvented by the use of multiple sensors. Advantages are small size, good frequency response, and wide dynamic range, in addition to being a stand-alone device not requiring a pressure transducer. Furthermore, because the operating principle does not involve pressure measurement, the output signal is not subject to artifacts from sudden pressure gradients, or from pressure spikes in the breathing circuit, as are pressure-dependent devices.

Other Flow-Resistive Type Sensors

Other flow-resistive–type sensors include the pitot tube,[33,34] fixed orifice, and variable orifice sensors.[35] These types of flow sensors also require a pressure measurement. Unlike with the laminar flow pneumotachometer, the flow-pressure relationship for these devices is nonlinear, and they require hardware or software linearization. Like the anemometer, these sensors typically are smaller and lighter and thus have a smaller dead space than the pneumotachometer. These devices are therefore more appropriate for long-term monitoring, especially as integral components of mechanical ventilators for flow monitoring and for breath detection and triggering.

Ultrasonic Beam Flow Sensors

The theory behind operation of the ultrasonic beam flow sensor is the fact that sound waves traveling through a medium in flux are either accelerated or slowed down in accordance with the velocity of the medium. By measuring the transit time of a sound wave pulsed through a gas stream in a rigid conduit, the flow rate of the gas can be calculated. The main advantage of the ultrasonic flow meter is a much lower sensitivity to changes in gas properties than that typical for a traditional pneumotachometer.[36,37]

Volume Measurement

When airflow is measured directly at the airway opening using a flow sensor, respiratory volume change may be obtained simply by integration of the flow signal over time—achieved by measuring the area under the flow curve. With the advent of computerized signal processing, electronic integration of the flow signal has been replaced by digital integration. Because inspiratory gas differs from expiratory gas in O_2 and CO_2 composition, amount of water vapor, temperature, and viscosity, the inspiratory and expiratory portions of the resultant tidal volume trace will necessarily be slightly different. Additionally, flow sensors do not always have perfectly symmetric inspiratory and expiratory response characteristics. For these reasons, most volume signal conditioning routines correct for any baseline drift by incorporating an automatic return to zero at end-expiration. Differences in inspiratory and expiratory tidal volumes larger than 10%, however, may be indicative of airflow leakage around the endotracheal tube or face mask. Large drifts also may potentially indicate faulty flow sensor readings, which may be due to factors such as water vapor condensation or buildup of secretions.

Indirect methods for measuring volume change, without connection to the airway opening, may also be implemented when flow measurement is not essential. Inductive plethysmography[38-40] and impedance pneumography[41,42] are two indirect techniques that make use of measurements derived from thoracic and abdominal excursions. Although not as accurate as direct airway measurements, these alternative methods have the advantage of allowing noninvasive continuous monitoring without the alterations in breathing pattern that have been shown to occur with use of facemasks or inline flow sensors[43,44] These modalities are thus especially useful for undisturbed measurements in spontaneously breathing, sleeping infants.[45]

Whole-body plethysmography, when used in a manner in which the subject's airway opening connects to the atmosphere, as a "face-out" device, can be used to assess tidal volume by relating pressure changes in the box with thoracic expansion and contraction to volume changes.[46-48]

LUNG VOLUMES

Static Lung Volumes

Definitions

The components of static lung volumes are illustrated in Figure 92-2. The classical definitions of relevance for lung function testing in neonates are as follows:

Residual volume: Volume of air remaining in the respiratory system at the end of maximum possible expiration.

Functional residual capacity: Volume of air in the respiratory system at the resting level at the end of a normal tidal volume expiration and which is in continuity with the airways. Lung volume at functional residual capacity (FRC) is maintained by the opposing forces of lung elastic recoil and chest wall elastic recoil.

Thoracic gas volume: The total amount of gas in the lung at end expiration; irrespective of whether or not the gas is in communication with the airways. In healthy infants without air trapping, FRC is equivalent to thoracic gas volume.

Expiratory reserve volume: Volume of air that is the difference between FRC and residual volume.

Total lung capacity: Volume of air that is in the respiratory system at the end of a maximum possible inspiration.

Functional Residual Capacity Measurement by Plethysmography

Whole-body plethysmography, first described by DuBois and colleagues in 1956, has been a standard modality for measurement of thoracic lung volume for many years.[49] From Boyle's law it is known that under isothermal conditions, the gas pressure in a closed rigid chamber is inversely proportional to the gas volume by a constant such that for initial and final conditions 1 and 2, respectively,

$$P_1V_1 = P_2V_2$$

For an infant placed in a rigid body plethysmograph and breathing against a momentarily occluded airway,

$$P_1V_1 = (P_1 + \Delta P)(V_1 + \Delta V)$$

where P_1 is initial mouth pressure, V_1 is the initial lung volume, and ΔP and ΔV are the resultant changes in pressure and volume from the respiratory efforts. For this measurement, the infant breathes through a facemask incorporating a remotely controlled shutter, a pneumotachometer to record flow, and a port for recording mouth pressure. When the occlusion is triggered at the end of a resting expiration, lung volume is at FRC, and because no airflow is present, mouth pressure equals

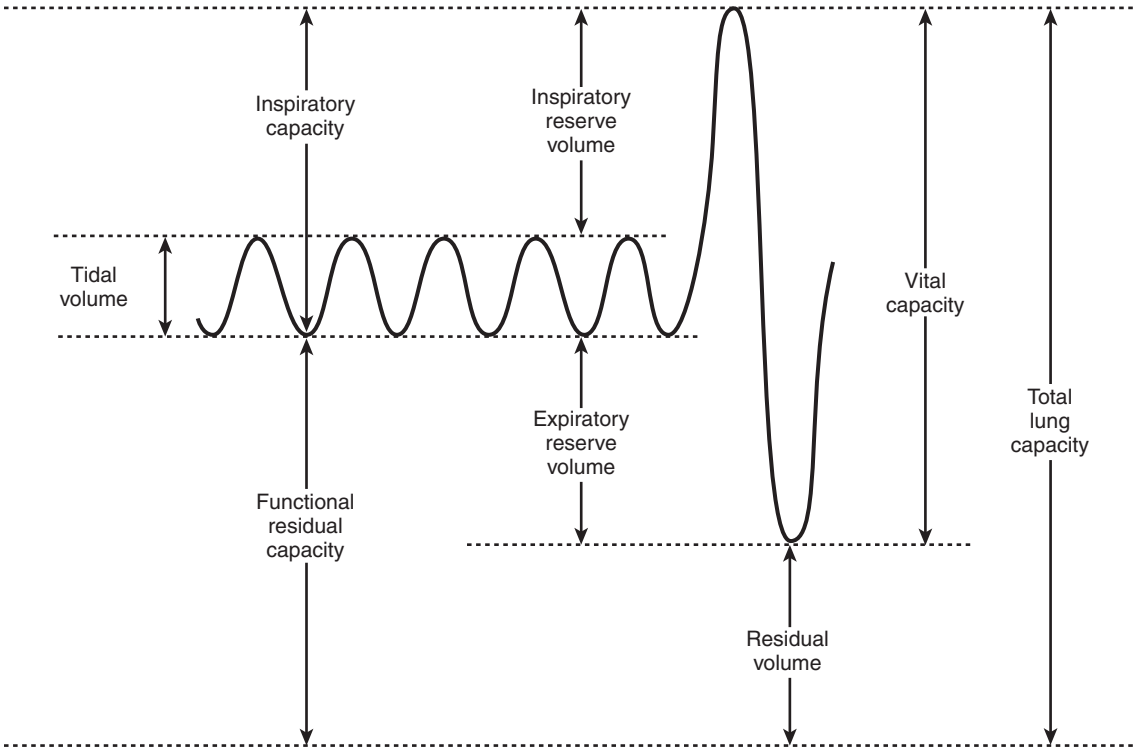


Figure 92-2 Static lung volumes.

alveolar pressure. As the infant breathes against the closed shutter, alveolar pressure changes owing to alternating gas compression and decompression are recorded, as are the concomitant changes in plethysmograph pressure, which are directly proportional to changes in alveolar volume. With appropriate corrections for temperature drift and apparatus dead space, as well as water vapor and body temperature and pressure saturated (BPTS) corrections, Boyle's law can be applied to solve for FRC. Of note, in contrast with tracer gas techniques, which measure only those volumes in communication with airways undergoing tidal ventilation, plethysmography measures total thoracic gas volume (TGV), including trapped air spaces in occluded airways.

Although several commercial infant body-box systems are now available,[50,51] whole-body plethysmography is not a practical technique for routine measurements in newborn infants in the intensive care nursery, and in particular infants receiving assisted ventilation. However, despite the complexities involved, the plethysmographic method has the advantage of allowing rapid repeat measurements within a period of only a few minutes. In addition, with proper BPTS conditioning of the respired gas, simultaneous measurement of airway resistance also is possible with whole-body plethysmography.[52]

Functional Residual Capacity Measurement by Helium Dilution

For measurement of FRC by helium dilution, the infant's airway is connected to a closed breathing circuit containing a known helium concentration (Figure 92-3). The connection to the closed breathing circuit is made at the end of a normal, restful expiration, and the infant then breathes from the circuit for 30 to 60 seconds, during which time the helium concentration is diluted to a lower value and measured.[53] If no helium is lost from the closed system, the amount of helium present before connection is equal to the amount after connection. The mass balance equation for helium is therefore

$$V_iC_i = (V_f + \text{FRC})\, C_f$$
$$\text{FRC} = V_iC_i / C_f - V_f$$

where

V_i = initial volume of the closed breathing circuit before patient connection
C_i = initial helium concentration in V_i
V_f = final volume of the closed breathing circuit after equilibration with the patient's FRC
C_f = final helium concentration in V_f + FRC

The volume V_f includes volume changes resulting from the patient's carbon dioxide production, oxygen consumption, and dead space in the patient connection. Carbon dioxide can be continuously removed from the system by passing the gas stream through carbon dioxide absorbent. To compensate for the patient's oxygen consumption, oxygen is gradually added to the breathing circuit to maintain a constant volume during the connection phase. In variations of the technique, the final system volume of the breathing circuit is measured directly.[54] Modifications of the rebreathing circuit have been used to measure FRC in mechanically ventilated infants.[55-57]

Technical Limitations

Sick infants with airway disease and poor distribution of ventilation have prolonged equilibration time and require long periods of rebreathing, which may lead to hypoxia and hypercapnia. Leaks around the endotracheal tube or face mask also are a frequent source of error, and methods have been developed that attempt to correct for this error.[58] To achieve maximum helium measurement accuracy, and to maximize the dilution effect, the total volume of the breathing circuit should be close to the approximate infant FRC[59,60]; this setting may be impractical, however, owing to the characteristically small lung volumes in premature and small infants. In addition, helium analyzers are not insensitive to other ventilatory gases, thereby potentially also affecting measurement accuracy.

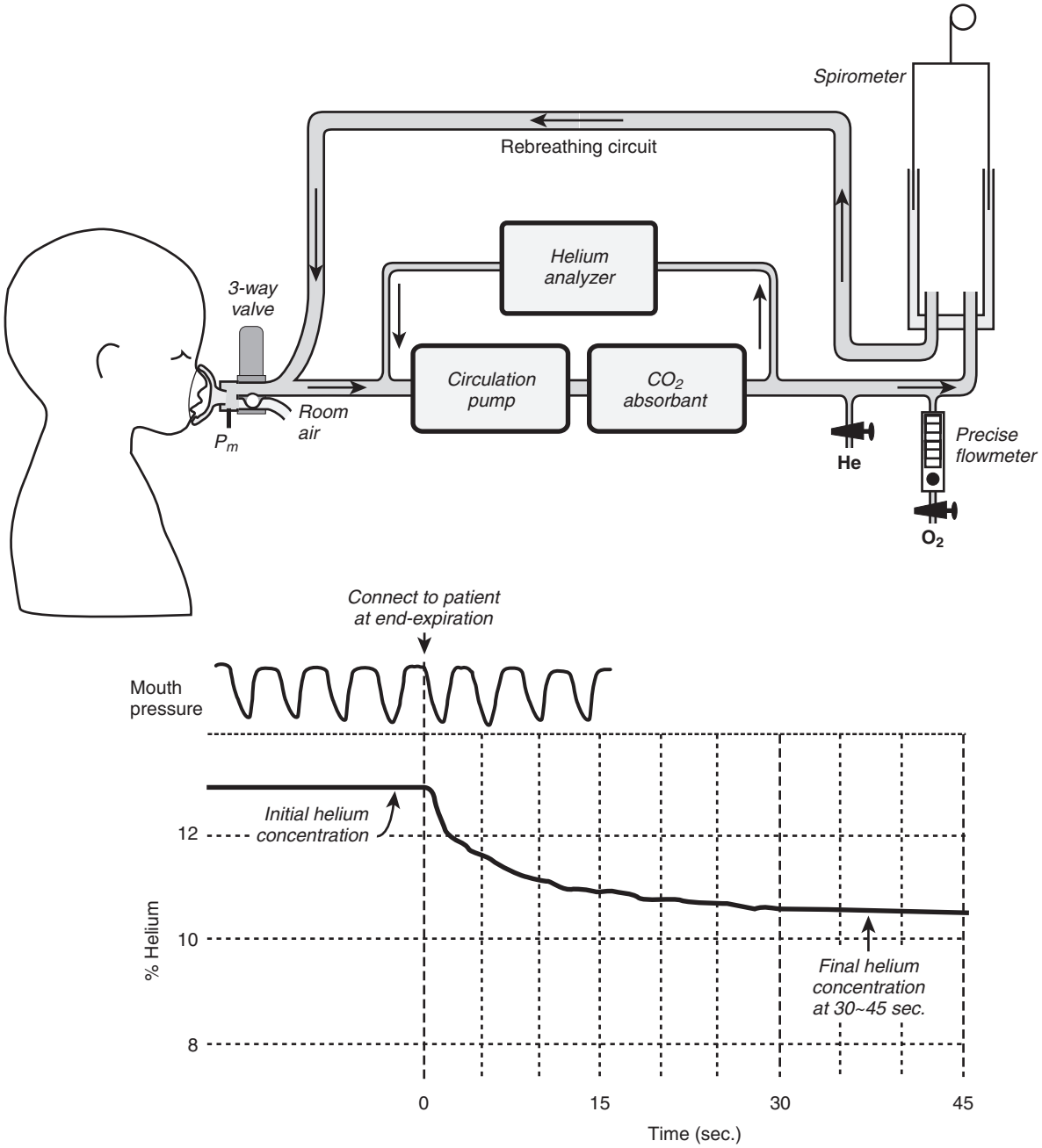


Figure 92-3 Determination of functional residual capacity (FRC) using helium dilution technique. Typical time course for helium concentration during FRC measurement.The mouth pressure signal is used to automatically switch a solenoid valve at end-expiration.

Functional Residual Capacity Measurement by Nitrogen Washout

FRC also can be determined by measurement of the total expired nitrogen "washed out" of the lungs while the infant breathes either 100% oxygen or an oxygen-helium mixture.The basic principle is described by the mass balance equation

$$\text{FRC} = V_{N2} / (C_{iN2} - C_{fN2})$$

where

V_{N2} = volume of nitrogen washed out

$C_{i\,N2}$ = initial, prewashout alveolar nitrogen fractional concentration (assumed to be 0.79)

$C_{f\,N2}$ = final, postwashout alveolar nitrogen fractional concentration (assumed to be 0.0)

The value V_{N2} can be obtained by measuring the nitrogen concentration of the entire expired volume, making this method impractical.Theoretically, V_{N2} could be calculated from the integration of the product of instantaneous flow (V') and the instantaneous nitrogen concentration (C_{N2}) signals over time:

$$V_{N2} = \int V'(t)\, C_{N2}(t)\, dt$$

For this method, known as the *multiple-breath nitrogen washout technique,* flow may be measured using a pneumotachometer. However, care must be taken to correct the flow for varying viscosity, temperature, and gas composition; most important, any phase lag between the flow and nitrogen concentration signals must be carefully synchronized.[61] Alternatively, to circumvent

these complexities, V_{N2} may instead be measured using a much-simplified method in which the infant breathes from a constant bias flow of 100% oxygen.[62] The expired gas is added to the bias flow, which is passed through a mixing chamber to produce an averaged nitrogen washout curve wherein the increase in flow during expiration is assumed to be equal to the decrease in flow during inspiration.[63] Thus the integrated nitrogen concentration signal is simply multiplied by the averaged bias flow (V'), which is treated as a constant in the following equation:

$$V_{N2} = V' \int C_{N2}(t)\, dt$$

The nitrogen washout technique has been widely implemented. When compared with the helium dilution technique, it was found to produce comparable estimates of FRC in infants and very young children.[64,65]

When compared with FRC determined by whole-body plethysmography in paired measurements, nitrogen washout FRC was consistently lower, the difference being larger for infants with BPD than for healthy infants.[66-69] This difference, previously attributed mainly to trapped gases in obstructed airways, recently has been reevaluated as possibly also attributable to methodologic factors.[70] The nitrogen washout technique also has been adapted for application in mechanically ventilated infants through use of a secondary washout ventilator.[71-73]

Technical Limitations

Breathing high oxygen concentration may result in hyperoxia or absorption atelectasis in areas of lung with low ventilation. To circumvent this problem, a helium-oxygen mixture has been used in place of 100% oxygen with favorable results.[74,75] In addition, accurate measurement of expired nitrogen in infants receiving inhaled oxygen greater than 70% is difficult owing to the very low levels of nitrogen present. And, as with the helium dilution method, the nitrogen washout technique is also prone to gas leakage errors.[76]

Functional Residual Capacity Measurement by Sulfur Hexafluoride Washout

An alternative washout technique that uses sulfur hexafluoride (SF_6) as the tracer gas can be used with either spontaneously breathing or ventilated infants on high oxygen concentrations.[77,78] For this technique, an SF_6 dispenser is connected to the inspiratory portion of the breathing circuit, and a low level of the inert gas SF_6 is washed into the lungs until a fixed alveolar level (usually less than 5%) is reached. The SF_6 dispenser is then switched off to initiate a washout period. A rapid-response SF_6 sensor in series with a flow sensor enables near-simultaneous calculation of the cumulative expired volume of SF_6. In a manner similar to the multiple-breath nitrogen washout method, FRC is determined from the breath-by-breath integration of the product of flow and C_{SF6}. In contrast with the nitrogen washout method, however, ventilator and Fio_2 settings need not be altered, and flow sensor accuracy is not compromised with significant changes in gas composition during the testing procedure. Moreover, unlike with the bias-flow nitrogen washout method, no additional ventilator or mixing chamber is needed. In contrast with the more conventional tracer gas techniques, infants on very high Fio_2 levels may be studied since oxygen levels up to 100%, as well as other ventilatory gases, have been shown to have negligible effect on the SF_6 analyzer accuracy.[79,80] More recently the SF_6 dispenser–flow sensor combination has been replaced by an ultrasonic flow meter capable of providing simultaneous measurements of airflow and SF_6 concentration in a single unit.[81-83]

The SF_6 washout method, as well the nitrogen multiple-breath washout method, also may be used for determination of ventilation distribution[84-86] and for estimation of trapped gas.[87] An alternative inert gas that is less expensive than SF_6 and is more widely accepted for clinical use is heptafluoropropane, whichalso has been tested and found to give reproducible results in FRC determinations in newborns.[86,88]

Dynamic Lung Volumes

Definitions

Tidal volume: Tidal volume (V_T) is defined as the volume of gas entering and leaving the respiratory tract with each breath. The volume varies with the activity of the baby.

Minute ventilation: Minute ventilation is defined as the total volume of gas expired over a period of one minute. Accurate measurement of tidal volume (no leak around endotracheal tube or face mask) and respiratory frequency (f_R) allows estimation of minute ventilation as: $V_T f_R$.

Measurement of dynamic lung volumes can be accomplished either directly by spirometry, pneumotachometry, or other flow sensing technology at the airway opening; or indirectly by one of the methods described earlier, such as induction plethysmography.

RESPIRATORY MECHANICS

Definitions

Compliance

Lungs, airways, and thorax are elastic; these tissues stretch due to changes in pressure during inspiration; when the pressure change ceases, the tissues return or recoil to their resting positions. The compliance of a closed elastic structure is defined as the change in volume resulting from a unit change in pressure (i.e., compliance = $\Delta V/\Delta P$).[89]

Static compliance: Static compliance is an accurate determinant of lung elastic properties only. It measures the change in volume for a given pressure during static, non-flow, conditions. A sufficient equilibration period is required to ensure that the actual static pressure change is recorded. Measurement of the systematic change in both pressure and volume during inflation and deflation allows a static pressure-volume curve of the lungs to be plotted. The curves that the lungs follow during inflation and deflation are different and are also influenced by surface tension and viscoelastic tissue resistances. This physical property of the lungs is known as hysteresis.

Dynamic compliance: Dynamic compliance is the determination of the change in volume and pressure during the respiratory cycle, either spontaneous breathing or mechanical ventilation, and is predominantly influenced by breathing frequency.[90,91] During rapid breathing, there may not be sufficient equilibration time to determine the actual change in pressure accurately.

Pulmonary compliance: Compliance of the lung only and as determined from measurement of transpulmonary pressure, that is, the difference between pleural and alveolar pressures. Under static conditions alveolar pressure is equivalent to airway pressure. Pleural pressure may be estimated from measurement of esophageal pressure.

Respiratory system compliance: Volume change of the entire respiratory system as related to the total driving pressure across the lungs and chest wall. During mechanical ventilation, the driving pressure is measured at the airway opening.

Chest wall compliance: Volume change related to the pressure across the chest wall, or trans-thoracic pressure. Transthoracic pressure may be measured as the difference between airway opening and pleural pressure. Alternatively, if the total compliance and lung compliance are known, the following

relationship may be used to calculate chest wall compliance (C_{cw}): total respiratory system elastance = elastance of the lungs + elastance of the chest wall, or terms of compliance:

$$1 / C_{rs} = 1 / C_L + 1 / C_{cw}$$

Specific compliance: Compliance normalized to the lung volume at which it is measured, usually at FRC, or compliance/FRC.

Resistance

Resistance is a frictional phenomenon that is due to the motion of both respiratory gases and tissues. Thus resistance is absent during static respiratory conditions. Tissue resistance is due to the viscoelastic properties of biologic tissues during respiratory movements. Airflow resistance is defined as the change in pressure associated with a unit change in flow of a gas through a conduit (i.e., resistance = $\Delta P/\Delta V'$) and is dependent on the length and diameter of the conduit, and the density and viscosity of the gas. The magnitude of airflow resistance also is dependent on the fluid dynamics of the air stream—for example, turbulent airflow will produce a greater resistance than laminar airflow. Respiratory airflow requires a driving pressure generated by changes in alveolar pressure. When alveolar pressure is less than or greater than atmospheric pressure, air flows into or out of the lungs accordingly, and the magnitude of this airflow is influenced by resistance.

Total resistance: The combined airway, lung tissue, and chest wall resistance of the respiratory system.

Pulmonary resistance: The pressure difference across the lung only—that is, transpulmonary pressure divided by the resultant airflow.

Airway resistance: Resistance of the airway alone, equal to the pressure gradient between alveolar and atmospheric pressure divided by the resulting airflow value. Airway resistance may be responsible for as much as 80% of the total pulmonary resistance in a normal lung and is influenced by the velocity and pattern of airflow, geometry of the airways, and the density or viscosity of the gas itself.

Tissue frictional resistance normally accounts for approximately 20% of the pulmonary resistance and is influenced by the configuration of the chest wall and the lung, as well as by the fluid content of the pulmonary tissue. Inertial resistance, which is less than 1% of pulmonary resistance, usually is more dependent on the breathing frequency and the density of the gas and typically is ignored within the normal range of breathing frequencies.[92,93]

Measurement of Respiratory Mechanics

Dynamic Pulmonary Mechanics

Pulmonary mechanics are calculated from measurements of transpulmonary pressure (the difference between the airway opening and pleural pressures) and flow and volume changes throughout the respiratory cycle. These are dynamic measurements obtained during either spontaneous breathing or mechanical ventilation. Airflow and volume are measured by pneumotachometry or other flow sensor technology as described previously. Pleural pressure can be estimated from esophageal pressure[94-96] measured by a pressure transducer connected to either an air-filled balloon[97,98] or a fluid-filled catheter,[99,100] or by a microtransducer–tipped catheter.[101] The accuracy of the esophageal pressure measurement can be evaluated by an occlusion test.[102-104]

The calculations of compliance and resistance are based on the assumption of a linear model.[3] Thus the driving pressure is always equal to the sum of the elastic and resistive pressures (see Figure 92-1). The calculations may be performed by the Mead-Whittenberger analysis, as shown in Figure 92-4, or by any of several computerized techniques such as least mean squares (LMS) analysis.[105-108]

Technical Limitations

Reliable esophageal pressure measurement by esophageal balloon catheter depends on proper size, positioning, and inflation of the balloon. Water-filled catheters need to be free of gas bubbles and have no accumulation of secretions at the tip. Placement of catheters may generate peristalsis, resulting in an initial pressure drift. Because measured esophageal pressure reflects pleural pressure only at a specific location, its accuracy depends on assuming that the pressure change at the site represents mean pleural pressure changes.[109] Altered patterns of inspiratory muscle contraction or esophageal muscle tone and distortion of the chest wall may potentially result in uneven pleural pressure distribution.[103,110,111]

Accuracy of measurements also is dependent on body position. Measurements should be done with the patient in supine neutral head position, and only during quiet sleep. Flexion or extension of the neck in small infants can affect the airflow resistance.[112] Feeding volume also may affect the measurements and should be standardized in repeated studies.[113,114] In mechanically ventilated infants, the ventilatory support should be kept the same during sequential studies to prevent shifting of the tidal volume to a different portion of the volume-pressure curve.

Commercially available equipment may exhibit variable frequency response, phase shift errors between signals, and drifting calibration values. Testing of such equipment with a calibrated lung simulator before clinical use will ensure accuracy at the expected infant tidal volumes and frequencies.[16]

Passive Respiratory Mechanics: Occlusion Techniques

Passive occlusion techniques constitute a relatively noninvasive method for determining total respiratory system compliance (C_{rs}), resistance (R_{rs}), and time constant (τ_{rs}) and therefore do not require measurement of esophageal pressure. These passive techniques rely on the Hering-Breuer inflation reflex[115-117] to relax both the inspiratory and expiratory respiratory muscles during an expiratory occlusion. Under the resultant condition of complete expiratory relaxation, driving pressure is zero, and the airway opening pressure comes into equilibrium with alveolar pressure.[118,119]

Multiple Occlusion Technique

A series of brief expiratory airway occlusions are performed on multiple spontaneous breaths. During each occlusion the elastic recoil pressure measured at the airway opening (P_{ao}) reaches a plateau as it equilibrates with alveolar pressure. An occlusion is repeated at each of several different volume points on separate breaths and over the first two thirds of expiration. A volume-versus-pressure plot is constructed from the resultant measurement pairs, and the slope of the linear regression fit through these points gives the C_{rs}.

Single-Breath Occlusion Technique

A single end-inspiratory occlusion is performed to invoke the Hering-Breuer inflation reflex to induce relaxation of the respiratory muscles during both the occlusion and the post-occlusion passive expiration. Elastic recoil pressure is measured during the occlusion and a passive flow-volume loop is recorded during the post-occlusion relaxed expiration (Figure 92-5). The reciprocal of the slope of the linear portion of the expiratory flow-volume relationship gives the passive expiratory time constant ($\tau_{rs} = \Delta V/\Delta V' = C_{rs}\, R_{rs}$). Extrapolation of this line to the zero flow point, that is, the elastic equilibrium volume (V_{EEV}), and the volume at start of occlusion (V_0), allows estimation of the occluded volume above the V_{EEV}. Knowing the relaxed plateau pressure, P_{ao}, during zero flow allows estimation of compliance as $C_{rs} = (V_0 - V_{EEV})/P_{ao}$ and resistance as $R_{rs} = \tau_{rs}/C_{rs}$.

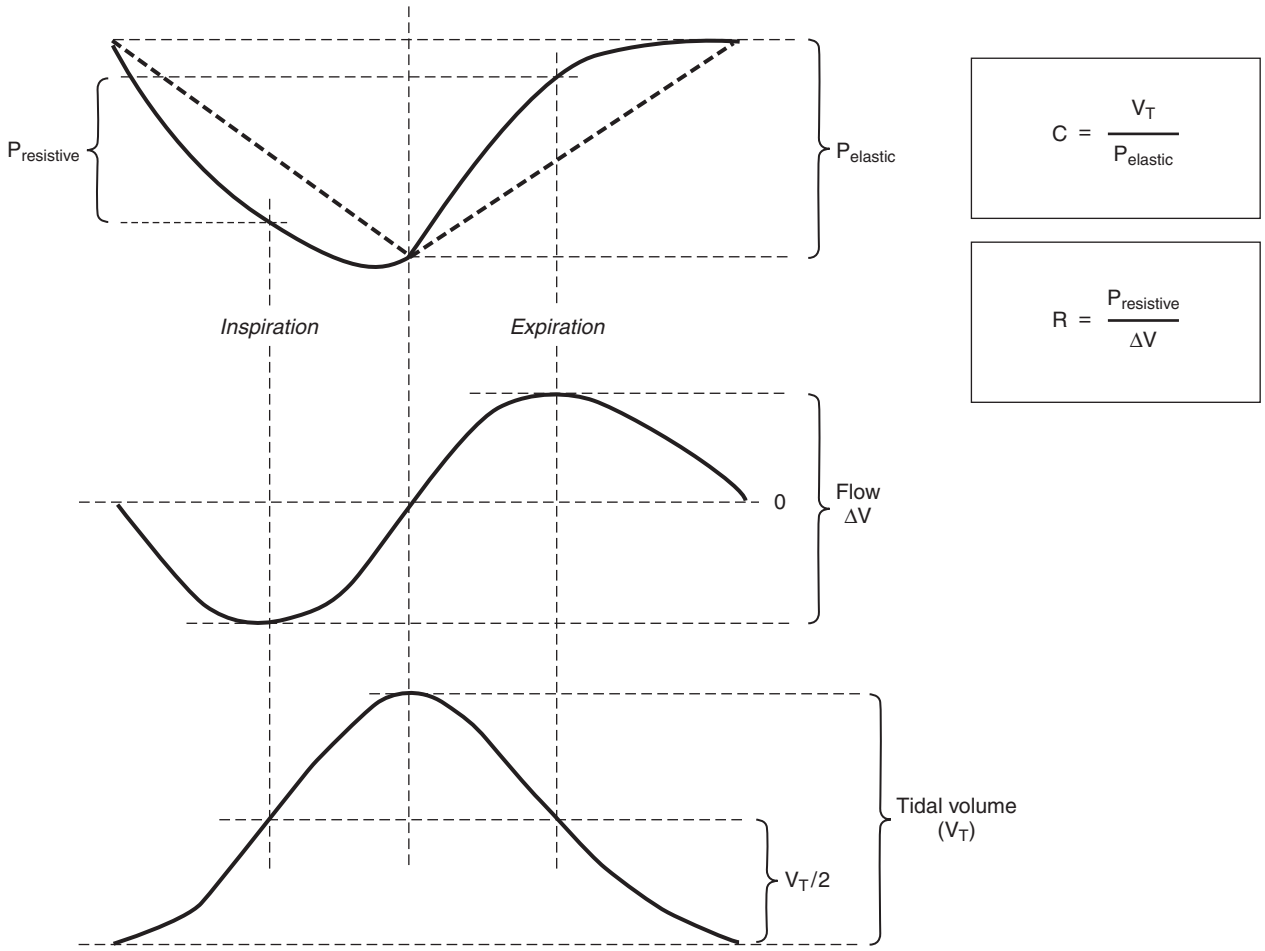


Figure 92-4 Determination of pulmonary mechanics by analysis of pressure, airflow, and volume signals using the Mead-Whittenberger method.

AIRWAY MECHANICS

The tone of tracheobronchial smooth muscle provides a mechanism to stabilize the airways and prevent airway collapse.[120] Plugging of the airways, edema, and weakening of the airway walls and the resulting tracheobronchomalacia alter airflow.[121-123] In addition, the increased driving pressure secondary to the high elastic and resistive loads during tidal breathing in infants with bronchopulmonary dysplasia often leads to self-induced expiratory flow limitation.

Measurement

Resistance to airflow is measured by calculation of pulmonary mechanics, as described. It also can be evaluated by the tidal flow-volume relationship obtained using a forced expiratory flow generated by rapid thoracic compression.

Forced Expiratory Flow

Forced expiration commonly is used to detect obstructive lung disease by determining the lung volume at which small airways begin to close. During expiration, flow is limited by airway compression, which in turn is determined by the elastic recoil of the lung, the resistance of the airway, and airway wall mechanics. In infants, forced expiratory flow is generated by rapid thoracic compression, or negative-pressure forced deflation. Rapid thoracic compression is preferred as a noninvasive technique that does not require intubation, paralysis, or deep sedation to generate a partial expiratory flow-volume (PEFV) loop. Parameters that are used to characterize PEFV include peak expiratory flow, forced expiratory flow at functional residual capacity (V'_{max} FRC), and the shape of the expiratory flow curve[124-127] (Figure 92-6).

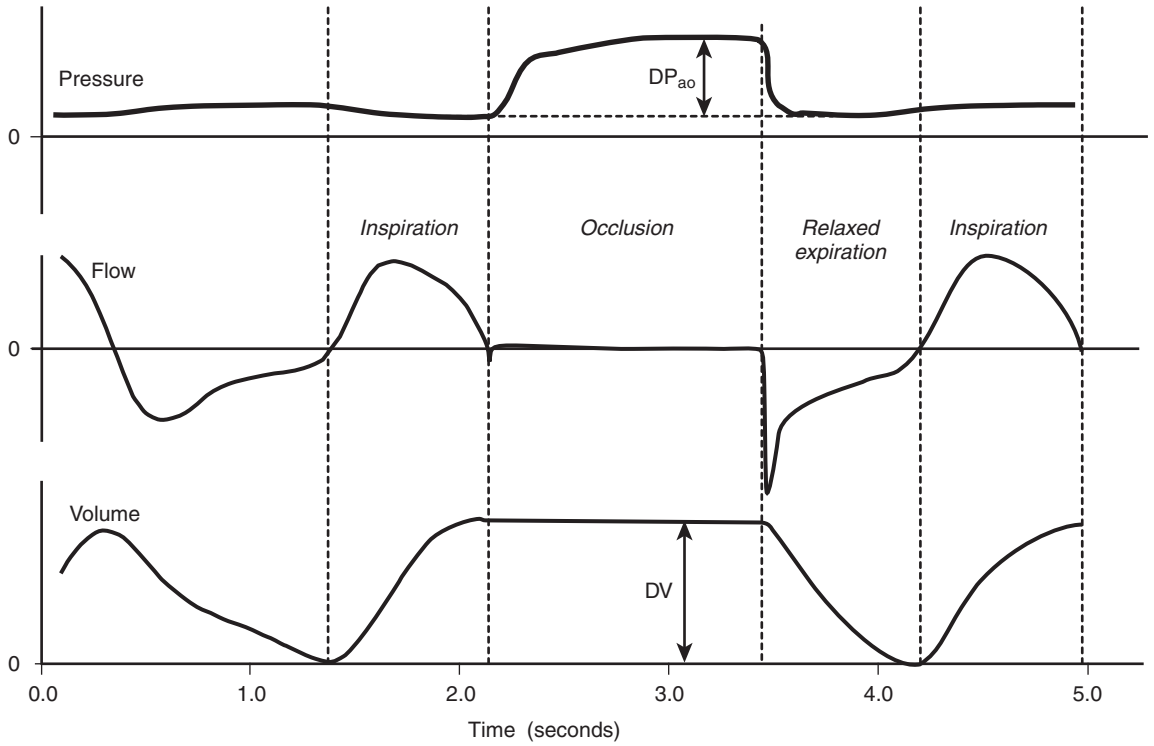
Technical Limitations

This technique generates only a partial rather than a complete flow-volume loop, which limits the evaluation of true flow limitation in response to the appropriate compression pressure for a particular infant. The main limitation is the variability of FRC in young infants, because inspiration may start before the completion of a full expiration.

NORMAL VALUES

Normal values for pulmonary function parameters are essential for meaningful clinical interpretation. In adults, these values have been predicted on the basis of age, gender, and relationship to height and weight, as well as race and ethnic background. Similar data for neonates have not yet been established. Values for normal term babies, obtained by different techniques, are presented in Table 92-1. Preterm neonates who are considered normal and who have not received mechanical ventilatory support[128-130] are infrequently encountered in clinical practice; the data for such infants also are summarized in Table 92-1.

Any comparison of values needs to take into account the study population and the conditions that affect the measurements such as body and neck posture, feeding, and sleep state.



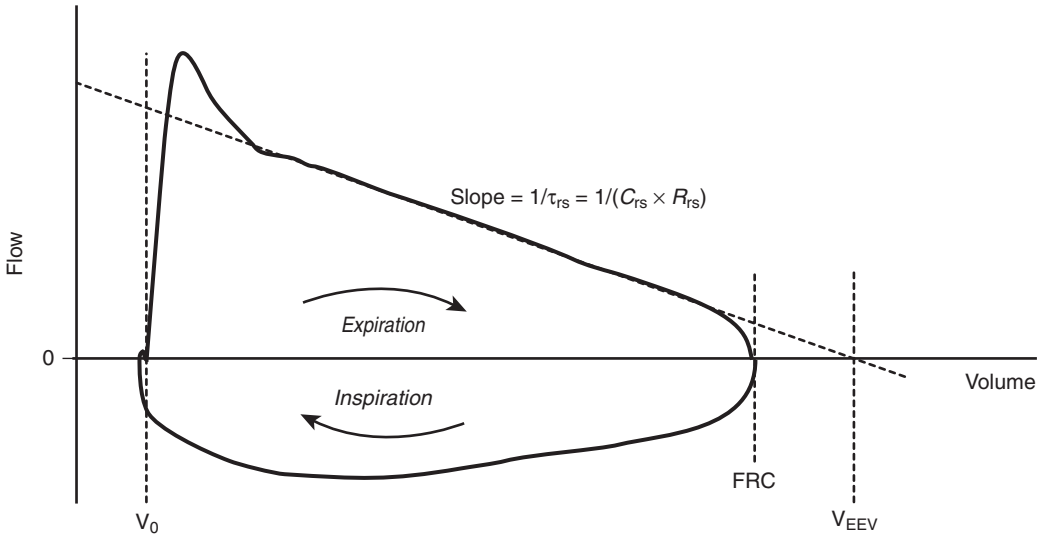


Figure 92-5 Passive respiratory mechanics determined using the single occlusion technique (SOT): End-inspiratory occlusion. ΔP_{ao}, Elastic recoil pressure plateau measured at the airway opening; ΔV, occluded volume change.

REAL-TIME PULMONARY GRAPHICS MONITORING

The addition of pneumotachometers and waveform graphics to the latest generation of infant ventilators provides clinicians with respiratory function information that previously was available only with specialized instrumentation requiring specifically trained operators and frequent calibration. These dedicated bedside instruments at best sampled only a few minutes' worth of data, as opposed to continuous monitoring; serial repeat measurements were therefore needed to observe the effect on pulmonary function resulting from changes in ventilator settings or other therapies. The newer graphics-based ventilators make use of improved sensor and microprocessing technology to provide continuous real-time display of the fundamental measurable parameters of respiration—namely, the pressure, flow, and volume signals. These signals may be displayed as waveforms along a time axis or as plots of *x-y* relationships such as volume-versus-pressure and flow-versus-volume loops. In addition to graphic display, measurements from the three signals are used to calculate a limited set of respiratory function data.

Volume-Pressure Loops

A volume-pressure loop is an *x-y* plot of tidal volume as a function of airway pressure. Figure 92-7, *A,* illustrates a volume-pressure loop from a mechanically ventilated neonate. The loop represents

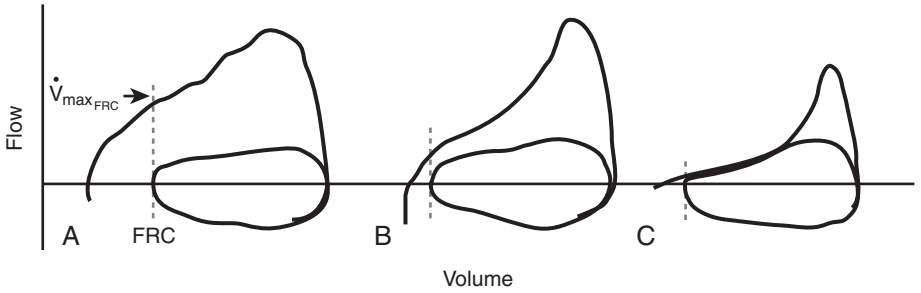


Figure 92-6 Schematics of partial forced expiratory flow relationship: Infants with normal expiratory flow, moderate flow restriction (partial limitation), and severe chronic lung disease (CLD).

TABLE 92-1

Pulmonary Function Parameters During the First Month of Life: Mean Normal Values

Study	GA (wk)	Age (days)	V_T (mL/kg)	FRC (mL/kg)	$C_{L,dyn}$ (mL/cm H_2O)	C_{rs} (mL/cm H_2O)	R_{rs} (cm H_2O/L/sec)	R_L (cm H_2O/L/sec)	WOB (g · cm/kg)	V'_{max} (mL/sec)	V'_{max}, FRC(mL/sec)
Abbasi and Bhutani, 1990[128]	28-30	2-3	5.9		2.0			Exp 50.1			
Abbasi and Bhutani, 1993[129]	28-34	2-3	6.3		2.4			Total 54.0	12.0		
Anday et al, 1987[130]	28-30	5-7	6.6		2.3			Exp 70.0			
Choukroun et al, 2003[131]	29-31	1-2				0.55 ± 0.13	130 ± 30				
Gerhardt et al, 1987[132]	31-36	3-30		16.7	2.2			Exp 86.8			
Ronchetti et al, 1975[133]	34	4-28		29.5							
Migdal et al, 1987[134]	34	1-28									
Nelson et al, 1963[135]	Preterm	1-16		38.7		2.4					
Multiple*	Term	1-30	53-7.2	17.1-52.6	3.2 - 5.7	3.2-3.8		Total 26-58	13.0-20.5	185-186	182-190

*Combined data from references 78, 83, 124, 128-130, 132, 134, 136-141.

$C_{L,dyn}$, Dynamic lung compliance; C_{rs}, total respiratory system compliance; *Exp,* expiratory; *FRC,* functional residual capacity; *GA,* gestational age; R_L, pulmonary resistance; R_{rs}, respiratory system resistancewmaximal flow; V'_{max} *FRC,* maximal flow at FRC; V_T, tidal volume; *WOB,* work of breathing per kg of body weight.

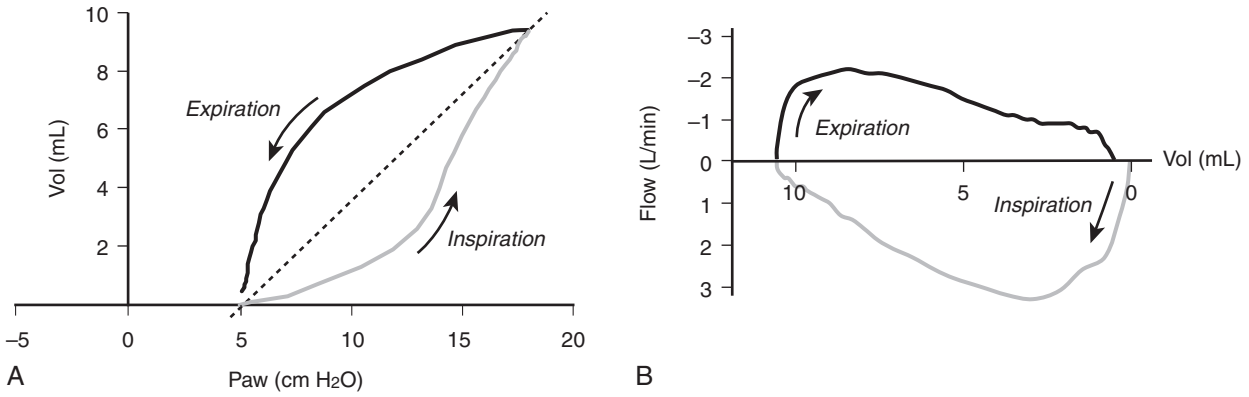


Figure 92-7 Schematic of a typical infant ventilator graphics display for a single breath. **A,** Volume-pressure loop. **B,** Flow-volume loop.

a single breath and is plotted in a counterclockwise direction as the breath progresses from inspiration to expiration. The slope of the dotted line, connecting the zero flow points at end-expiration and end-inspiration, is a good estimate of the overall elasticity or dynamic respiratory system compliance, defined as the ratio of tidal volume to driving pressure, or V_T(PIP − PEEP). Tilting of the loop toward the horizontal or toward the vertical is indicative of either decreased or increased compliance, respectively (Figure 92-8, *A*). Of note, the volume-pressure relationship follows a different course during expiration from that during inspiration. The resulting hysteresis, depicted as the area within the loop, is due to resistive pressures. Increased inspiratory or expiratory resistive work expands the hysteresis to the right or left, respectively (see Figure 92-8, *B*).

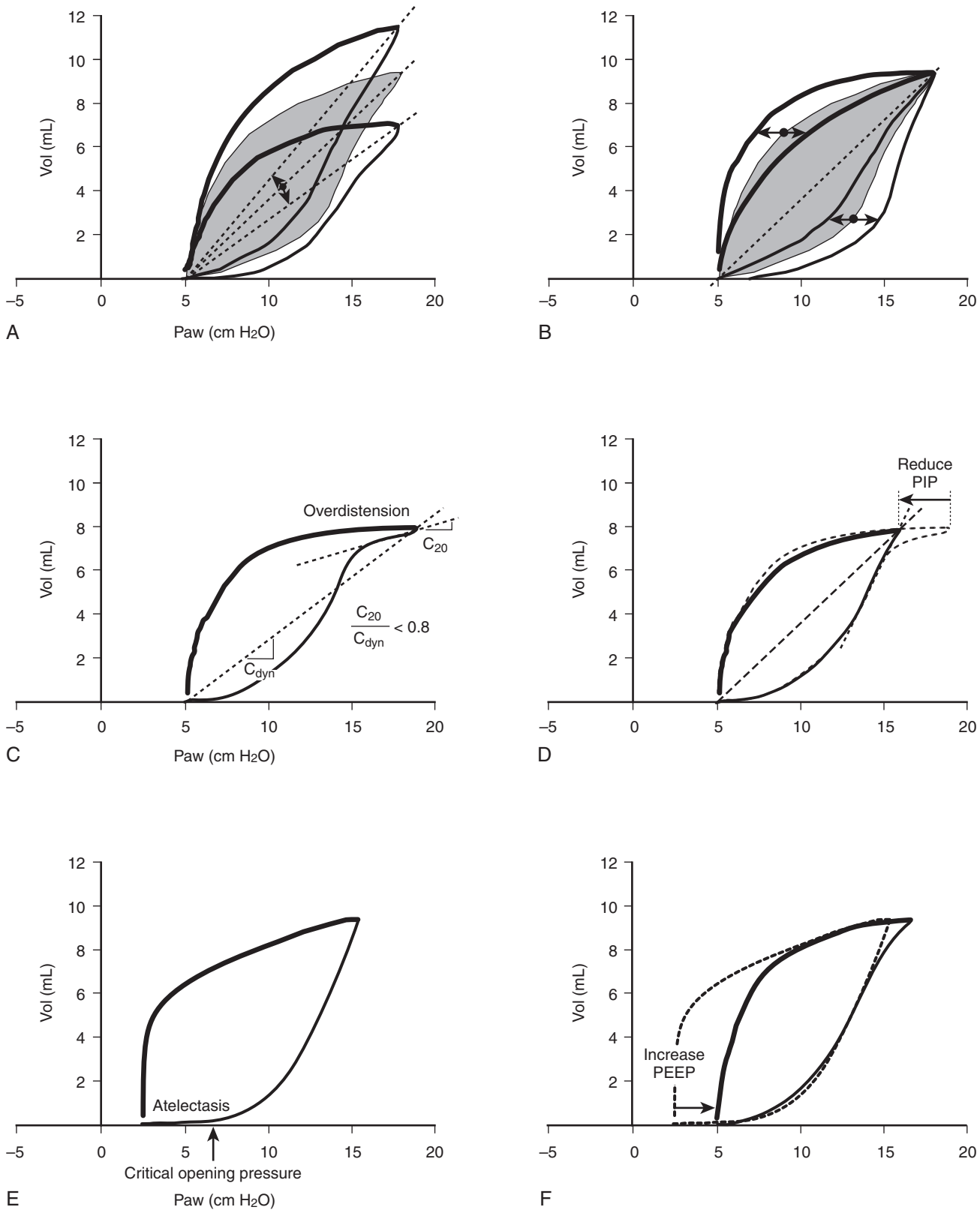


Figure 92-8 Volume-pressure loop characteristics. A, Compliance change with fixed PIP and PEEP. **B,** Inspiratory and expiratory resistance changes. **C,** "Beaking" effect seen with overdistention. **D,** Effect of reducing PIP on overdistention. **E,** Atelectasis due to insufficient FRC. **F,** Increasing PEEP to reduce atelectasis. *FRC,* Functional residual capacity; *PEEP,* positive end-expiratory pressure; *PIP,* peak inflation pressure.

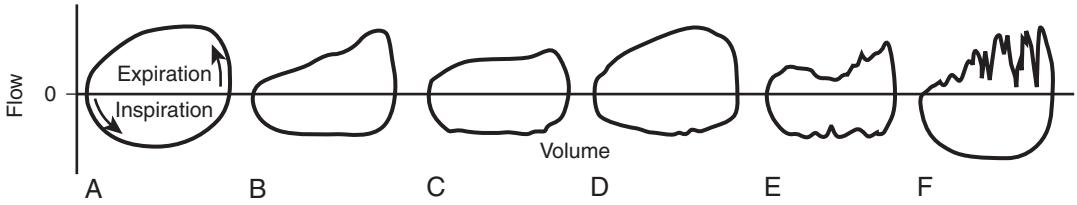


Figure 92-9 Tidal flow-volume loops illustrating various types of flow limitation. A, Healthy newborn. **B,** Obstructive airway disease—expiratory airway collapse. **C,** Fixed upper airway obstruction—inspiratory and expiratory flow limitation. **D,** Extrathoracic inspiratory flow limitation. **E,** Bronchomalacia—severe flow limitation. **F,** Airflow turbulence due to secretions in the airway from crying.

Volume-pressure loops are useful in clinical practice for detection of ventilatory overdistention, evidenced by the "beaking" effect as illustrated in Figure 92-8, *C.* In this breath, the compliance of the flattened area within the final 20% of the inspiratory limb (C_{20}) is less than the dynamic compliance of the entire breath (C_{dyn}). It has been suggested that a C_{20}-C_{dyn} ratio less than 0.8 may be an indicator of lung overdistention.[142] Any further pressure increase in the flattened portion of the volume-pressure loop produces negligible volume change, and a small decrease in PIP may be used to reduce overdistention while maintaining the same tidal volume (see Figure 92-8, *D*).

Another abnormality easily detected from observation of the volume-pressure loop is lung atelectasis (see Figure 92-8, *E*), evidenced by a prolonged period of negligible volume recruitment at the start of inspiration. Figure 92-8, *F*, illustrates how an increase in PEEP may be used to shift the FRC upward along the lung's sigmoidal volume-pressure characteristic to a point above the critical opening pressure, thereby preventing alveolar collapse at end-expiration.[143-145] The volume-pressure loop also is useful for detecting other ventilatory abnormalities such as endotracheal tube leaks and air trapping, both of which result in incomplete closure of the graphical volume-pressure loop during end of expiration.

Flow-Volume Loops

A flow-volume loop is a plot of airflow versus tidal volume, as illustrated in Figure 92-7, *B*, which also shows the corresponding volume-pressure loop from the same mechanically ventilated breath. Each loop represents one breath as it progresses downward with inspiration to an upward expiration in a clockwise direction (of note, the direction and orientation may vary among ventilators and monitors from different manufacturers). The tidal flow-volume loop is useful for evaluating changes in inspiratory and expiratory airflow-to-volume response during tidal breathing. In addition to determination of the peak flow values, the patterns of airflow and tidal volumes indicative of turbulence or obstructive flow limitation also may be evaluated.[146-150] A typical flow-volume loop for a healthy newborn infant is shown in Figure 92-9, *A.* Preterm neonates with high chest wall compliance and greater expiratory airway collapsibility, as well as babies with CLD, typically will show an early peak expiratory flow followed by a concave profile (see Figure 92-9, *B*).[151-154] Other loop shapes that are characteristic of intrathoracic and extrathoracic flow limitation are shown in Figure 92-9, *C* to *F*.

CLINICAL APPLICATIONS

The clinical usefulness of neonatal pulmonary function testing is that the functional parameters define the magnitude and pattern of respiratory compromise. Individual disease processes are not differentiated by pulmonary function data. Infants with hyaline membrane disease, pulmonary edema, or pneumonia have low lung compliance, whereas infants with increased airway resistance may have an obstructive disease process such as meconium aspiration syndrome or bronchopulmonary dysplasia. However, pulmonary function data can be used to determine the severity of the pathologic condition, its resolution, and the effect of treatment.

The continuous real-time dynamic time flow-volume and volume-pressure loops and scalar tracings data, along with measurement of lung mechanics, can be used to assist in management of ventilatory support. Potentially useful measures and strategies include adjustment of tidal volume to prevent overdistention,[155,156] determination of optimal inspiratory time,[157] estimation of optimal end-distending pressure, assessment of the appropriateness of manual ventilation,[158] testing for airways reactivity,[159,160] and evaluation of the effect of therapeutic interventions.[161-168]

The serial evaluation of pulmonary outcome in the immediate postnatal period[169,170] and during infancy[171-174] provides the information necessary for detection of the late-onset complications or for resolution of the acute lung injury and adjustment of the treatment.

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93 Pathophysiology of Hyaline Membrane Disease (Excluding Surfactant)

Robert B. Cotton

Hyaline membrane disease is a disorder of newborn premature infants that is characterized by respiratory failure beginning at birth. Unless modified by surfactant treatment, the disease progresses to maximal severity by 24 to 48 hours after birth. Almost all of the 5% to 10% mortality associated with hyaline membrane disease is limited to extremely immature infants weighing less than 1000 g at birth. Of those who survive the initial course of acute respiratory failure, 15% to 30% experience a prolonged course of ventilatory failure known as *bronchopulmonary dysplasia* or *chronic lung disease of prematurity,* which may last months or longer and occasionally ends in death. Figure 93-1 illustrates the strong influence of birth weight on the outcome of hyaline membrane disease.

The clinical course of hyaline membrane disease has changed remarkably since the introduction of surfactant replacement therapy on a routine basis in the early 1990s. With surfactant therapy, oxygen and airway pressure requirements during the classic 3- to 5-day course of respiratory failure have been markedly attenuated. In extremely-low-birth-weight infants, the transition in clinical findings from one of respiratory insufficiency due to inadequate surfactant to one of respiratory insufficiency due to continuing lung injury of the immature lung resulting from oxygen therapy, mechanical ventilation, and inflammatory processes may not be obvious.

Hyaline membrane disease, respiratory distress syndrome, and idiopathic respiratory distress syndrome are interchangeable labels for the same disease. By calling attention to an important histopathologic feature of the disorder, the term *hyaline membrane disease* is a useful reminder that its pathogenesis includes destruction of terminal airway epithelium. This finding is not seen in the various syndromes of transient respiratory distress, which largely have run their course by 24 to 48 hours after birth. *Respiratory distress syndrome* is the most commonly encountered label for this disease.

The stage was set for our current understanding of the pathophysiology of hyaline membrane disease by investigations in the 1950s and 1960s that described the disease based on classic models of pulmonary function. It was also during this era that the role of persistent fetal circulatory pathways in hyaline membrane disease was discovered. Further understanding of the mechanisms underlying impaired gas exchange during hyaline membrane disease came in the 1970s, as investigators sought to explain the spectacular success of constant distending airway pressure on oxygenation and survival. In the 1980s, conclusive demonstrations of the efficacy of surfactant replacement validated the idea put forth by Avery and Meade[1] in 1959 that "lack of a normal lining material in the lungs of infants would contribute to the atelectasis seen in hyaline membrane disease."

This chapter describes the mechanisms involved that underlie the clinical manifestations of hyaline membrane disease. In regard to surfactant, this chapter limits the discussion to the major pathophysiologic consequences of deficiency of this material. The biochemistry of surfactant, mechanisms governing its production, release, and action, and issues surrounding its use in prevention and treatment of hyaline membrane disease are discussed elsewhere in this volume.

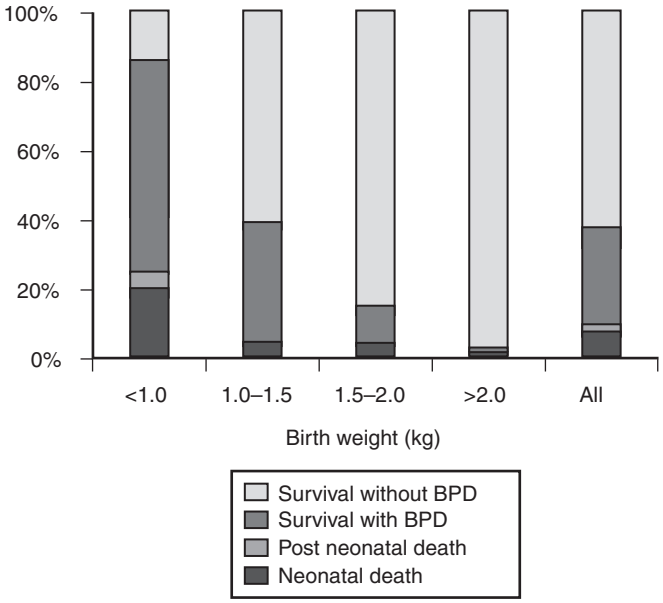


Figure 93-1 Outcome of 1184 infants with hyaline membrane disease who were admitted to the Vanderbilt Newborn Intensive Care Unit in Nashville, Tennessee, during the 5-year period 2001 to 2005. *BPD,* Bronchopulmonary dysplasia.

PATHOLOGY

Total lung volume is not decreased in hyaline membrane disease. Postmortem examination of human infants dying during the acute phase of this disorder reveals lungs that are described as full[2] or even voluminous.[3] They are often compared with liver to emphasize their appearance as a solid, airless organ that is congested and dark purplish red. Microscopic examination reveals marked capillary and venous congestion. Interstitial edema is widespread and is especially prominent in the adventitia surrounding small arterioles, which are markedly constricted early in the disease. Dilated lymphatic spaces are frequently seen adjacent to respiratory bronchioles and arterioles. Hyaline membrane formation, which represents a coagulum of sloughed cell debris in a protein matrix, occurs characteristically at the junction of respiratory bronchioles and alveolar ducts. Hyaline membranes are visible evidence that this disease involves a massive exudation of plasma proteins occurring in association with a destructive injury of the epithelial lining of the terminal conducting airways. The fully formed membrane often has the appearance of an eschar plastered against the denuded epithelial basement membrane. The respiratory bronchioles and alveolar ducts are frequently dilated and may be filled with protein-rich edema fluid in association with membrane formation early in the course of the disease.

The pathologic appearance of the lung of infants dying early in the course of hyaline membrane disease presents a picture of lung injury characterized by vascular engorgement and

interstitial edema in association with terminal airways filled with protein-containing fluid. These features are consistent with physiologic findings that include delayed clearance of fetal lung liquid,[4-7] increased permeability of both epithelial[4,8,9] and endothelial barriers,[6] delayed lung lymph protein clearance,[5,6] and a grossly increased pulmonary blood volume.[10] These physiologic observations confirm that the pathologic appearance of the lung in hyaline membrane disease results from disruption of the normal segregation of gas and liquid compartments.

LUNG INJURY AND PERMEABILITY PROBLEMS

In the presence of hyaline membrane disease, total lung water, which includes water in the airways and vascular spaces as well as interstitial and intracellular water, is increased almost 50% in premature lambs. When compared with control animals, lambs with hyaline membrane disease have more than twice as much extravascular water and 50% more intravascular blood volume (Figure 93-2).[10] The increased extravascular water represents both delayed clearance of fetal lung liquid and an accumulation of water resulting from increased transvascular filtration of fluid across the pulmonary capillary bed. The marked increase in lung lymph protein clearance, the appearance of proteinaceous edema fluid within the terminal conducting airways, and the subsequent widespread development of hyaline membranes signify the escape of protein as well as water from the microvascular circulation in a process involving increased vascular permeability.[6]

Permeability of the epithelial lining of terminal airways is also increased in hyaline membrane disease.[4,8,9] A large bidirectional protein flux can be demonstrated between the airways and circulation of prematurely delivered lambs,[9] a finding consistent with the microscopic findings of widespread destruction of terminal airway epithelium at the respiratory bronchioles and alveolar ducts that leaves only the basement membrane as a barrier between air space and interstitium. Surfactant deficiency and ischemia are two mechanisms that contribute to this injury.

Surfactant Deficiency

Evidence indicates that *surfactant deficiency* per se is responsible for the epithelial lesion of hyaline membrane disease. Endotracheal surfactant treatment of premature newborn rabbits before positive pressure ventilation prevents the widespread necrosis and desquamation of bronchiolar epithelium seen in untreated littermates.[11] In the presence of inadequate surfactant at gas-liquid interfaces, the high pressure required to overcome surface tension forces in terminal conducting airways leads to epithelial injury and necrosis. The respiratory bronchioles and alveolar ducts are poorly supported structures in the immature lung and, under these conditions, are particularly susceptible to disruption as a result of overdistention. Based on comparisons between surfactant-deficient rabbits ventilated with conventional mechanical ventilation and those receiving high-frequency ventilation, the degree of epithelial damage appears to be related to the magnitude of pressure swings, rather than mean pressure within the airways.[12] In addition, surfactant deficiency and accompanying high surface tensions at gas-liquid interfaces in terminal conducting airways may cause hydrostatic pressure gradients that interfere with the expedient disposal of fetal lung liquid or promote interstitial accumulation of edema fluid filtered from the microvascular circulation.

Injury to the terminal airway units induced by mechanical ventilation of the surfactant deficient lung can involve several mechanisms. High airway pressure, distention of terminal airways, repeated lung collapse and re-expansion, and the inflammatory process that is evoked by the injury itself all contribute to lung damage.[13-15] The extent of this injury that occurs with hyaline membrane disease in the early neonatal period is a significant predictor of the severity of chronic lung disease (bronchopulmonary dysplasia) that occurs as a long-term complication of this disorder.

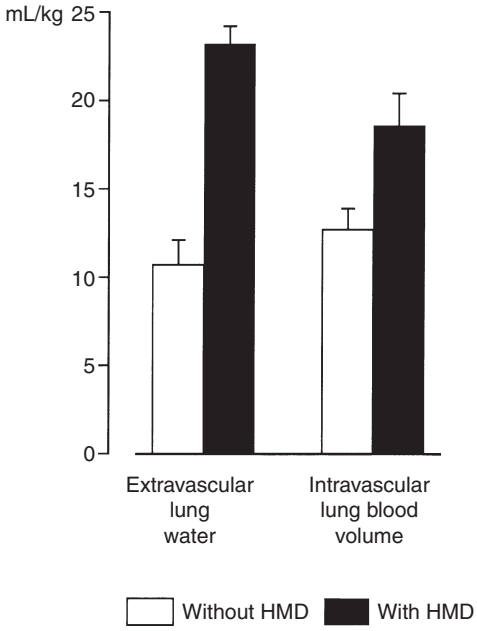


Figure 93-2 Extravascular lung water and intravascular lung blood volume measured in premature lambs with hyaline membrane disease using a multiple indicator dilution method. *(Data from Sundell HW, Harris TR, Cannon JR, et al: Lung water and vascular permeability-surface area in premature newborn lambs with hyaline membrane disease,* Circ Res *60:923-932, 1987.)*

Ischemic Damage

Under some circumstances the pathologic lesion of hyaline membrane disease may represent *ischemic necrosis* of the epithelial lining of terminal airways at the junctions of respiratory bronchioles and alveolar ducts. Both experimental and epidemiologic findings link acute asphyxia to the incidence and severity of this disease. Occasionally hyaline membrane disease occurs in the near-term infant with a mature amniotic fluid lecithin-sphingomyelin ratio. Similarly, premature infants with immature lecithin-sphingomyelin ratios do not always have hyaline membrane disease. Infants with a prolapsed umbilical cord, bleeding placenta previa, or placental abruption have an increased incidence of hyaline membrane disease. Incidence and severity of the disease are increased in the second-born twin. These factors point to the possible role of acute perinatal stress in the pathogenesis of hyaline membrane disease. Moreover, the tightly constricted arteriole characteristically adjacent to a respiratory bronchiole that is undergoing epithelial slough provides histopathologic evidence that ischemia may be involved in a process in which surfactant deficiency may be a secondary consequence of injury and necrosis of the epithelial lining.[16]

PERSISTENT FETAL VASCULAR PATHWAYS

During the 1950s many investigators considered hyaline membrane disease to be the result of left ventricular failure and pulmonary congestion caused by left-to-right shunting through the ductus arteriosus.[17-19] The presence of this shunt during the course of hyaline membrane disease, suspected on the basis of physical and radiographic findings, was confirmed by indicator dilution studies.[20,21] In 1960, Dr. Clement Smith[22] stated, "Although the primary cause [of hyaline membrane disease]

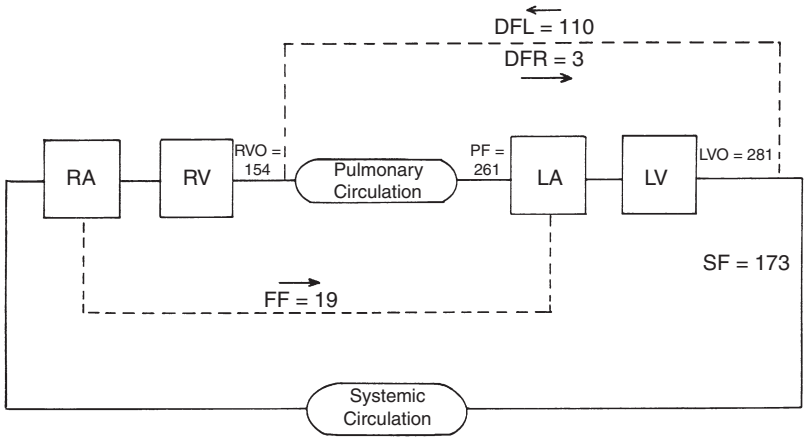


Figure 93-3 Mean values for components of cardiac output in 10 premature lambs with hyaline membrane disease. Flows are expressed as mL/kg/min. *DFL* and *DFR,* Left-to-right and right-to-left flow through the ductus arteriosus; *FF,* right-to-left flow across the foramen ovale; *LA,* left atrium; *LV,* left ventricle; *RA,* right atrium; *RV,* right ventricle; *SF,* systemic flow. *(From Cotton RB, Lindstom DP, Kanarek KS, Sundell H, Stahlman MT. Quantitation of cardiac output components in the presence of bidirectional shunts,* J Appl Physiol *43:352-356, 1977.)*

may be elsewhere, we continue to think that circulatory factors lie somewhere near the center of this puzzling and important problem."

During the acute phase of hyaline membrane disease, traditional clinical signs of a patent ductus arteriosus such as prominent murmur and precordial hyperactivity are usually absent. Nevertheless, left-to-right ductus shunting can be demonstrated in this setting by indicator dilution studies[21] or by Doppler ultrasound examinations[23,24] in most, if not all, newborn infants with hyaline membrane disease. A quantitative description of flows at different sites in the central circulation during hyaline membrane disease is shown in Figure 93-3.[25] In this composite example, which is based on studies of newborn premature lambs with the disease, 39% of left ventricular output is diverted back across the ductus and is recirculated through the pulmonary vascular bed. Figure 93-3 also illustrates the possibility of simultaneous right-to-left shunting across the foramen ovale. In fact, bidirectional shunting (right-to-left at the foramen ovale and left-to-right at the ductus arteriosus) is probably not uncommon in infants making strong respiratory efforts. Right-to-left flow across the foramen ovale is thought to occur during the inspiratory phase of spontaneous ventilation. When the infant with hyaline membrane disease makes vigorous inspiratory efforts against poorly compliant lungs, intrathoracic negative pressure deflections are created that promote blood flow from the inferior vena cava across the foramen ovale into the left atrium.[26]

The lungs of infants with hyaline membrane disease are characteristically fluid laden for reasons described earlier in this section. As discussed later, hypoxemia occurs in large part because of venous admixture resulting from right-to-left shunts through terminal airway units that are fluid filled or collapsed. The problem of fluid and protein accumulation by the lungs is further exacerbated by left-to-right ductus shunting.[9] In the past some investigators considered that the clinical expression of hyaline membrane disease in extremely immature infants was dominated by the consequences of left-to-right ductus shunting, rather than by surfactant deficiency.[27]

MECHANISMS OF HYPOXEMIA IN HYALINE MEMBRANE DISEASE

The principal mechanism of *hypoxemia* in hyaline membrane disease is venous admixture resulting from both intrapulmonary shunting and right-to-left shunting across the foramen ovale. When hyaline membrane disease is complicated by pulmonary vasoconstriction, right-to-left shunting may also occur across an open ductus arteriosus. Venous admixture is quantified by assuming that the oxygen content of arterial blood is the result of adding venous blood to blood equilibrated with alveolar gas. Venous admixture, an expression of the percentage of this mixture made up of venous blood, can be derived from the standard shunt equation:

$$VA(\%) = (CcO_2 - CaO_2)/(Cc'O_2 - CvO_2) \times 100$$

where VA = venous admixture, $Cc'o_2$ = the end-pulmonary capillary blood oxygen content, Cao_2 = the arterial blood oxygen content, and Cvo_2 = the mixed venous blood oxygen content.[28] Figure 93-4 shows how arterial partial pressure of oxygen (Pao_2) responds to changes in the fraction of inspired oxygen (Fio_2) at selected amounts of venous admixture.[29] When venous admixture is less than 40%, increasing the Fio_2 will usually result in an adequate Pao_2. When venous admixture is 50% or more, increasing Fio_2 from room air to 1.0 increases Pao_2 by only a few millimeters of mercury, and adequate oxygenation is not achieved.

Intrapulmonary Shunting

In hyaline membrane disease, intrapulmonary venous admixture occurs largely as a result of the perfusion of terminal airways that are partially collapsed, collapsed, or fluid filled. For the most part these terminal airways are either extremely underventilated or not ventilated at all as a result of epithelial slough, exudation, and hyaline membrane formation at the junction of the respiratory bronchioles and alveolar ducts. Based on studies of alveolar-arterial gradients of nitrogen and carbon dioxide, the lung in hyaline membrane disease can be modeled as comprising three compartments.[30,31] One compartment is made up of terminal gas exchange units that are intact and are adequately ventilated and perfused. The second compartment is characterized by terminal airways that are open but markedly underventilated; these units may be perfused, but only if local alveolar hypoxia can be relieved by breathing 100% oxygen. The third compartment is totally unventilated but may be perfused. It includes those units with terminal conducting airways that are severely affected by epithelial damage, exudate, and slough. In addition, this compartment also represents any present anatomic right-to-left shunt.[32]

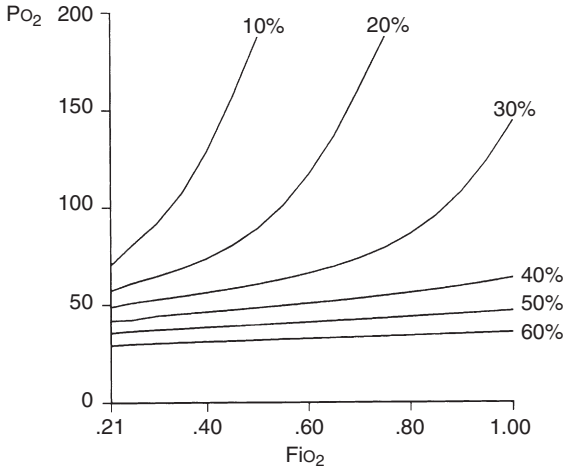


Figure 93-4 Arterial oxygen pressure (P_{O_2}) response to changes in the fraction of inspired oxygen (F_{IO_2}) at selected amounts of venous admixture between 10% and 60%. *(Data obtained using computer software provided by Daniel P. Lindstrom and Torsten Olsson.)*

Extrapulmonary Shunting

A complex but predictable pattern of shunting through persistent fetal circulatory pathways evolves during the natural course of hyaline membrane disease.[21] During the first 12 hours after birth, the predominant shunt is from right to left, from the inferior vena cava across the foramen ovale into the left atrium. Even though the ductus is widely patent, there is little flow through it in either direction until after 12 to 24 hours of age, when a left-to-right shunt of increasing magnitude occurs as a result of falling pulmonary vascular resistance. In some cases pulmonary vascular resistance remains high beyond the immediate period after birth and may lead to severe hypoxemia, especially in the presence of hypercarbia, acidosis, or severe systemic hypotension. Low-dose inhaled nitric oxide has been shown to improve oxygenation in such infants by either decreasing pulmonary vascular resistance and extrapulmonary shunting or by improving ventilation-perfusion matching. However, overall survival is not improved in these infants.[33]

PULMONARY FUNCTION IN HYALINE MEMBRANE DISEASE

Functional Residual Capacity

Functional residual capacity (FRC) is decreased in hyaline membrane disease.[34-38] In newborn infants without lung disease, FRC is approximately 30 mL/kg,[35,38] a volume coinciding with the volume of fetal lung liquid present in the airways before birth.[39] Loss of FRC occurs in hyaline membrane disease as a result of surfactant deficiency and displacement of gas volume by pulmonary vascular congestion, interstitial edema, and airway flooding with proteinaceous fluid.

During the course of hyaline membrane disease FRC is influenced by numerous factors including the patient's spontaneous ventilatory effort, the stability of the chest wall, and the patient's ability to muster an effective grunt. Defense of FRC against encroachment by a rising tide of lung water is the principal strategy of management for this disorder. When the patient's own defenses are overcome, FRC can be recovered and protected mechanically by the addition of distending airway pressure in the form of continuous positive pressure or intermittent mandatory ventilation.

Changes in FRC mirror the improvement in oxygenation that occurs with the addition of distending airway pressure or with spontaneous recovery beginning on the second or third day after birth.[36,40-42] During recovery there is a striking correlation between improving arterial oxygenation and increasing FRC and diuresis as excess lung water recedes into the circulation and is cleared by the kidneys.[40,43]

Mechanical Properties of the Lung

The abnormal mechanical properties of the lung in hyaline membrane disease are the consequence of interacting pathophysiologic events.[44] The marked decrease in *lung compliance,* one of the hallmarks of hyaline membrane disease, is the consequence of two factors: (1) a decrease in the number of ventilated terminal air spaces (i.e., *de-recruitment*); and (2) an increase in the recoil pressure of ventilated terminal air spaces. In addition, dynamic, but not static, compliance is further reduced in hyaline membrane disease owing to changes in the viscoelastic properties of lung tissue and the presence of ventilation inhomogeneity.[45]

De-recruitment and the consequent loss of FRC occur as a result of the collapse of unstable terminal air spaces or as a result of alveolar flooding and tissue injury. The effects of air space instability on compliance and lung volume are discussed later. The elastic recoil pressure of ventilated air spaces in hyaline membrane disease may be increased for a variety of reasons. Surface elastic forces are increased in hyaline membrane disease as a result of surfactant deficiency, inhibited surfactant function, or both (see Section XIII of this text). Alveolar flooding[46,47] may also increase surface elastic forces. Tissue elastic forces may be increased with engorgement of the pulmonary vascular bed[48] but it is unclear that this is the mechanism of decreased compliance observed in patients with hyaline membrane disease complicated by symptomatic patent ductus arteriosus.[49] Increased elastic recoil resulting from both surface forces and tissue elastic forces occurs when the infant with hyaline membrane disease is ventilated high on the volume axis of the lung's pressure-volume curve, either inadvertently or out of necessity. In the presence of extensive de-recruitment, the small compartment of ventilated air spaces that remains must provide more than its normal share of ventilation to achieve adequate gas transport. The smaller the ventilated compartment is, the more distended will its terminal air spaces become at end inspiration for a given tidal volume. At high volume, surface forces are minimally reduced by surfactant, and tissue elastance increases as the elastin network, which dominates at low- and middle-volume ranges, gives way to the structural influence of poorly compliant collagen.[50]

Like FRC, compliance also mirrors the course of pulmonary insufficiency in hyaline membrane disease.[40] Intercostal and sternal retractions in the infant capable of a spontaneous ventilatory effort provide a visual assessment of compliance at the bedside. As compliance improves, with manipulations of mechanical ventilation, surfactant replacement therapy, or evolution of the natural course of disease, chest wall retractions diminish, a finding indicating that less transpulmonary pressure is required to expand the lung.

Lung resistance measured in hyaline membrane disease during spontaneous breathing without mechanical ventilation is not substantially different from that in newborn infants without lung disease.[44] However, lung resistance is higher in infants with hyaline membrane disease requiring mechanical ventilation with an endotracheal tube in place.[44] Some of the increased resistance in these infants may include the resistance of the endotracheal tube, but this increment does not appear sufficient to account for all of the increased resistance measured in patients with hyaline membrane disease.[51] It has also been suggested that some of the increase in resistance results from damage to conducting airways or from peribronchial edema.[52] In addition, conductance of the

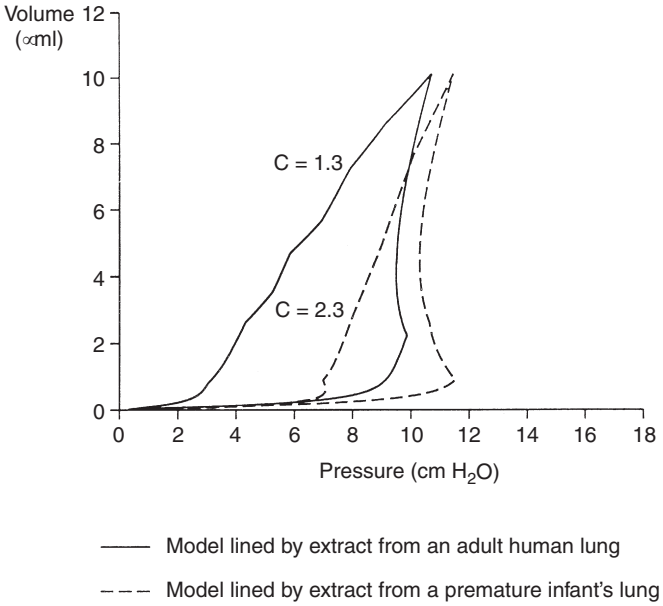


Figure 93-5 Pressure volume characteristics of a model alveolus when lined by extract from a premature infant's lung *(broken line)* or when lined by extract from an adult human lung *(solid line). (The data and model used to construct these pressure volume curves were taken from Clements JA, Hustead RH, Johnson RP, Gribetz I: Pulmonary surface tension and alveolar stability,* J Appl Physiol *16:444-450, 1961 and Cotton R: A model of the effect of surfactant treatment on gas exchange in hyaline membrane disease,* Semin Perinatol *18:19-22, 1994.)*

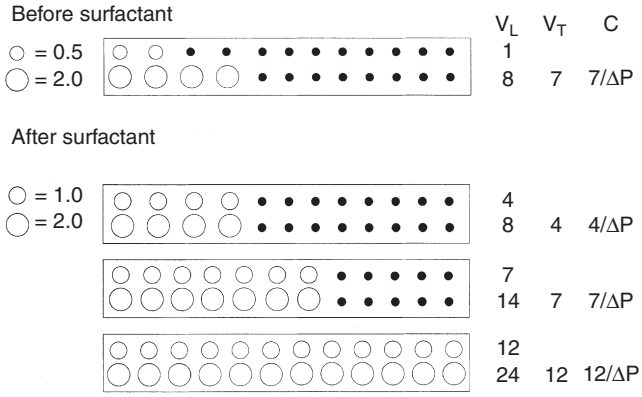


Figure 93-6 Alveolar stabilization and recruitment. Model of a 12-alveolus lung in which the top row of each panel represents the volume of each alveolus at end expiration and the bottom row represents the volume of each alveolus at end inspiration. The solid circles indicate alveolar collapse. The open circles indicate alveoli that are not collapsed. The end-expiratory volume and end-inspiratory volume of an individual alveolus are shown to the left of panels 1 and 2. Before surfactant treatment, two ventilated alveoli are stable, two ventilated alveoli are unstable, and eight collapsed alveoli constitute an unventilated compartment. In panel 2, after surfactant, the two ventilated alveoli that were unstable before surfactant have become stabilized. The unventilated compartment of eight collapsed alveoli remains the same as before surfactant. In panel 3, three alveoli from the unventilated compartment have been recruited and stabilized. In panel 4, the unventilated compartment has been fully recruited, and all alveoli are ventilated and stable. The stepwise effect of this process of stabilization and recruitment on lung volume (V_L), tidal volume (V_T), and compliance (C) is shown to the right of the panels. *(From Cotton R: A model of the effect of surfactant treatment on gas exchange in hyaline membrane disease,* Semin Perinatol *18:19-22, 1994.)*

respiratory system is highly correlated with lung volume in hyaline membrane disease (calculated from previously reported results),[53] implying that a major cause of increased resistance of the respiratory system in infants with hyaline membrane disease is related to a decrease in the cross-sectional area of distal conducting airways.

Effects of Alveolar Instability on Lung Volume and Compliance

Alveolar instability refers to the tendency for an alveolus to switch abruptly between the inflated state and the collapsed state.[54] When pressure across the alveolus exceeds the critical opening pressure, an unstable alveolus inflates suddenly. During deflation an unstable alveolus collapses abruptly when transalveolar pressure falls to less than the critical closing pressure. Figure 93-5 illustrates the stabilizing effect of surfactant treatment on an unstable alveolus with a critical opening pressure of slightly more than 11 cm H_2O and a critical closing pressure of just less than 7 cm H_2O.

Because the dimensions of the terminal air spaces are not uniform throughout the lung, all alveoli do not behave uniformly in surfactant deficiency. Under these conditions there is a range of critical opening and closing pressures. As a result there are several populations or compartments of terminal air spaces. One compartment remains collapsed throughout inspiration and expiration, another is ventilated during inspiration but collapses during each expiration, and a third is ventilated and stable to the extent that gas volume is retained at end expiration. The distribution of these compartments is affected by a variety of factors, especially treatment with surfactant replacement or with distending airway pressure. How changes in the relative size of these compartments affect lung volumes and compliance during fixed conditions of pressure-limited mechanical ventilation is illustrated in Figure 93-6. In this model, stabilization alone results in increased FRC and decreased compliance. Tidal volume and compliance do not improve until recruitment of new terminal air spaces from the previously unventilated compartment.

Ventilation

Infants with hyaline membrane disease who can be managed without intermittent positive pressure ventilation have spontaneous tidal volumes of 4 to 6 mL/kg, the same or only slightly less than the tidal volume of newborn infants with normal lung function. In contrast to infants with normal lungs, dead space makes up a much larger portion of tidal volume, 60% to 80% versus 30% to 40%.[55-57] After subtracting dead space from tidal volume, infants with hyaline membrane disease are left with a decreased alveolar portion of tidal volume for which they compensate by breathing faster. As a result of increasing respiratory rate, a nearly normal alveolar ventilation and arterial carbon dioxide tension can be attained. A decreased alveolar portion of tidal volume is the expected pathophysiologic consequence of encroachment on terminal air spaces by lung water and is consistent with the well-documented loss of FRC in this disease.

Physiologic dead space is derived from systemic arterial carbon dioxide tension; *anatomic dead space* is derived from an estimate of alveolar carbon dioxide tension obtained from end-tidal gas analysis. The difference between the two dead spaces is referred to as *alveolar dead space.* (Physiologic dead space = anatomic dead space + alveolar dead space.[57]) In newborn infants with normal lungs, there is no detectable difference between physiologic and anatomic dead space because their arterial-alveolar carbon dioxide gradient is close to zero. Infants

with hyaline membrane disease have a significant gradient of 12 to 16 mm Hg in carbon dioxide tension between arterial blood and alveolar gas, which results in an increase in measured physiologic dead space. This gradient has been explained as the result of a relative underperfusion of ventilated alveoli.[30,57] When terminal airway units are totally obstructed by epithelial slough and exudate, dilated respiratory bronchioles proximal to the obstruction behave as unperfused but ventilated "alveoli."

TREATMENT: PATHOPHYSIOLOGIC CONSIDERATIONS

Surfactant replacement therapy has become a standard part of the treatment of hyaline membrane disease and has proven to be enormously successful in bringing about highly significant reductions in mortality and pulmonary complications, especially air-leak syndromes (Chapters 104 and 106 are devoted to the subject of surfactant replacement therapy). The following discussion of treatment of hyaline membrane disease is limited to the role of oxygen therapy and the effects of mechanical ventilation and distending airway pressure on the pathophysiology of this disorder.

The primary challenge in managing the pulmonary insufficiency of hyaline membrane disease is achieving an adequate Pao_2. If oxygenation can be achieved, carbon dioxide removal is only a matter of increasing minute ventilation sufficiently to make up for an inadequate alveolar portion of tidal volume. Management of the hypoxemia of hyaline membrane disease should be approached by recognizing the impact of venous admixture on Pao_2 as a function of Fio_2 (see Figure 93-4). Oxygenation can be achieved under conditions of modest venous admixture up to 30% or 40% by increasing Fio_2. With severe disease characterized by venous admixture greater than 40%, something must be done to reduce right-to-left shunt before adjustments in Fio_2 have any significant effect on Pao_2.

Oxygen Administration

Some reduction in venous admixture can be realized by increasing the Fio_2 to 100%.[30,31] In part, this reduction in venous admixture reflects events in a lung compartment made up of gas exchange units that are extremely poorly ventilated. When the patient breathes 100% oxygen, local alveolar hypoxia is relieved, and perfusion is restored to those units that are open. In addition to reducing right-to-left intrapulmonary shunting, it is also possible to manipulate the magnitude of right-to-left shunt across fetal pathways by changing Fio_2.[21]

Even though simple oxygen administration without adding positive airway pressure can lead to a reduction in venous admixture, oxygen therapy by itself is often of limited efficacy. Before use of mechanical ventilation, survival from hyaline membrane disease was predicated more on effective grunting and increased respiratory effort than on oxygen therapy. Unless a patient could sustain adequate spontaneous ventilation and an effective grunt, the hypoxemia of hyaline membrane disease could not be overcome, even by hyperbaric oxygen therapy (Figure 93-7).[58]

Distending Airway Pressure

Application of distending airway pressure to the airway of patients with hyaline membrane disease brings about a prompt reduction in venous admixture. The reduction in venous admixture results from a decrease in intrapulmonary right-to-left shunting as well as a decrease in right-to-left shunting across the foramen ovale.

Distending airway pressure may be applied in a variety of ways. In the original report[59] of its successful use in hyaline membrane disease, constant distending pressure was applied to an indwelling endotracheal tube by means of a continuous flow through an anesthesia bag that had an adjustable leak in its tail. Continuous positive airway pressure can also be applied by face mask, nasal prongs, or head chamber. All these methods deliver a continuous distending pressure during which ventilation depends on spontaneous breathing by the patient.

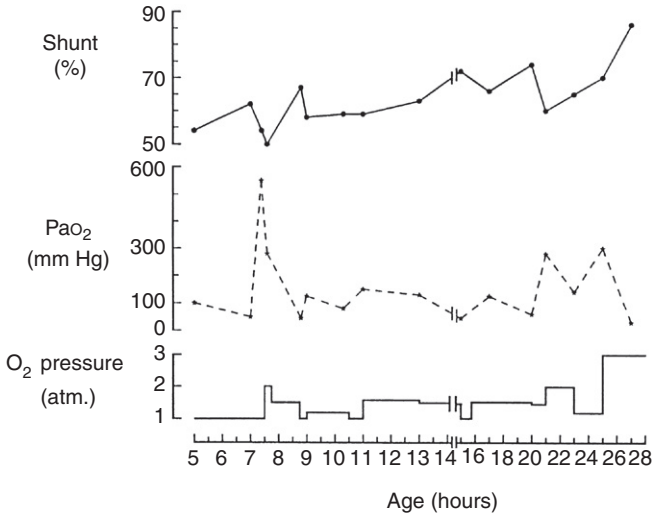


Figure 93-7 Serial arterial oxygen tensions in an infant with hyaline membrane disease treated with hyperbaric oxygen. The increase in arterial partial pressure of oxygen (Pao_2) after each increment in inspired oxygen pressure is transient. The overall course is one of progressive venous admixture (shunt %) despite the administration of 100% oxygen at pressures up to 3 atmospheres. *(From Cochran WD, Levison H, Muirhead DM, et al: A clinical trial of high-oxygen pressure for the respiratory-distress syndrome,* N Engl J Med *272:347-351, 1965. Reprinted by permission of the New England Journal of Medicine.)*

With increasing distending airway pressure, FRC increases[36] and venous admixture decreases as a result of stabilization and recruitment of effective ventilatory units. There is a significant decrease in the gradients of carbon dioxide[30] and nitrogen[60] between alveolar gas and arterial blood, indicating an improved perfusion of ventilated units and an improved ventilation of perfused units, respectively. In patients able to sustain spontaneous ventilation, application of distending airway pressure is followed by a reduction in minute ventilation. For the most part, this reduction can be accounted for by a decrease in tidal volume[41,59]; to a lesser extent, a decrease in the rate of breathing plays a role.[41] When distending airway pressure is applied, there is little or no change in arterial partial pressure of carbon dioxide,[41,59] signifying that alveolar ventilation is maintained in spite of the reduction in minute ventilation. This suggests that the dead space–tidal volume ratio is also decreased by distending airway pressure. The decrease in dead space–tidal volume ratio results from an increase in the alveolar portion of tidal volume caused by stabilization and recruitment of terminal air spaces.

Although application of distending airway pressure causes a prompt increase in arterial oxygen tension as a result of decreasing right-to-left shunt, there is no corresponding increase in dynamic lung compliance.[59] In fact, dynamic compliance may decrease after the application of distending airway pressure.[41] This response in dynamic compliance, which is similar to that reported after surfactant replacement therapy,[53,61,62] can be explained by the stabilizing effect of distending airway pressure on terminal air spaces. Figure 93-6 illustrates the effects of stabilization and recruitment on lung volumes and compliance. The results are similar, regardless of whether those effects are mediated by surfactant replacement or by increased end-expiratory pressure.

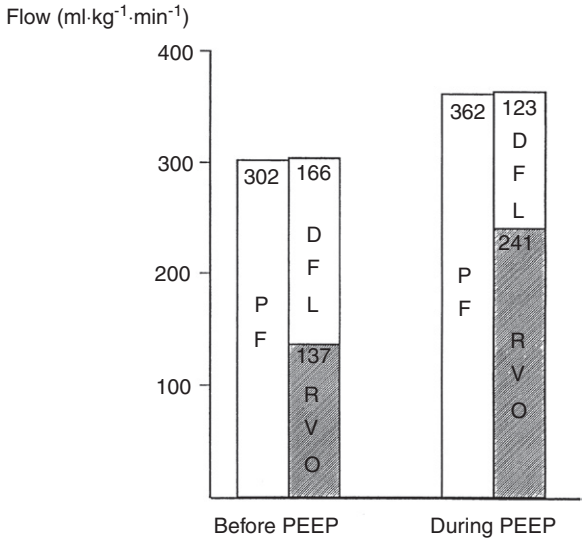


Figure 93-8 Effect or positive end-expiratory pressure *(PEEP)* on the components of pulmonary blood flow *(PF)* in premature lambs with hyaline membrane disease. *DFL,* Left-to-right blood flow across the ductus arteriosus; *RVO,* right ventricular output. *(From Cotton RB: In Stern L [ed]:* Hyaline Membrane Disease: Pathogenesis and Pathophysiology. *Orlando, FL, Grune & Stratton, 1984, p 184.)*

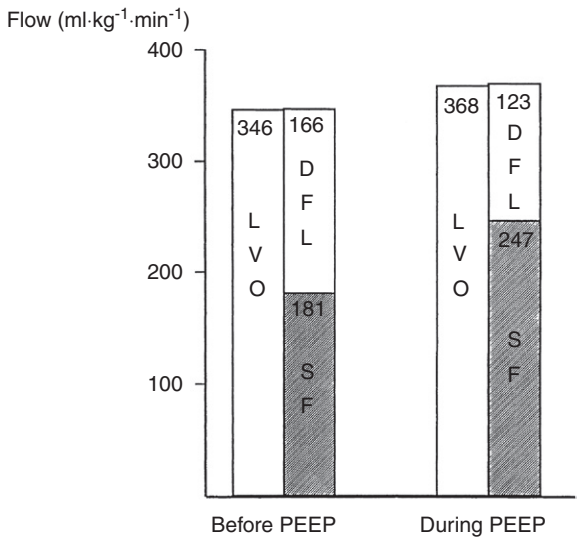


Figure 93-9 Effect or positive end-expiratory pressure *(PEEP)* on the components of left ventricular output *(LVO)* in premature lambs with hyaline membrane disease. *DFL,* Left-to-right blood flow across the ductus arteriosus; SF, systemic blood flow. *(From Cotton RB: In Stern L [ed]:* Hyaline Membrane Disease: Pathogenesis and Pathophysiology. *Orlando, FL, Grune & Stratton, 1984, p 184.)*

It has been recommended that the optimal level of distending airway pressure should be that level at which compliance increases.[63,64] Above this optimal level of distending airway pressure, compliance decreases, signifying that the dominant effect has become an over-distention of open gas exchange units rather than recruitment of new units. Even though compliance does not necessarily increase when distending airway pressure is applied to the airway of infants with hyaline membrane disease,[41,59] the pattern of breathing may still become more regular and the chest wall may appear more stable as a result of a decrease in tidal volume and respiratory rate.

Outside the lung, distending airway pressure has a beneficial effect on the distribution of cardiac output and on right-to-left shunting through the foramen ovale. In hyaline membrane disease, left ventricular output exceeds right ventricular output as a result of the bidirectional shunting discussed earlier. One increment in the extra volume load of the left ventricle can be accounted for by the portion of left ventricular output that is diverted back through the pulmonary circulation by way of the ductus arteriosus. A second increment results when a portion of systemic venous return is shunted from the inferior vena cava across the foramen ovale into the left atrium during vigorous inspiratory efforts. When distending airway pressure in the form of positive end-expiratory pressure (PEEP) is added to the positive pressure ventilation of newborn premature lambs with hyaline membrane disease, a series of effects on the circulatory pattern can be demonstrated (Figures 93-8 and 93-9).[26] The triggering event is thought to be a reduction in right-to-left shunting across the foramen ovale. The rerouted right-to-left foramen shunt appears as additional right ventricular output, which displaces a major part of the left-to-right ductus shunt from the pulmonary circulation forward into the systemic circulation. Pulmonary vascular resistance, pulmonary blood flow, and left ventricular output do not change appreciably. Right ventricular output and systemic blood flow increase. The net result of these changes is an increase in systemic arterial Po_2 caused by reduced venous admixture at the foramen ovale, as well as decreased lung congestion and protein leak subsequent to the reduction in left-to-right ductus shunting.

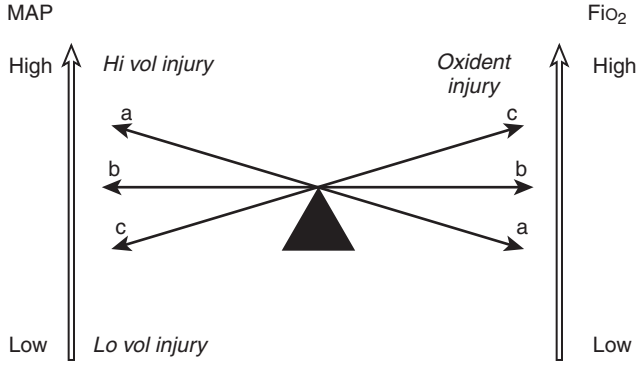


Figure 93-10 There is an inverse relationship between the intensity of positive pressure ventilation measured by mean airway pressure *(MAP)* and the fraction of inspired oxygen *(Fio_2)* required to achieve acceptable oxyhemoglobin saturation. Although a high MAP allows oxygenation at a low Fio_2, this combination increases the risk of high-volume lung injury *(line a).* On the other hand, using a low MAP in combination with a high Fio_2 may increase the risk of low volume lung injury as well as oxidant injury *(line c).* Theoretically, MAP and Fio_2 both should be set as low as possible to minimize both high volume lung injury and oxidant injury *(line b).*

Mechanical Ventilation

An understanding of the pathophysiology underlying impaired gas exchange in hyaline membrane disease provides a rational basis for the use of mechanical ventilation in the management of this disorder. The positive intermittent pressure (PIP) provided by conventional mechanical ventilation can be regarded as the "recruiting tool," and the PEEP can be regarded as the "stabilizing tool." The area under the pressure wave form applied to the airway, that is, mean airway pressure (MAP), can be regarded as an index of the overall intensity of positive pressure ventilation being applied. As recruitment progresses, lung compliance and

alveolar ventilation improve, allowing PIP to be lowered. When newly recruited terminal airways are stabilized by PEEP, they become more easily inflated than if they are allowed to collapse during expiration.

There is an inverse relationship between the intensity of positive pressure ventilation measured by MAP and the Fio_2 required to achieve acceptable oxyhemoglobin saturation (Figure 93-10). An attempt to minimize the required Fio_2 by using high MAP increases the risk of high volume lung injury. However, using a low MAP combined with high Fio_2 may increase the risk of low volume lung injury, which occurs due to the repetitive reinflations of unstable terminal airways that collapse during expiration.[13] In order to minimize lung injury during mechanical ventilation, it would seem reasonable that ventilator settings be based on the magnitude of applied MAP required to achieve acceptable oxygenation, so that high MAP is reserved for cases that have the greatest intrapulmonary shunting and that low MAP be used when oxygenation can be achieved with low inspired oxygen concentrations.

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94 Mechanisms of Neonatal Lung Injury

Richard L. Auten, Jr.

At birth the term newborn lung is at the alveolar phase of lung development, with a smaller number of alveoli normalized to body weight than an adult has. The lung must make the rapid transition from a fluid-filled structure with low stretch frequency and low strain to an air-filled structure subjected to high strain at variable frequency in order to support gas exchange. Spontaneous breathing efforts just after delivery at 40 weeks' gestation can generate high transpleural or transpulmonic pressures during the first few breaths. Failure to reabsorb amniotic fluid can lead to uneven gas distribution during spontaneous breathing or positive pressure ventilation, generating stress points that contribute to strain, injury, alveolar rupture, and pneumothorax (Figure 94-1). The prevalence of spontaneous pneumothorax is undoubtedly higher than recognized because many are clinically insignificant. In case-control studies, pneumothorax is associated with meconium-stained amniotic fluid[1,2] and elective repeat cesarean deliveries.[3]

In babies born very prematurely, at less than 26 weeks, the fetal lung is completing the transition from the cannalicular stage to the saccular stage of development. Air sacs are relatively thick walled, and pulmonary capillaries are incompletely branched and do not extend to the ends of the alveolar septa. Surfactant synthesis and secretion are decreased. Enzymatic antioxidants, superoxide dismutase, catalase, and glutathione peroxidase are relatively low, and less inducible than at term. Nutritional antioxidants such as vitamin E and retinoids are also relatively low.[4] In order to support ventilation, the extremely premature newborn lung with these liabilities is often subjected to supplemental oxygen, endotracheal intubation, and positive pressure ventilation, all of which can injure developing lung, leading to impaired alveolar development and chronic lung disease of prematurity, or bronchopulmonary dysplasia (BPD) (Figure 94-2).

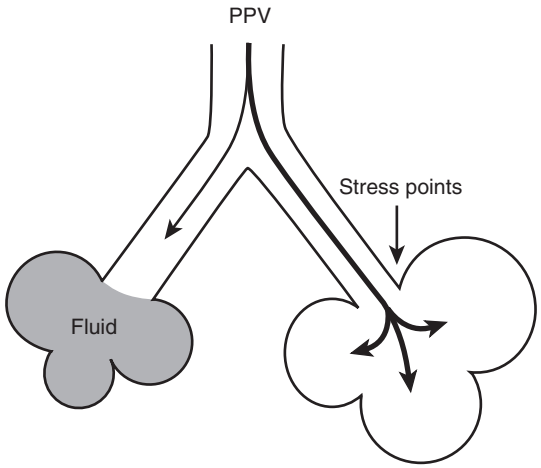


Figure 94-1 During inspiration, fluid filled atelectatic alveoli *(gray)* allow gas to be shunted to more compliant alveoli, leading to overdistention and alveolar wall stress.

MECHANICAL MECHANISMS OF LUNG INJURY

Mechanical Lung Injury at Birth

Vulnerability to mechanical lung injury is significant immediately after birth.[5] Animal studies have shown that structural damage and inflammatory response can be generated after only a few overdistending breaths.[6,7] Significant lung injury can take place after brief periods of mechanical ventilation of the incompletely recruited lung. Forced "recruitment" maneuvers may be harmful. In preterm, surfactant-treated lambs, initial recruitment maneuvers with tidal volumes varying from 8 to 30 mL/kg had no benefit, and caused "dose-dependent" histologic damage with increasing tidal volumes.[8,9] Lung volumes at less than optimal functional residual capacity (FRC) require higher than optimal peak pressures in later breaths to fully re-recruit alveoli. Maneuvers designed to avoid de-recruitment, such as use of continuous positive airway pressure (CPAP),[10] have decreased injury markers in experimental models of prematurity and respiratory distress syndrome (RDS). Choosing and delivering the appropriate tidal volume during delivery room resuscitation remain important, but as yet unrealized goals in the quest to minimize lung injury, particularly in the extremely premature newborn at high risk for developing BPD.

De-recruitment

Trauma to delicate airways can easily result if the lung is de-recruited to volumes at less than FRC (Figure 94-3), resulting in alveolar collapse (atelectasis). Higher airway pressures are then

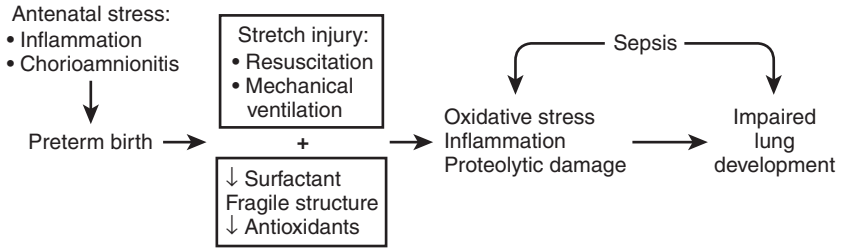


Figure 94-2 Developmental lung vulnerabilities superimposed with mechanical injury and maladaptive inflammation, leading to disrupted lung development.

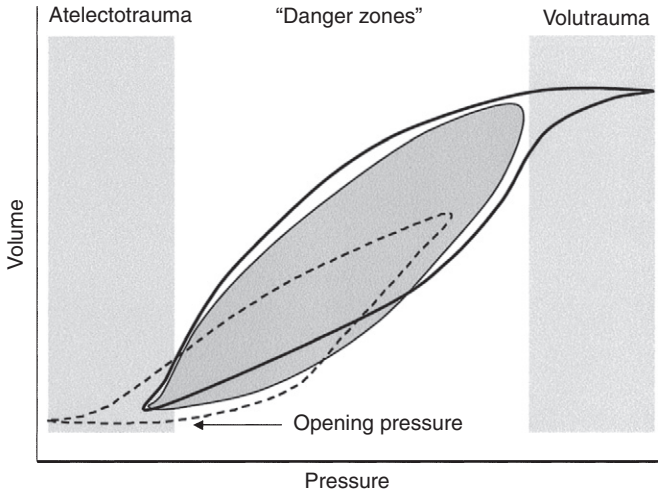


Figure 94-3 Static P-V loop after recruitment *(solid line, gray fill)* of lung volume above functional residual capacity (FRC). After derecruitment the P-V loop demonstrates decreased hysteresis *(dashed line),* decreased slope, and loss of lung volume at end-deflation, predisposing to "atelectotrauma." Overinflation *(dark solid line)* of the lung leads to increased luminal pressure without increased accumulation of lung volume, increasing shear stress to airways, and predisposing to "volutrauma."

required during subsequent mechanical breaths in order to re-recruit alveoli, leading to stretch injuries in terminal airways.[11] The spontaneously breathing patient must generate greater negative pressures to achieve the same goal, also leading to the same stretch injury, higher work of breathing, and fatigued muscles of respiration. Considerations for mechanical ventilation strategies designed to avoid lung injury due to de-recruitment and atelectasis are discussed in Chapters 97 and 98.

Overexpansion

As the mechanical breath volume increases to approach the total lung capacity, increased airway pressure and mechanical shear force are applied to the airways and alveoli without a proportional increase in lung volume at the upper inflection point (see Figure 94-3). The widespread use of in-line flow or pressure sensors capable of displaying the pressure-volume relationship can detect overinflation, demonstrating the flattened inspiration and expiration limbs and decreased pressure-volume loop hysteresis at the end of inspiration. Overinflation is more likely to occur when patients are receiving time-cycled, pressure-limited ventilation at a time when compliance rapidly improves, for example, immediately after surfactant therapy, or on achieving full alveolar recruitment.

Balancing the risks of lung injury due to de-recruitment or atelectotrauma against the risks of overinflation can be complex, particularly when the pathophysiology that affects the lung microenvironment is nonuniform, and produces underinflated and overinflated regions. Experimental studies have blended the so-called low lung volume approach with intermittent recruiting breaths, or "sighs," and have demonstrated in adult mice that an optimal sigh frequency can minimize evidence of cellular injury.[12]

Use of Continuous Positive Airway Pressure to Avoid Lung Injury

Because the accurate determination of a "safe" tidal volume for use in mechanically ventilated newborns is problematic, less invasive approaches to assist respiration have gained wider use in an effort to avoid injury. Early use of CPAP, within a minute or two of delivery (in the spontaneously breathing infant), may avert lung injury by allowing gradual recruitment and avoiding the need for mechanical ventilation. Although lower rates of surfactant use and mechanical ventilation have been reported in some centers, it is unclear whether the use of CPAP as the initial ventilatory approach avoids lung injury and BPD. Preclinical investigations have shown improved lung development in preterm lambs[10] and baboons[13] treated with CPAP. Pilot studies suggest that initial use of CPAP does not increase the need for later mechanical ventilation.[14,15] However, a large, multicenter, randomized, controlled clinical trial comparing the use of CPAP with intubation and prophylactic surfactant demonstrated that CPAP was equal to or slightly better than intubation and surfactant for prevention of BPD.[16]

Surfactant to Prevent Injury

Nonuniform compliance and resistance can promote mechanical strain, so it follows that achieving more uniform compliance by correcting surfactant deficiency common in those prematurely born should prevent mechanical injury. For short-term lung injury outcomes such as pneumothorax, and duration of mechanical ventilation, and for survival in some subgroups, surfactant treatment has been shown to be beneficial in multicenter randomized trials (see reference 17 for review). Early use of selective, empiric surfactant therapy of very premature newborns, typically less than 26 weeks estimated gestational age, has been shown to reduce the risk of pneumothorax and BPD.[18] However, uneven or asymmetric surfactant distribution may contribute to the heterogeneous clinical response, and may further contribute to lung injury by directing positive airway pressure preferentially to the compliant parts of the lung. This often injures the compliant lung and spares (for a while) the noncompliant, non–surfactant-treated lung. In an effort to avoid mechanical ventilation, some have used immediate surfactant treatment followed by CPAP, but there is no clear evidence that this prevents BPD as typically defined,[19] with its multifactorial etiology (see Chapter 97).

Lung Water, Patent Ductus Contribute to Mechanical Lung Injury

Patent ductus arteriosus (PDA) in the setting of low pulmonary vascular resistance contributes to pulmonary edema by allowing pulmonary overcirculation, which results in elevated left atrial

pressures. PDA has been associated in numerous clinical studies with the development of chronic lung disease of prematurity, and is discussed in detail in Chapters 73 and 97. Persistence or recurrence of a PDA is highly correlated with the development of BPD, probably because of uneven effects on lung compliance. Alternatively, recurrence of the PDA is often associated with sepsis, which itself may contribute to inflammatory lung injury.[20] Chorioamnionitis and fetal inflammation may also contribute to the production of prostaglandins that promote ductal patency by the same mechanisms, and may contribute to lung injury via inflammation, making it difficult to separate the physiologic contribution of the PDA to lung injury in the premature newborn.

Intravenous fluid management using rates exceeding 200 mL/kg/day in the first days of life for extremely premature newborns has been linked to later development of BPD,[21] but the precise role in lung injury remains unclear because pulmonary lymphatic drainage appears to compensate for increased pulmonary blood flow in experimental animals given similarly high rates of intravenous fluids.[22] High fluid rates have been linked to arterial ductus patency in clinical trials and observational studies and may contribute to lung injury through this mechanism.

HUMORAL LUNG INJURY: INFECTION AND INFLAMMATION

Congenital, Perinatal, and Postnatal Sepsis

Premature newborns are likely to have been exposed to infection in utero because infection often precipitates preterm labor.[23] Epidemiologic evidence correlates fetal exposure to chorioamnionitis with development of BPD.[24] Infection and inflammation damage lung cells as a result of the maladaptive inflammatory response that amplifies the cycle of mechanical or oxidative injury, which leads to inflammation and additional injury. Fetal exposure to inflammatory or immunologic challenge can directly injure lung cells without the additional burdens of oxygen therapy and mechanical ventilation.[25]

Postnatal sepsis may provoke a systemic inflammatory response, accompanied by significant pulmonary neutrophil influx, alveolar-capillary leak, and in some cases, necrotizing tracheobronchitis. Premature newborns are more likely to develop BPD if they develop postnatal sepsis.[26,27] Death or dysregulation of lung progenitor cells,[28] ordinarily destined to complete repair and development, may interrupt normal alveolar and capillary development. Exposure to proinflammatory cytokines or endotoxin components without actual infection can disrupt alveolar development (see Chapter 96).

Ureaplasma infection has been implicated in the pathogenesis of BPD and may impair lung development through its provocation of inflammation. Autopsy studies of premature newborns with pneumonia have linked the presence of *U. urealyticum* in the lung to fibrotic histologic changes often found in advanced BPD. These studies linked *Ureaplasma* infection with a "pro-fibrotic" phenotype in alveolar macrophages that strongly expressed transforming growth factor β, a cytokine linked to fibroblast proliferation.[29] Experimental infection with *Ureaplasma* in premature baboons caused lung injury and abnormal alveolar development.[29] Whether it does so in humans remains to be determined. The role of *U. urealyticum* in apparent exacerbations of respiratory failure in very-low-birth-weight prematures may be underestimated.

Late-onset sepsis in prematures has been strongly predictive of later development of BPD.[20] In the premature newborn lung subjected to oxidative and mechanical stress, infection may represent another instance of the "second hit" that further amplifies inflammation and contributes to dysregulated lung development and repair.

ASPIRATION INJURY

Meconium Aspiration

Passage of meconium is associated with compromised uteroplacental blood flow at or near full-term gestation, and under certain circumstances may be aspirated in utero or at delivery. Animal models using human meconium routinely demonstrate lung injury, but lack the in utero pulmonary vascular remodeling that may accompany clinical meconium aspiration syndrome. Mechanical obstruction of small airways with particulate meconium can lead to air trapping and pneumothorax. Meconium present at birth is associated with pneumothorax as noted previously.

Meconium components are toxic to the lung. Meconium contains fatty acids, and oleic acid in particular can be directly toxic to epithelial membranes. In vitro, neutrophils exposed to meconium release interleukin (IL)-8,[30,31] and in vivo aspiration models also demonstrate elevated IL-8 levels in tracheal aspirates.[32] In addition, oleic acid, hemoglobin, and other components in meconium may inactivate lung surfactant, leading to alveolar instability that contributes to shear stress and cellular injury.[17] Experimental and clinical treatment with exogenous surfactant restores minimum surface tension, improves oxygenation,[33] and reduces the need for extracorporeal membrane oxygen (ECMO) in patients with meconium aspiration syndrome.[34]

Amniotic Fluid Aspiration

At first glance it may seem paradoxical that amniotic fluid, which is produced in part by the fetal lung, would be toxic to pulmonary epithelium. However, the aspiration of cellular debris and the components of dead cells are likely to provoke an inflammatory response by the same mechanisms attributed to meconium aspiration: direct cellular toxicity, release of humoral mediators, and surfactant inactivation. Although the net flux of pulmonary fluid in fetal life is toward the mouth and away from the lung, mixing occurs during labor, particularly if the fetus gasps in response to hypoxia. Infected amniotic contents have been reported frequently in postmortem examinations. In addition, experiments in fetal lambs have demonstrated the exposure of the fetal lung to proinflammatory cytokines[10] derived from inflamed fetal membranes.

LEUKOCYTE-MEDIATED INJURY

Neonatal Pulmonary Inflammatory Responses

Inflammation—regardless of its initiating cause—exacerbates newborn lung injury. Different injuries or exposures can alter the timing and sequence of inflammatory cell influx. In neonatal surfactant deficiency and in experimental oxygen toxicity in newborn rats, resident alveolar macrophages[35] both precede and succeed the influx of neutrophils[36] that are isolated from bronchoalveolar lavage fluid of newborns with respiratory distress syndrome. Cellular inflammatory responses to mechanical or oxidative injury in the lung are prompted by release of prostanoids, or by the binding of pathogens to signal molecules that activate proinflammatory cytokines, such as IL-1β or tumor necrosis factor-alpha (TNFα). These stimulate expression of monocyte chemokines, such as monocyte chemoattractant protein-1, known to be expressed in alveolar epithelium, which in turn recruits macrophages into the lung.[37] The recruitment of leukocytes, particularly neutrophils, into the lung hinges on the complex interplay of cytokines, chemokines, and cellular adhesion molecules summarized in Figure 94-4.

Large animal models designed to mimic clinical BPD (use of appropriate oxygen therapy, moderate ventilation, and surfactant treatment after premature delivery) have shown a similar sequence of the development of injury and inflammation even

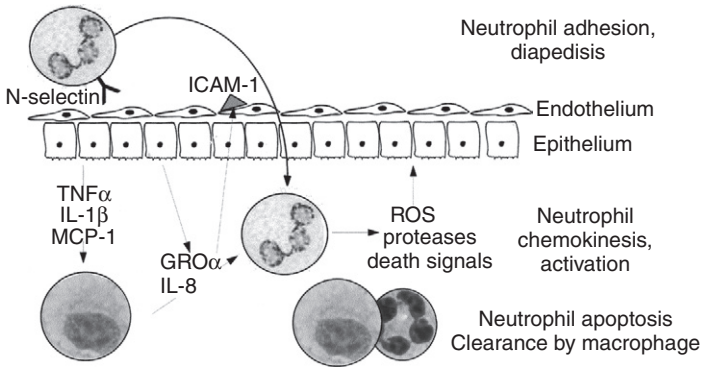


Figure 94-4 Damaged cells release proinflammatory cytokines that up-regulate adhesion molecules on circulating leukocytes and endothelium. Both epithelium and macrophages produce neutrophil chemokines, for example, GROα and interluekin 8 (IL-8), that further up-regulate adhesion of neutrophils and regulate chemotaxis, chemokinesis, and the reactive oxygen-producing respiratory burst. On activation, the neutrophil requires continuous chemokine signal transduction without which the neutrophil undergoes apoptosis and is cleared by alveolar macrophages. Activated neutrophils release reactive oxygen species, proteases, and other toxic materials that can damage adjacent cells.

though they lack antecedent labor.[38] Premature newborns that later develop BPD are likely to have elevations of proinflammatory cytokines and chemokines in tracheal aspirate samples.[39] These elevations may be present at birth, suggesting that the inflammatory response was begun antenatally, or shortly after delivery, perhaps during the initial resuscitation (Figure 94-5).

Inflammatory Processes Interrupt Lung Development

Damage to the developing lung may be sustained during a critical period, leading to death of particular progenitor cells necessary to orderly transition from the cannalicular to the alveolar stage of lung development. Indirect effects of reactive oxygen species produced by leukocytes following oxidative stress in newborns[40] may damage lung cell deoxyribonucleic acid (DNA) in certain proliferating cell types,[41] necessitating DNA repair and thereby delaying orderly mitosis and tissue development.[42] This may occur by disrupting the normal order of cell proliferation or by accelerating pathologic lung cell apoptosis.[43] Therapies aimed at selective modification or prevention of maladaptive inflammatory responses may attenuate lung injury and permit orderly postnatal lung development.[44]

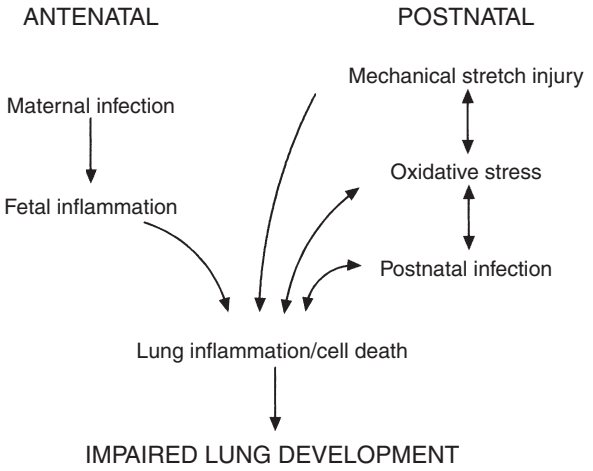


Figure 94-5 Combined antenatal and postnatal cellular injuries at critical phases of development contribute to bronchopulmonary dysplasia (BPD).

OXIDATIVE STRESS, ACUTE NEONATAL LUNG INJURY, AND BRONCHOPULMONARY DYSPLASIA

Deficiency of Antioxidant Defenses in Prematurity

Numerous oxygen radicals including superoxide, hydrogen peroxide, peroxyl radical, singlet oxygen, and hydroxyl radical can develop in particular compartments (in vitro) within the lung and inside lung cells. These compounds can damage lipid, protein, carbohydrate, and nucleic acid cell components. What is unknown at present, is whether they *do* occur in sufficient measure to cause enough lung cell injury to lead to BPD. The strength of the evidence implicating each reactive species in the pathology of newborn lung injury is variable and has been reviewed elsewhere.[45] Effects of reactive oxygen and nitrogen species on cell viability, function, and their ultimate effects on lung injury and development depend on the microenvironment in which they are formed and metabolized.

Premature newborns have relatively low chemical and enzymatic or inducible antioxidant systems compared with term and adult animals and are subjected to relatively high oxidative stress. The transition from the fetal milieu, wherein the oxygen delivery at relatively low arterial Po_2 is adequate to meet the metabolic demands, to the extrauterine existence with its significantly higher metabolic demands of thermogenesis and work of breathing, is typically accompanied by supplemental oxygen treatment. Studies in premature animals have shown that gene expression and the enzymatic activities of superoxide dismutases, catalase, and glutathione peroxidase are decreased.[4] This suggests the circumstance of antioxidant deficiency in the face of relatively increased oxidative stress, leading to impaired cellular metabolism, direct biomolecular damage, and structural cellular damage. However, the nature of the contribution of oxidative stress depends on the relevant biologic compartments and relationships among potential molecular substrates.[46] Serum glutathione stores are diminished (relative to adults) in premature newborns at risk to develop BPD. Oxidation of proteins isolated from tracheal aspirates of newborns destined to develop BPD provides indirect evidence of inadequate antioxidant defense.[47] Treatment with exogenous antioxidants such as vitamin A[48] or superoxide dimutase[49] has been shown to reduce bronchopulmonary dysplasia or its sequelae.

Metals and Oxidant-Mediated Lung Injury

Likely oxidant-stress mechanisms involved in the initiation and propagation of lung injury include oxidation and or nitrosylation of proteins, lipids, and nucleic acids. In addition, free metals such as iron or complexed iron can take part in a wide variety of reactions that are likely to produce reactive oxygen species or to damage critical catalytic components of enzymes or cofactors, for example:

I. $Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$ formation of ferrous iron
II. $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$ formation of hydroxyl radical
III. $Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\bullet} + OH^-$ catalysis of lipid peroxidation "chain reaction"

Normally iron is handled very carefully by cells as a result of its powerful redox capacity for catalyzing these potentially damaging reactions. Direct and indirect evidence points to this pathway as a contributor to lung injury. Free iron contributes to lipid peroxidation in ischemia-reperfusion injury.[50] Newborns that developed chronic lung disease were found to have higher serum ceruloplasmin and ferritin levels, and had twice as many blood transfusions as age-matched nonchronic lung disease control patients.[51] Low plasma antioxidant activity and frequent red blood cell transfusions were associated with bronchopulmonary dysplasia.[52] Erythropoietin treatment in hyperoxia-exposed rabbits increased the resistance of blood and tracheal aspirates to lipid peroxidation.[53] This coupled with the relatively decreased inducibility of heme oxygenase-1 in newborns, a principal chelator of free iron, suggests that iron may contribute to oxidant-mediated lung damage in newborns.[54]

SUMMARY

Lung injury with lifelong consequences for pulmonary function and cognitive development may occur during fetal life. Mechanical injury and resulting inflammation that may lead to nonuniform lung mechanics invite repetitive stretch injury in mechanically ventilated patients, and may begin at birth during initial resuscitation. Individual differences in host antioxidant defenses, and in inflammatory responses and their regulation, very likely contribute substantially to variability of resistance to initial injury, and to the variability of successful repair in the vulnerable newborn lung (see Chapters 96 and 97). Direct cytotoxic injuries—oxygen, aspiration, sepsis—provoke inflammation, which is often maladaptive and contributes to further injury.

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95 Impaired Lung Growth After Injury in Premature Lung

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Premature birth is any delivery, regardless of birth weight, that occurs before 37 completed weeks from the first day of the last menstrual cycle (term is 40 weeks in humans).[1] In this regard, *gestational age* is dated from the first day of the last menstrual cycle. Pregnancies that end before 20 completed weeks gestation are termed *abortions.* A reasonable definition of premature birth, therefore, is any delivery that occurs between 20 and 37 weeks gestation. In the United States and Canada, the overall rate of prematurity is approximately 10%,[2,3] despite the extensive use of tocolytic agents to arrest premature labor.[4-6]

Today, infants who are born as early as 22 weeks gestational age may survive if supported by intensive medical care, important components of which are antenatal steroids and postnatal surfactant replacement, and ventilatory support with supplemental oxygen. These and other supportive measures are required because the gas exchange regions of the lungs of the prematurely born infant are not sufficiently developed, either structurally or functionally, for extrauterine life. This chapter describes the impact of premature birth on lung growth, with emphasis on alveolar formation.

STRUCTURE OF DISTAL LUNG IN CONTEXT OF PRINCIPAL LUNG FUNCTION

Exchange of oxygen and carbon dioxide in the mature lung occurs in relatively large units that are referred to as *terminal respiratory units.* Each such unit—approximately 5 mm in diameter with a volume of around 0.02 mL at functional residual capacity—consists of all of the alveolar ducts, together with their accompanying alveoli, that stem from a single respiratory bronchiole.[7] In the adult human lung, one terminal respiratory unit contains approximately 100 alveolar ducts and 2000 anatomic alveoli. Together, the two lungs of the adult human contain approximately 150,000 of these specialized units.[8]

The function of the terminal respiratory unit is such that diffusion of oxygen and carbon dioxide in the gas phase is so rapid that the partial pressures of each gas are uniform throughout the unit. From each inspired breath, oxygen that reaches the alveolar duct gas diffuses into all of the associated alveoli, because the incoming air has a higher oxygen concentration than that of the alveolar gas. Oxygen subsequently diffuses through the air-blood barrier into red blood cells, where oxygen combines with hemoglobin as the red blood cells flow along the capillaries. Carbon dioxide diffuses in the opposite direction.

In the normal adult human lung, the air-blood barrier is exceedingly thin, which facilitates gas diffusion through the barrier. The average width of the air-blood barrier is approximately 1.5 μm.[8,9] For oxygen, the air-blood barrier's structural components are, in the order in which they are encountered by incoming air, alveolar epithelium and its subjacent basal lamina, alveolar wall interstitium, basal lamina of the capillary endothelium and the capillary endothelium, plasma, the membrane of red blood cells, and finally, hemoglobin molecules. For carbon dioxide, the obstacles to diffusion are encountered in the opposite sequence.

STRUCTURE OF THE PREMATURE LUNG

In the prematurely born infant, the terminal respiratory units are incompletely developed, and the air-blood barrier is too thick to allow efficient exchange of oxygen and carbon dioxide. This structural problem is greater with earlier preterm birth, because development of the terminal respiratory units occurs during the second half of gestation and the thickness of the air-blood barrier is inversely related to gestational age. A brief review of the stages of lung development in humans is presented next to illustrate these points[10] (Figure 95-1).

Branching of the bronchial tree and major pulmonary vessels is complete by 16 weeks gestation in humans. Before 17 weeks, the future bronchi and bronchioles extend into a core of relatively undifferentiated mesenchyme that contains few blood vessels (see Figure 95-1, *A*). This period of lung development is called the *pseudoglandular* stage. From weeks 16 to 26 gestation, the human fetal lung is at the *canalicular* stage. Lung architecture at this stage is characterized by more elongated profiles of the

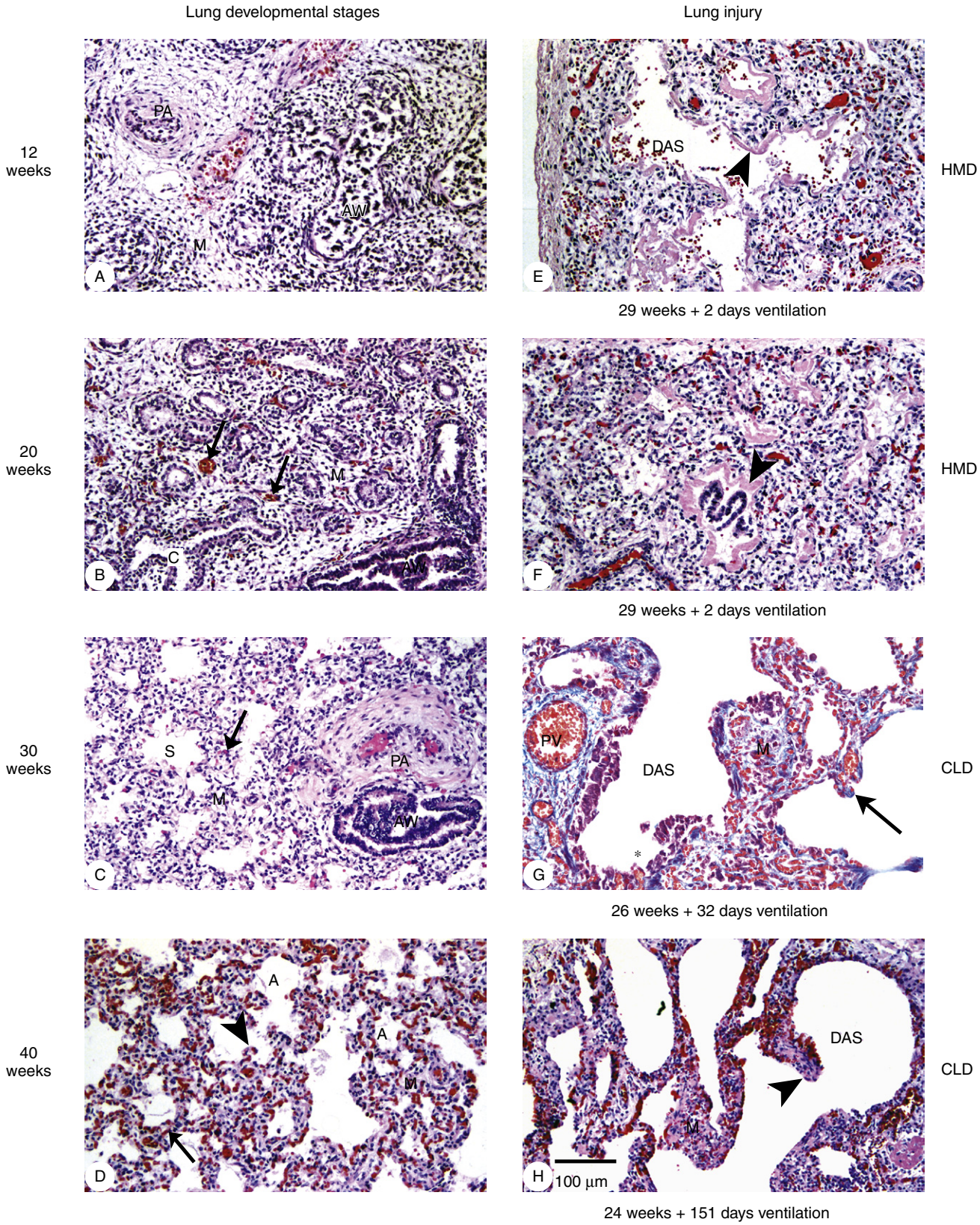

Lung developmental stages
Lung injury
12 weeks
PA
AW
M
A
DAS
E
HMD
29 weeks + 2 days ventilation
20 weeks
M
C
AW
B
F
HMD
29 weeks + 2 days ventilation
30 weeks
S
PA
M
AW
C
PV
DAS
M
*
G
CLD
26 weeks + 32 days ventilation
40 weeks
A
A
M
D
DAS
M
H
100 µm
CLD
24 weeks + 151 days ventilation

Figure 95-1 Comparison of normal lung histology during development in humans to lung histopathology associated with acute lung injury or neonatal chronic lung disease. All of the lungs were fixed at low lung volume when fixed at autopsy. **A,** Infant stillborn at 12 to 14 weeks gestation. Although considerable autolysis (postmortem degeneration) is evident, larger airways *(AW)* are lined by columnar epithelium that is largely desquamated. There is no development in the intervening mesenchyme *(M)*, which is devoid of blood vessels in this tissue section. **B,** Infant stillborn at 20 weeks gestation. Canaliculi *(C)* lined by low cuboidal epithelium are visible beyond the larger airway *(AW)*. Capillaries *(arrows)* are visible deep within the developing mesenchyme. **C,** Infant stillborn at 29 weeks gestation. Saccules *(S)* and capillaries *(arrows)* are present in the thick primary septa. A larger airway *(AW)* is visible near the edge of the panel. **D,** Infant stillborn at 40 weeks gestation (term). Alveolar development has progressed to where the primary septa are thinner and secondary septa (crests; *arrowhead*) protrude into the developing alveoli *(A)*. The alveolar walls have numerous capillaries *(arrow)*. **E** and **F,** Examples of hyaline membrane disease *(HMD)*. **E,** Birth at 25 weeks gestation followed by 6 hours of mechanical ventilation. In the first few hours of life, the premature, mechanically ventilated lung shows uneven expansion of the distal air spaces *(DAS)*, vascular congestion, and interstitial edema. Hyaline membranes *(arrow)* are focally present. Some patchy hemorrhage also may be present. **F,** Birth at 29 weeks gestation followed by 2 days of mechanical ventilation. The distribution of ventilation is uneven, with centriacinar expansion and peripheral collapse, and hyaline membranes *(arrowhead)* are more extensive. **G** and **H,** Bronchopulmonary dysplasia *(BPD)*. **G,** Birth at 22 weeks gestation followed by 44 days of mechanical ventilation (28 to 29 weeks postconceptual age). The distal air spaces *(DAS)* have cellular debris *(*)*. Thick, cellular mesenchyme *(M)* separates the adjacent distal air spaces. **H,** Birth at 24 weeks gestation followed by 151 days of mechanical ventilation (45 weeks postconceptual age). Simplification and overdistension of the distal air spaces *(DAS)* is clearly evident. Primary septa are thick and cellular. Secondary septa (crests; *arrowhead*) are infrequently visible; those that are visible are short, thick, and devoid of capillaries near their tips. **A** to **H** are the same magnification (the scale bar is 100 μm in length). **A** to **F** and **H** were stained with hematoxylin and eosin; **G** with Masson's trichrome stain.

distalmost airways and proliferation of capillaries. Although capillary proliferation is robust, the developing capillaries are distant from the distal canals (see Figure 95-1, *B*). During this period, the cells lining the distal canals become cuboidal and show ultrastructural evidence of differentiation toward alveolar type II epithelial cells, in the form of osmiophilic lamellar bodies.[11] Arising from cuboidal epithelial cells are alveolar type I (squamous) epithelial cells. These terminally differentiated cells, although fewer in number than their cuboidal counterpart, eventually cover approximately 90% to 95% of the alveolar surface area of the peripheral lung in the adult.[12] Both structural attributes of alveolar type I epithelial cells provide a large, thin cellular barrier specialized for gas exchange.

From approximately weeks 24 to 38 gestation, lung development is at the *saccular* stage. During this stage, the initially cylindrical distal canals become divided by primary septa into which capillaries extend, resulting in greater surface area. Continual growth results in thinning of the mesenchyme. The result of these structural changes is a marked increase in the potential gas exchange surface of the lung. From around 36 weeks gestation and continuing through the first 18 to 24 months of postnatal life, alveoli are formed by progressive expansion and subdivision of the terminal air sacs (see Figure 95-1, *C*). Subdivision occurs during the process of secondary septation (also called secondary crest formation). During the alveolar stage, the air-blood barrier attains its adult thinness by further reduction of the connective tissue compartment in the developing alveolar walls and juxtaposition of the alveolar walls and alveolar capillaries.

Alveolar type II epithelial cells, and the surfactant phospholipids and apoproteins that they synthesize, secrete, and recycle are essential for normal lung function. In the human lung, type II cells containing lamellar bodies are histologically recognizable at the transition from the canalicular to the saccular stages of lung development. Surfactant synthesis normally begins later, during the saccular stage (around 30 weeks gestation),[13] but can be induced earlier, as discussed further on. From the saccular stage to the end of term gestation, the concentrations of dipalmitoyl phosphatidylcholine and other surfactant phospholipids increase in lung tissue, lung lavage liquid, and amniotic fluid. Surfactant apoproteins SP-A, SP-B, and SP-C, expressed only in lung tissue, also are developmentally regulated but not concordantly. Human SP-A messenger RNA (mRNA) and protein in lung tissue are not detectable until the saccular stage of lung development (around 30 weeks gestation).[13-16] Human SP-B and SP-C mRNAs are detectable in lung tissue at very low levels earlier than SP-A, during the transition from the canalicular stage to the saccular stage (around 24 weeks gestation).[17] Detection of SP-B and SP-C proteins, however, is later in development, during the saccular stage (about 30 weeks gestation).[16] Human SP-D protein has been detected only in amniotic fluid during the transition from the canalicular stage to the saccular stage of lung development (around 24 weeks gestation).[18,19] Thus the cellular and biochemical machinery to reduce surface tension at air-liquid interfaces in the future air spaces develops during the third trimester in humans. Many premature infants are born before the third trimester and are therefore at risk for respiratory failure because their lungs are deficient in surfactant. An approach to assessment of lung development before birth is to measure these secretory products in amniotic fluid.[20]

Another impediment to gas diffusion in the lungs of premature infants is the presence of liquid in the interstitium and potential air spaces. Fluid is an impediment because diffusion is much faster in the gas phase than in water. For example, the solubility of oxygen in water is low (0.03 mL/L × mm Hg Pa_{O_2}) compared with carbon dioxide, the solubility of which is approximately 20 times more than oxygen (0.7 mL/L × mm Hg Pa_{CO_2}). The advantage for carbon dioxide persists even though the driving pressure for carbon dioxide diffusion is only one-tenth that for oxygen entering the blood. Therefore oxygen diffusion is much slower in the relatively hydrated environment of the premature lung.

Normal intrauterine lung growth requires appropriate distension by lung luminal liquid.[21] The fetal lungs are filled with liquid that flows from the pulmonary vascular compartment, through the mesenchyme, to the potential air spaces. Liquid flows to the fetal potential air spaces because chloride is secreted across the fetal respiratory epithelium into the potential air spaces,[22] causing an osmotic concentration gradient that promotes flow of water into the potential air spaces. Lung luminal liquid is propelled centrally along the conducting airways to the oropharynx, where the liquid is either swallowed or expelled into the amniotic sac. Balance between production and drainage of lung luminal liquid is required for normal intrauterine lung growth.[21] When drainage exceeds production, the fetal lung is not exposed to continuous liquid-distending pressure; consequently, lung growth is inhibited.[23] Near or at the time of term birth, the

secretory activity of the respiratory epithelium switches from a predominantly chloride-secreting membrane to a predominantly sodium-absorbing membrane.[24-26] This molecular switch reverses water movement, thereby drying the potential air spaces for gaseous diffusion.

Structural and functional characteristics of the airways also may be affected by premature birth. During the canalicular and saccular stages of lung development, the airways have little smooth muscle in their wall, the epithelium is immature, and cell-cell adhesion is weak. Physiologic studies of human tracheobronchial segments ex vivo indicate that pressure-volume relations are affected by maturity,[27,28] such that airway compliance is inversely related to maturity. Less compliance of the airways of premature infants may explain the need for higher airway pressures than those adequate in term infants.[29] Furthermore, increased volume may be needed to inflate the collapsed gas exchange regions of premature infants compared to term infants.[29] The dearth of smooth muscle and weak adhesion between cells of the immature airway serve to increase the premature infant's airway susceptibility to injury by the increased pressures and volumes that are required to effect ventilation.

Acute Lung Injury of Prematurity (Respiratory Distress Syndrome)

When an infant is born prematurely, the structurally immature lungs are deficient in surfactant. Therefore, surface tension forces are high and the alveoli are unstable.[30] Alveolar instability leads to alveolar collapse (atelectasis). This atelectasis results in ventilation-perfusion mismatch. Such mismatch results in turn in intrapulmonary shunting, thereby contributing to poor oxygenation.[31] Opening the collapsed air spaces requires transmission of high ventilatory pressures to the immature distal airways and gas exchange regions of the lung. In effect, the extreme effort required to expand the lungs with the first breath must be repeated with each breath, because surfactant is not present to prevent collapse of the distal air spaces. Prematurely born infants who have these characteristics tire quickly, leading to the development of respiratory distress syndrome (RDS).

RDS affects approximately 30,000 infants annually in the United States. The incidence is around 50% to 60% in infants who are born before 30 weeks of gestation.[32] The incidence increases with decreasing gestational age.[33-35] RDS is more prevalent and severe in male than in female premature infants, for reasons that remain unclear.

Because RDS is a predictable consequence of lung immaturity, strategies have been developed to accelerate lung development before premature delivery. One such strategy is administration of hormones, such as glucocorticoids.[36-39] Seminal studies by Liggins[40] and, subsequently, Liggins and Howie[41] demonstrated that glucocorticoids accelerate lung maturation of fetal sheep and decrease the incidence of RDS in human infants after antenatal corticosteroid therapy.[40,41] The maturational effect of corticosteroid treatment on lung structure was first described by Kikkawa and colleagues.[42] Corticosteroids (betamethasone or dexamethasone) accelerate maturation of alveolar type II epithelial cells, which are the source of pulmonary surfactant. For these reasons, mothers who are in premature labor and are at risk of giving birth to a premature infant between 24 and 35 weeks gestation are treated antenatally with glucocorticoids.[43]

A treatment strategy to reduce lung stiffness (increase lung compliance) after premature birth is to instill surfactant into the airways.[44] Surfactant replacement therapy is beneficial because once it becomes widely and thinly distributed in the lung, the exogenous surfactant reduces surface tension at air-liquid interfaces, thereby stabilizing alveoli when they are deflated. After surfactant replacement, oxygenation improves swiftly, followed for several hours by progressive improvement in gas exchange and lung mechanics.[45-47] Other improvements, at least in premature lambs, are reduced vascular injury and edema.[48] Thus surfactant replacement therapy reduces the incidence and severity of RDS after premature birth. Instilled surfactant is not recycled as efficiently as native surfactant, but by reducing the lung injury associated with management of RDS, this therapy improves the opportunity for the premature lung to repair and grow.

The pathologic findings in RDS are similar to those in adults with acute respiratory distress syndrome (ARDS),[49] superimposed on the immature lung.[50] Lesions in the lungs reflect both the disease and its treatment with mechanical ventilation and supplemental oxygen. Within the first 3 to 4 hours, the lungs may only show uneven ventilation of distal air spaces resulting from surfactant deficiency, interstitial edema resulting from incomplete removal of fetal lung liquid or acute injury to microvascular endothelial cells, and congestion of capillaries (see Figure 95-1, *E*). By 12 to 24 hours, necrosis of alveolar and bronchiolar epithelial cells develops, and the denuded walls become coated by characteristic *hyaline membranes,* which are eosinophilic transudates of plasma proteins admixed with necrotic epithelial cells and fibrin (see Figure 95-1, *E* and *F*).[51] These histopathologic features are similar to those in the exudative phase of ARDS in adults, but the alveolar type II epithelial cell hyperplasia that constitutes the proliferative phase of ARDS in adults is not as prominent in hyaline membrane disease seen in autopsy slides from premature infants.

Mechanical ventilation of premature infants can result in ventilator-induced lung injury,[52] even after repeated doses of exogenous surfactant. The injurious effects of mechanical ventilation depend on a number of factors, among which are the magnitude of airway pressure (barotrauma) and lung volume (volutrauma)[53,54] and the concentration of inspired oxygen (oxygen toxicity). Several experimental animal studies have compared indices of ventilator-induced lung injury between conditions that raised airway pressure and increased lung volume.[55-57] Those studies suggest that large lung volumes, rather than high peak airway pressures per se, are the cause of ventilator-induced lung injury. For this reason, the term *volutrauma* is used to describe the injury that is associated with mechanical ventilation.

How does volutrauma induce lung injury, especially after premature birth? Evidence suggests that some of the circumstances described above are exacerbated. For example, regional overinflation increases microvascular permeability, either directly or indirectly (through release of inflammatory mediators from sequestered leukocytes in the lung), which leads to alveolar flooding. Alveolar flooding is in turn associated with inactivation of surfactant, leading to atelectasis and decreased lung compliance. If these cycles are not broken, lung injury recurs, necessitating higher airway pressure, larger tidal volume, and more supplemental oxygen. As the extent and duration of mechanical ventilation with supplemental oxygen increase, and lung volutrauma persists or increases, acute lung injury may progress to chronic lung disease (CLD).

CHRONIC LUNG DISEASE OF PREMATURITY

Survival of prematurely born infants with RDS has significantly improved since the introduction of antenatal steroid and postnatal surfactant replacement therapies and use of gentler ventilation strategies.[34,58-60] As described earlier, 84% of premature infants weighing 500 to 1500 g at birth now survive. Unfortunately, in approximately 8,000 to 10,000 premature infants in whom RDS develops in the United States annually, and who receive antenatal glucocorticoids and postnatal surfactant replacement, the disease progresses to CLD of prematurity (i.e., bronchopulmonary dysplasia [BPD]). The reported incidence of CLD in premature infants ranges between 15% and 60%.[61-63] As expected, compared with larger premature infants, smaller infants are at greater risk

for this disorder. After adjustment for a number of risk factors, development of CLD most frequently is associated with need for high inspired oxygen concentration 96 hours after birth, high peak inspiratory pressure, and high fluid intake.

The original description of BPD was by Northway and colleagues.[64] The principal clinical criterion was the requirement for supplemental oxygen for at least 28 days after birth (a duration selected because the first preterm neonates were managed during the month of February (Northway WH Jr, personal communication, 2009). The major radiologic criteria were cyst formation and hyperexpansion mixed with atelectasis. The characteristic histopathologic findings at autopsy depended on the duration of disease before death: Classic hyaline membrane disease is seen in the first 2 to 3 days. A regenerative phase is seen over the next week that consists of necrosis and regeneration of bronchiolar and alveolar epithelium, in addition to hyaline membranes. Over the next 10 days, hyperplasia of bronchiolar and arteriolar smooth muscle, fibrosis, and dilation or collapse of distal air sacs ensue. In the final stage, these alternating areas of overdistention and atelectasis become fixed. The term *bronchopulmonary* was chosen to identify airway and blood vessel involvement; *dysplasia* was chosen to describe the failure of the distal air sacs to develop into normal anatomic alveoli (Northway WH Jr, personal communication, 2009). Today, CLD usually is defined clinically as either oxygen dependency at 28 days of life with appropriate radiologic findings or oxygen dependency at 36 weeks postconceptual age.[65,66]

BPD as originally described by Northway and colleagues[64] was diagnosed in premature infants who did not recover from RDS. However, treatment of respiratory failure from other causes, including meconium aspiration pneumonia,[67] neonatal pneumonia,[68] congestive heart failure,[69] or congenital diaphragmatic hernia,[70] may be associated with CLD.

Prematurely born infants diagnosed with CLD today are much younger (23 to 26 weeks gestation) and smaller (less than 1000 g) than those with BPD as described in early reports (31 to 34 weeks gestation and approximately 2000 g at birth), and the radiologic and pathologic features of their lung disease are less severe. Today, the radiologic abnormalities include lung hyperinflation, emphysema, and interstitial densities. The principal histopathologic finding at autopsy is dilated and simplified distal air sacs[71,72] (see Figure 95-1, *G* and *H*). Usually missing are the classic histopathologic changes in the central airways. Recognition of these distinctions between contemporary CLD and BPD, as originally described, has prompted some clinicians and investigators to call today's CLD "new BPD." Debate continues about whether the disease that is seen today is in fact new or represents a less severe variant of BPD.

Impaired Alveolar Formation After Injury in Premature Lung

Simplification of distal air sacs is characteristic of the histopathologic pattern in premature lungs. Simplification is related to failure of alveolar secondary septa (crests) to sprout into the distal air sacs. The mechanisms that participate in normal or abnormal secondary septation of alveoli are not known and are a topic of intense investigation today.[65]

Alveolar formation is initiated near the end of the fetal period and continues postnatally after term gestation.[10,73-77] At term gestation, alveolar formation is less complete in humans, baboons, and mice.[10,73-77] than in sheep and other ruminants.[78-80]

Alveolar formation is structurally manifested by evagination of partitions along the smoothly curved saccular walls during late gestation and early postnatal life.[10] Regardless of the speed with which alveolar formation occurs among species, low ridges elongate into secondary septa. Capillary loops accompany these septal evaginations, resulting in a thin air-blood interface on either side of the septa, and attenuation of the mesenchymal core through apoptosis.[81] These processes increase surface area and thinness of air-blood barriers, resulting in minimal impediment to the diffusion of oxygen and carbon dioxide.[82]

Because of the late initiation of and prolonged period for alveolar formation, success with use of knockout or transgenic mice as models to identify regulatory molecules during alveolar formation has been limited. With such approaches, the earliest stages often are affected profoundly, sometimes resulting in embryonic or fetal death before alveolar formation is initiated. An important exception is the platelet-derived growth factor-A (PDGF-A)–null mouse.[83] Some of the PDGF-A$^{-/-}$ mice survive through the postnatal period, but these mice lack both alveolar myofibroblasts and alveolar elastin. Their lungs have simplified distal air spaces. Another exception is the elastin-null mouse.[84] The elastin$^{-/-}$ mice survive through the first several days of postnatal life, but they are cyanotic. Their lungs have reduced airway and vascular generations, with arrest of distal air spaces at the saccular stage.

Because CLD occurs almost exclusively after premature birth and weeks of mechanical ventilation with supplemental oxygen, reproducing the human disease in animal models requires intensive, large-animal protocols. Coalson and co-workers developed the baboon (primate) model that has the pathologic features of CLD, including alveolar simplification.[85-88] These investigators showed that early application of high-frequency ventilation may reduce the severity of the histopathologic changes.[89-91] and speculated that hyperoxia and tidal ventilation of the premature lung leads to lung overstretch and release of inflammatory mediators that cause loss of alveoli and alveolar capillary surface area.[86,92] Bland and colleagues also developed a model of CLD using preterm lambs.[93-96] Mechanical ventilation of preterm lambs for 3 to 4 weeks, using large tidal volumes (15 mL/kg of body weight), resulted in inhibition of alveolar formation and alveolar capillary growth, disruption of gas exchange, diminished numbers of microvessels, persistent muscularization of resistance arterioles, increased pulmonary vascular resistance, and decreased expression of endothelial nitric oxide synthase protein.[93-96] Management of premature lambs with high-frequency jet ventilation, on the other hand, was associated with improved structural formation of alveoli and alveolar capillary growth.[97]

A consequence of failed septation is reduced surface area for gas exchange, including the capillary surface area.[92,94] However, the latter observation in experimental animals with evolving CLD has not been confirmed in autopsy tissue from infants who died with established CLD.[98] One explanation for this discordance is the time frame of CLD studies. Experimental animal studies are relatively short (up to 4 weeks, with evolving CLD), whereas premature infants with CLD may be managed with mechanical ventilation and supplemental oxygen for much longer periods (months, with established CLD), suggesting that capillary proliferation may be an adaptive response to impaired septation in CLD.

Studies using premature lambs with evolving CLD also indicated that the elastin gene is excessively and continuously upregulated.[96] Up-regulation occurs on the third day of continuous mechanical ventilation[99] and persists for 3 to 4 weeks. Although the results from the premature lambs with evolving CLD and elastin-knockout mice[84] (described earlier) seem contradictory, they actually suggest that tropoelastin gene expression has to be tightly regulated for normal septation. Too little or too much tropoelastin gene expression is detrimental to normal septation.

Appropriate stretch of the developing lung appears to be of key importance for formation of normal alveoli. Overstretch leads to distortion of cells and extracellular matrix in the developing lung. The distortion may lead to the local synthesis and release of regulatory molecules that affect cellular division, phenotype, and metabolic activity. High lung inflation (overstretch), for example, increases mRNA levels for transforming growth factor (TGF)-β_1

four-fold and basic fibroblast growth factor (bFGF) (i.e., FGF2) by 60% compared with values obtained for low lung inflation in adult rabbits.[100] In this regard, PDGF is involved in stretch-mediated signaling, and elastin also is a stretch-responsive gene. These data emphasize the importance of mechanical stretch in alveolar formation.

Among the mediators proposed to link overstretch and arrested alveolar formation are growth factors, cytokines, and matrix-degrading proteinases released by epithelial and mesenchymal cells, as well as resident and infiltrating inflammatory cells. For example, the developing lung has the ability to synthesize and release vascular endothelial growth factor (VEGF),[101] FGFs,[102] PDGFs,[103,104] TGFs,[105,106] insulin-like growth factors,[107,108] and epidermal growth factor (EGF),[109,110] as well as their receptors. Of these growth factors, VEGF[111-115] and related vascular growth–signaling molecules,[116,117] FGF signaling,[118,119] and PDGF-A[83,103] are implicated in alveolar formation.

VEGF expression is regulated by other growth factors, such as TGF-β[120] and PDGF,[121] and dexamethasone.[101] The biologic function of VEGF in disease states may rely on altered amounts of VEGF protein, as well as altered VEGF receptor (VEGF-R) protein and affinity. For example, hypoxia induces pulmonary production of VEGF and its receptor, VEGF-R2 (also named FLK-1 [fetal liver kinase-1] or KDR [kinase insert domain–containing receptor]).[101,122] The latter paracrine ligand/receptor pair is responsible for differentiation of angioblasts into capillary endothelial cells in the lung.[123] The converse is true for hyperoxia,[124] which is a risk factor for failed alveolar formation.[124,125] In this regard, disruption of pulmonary blood vessel formation in rats, by blocking binding of VEGF to its receptor, results in lung histopathologic changes that look like CLD.[113,114] Recovery of neonatal and adult rabbits from hyperoxia is associated with VEGF message expression by alveolar epithelial cells.[124,126] These findings suggest a role for VEGF in regulating alveolar capillary proliferation normally and after oxidant injury; the latter plays a central role in the pathogenesis of CLD.

Deficiency of retinol or retinol-binding protein after premature birth may contribute to the development of CLD in neonates.[127-130] Clinical trials of retinol treatment of neonates at risk for CLD, however, have yielded conflicting results. In a randomized, double-blind controlled trial,[131] infants who received vitamin A had a significantly lower incidence of CLD and reduced need for supplemental O_2, mechanical ventilation, and intensive care. In a subsequent study, retinol supplementation had no effect on the incidence of CLD in preterm infants.[132] More recently, however, large multicenter studies of retinol versus placebo treatment of tiny preterm infants have shown a significant reduction in the incidence of CLD among the retinol-treated infants.[133,134]

Experimental animal studies have provided promising results in this regard. One study showed that retinoid signaling is required for alveolar formation.[135] Another study used pregnant rats treated with dexamethasone repeatedly to inhibit lung development in utero.[136] The newborn pups, treated with all-*trans*-retinoic acid postnatally, demonstrated greater alveolar formation than that in the untreated control rat pups. A third study showed that retinoids reversed steroid-induced suppression of lung growth and epithelial cell proliferation in cultured fetal rat lung.[137] A fourth study showed that retinoic acid treatment of adult rats with elastase-induced pulmonary emphysema was associated with reversal of emphysematous lesions.[138] Initial studies from our own clinical laboratory using premature lambs with evolving CLD of prematurity have shown that daily treatment with vitamin A (Aquasol) is associated with enhanced formation of alveoli and alveolar capillaries and increased expression of VEGF and VEGF-R2.[112] The molecular mechanisms by which vitamin A up-regulates expression of VEGF and VEGF-R2 are being investigated. More recent studies suggest involvement of retinoic acid receptors.[139]

Ongoing research is providing exciting evidence that nasal continuous positive airway pressure (nasal CPAP) permits alveolar formation. From a clinical standpoint, the incidence of CLD of prematurity is lower in preterm neonates managed by nasal CPAP than in those receiving mechanical ventilation.[140,141] Why the incidence is less is starting to become evident, through studies using chronically ventilated preterm lambs or baboons. For example, Jobe and colleagues compared the inflammatory milieux in two groups of preterm lambs that were managed for 2 hours by mechanical ventilation or nasal CPAP. Preterm lambs managed by conventional mechanical ventilation had almost seven times more neutrophils and hydrogen peroxide in alveolar washings than in those from preterm lambs managed by nasal CPAP.[142] A subsequent study by Thomson and colleagues examined indices of alveolar formation in preterm baboons that received nasal CPAP for 28 days.[143] Alveolar complexity was greater in the preterm baboons than in historical control baboons managed by mechanical ventilation. The lungs of the nasal CPAP–managed preterm baboons had enlarged, thin-walled air spaces, with greater immunolocalization of capillary endothelial cells (on platelet endothelial cell adhesion molecule [PECAM] immunostaining), scattered secondary septa, and minimal fibroproliferation. Internal surface area and surface-to-volume ratio dimensions were similar to those in 156-day gestational control lungs, the intrauterine developmental control.

In preliminary studies from our laboratory using chronically ventilated preterm lambs, comparable results have been obtained.[144] For instance, management of preterm lambs by nasal CPAP for 21 days resulted in three- to four-fold enhancement of alveolar septation and capillary development, as well as up-regulation of VEGF and FLK-1 gene expression, compared with mechanical ventilation for 21 days. In addition, the fractional inspired O_2 to achieve a target Pa_{O_2} of 60 to 70 mm Hg was significantly less in the nasal CPAP group than in the mechanically ventilated group at 1, 2, and 3 weeks of life. For the target Pa_{CO_2} (50 to 60 mm Hg), peak inspiratory pressure, positive end-expiratory pressure, and mean airway pressure also were significantly less in the nasal CPAP group than in the mechanically ventilated group at weeks 1, 2, and 3 of life.

Our more recent studies suggest that preterm lambs managed by nasal CPAP have thinner distal air space wall thickness, less cell proliferation of mesenchymal cells, and more apoptosis of mesenchymal cells.[3,145] Thus results such as these from the only two large-animal models of CLD of prematurity are providing new mechanistic insights into how and why outcomes appear to be better for preterm neonates managed by nasal CPAP than for those managed by mechanical ventilation. The common theme appears to be that nasal CPAP permits alveolar formation, whereas mechanical ventilation arrests alveolar formation.

SUMMARY

The structural and functional immaturity of the premature infant's lung necessitates use of antenatal steroids, perinatal surfactant replacement therapy, postnatal mechanical ventilation and oxygen supplementation, and other supportive measures. Acute lung injury often occurs as a result of lung immaturity and the therapy that it necessitates. If recovery does not ensue, the affected premature infants progress to CLD. Although CLD continues to be one of the most serious of today's pediatric public health issues, important progress has been made. For example, CLD of prematurity now occurs infrequently in larger (2 kg or greater) and more mature (32 weeks and beyond) premature infants, the population that Northway and colleagues described in their original report on BPD.[64] Moreover, the severity of CLD is less today than at the time of that report. These encouraging changes reflect better understanding of lung developmental biology and improvements in clinical management of prematurely born infants, such as use

of nasal CPAP. Nonetheless, challenges remain, because the incidence of premature birth is increasing, the size of the premature infant who can be supported is decreasing, and the incidence of CLD among the smallest premature infants remains very high.

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Suhas G. Kallapur and Alan H. Jobe

Antenatal Factors That Influence Postnatal Lung Development and Injury

SPECTRUM OF LUNG DISEASE IN PRETERM INFANTS

The fetal lung at 26 weeks gestation is in an early saccular stage of development and surfactant deficient. Surfactant treatment and the use of more gentle modes of assisted ventilation have resulted in populations of very preterm infants with a wide range of lung diseases not easily classifiable into the traditional categories of respiratory distress syndrome (RDS), lung immaturity, transient tachypnea, and normal. In one large series, the mortality rate for infants weighing 501 to 1500 g at birth was only 14%, but *bronchopulmonary dysplasia* (BPD), defined as the need for supplemental oxygen at 36 weeks, occurred in 23% of these infants.[1] For the population of very preterm infants in the 750- to 1000-g birth-weight range, 37% of the infants did not have RDS, but 42% ultimately had BPD. In some instances, then, BPD may develop without preceding RDS.

In a series of 177 surviving infants with birth weights less than 1250 g, 115 infants had RDS, which progressed to BPD in 61 infants.[2] Of 88 infants with BPD, the disease developed in 27 infants, either after resolution of the RDS or in the absence of preexisting lung disease. Thus, in 30% of the infants with BPD, the clinical course was not characteristic of the usual progression from RDS to BPD associated with assisted ventilation in the neonate.

A complex interplay of antenatal and postnatal factors is now recognized to affect postnatal lung development after premature birth and, ultimately, the incidence of BPD (Figure 96-1). This chapter explores some of the antenatal factors that may modulate lung development and thereby alter the postnatal clinical course of lung disease in preterm infants.

CHORIOAMNIONITIS

The fetal lungs are protected from the traditional mechanisms of lung injury, but infection and inflammation can reach the fetal lung. Infective organisms from the lower genital flora can ascend through the cervix, with subsequent spread of infection between the chorioamnion and the uterine wall; this mode of transmission is commonly associated with early preterm labor.[3] Inflammation of the chorioamnion, termed *chorioamnionitis,* can be confirmed only by histopathologic examination, for a diagnosis of "histologic chorioamnionitis." In clinical practice, chorioamnionitis is diagnosed using a combination of indicators such as elevated maternal temperature, uterine tenderness, malodorous vaginal discharge, maternal leukocytosis, and fetal tachycardia.[4] Spread of the inflammatory process to the amniotic fluid can be evaluated by amniocentesis to obtain amniotic fluid for culture

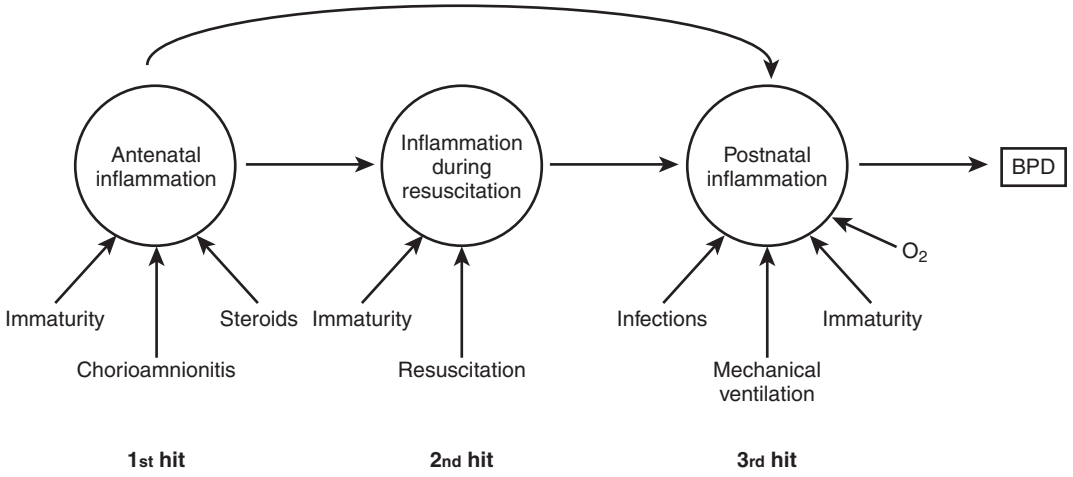


Figure 96-1 A model for the interplay of fetal exposures on early postnatal events that may contribute to altered lung development and injury.

or cell counts, or by measurement of proinflammatory mediators. Further spread of the infection or inflammation to the fetus can occur by either of two routes[5]: Normal fetal swallowing may lead to aspiration of the inflammatory products in the amniotic fluid, with subsequent development of a pneumonia-sepsis syndrome, or the fetus may mount an inflammatory response to signals from the inflamed fetal placenta and chorioamnion. The fetal inflammatory response in chorioamnionitis has been defined as an elevation of interleukin (IL)-6 in fetal cord blood obtained by cordocentesis or in cord blood after delivery.[6] Funisitis, or inflammation of the cord, correlates with elevated cord blood IL-6 levels and signs of generalized fetal inflammation.

The rapidly progressive chorioamnionitis or bloodstream infection caused by organisms such as *Escherichia coli, Listeria monocytogenes,* or group B streptococci is relatively infrequent in the preterm population. By contrast, clinically asymptomatic histologic chorioamnionitis caused by low-virulence or commensal organisms increases in frequency as gestational age at delivery decreases. In one series, approximately 70% of infants (born before 30 weeks gestation) delivered by cesarean section with intact membranes had histologic chorioamnionitis.[3] This clinically inapparent chorioamnionitis is associated with recurrent preterm deliveries, and indicators of inflammation may be present before midgestation.

The diagnosis of chorioamnionitis can be problematic. In general, various markers of chorioamnionitis—amniotic fluid cytokines, neutrophils in amnion or chorion, and positive microbiologic findings—correlate with one other. However, in a study involving preterm infants born at 23 to 32 weeks gestation, evidence of histologic chorioamnionitis was lacking in 25% of cases with positive amniotic fluid culture, whereas in 20% of the cases with histologic chorioamnionitis, amniotic fluid culture result was negative.[7] Similarly, in 35% of the cases with evidence of histologic chorioamnionitis, no elevation of amniotic fluid IL-6 level was noted. The correlation of clinical chorioamnionitis with histologic chorioamnionitis is even more tenuous, with absence of clinical manifestations in more than 75% of cases with positive histopathologic findings.[7] Even in cases with negative amniotic fluid culture, evidence of bacterial RNA was present in the placenta of most preterm infants adding another dimension of complexity in the definition of chorioamnionitis.[8]

Growing evidence suggests that endometrium is not normally sterile. In a clinical study using careful techniques to prevent contamination, at least one organism was isolated from endometrial cultures in 85% of nonpregnant women.[9] Amniotic fluid sampled before 20 weeks gestation for genetic diagnostic indications can contain elevated proinflammatory mediators and be culture-positive for low-virulence pathogenic organisms.[10] Often the infants born to mothers with such amniotic fluid markers have no overt signs of infection at delivery. Thus a majority of fetuses may be exposed to bacterial products for a long time in utero without clinically apparent complications. Nevertheless, the most common cause of fetal death or death soon after birth for very low-birthweight infants was sepsis or pneumonia in an autopsy series.[11] Evidently, then, the effects of chorioamnionitis on the preterm fetus may range from severe infection or death to normal fetal development, with histologic chorioamnionitis as an incidental finding. Attempts to secure a definitive diagnosis of chorioamnionitis typically are limited by a lack of information about its duration, intensity, or extent or the organisms responsible for the clinical or histopathologic findings. In the face of exposure to commonly encountered infectious agents, down-regulation of inflammatory responses in utero may be a key coping mechanism of the fetus.

CHORIOAMNIONITIS AND FETAL LUNG INJURY

The traditional concept is that fetal infection or inflammation associated with chorioamnionitis can result in an aspiration pneumonia syndrome and sepsis. Gomez and colleagues[6] proposed that the fetal inflammatory response was equivalent to acute RDS in adults, with systemic inflammatory mediators targeting the lungs. In other fetuses exposed to inflammation in the amniotic fluid, however, elevation of plasma IL-6 levels is not seen. Elevated IL-1β, IL-6, and IL-8 levels in amniotic fluid measured within 5 days of preterm delivery predicted the subsequent development of BPD.[12] Histologic chorioamnionitis was not useful retrospectively in the reported series because it was present in 92% of infants with subsequent BPD and in 62% of those without BPD. RDS also was not a good predictor of BPD. In another report, severe histologic chorioamnionitis and elevated tumor necrosis factor-α (TNF-α) concentration in amniotic fluid were associated with an increased incidence of RDS and subsequent BPD.[13] In a comparison of the predictive values for the development of BPD, elevated cord plasma IL-6 was a better predictor than elevated IL-6 in the amniotic fluid.[14] Fetal exposure to inflammation was associated with a lung injury process that results in an increase in the incidence of BPD. A graded increasing risk of BPD was observed, ranging from histologic chorioamnionitis, to elevation of inflammatory mediators in amniotic fluid, to a fetal inflammatory response with elevated IL-6 in fetal blood. This association, however, was not apparent in a more recent series of 466 consecutive singleton births before 32 weeks gestation[7] (Table 96-1).

TABLE 96-1

Associations Between Histologic Chorioamnionitis and Outcomes for 446 Consecutive Singleton Deliveries Before 32 Weeks Gestation

	MEMBRANES		CORD		
Clinical Outcome	**Yes (%)**	**No (%)**	**Yes (%)**	**No (%)**	**Statistically Significant**
Ureaplasma + *Mycoplasma*	81	32	49	21	•
RDS	61	73	57	72	•
BPD (need for O_2 at 36 weeks)	7	9	7	8	
IVH (grade 3 or 4)	10	8	11	9	
NEC	18	14	22	14	•
SIRS	44	18	55	20	
Death	10	9	11	10	•

From Andrews WW, et al: The Alabama Preterm Birth study: Polymorphonuclear and mononuclear cell placental infiltrations, other markers of inflammation, and outcomes in 23- to 32-week preterm newborn infants, *Am J Obstet Gynecol* 195:803, 2006.

BPD, Bronchopulmonary dysplasia; *IVH,* intravascular hemorrhage; *NEC,* necrotizing enterocolitis; *RDS,* respiratory distress syndrome; *SIRS,* systemic inflammatory response syndrome.

The lack of consistent association probably reflects the poor precision of the diagnosis of chorioamnionitis and the multiple contrasting effects of fetal exposures that can both injure the lungs and induce their maturation.

CLINICAL LUNG MATURATION

Although chorioamnionitis generally is considered to have adverse effects on the fetus, some reports indicate a clinical benefit. Watterberg and colleagues[15] reported that histologic chorioamnionitis and high IL-1β in airway samples determined on the first postnatal day predicted a decreased incidence of RDS but an increased incidence of BPD. Infants in whom blood cultures were positive for *Ureaplasma urealyticum* also exhibited a lower incidence of RDS but a higher incidence of BPD than in infants with negative blood cultures.[16] Higher cord plasma levels of IL-6 predicted a lower incidence of RDS in another report.[17] Although these small clinical series may reflect a reporting bias, the presence of chorioamnionitis was associated with improved survival for 881 infants born at less than 26 weeks in the United Kingdom and Ireland in 1995.[18] Histologic chorioamnionitis also was associated with less RDS in 446 consecutive singleton births before 32 weeks gestation (see Table 96-1).

The associations of chorioamnionitis with outcomes such as RDS and BPD are likely to be quite complex. In a large epidemiologic report, histologic chorioamnionitis predicted a decreased incidence of BPD unless the infant received mechanical ventilation or developed postnatal sepsis, which in combination with histologic chorioamnionitis increased the risk of BPD.[19] This study suggests that fetal exposure to inflammation can induce clinically apparent maturation of the lung, but that subsequent injury (as from ventilation or sepsis) may increase the injury response, thereby resulting in BPD.

ANIMAL MODELS OF CHORIOAMNIONITIS

Bry and colleagues[20] reported that intraamniotic injections of recombinant IL-1α induced messenger RNA (mRNA) for surfactant proteins SP-A and SP-B and improved the pressure-volume curves in preterm rabbits. IL-1α also increased mRNA for SP-A, -B, and -C in early-gestation explants of rabbit lung.[21] To further explore the relationships between inflammation and maturation, *E. coli* endotoxin was given by intraamniotic injection in sheep.[22] The fetal sheep lung responded to intraamniotic endotoxin with a large increase in compliance and lung gas volume within 5 to 7 days. These improvements in lung function after preterm birth were accompanied by persistent increases in the mRNA for SP-A, -B, -C, and -D, and by hundred-fold increases in the surfactant proteins in the air spaces.[23] The processing of SP-B was induced, the amount of surfactant lipid increased, and the antioxidant enzymes increased.[24]

In the preterm lamb model, "clinical maturation" was a late response of the lung to inflammation (Figure 96-2). Within hours of the intraamniotic injection of endotoxin, white cells producing the mRNA for the proinflammatory cytokines IL-1β, IL-6, and IL-8 infiltrated the chorioamnion and subsequently appeared in the amniotic fluid.[25] The cells in amniotic fluid expressed the mRNA for IL-1β and IL-8 for at least 1 week after the endotoxin exposure. Within 5 hours of intraamniotic endotoxin administration, the fetal lung contained activated granulocytes, and the airways expressed heat shock protein 70 (HSP70).[26] The amount of H_2O_2 in cells lavaged from the lungs was maximal at 24 hours, and both the lung tissue and lavage cells expressed proinflammatory cytokines.[27] Apoptosis increased at 24 hours and cell replication increased by 72 hours. The fetal lung exhibited an acute but modest generalized inflammatory response that resolved to "clinical maturation" in that lung function was strikingly improved after preterm delivery and ventilation.

The lung maturational response in the preterm lamb model did not occur in the absence of chorioamnionitis and lung inflammation. A low dose of endotoxin that induced very mild inflammation did not result in lung maturation.[25] When inflammatory cell recruitment to the lung was blocked with an anti-CD18 antibody, the lung maturational changes did not occur.[28] The inflammation in the chorioamnion and lung did not result in increased plasma cortisol or a significant systemic inflammatory response.[22] Therefore the inflammation-mediated mechanisms for inducing lung maturation were independent of the adrenal axis. The fetal sheep responded to intraamniotic administration of recombinant ovine IL-1α or IL-1β with chorioamnionitis and lung maturation.[29] Similarly, endotoxin from periodontal organisms and chronic fetal colonization with live *Ureaplasma* organisms caused lung inflammation with improved lung function.[30,31] When chorioamnionitis was induced by intraamniotic endotoxin, it had no effect on the fetal lung when it was surgically isolated from the amniotic fluid.[32] By contrast, endotoxin infused over 24 hours into the fetal lung fluid resulted in striking lung maturation. These findings show that endotoxin, other inflammatory mediators, or the products of inflammation can induce lung maturation by direct contact with the fetal lung, indicating that lung maturation seems to be a consistent response of the fetal lung to inflammation.

CYTOKINES AND ALTERED LUNG DEVELOPMENT

Preterm infants in whom proinflammatory cytokines are increased in bronchoalveolar lavage fluid are at increased risk for the development of BPD.[33] A number of animal experiments show

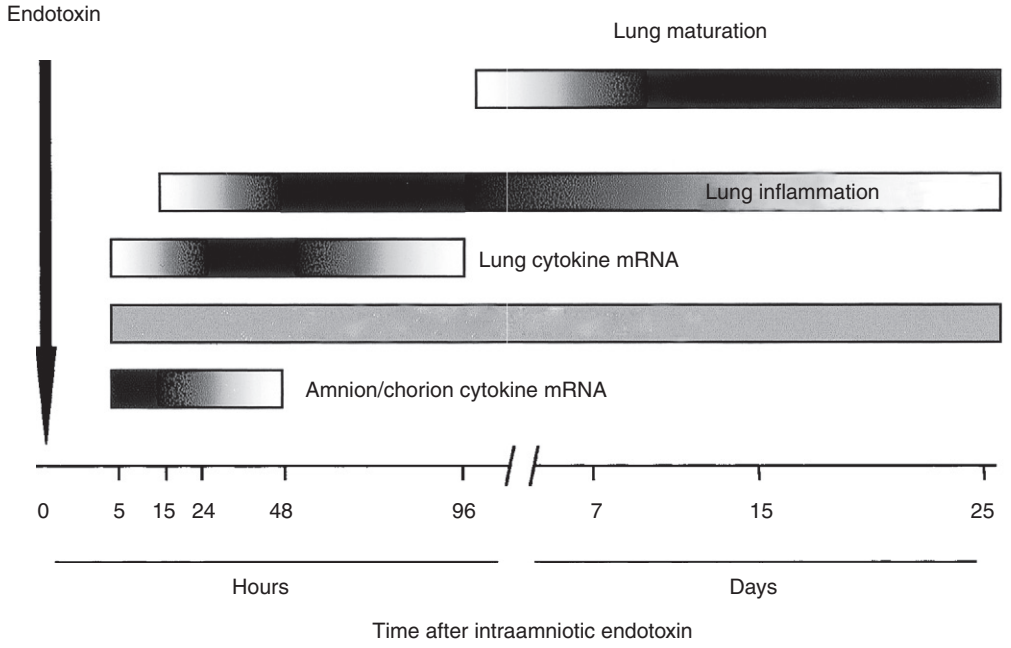


Figure 96-2 The time course of lung inflammation and maturation after intraamniotic endotoxin in preterm lambs.The *shading* within each box is proportional to the intensity and magnitude of the responses. *(Data from Kramer BW, et al: Dose and time response after intra-amniotic endotoxin in preterm lambs,* Am J Respir Crit Care Med *164:982, 2001; Kramer BW, et al: Injury, inflammation and remodeling in fetal sheep lung after intra-amniotic endotoxin,* Am J Physiol Lung Cell Mol Physiol *283:L452, 2002; and Kallapur SG, et al: Intra-amniotic endotoxin: Chorioamnionitis precedes lung maturation in preterm lambs,* Am J Physiol *280:L527, 2001.)*

that exposure of the developing lung to cytokines alters both the lung architecture and expression of components of the surfactant system.Transgenic mice that overexpress TNF-α,[34] transforming growth factor-α (TGF-α),[35] IL-6,[36] IL-13, and interferon-γ[37] have fewer and larger alveoli.Although overexpression of each of these cytokines resulted in different associated lung abnormalities, the common theme was interference with alveolar development. Proinflammatory cytokines also are uniformly elevated in the BPD caused by chronic ventilation of preterm sheep and baboons.[38] Oxidant exposures that disrupt alveolar septation cause increases in proinflammatory cytokines,[39] also identifying proinflammatory cytokines as a consistent association with BPD-like changes in animal models. Cytokines also regulate surfactant homeostasis. Mice that lack granulocyte-macrophage colony-stimulating factor (GM-CSF) or the GM-CSF receptor have alveolar proteinosis as a consequence of decreased catabolism of saturated phosphatidylcholine and SP-A.[40]

Preterm fetal rabbits or lambs exposed to intraamniotic IL-1α or endotoxin exhibit increased surfactant pool sizes and improved lung compliance.[20] IL-1 and endotoxin injected in amniotic fluid induce comparable maturational changes in fetal lamb lung.[29] The mechanism of lung maturation in this model is not known, but both IL-1 and the Toll-like receptors involved in endotoxin signal transduction share common components.[41] Although the endotoxin-induced lung maturation is striking in the preterm lamb, these changes also are accompanied by structural changes of fewer and larger alveoli—a pathologic hallmark of BPD.[32,42]

The fetal lung response to a proinflammatory stimulus also is dependent on the gestational age at exposure and the developmental milieu of the fetus. Exposure of explants of preterm fetal rabbit lung to IL-1 caused increased surfactant protein mRNA, whereas IL-1 inhibited surfactant protein mRNA in explants from term lungs.[21] Similarly, postnatal exposure to endotoxin decreased SP-B mRNA synthesis in mouse lung, whereas intrauterine exposure to endotoxin increased SP-B synthesis in lamb lungs.[23] Thus the preterm infant probably responds to proinflammatory stimuli in a complex fashion with changes in the developmental program of the saccular lung. Some of these changes are beneficial to ex-utero survival of the preterm, whereas others may predispose the fetus to development of BPD.

ANTENATAL INFECTION AND VASCULAR INJURY

Infants with BPD have a decreased and disorganized lung microvasculature with decreased expression of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and its receptors[43,44] (Figure 96-3). This decreased microvascular development also was observed in preterm lamb and baboon models of BPD induced by mechanical ventilation.[38,45] In recombination experiments using rat embryo tissue, epithelial cells were required to maintain expression of fetal liver kinase-1 (Flk-1), a marker of endothelial cells, in the distal lung mesenchymal cells.[46] Alveolar development was disrupted in transgenic mice that overexpressed VEGF or in mice lacking the higher-molecular-weight heparin-binding VEGF isoforms.[47,48]

Abnormalities of pulmonary circulation were associated with lung hypoplasia in animal models.[49,50] In neonatal rat pups in which VEGF signaling was disrupted with pharmacologic inhibitors of the Flk-1/KDR receptor, impaired alveolarization was observed.[51] These experiments demonstrate the phenomenon of cross-talk between the mediators of vascular and alveolar development in the lung. Intraamniotic endotoxin caused impaired alveolarization in lambs[42,52] (Figure 96-4). The chemokines IP-10 (*i*nterferon-γ–inducible *p*rotein-10) and MIG (*m*onokine *i*nduced by interferon-*g*amma), known to inhibit vascular development in other animal models, are robustly induced in the lungs of preterm lambs exposed to intraamniotic endotoxin.[53] The lung inflammatory response in these preterm lambs is

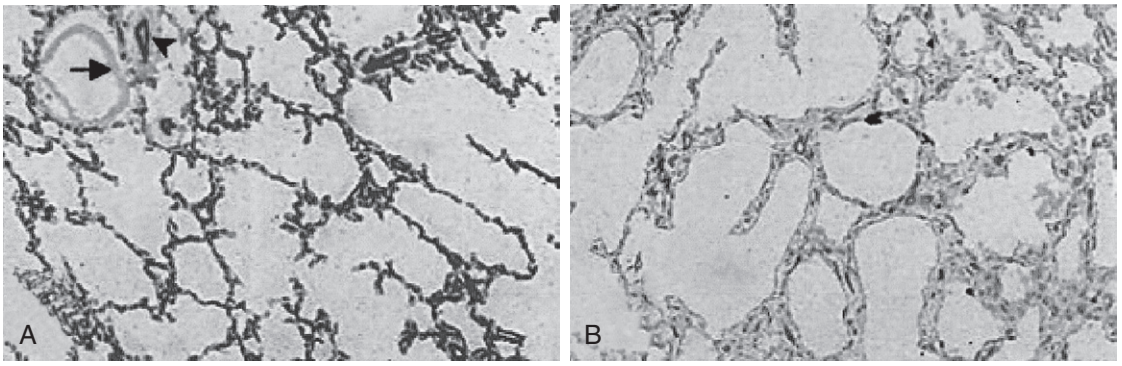


Figure 96-3 Lung sections from infants who died from nonlung causes **(A)** or bronchopulmonary dysplasia (BPD) **(B).** The sections were immunostained for PECAM-1 (CD31) antigen, which is specific for vascular endothelium. Staining for platelet–endothelial cell adhesion molecule (PECAM) was decreased in lungs from infants with BPD, indicating impaired vascular growth. *Arrowhead* shows PECAM staining in the alveolar wall. *(From Bhatt AJ, et al: Disrupted pulmonary vasculature and decreased vascular endothelial growth factor, Flt-1 and Tie-2 in human infants dying with bronchopulmonary dysplasia,* Am J Respir Crit Care Med *164:1971, 2001.)*

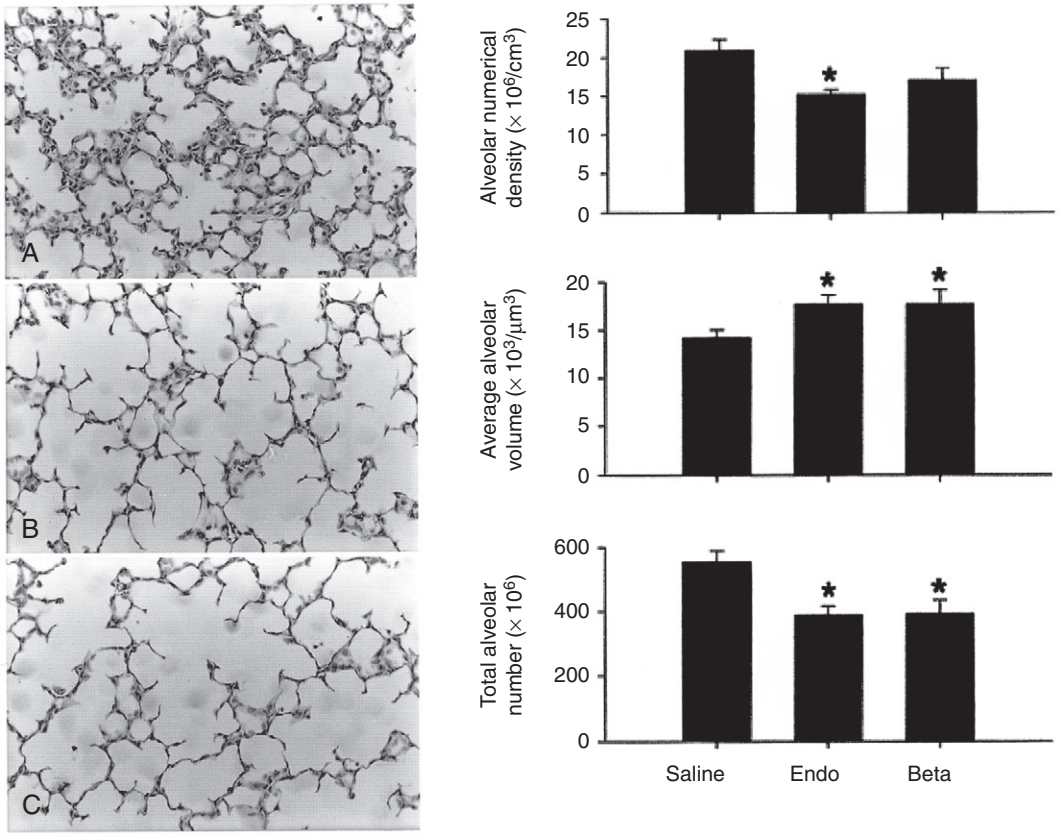


Figure 96-4 Preterm lamb lungs at 125 days gestation, 7 days after administration of control intraamniotic saline **(A),** intraamniotic endotoxin *(Endo)* **(B),** or maternal betamethasone *(Beta)* **(C).** *Bar graphs* give indices of alveolar development in treated versus control groups of preterm lambs. Note that both endotoxin and maternal betamethasone impaired alveolar development. *$p < .05$ versus control data. *(From Willet KE, et al: Antenatal endotoxin and glucocorticoid effects on lung morphometry in preterm lambs,* Pediatr Res *48:782, 2000.)*

accompanied by decreased expression of endothelial proteins in the lung important for blood vessel formation (e.g., VEGF receptor-2 [VEGFR-2], endothelial nitric oxide synthase [eNOS]).[54] The preterm fetal lambs exposed to intraamniotic endotoxin developed smooth muscle hypertrophy in resistance lung arterioles and adventitial fibrosis in large blood vessels. These vascular remodeling changes are similar to those described in human infants with BPD.[55]

IMMUNOLOGIC RESPONSES AFTER FETAL LUNG INFLAMMATION

The inflammatory responses of the fetal lung differ from adult responses in that multiple components that make up a mature inflammatory response to injury are deficient in the former. The preterm fetal lung does not respond to bioactive TNF-α in the amniotic fluid or to TNF-α given intravenously.[56] The preterm

fetal lung contains almost no macrophages or granulocytes, and normal host defense proteins such as SP-A and SP-D are present in very low amounts.[57] Although multiple components of innate immune response are deficient, preterm fetal sheep at 50% gestation can recruit inflammatory cells in the airway and mount an inflammatory response to intraamniotic endotoxin.[52]

Endotoxin tolerance is a state in which the cells have a diminished ability to respond to repeated challenges with endotoxin. Whereas endotoxin tolerance is well described in adult monocytes, little information about endotoxin tolerance in fetuses is available. Exposure of fetal sheep to intraamniotic endotoxin induces an endotoxin tolerance response in systemic blood monocytes early after exposure, followed by an amplification of endotoxin responsiveness by 7 days.[58] Consistent with the endotoxin tolerance response, repeated doses or continuous intraamniotic infusions of endotoxin did not result in severe or progressive lung inflammation and induced endotoxin tolerance in blood monocytes.[52,58,59] As a result, and in contrast with effects of exposure to a single-dose endotoxin, chronic or multiple doses of intraamniotic endotoxin did not increase inhibition of alveolar development or cause any sustained structural abnormalities in the fetal lung.[59] The complex effects of chronic exposure of the fetal lung to antenatal inflammation probably will alter postnatal lung and systemic inflammatory responses. Chronic exposure to inflammation may be quite common in infants born before 30 weeks gestation.

ANTENATAL INFLAMMATION AND POSTNATAL INFLAMMATION

BPD in preterm infants is multifactorial in etiology and results from the interaction of antenatal and postnatal risk factors.[60] In epidemiologic and clinical studies, chorioamnionitis was associated with BPD in some studies[15] but not in others.[7] Multiple reasons for this discrepancy of association between chorioamnionitis and BPD are recognized: (1) Definitions of chorioamnionitis vary (clinical chorioamnionitis versus histologic chorioamnionitis); (2) different consequences may result from chorioamnionitis with different organisms (*Ureaplasma* versus gram-negative organisms); and (3) differing degrees of antenatal and postnatal inflammation are seen in different studies. A way to interpret the interaction between antenatal and postnatal inflammation is by analogy to the "multiple-hit hypothesis" (see Figure 96-1). Antenatal inflammation may "prime" the inflammation, leading to exacerbation of injury from postnatal inflammation.

Experimental evidence from the fetal sheep model supports a "multiple-hit" mechanism. Exposure to intraamniotic endotoxin 30 days before preterm delivery increased postnatal lung inflammation induced by mechanical ventilation in preterm lambs, whereas exposure to endotoxin 4 days before delivery did not amplify the ventilation-induced lung injury response.[61,62] The timing of the onset of antenatal inflammation may be important for modulating injury responses to postnatal inflammation. In another experiment, intraamniotic endotoxin caused a tolerance-type or "down-regulated" endotoxin responsiveness of blood monocytes 2 days after exposure but an amplified endotoxin response 7 to 14 days after exposure.[58] Thus, in a developing fetus, exposure to antenatal infection may cause either innate immune paralysis or augmentation of innate immune responses, depending on the timing of the insult.

Another variable that may contribute to lung injury is the fate of inflammatory mediators that are in the lungs of the infant at delivery. The uninjured fetal lung is quite impermeable to proteins and large-molecular-weight substances such as endotoxin. In two different studies, however, gentle mechanical ventilation of the preterm lamb lung with endotoxin or IL-1 in the air spaces resulted in transfer of the proinflammatory mediators to the systemic circulation, which caused a systemic inflammatory response.[63,64] In an epidemiologic study, the risk of BPD did not increase on exposure to chorioamnionitis but was increased after exposure to chorioamnionitis and mechanical ventilation after birth.[19]

GLUCOCORTICOID EFFECTS ON THE FETAL LUNG

Although antenatal glucocorticoids decrease the incidence of RDS, they do not decrease the incidence of BPD in survivors.[65] This lack of clear benefit for prevention of BPD has been attributed to the increased survival of very preterm infants, who are most at risk for BPD. An alternative possibility, however, is the diminished alveolar septation observed after antenatal glucocorticoid treatment. Exposure of fetal monkeys or sheep to glucocorticoids decreased the lung mesenchyme, thinned the alveolus-capillary barrier, increased potential lung gas volume, but inhibited alveolar septation[42,66] (see Figure 96-4). Monkeys exposed to a relatively high dose of antenatal glucocorticoids had functionally more mature lungs that held more gas after preterm birth. However, when delivery was at term, the lungs were smaller, with fewer and larger alveoli.[67] Alveolar septation also was decreased in the fetal sheep lung by maternal betamethasone.[68] The magnitude of the arrest in alveolar septation was similar for both antenatal intraamniotic endotoxin and maternal betamethasone in sheep.[42] Although the mechanisms resulting in altered alveolar septation are not known, it is provocative that both proinflammatory and antiinflammatory stimuli that induce clinical lung maturation also have as an adverse effect the inhibition of alveolar septation. As with endotoxin, antenatal glucocorticoids may alter the fetal lung responses to injury and initiate the anatomic changes characteristic of BPD.

INTERACTIONS OF ANTENATAL GLUCOCORTICOIDS AND INFLAMMATION

Most women at risk for very preterm delivery with chronic histologic chorioamnionitis and low-grade infection will be asymptomatic. On the basis of current recommendations, such women will receive antenatal glucocorticoids without an amniocentesis to evaluate for infection or inflammation. This clinical practice is based on data from well-performed studies indicating that antenatal glucocorticoids improved neonatal outcomes despite presence of histologic chorioamnionitis or preterm rupture of membranes.[17,69,70] Antenatal glucocorticoids decreased plasma IL-6 and IL-8 in newborns born to mothers with chorioamnionitis, suggesting that the fetal inflammatory response can be suppressed.

Interactions between experimental chorioamnionitis and antenatal glucocorticoids have been minimally explored. Fetal sheep responded to antenatal betamethasone with improved lung function and decreased body weight and thymus gland weight.[71] By contrast, the more striking lung maturation induced by intraamniotic endotoxin occurred without a decrease in body or thymic weight. Exposure of the fetus to antenatal glucocorticoids and intraamniotic endotoxin resulted in a robust lung maturation response without adverse effects on body and thymic weights. In contrast with the additive effects of maternal betamethasone and intraamniotic endotoxin on fetal sheep lung maturation, the two exposures had opposing effects on fetal lung inflammation. Consistent with its widely known role as an antiinflammatory agent, maternal betamethasone initially suppressed the endotoxin-induced inflammation in the fetal lungs.[72] The new and counterintuitive information from experiments in fetal sheep was that maternal betamethasone caused a subsequent rebound in endotoxin-induced lung inflammation (Figure 96-5). The implications of this finding for clinical practice are not clear, but the emerging evidence highlights the complexity of interactions between these common exposures in preterm neonates. No experimental information is available regarding how these combined mediator systems may potentially influence postnatal lung development.

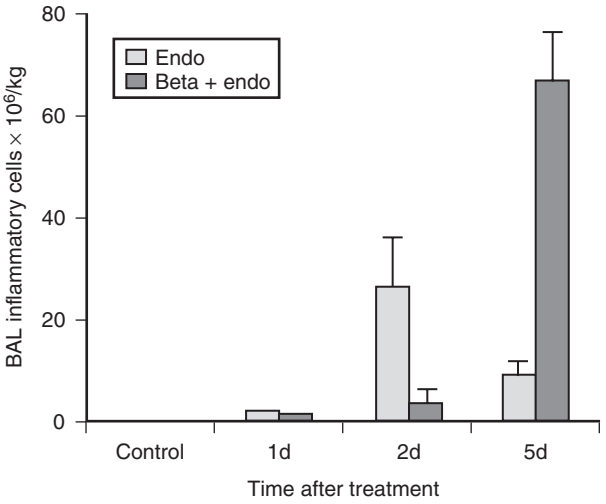


Figure 96-5 The time course of lung inflammation after exposure to intraamniotic endotoxin *(endo)* or maternal betamethasone and endotoxin *(beta + endo).* Of note, betamethasone initially suppressed endotoxin-induced lung inflammation but at 5 days increased the inflammation. *(Data from Kallapur SG, et al: Maternal glucocorticoids increase endotoxin-induced lung inflammation in preterm lambs,* Am J Physiol Lung Cell Mol Physiol *284:L633, 2003.)*

The consequence of less RDS may be more BPD, or greater severity of BPD, if inflammation is accompanied by persistent adverse effects on alveolar septation.

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Eduardo Bancalari

Pathophysiology of Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia (BPD) is one of the most frequent sequelae in preterm infants who survive after prolonged mechanical ventilation. It is characterized by chronic respiratory failure, and in more severe cases it frequently is associated with pulmonary hypertension and cor pulmonale. The infants with BPD, as described originally, required prolonged mechanical ventilation because of severe respiratory failure resulting from hyaline membrane disease.[1] Today, most infants who have BPD are small premature babies who require prolonged mechanical ventilation because of poor respiratory effort or apnea.[2] Important pathogenic factors that contribute to chronic lung damage include incomplete lung development associated with prematurity, mechanical trauma from positive-pressure ventilation, oxygen toxicity, prenatal and postnatal infections, increased pulmonary blood flow and pulmonary edema secondary to patent ductus arteriosus (PDA), and airway damage and obstruction. The interaction of these and other possible factors leads to the severe morphologic and functional alterations of the lungs observed in these infants. Whereas in the originally described form of BPD the main alterations in lung morphology are emphysema, fibrosis, and marked airway epithelial changes, the more common milder forms of BPD seen today are characterized mainly by an arrest of alveolar and vascular development.[3-7]

Although mechanical trauma and oxygen toxicity are still considered important pathogenic factors in the development of the more severe form of BPD, in recent years, with the increased use of prenatal steroids and the administration of exogenous surfactant, the initial respiratory illness usually is milder, so infants are exposed to much lower airway pressures and oxygen concentrations. Nevertheless, many of the more immature infants, with relatively higher oxygen requirements, will show persistent radiographic changes compatible with BPD. Although positive airway pressure and oxygen exposure at these lower levels may still be sufficient to damage the immature lung, it is likely that other factors also play an important role in the development of the milder lung damage observed in infants today.

Substantial evidence indicates that inflammation plays a major role in the pathogenesis of BPD.[8] The pulmonary inflammatory response can be triggered by a number of factors, including ventilation with excessive tidal volumes,[9] oxygen free radicals,[10-12] increased pulmonary blood flow secondary to a patent ductus arteriosus,[13] and a variety of antenatal and postnatal infections.[14-20] The possible role of antenatal infections has been suggested by published studies showing an increased risk for BPD in infants born to mothers with evidence of chorioamnionitis.[21-23] Several inflammatory cytokines were found in higher

concentrations in the amniotic fluid of mothers of infants who subsequently developed BPD.[24,25] In addition, several reports have suggested a higher risk for BPD in infants whose airways are colonized with *Ureaplasma urealyticum* or other bacteria.[26-29] A significant increase in inflammatory cells, eicosanoids, and various cytokines has been demonstrated in the airways of infants in whom BPD develops subsequently.[30-37] The increase in cytokine concentrations has been documented early after birth, supporting the contention that the inflammatory process can start before birth and is secondary to an infection of the amniotic cavity and the fetal lung. Among the markers of inflammation that have been found in high concentrations in tracheobronchial secretions of infants with later BPD are neutrophils,[30,31] macrophages,[32] leukotrienes,[34] platelet-activating factor (PAF),[33] interleukin (IL)-6,[35,37] IL-8,[36] and tumor necrosis factor (TNF-α).[37] Evidence of pulmonary alveolar macrophage (PAM) activation also has been reported in infants in whom BPD developed subsequently.[38] These activated PAMs have been suggested as a source of neutrophil chemoattractants, especially when exposed to hyperoxia.[39] Neonates with subsequent BPD also have elevated concentrations of fibronectin in lung lavage fluid.[40,41] This large-molecular-weight protein is released from PAMs, epithelial and endothelial cells, and fibroblasts and is associated with the development of pulmonary fibrosis. An increase in elastase and an imbalance between elastase and α_1-proteinase inhibitor (α_1-PI) in the lung also has been postulated as a possible mechanism for the development of neonatal lung injury.[42-45] Urine excretion of elastin degradation products is greater in neonates with subsequent BPD than in control infants.[46] This finding is of particular concern in light of the evidence of a marked reduction in alveolar septation in lungs of infants with BPD.[47,48]

In extremely low-birth-weight infants, the presence of hospital-acquired systemic infections and a symptomatic PDA are strong predictors for the development of BPD.[2,49,50] Furthermore, occurrence of both infection and PDA at the same time produces a synergistic interaction that further increases the risk for developing BPD[51] (Figure 97-1). As a consequence of the left-to-right shunting through the PDA, pulmonary blood flow and lung fluid increases, negatively affecting lung mechanics and gas exchange, thereby increasing the risk for BPD.[52] The presence of a PDA also has been associated with elevated concentrations of myeloperoxidase in the tracheobronchial fluid, suggesting that

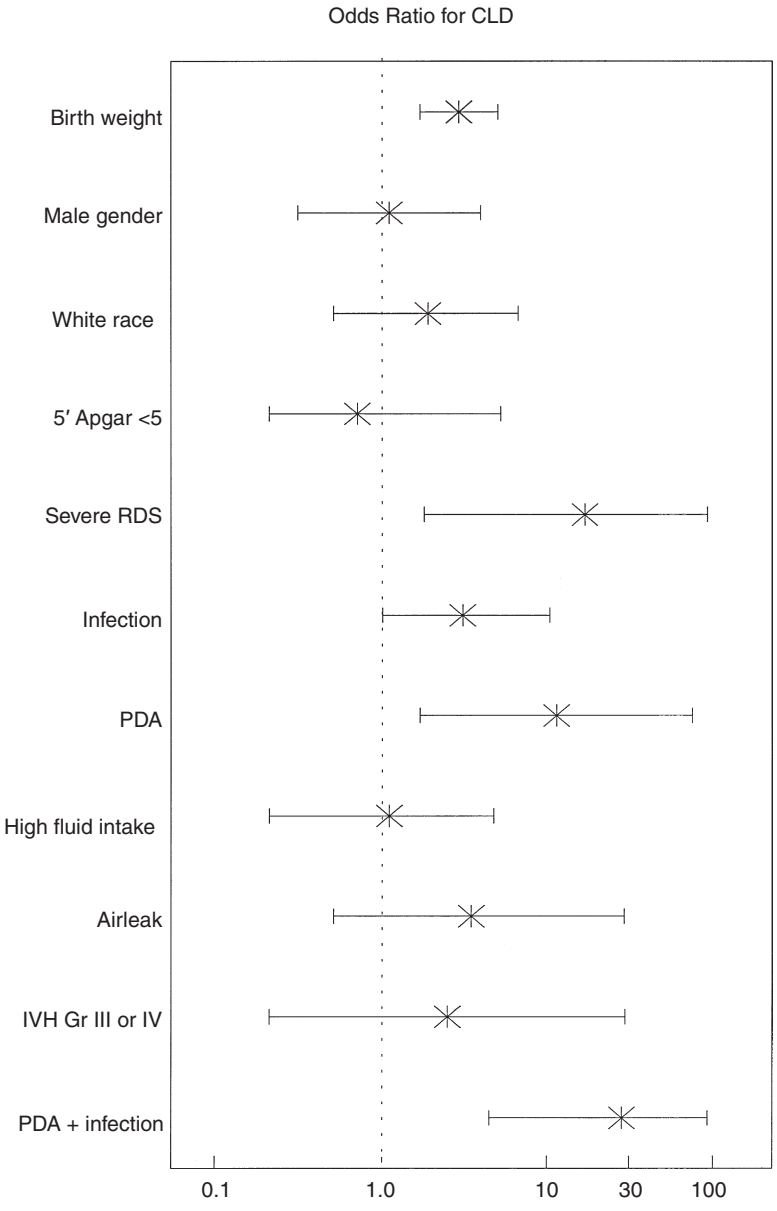


Figure 97-1 Odd ratios (∗) and their 95% confidence intervals for bronchopulmonary dysplasia (BPD), with logistic regression analysis of data for infants who survived 28 or more days after birth (n = 105). *(From Gonzalez A, et al: Influence of infection on patent ductus arteriosus and chronic lung disease in premature infants weighing 1000 grams or less,* J Pediatr *128:470, 1996.)*

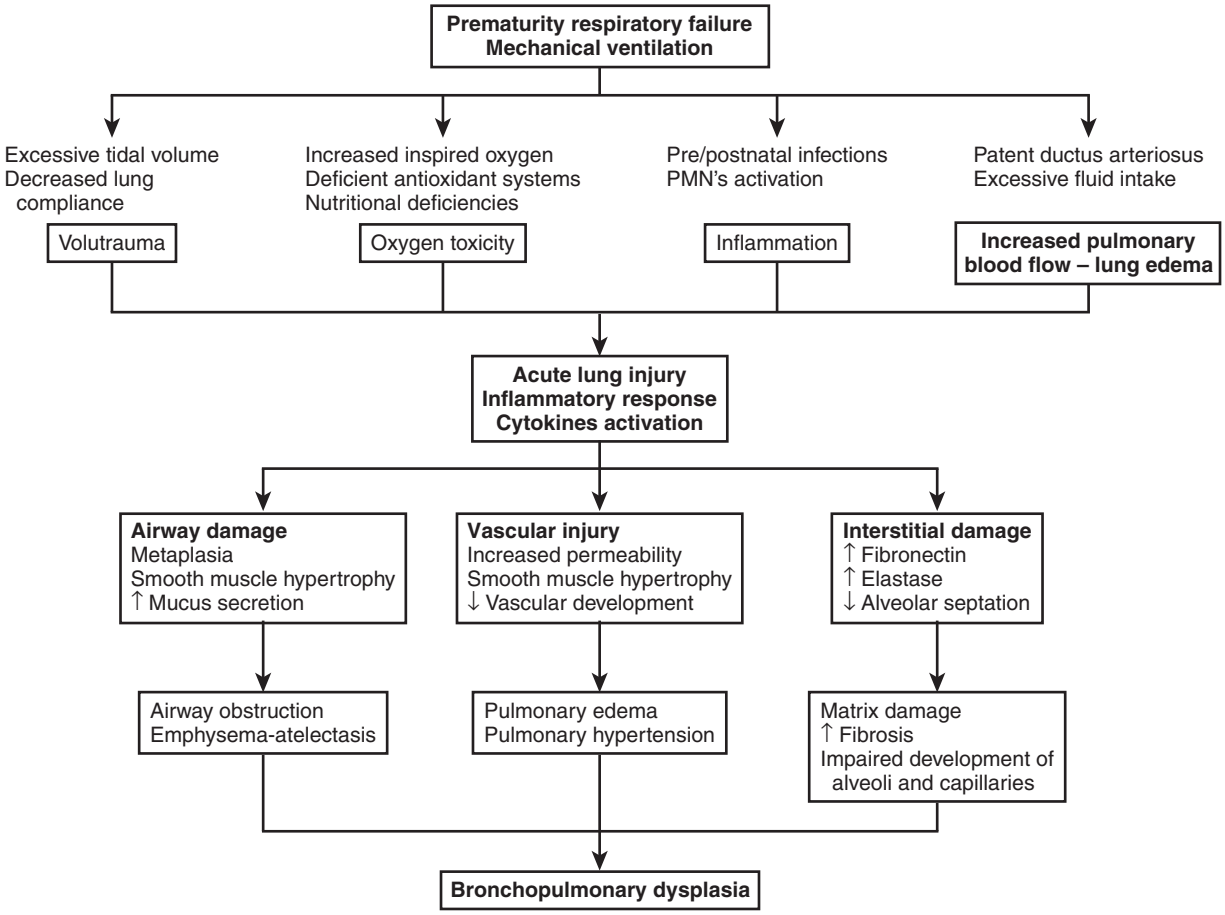


Figure 97-2 Pathophysiology of bronchopulmonary dysplasia.

the increased pulmonary blood flow may result in damage to the pulmonary endothelium with adhesion and migration of polymorphonuclear leukocytes (PMNs [i.e., polymorphonuclear neutrophils]) into the lung tissue.[13]

Other factors have been proposed to have a role in the pathogenesis of BPD. Because of evidence that vitamin A levels are lower in infants in whom BPD develops than in those who recover without chronic lung damage, vitamin A deficiency has been proposed to play a role in BPD.[53] This possible association is supported by the similarities between some of the airway epithelial changes observed both in BPD and in vitamin A deficiency, and by clinical evidence that vitamin A administration in the first weeks of life affords some degree of protection against BPD.[54,55] Another factor suggested to play a role in the development of BPD in extreme premature infants is early adrenal insufficiency. Among infants with lower cortisol levels in the first week of life, the incidence of lung inflammation and BPD is increased, and early treatment with low-dose hydrocortisone increases survival without BPD.[56-58]

The major difficulty in the prevention of BPD is that as the lung damage progresses, a deterioration in lung mechanics and gas exchange occurs that makes necessary the use of mechanical ventilation with higher peak airway pressures and inspired oxygen concentrations. A vicious circle is thereby created in which the required treatment for the respiratory failure, mechanical ventilation and increased inspired oxygen, induces more lung damage and exacerbates the respiratory impairment. Figure 97-2 summarizes some of the most important pathogenic factors in BPD.

PULMONARY FUNCTION IN INFANTS WITH BRONCHOPULMONARY DYSPLASIA

The alterations in pulmonary function in infants with BPD are nonspecific and reflect the severe disruption of lung growth and architecture that occurs in these infants. The degree of abnormality in lung function may range from mild to severe, paralleling the clinical and radiographic presentation. Very few studies have described the early changes in lung function in infants who subsequently develop BPD, or that have followed longitudinally the course of lung function in these infants.

Lung Function in Early Bronchopulmonary Dysplasia

Infants in whom BPD ultimately develops appear to have worse lung function early in the evolution of their respiratory failure than infants who recover without lung sequelae after their initial RDS. Higher airway resistance was noted during the first weeks of life in infants in whom BPD developed subsequently than in infants who recovered without lung damage.[59,60] This finding suggests that early airway obstruction may be a marker or a predisposing factor for the development of more severe pulmonary damage. Measurements of lung compliance during the first week of life also have shown lower values in infants in whom BPD developed later on.[61-63] For this reason, lung mechanics measurements during the first week of life have been used to improve the accuracy of BPD prediction models.[64,65] Although the added predictive value of pulmonary resistance has been inconsistent, models that include gestational age and

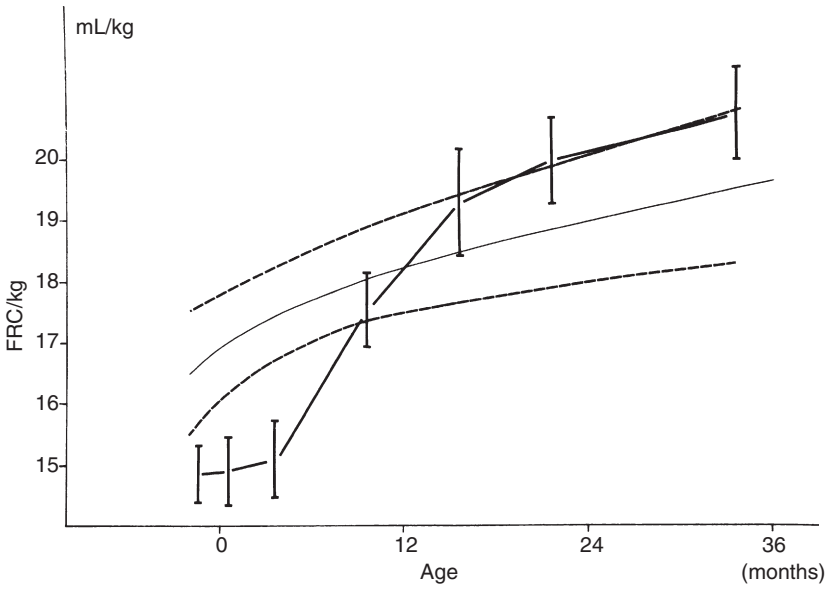


Figure 97-3 Sequential measurements of functional residual capacity *(FRC)* per kilogram in infants with chronic lung disease (mean ± SE) *(heavy line)*. Measurements were shifted to left to correct for preterm birth. For comparison, curve for normal infants *(thin line)* and its 95% confidence limits *(dashed lines)* are shown. *(From Gerhardt T, et al: Serial determination of pulmonary function in infants with chronic lung disease,* J Pediatr *110:448, 1987.)*

pulmonary compliance have displayed the highest predictive accuracy. The worse initial respiratory function in infants with subsequent BPD is most likely to reflect the severity of the initial acute respiratory illness.

Lung Function in Established Bronchopulmonary Dysplasia

Ventilation

Tidal volume is normal or reduced and respiratory rate is increased in most of these infants, resulting in a normal or increased minute ventilation.[66] The increased dead space ventilation that can result will contribute to the hypercapnia observed in infants with severe BPD.

Distribution of Ventilation

The morphologic alterations in the lungs of infants with BPD result in a severe disruption of the relationship between ventilation and perfusion. The damage to the small airways produces different time constants in different areas of the lungs, thereby altering the distribution of the inspired gas.[67] The disturbed ventilation-perfusion relationships lead to an increased arterial-alveolar gradient for carbon dioxide in areas that are ventilated but poorly perfused (high V_A/Q ratio) and an increased alveolar-arterial oxygen gradient caused by poorly or nonventilated areas that receive blood flow (low V_A/Q ratio).[68] Studies evaluating regional excretion of xenon have demonstrated very limited ventilation of the overdistended areas, whereas perfusion was relatively preserved.[69] Nitrogen clearance delay studies have confirmed the presence of slowly ventilated compartments in infants with severe BPD.[67]

Lung Volume

Lung volume measurements in infants with BPD have yielded variable results depending on the age of the infant. Functional residual capacity (FRC) values below normal have been reported during the first month of life, but values increased to above normal by the age of 6 to 16 months[70-72] (Figure 97-3). The later increase in lung volume in infants with BPD may reflect gas trapping secondary to small airway obstruction.

Lung Compliance

Dynamic lung compliance is consistently decreased in infants with BPD[70,71] (Figure 97-4). This decrease may reflect changes in the elastic properties of the lung secondary to fibrosis and interstitial fluid accumulation. Compliance also may be reduced by alterations in surfactant metabolism, which have been described in infants with BPD.[73] The decrease in dynamic compliance also reflects the increase in small airway resistance that produces frequency dependence of compliance. Overdistention of portions of the lung from gas trapping can further contribute to the decrease in compliance.

Therapeutic intervention with diuretics, bronchodilators, and systemic steroids results in a reduction in airway resistance with improved lung compliance in these infants.[74-78] Similar results have been reported after the administration of inhaled nebulized furosemide[79] or beclomethasone.[80]

Airway Resistance

A consistent change in lung function in infants with severe BPD is increased airway resistance (see Figure 97-4). This can lead to severe maldistribution of the inspired gas,[67] lung overdistention from gas trapping, and hypoventilation with hypercapnia secondary to increased work of breathing.

The mechanisms for increased airway resistance include several factors that contribute to airway damage and obstruction. During the initial stages of BPD, histopathologic changes include epithelial cell damage with hyperplasia and squamous metaplasia of the airway epithelial lining. Infection and inflammatory reaction in the airways increase mucus secretion. In addition, clearance of mucus may be decreased as a result of depression of ciliary activity. Infants with severe BPD exhibit hypertrophy of the smooth muscle surrounding the airways[1,81]; this development plays an important role in the increased airway resistance and airway hyperreactivity observed in these infants. Increased production of inflammatory mediators, such as leukotrienes and PAF, also may contribute to airway smooth muscle constriction and increased pulmonary resistance.[34,82,83] A more frequent family history of asthma in infants with BPD raises the possibility that a genetically determined airway hyperreactivity also may contribute to the development of BPD in some cases.[84,85] The increase in airway resistance is not specific to infants with BPD but is observed in most premature infants who receive mechanical ventilation, regardless of whether or not BPD develops subsequently.[86] The increase in airway resistance combined with lower compliance is responsible for a marked increase in the work of breathing and for the alveolar hypoventilation and carbon dioxide retention that are characteristic of these infants. The degree of airway damage is not uniform throughout the lung; this

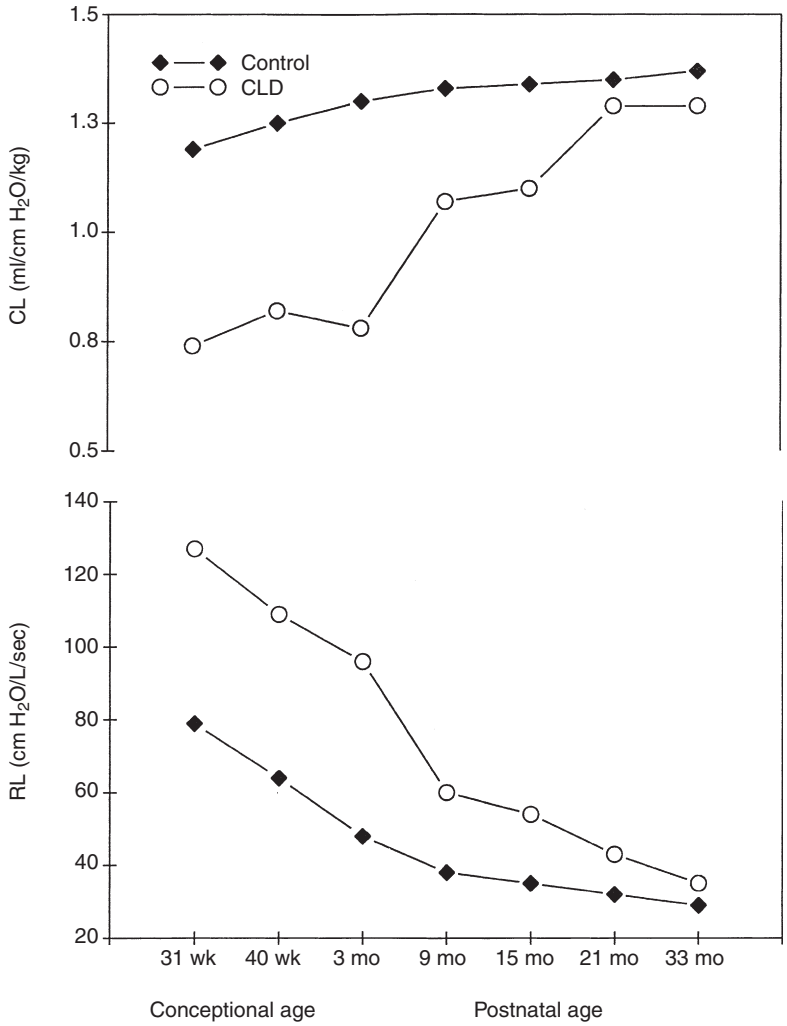


Figure 97-4 Sequential measurements of lung compliance and resistance in infants with chronic lung disease *(CLD)* and in normal controls. *(Modified from Gerhardt T, et al: Serial determination of pulmonary function in infants with chronic lung disease,* J Pediatr *110:448, 1987.)*

variability contributes to the abnormal distribution of ventilation and the development of areas of overdistention alternating with areas of collapse observed in cases of severe BPD.

The exact location of the obstruction has not been defined, but forced expiratory flows at FRC are decreased, suggesting that the main obstruction is at the level of the small airways.[87-89] In infants with severe BPD, large airways also are compromised and may demonstrate increased collapsibility that manifests during active expiration as a result of bronchomalacia.[90,91] This alteration is responsible for the severe deterioration in gas exchange observed in some of these infants during periods of agitation, which results in generation of positive intrathoracic pressure. These episodes can be ameliorated with use of sedation or by maintaining a relatively high positive end-expiratory airway pressure.[92,93]

Of interest are observations that demonstrate increased airway resistance and FRC in infants with BPD in response to a reduction in their inspired oxygen concentration.[94,95] This physiologic response also might explain the episodic airway obstruction often observed in infants with BPD. Cold air or methacholine challenge also produces a marked increase in pulmonary resistance in these patients.[96-98]

The increased airway resistance in infants with BPD is partially reversible with bronchodilators[99-105] and after diuretic therapy.[76,106-110] This dual effect suggests that the increased airway resistance is due to a combination of airway hyperreactivity and interstitial pulmonary edema, both of which may play a role in the obstruction of the small airways. The administration of systemic or inhaled steroids also has been demonstrated to reduce the airway obstruction in infants with BPD.[75,78,80]

Gas Exchange

Most infants with severe BPD exhibit marked hypoxemia and hypercapnia and require supplemental oxygen to maintain a normal Pa_{O_2}. This hypoxemia is due mainly to a reduced ventilation-perfusion ratio and alveolar hypoventilation.[68] The oxygen requirement decreases gradually as the disease process subsides but may increase during sleep, feedings, physical activity, or episodes of pulmonary infection or edema.

The increased Pa_{CO_2} is secondary to alveolar hypoventilation and to an increased arterial-alveolar carbon dioxide gradient produced by a mismatch of ventilation and perfusion and increased alveolar dead space. The chronic hypercapnia is often accompanied by an increase in serum bicarbonate concentration that tends to compensate for the respiratory acidosis. This increase in plasma base can be exaggerated by administration of diuretics, which frequently are used in infants with BPD.

Metabolic Rate

Infants with BPD have an increased metabolic rate.[111] This increase in oxygen consumption is due, at least in part, to the increased work of breathing and is one of the factors that may interfere with normal growth in these infants.[112] The increased metabolic rate also can impose an extra load on the respiratory system, contributing to respiratory failure in infants with severe BPD. This impairment may become evident when carbon dioxide

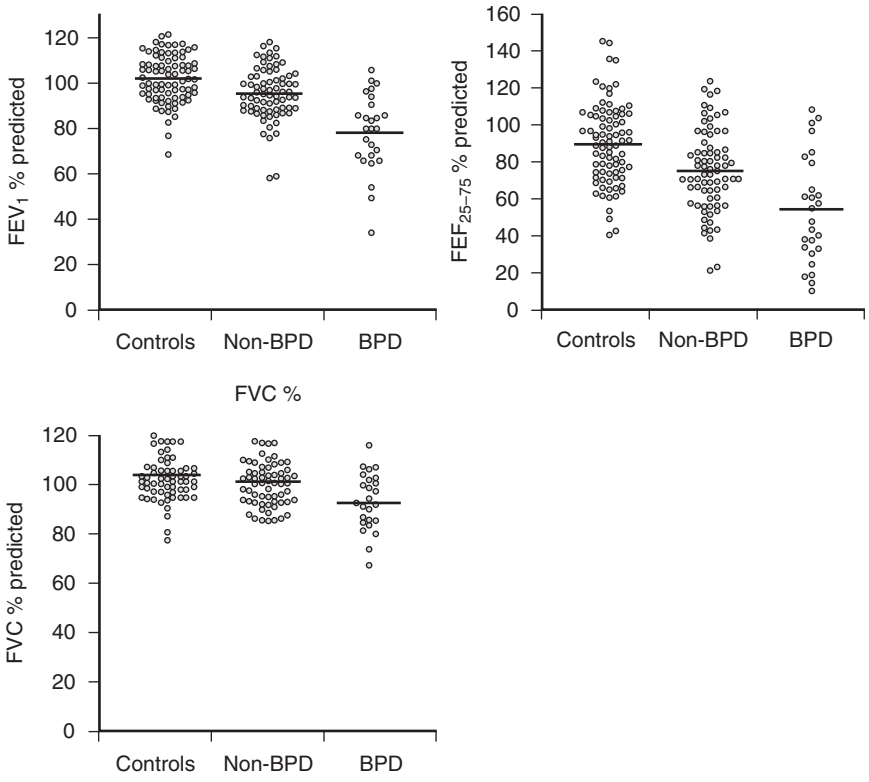


Figure 97-5 Spirometric values, expressed as percent predicted for children in the three study groups: BPD, non-BPD, and controls. Each *point* represents an individual subject, and the *horizontal lines* are the mean values. *BPD,* Bronchpulmonary dysplasia. *(Modified from Kennedy JD, et al: Effects of birthweight and oxygen supplementation on lung function in late childhood in children of very low birth weight,* Pediatr Pulmonol *30:32, 2000.)*

production is suddenly increased, such as during glucose loading, which can lead to aggravation of hypercapnia in these infants.[113]

Long-Term Lung Function in Infants With Bronchopulmonary Dysplasia

Several studies have evaluated long-term lung function in infants with BPD. Because the initial damage occurs at a critical time when the lung is in one of the most active phases of growth and development, it is now clear that many of the alterations in lung structure and function persist through adulthood.

Gerhardt and colleagues[71] performed serial pulmonary function evaluations in a group of infants up to 3 years of age who had relatively mild BPD. Lung function showed gradual improvement over time, and at the end of the follow-up period, the values for compliance and resistance were close to the normal range (Figure 97-4). In infants who had severe BPD, persistent alterations in lung function have been reported beyond 3 years of age[114-119] whereas in some patients, abnormalities were still present at the end of the first and second decades of life, indicating that the lung damage, when severe, persists into adulthood[96,97] (Figure 97-5).

Morphometric studies performed in lungs from children who died with severe BPD between the ages of 2 and 28 months revealed a striking reduction in the number of alveoli from that typical for the lungs of control subjects.[48] This abnormality results in a significant decrease in the lung surface area available for gas exchange. By contrast, pulmonary function studies done in children with a history of BPD who have reached school age suggest continued lung growth, as indicated by an increase in total lung capacity and improved airway function.[115] Although the decreased lung compliance and changes in lung volume usually normalize over time, the increased airway resistance and flow limitations seem to persist longer and through adulthood in those infants with more severe lung disease.[88,72,116-118] The degree of flow limitation at the age of 11 years has been correlated with the duration and amount of supplemental oxygen received during the neonatal period[119] (Figure 97-6).

Surprisingly, despite these persistent abnormalities in lung function, survivors with BPD do not always have a significant reduction in exercise capacity when compared with healthy term infants or preterm babies without lung disease.[116,120] However, reduced soluble gas transfer at rest and during exercise has been reported in school-aged children with a history of BPD.[121]

Cardiovascular Function in Infants With Bronchopulmonary Dysplasia

Pulmonary Hypertension—Cor Pulmonale

Pulmonary hypertension and right ventricular hypertrophy commonly are observed in infants with severe BPD, and in many instances, pulmonary hypertension is responsible for a gradual deterioration in the clinical condition or the demise of these infants.[81,122] Pulmonary hypertension has been documented by direct pressure measurements during cardiac catheterization and indirectly by echocardiography.[122-124] The specific mechanisms that result in increased pulmonary vascular resistance in these infants are many and include alveolar hypoxia, increased alveolar pressure and overdistention, and release of mediators of inflammation such as PAF and leukotriene B_4.[33]

Postmortem examination of the lungs of infants with severe BPD frequently reveals hypertrophy of the smooth muscle in the pulmonary vessels and muscle extension into intraacinar arteries.[81] Some cases also feature evidence of thromboembolism in the lumina of pulmonary vessels.[125] A derangement in the growth and development of the pulmonary vessels also has been described in these infants and is likely to play a major role in the increased pulmonary vascular resistance observed with severe BPD.[126] The bronchial arteries in infants with BPD are abnormally prominent, and communications exist between those arteries and the pulmonary circulation through precapillary collaterals.[127] These anastomoses may develop in preexisting channels that subsequently dilate, or they may occur in granulation tissue seen in the lungs of patients with BPD. These systemic-to-pulmonary communications may aggravate the existing pulmonary hypertension.

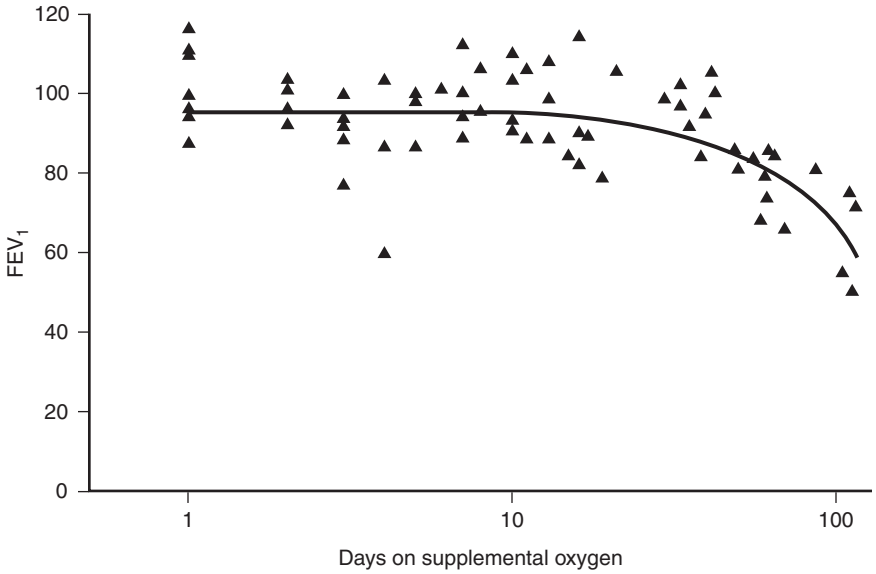


Figure 97-6 Relation between forced expiratory volume in 1 minute (FEV_1) and duration of supplemental oxygen (in log days) during the neonatal period. *Solid line* shows fitted values derived by use of the power function $FEV_1 = \beta_0 + \beta_1 r^{(\text{days on oxygen})}$. *(Modified from Kennedy JD, et al: Effects of birthweight and oxygen supplementation on lung function in late childhood in children of very low birth weight,* Pediatr Pulmonol *30:32, 2000.)*

The pulmonary hypertension in infants with BPD initially is responsive to an increase in the inspired oxygen concentration, demonstrating a reactive pulmonary vascular bed.[123,124,128] Nevertheless, the values frequently remain substantially above normal, reflecting the loss in vascular cross-sectional area secondary to inadequate development or structural remodeling of the pulmonary vascular bed, and in advanced BPD the hypertension becomes unresponsive to all available therapeutic interventions.

Systemic hypertension also has been described in infants with BPD.[129] The mechanism for the systemic hypertension is not clear, but the derangement may contribute to the left ventricular dysfunction and hypertrophy observed in many of these patients.[130] This hypertrophy also can be secondary to an increased afterload of the left ventricle. Left ventricular overload results from the higher negative intrathoracic pressure generated by these infants to overcome the increased mechanical impedance of their respiratory system. Right ventricular hypertrophy also can interfere with left ventricular function, contributing to the hypertrophy of this ventricle. A decreased pulmonary vascular clearance of catecholamines has been reported in infants with severe BPD and also may contribute to the systemic hypertension observed in some of these patients.[131]

The use of vasodilators such as the calcium channel blocker nifedipine can be effective in improving cardiovascular function in infants with severe BPD.[132,133] Inhaled nitric oxide (NO) and phosphodiesterase inhibitors also have been used in infants with BPD to decrease pulmonary vascular resistance and improve ventilation perfusion matching. Although results have not been consistent, some studies have shown an improvement in oxygenation during NO administration.[134-136] NO also may modulate the inflammatory process in the lung, and its early administration in preterm infants with respiratory failure has resulted in reduced incidence of BPD.[137,138] These results are not consistent enough, however, to recommend the routine administration of NO in preterm infants.

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98 Liquid Ventilation

Thomas H. Shaffer and Marla R. Wolfson

Respiratory insufficiency during early development, whether resulting from immaturity of pulmonary structure and biochemical processes or from aspiration syndromes, calls for the use of assisted ventilation techniques. Exogenous surfactant replacement therapies have demonstrated improvement in gas exchange, decreased ventilatory requirements, and reduction in mortality. Pulmonary function–guided alterations in conventional ventilatory techniques and high-frequency ventilation can facilitate gas exchange at reduced inflation pressures; aggressive measures such as extracorporeal life support eliminate the requirement for pulmonary inflation pressures. In spite of improved survival of the infant with respiratory insufficiency, significant morbidity persists, resulting from barotrauma leading to chronic pulmonary sequelae.[1] Alternative means to support pulmonary gas exchange while preserving lung structure and biochemical development are still required.

Fluid breathing (liquid-assisted ventilation) uses a liquid to replace nitrogen gas as the carrier for oxygen (O_2) and carbon dioxide (CO_2). The air-liquid interface at the alveolar-capillary surface is eliminated, reducing pressure requirements to inflate the lung. To date, perfluorochemicals (PFCs) are the most promising breathing media liquids because of their specific physiochemical properties of low surface tension and high solubility for respiratory gases, as well as their chemically and biologically inert nature. After more than 30 years of neonatal and adult animal studies of liquid ventilation,[2-8] the clinical investigations have yielded promising results.

With the evolution of gas breathing in land-dwelling vertebrates, the lung replaced the gill as the gas exchange organ. Mammals make the transition from fluid-filled lungs during fetal life, when gas exchange is performed by the placenta, to gas breathing at birth, when the lungs assume responsibility for breathing. If premature delivery, antenatal or perinatal incident, or postnatal pulmonary insult precludes adequate maturation or impedes function of the pulmonary system, gas exchange insufficiency ensues. Because the lungs developed within a fluid environment, it appears logical that restoration of the fluid-filled environment would provide a natural transitional adjunctive therapy, and the fluid-filled lung might provide an experimental tool to understand lung function. This chapter traces the development of fluid breathing and extended applications from its origin to current areas of investigation.

FLUID AS A VENTILATORY MEDIUM

Saline Solution

Saline solution was the first liquid used as a respiratory medium,[9] and it effectively eliminates the gas-liquid interface within the lung. It has been fundamental in elucidating factors that influence alveolar structure, distensibility, stability, ventilation, and pulmonary blood flow.[10-16] Although the detergent-like action of saline solution has proved useful in removing pulmonary debris, it also removes pulmonary surfactant, which is necessary for lung stability. Furthermore, low respiratory gas solubility and excessive diffusion gradients at atmospheric conditions have limited the applicability of saline solution to support pulmonary gas exchange.[17-21] Because the O_2 content of saline solution saturated at 1 atm is only approximately 3 vol%, it cannot support oxygenation under atmospheric conditions. In 1950, it was suggested that saline solution saturated with O_2 dissolved under pressure could potentially sustain submersed mammals.[22] Practical application of this concept was first demonstrated in 1962. Additional studies demonstrated that mammals submerged in hyperbaric-oxygenated saline solution could breathe liquid and resume gas breathing.[18,23-25] Although these studies demonstrated adequate oxygenation, CO_2 retention and profound acidosis occurred, thus rendering saline ventilation physiologically unsound during either normobaric or hyperbaric conditions.

Perfluorochemical Liquids

As an alternative to saline solution, the utility of other liquids as respiratory media was investigated. Experiments with silicone, vegetable oils, and animal oils demonstrated toxic effects.[26,27] PFCs were first produced during World War II as part of the Manhattan Project and, on the basis of the high solubility of this medium for respiratory gases, were first used to support normobaric respiration in 1966 (Figure 98-1).[28] This marked the beginning of the use of PFC as an alternative respiratory medium.

A true PFC is formulated from common organic compounds such as benzene, by replacing all the carbon-bound hydrogen atoms with fluorine atoms through techniques such as vapor-phase fluorination, cobalt trifluoride agitation, or electrochemical fluorination.[27] PFC liquids have a strong carbon-fluorine bond that provides them with extreme thermal, chemical, and physical stability. They can be stored at room temperature indefinitely, they can be subjected to antiseptic conditioning without alteration (i.e., autoclave, small-pore filtering), and they are clear, odorless, nonbiotransformable, and generally insoluble in aqueous media and nonlipid biologic fluids.

The physicochemical profile and structure of various PFC liquids for PFC ventilation are shown in Table 98-1 and Figure 98-2. As a class of liquids, they are unique in two ways: (1) they have the lowest sound speeds of any liquid, and (2) gases are exceptionally soluble in the PFC liquids. PFC fluids are characterized by a cyclic or aliphatic structure consisting of 6 to 14 carbon atoms; the physicochemical profile of each compound is influenced by the particular arrangement of the carbon-fluorine bonds.[27,29-31] Gas solubility differs across PFC liquids and type of gas. It is dependent on the sizes of and steric relationships between the PFC and gas molecules.[32-34] Specific to respiratory gas exchange, O_2 and CO_2 are carried only as dissolved gases with solubilities as much as 16 and three times greater, respectively, in PFC than in water. O_2 solubility ranges from 35 to 70 mL gas per deciliter at 25° C.[35] Although the carrying capacity for CO_2 is known for fewer compounds, reported values of CO_2 solubility are approximately four times greater than those for O_2, ranging from 122 to 225 mL/dL (i.e., perfluorodecalin [APF-140] = 122 mL/dL, perfluoroalkylpentane [FC-75] = 192 mL/dL, perfluorooctylbromide [PFOB] = 225 mL/dL). Fewer data are available regarding the diffusivity of specific gases within the PFC liquids.[34,35] It is known that gases diffuse more slowly in liquids than in a gaseous

medium and that the gas solubility is not related to the diffusivity within the PFC liquid. Although some properties of these liquids vary, their dielectric strength and resistivity are high, they are denser than both water and soft tissue, and they have relatively low surface tension and viscosity.

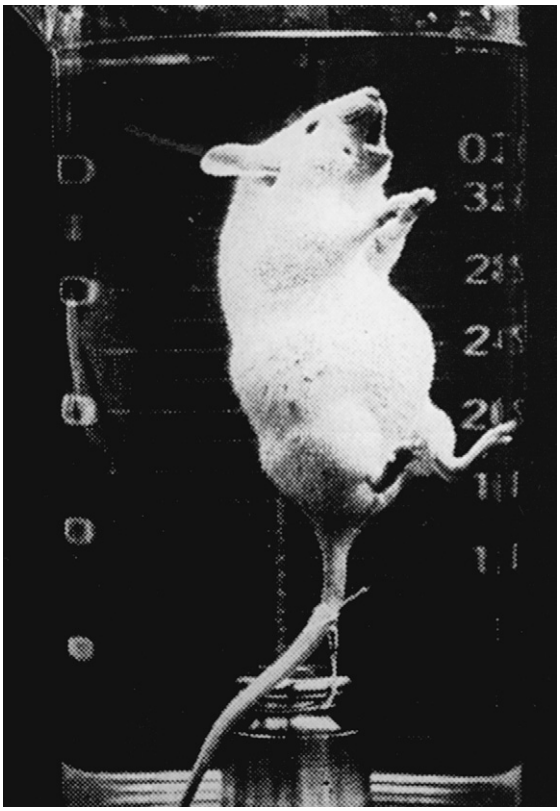


Figure 98-1 The liquid-breathing mouse. This historic mouse is submersed in and breathing a PFC liquid (FC-75; 3M Corporation, St. Paul, Minnesota) saturated with 100% oxygen. *(Courtesy L. C. Clark, Jr., Ph.D.)*

Differences in several of the physicochemical properties of the PFC fluids have relevance to their performance as respiratory media. Fluids of higher vapor pressure (e.g., lower boiling points) tend to volatilize from the lung faster than fluids of lower vapor pressure (e.g., higher boiling points). Fluids of higher spreading coefficients (a parameter related to the surface tension of the liquid and gas-liquid or liquid-liquid interfacial surface tension) may be distributed more readily in the lung than fluids of lower spreading coefficients (i.e., FC-75 > PFOB > APF-140).[36,37] Fluids of higher viscosity or kinematic viscosity (e.g., viscosity-density) may resist redistribution in the lung and therefore maintain contact with more of the alveolar surface and for a longer time than those that stratify more quickly[38,39]; however, they may offer greater flow resistance. Partial liquid ventilation (PLV) with a higher-viscosity, lower–vapor pressure liquid resulted in significant improvement in gas exchange and lung mechanics, as well as significant reduction in inflammatory mediators in the lung tissue after repetitive saline injury compared with conventional mechanical ventilation and PLV with lower-viscosity, higher–vapor pressure fluids.[40,41] Given that PFC evaporative loss and redistribution of PFC is minimized by lower vapor pressure and higher viscosity,[39] these data suggest that, in addition to enhancing lung function, greater lung protection by way of both mechanoprotection and cytoprotection is conferred during PLV with a PFC liquid that remains distributed throughout the entire lung for a longer duration. Both the carrying capacity (i.e., solubility) and the transfer capacity (i.e., diffusivity of gases within the PFC liquid as determined by the diffusion coefficients) of the liquid are important functional criteria in liquid ventilation. Smaller partial-pressure gradients and less time are required for gases to saturate or leave liquids with higher gas diffusion coefficients. The higher the gas solubility, the less fluid volume required to remove or deliver gases. Fluids of higher CO_2 solubility may provide a greater carrying capacity for CO_2 but may require additional ventilation to deplete CO_2 from the reservoir.[42] Each of the physicochemical properties should be considered in the selection of a PFC liquid for specific ventilatory techniques.

Although these fluids are innately nontoxic and weak solvents, material compatibility with ventilator circuitry and endotracheal tubes has been investigated to rule out potential complications associated with leaching of plastics into the PFC and the effect of PFC on the structural integrity of plastic components.

TABLE 98-1

Physicochemical Profile of Various Perfluorocarbons

Perfluorocarbon	Formula	Orientation	O_2 solution (mL/100 mL) (25° C)	Vapor pressure (mm Hg) (37° C)	Boiling point (° C)	Viscosity (cSt) (25° C)	Mol wt (g/mol)	Density (g/mL) (25° C)
PP2	C_7F_{14}	Cyclic	57.2	180	76	0.88	350	1.788
PFOB	$C_8F_{17}Br$	Aliphatic	52.7	11	140.5	1	499	1.89
PCI	$C_7F_{15}Cl$	Aliphatic	52.7	48.5	108	0.82	404.5	1.77
P12F	$C_9F_{20}O$	Aliphatic	52.5	39	121	0.95	504.1	1.721
FC-75F	$C_8F_{16}O$	Cyclic	52.2	51	102	0.85	416.1	1.783
FC-75P	$C_8F_{16}O$	Cyclic	52.2	51	102	0.85	416.1	1.783
PFDMA	$C_{12}F_{18}$	Cyclic	39.4	2.6	177.5	4.35	524.1	2
FC47	$C_{12}F_{27}N$	Aliphatic	38.4	2.5	174	2.52	671.1	1.9
PP9	$C_{11}F_{20}$	Cyclic	38.4	5.2	160	3.32	512.1	1.972
APF-57	C_6F_{14}	Cyclic	70	356.4	57.3	—	338	1.58
APF-100	C_8F_{16}	Cyclic	42.1	64.6	98.6	1.11	400	1.84
APF-125	C_9F_{18}	Cyclic	47.7	30	116.6	1.17	450	1.86
APF-140	$C_{10}F_{18}$	Cyclic	49	13.6	142	2.9	462	1.93
APF-145	$C_{10}F_{20}$	Cyclic	45.3	8.9	142.8	1.44	500	1.9
APF-175	$C_{12}F_{22}$	Cyclic	35	1.4	180	3.5	562	1.98
APF-200	$C_{13}F_{24}$	Cyclic	41	1.26	200	5.3	612	1.99
APF-215	$C_{14}F_{26}$	Cyclic	37	0.2	215	8	662	2.02

Data from references 21, 27, and 34.

PFC liquids have been shown to cause plastic embrittlement of polyvinyl chloride plastics, minimal swelling of polytef (Teflon)–based products, compatibility with medical-grade polyurethane, and solubilization of silicone oil–based materials.[36]

PFC liquids have formed the basis for biologic applications including synthetic bloods, imaging agents, and replacement fluid for the eye.[43-49] Pure medical-grade PFC liquids currently exist for the purpose of liquid ventilation. Various PFCs have been explored in patients as gas transporters in the lung and blood; as ultrasound, oral, and lung contrast agents; to provide intraocular tamponade after vitreoretinal surgery; to enhance oxygenation via peritoneal lavage, for organ preservation and wound healing; and to distend and grow the lungs in patients with congenital diaphragmatic hernia.[2,47,50-66] Over the past three decades, we have pioneered, developed the relevant technology, and used intrapulmonary PFCs in both animal and clinical studies to safely support lung volume recruitment, support gas exchange, decrease pulmonary inflammation and oxidative stress, and preserve lung architecture in multiple causes of respiratory distress.[41,59,67-82] In addition, intrapulmonary use of thermally conditioned PFCs effectively and rapidly induces systemic heating or cooling because of expansive surface area of the pulmonary circulation and thermal capacity of the PFC liquids.[83-87] The feasibility, safety, and efficacy of aerosolizing PFC through the nasopharynx in normal sheep to preferentially induce and maintain brain cooling at rates as great as 10 times faster than by conventional means has been recently demonstrated.[88] This approach minimizes systemic cooling and subsequent systemic adverse effects while selectively providing central hypothermic neuroprotection. Aerosolized PFC significantly increases the heat-carrying capacity of air passing through the nasopharynx. In addition, because of proximity to the cerebral circulation, the nasopharynx is uniquely suited for preferential and homogenous brain cooling. In summary, the nasopharyngeal-PFC approach uses latent heat of vaporization and hematogenous and conductive mechanisms to induce and maintain brain cooling.

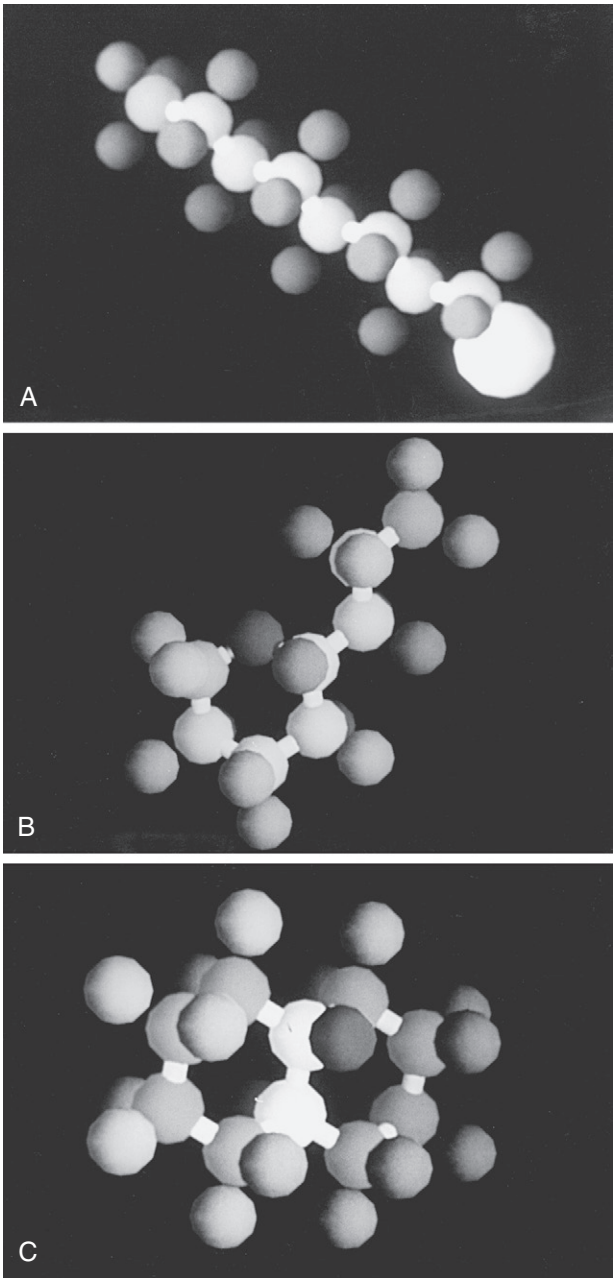


Figure 98-2 Molecular models of PFCs suitable for liquid ventilation. **A,** *Perfluorooctylbromide* (i.e., LiquiVent; Alliance Pharmaceutical Corporation, San Diego, California) consists of an eight-carbon skeleton with surrounding fluorine atoms. Carbon 8 is also bound to a terminal bromide atom. **B,** *Perfluoroalkylpentane* (i.e., a component of RIMAR 101; Miteni Corporation, Milan, Italy; FC-75P; 3M Corporation) consists of a cyclic portion of five fluorinated carbon atoms and one oxygen atom and a chain of three fluorinated carbon atoms. **C,** *Perfluorodecalin* (APF-140; Air Products and Fine Chemicals Corporation, Allentown, Pennsylvania) consists of a bicyclic molecule of 10 fluorinated carbon atoms.

BIOPHYSICAL PROFILE AND PHYSIOLOGIC RESPONSES OF THE FLUID-FILLED LUNG

Figure 98-3 provides a summary of the biophysical profile and physiologic responses of the fluid-filled lung.

Pulmonary Structure and Function

The differential effects of tissue forces from surface-active forces on alveolar distensibility have been identified. One study found that more volume was displaced for the same change in pressure in the saline-filled lung than in the gas-filled lung.[16] This effect was exaggerated when the gas-filled lung with elevated surface tension was filled with saline solution (Figure 98-4). On the basis that saline solution eliminated the gas-liquid interface, and thus

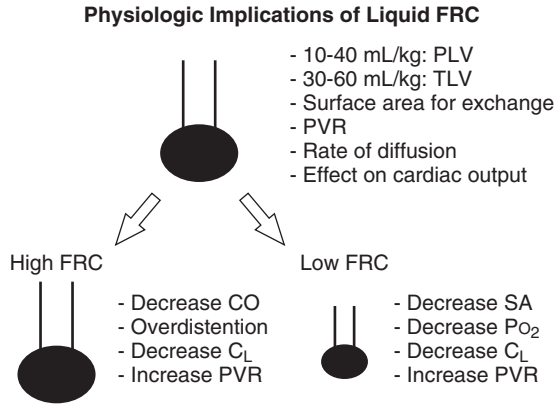


Figure 98-3 Biophysical profile and physiologic responses of the fluid-filled lung. C_L, Lung compliance; *FRC,* functional residual capacity; *PLV,* partial liquid ventilation; *PVR,* pulmonary vascular resistance; *SA,* surface area; *TLV,* tidal liquid ventilation.

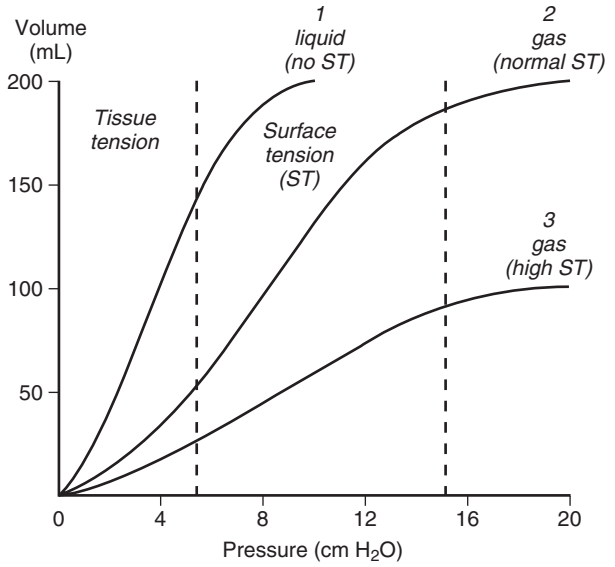


Figure 98-4 Pressure-volume relationship of the gas-filled or liquid-filled lung. *ST,* Surface tension. *(Redrawn from Comroe JH:* Physiology of Respiration*, Chicago, 1977, Year Book Medical Publishers.)*

interfacial tension, it was concluded that the greater pressure requirements in the air-filled normal and abnormal lung resulted from surface forces.

In the gas-filled lung, alveolar pressures are uniform, and vascular pressures are subject to a hydrostatic gradient, but in the saline solution–filled lung, alveolar and vascular pressures are relatively balanced throughout.[89,90] This finding suggests that pulmonary blood vessels would be uniformly distended, resulting in more uniform distribution of blood flow and improved matching of ventilation to perfusion in the saline-filled as opposed to the gas-filled lung. In that regard, ultrastructural studies have shown that the capillaries bulge into the alveolar space in the saline-filled lung, whereas the alveolar surface is relatively smooth in the gas-filled lung.[91] The irregular alveolar surface area is larger in the fluid-filled than in the gas-filled lung of the same volume.

Pulmonary Mechanics

Factors that play a predominant role in maintaining lung volume stability include those attributable to the tissue properties and surface forces acting at the alveolar-capillary interface. Elastin and collagen confer structural integrity, whereas surface forces dictate stability of gas exchange regions over a wide range of lung volume.

Surface tension at the alveolar-capillary interface created by the interface between two phases (i.e., gas and the lung surface) is the force that resists expansion and determines the amount of work required to create additional surface for gas exchange. Because the cohesive forces of molecules within a liquid are stronger than those between liquid (i.e., lung surface) and gas (i.e., within alveolus), more work is required to move molecules from within the liquid to a gas-liquid interface than to a liquid-liquid interface. Addition of a surface-active agent, or *surfactant,* to liquid decreases surface tension and reduces the work needed to increase surface area. Replacement of gas with liquid at the lung surface eliminates surface tension. Applying Laplace's law and the bubble model to the lung, $P = 2T/R$, the radius *(R)* of the alveolus is determined by the pressure gradient *(P)* across the alveolar wall and surface tension *(T)* acting on the alveolus; the pressure required to maintain alveolar size is decreased by lowering surface tension. According to this theory, if alveoli at different sizes are subject to the same transmural pressure, surface tension must vary with size to prevent collapse of smaller into larger alveoli. On this basis, an effective pulmonary surfactant must demonstrate a surface tension that varies with surface area.[92]

Alternatively, evidence from morphometric studies has disputed the bubble model theory. If interior alveoli of different sizes share a common wall, the two alveoli have the same internal pressure; thus there is no driving force to collapse the smaller alveolus. Thus changes in alveolar size over the respiratory cycle are achieved by unfolding of alveolar membranes.[91] In this situation, any agent capable of lowering surface tension would serve to decrease membrane adhesion and allow membrane unfolding; a variable surface tension with varying surface area would not be required.[91,93-95] These principles identify requirements of a modality that would facilitate alveolar stability.

The stabilizing role of pulmonary surfactant originated in 1929. It was shown that less pressure (i.e., work) was required to inflate the saline-filled than the air-filled animal lung. This difference resulted from surface forces at the air-liquid interface of the air-filled lung.[16] It was suggested later that phospholipids in the alveolar surface forces reduced surface forces at the air-liquid interface.[96] Subsequently, it was demonstrated that surface tension changed in relation to surface area,[97] and alveolar material obtained from infants dying of respiratory distress syndrome had decreased surfactant activity.[10] These findings suggested that the lack of pulmonary surfactant predisposed infants to atelectasis. Taken together, these studies established the fundamental basis of subsequent work toward exogenous surfactant replacement therapy to reduce inflation pressures for the treatment of respiratory distress syndrome.

Because of the biophysical similarity between the alveolar lining fluid and saline solution, interfacial tension and surface forces are essentially eliminated in the saline-filled lung. Similar to a completely saline-filled lung, complete insufflation of the lung with a PFC liquid in which gas is carried in the dissolved state eliminates the gas-liquid interface in the lung and reduces, but does not abolish, surface forces. Unlike in the saline-filled lung (and because of physicochemical differences between the alveolar lining fluid and PFC), interfacial tensions remain between alveolar lining fluid and PFC. Therefore alveolar inflation pressures with PFC are lower than those with gas; however, more pressure is required to inflate the PFC-filled lung than the saline-filled lung. Impairment of lung mechanics after PFC ventilation of healthy, surfactant-sufficient adult animals may be attributable to the additional interfacial tension at the PFC-lung surface and the air-PFC surface.[18,80,98-100]

In vivo pulmonary compliance determinations performed in gas-ventilated or PFC-ventilated preterm lambs ranging in age from 106 to 136 days gestation (term approximately 150 days) demonstrated significant age-related differences.[5] Although pulmonary compliance of the PFC-ventilated lamb was consistently higher than that of the gas-ventilated lamb, this difference was not significant until after approximately 120 days gestation. This finding suggests that morphologic and tissue properties predominate over surface properties in determining lung distensibility at the earlier stages of development. In addition, it suggests that the structural development (i.e., elastic, collagen, parenchymal interdependence) of the lung limits the effect of a reduction in interfacial surface tension on increasing lung distensibility.

Numerous in vivo studies in animals and humans with surfactant deficiency or dysfunction, with or without surfactant pretreatment, have demonstrated improvement in respiratory compliance during tidal liquid ventilation (TLV),* on recovery to gas ventilation,[50,111,114,118-120] or during gas ventilation after

*References 5, 6, 72, 76, 79, 101-117.

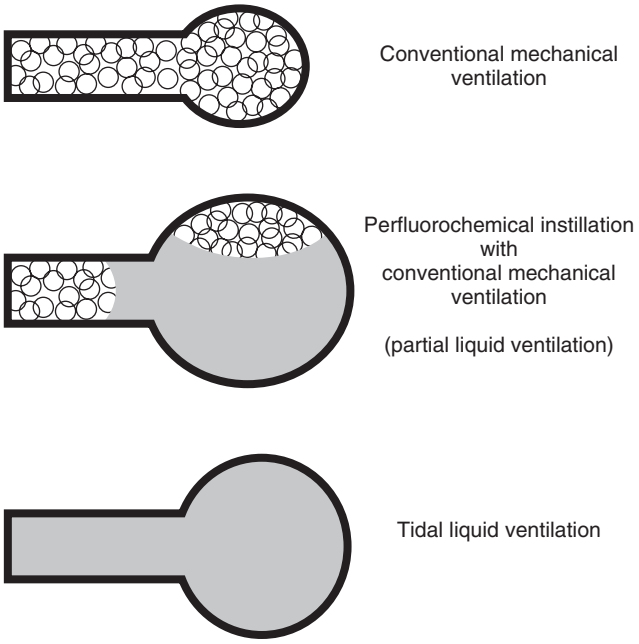


Figure 98-5 Schematic of potential sources of interfacial tensions in the PFC-treated lung during ventilation with tidal volumes of gas (PLV) or PFC liquid (TLV).

instillation of PFC liquid (PLV).[†] The greatest reduction in ventilatory pressure requirements and improvement in specific respiratory compliance were noted after (1) the lung was insufflated with PFC liquid to functional residual capacity (FRC), (2) free gas was vented, and (3) ventilation was achieved by moving tidal volumes of PFC.[79] Figure 98-5 illustrates schematically the various interfacial tensions that may exist in the PFC-treated lung during ventilation with tidal volumes of gas or PFC liquid.

With use of surfactant-deficient excised lungs of preterm lambs, studies have shown that net interfacial tension is lower in the lung filled with an FRC of PFC liquid than in the gas-filled lung; surfactant pretreatment further reduces net interfacial tension in the PFC-filled lung.[146,147] When free gas is introduced into the PFC-treated lung, additional interfacial tensions are created at the free gas–PFC and gas-lung surfaces. Although net interfacial tension is increased in the PFC-treated and gas-ventilated lung relative to the liquid-ventilated lung, tension remains lower than in the gas-filled lung and is reduced by surfactant pretreatment.

Gas Exchange

As reviewed earlier, the need for hyperbaric conditions to oxygenate saline solution adequately, the detergent action of saline solution (removing endogenous surfactant), and the inability to remove CO_2 limit the utility of saline solution as a ventilatory medium. Inadequate CO_2 elimination during saline ventilation has been linked to the slower diffusion rate (greater diffusion coefficients) of CO_2 and the lower maximal expiratory flow associated with elevated density and viscosity in liquids compared with those in gases. These mechanisms were elucidated by theoretic models and experimental data obtained in the saline-filled lung.[17-19,148,149]

One theoretic model viewed the lung as multiple and identical spherical exchange units.[17,18] With each respiratory cycle, the tensions of O_2 and CO_2 change as a function of radial distance from the center of each sphere, diffusion coefficient for the respective gas, and time for diffusion. The partial pressure gradient for each gas decreases over time, resulting in regional differences within each sphere and diffusional dead space (i.e., the center of the sphere has lower CO_2 tensions and higher O_2 tension). Relative to liquid versus gas flow rates, it has been shown that maximal expiratory flow in excised saline-filled animal lungs was one hundredth of that obtainable in the gas-filled lung.[13,14] The reduction in maximal expiratory flow was linked to the increase in density and viscosity of saline solution compared with those of gas and elevated resistive pressures when moving saline solution and gas at comparable rates. Therefore, to overcome such limitations, the respiratory cycle would ostensibly have to be increased in length and depth for adequate alveolar ventilation to eliminate CO_2. Although the potential for adequate ventilation was identified, strategies for ventilating with saline solution were not explored further because of the dependence on achieving hyperbaric oxygenation and the detergent-like action of saline solution in removing native pulmonary surfactant.

There are numerous similarities and differences in the characteristics of PFC liquid and saline solution relative to gas exchange. Although high respiratory gas solubility of the PFC liquids distinguishes these fluids from saline solution to support pulmonary gas exchange, theoretic analysis and in vivo experimentation demonstrated that the low diffusion coefficient for respiratory gases (e.g., gases diffuse into liquids several orders of magnitude slower than into gas) and elevated density and viscosity of liquid (e.g., 10^3 and 10^2 times greater than air) impose rate limitations on alveolar diffusion and expiratory flow.[34,35] Low gas diffusion coefficients and solubilities in PFC have little impact on O_2 exchange because the partial pressure of inspired O_2 can be increased to approximately 700 mm Hg (thereby establishing a sufficient alveolar-arterial O_2 gradient and O_2 content in PFC to meet most metabolic demands). In contrast, the amount of CO_2 removed from the lungs depends primarily on the partial pressure of CO_2 in the blood (usually, 40 mm Hg), thereby limiting the alveolar-arterial CO_2 gradient and CO_2 content. This has been demonstrated experimentally in preterm lambs that showed that the low diffusion coefficient of O_2 in PFC liquid yielded a relatively large gradient between the mean inspired O_2 (496 mm Hg) and the mean alveolar O_2 (335 mm Hg), thus explaining the mean arterial O_2 tensions (170 mm Hg).[67]

Factors contributing to improved matching of ventilation and perfusion in the completely fluid-filled lung also contribute to effective gas exchange during tidal PFC ventilation (see the earlier discussion of pulmonary structure and function). During tidal PFC ventilation, PFC is evenly distributed throughout the lung, and pulmonary blood is redistributed in the completely PFC-filled lung, creating a more homogeneous pattern than during gas breathing.[78,120] The adequacy of gas exchange during gas ventilation of a PFC-filled lung (e.g., PFC-associated gas exchange or PLV) has been related to the "evenness" of bubble oxygenation of PFC and matching of any maldistribution of this pattern to concomitant maldistribution of pulmonary perfusion.[150] Radiographic studies indicate that PFC liquid during gas ventilation is not evenly distributed; stratification occurs, with a primary pattern of gas rising to the nondependent regions and PFC settling to the dependent regions of the lung despite frequent changes in position. Under these conditions, it is currently unknown how much gas exchange occurs in the combined PFC-filled regions of the lung. It is also not known whether improvement in gas exchange in the immature or injured lung occurs in lung regions with only small volumes of PFC liquid (however, these units still may have improved mechanics). Although the high CO_2 solubility in PFC liquids provides an efficient reservoir for CO_2, effective CO_2 elimination during gas ventilation of a PFC-filled lung depends on the distribution of the gas. Nonventilated or poorly ventilated regions and uneven emptying of the lung because of

[†]References 12, 58, 66, 105, 114, 121-145.

airway obstruction or changing lung mechanics present challenges to this ventilatory approach. Studies of gas ventilation of the PFC-filled normal, immature, or injured lung have demonstrated that inspiratory resistance to gas flow may be normal or increased,[119,142,151] and expiratory resistance to gas flow may be elevated.[136] The expiratory flow profile suggests that the lung empties as multiple compartments.[119,150]

Cardiovascular and Metabolic Responses

Less information is available regarding cardiovascular and metabolic responses during liquid ventilation. Early studies performed in normal adult animals reported decreases in cardiac output, O_2 consumption, CO_2 production, and pH and increases in pulmonary vascular resistance (assessed in the isolated-perfused lung), as well as redistribution of cardiac output and pulmonary blood flow.[152-158] These studies suggested that although liquid ventilation techniques could support effective gas exchange, cardiovascular compromise may be an issue when liquid lung volumes and alveolar pressures are increased to more than normal levels. Similar complications have been noted with excessive use of positive end-distending pressure in the gas-filled lung.[159] It was speculated that most of these findings in the PFC-filled lung were related to the effect of the weight of the PFC to compress the heart, great vessels, or pulmonary vasculature in the dependent region of the lung. More recent studies, performed in adult, neonatal, and premature animals with normal or abnormal lungs, have reported less compromise of cardiopulmonary interaction in the PFC-filled lung.[5,11,120,160-162] A recent multicenter trial of TLV in oleic-acid lung–injured adult sheep demonstrated significant improvement in oxygenation, respiratory compliance, cardiac output, stroke volume, physiologic shunt, oxygen delivery, ventilatory efficiency index, lung histomorphology, and reduced lung inflammation (interleukin [IL]-6, IL-8, myeloperoxidase) compared with animals treated with 4 hours of lung-protective mechanical gas ventilation.[59] In a 12-hour study of very-preterm lambs treated with either TLV or repeated dose of surfactant and lung-protective mechanical gas ventilation, animals treated with TLV demonstrated significantly improved compliance, oxygenation index, and ventilatory efficiency index. They required lower ventilatory pressures, had markedly improved histologic outcomes, and demonstrate reduced lung (tumor necrosis factor alpha [TNF-α], IL-6, IL-8, myeloperoxidase) and systemic (plasma TNF-α, IL-6, IL-8)[163] cytokine levels.

Differences between the earlier and more recent studies reflect improvements in ventilator designs and lung volume management during ventilation. In the earlier studies, measurements were performed shortly after PFC instillation, raising the issue of whether the cardiovascular compromise was related to elevated alveolar or intrathoracic pressures associated with liquid compression of resident gas volumes. Preexisting metabolic acidosis complicated interpretation of several of these studies.[152,153,155,157] In a study of normal neonatal piglets, initial elevations in pulmonary artery pressure and pulmonary vascular resistance (which resolved within 40 minutes) were related to early respiratory acidosis rather than to vascular compression.[160] This study demonstrated a small decrease in cardiac output in several animals with no difference in cardiac output between gas and tidal liquid breathing in the remaining animals. It was suggested that application of liquid ventilation of the normal animal may require only modest volume expansion (i.e., a single 10 mL/kg bolus) to support normal cardiac output. Furthermore, the earlier studies were conducted in the normal lung, which transmitted ventilatory pressures more readily to the vasculature than would a stiff lung. In contrast, studies of adult animals with induced acute respiratory distress syndrome have reported no changes in cardiac index during gas ventilation after PFC instillation.[119,161]

Ventilation-perfusion matching is improved during TLV of the poorly compliant lung of preterm animals.[72,76,113,116,120] Increased oxygenation and reduction in preductal to postductal O_2 tension differences indicate improvement in pulmonary blood flow and reduction of right-to-left ductal shunting. Developmental changes in cardiovascular and metabolic function of the TLV-treated lamb (102 days gestation to term) parallel developmental changes reported for fetal or gas-ventilated animals.[5] Left ventricular output, absolute pulmonary blood flow, ductal flow, systemic arterial pressure, heart rate, left ventricular ejection and shortening fraction, and velocity of circumferential fiber shortening remain unchanged, whereas meridional wall stress is reduced during gas ventilation of the PFC-filled lung (10 mL/kg).[11,154] Therefore it is unlikely that the weight of the PFC liquid presents a risk of cardiovascular compromise in the preterm lamb with respiratory distress. As the anteroposterior dimension of the lungs increases, however, hydrostatic pressure gradients may be an issue. Thus, in larger lungs, stratification of gas and PFC liquid during PLV and regional differences in hydrostatic pressure during TLV could play a role in cardiovascular performance.

EXPERIMENTAL AND CLINICAL TECHNIQUES FOR LIQUID-ASSISTED VENTILATION

Figure 98-6 and Table 98-2 illustrate techniques and protective mechanisms associated with liquid-assisted ventilation. The earliest and purest form of respiratory support with PFC liquids is TLV. Early investigations of fluid breathing used either total body immersion or gravity-assisted ventilation, which delivered PFC liquid from a reservoir to an intubated animal. These methods were inadequate for extended ventilation of experimental animals because of problems attributed to the differences between gas and liquid physical properties, such as density, viscosity, and gas diffusion rates.[2,18,28,148] Despite more uniform distribution of blood flow in the liquid-filled lung,[90,158] it was demonstrated that expiratory liquid flow and diffusion limitations could preclude sufficient removal of CO_2 when earlier mechanical ventilation schemes were applied.[69,148]

The concept of demand-regulated liquid ventilation was introduced in the early 1970s.[80-82,164,165] This technique used the respiratory centers of the experimental animal to control the cycling of the respirator, which circulated oxygenated liquid to and from the lungs. This approach reduced the work of breathing. Early experiments with this type of ventilation reported effective oxygenation and better CO_2 removal than immersion or gravity-assisted techniques. Modifications of liquid ventilation systems have evolved, incorporating various designs such as manually controlled flow-assist pneumatic systems[80-82,164]; roller pumps with pneumatic, fluidic, and electronic controls[5,76,116]; gravity-driven systems[110,166,167]; and modified extracorporeal membrane oxygenation circuits.[168] Control strategies incorporating constant pressure or constant flow, time cycling, and pressure (system, airway, or alveolar) or volume (lung volume, tidal volume) limitation have been explored, with the current approach emphasizing microprocessor-based feedback control (Figure 98-7).[42,101]

In general, TLV is achieved by cycling fluid from a reservoir to and from the lung. Warmed and oxygenated PFC liquid is pumped from a fluid reservoir into the lung. During expiration, fluid is pumped from the lung with passive assist of the lung recoil; it is filtered and returned to a gas exchanger for oxygenation and CO_2 scrubbing. O_2 concentration is titrated from the inspired O_2 tension,[42] and fluid is returned to the fluid reservoir. PFC vapor in the expired gas is condensed with high efficiency (at 70% to 80%), and noncondensed PFC loss caused by evaporation is measured and returned to the system.[74,169] During TLV, gas is transported to the alveolar-capillary membrane in dissolved form; O_2 and CO_2 diffuse through the liquid, and the fluid

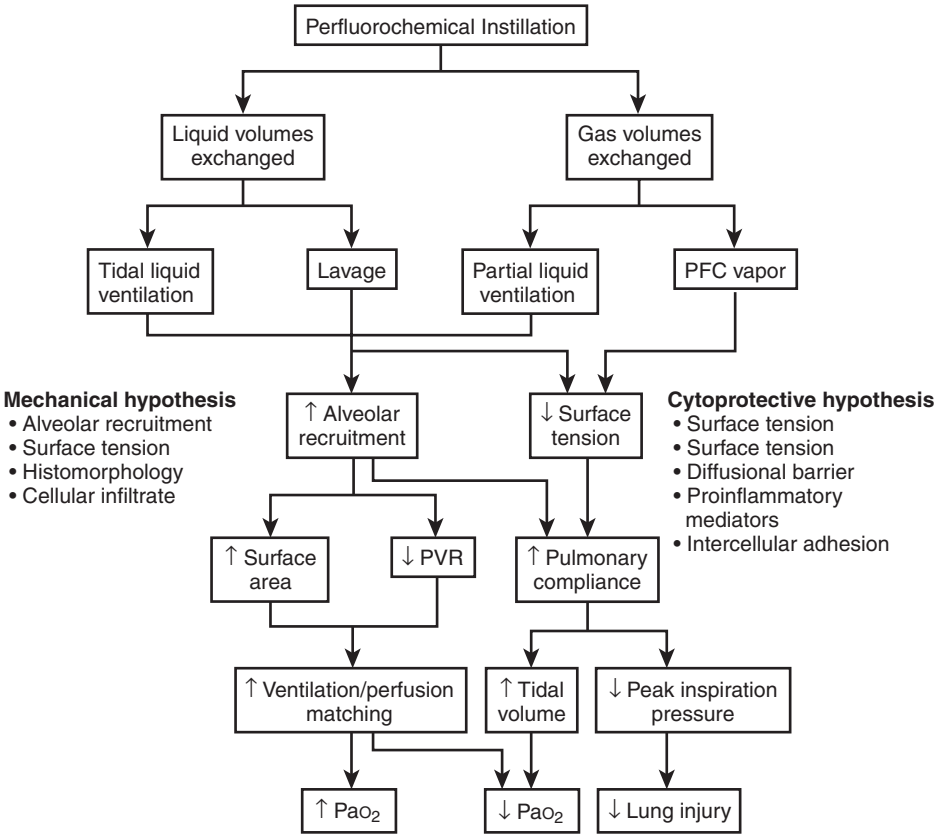


Figure 98-6 Liquid-assisted ventilation techniques and associated lung-protective mechanisms.

TABLE 98-2

Comparison Between Partial and Tidal Liquid Ventilation

Parameter	Partial liquid ventilation (PLV)	Tidal liquid ventilation (TLV)
Dosing—How much perfluorocarbon is in the lung at any given time?	*Unknown* because the perfluorocarbon will evaporate and be expelled with each patient expiration	*Controlled* as a result of the closed circuit established by the liquid ventilator
Pressure changes within the alveoli	*Unknown* because the pressure waveform characteristics vary from the compressible air column in the air ventilator through the incompressible fluid column in the lung	*Controlled* by the liquid ventilator through the incompressible column of perfluorocarbon
Volume changes within the alveoli	*Unknown* as a result of air/fluid compressibility issues noted under pressure changes above	*Controlled* by the liquid ventilator through the incompressible column of perfluorocarbon
Oxygenation at the alveoli	*Unknown* because the transferrence of oxygen delivered by the air ventilator at the meniscus of the perfluorocarbon in the upper airways to the alveoli is indeterminate	*Controlled* by the liquid ventilator which cycles the fluid, scrubbing out CO_2 and establishing O_2 levels
Clearing of debris such as mucus	*Unknown* because any material in suspension within the perfluorocarbon fluid is likely redeposited in the lung when the patient is removed from liquid ventilation and the perfluorocarbon evaporates	*Controlled* by the liquid ventilator which cycles the fluid, filtering any debris from the perfluorocarbon fluid before reintroducing it back into the lung
Equipment	Gas ventilators, PFC vapor condenser, PFC regenerator, PFC vapor analyzer, infusion device	Tidal liquid ventilator

From Wolfson MR, Shaffer TH: Pulmonary applications of perfluorochemical liquids: ventilation and beyond, *Paediatr Respir Rev* 6:117-127, 2005.
PFC, Perfluorochemical.

is regenerated extracorporeally. Because the lung is entirely fluid filled, heart sounds are more clearly discerned during TLV. Periodic rotation of the patient and suctioning of secretions may be used to facilitate removal of debris. Ventilation-perfusion matching is improved in the lung of a preterm infant with this form of ventilation independent of rotation; however, periodic rotation may further support homogeneous distribution of ventilation and perfusion. This may be more important in larger lungs, in which the effect of hydrostatic pressure has a greater influence on the distribution of pulmonary blood flow.

Repeated trials by different investigators confirmed that when mechanical ventilation is performed properly, oxygenation and ventilation can be maintained effectively in the liquid-filled lung. There is no known physiologic limitation that precludes the

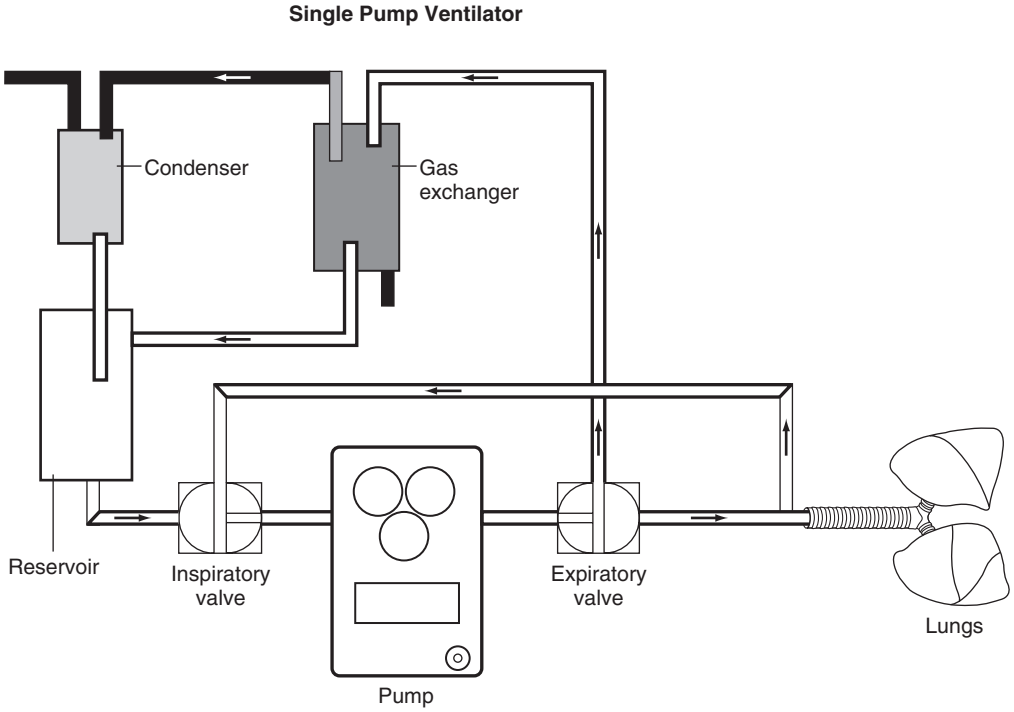


Figure 98-7 Single-pump tidal liquid ventilator. Heater omitted for clarity. *Solid line,* Gas flow; *open line,* PFC liquid flow.

duration of TLV. Term lambs have been ventilated successfully with physiologic gas exchange for up to 24 hours. Gas exchange data from studies of normal or lung-injured preterm, term, and adult animals have reflected improved liquids and ventilation methods. Along these lines, several researchers have reported alternating conventional ventilator therapy with PFC ventilation in various animal preparations and in human patients with respiratory distress ranging in age from extremely premature infants to adults.* This form of combined therapy was well tolerated and in many cases demonstrated improvement in lung mechanics.

This combined ventilation scheme (liquid ventilation and gas ventilation) has since been described as PLV,[66,136] or PFC-associated gas exchange,[154] and is characterized by filling and maintaining the lung with an FRC of PFC liquid while conventional gas ventilation is performed. It has been proposed that the residual PFC liquid within the lung is oxygenated and CO_2 is exchanged in the lung by means of gas movement provided by the conventional gas ventilator. PLV resembles the situation after the TLV-treated lung is returned to gas ventilation.

BIOCHEMICAL, HISTOLOGIC, AND MORPHOLOGIC FINDINGS IN THE LIQUID-VENTILATED LUNG

Since the 1970s, the aftereffects of liquid breathing have been given a substantial amount of attention. In this regard, it has been demonstrated that adult, newborn, and preterm mammals ventilated with PFC liquids for extended periods were able to return safely to breathing gas without long-term negative effects.

Biochemical Findings

Small but significant levels of PFC have been detected in blood samples from animals either ventilated with PFC liquid or after intratracheal instillation of PFC liquids. Across studies, PFC concentration in the blood ranged from 0.25 to 10 μg PFC per milliliter of blood and reached a plateau by 15 to 120 minutes of liquid ventilation, depending on the physical properties of the PFC liquid, the technique of PFC administration, and the species or animal preparation studied (Table 98-3). Although there are differences in experimental conditions across studies, results were similar in that they all noted low concentrations of different PFC compounds in the blood after pulmonary exposure to PFC.[145,172-175]

As reported, the concentration of PFC in the tissues with liquid ventilation is organ dependent, with the highest PFC level typically found in the lungs. In contrast to amounts found in the lungs, there were smaller amounts of PFC reported in other organs. The lowest levels were typically found in organs with low lipid content, and the highest were found in organs with high lipid content.[176] As noted by several investigators,[145,173,174] after return to gas ventilation, there was an overall reduction in PFC levels in organs as a function of gas ventilation duration. This reduction in PFC concentration in tissue was paralleled by a decrease in PFC vapor in expired gas as a function of time after return to gas ventilation.[145,174]

PFC uptake, biodistribution, and elimination were assessed in several infants after liquid ventilation.[175] Similar to results from animal studies, the data demonstrated that PFC uptake and elimination were organ dependent, PFC concentration in the expired gas samples decayed exponentially to within control range by 8 hours after liquid ventilation, and PFC concentration in the blood was relatively saturated by 15 minutes of liquid ventilation. Tissue values revealed highest residual PFC levels in the lung, with variable but small levels in other organs. In infants surviving for an extended period, levels in the lung markedly diminish, and levels in other organs approach control values.

The overall process of PFC uptake, biodistribution, and elimination has been supported by human studies, experimental animal models, and mathematical models of liquid ventilation.* Based on the understanding to date, a conceptual interpretation of this

*References 5, 50, 118-120, 127, 128, 150, 170, 171.

*References 45, 46, 145, 160, 173-175, 177-179.

TABLE 98-3

Perfluorochemical Uptake Levels in Arterial Blood After Pulmonary Administration

PFC compound*	Ventilation strategy	Exposure time (hr)	Species	PFC level	Reference number
LiquiVent	TLV	24	Normal lambs	2.0-3.5 nL/mL	180
LiquiVent	TLV	4	Normal lambs	8.29 ± 0.68 μg/mL	180
LiquiVent	TLV	4	Injured lambs	7.35 ± 0.36 μg/mL	180
LiquiVent	PLV	4	Injured lambs	2.78 ± 0.24 μg/mL	180
LiquiVent	TLV	2	Preterm lambs	1.92-5.97 μg/mL	67
LiquiVent	PLV	2	Preterm lambs	2.88-9.55 μg/mL	79
RIMAR 101	TLV/GV	<1	Preterm humans	0.20-0.69 nL/mL	175
APF-140	TLV	1-3	Adult rats	0.14-2.70 nL/mL	145
APF-140	TLV	1	Preterm lambs	1.38-1.86 nL/mL	145

GV, Gas ventilation; *PFC,* perfluorochemical; *PLV,* partial liquid ventilation; *TLV,* tidal liquid ventilation.
*LiquiVent, Alliance Pharmaceutical Corporation; RIMAR 101, Miteni; APF-140, Air Products and Fine Chemicals.

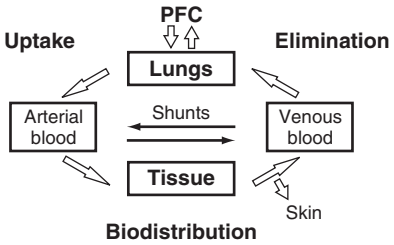


Figure 98-8 Schematic illustration of circulatory processes involved with uptake, biodistribution, and elimination of PFC liquid after intratracheal instillation.

process is diagrammed in Figure 98-8, and factors influencing this process are outlined in Table 98-4. Because PFC liquid in general is practically insoluble in water, essentially all the PFC in the blood and tissues is dissolved in lipid.[173,174] Therefore small quantities of PFC diffuse into the blood passing through the lungs, where PFC dissolves in blood lipid. The uptake process in the blood is dependent on PFC vapor pressure, permeability coefficients in the lung, PFC solubility in blood lipid, and ventilation-perfusion matching. Similar to the distribution of drugs in the body, biodistribution of PFC to the various organs depends on tissue-blood partition coefficients, tissue blood flow, and tissue compartment volume.[175] Therefore, as shown in previous animal and human studies,[145,175,180] vessel-rich organs with high blood flow are saturated more rapidly, and fat-rich organs have a higher capacity for PFC storage. Finally, PFC liquids may be scavenged by macrophages, and, because of the unique chemical structure of PFC liquids, they are not metabolized. The essential route of elimination is through the lungs by volatilization in the expiratory gases with the potential for transpiration through the skin. In a follow-up study of dogs treated with PFC ventilation,[181] for the first 5 years there were diminishing amounts of PFC as, detected histologically as persistent vacuolated macrophages in the alveoli, interstitium, and hilar lymph nodes, as well as supported by chemical analysis. By 10 years, no evidence of residual PFC was seen, and at no time was evidence seen of fibrosis or untoward structural alterations associated with the residual PFC.

The elimination of PFC liquid from the respiratory system is based on numerous factors and is still under extensive investigation. With use of a PFC vapor analyzer, studies showed that PFC liquid volume loss and evaporation rate from the lungs correlated with time after instillation (Figure 98-9) and were modulated by changes in PFC physical properties, ventilation strategy, lung pathophysiology, distribution of fluid in the lung, repositioning of the subject, and administration of supplemental PFC doses to the lungs.[169]

TABLE 98-4

Factors Influencing Perfluorochemical Uptake, Systemic Biodistribution, and Elimination

Perfluorochemical physicochemical factors	Physiologic factors
Uptake	
Vapor pressure	Blood lipid content
Lipid solubility	Lung surface area
Density?	Lung mechanics
Viscosity?	Lung permeability
	Pulmonary blood flow
	Hematocrit
Biodistribution	
Vapor pressure	Blood-organ partition coefficient
Lipid solubility	Organ lipid content
	Cardiac output
	Organ blood flow
Elimination	
Vapor pressure	Alveolar ventilation
Density?	Lung mechanics–pathophysiology
Viscosity?	Positioning
Surface tension?	Dosing requirements
	Duration and type of gas ventilation

Histologic and Morphologic Findings

Morphologic studies of newborn rabbits and immature lambs suggest that liquid ventilation actually seems to minimize damage typically incurred as a result of gas ventilation.[5,116,182] Fine structure studies of newborn rabbit lungs ventilated with PFC liquid revealed no indication of damage (Figure 98-10); the integrity of the epithelial cell membranes and bilaminar plasmalemma suggested maintenance of lung ultrastructure.[183] In addition, it is particularly noteworthy that after liquid ventilation, there was thinning of the epithelial septa, and the epithelial cells appeared flattened and more symmetric. As shown in an electron photomicrograph of lung tissue, these findings were reconfirmed in preterm lambs ventilated with PFC (Figure 98-11). The exchange spaces are clear, the air-blood interface is thin walled, the capillaries are intact and thin walled, and the cytoplasm of epithelial cells is relatively smooth. Abundant surfactant exists in lamellar bodies. The salutary effect of PFC ventilation has also been demonstrated in animal preparations of meconium aspiration or hydrochloric acid–induced or oleic acid–induced acute respiratory distress syndrome.[101,103,105,108,109] Histologic differences between the gas-ventilated lung and the TLV-treated lung were profound, reflecting a substantial reduction in pulmonary consolidation, atelectasis, hemorrhage, disruption of the alveolar

capillary membrane, and absence of inflammatory infiltration in the TLV-treated lung. The histology and morphology of lungs from healthy full-term animals after either prolonged TLV or PLV reflected preserved architecture.[79,184,185] Alterations associated with short-term exposure to intrapulmonary PFC liquid could not be detected by histologic or morphologic analysis in human lungs or other tissues.[186] Marked regional differences in pulmonary histology and morphology were noted in the lungs of preterm lambs with respiratory distress syndrome and in neonatal piglets with induced adult respiratory distress syndrome after PLV.[79,184,185] These changes were associated with stratification of the PFC and gas to the dependent and independent regions of the lung. Prolonged (96 hours) PLV of premature baboons resulted in gas exchange and lung mechanics improvements. However, a trend was noted in this study toward delayed pulmonary recovery (based on airway pressures and type II cell immaturity) in animals ventilated with PLV as compared with historical control animals.[187]

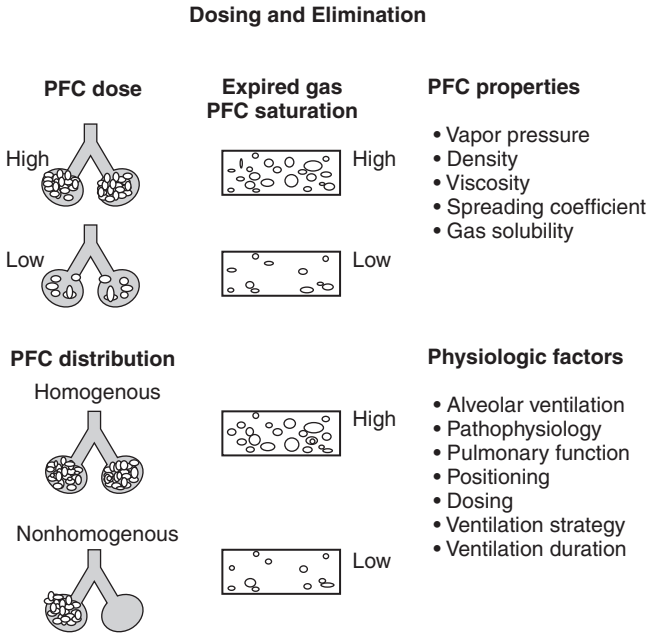


Figure 98-9 Influence of the physicochemical properties, dose, distribution, and physiologic factors of PFC liquids on PFC elimination.

Cellular Findings

In addition to improvement in lung distensibility and gas exchange, studies indicated that, whereas PFC liquids are chemically inert substances, their presence appears to modify certain biologic activity that may reduce pulmonary inflammation and injury associated with respiratory distress syndrome. Pulmonary neutrophil infiltration in rat lungs injured with cobra venom factor or resulting from hemorrhagic shock was significantly reduced during PLV when compared with conventional gas ventilation support.[188,189] Exposure of alveolar macrophages from rabbit and piglet lungs demonstrated that perflubron liquid in vitro decreased the responsiveness of macrophages to potent stimuli.[190] The effects of PFC on neutrophil–epithelial cell interactions have also been evaluated.[191] Neutrophils that were exposed to PFC, washed, and then stimulated retained their ability to generate oxidants, to release proteolytic enzymes, and to adhere to epithelial cells. However, when neutrophils and epithelial cells were simultaneously exposed to PFC, adhesion and target cell injury after stimulation were reduced. The oxidant-generating capacity of cells obtained from PFC-treated humans with acute respiratory distress syndrome was similar to that of peripheral blood neutrophils. Collectively, these findings suggest that the presence of PFC may provide a mechanical barrier to reduce neutrophil-mediated lung inflammation and injury.

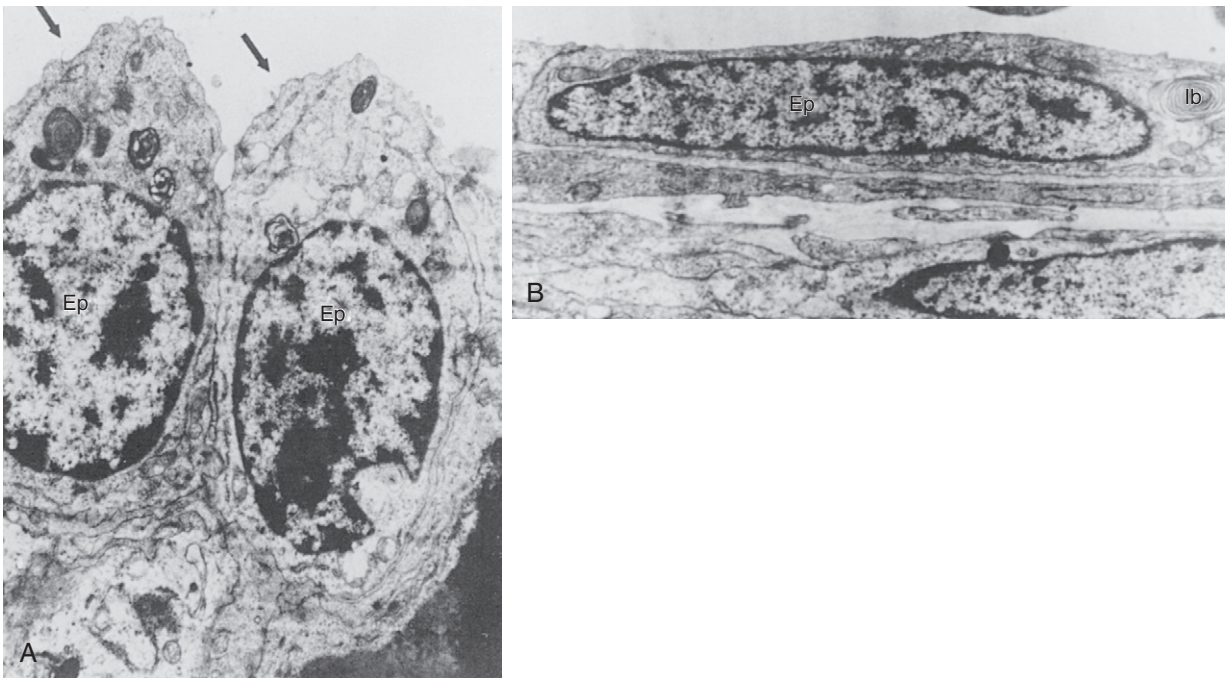


Figure 98-10 Electron micrographs of the lung of a preterm rabbit (27 days gestation). **A,** Unventilated lung. Note rounded contour of lung epithelium borders *(Ep)* (×7752). **B,** Liquid-ventilated lung. Note evenness of the luminal border, the flattened and elongated cells with early differentiation, and the lamellar body *(lb)* (×11,172).

More recent studies compared the cellular effects of PFCs of different properties on human blood cellular elements.[192,193] At the lowest PFC concentrations, which are comparable to the concentrations found in blood during liquid ventilation, PFC liquids seem to be inert and do not induce leukocyte markers associated with inflammation. In the presence of high perflubron concentrations, simulating the condition within the airways and lungs, monocytes may be induced to produce reactive O_2 species.

The permeability of the alveolocapillary membrane was studied in healthy adult rabbits ventilated with gas alone (control) or by PLV with perflubron.[194] Pulmonary clearance of inhaled technetium-labeled diethylenetriamine pentaacetic acid occurred significantly faster in animals treated with PLV than in animals treated with conventional ventilation alone. The investigators concluded that the faster clearance reflected greater alveolocapillary permeability associated with reversible changes in the surfactant system of healthy animals treated with PLV.

The effect of PFC liquid on surfactant function and metabolism has been examined in healthy and injured animal models. In vitro studies have shown that PFC-exposed exogenous surfactant maintains normal surface tension properties.[136,195] In vivo studies of healthy puppies have shown that most of the endogenous surfactant is retained in the lung after a 5-minute PFC lavage.[195] In one study, surfactant synthesis and secretion in surfactant-sufficient lungs were shown to increase after 5 hours of PLV when compared with gas-ventilated controls,[196] whereas the surfactant pool size was unchanged in another study of PLV-treated healthy or injured animals.[197] Preliminary results indicate increased endogenous surfactant phospholipids in bronchoalveolar lavage fluid after 5 hours of PLV in surfactant-deficient lambs.[198]

The effect of PFC ventilation techniques on markers of cell injury, as compared with scheduled physiologically regulated cell death (apoptosis), has been studied in very premature lambs supported with either TLV or conventional mechanical ventilation techniques.[199-201] Animals supported with TLV demonstrated a different pattern of apoptosis than those treated with conventional mechanical ventilation.[199] After conventional mechanical ventilation, apoptotic cells were observed to occlude the lumen of immature air spaces; in contrast, during TLV, apoptosis was limited to small numbers of epithelial cells lining the air spaces, with only a few cells observed within the lumen. No differences in antioxidant enzyme activity were observed.[200] Histologic evidence of barotrauma was less frequent and urinary desmosine levels, an index of elastin turnover, were significantly lower in animals supported with TLV as compared with conventional mechanical ventilation.[77,201] Collectively, these studies suggest that the reduction of barotrauma in premature animals supported with TLV as compared with conventional ventilation could be related to cell-specific apoptosis and appears to be independent of antioxidant enzyme activity.

BIOMEDICAL APPLICATIONS

Treatment of Respiratory Insufficiency

Animal Studies

Respiratory Distress

The hypothesis that PFC liquid would minimize surface forces, recruit and promote alveolar stability, and improve gas exchange at lower inflation pressures than with gas ventilation of the surfactant-deficient lung was first tested in 1976 (Figure 98-12).[113] The first studies were performed with preterm lambs of 132 to 138 days gestation[113,120] with respiratory distress syndrome. The lambs were delivered by cesarean section, initially supported with gas ventilation, and then briefly ventilated with PFC liquid (≤3 hours) and returned to conventional gas ventilation. Inflation pressures and alveolar-arterial O_2 gradients were reduced, and Pa_{O_2} and pulmonary compliance improved during and after rescue liquid ventilation. Pa_{CO_2} remained lower on return to gas ventilation, and the youngest animals with the lowest initial pulmonary compliance and Pa_{O_2} showed the most remarkable improvement in Pa_{O_2} and alveolar-arterial O_2 gradient with TLV. In contrast to the gas-filled lung, the low surface tension and spreading capabilities of PFC allow recruitment of lung tissue, which in itself increases lung compliance. Furthermore, because PFC liquid is incompressible and is not absorbed across the lung surface as is gas, alveolar stability is enhanced, thereby providing a liquid positive end-expiratory pressure. These results indicated that TLV could result in a sustained decrease in intrapulmonary shunting and atelectasis.

The ability to support pulmonary gas exchange while protecting the delicate alveolar epithelium of the immature lung with PFC liquid after delivery was demonstrated in several studies using preterm lambs of 102 to 137 days gestation.[72,76,116] TLV was initiated immediately after birth and was maintained for at least 3 hours. Effective gas exchange and physiologic acid-base status were demonstrated, with little age-related variation observed.[76]

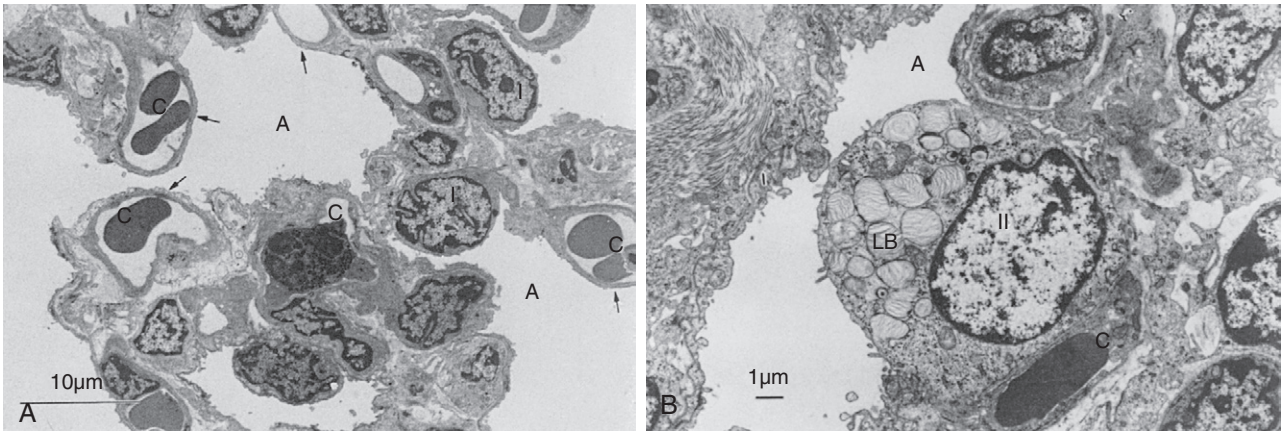


Figure 98-11 Electron micrographs of lung from preterm lamb that was liquid ventilated with perflubron for 1 hour. **A,** Note the clear exchanged spaces *(A),* thin-walled air-blood interface (red blood cell *[C]*), and smooth cytoplasm of the epithelial cell. **B,** Abundant surfactant in lamellar bodies *(LB)* of an alveolar type II cell. *(Courtesy Emil Chi, Ph.D., and J. Craig Jackson, M.D., University of Washington, Seattle, Washington.)*

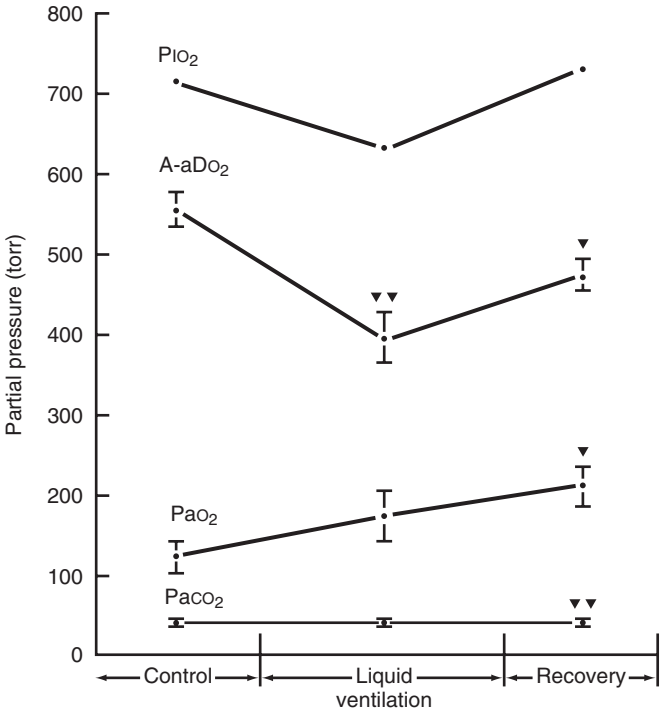


Figure 98-12 Inspired oxygen tension *(P_{IO_2}),* alveolar-arterial oxygen gradient *(A-aD_{O_2}),* arterial oxygen tension *(P_{aO_2}),* and arterial carbon dioxide tension *(P_{aCO_2})* during control gas ventilation, TLV, and recovery to gas ventilation. *(Redrawn from Shaffer TH, Douglas PR, Lowe CA, Bhutani VK: The effects of liquid ventilation on cardiopulmonary function in preterm lambs,* Pediatr Res *17:303, 1983.)*

These studies showed that the alveolar-arterial O_2 gradient during TLV was lower than that reported for gas-ventilated lambs of comparable gestational age, and pulmonary compliance approximated that of older gas-ventilated lambs.

Distinct differences in the clinical, physiologic, and histologic profile between conventional gas-ventilated and liquid-ventilated preterm lambs have been demonstrated (Figures 98-13 and 98-14).[116] Prophylactic TLV techniques supported gas exchange, acid-base balance, and cardiovascular stability throughout the duration of the experiment and improved survival; gas-ventilated animals demonstrated progressive deterioration, high morbidity, and poor survival. Differences in profiles were related to the mechanical characteristics of the respiratory system; respiratory compliance was greater, requiring lower inflation pressures in the liquid-ventilated than in the gas-ventilated animals. Thus maintenance of a liquid-liquid alveolar interface promoted effective cardiopulmonary function and maintenance of the integrity of the pulmonary architecture. These studies suggest that prophylactic TLV (implemented at birth) may decrease intrapulmonary and extrapulmonary shunting and may attenuate the complications associated with perinatal cardiopulmonary instability of the preterm infant. Evidence obtained in preterm lambs (112 to 128 days gestation), ventilated prophylactically with PFC liquid at birth and subsequently ventilated with gas, further supports this notion.[118] These animals demonstrated effective pulmonary gas exchange, acid-base status, and cardiovascular stability during TLV. Furthermore, during the period of gas ventilation, pulmonary compliance was noted to be significantly higher than that reported for animals of comparable or older age that had been gas ventilated since birth. Pulmonary compliance was also greater than that of equivalent-age animals rescue-treated with natural surfactant,[202,203] PFC,[114] or artificial fluorinated surfactant.[204,205] More recent studies performed in very-preterm lambs treated for 12 hours with either TLV or repeated doses of surfactant and lung-protective gas ventilation and in near-term lambs treated with TLV for 72 hours have demonstrated improvement in physiologic markers of gas exchange and lung mechanics, reduced lung inflammation, and improved lung histologic findings.[67,163,180]

In addition to the efficacy of tidal PFC ventilation, that of intratracheal instillation of PFC combined with conventional mechanical ventilation has been explored in animals with respiratory distress. Similar to total liquid ventilation, or the recovery phase of liquid ventilation (i.e., return to gas ventilation), PLV uses the alveolar recruitment capabilities of a fluid of low surface tension to establish an adequate FRC, as well as the ability of residual PFC to stabilize FRC in a surfactant-deficient or impaired lung. This experimental approach has been studied in acute lung injury models (Figure 98-15)* and in neonatal respiratory distress syndrome models (Figure 98-16).† Although the magnitude of responses varied within the different studies (possibly as a result of differences in techniques and degree of lung injury), it was generally shown that maintenance of an FRC with use of PFC combined with conventional gas ventilation provided adequate or improved gas exchange at relatively lower airway pressures than conventional gas ventilation alone. In many cases, the histologic pictures of the lungs were greatly improved compared with use of other forms of respiratory support or with untreated control groups.[143]

Aspiration Syndromes

Efficacy of PFC ventilation for the management of aspiration syndromes has also been demonstrated in neonatal and adult animals.[103,120,121,208] The foundation for this approach is related to removal of caustic substances (i.e., meconium) from the airways and alveolar surface, lung volume recruitment, reduction of surface forces, and improvement in ventilation-perfusion matching. Poor gas exchange, acidosis, and low pulmonary compliance present at birth and during gas ventilation of preterm lambs or induced term animals significantly improved during tidal PFC ventilation[120] or gas ventilation after instillation of PFC liquid (e.g., PLV- or PFC-associated gas exchange).[103,208] Meconium was observed in the expired liquid during tidal PFC ventilation and floated to the top of the endotracheal tube during PLV. Improvements were noted during tidal PFC ventilation in P_{aO_2}, alveolar-arterial O_2 gradient, and pulmonary compliance. Pulmonary blood flow was more uniform and P_{aCO_2} was lower during liquid ventilation. On the basis of these findings, it was concluded that tidal PFC ventilation improved pulmonary perfusion and therefore ventilation-perfusion matching. Similar improvements in pulmonary gas exchange and function have been reported in PLV of term animals with gastric acid–induced or hydrochloric acid–induced acute respiratory distress syndrome with inflammatory processes.[106,121] Similarly, it appears that PFC ventilation has a role in the management of aspiration syndromes. Early intervention may be useful in breaking the cycle of surfactant inactivation and inhibition and inflammatory processes.

Pulmonary Hypoplasia

Several studies have shown the potential of liquid ventilation for supporting gas exchange and lung mechanics in the presence of pulmonary hypoplasia. The basis of this application is related to low-pressure alveolar recruitment and improved ventilation-perfusion matching. Investigation of a lamb preparation of congenital diaphragmatic hernia supported with PLV, either prophylactically at birth or rescued after a period of gas ventilation,

*References 119, 121, 127, 128, 140-144, 161.

†References 79, 105, 122, 129, 136, 162, 187, 206, 207.

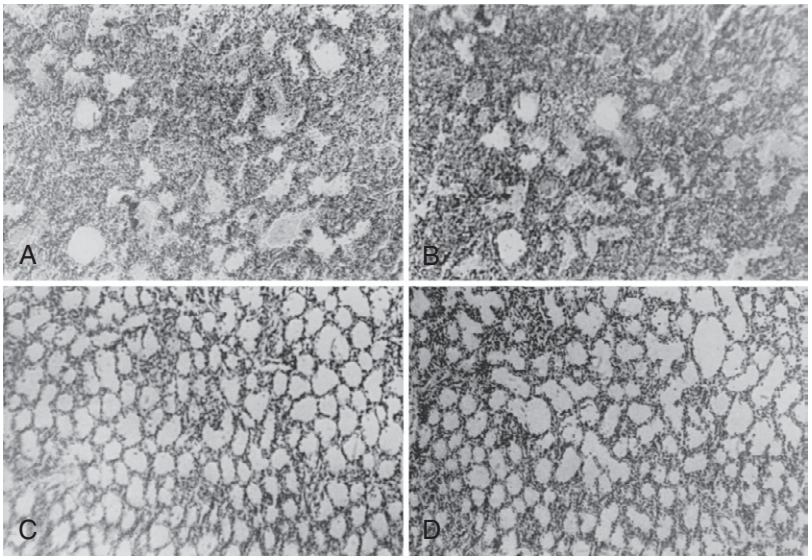


Figure 98-13 Light microscopy of lung sections (**A** and **C,** upper lobes; **B** and **D,** lower lobes) from preterm lambs (110 days gestation) after 3 hours of gas (**A** and **B**) or tidal liquid (**C** and **D**) ventilation. *(From Wolfson MR, Greenspan JS, Deoras KS, et al: Comparison of gas and liquid ventilation: clinical, physiological, and histological correlates,* J Appl Physiol *72:1024-1031, 1992.)*

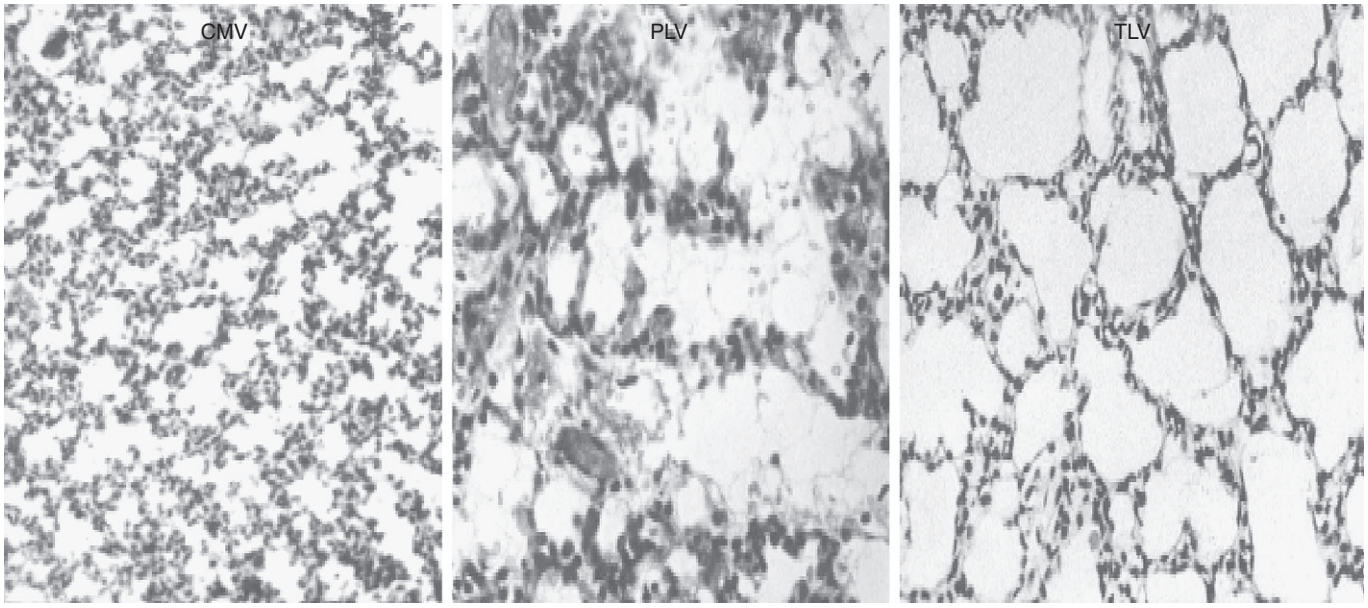


Figure 98-14 Lung histomicrographs of matched regional sections obtained from the surfactant-treated preterm lambs described in Figure 3, after 4 hours of conventional mechanical ventilation *(CMV),* partial liquid ventilation *(PLV),* or tidal liquid ventilation *(TLV).* Ventilation with PFC liquid improved overall expansion; however, marked heterogeneity and alveolar debris were noted after PLV. In comparison, after TLV, lungs were homogenously expanded and free of debris, and had less evidence of inflammatory cells. *(From Wolfson MR, Shaffer TH: Pulmonary applications of perfluorochemical liquids: ventilation and beyond,* Paediatr Respir Rev *6:117-127, 2005.)*

showed improved gas exchange and compliance compared with that of conventional gas ventilation support.[137,209] The congenital diaphragmatic hernia lamb preparation treated prophylactically with PFC at delivery demonstrated improved function and histologic findings compared with rescue treatment.[12,209]

Use of Perfluorochemicals in Infants With Respiratory Distress Syndrome

The first human studies of PFC liquid ventilation for the treatment of respiratory distress were performed in near-death infants with severe respiratory failure in 1989.[9,171] TLV was performed in two 3- to 5-minute cycles separated by 15 minutes of gas ventilation. Although all six infants in these studies ultimately died of their underlying respiratory disease, it was shown that liquid ventilation was able to support gas exchange and residual improvement in pulmonary function after return to gas ventilation in these critically ill patients.[210] Further clinical trials were limited by the need for a medically approved liquid ventilator and medical-grade breathing fluid.

The results of this first human trial and continued success of neonatal and adult animal trials with liquid-assisted gas ventilation promoted initiation of a corporate-sponsored multicenter

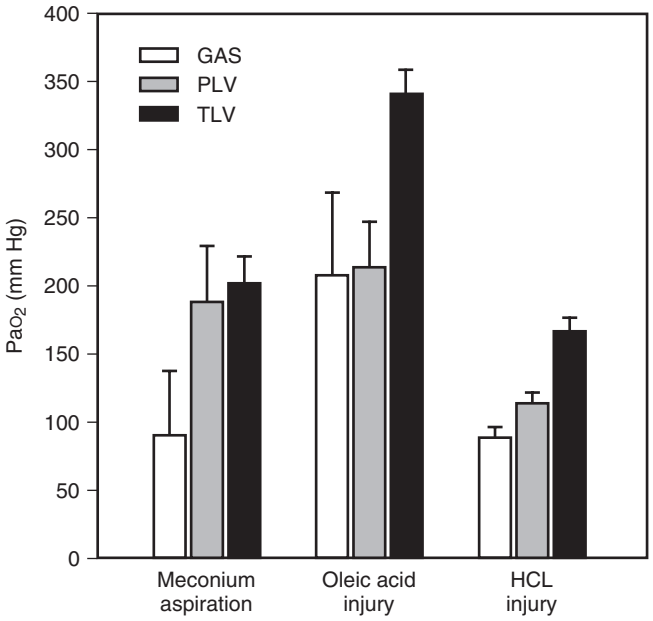


Figure 98-15 Arterial oxygen tension *(Pa_{O_2})* during gas *(GAS),* partial liquid *(PLV),* and tidal liquid *(TLV)* ventilation in animal models of aspiration syndromes and acid-induced adult respiratory distress syndrome. *(Data from references 6, 72, 99, 101, 117, and 169.)*

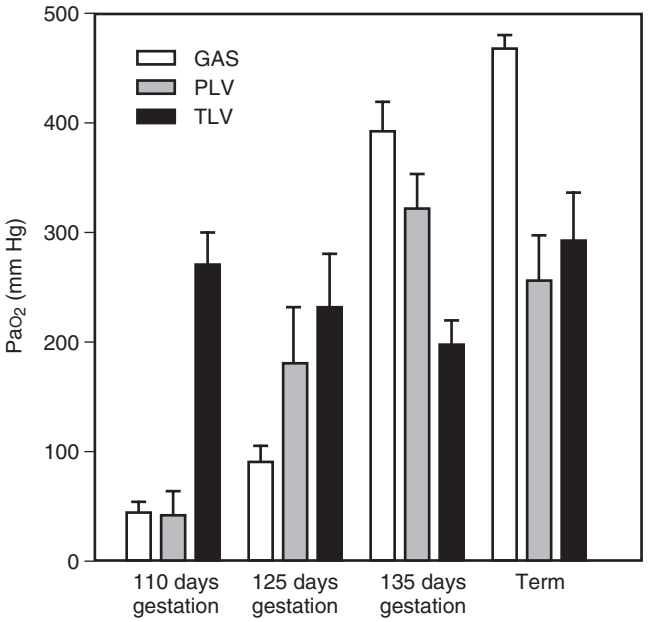


Figure 98-16 Representative "best" arterial oxygen tension *(Pa_{O_2})* during gas *(GAS),* partial liquid *(PLV),* and tidal liquid *(TLV)* ventilation as a function of age in the developing sheep. *(Data from references 5, 6, 11, 67, 72, 76, 77, 79, 103, 111, 113, 124, 136, 151, and 180.)*

trial. This trial was designed to examine the safety and efficacy of PLV with LiquiVent (Alliance Pharmaceutical Corporation, San Diego, California) in the treatment of premature infants with severe respiratory failure in whom conventional therapy had failed.[66] Seven preterm infants 5 days of age or younger (27.8 ± 1.3 weeks gestation; 1000 ± 166 g) who had received two or more doses of surfactant and were at high risk of death were enrolled. During the PLV treatment (Figure 98-17), all patients demonstrated some improvement in oxygenation, and five of seven patients demonstrated improved CO_2 removal and lung compliance; patients with hypercarbia were transferred to high-frequency ventilation. No serious adverse events were related to PLV, and two of the seven patients survived. The remaining infants died of intracranial hemorrhage (n = 2) and bronchopulmonary dysplasia (n = 1). It was concluded that PLV could be achieved for up to several days in critically ill infants without serious adverse events. Additional patients were enrolled in this protocol, and, from the series of 13 infants observed to date, 10 infants received 24 to 76 hours of PLV and 3 infants were withdrawn after a short course to receive high-frequency ventilation with residual PFC in the lung (Alliance Pharmaceutical Corporation, personal communication, 1995). Eight infants were survivors at 28 days, and 7 were discharged. It appears that clinical improvements were clearly demonstrated, with survival in some patients not expected to survive. A number of infants enrolled in this study are currently in their teenaged years. As an adjunct to extracorporeal life support in term neonates (i.e., congenital diaphragmatic hernia, acute respiratory distress syndrome), PLV for up to 7 days has been shown to gradually improve gas exchange and pulmonary compliance without adverse events related to the drug or technique.[109] It is unknown why the response to PLV of the sick term infant is more gradual than in the preterm infant with respiratory distress syndrome. On the basis of the mechanisms of liquid-assisted ventilation (see Figure 98-6), differences in this temporal response are likely due to a relatively rapid reduction in surface tension and volume recruitment in the preterm (within hours) as compared with the term infant in whom improvement in lung function requires removal of debris, which occurs more gradually over days. During the late 1990s, Alliance Pharmaceutical Corporation conducted several clinical trials of PLV with LiquiVent PFC fluid for the treatment of acute respiratory distress syndrome in adults.[58,130,131] In May 2001 Alliance announced the preliminary results from its phase 2 to 3 clinical study, which enrolled 311 adult patients at 56 medical centers in the United States, Europe, and Canada. Results of this trial have only been discussed publicly and not published in complete form to date. Safety data demonstrated that LiquiVent was well tolerated by the patients. Efficacy data revealed that PLV with LiquiVent did not meet the primary study end point for improvement in "ventilator-free days" or the secondary end point of improvement in 28-day mortality. The results of this adult trial suggested that PLV did not provide incremental benefit when compared with the ventilation strategies outlined in the ARDSnet Trial. Given the low mortality in both arms of the adult LiquiVent trial (i.e., 15%-19%) relative to mortality rates between 30% and 50% in most all other acute respiratory distress syndrome studies, failure to meet these end points is not surprising. According to an Alliance Pharmaceutical spokesperson, failure to meet these end points in adults and financial pressures, despite equivalency to existing therapies, precluded further investment in this effort. Although techniques used in the trials in adults had minimal relevance to the neonatal population, all industry-organized trials including those in the neonatal and pediatric groups were suspended. Because there has not been full disclosure of the adult trial results and because corporate support was withdrawn, a sense of uneasiness resulted as an unfortunate consequence of these trials. However, through further questioning, it is important to note that the adult trial continued until completion and was not terminated early on the basis of adverse events, risks associated with this technology, or concerns of the data safety monitoring board (Mark Weidel, personal communication, 1997). Lessons learned from history are applicable: lack of efficacy of exogenous surfactant demonstrated in adult trials did not predict efficacy demonstrated in the neonate. In this regard, equivalency of liquid

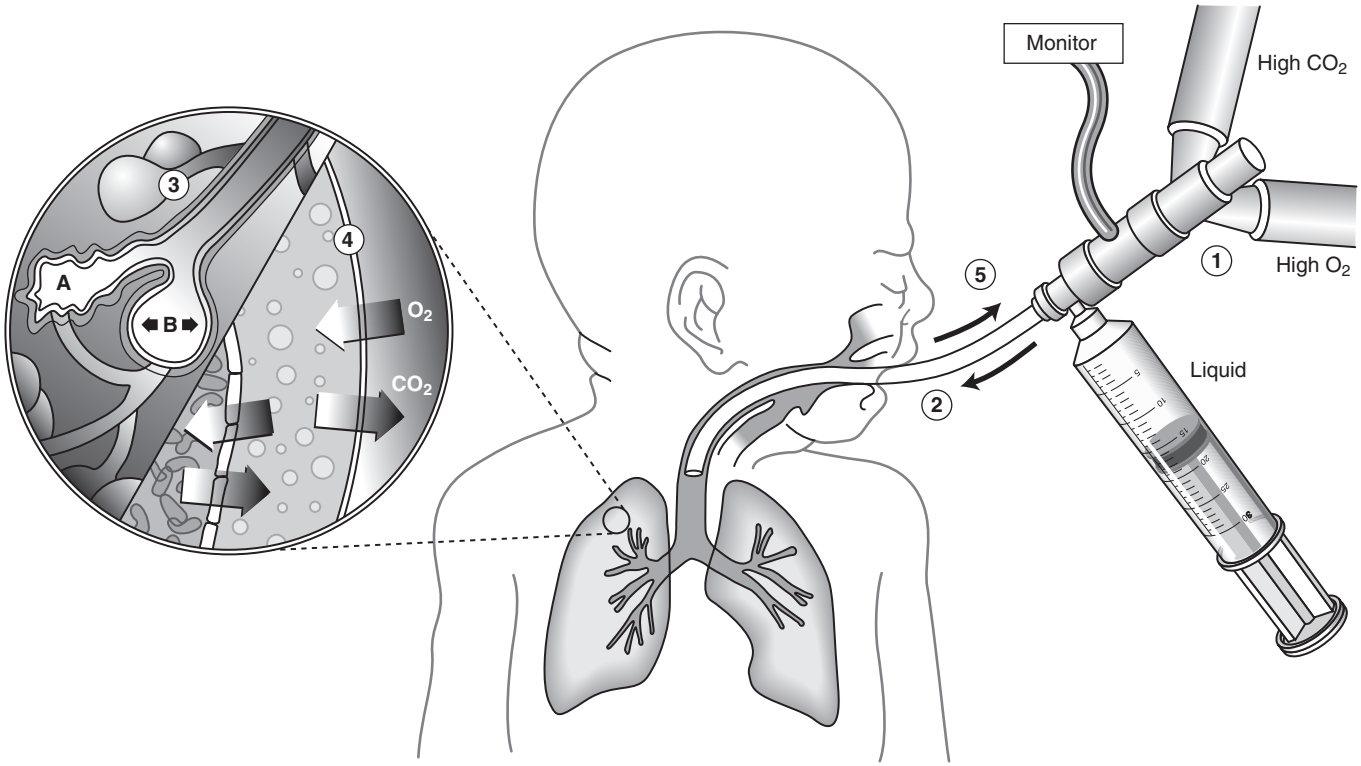


Figure 98-17 Schematic of PFC dosing and conceptualized gas exchange processes during gas ventilation (i.e., PLV). *(Courtesy Jay S. Greenspan, M.D., Thomas Jefferson University Hospital, Philadelphia.)*

ventilation to standard of care in adults rather than exceeding end points should not be used as a deterrent for resumption of clinical trials in the neonate. More recently, Lindemann and colleagues[132] demonstrated that bronchoalveolar lavage with PFC liquid could be performed safely during conventional mechanical ventilation without the additional support of extracorporeal membrane oxygenation in infants with severe alveolar space-occupying material.

Follow-up studies in humans up to 12 years after treatment with PLV have demonstrated no untoward effects of this treatment.[133-135] These imaging studies have demonstrated evidence of residual PFC in the lung, thorax, mediastinum, and retroperitoneum. In addition, they caution against interpreting the high-density opacifications associated with the Hounsfield unit density of some PFCs used with any intrapulmonary application as pulmonary calcification[134] and emphasize the importance of obtaining clinical history when faced with unusual radiographic findings.[135]

Liquid Ventilation as Adjunctive Therapy

The utility of liquid ventilation in the treatment of pulmonary dysfunction extends beyond the lowering of surface tension. In addition to recruiting atelectatic regions of the lung, filling the lungs with PFC has a potential role in delivering both active and inactive agents for treating or diagnosing pulmonary disorders, in preventing further collapse of the lung, and in cleansing the lung, thereby providing a means for improved pulmonary gas transport and mechanics. For these reasons, several investigators have explored the use of liquid ventilation in combination with other forms of respiratory support.

Drug and Tracer Delivery

Several studies have demonstrated the feasibility of using PFC liquid ventilation techniques to deliver aqueous-based pharmacologic agents (i.e., vasopressors, antibiotics) to the lung. Physiologic responses and homogeneous distribution of these agents were demonstrated when they were delivered during tidal PFC ventilation in preterm lambs with respiratory distress syndrome and normal or lung-injured term lambs[78,106,211-216]; limited responses were noted when these agents were delivered during PLV.[212] Despite the lack of miscibility of the aqueous agents in the PFC, the physiologic responses demonstrate that tidal PFC ventilation provided a vehicle to deliver vasoactive agents to the pulmonary and systemic circulation. The delivery of these agents within the PFC tidal volume is consistent with the convective-exchange mechanism[217]; this mechanism is ostensibly absent during PLV.

The physiologic responses to inspired nitric oxide (NO) during PLV were investigated. NO administration in combination with PLV in surfactant-depleted adult pigs produced a significant improvement in gas exchange and decrease in pulmonary artery pressure without deleterious effects on systemic hemodynamic conditions.[218] A study of a congenital diaphragmatic hernia lamb preparation treated prophylactically with PLV demonstrated that NO improved oxygenation and reduced pulmonary hypertension.[209] The ability to deliver NO during PLV is probably related to distribution of NO in the gas-ventilated regions of the lung, the solubility and diffusion of this gas in the PFC, and recruitment of lung volume. These findings on the effective delivery of NO in PFC liquid are consistent with those of earlier studies demonstrating the use of PFC liquid as a vehicle for the delivery of other biologic agents. On the basis of the transport principles previously described, it appears that the absolute amount of NO delivered to the pulmonary structures is dependent not only on the NO in parts per million in the inspired gas, but also on the amount dissolved in the PFC liquid (PFC liquids have high solubility for most gases; solubility data of NO in PFC have not been reported), the stratification pattern of gas and PFC liquid, the amount and distribution of pulmonary blood flow, and ventilation-perfusion matching. Therefore, for equivalent inspired NO

(parts per million), the amount of NO delivered to the pulmonary structures of the PFC-filled lung may be different from the amount delivered by gas ventilation. In addition, the clearance of NO from the PFC-filled lung and potential formation of NO_2 during liquid ventilation may also be different as compared with the gas-filled lung.

These studies suggest that PFC-assisted ventilation may be a useful adjunct in delivering therapeutic levels of other agents (i.e., bronchodilators, exogenous surfactant, antibiotics, steroids, chemotherapeutics, mucolytics, antioxidants, and gene therapy products) directly to the lung while protecting nontargeted organs from pharmacologic effects. Alternate combinations of PFC with biologic or nonbiologic agents such as suspensions, micelles, or liposomes may enhance the therapeutic and diagnostic potential of PFC pulmonary delivery techniques. Overall, it appears that this approach has a promising therapeutic role in the management of various neonatal problems, such as surfactant deficiency, consolidation, exudative processes, persistent or acquired pulmonary hypertension, pneumonia, and airway reactivity.

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SECTION XIII

Surfactant

99 Historical Perspective

Mary Ellen Avery

In 1929, a Swiss physician, Kurt von Neergaard,[1] wrote, "New notions on a fundamental principle of respiratory mechanics: The retractile force of the lung, dependent on the surface tension in the alveoli." He speculated that atelectasis of the newborn could result from "considerable retractive force of surface tension in the lungs." He carried out experiments demonstrating that an excised lung inflated with air had considerably greater transpulmonary pressure than the same lung extended to the same volume with liquid.[1] That simple, straightforward experiment was reproduced independently in the 1950s by Mead and associates[2] at the Harvard School of Public Health. The stage was set for understanding what physical and chemical forces were involved in incurring the unique properties of the alveolar lining layer, namely, the ability to augment elastic recoil of the lung at large lung volumes and the ability to reduce surface tension and stabilize air-filled spaces arranged in parallel at low lung volumes (and thus to act as an antiatelectasis factor).[2]

In the late 1940s, Peter Gruenwald[3] and George Anderson with a colleague,[4] both pathologists at Johns Hopkins University, studied lungs at autopsy and commented on the airless state that was seen. They described the lungs as "liver-like" and, in the case of Gruenwald, noted that the lungs could be expanded with liquid without significant change, but when they were expanded with air, they achieved a "Swiss cheese pattern" of overdistended air spaces and atelectasis.[3,4] I had the privilege as a medical student and, later, as a resident of hearing from both Gruenwald and Anderson, and I realized how very important was the contribution of pathologists in stimulating clinicians to relate the baby's clinical course to the findings at autopsy. It was not until the early 1950s that Herbert Miller and colleague[5] at Yale University and Blystad and associates[6] in Boston described the clinical correlations of elevated respiratory rates and retractions that small premature infants showed before their demise from atelectasis with hyaline membranes. It was Herbert Miller and colleagues[5] who first said that membranes could not result from the aspiration of amniotic fluid, as had been previously surmised, because they were not seen in babies who died in the first hours of life but took time after birth to develop. Gitlin and Craig,[7] working in Boston, demonstrated the fibrin content of the membranes and deduced that the proteinaceous material had to come from the circulation.

Independently of these clinical and pathologic observations, the respiratory physiologists at the Harvard School of Public Health were repeating von Neergaard's experiments and were making estimates of lung surface areas on the basis of an assumed surface tension of 50 dyne/cm (the equivalent of serum). The results of those calculations revealed the estimated areas to be one tenth that estimated by histologists, and it was clear a better explanation for the differences had to be found.[8]

The better explanation came from an unlikely source, the Chemical Defense Establishment in Porton, England, where Pattle[9] was assigned. Pattle[9] had been stationed there to study the role of antifoam agents in the prevention of pulmonary edema induced by certain war gases. He needed a measure of bubble stability and expressed bubbles from the lung into a drop of saline to measure their lifespan. He noted that bubbles expressed from the lung seemed to have a much longer lifespan than bubbles from serum, from other tissues, or even from ordinary detergents. He wrote a description of his findings in a now famous article entitled "Properties, Function, and Origin of the Alveolar Lining Layer," published in the *Proceedings of the Royal Society of London* in 1958.[10] He had noted that bubbles expressed from the lungs of fetal guinea pigs did not have the stability of those found in term mammalian lungs and then suggested that one of the difficulties with which a premature baby has to contend is the increased surface forces in the immature lung.

The turning point in all this was the work of John Clements and colleagues,[11] Clements was working at the Army Chemical Center in Edgewood, Maryland. Clements and colleagues proceeded to measure surface tension in a more sophisticated way with a Wilhelmy balance that allowed them to ascertain the changes of surface tension with surface area. Around Christmas-time 1957, I drove to Edgewood and visited Clements. I saw the surface balance, returned to Boston, and decided to explore the reason why lungs of infants who died of hyaline membrane disease never had foam in their airways. It is obvious that the reason they lacked foam was that they did not have surfactants with the capacity to reduce surface tension when surface area was reduced. It was straightforward to demonstrate the deficiency in surfactants on a surface film balance modeled after that of Clements. The following is a quotation from the publication I co-wrote with Mead[12]:

> Low surface tension in the lining of the lung permits stability of the alveoli at end-expiration. Lacking such a material, the lung is predisposed to atelectasis. Measurements of the surface tension of lung extracts confirm the presence of a very surface-active substance in lungs of infants over 1,000 - 2,000 gm and in children and adults. In lung extracts of immature infants and infants dying with hyaline membrane disease, surface tension is higher than expected. This deficiency of surface-active material may be significant in the pathogenesis of hyaline membrane disease.

Meanwhile, Sue Buckingham joined me[13] with the idea that the alveolar type II cells could be the source of production of surface-active lipids because these cells contained osmiophilic lamellar inclusions. We showed a temporal correspondence in mice between the appearance of the osmiophilic bodies and the capacity of lung extracts to lower surface tension. Many studies since then support the concept that the osmiophilic or lamellar bodies are sites of storage for the phospholipids, which are attached to apoproteins that facilitate secretion and spreading on the alveolar surface.

The subsequent years were characterized by work from many laboratories, analyzing the lipid composition of materials expressed from the lung. The finding that lung lipid could be identified in amniotic fluid suggested to Gluck and Kulovich[14] that sampling amniotic fluid in the last trimester of pregnancy may allow ascertainment of the maturity of the lung. The use of the lecithin-sphingomyelin ratio led to the development of an important and clinically useful test to guide obstetricians in the likelihood of an infant being born with or without adequate quantities of pulmonary surfactants. That same year, 1973, Richard King and colleagues[15] identified a surfactant-specific protein that subsequently led to extensive exploration of the three or more proteins that are associated with lipids and can be recovered from lung lavage liquid.

As of early 1995, four surfactant-associated proteins had been discovered and fully defined by identification of their respective genes. Surfactant protein (SP) A is hydrophilic and extensively modified after translation. SP-B is hydrophobic and has been found deficient in congenital alveolar proteinosis. SP-C, the smallest of the peptides, has a covalent linkage with the phospholipids and enhances their uptake by the alveolar type II cells. SP-D resembles SP-A and serves as an opsonin to enhance uptake of bacteria and viruses by macrophages. The sites of synthesis, methods of degradation, and aspects of hormonal regulation have been explored and reviewed extensively.[16-18]

SURFACTANT REPLACEMENT

Glucocorticoids play an essential role in both lung maturation and the synthesis of lipid and protein components of pulmonary surfactants.[19,20] Knowledge of hormonal influences in the timing of organ maturation began with the observations of Moog,[21] who in 1953 described the influence of the pituitary-adrenal system on the differentiation of phosphatase in the duodenum of the suckling mouse. Extensive experience with the use of antenatal glucocorticoids to accelerate lung maturation followed the pioneering observations of Liggins,[22] who in 1969 noted the survival at a younger age of premature lambs of ewes that had received glucocorticoids before delivery. Many controlled clinical trials, which were reviewed in 1990 by Crowley and colleagues,[23] established both the safety and the efficacy of antenatal corticosteroids. It appears that the effects of the combined use of prenatal corticosteroids and postnatal surfactant replacement are additive. It is as if the steroids "condition" the lung by increasing the surface area over which the surfactants, which are instilled as a liquid into the trachea, can spread and exert their effects.

A new chapter in the history of surfactant discovery was written when Fujiwara and colleagues[24] presented their initial experience in an article in *Lancet* in 1980. They and others, notably Forrest Adams in Los Angeles and Goran Enhorning in Toronto,[25,26] spent the previous decade analyzing various mixtures of phospholipids in animal models and then testing them in vitro. They finally decided that mixtures composed only of phospholipids would not have the requisite surface properties to stabilize airways effectively. Fujiwara and colleagues decided to use artificial surfactant (material derived from minced cow lungs), which consisted of lipids and lipid-associated proteins. He enriched the material with added dipalmitoylphosphatidylcholine (DPPC) and called it *TA surfactant,* after Tokyo, where the pharmaceutical house was located, and Akita, where he was working at the time.

The experience of Fujiwara and colleagues was distinguished from that of many investigators who attempted to treat surfactant-deficiency states with aerosolized DPPC; they used natural surfactants from cow lungs, and they instilled them as a liquid into the trachea and enhanced distribution by changing the position of the infant from one side to the other. After the instillation of 3 to 5 mL of material, they could demonstrate distribution in all the lobes of the lung and, impressively, a prompt increase in oxygenation. Thus it was evident that the material was distributed to the gas-exchanging surfaces of the lungs because it resulted in a prompt and sustained improvement in oxygenation. Fujiwara and colleagues demonstrated that, in some infants, a single instillation in the first hour of life or after the diagnosis of hyaline membrane disease could produce improved oxygenation that persisted for 2 to 3 days, which was long enough for the baby to acquire the capacity to synthesize endogenous surfactant.

These observations were not immediately acclaimed because the experience was not a prospective, randomized, controlled study. Nevertheless many investigators in various parts of the world in the mid-1980s were encouraged to conduct prospective, randomized, controlled trials, not only with TA surfactant but also with other mixtures of materials, mostly those derived from calf lung lavage. Other sources of surfactant have included human amniotic fluid, as evaluated by Merritt and colleagues,[27] and porcine lung extracts, which were studied by the Collaborative European Multicenter Study Group[28] in Europe. Meanwhile, TA surfactant was licensed in Japan in 1988 and is widely used. Other preparations, including a totally synthetic mixture, colfosceril, cetyl alcohol, and tyloxapol, also known as colfosceril palmitate, are licensed in the United States. Exosurf was invented by John Clements, who proposed that an alcohol (hexadecanol) be added to the principal phospholipid in pulmonary surfactant, DPPC. He also added tyloxapol to facilitate dispersion.

The natural surfactants from bovine or porcine sources have been used extensively. Short-term benefits are evident with early administration, and continuing evaluation is under way to assess long-term benefits and safety.

Thus, in the years from identification of a deficiency of surfactants in the lungs of infants who died of hyaline membrane disease, to clinical trials, and now to the wide availability of natural and synthetic surfactants for treatment, the odyssey from bench to bedside to pharmacy has been accomplished.

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Jeffrey A. Whitsett

Composition of Pulmonary Surfactant Lipids and Proteins

In vertebrates, adaptation to a nonaqueous respiratory environment was achieved by the development of lungs, which provide an extensive surface area for gas exchange. The unique physicochemical boundary between respiratory gases and the respiratory epithelium creates a region of high surface tension, generated by the unequal distribution of molecular forces on water molecules at the air-liquid interface. Unopposed, this surface tension creates collapsing forces that cause atelectasis and respiratory failure. Pulmonary surfactant creates lipid layers separating alveolar gas from the aqueous phase, decreasing these surface forces. It is not surprising that pulmonary surfactant is found in all air-breathing vertebrates studied, including animals as phylogenetically divergent as the lungfish and humans.

Synthesis and secretion of an abundance of phospholipid-rich material accompany the maturation of lung before birth in the human. The lack of pulmonary surfactant in premature infants results in respiratory distress syndrome (RDS) after birth. Likewise, loss of surfactant function related to lung injury causes acute respiratory failure postnatally. Hereditary disorders of surfactant homeostasis cause respiratory failure in newborn infants and children. The specifics of structure and function of the surfactant complex have important implications for treatment of RDS and other pulmonary diseases. This chapter considers the phospholipid and protein components that play critical roles in surfactant function and pulmonary homeostasis.

FORMS OF PULMONARY SURFACTANT

Pulmonary surfactant is composed primarily of phospholipids but also contains proteins and carbohydrates. Pulmonary surfactant is present in distinct macromolecular aggregates whose structural forms probably are conferred by the relative abundance of proteins and phospholipids, as well as by the impact of mechanical forces on the surfactant material accompanying the respiratory cycle. Tubular myelin is a highly surface-active material sedimenting at relatively low gravitational forces and consists primarily of phospholipids and proteins (Figure 100-1). Various lamellar and vesicular forms of surfactant that lack the highly organized structures of tubular myelin also can be isolated from the lung. Although the forms of surfactant may be influenced by procedures designed to isolate or identify them (and thus represent artifactual structures), it is likely that these diverse structures are generated in vivo and play distinct roles in surfactant function and homeostasis.

LIFE CYCLE OF PULMONARY SURFACTANT

Surfactant lipids and proteins are synthesized, stored, and secreted by type II alveolar epithelial cells (Figure 100-2). Surfactant proteins are routed through the endoplasmic reticulum, Golgi complex, and multivesicular bodies before storage in lamellar bodies; the pathway involved in trafficking of surfactant lipids from the site of synthesis in the endoplasmic reticulum to the lamellar body is less clear. The contents of lamellar bodies are secreted into the air space in response to stretch, as well as various secretagogues, including purinoreceptor and adrenergic agonists. As secreted surfactant unravels in the air space, tubular myelin is formed in a process requiring extracellular calcium ions, surfactant lipids, and the surfactant proteins SP-A and SP-B. Tubular myelin is an abundant, large aggregate form of surfactant, probably serving as a reservoir from which surfactant monolayers or multilayers are generated at the alveolar surface. Rapid spreading and stability of these lipid films are determined by the influence of SP-B and the surfant protein SP-C, which serve to maintain phospholipids at the air-liquid interface, thereby reducing surface tension. Small vesicular forms, likely produced by the molecular forces produced during compression and decompression of surfactant during the respiratory cycle, are taken up by type II cells and either reutilized or catabolized. A fraction of surfactant is taken up by alveolar macrophages and degraded in a process requiring signaling by way of granulocyte-macrophage colony-stimulating factor (GM-CSF). Recent evidence demonstrates the important role of both GM-CSF and surfactant protein D (SP-D) in the regulation of surfactant phospholipid concentrations in the alveolus.[1]

ISOLATION OF PULMONARY SURFACTANT

Surface-active material usually is isolated by differential sedimentation of material collected by washing the lung with isotonic saline solutions.[2-4] Centrifugation at low forces is used to remove mononuclear cells, which in the normal lung are



Figure 100-1 Aggregate forms of alveolar surfactant. Lamellar bodies, the intracellular form of surfactant, are secreted into the alveolar lumen as concentrically arranged layers of tightly packed, phospholipid-rich membranes *(large arrow).* Here they are converted into tubular myelin, a lattice-like arrangement of intersecting tubules *(small arrows)* and associated matrix material *(arrowheads).* Image is a transmission electron micrograph of glutaraldehyde–tannic acid–osmium tetroxide fixed material from the neonatal mouse lung. (Magnification ×124,200.)

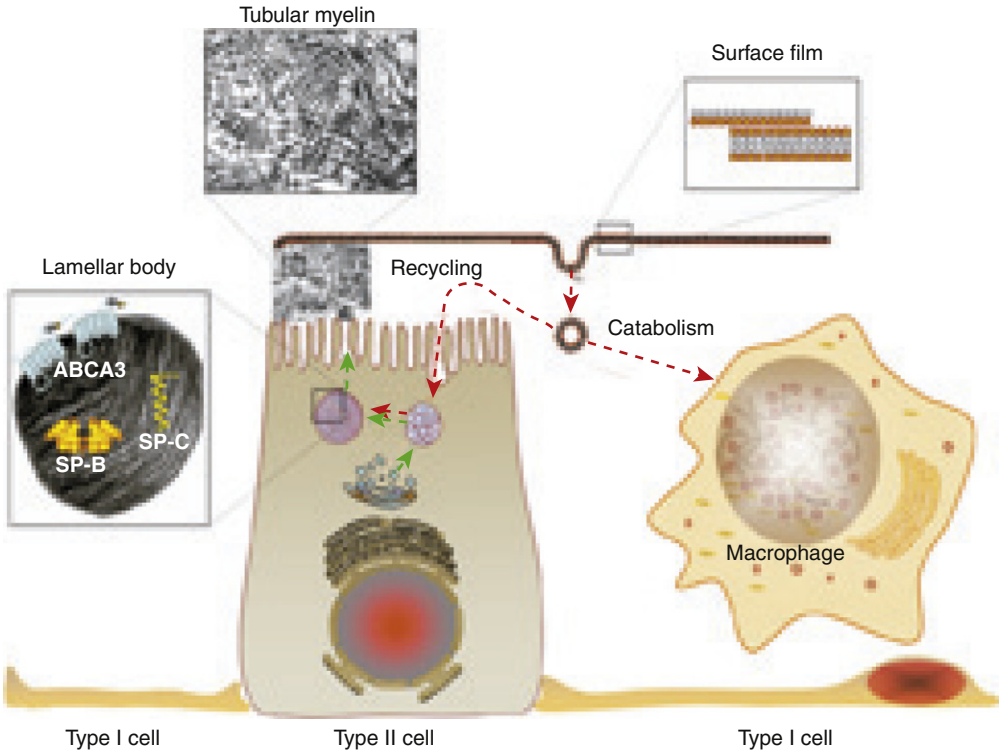


Figure 100-2 Life cycle of pulmonary surfactant. Pulmonary surfactant lipids and proteins are synthesized by type II epithelial cells. Surfactant proteins are transported from endoplasmic reticulum, Golgi complex, and multivesicular bodies to lamellar bodies. Surfactant proteins SP-B and SP-C are proteolytically processed during transit to lamellar bodies, where the active peptides are stored with phospholipids before secretion. After secretion, surfactant membranes unwind and in the presence of extracellular Ca^{2+} and SP-A from tubular myelin (large aggregate surfactant) from which monolayers and multilayers of phospholipids are produced to lower surface tension at the air-liquid interface. Small vesicles produced during the respiratory cycle are taken up and recycled by type II cells or catabolized by alveolar macrophages.

primarily alveolar macrophages. Some surface-active material—specifically, large tubular myelin forms—generally sediments at low gravitational forces. Higher-speed centrifugation or buoyant density separation is then used to isolate subfractions of surfactant containing various physical forms. The organization and separation of pulmonary surfactant are highly dependent on the concentration of calcium used during preparation. Calcium enhances the aggregation of surfactant, creating material with higher buoyant density.[5,6] The dense, tubular myelin-rich material (large aggregate surfactant) is enriched in surfactant-associated proteins SP-A, SP-B, and SP-C and is highly active as a pulmonary surfactant.[7,8] Less surface-active fractions containing primarily smaller or less dense vesicular forms (small aggregate forms) are relatively depleted of protein and are less surface-active than tubular myelin. SP-D is relatively enriched in the small aggregate fraction. These vesicular forms may

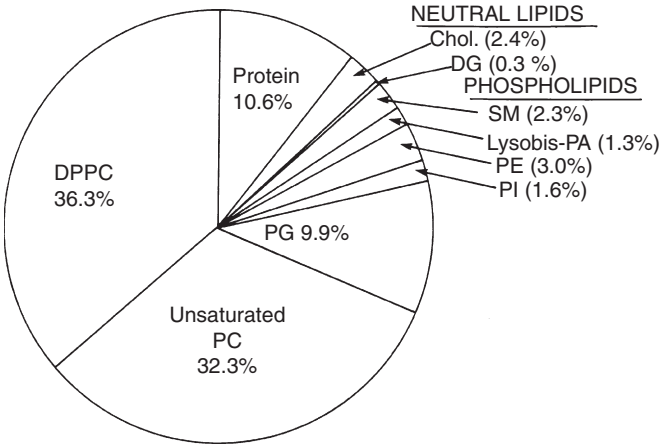


Figure 100-3 Composition of bovine pulmonary surfactant obtained from lung lavage fluid. Components are expressed as % wt. *Chol,* Cholesterol; *DG,* diacylglycerol; *DPPC,* dipalmitoylphosphatidylcholine; *PA,* phosphatidic acid; *PC,* phosphatidylcholine; *PE,* phosphatidylethanolamine; *PG,* phosphatidylglycerol; *PI,* phosphatidylinositol; *SM,* sphingomyelin. *(Modified from Possmayer F, et al: Pulmonary surfactant,* Can J Biochem Cell Biol *62:1121, 1984.)*

represent catabolic products generated from tubular myelin or from the lipids of the surface multilayers and monolayers that are generated during the respiratory cycle and are destined for uptake and catabolism by alveolar macrophages or reutilization by type II epithelial cells.

COMPOSITION OF SURFACTANT

The general composition of lung surfactant has been determined in numerous species, including reptiles, fish, birds, amphibians, and mammals. The composition of pulmonary surfactant is, in general, well conserved among diverse species and is rich in phosphatidylcholine and other lipids; proteins generally contribute less than 10% of its mass. The general composition of mammalian surfactant[9-11] is represented in Figure 100-3.

LIPID COMPONENTS

Surfactant is composed primarily of phospholipid, which represents approximately 80% to 90% of its mass. Lesser amounts of glycolipids and neutral lipids are detected in approximately equal amounts. Phospholipid is the primary surface tension–lowering component of pulmonary surfactant. The phospholipids form multilayered sheets that are derived from tubular myelin or other aggregate forms present in the alveolus. Phosphatidylcholine is the most abundant phospholipid in surfactant and is uniquely enriched in disaturated forms of palmitoylphosphatidylcholine (Table 100-1). In human surfactant isolated from lung minces, phosphatidylcholine represents 80% of the total phospholipid, of which 70% is present as the palmitoylphosphatidylcholine; 55% of this lipid species is in the form of disaturated palmitic acid acyl groups or disaturated phosphatidylcholine (DSPC).[11] Phosphatidylglycerol generally represents 5% to 10% of surfactant phospholipids. Phosphatidylglycerol also is capable of reducing surface tension at an air-liquid interface; however, its precise role in surfactant function remains unclear. Phosphatidylglycerol is not a prerequisite for surface tension–lowering, because surfactant from newborn rabbits lacking this phospholipid is highly functional at birth. It is possible that phosphatidylinositol provides the acidic phospholipid components required for optimal surface properties in the phosphatidylglycerol-deficient lung. Other phospholipids, including phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, lysophosphatidylcholine, and sphingomyelin, are present in relatively low amounts in pulmonary surfactant. Glycolipids also are present in pulmonary surfactant and have been partially characterized in rabbit surfactant. Neutral lipids are present primarily as cholesterol esters and acylglycerol fatty acids. The biologic functions of these components, present in relatively low amounts, have not been determined with certainty.[9-12]

TABLE 100-1

Phospholipid Composition of Human Surfactant and Lamellar Bodies (% wt/wt)

Component(s)	Surfactant	Lamellar Bodies
Phosphatidylcholine	73	71
Phosphatidylethanolamine	3	8
Phosphatidylglycerol	12	10
Phosphatidylinositol and phosphatidylserine	6	6
Sphingomyelin	4	2
Others	2	3

Modified from Harwood JL: Lung surfactant, *Prog Lipid Res* 26:211, 1987.

The molecular structures of phosphatidylcholine and phosphatidylglycerol are represented in Figure 100-4. Several aspects of their structures are critical for surface tension reduction at the alveolar-air interface. Each molecule consists of a three-carbon glycerol backbone. The C_1 carbon is modified by the addition of polar head groups (relatively more hydrophilic residues). In the case of pulmonary surfactant, the most abundant head groups are choline and glycerol. The C_2 and C_3 carbons of the glycerol backbone contain acyl groups of long-chain fatty acids, which are highly hydrophobic and lacking in significant charge. The polar head groups—choline, glycerol, and inositol—of the phospholipids produce charge-dependent interactions among neighboring phospholipid molecules and with water. By contrast, the acyl groups are energetically more stable in a nonaqueous environment and are tightly associated with neighboring phospholipid molecules by interactions between carbon and hydrogen atoms of the acyl chains. Hence these molecules are inherently insoluble in aqueous environments and form a variety of complex structures that include membrane monolayers, bilayers, multilayers, micelles, inverted micelles, and vesicles.

The surface properties of surfactant phospholipids (spreading, stability, and surface tension reduction) are influenced by a number of factors, including the degree of saturation of the acyl chains, which alter the tightness of packing of phospholipid molecules in membranes. The fatty acid composition of the phospholipids in pulmonary surfactants has been determined for various species[10] (Table 100-2). The structure of the acyl chains and the composition of the major phospholipids are important determinants of the organization of the membranes. The acyl chains present in phosphatidylcholine isolated from pulmonary surfactant are uniquely enriched in palmitic acid (C16) with disaturated acyl chains. Enrichment of these phospholipid species at the surface would result in a densely packed monolayer, creating an interface with extremely low surface tension. Saturated acyl chains contain no methylene (C═C) bonds, and the carbon atoms are fully hydrogenated. Membranes containing such lipids pack densely through the hydrophobic interactions of the acyl chains. The ordering of phospholipid molecules in the surfactant membrane also is highly dependent on temperature. Surfactant lipids are present in a gel or crystalline state at the physiologic temperatures of homeothermic

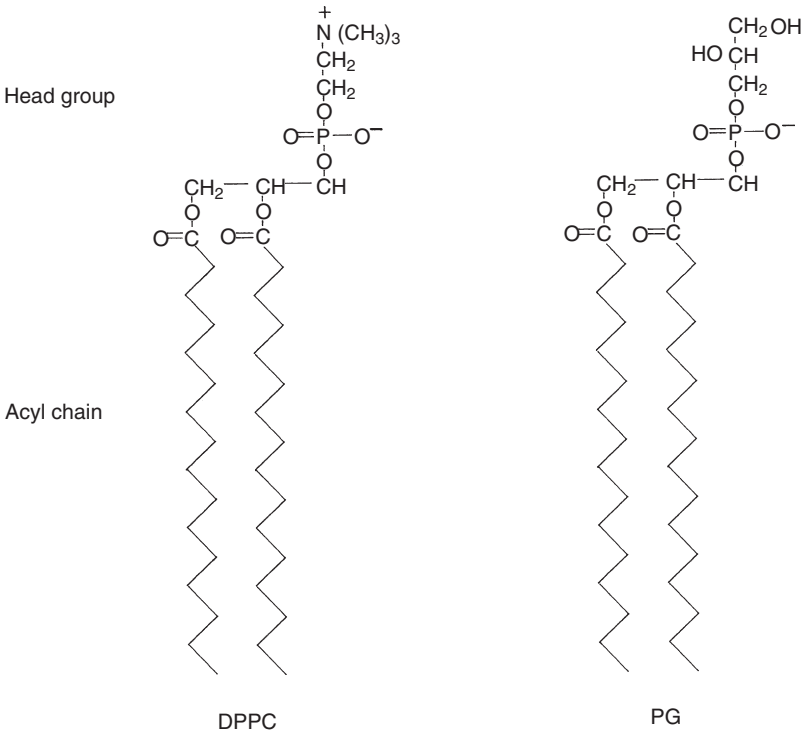


Figure 100-4 Molecular structures of dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerol (PG). Phospholipid molecules pack densely, forming membrane monolayers, bilayers, and vesicles and other aggregate forms. Strong molecular interactions occur between polar head groups. Distinct interactions occur between atoms composing the more hydrophobic acyl chains.

TABLE 100-2

Fatty Acid Composition of Phospholipids in Human Surfactant

Acyl Chains	Percentage of Weight/Weight (%)
14:0	3
16:0 (dipalmitoylphosphatidylcholine [DPPC])	81
16:1	6
18:0	3
18:1	5
18:2	2
Others	Trace

Modified from Harwood JL: Lung surfactant, *Prog Lipid Res* 26:211, 1987.

organisms because the transition temperature (temperature of melt) of DPPC is approximately 41° C. Therefore DPPC would be present in a relatively rigid state at 37° C. The presence of minor lipids, proteins, and unique phospholipid acyl chains, however, alters the packing characteristics of the phospholipids. The relative abundance of the major lipid classes and their acyl chain length and specific composition, including the proportion of molecular species with unsaturated acyl chains, therefore result in a unique pulmonary surfactant mixture that may alter the surface properties of the surfactant film. The characteristics of rapid adsorption and stability during compression of pulmonary surfactant are not properties inherent in the phospholipids alone, requiring the presence of surfactant proteins as well. The hydrophobic surfactant proteins SP-B and SP-C play critical roles in the organization of phospholipids and are required for full surface-active properties of the lipids in surfactant.

COMPOSITION OF LAMELLAR BODIES

Surface-active material can also be isolated from its primary intracellular storage site in lamellar bodies of alveolar type II cells. Lamellar bodies are highly enriched in phospholipids, generally containing approximately 10 to 12 mg of phospholipid per mg of protein. The limiting membrane of the lamellar body contains at least one ABC transporter, ABCA3, which probably plays an important role in importing surfactant lipids into the lamellar body.[13,14] Mutations in the ABCA3 transporter block the formation of lamellar bodies and cause severe lung disease in newborn infants.[15] Lipid composition of lamellar bodies is similar to that for surfactant isolated from lung lavage fluid.[10] SP-B and SP-C are highly enriched in lamellar bodies and are co-secreted with phospholipids into the air space.

DEVELOPMENTAL CHANGES IN PHOSPHOLIPID COMPOSITION

The phospholipid composition of alveolar lavage material changes during perinatal development. Increased phospholipid synthesis and secretion occur with advancing gestation and are influenced by a variety of hormonal and cellular factors.[12] Because pulmonary secretions contribute a significant volume to amniotic fluid, increased phospholipid in amniotic fluid accompanying advancing gestation has been used for determining the relative maturity of the fetal lung and thus predicting the risk of RDS in premature infants. The phosphatidylcholine content of amniotic fluid increases during the last third of human gestation. The ratio of lecithin (phosphatidylcholine) to sphingomyelin, otherwise known as the L/S ratio, has been useful in the clinical assessment of risk for RDS.[16]

Surfactant content in amniotic fluid can be determined by a number of procedures that predict pulmonary maturity.[17,18] Various amniotic fluid assays are useful in predicting surfactant function or lack of respiratory distress in preterm infants, including the L/S ratio; lamellar body counts; quantitation of phosphatidylglycerol, DSPC, or phosphatidylcholine; and fluorescence anisotropy. Changes in total phospholipid content and in the relative abundance of phospholipid species also accompany respiratory failure in infants and adults.[19]

TABLE 100-3

Human Surfactant Proteins

Gene	Protein	Protein Monomer (kDa)	Chromosome	Function
SFTPA	SP-A	30,000-36,000	10	Innate defense/tubular myelin
SFTPB	SP-B	8000	2	Surface activity, lamellar body formation
SFTPC	SP-C	3800	8	Surface activity
SFTPD	SP-D	43,000	10	Innate defense, surfactant lipid homeostasis

SP-A, -B, -C, -D, Surfactant proteins A to D.

PROTEINS

The characteristics and nomenclature of surfactant proteins are the subject of several reviews.[20-22] Surface-active fractions isolated from lung lavage by differential sedimentation contain numerous polypeptides, which include both serum and nonserum proteins. At present, four distinct surfactant-associated proteins have been purified from surfactant and their primary structures discerned. These proteins have been termed surfactant proteins SP-A, SP-B, SP-C, and SP-D (Table 100-3). Complementary DNAs and genes encoding these surfactant proteins have been isolated and characterized. The human genes are termed *SFTPA, SFTPB, SFTPC,* and *SFTPD.* The role of each of the proteins in surfactant function and pulmonary homeostasis have been clarified in gene-targeted mice and in clinical observations in children and adults. The precise abundance of each of the proteins in pulmonary surfactant or surfactant subfraction has not been determined with certainty. Nevertheless, SP-A, SP-B, SP-C, and SP-D account for most of the nonserum proteins present in the lipid-associated fraction isolated from lung lavage.

Surfactant Protein A

SP-A is an abundant nonserum lipid-associated protein in pulmonary surfactant.[4] SP-A is a member of a related family of polypeptides termed the *collectins* that includes SP-D, mannose-binding lectin, conglutinin, and collectin-43 (CL-43). These proteins share collagenous and lectin-containing domains that bind complex carbohydrates. SP-A is a glycoprotein of 26,000 to 35,000 Da that undergoes sulfhydryl-dependent oligomerization and other post-translational modifications, accounting for the significant molecular heterogeneity of its isoforms in pulmonary surfactant. Two human *SFTPA* genes have been identified, each consisting of five exons contained within approximately 4.5 kilobases (kb) of DNA.[23] The *SFTPA* locus consists of two coding sequences and a noncoding sequence on human chromosome 10.[24,25] The entire polypeptide sequence of SP-A has been deduced from the gene and complementary DNAs (cDNAs) encoding the human proteins[23] (Figure 100-5). SP-A is first synthesized as a 248 amino-acid precursor peptide from which a small signal sequence is cleaved. The remaining amino-terminal (N-terminal) domain of the mature SP-A peptide contains an extensive collagen-like region of approximately 10,000 Da. A discrete carboxyl-terminal (C-terminal) globular domain is homologous to other mammalian lectins (carbohydrate-binding proteins) and is structurally related to those in SP-D, mannose-binding lectin, and other members of the collectin family of polypeptides.[26] SP-A binds carbohydrates, phospholipids, and glycolipids that are surface components of numerous pathogens, including bacteria, viruses, and fungi.[27,28]

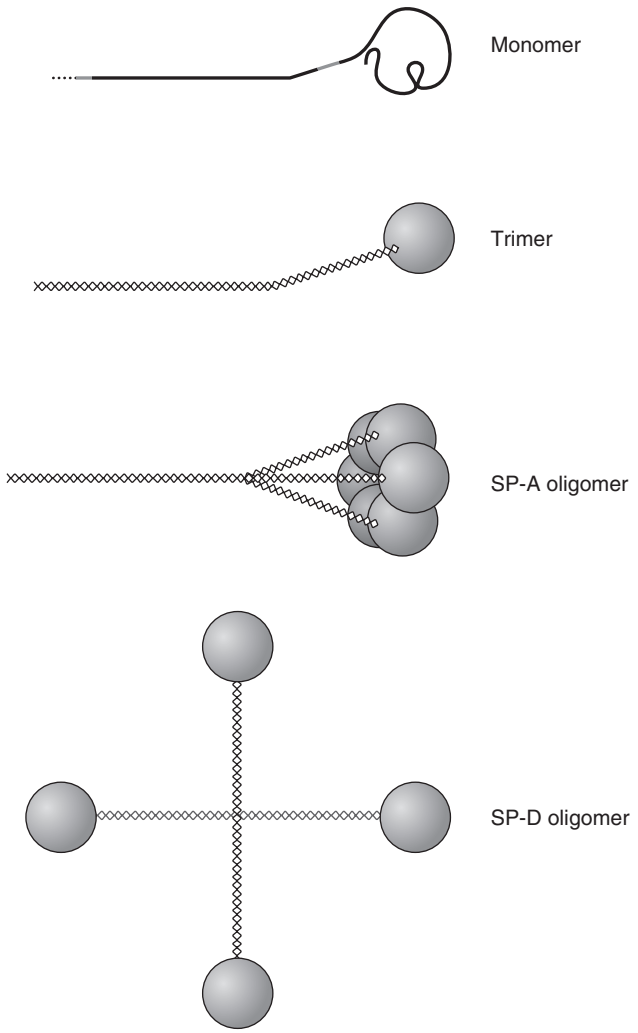


Figure 100-5 Human SP-A is encoded by genomic DNA on chromosome 10. Translocation of the mRNA produced a monomer that forms trimers. Furthermore, oligomerization through the amino-terminal (N-terminal) collagen-like domain *(hatched area)* results in hexamers and larger assembled forms such as octadecamers found in association with tubular myelin in the alveolus. SP-D is formed by a similar process from the SP-D gene locus, also located on chromosome 10; SP-D monomers form trimers that are organized into tetramers and dodecamers.

Functions of Surfactant Protein A

Although the functions of SP-A have not been clarified precisely, in vitro and in vivo studies have demonstrated a wide variety of biologic activities intrinsic to the molecule. SP-A binds carbohydrates and aggregates phospholipids in a calcium-dependent manner.[29-31] Mixtures of phospholipids, SP-A, and SP-B form tubular myelin-like structures in vitro.[32] SP-A enhances the biophysical activity of surfactant phospholipid-rich extracts.[29] In vitro, SP-A binds type II epithelial cells and macrophages and is internalized by receptor-mediated endocytosis.[33,34] After uptake, SP-A is

detected in multivesicular bodies and vesicles closely associated with lamellar bodies.[33] Receptor binding and internalization support the concept that SP-A is reutilized or is involved in cell signaling. Although in vitro findings support a role for SP-A in surfactant homeostasis,[35-37] gene targeting experiments in transgenic mice do not support its importance in surfactant metabolism in vivo.[38] By contrast, a number of studies support a critical role for SP-A in innate host defense of the lung.[27] SP-A is a member of the Ca^{2+}-dependent lectins or "collectins" that serve as opsonins, enhancing binding, phagocytosis, and killing of a variety of bacterial, fungal, and viral pathogens. SP-A enhances binding or uptake of group B streptococci, subtypes of *Haemophilus influenzae, Staphylococcus aureus,* and *Pseudomonas aeruginosa* by alveolar macrophages and is therefore an important defense molecule in the both neonatal and mature lung.[27] SP-A activates alveolar macrophages in vitro and enhances their opsonin-type functions, oxidant production, and killing of pathogens.[39]

Transgenic SP-A–deficient mice, *Sftpa* gene–targeted mice, in which the SP-A gene was disrupted by homologous recombination, survive postnatally.[38] Although SP-A deficient mice do not form tubular myelin, intracellular and extracellular surfactant phospholipid content, uptake of PL by lung tissue, PL secretion, as well as surfactant function are not altered by the absence of SP-A. Thus SP-A plays a critical role in innate host defense of the lung and in the organization of tubular myelin but does not play an important role in surfactant function or homeostasis in vivo.

Control of Surfactant Protein A Content

Like other surfactant proteins, SP-A is synthesized by respiratory epithelial cells in the developing fetal lung and its expression increases in late gestation (reviewed by Ballard[40]). SP-A is detected in nonciliated cells in tracheal bronchial glands, as well as in bronchiolar and alveolar epithelial cells of the lung. In the human, expression of SP-A increases with advancing gestational age in association with the maturation of type II epithelial cells occurring in the latter part of gestation. SP-A appears in increasing concentrations in amniotic fluid during advancing gestation and, like the L/S ratio, is a useful marker of fetal lung maturity in humans.[41,42] SP-A synthesis in human fetal lung cultures is enhanced by a number of hormonal factors, including epidermal growth factor, interferon-γ, interleukin-1, and cyclic adenosine monophosphate (cAMP).[40,44] Glucocorticoids both stimulate and inhibit human SP-A synthesis by distinct mechanisms, which include transcriptional enhancement and decreased messenger RNA (mRNA) stability in vitro.[44] Transcription of the SP-A gene, as well as the genes encoding SP-B and SP-C, are regulated by elements located in the 5′ region of the gene that bind to nuclear transcription factor thyroid transcription factor-1 (TTF-1), a homeodomain–containing member of the Nkx2.1 family of proteins. Binding of TTF-1 confers lung epithelial specificity to SP-A gene transcription. TTF-1 also regulates the transcription of genes expressed exclusively in the respiratory epithelium including SP-A, -B, and -C and the Clara cell secretory protein (CCSP).[45] Inhibitory effects of TGF-β and insulin on SP-A expression have been observed in human fetal lung explant culture.[46,47] SP-A content in alveolar lavage is induced by oxygen exposure, interleukin-4, and silicosis, and SP-A accumulates in the lavage fluid from patients with pulmonary alveolar proteinosis.[48-50] Although GM-CSF–deficient mice develop severe pulmonary alveolar proteinosis with increased surfactant protein content, the relative abundance of surfactant protein mRNAs are not altered, supporting the concept that GM-CSF plays an important role in surfactant clearance.[1,51] Studies in humans demonstrated that pulmonary alveolar proteinosis is associated with neutralizing antibodies against GM-CSF or mutations in the GM-CSF receptor.[52]

In summary, SP-A is an abundant pulmonary host defense protein that is strongly associated with surfactant phospholipids in tubular myelin. SP-A plays important roles in innate defense against bacterial, fungal, or viral pathogens, enhancing opsonization and killing of respiratory pathogens by alveolar macrophages.[27] SP-A also is expressed at lower levels in extrapulmonary cells, and infection of newborn SP-A–deficient mice results in significant mortality associated with gastrointestinal tract disease.[53]

Surfactant Protein D

SP-D is another member of the collectin family of polypeptides, sharing structural motifs with SP-A and other family members.[27,28] A single human SP-D gene *(SFTPD)* is located in close proximity to the SP-A genes on chromosome 10. SP-D is slightly larger than SP-A, being composed of 43-kDa monomers containing an approximate 15,000-kDa collagenous region that forms trimers and higher-ordered complexes that are found in the alveoli (see Figure 100-5).

Unlike that of SP-A, SP-B, and SP-C, the expression of SP-D is not restricted to cells within the lung. SP-D mRNA and protein have been detected in various organs, including the gastrointestinal tract, pancreas, bile duct, cervical glands, and other sites.[54] In the lung, SP-D is expressed by type II epithelial cells in the alveoli, nonciliated bronchiolar, and tracheal-bronchial epithelial cells, including cells lining tracheal-bronchial glands. SP-D is less abundant and is not associated with large aggregate forms of surfactant. SP-D is, however, associated with small lipid vesicles (small aggregate surfactant) but is not required for the formation of tubular myelin or lamellar bodies.[55]

Role of Surfactant Protein D in Innate Host Defense of the Lung and Regulation of Surfactant Homeostasis

The C terminus of SP-C consists of a globular carbohydrate recognition domain (CRD) that binds molecules on the surface of bacterial, viral, and fungal pathogens. SP-D has high affinity for various bacterial lipopolysaccharides (LPSs), complex carbohydrates, and phosphoinositol.[28] SP-D binds and agglutinates various bacteria (including *Escherichia coli*, *Salmonella*, and *Pseudomonas*), fungal, as well as viral pathogens (influenzavirus A, adenovirus, and respiratory syncytial virus), enhancing their uptake and killing by alveolar macrophages.[27,28] Thus SP-D serves an important role in pathogen recognition critical to innate defense of the lung against infection. In vivo, SP-D–deficient (*Sftpd* gene–targeted) mice are susceptible to various bacterial and viral pathogens. SP-D enhances the clearance and suppresses inflammatory responses following viral and bacterial infection of the lung.[27] SP-D also plays important roles in the regulation of surfactant phospholipid pool size and in the suppression of oxidant production by alveolar macrophages in the lung.[56] Deletion of SP-D in transgenic mice caused emphysema, macrophage activation, accumulation of oxygen reactive species, and a pulmonary lipidosis. Alveolar and tissue surfactant lipid pool sizes were markedly increased in the absence of SP-D. Thus SP-D plays a critical role in the regulation of surfactant lipid homeostasis, the inflammatory responses, and innate host defense of the lung. Because SP-D influences inflammation and cytokine responses after exposure to various pulmonary pathogens, it also is highly likely that SP-D influences subsequent acquired immune responses after infection.

Regulation of Surfactant Protein D

SP-D is expressed at high levels in the lung, where it is regulated by C/EBPα (*C*CAAT/*e*nhancer-*b*inding *p*rotein-α), nuclear factor of activated T cells (NFAT), and TTF-1.[57] SP-D also is expressed in many other organs and in various cell types.[54] SP-D content in fetal lung and amniotic fluid increases with advancing gestational age, and its content is enhanced by glucocorticoids in experimental models. SP-D expression is enhanced by allergens and interleukins (IL-4 and IL-13). SP-D concentrations in alveolar

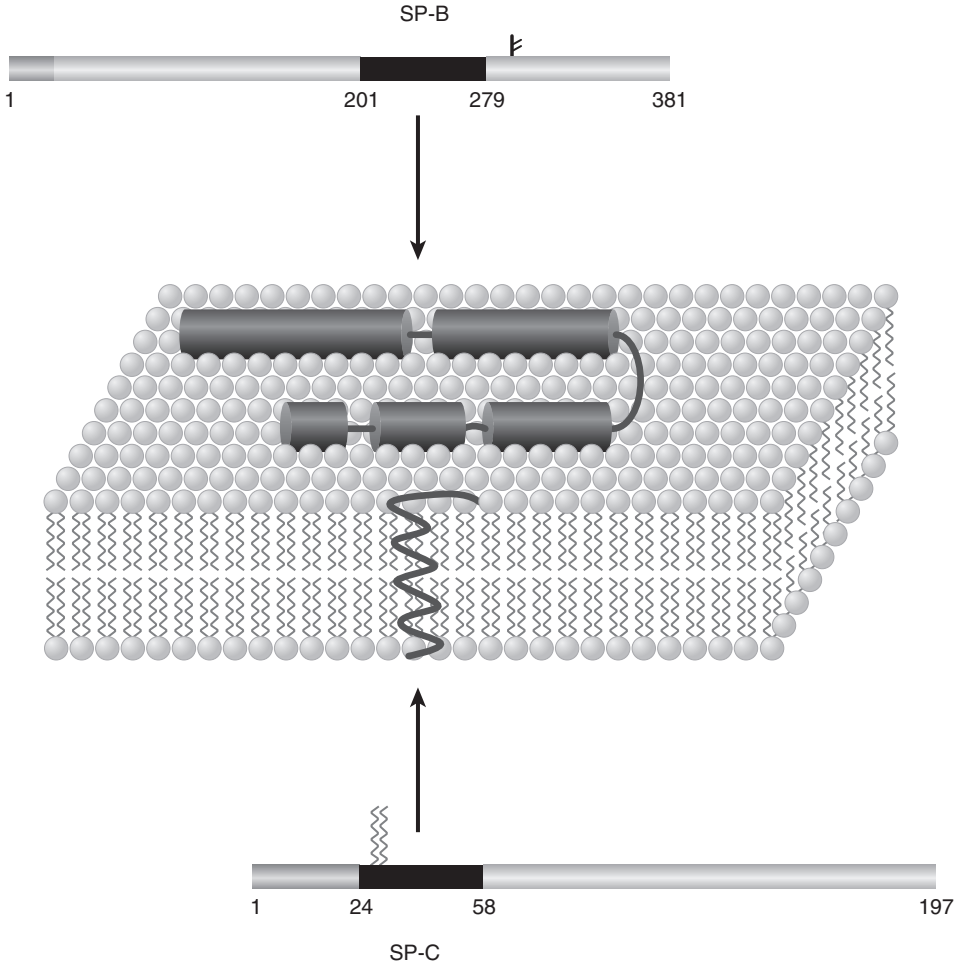


Figure 100-6 Human SP-B is encoded by a single gene located on chromosome 2 and comprises approximately 9.5 kb of genomic DNA. The active peptide comprises 79 amino acids. The messenger RNA (mRNA) is approximately 2.0 kb in length and is translated to a preproprotein of approximately 39,000 Da that is proteolytically processed and glycosylated. The active peptide is generated from proSP-B during proteolytic processing to form the 79–amino acid peptide (M_r of 8000), that forms oligomers are tightly associated with phospholipids in the airway. Human SP-C is encoded by a gene located on chromosome 8 that compries approximately 3.5 kb of genomic DNA. The active peptide, comprising 35 or 36 amino acids, is encoded by a single exon and is palmitoylated. The mRNA is approximately 0.9 kb long and is translated to proSP-C (M_r of approximately 22,000), which is proteolytically processed to an M_r = 3800 monomer, and its oligomers found in the airway. SP-B and SP-C are tightly associated with phospholipid membranes in the alveolus. M_r, Molecular weight.

fluid are decreased in various clinical conditions associated with pneumonitis, including cystic fibrosis.[27,58] Like other surfactant components, SP-D accumulates in the pulmonary alveolar proteinosis associated with GM-CSF deficiency and silicosis.[1]

Surfactant Protein B

SP-B is a small hydrophobic polypeptide comprising 79 amino acids (Figure 100-6). SP-B structure and function have been reviewed in detail by Weaver and Conkright[59]; a brief summary follows.

A single human SP-B gene *(SFTPB)* is composed of 10 exons, spanning approximately 10 kb of DNA located on chromosome 2.[60] Analysis of cDNAs encoding SP-B demonstrate that the SP-B polypeptide is produced by proteolytic processing of an approximately 40,000- to 46,000-Da glycosylated precursor comprising 381 amino acids.[61-63] The active peptide found in the airway forms sulfhydryl-dependent oligomers that include dimers and tetramers. Proteolytic processing of the precursor proSP-B occurs in type II epithelial cells, during routing of the proSP-B from endoplasmic reticulum to multivesicular bodies and during transport from MVB and lamellar bodies before secretion. Immunostaining with antibodies generated against SP-B co-localize with apical intracellular inclusions in type II cells and stain material in the lumen of alveolar and airway structures.[64,65] Although the functions of SP-B have not been elucidated with certainty, SP-B is tightly associated with surfactant phospholipids and is required for formation of tubular myelin in the presence of SP-A, phospholipids, and calcium.[32] SP-B is highly fusogenic,[66] generating phospholipid membranes from vesicular lipid forms and enhancing spreading and stability of surfactant. SP-B is required for conversion of vesicular lipids in the lumen of multivesicular bodies to the tightly packed membrane sheets observed in the lamellar bodies.[67] Although SP-B enhances the uptake of phospholipid vesicles by type II epithelial cells in vitro, when present with SP-A, SP-B enhances formation of tubular myelin, a form of surfactant that remains in the alveolus.[68] Increasing evidence supports the concept that SP-B is critical for the enhancement of surface properties of surfactant phospholipids. SP-B is an important component of surfactant replacement mixtures made by organic solvent extraction of pulmonary surfactant or lung minces.

Surfactant Protein B Is Required for Lung Function at Birth: Hereditary SP-B Deficiency

Studies in SP-B gene (*Sftpb)*–targeted mice, and in full-term infants bearing mutations in *SFTPB*, demonstrated that SP-B is required for pulmonary function at birth.[67,69] Although pulmonary structure is normal, mice lacking SP-B die in respiratory distress immediately after birth. Decreased lung volumes, lack of hysteresis and atelectasis were associated with the lack of lamellar bodies and accumulation of aberrant multivesicular bodies with type II cells. Decreased surfactant activity, lack of tubular myelin, and the synthesis of an abnormal proSP-C precursor demonstrated that SP-B is required for both intracellular and extracellular routing of surfactant lipids and proteins. Full-term human infants with mutations in *SFTPB* generally demonstrate respiratory distress within hours after birth, with clinical and radiologic features typical for RDS in preterm infants. Respiratory failure generally is progressive and is not responsive to exogenous surfactant replacement therapy, affected infants dying of respiratory failure despite intensive ventilatory support. In SP-B–deficient infants, alveolar spaces are filled with proteinaceous material[69] that consists primarily of surfactant proteins and abnormally processed proSP-C. The disorder generally is inherited in an autosomal recessive manner.

The most common mutation, $SP\text{-}B_{121ins2}$, creates an unstable mRNA, resulting in lack of SP-B protein production. More than 15 distinct mutations in the SP-B gene have been associated with respiratory failure in infants.[70] The disorder generally is fatal in the first months of life but has been treated by lung transplantation. SP-B is required for lung function and survival in adult animals.[71]

In summary, SP-B is required for packaging and processing of surfactant lipids and proteins intracellularly and for organization, function, and homeostasis of surfactant lipids and proteins in the alveolar space.

Regulation of Expression of Surfactant Protein B

The abundance of SP-B mRNA increases with advancing gestation in the human fetal lung, being expressed in both type II alveolar cells and nonciliated respiratory epithelial cells lining distal portions of the respiratory tract.[72] In fetal lung, SP-B synthesis is stimulated by cAMP, IL-1β, and glucocorticoids.[40,43,73,74] The concentration of SP-B in human amniotic fluid increases with advancing gestation in association with increased L/S ratio and phosphatidylglycerol.[75] As with other surfactant proteins, the SP-B expression is regulated by the homeodomain-containing nuclear protein TTF-1, that controls transcription of the SP-B gene.[45,57]

Surfactant Protein C

SP-C is the most hydrophobic protein isolated from pulmonary surfactant[21,22] (see Figure 100-6). Like SP-A and SP-B, SP-C is relatively abundant in the dense surfactant fractions obtained by lung lavage. SP-C consists of 35 amino acids, most of which are hydrophobic residues valine, leucine, and isoleucine.[76] Complementary DNAs and genomic DNA encoding SP-C *(SFTPC)* have been isolated and characterized.[77,78] In humans, the SP-C gene comprises approximately 3 kb of contiguous DNA and consists of 6 exons located on chromosome 8. Allelic variations and differential RNA splicing are likely to account for the molecular heterogeneity at these loci.[79] The precursor contains neither an N-terminal signal sequence nor amino acid sequences predicting the addition of asparagine-linked carbohydrate. SP-C is palmitoylated and is transported through the endoplasmic reticulum to multivesicular bodies with proSP-B. Both proSP-C and proSP-B are processed during transport to lamellar bodies, SP-B being required for proteolytic processing of proSP-C. The active 35–amino acid SP-C peptide is stored in lamellar bodies and is associated with surfactant lipid in the alveolus. In the lung, synthesis of SP-C is restricted to type II alveolar cells.

SP-C or mixtures of SP-B and SP-C enhance the rate of absorption of surfactant phospholipids and confer important surfactant-like properties to the lipids.[21,22,59,80] Evidence from studies in *Sftpc* gene–targeted mice demonstrates that SP-C is not required for formation of lamellar bodies or tubular myelin. SP-C is taken up by alveolar epithelial cells; addition of SP-C peptides enhances uptake of phospholipid vesicles by type II epithelial cells in vitro.[68,81] SP-C is highly enriched in organic solvent extracts of surfactant and, like SP-B, is present in the surfactant extracts used for replacement therapy of RDS in infants.

Role of Surfactant Protein C in Surfactant Function and Homeostasis

Studies in SP-C gene–targeted mice and in humans bearing mutations in the SP-C gene demonstrate the important role of SP-C in surfactant function and pulmonary homeostasis.[80] Although SP-C gene–targeted mice survive perinatally, abnormalities in the stability of surfactant film formed from SP-C–deficient mice, demonstrate the role of SP-C in recruiting phospholipids to monolayers/multilayers, SP-C is required for the stability of phospholipid films during dynamic compression.[82] Formation of lamellar bodies and tubular myelin are not perturbed in SP-C deficient mice. However, SP-C–deficient mice develop strain-dependent interstitial lung disease associated with emphysema, epithelial cell dysplasia, and inflammation.[80] Humans bearing mutations in the SP-C gene *(SFTPC)* develop various forms of acute and chronic pulmonary disease including acute respiratory distress syndrome (ARDS) and idiopathic pulmonary fibrosis.[83,84] *SFTPC* mutations generally are inherited as an autosomal dominant trait and have been associated with the histopathologic diagnoses of usual interstitial pneumonitis, nonspecific interstitial pneumonitis, and desquamating interstitial pneumonitis.

Regulation of Surfactant Protein C Synthesis

SP-C synthesis and mRNA content increase in association with type II cell maturation in fetal lung, and its expression is restricted to type II cells in the postnatal lung.[40,73,74] SP-C mRNA is detected early in embryonic lung development—by postconception day 10 in the mouse and at 10 to 12 weeks gestation in the human.[85] SP-C mRNA are increased by glucocorticoids and cAMP.[73,74] The transcription of the *Sftpc* gene is lung epithelial cell–specific and requires TTF-1.[45,57]

Role of Surfactant Proteins in Surfactant Replacement Preparations

The structures of each of the major surfactant proteins are quite distinct, supporting the concept that each plays a unique role in the structure and function of surfactant. Surfactant phospholipids themselves, although providing the molecules critical for the reduction of surface tension in the alveolus, do not have the properties inherent in pulmonary surfactant. At physiologic temperatures, surfactant lipid is in a highly organized gel-crystalline state that is not capable of rapidly forming a surface film. Phospholipid molecules alone fail to rapidly spread and respread during compression and decompression. Their adsorption rates to surfaces are slow and do not generate the surface tension–lowering film necessary to maintain surface forces during the respiratory cycle. SP-B and SP-C interact with surfactant phospholipids to produce surfactant with unique physicochemical properties, allowing formation and stability of the surfactant film during the respiratory cycle.

Both SP-B and SP-C confer important surfactant-like activity to phospholipids and are included in preparations used for treatment of surfactant-deficient states.[86-89] Preparations containing SP-A have not been used widely for clinical studies. Human surfactant isolated from amniotic fluid, containing SP-A, SP-B, or

SP-C, as well as a variety of other human proteins, was previously used for exogenous surfactant replacement. Surfactant extracts based on organic solvent extracts of lung or surfactant preparations contain SP-B and SP-C (but not SP-A).[86] Survanta, Curosurf, Alveofac, and Infasurf are examples of such preparations.[90] SP-B and SP-C, when mixed with phospholipids, appear to be sufficient to generate the surface-active properties of pulmonary surfactant and may therefore be useful in future development of surfactant replacement preparations for treatment of surfactant-deficient states. Protein-free (Exosurf, Coltosceril) and synthetic peptide–based (Lucinactant) surfactant preparations have been developed, although their current use is limited.[91]

DISORDERS OF SURFACTANT HOMEOSTASIS

Mutations in genes encoding the surfactant proteins SP-B and SP-C (*SFTPB* and *SFTPC*) and the lamellar body–associated lipid transporter protein *(ABCA3)* are a rare cause of severe pulmonary disease in newborn infants and children,[15] as reviewed by Whitsett and associates.[92] Although each protein has distinct structures and functions, these mutations generally cause acute respiratory failure in newborn infants or chronic interstitial lung disease in children. Most infants with mutations in *SFTPB* or *ABCA3* are born at term and present in the immediate neonatal period with progressive respiratory failure and clinical and radiologic abnormalities consistent with respiratory distress and typically fail to respond to surfactant replacement therapy and supportive care. *SFTPB*- and *ABCA3*-related diseases are inherited as autosomal recessive traits. Most patients with mutations in *SFTPC* present with the respiratory signs and symptoms later in infancy, but some have been symptomatic at birth.

Histopathologic analysis of lung tissue from patients with mutations in *SFTPB, SFTPC,* or *ABAC3* reveals interstitial lung disease, consistent with chronic pneumonitis of infancy, nonspecific interstitial pulmonary disease, or desquamating interstitial pneumonitis. Abnormalities in surfactant lipid content associated with these disorders include decreased phosphatidylcholine and phosphatidylglycerol content. Mutations in *SFTPC* generally are inherited as an autosomal dominant trait with variable penetrance. These mutations cause the synthesis of a misfolded protein that in turn causes alveolar type II cell injury, disrupting the production of the active SP-C peptide. Although *SFTPC* mutations may be associated with disease manifestations in the newborn period, most affected patients present in infancy or childhood with chronic respiratory disease, often exacerbated by respiratory infection. Clinical, histopathologic, and immunohistochemical criteria are useful for identifying patients with disorders of surfactant homeostasis. Definitive diagnosis requires identification of the mutations underlying the disorder.

OTHER ALVEOLAR PROTEINS

Detailed analysis of material obtained at lung lavage, such as by two-dimensional gel electrophoresis or sensitive silver staining techniques, reveals hundreds of serum and nonserum proteins. Precise abundance, the cellular source, and the role of each of these proteins in lung function have not been determined. The alveolar fluid contains a number of molecules that may play a role in host defense: fibronectin, lysozyme, antiproteases, immunoglobulins (particularly IgA), defensins, mucins, and Clara cell proteins. Products from type I and type II epithelial cells, alveolar macrophages, and lymphocytes are likely to contribute to the heterogeneity of proteins found in alveolar lavage fluid from the normal lung; however, the identity and functions of these proteins have not been clarified. It also is increasingly apparent that surfactant homeostasis may be disrupted by the presence of blood or serum proteins, including albumin and fibrin.[93-95] Thus homeostatic mechanisms that exclude nonsurfactant proteins from the alveolus are likely to be highly critical for the function of surfactant and thus for gas exchange after birth.

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Fred Possmayer

Physicochemical Aspects of Pulmonary Surfactant

When an infant is born, he or she must clear the lungs of fetal pulmonary fluid, inflate the alveoli with air, and establish air breathing. These functions are facilitated by the presence of pulmonary surfactant. By lowering the surface tension in the lung to low values, surfactant reduces the work of breathing and stabilizes the terminal air spaces, particularly at low lung volumes. The net effect is to increase air space and thereby to enhance gaseous exchange. The obvious success of surfactant replacement therapy for prematurely delivered infants with respiratory distress syndrome (RDS), who lack sufficient surfactant of their own, emphasizes the critical role of surfactant during the neonatal period.[1-3] Surfactant is also being used to treat meconium aspiration and to help wean infants during treatment with extracorporeal membrane oxygenation[4-8] (see Chapter 106). Considerable evidence indicates that surfactant dysfunction contributes to pulmonary instability in acute lung injury and acute RDS and that surfactant therapy could prove beneficial.[6,9-15]

It is generally appreciated that the physicochemical properties of pulmonary surfactant are critical for normal lung function. However, the mechanisms by which this lipid-protein complex adsorbs to form a surface active film that stabilizes the lung are still not completely understood. Improved insights into the mechanisms by which surfactant lowers surface tension in the lung could lead both to superior "designer" surfactants and to improved applications of clinical management. Since the 1960s, it has generally been concluded that surfactant stabilizes the lung by generating a surface monolayer highly enriched in the disaturated lecithin, dipalmitoyl-*sn*-phosphatidylcholine (DPPC). More recently new techniques and approaches have combined to indicate that this paradigm not only is oversimplified but may be flawed. As elaborated in a later section, certain surfactant properties can best be explained in terms of multilayers rather than monolayers. In addition, it has become apparent that under appropriate circumstances, the low surface tensions required to stabilize terminal air spaces can be attained with unsaturated phosphatidylcholine (PC) in the absence of gel-phase disaturated phospholipids such as DPPC. This chapter summarizes current understanding of pulmonary surfactant compositional function in vitro and in vivo.

SURFACTANT COMPOSITION

Natural Pulmonary Surfactants

Surfactant can be isolated using bronchoalveolar lavage followed by centrifugation. Although some variation exists among species and with different disease states,[16-20] the composition shown in Figure 101-1 for bovine surfactant is representative. Mammalian surfactants normally contain 80% to 85% phospholipid, 5% to 10% neutral lipids, and 5% to 10% weight/weight-specific surfactant apoproteins. Phospholipid classes include PC 80%, phosphatidylglycerol (PG) plus phosphatidylinositol 10% to 15%, and phosphatidylethanolamine 1% to 3%, with lesser amounts of sphingomyelin, lyso-*bis*-phosphatidic acid, and lyso-PC. The neutral lipid fraction includes cholesterol, cholesterol esters, diacylglycerol, triacylglycerol, and some free fatty acids.[18,20] The surfactant-associated proteins (SPs) consist of SP-A and SP-D, which are hydrophilic glycoproteins, and SP-B and SP-C, which are low-molecular-weight hydrophobic proteins that dissolve with the lipids in organic solvents such as chloroform:methanol.[21-24]

Electrospray ionization mass spectroscopy and high-pressure liquid chromatography analyses have been used to estimate molecular species distribution of individual phospholipids in pulmonary surfactant for numerous animal species.[20,25,26] PC and PG molecular species for bovine surfactant are listed in Table 101-1. Such analyses show the PC fraction for different animal surfactants is high in DPPC (35% to 55%), with significant amounts (5% to 20%) of palmitoyl-myristoyl PC, palmitoyl-palmitoleoyl PC, palmitoyl-oleoyl PC (POPC), and palmitoyl-linoleoyl PC. PG molecular species are high in palmitoyl-oleoyl PG (POPG) (25% to 35%), with substantial amounts of dipalmitoyl PG (DPPG) and palmitoyl-linoleoyl PG.

Surfactant Proteins

The SPs are described in detail in Chapter 100. In the human, SP-A is a 228–amino acid hydrophilic protein. As a result of glycosylation and other secondary modifications, apparent molecular weights of 28 to 36 kDa are observed with polyacrylamide gel electrophoresis under reducing conditions. SP-A contains four functional domains: a short amino-terminal (N-terminal) region involved in sulfhydryl-dependent oligomerization, a collagen-like region, a neck region that forms coiled-coil oligomers, and an approximately 130–amino acid carboxyl-terminal (C-terminal) globular carbohydrate recognition domain.[27] SP-A forms trimers resulting from triple helical formation within the collagen-like domain, coiled-coil interactions within the neck region, and sulfhydryl-dependent and perhaps other interactions in the short N-terminal region. Furthermore, six of these triple-helix subunits combine to form a bouquet-like structure containing 18 monomers. Collagen consists of Gly-X-Y repeats where Y is often hydroxyproline. The collagen-like region of SP-A contains an extra amino acid that produces a kink in this structure. As a result of this kink, the collagenous region forms the stalks and stems of the bouquet. The C-terminal carbohydrate recognition domain forms structures resembling three-petaled flowers. The resulting oligomeric form is approximately 20 nm long and at its widest point is approximately 20 nm wide.[28] Polyacrylamide gel electrophoresis of SP-A under nonreducing conditions shows that SP-A recovered from the alveolus is predominantly present as large oligomers of up to 640 kDa. SP-A is a member of the C-type lectin (carbohydrate-binding) superfamily, which contains mannose-binding protein and the collectins (*col*lagen-*lectins*).[27,29] SP-A binds lipids and is isolated with natural surfactant. Its role in the biophysical functions of surfactant is discussed in a later section.

SP-D also contains collagenous and calcium-dependent lectin domains, and like SP-A, is a member of the C-lectin superfamily.[21,30,31] As in the case of SP-A, the collagen-like and neck-like regions of SP-D promote trimerization. SP-D contains four of these trimers, which are bound at their N terminus through disulfide bonds. The resulting structure of SP-D forms a cruciform oligomer consisting of 4 trimers in a total of 12 subunits.

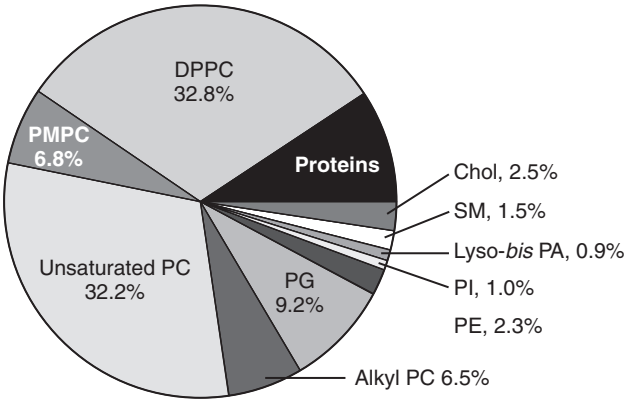


Figure 101-1 The composition of bovine pulmonary surfactant. *Alkyl-PC,* Alkylphosphatidylcholine; *Chol,* cholesterol; *DPPC,* dipalmitoylphosphatidylcholine; *PA,* phosphatidic acid; *PC,* phosphatidylcholine; *PE,* phosphatidylethanolamine; *PG,* phosphatidylglycerol; *PI,* phosphatidylinositol; *PMPC,* palmitoylmyristoylphosphatidylcholine; *SM,* sphingomyelin. *(Modified from Possmayer F, Yu SH, Weber JM, Harding PG: Pulmonary surfactant,* Can J Biochem Cell Biol *62:1121, 1984; unpublished data courtesy Dr. K. Rodriguez Capote.)*

TABLE 101-1

Major Molecular Species for Phosphatidylcholine and Phosphatidylglycerol in Bovine Lipid Extract Surfactant*

Molecular Species	% Total
Phosphatidylcholine (PC)	
Palmitoyl, myristoyl-PC (16:0, 14:0)†	9.2 ± 0.3
O-palmityl, palmitoyl-PC (16:0a,14:0)‡	5.4 ± 0.7
Palmitoyl, palmitoleoyl-PC (16:0, 16:1)	13.0 ± 1.0
Palmitoyl, palmitoyl-PC (16:0, 16:0)	40.0 ± 1.7
Palmitoyl, linoleoyl-PC (16:0, 18:2)	4.6 ± 0.7
Palmitoyl, oleoyl-PC (16:0, 18:1)	22.8 ± 1.0
Stearoyl, linoleoyl-PC (18:0, 18:2), plus oleoyl, oleoyl-PC (18:1, 18:1)	2.7 ± 0.7
Stearoyl, oleoyl-PC (18:0, 18:1)	1.8 ± 0.4
Phosphatidylglycerol (PG)	
Palmitoyl, palmitoleoyl-PG (16:0, 16:1)	6.4 ± 0.6
Palmitoyl, palmitoyl-PG (16:0, 16:0)	23.1 ± 0.6
Palmitoyl, linoleoyl-PG (16:0, 18:2)	7.6 ± 0.5
Palmitoyl, oleoyl-PG (18:0, 18:1)	48.2 ±3.4
Stearoyl, linoleoyl-PG (18:0, 18:2) plus oleoyl, oleoyl-PG (18:1, 18:1)	7.0 ± 0.7
Stearoyl, oleoyl-PG (18:0, 18:1)	7.7 ± 0.7

Unpublished data from the work of Capote KR, Possmayer F.
*As determined by electrospray ionization spectrometry.
†Numeric representations for the fatty acyl groups are given in *parentheses.*
‡Alkyl, acyl-PC.

Some SP-D is present in larger oligomeric forms (6 to 8 trimers). Each monomer has a molecular weight of approximately 43 kDa, yielding an overall molecular weight of 500 kDa or more. SP-D does not bind to surfactant lipids as well as SP-A and is predominantly found in the supernatant arising after centrifugation of lung lavages. SP-D exhibits different lectin properties from SP-A and selectively binds phosphatidylinositol rather than DPPC. SP-D has a relatively weak biophysical role compared with SP-A. SP-A and SP-D have important roles in the innate host defense system.[21,32]

SP-B is a low-molecular-weight hydrophobic protein, 79 amino acids long, present in surfactant as a disulfide-dependent dimer of approximately 18 kDa. Mature SP-B is produced by N-terminal and C-terminal proteolytic processing of a larger proprotein of approximately 42 kDa.[33-38] SP-B is cysteine rich and is very basic (positively charged). The 18-kDa SP-B dimer appears to be a membrane-associated protein that binds to the surface of lipid bilayers.[24,35,39] SP-B deficiency arising from natural human mutations or deliberate gene disruption in mice results in neonatal respiratory distress leading to death.[10,24,40,41]

Pro–SP-C (approximately 21 kDa) is also proteolytically processed. Mature SP-C is present in surfactant as a monomer of 35 amino acids with a molecular weight of 4.2 kDa. SP-C is not only the most hydrophobic protein known, but it also contains two palmitic acid moieties that are acylated to two adjacent cysteine groups near the N terminus. In some species, such as mink and canine, SP-C contains only one cysteine and, corespondingly, a single palmitate. SP-C has a short, 12–amino acid moderately hydrophilic N-terminal region, terminating in positively charged arginine and lysine groups. The remaining 23 amino acids form a hydrophobic α-helix composed primarily of aliphatic amino acids (leucine, isoleucine, and valine). This α-helix is of appropriate length to act as a membrane-spanning domain.[24,42,43] SP-C appears to be unique in that no similar protein has yet been identified.

Therapeutic Surfactants

As indicated previously, SP-B and SP-C can dissolve in organic solvents and are isolated with the lipids in lipid extracts of pulmonary surfactant. Organic solvent lipid extracts, containing SP-B and SP-C but not SP-A, are the basis of the so-called second-generation clinical preparations. These surfactants should not be called "natural" or "animal" surfactants. Rather, they should be referred to as "modified natural" or "animal-based" surfactants,[44] and because of processing differences, such preparations are not equivalent.[1,13,45] Survanta, also known as Beractant (Abbott Laboratories, United States), Curosurf (Chiesi Pharmaceuticals, Italy), and HL-10 (Leo Pharmaceuticals, Denmark) are obtained from minced bovine or porcine (Curosurf) lung. Alveofact (Boehringer-Ingelheim, Germany), BLES (bovine lipid extract surfactant) (BLES Biochemicals, Canada), and Infasurf, also referred to as CLSE (calf lung surfactant extract) (Forest Laboratories, United States) are obtained by lavage or foaming (BLES) techniques. Infasurf is obtained from neonatal calves, whereas the other bovine preparations are from mature calves or adult cows. Because neutral lipids (mainly cholesterol) reduced the ability to attain low surface tensions during compression with the available techniques, they were removed from BLES, Curosurf, and Survanta. Survanta has little SP-B compared with the other animal-based surfactants, but palmitic acid and tripalmitin are added to enhance surface film formation.[46-49] The available animal experimental literature would suggest that Infasurf promotes a faster and more effective response than Survanta, although a head-to-head clinical trial comparing these surfactants showed no difference in death or incidence of pneumothorax (air leak).[50]

In addition to these modified animal surfactants, two protein/peptide-based surfactants have been developed.[51] Surfaxin (Discovery Laboratories, United States) contains KL4 (a 21–amino acid peptide containing a series of 5 amino acid repeats, each with a lysine followed by 4 leucines), DPPC, dioleoyl-PC, and palmitic acid. Although designed to mimic SP-B, a surface membrane protein, KL4 acts as a transmembrane peptide.[52] Venticute (Byk Gulden Pharmaceuticals, Germany) contains recombinant human SP-C (rSP-C) produced in *Escherichia coli*, plus DPPC, dioleoyl-PC, and palmitic acid. Palmitic acid enhances phospholipid adsorption and can enhance the ability of these preparations to attain low surface tensions in vitro. However, the effectiveness of adding palmitate has been questioned because this fatty acid is taken up almost instantaneously by lung tissue.[53]

At least two artificial, wholly synthetic, protein-free surfactants, Pneumactant, also known as ALEC (artificial lung expanding compound, Britannia Pharmaceuticals, United Kingdom) and Exosurf (Burroughs-Wellcome, United States) are available. ALEC, which contains DPPC:POPG (7:3), was named for Dr. Alec Bangham of Cambridge, who developed this artificial preparation. Exosurf, developed by Dr. John A. Clements, a pioneer in the surfactant field, consists of DPPC, hexadecanol (a long-chain alcohol that acts as a spreading agent), and tyloxapol, a detergent introduced to ensure consistent formulations. Clinical trials show that these surfactants have biologic activity with premature neonates, but the effects are slow compared with those of the lipid extract surfactants, a finding consistent with a secondary effect arising from phospholipid uptake and recycling by type II cells.[51] Exosurf recovered from premature lungs possesses enhanced surface activity consistent with the equilibration with endogenous surfactant apoproteins.[54] Comparisons indicate that natural surfactant-based extracts are more effective than the wholly synthetic preparations in reducing death and morbidity.[2,55]

LUNG BIOLOGY OF PULMONARY SURFACTANT

The present view of the life cycle of pulmonary surfactant is depicted in Figure 101-2. Surfactant is produced in the type II cells of the alveoli and assembled and stored in lamellar bodies.[56-61] These organelles are composed of a limiting membrane and concentric or parallel phospholipid lamellae apparently composed primarily of phospholipid bilayers. Lamellar bodies contain surfactant lipids, SP-B, and SP-C, but relatively low amounts of SP-A.[62-64] SP-A is also secreted from type II cells via the normal constitutive secretory pathway, which bypasses lamellar bodies.[65] Once secreted into the alveolar hypophase, lamellar bodies interact with calcium and SP-A to form the unique structure known as *tubular myelin*.[56,59,66-68] Tubular myelin consists of long, rectangular, stacked tubes composed mainly of phospholipid bilayers. The corners of these tubes appear fused, giving cross-sections of tubular myelin a lattice-like appearance (Figure 101-3). Tubular myelin-enriched surfactant fractions adsorb readily to form a film at the air-liquid interface, a finding suggesting that this structure acts as an important monolayer-generating source.[61,69] The surfactant monolayer is ultimately responsible for lowering surface tension at the air-liquid interface.

As described elsewhere, surfactant obtained through lung lavage (washing) can be separated into numerous fractions by density gradient or differential centrifugation. Two major fractions, known as large surfactant aggregates (LAs) and small surfactant aggregates (SAs), also known as heavy and light surfactant subtypes, are readily isolated.[61,70,71] The heavy LA subtype contains lamellar bodies, tubular myelin, and multilamellar vesicles of various sizes. The light subtype is composed mainly of small unilamellar vesicles. Time course studies conducted shortly after birth and pulse-labeling experiments have established that the small vesicles are metabolic products of the larger, heavier forms. These observations support the view that SAs, presumably generated from the surface film during repeated compression and expansion, represent spent surfactant in transit to the type II cells. Surfactant lipids are taken up by type II cells and are partially degraded, presumably by lysosomes, but they are also recycled into lamellar bodies. Considerable recycling of surfactant constituents occurs with newborn animals, and this feature likely explains in part the success of surfactant therapy for RDS. Certainly, without recycling, more frequent retreatment would be required. Compositional studies have shown the LA and SA surfactant subtypes exhibit similar phospholipid profiles, but only the heavier subtype possesses significant amounts of the surfactant apoproteins.[72,73] Not surprisingly, the heavier LA forms show superior surface activity.[74,75]

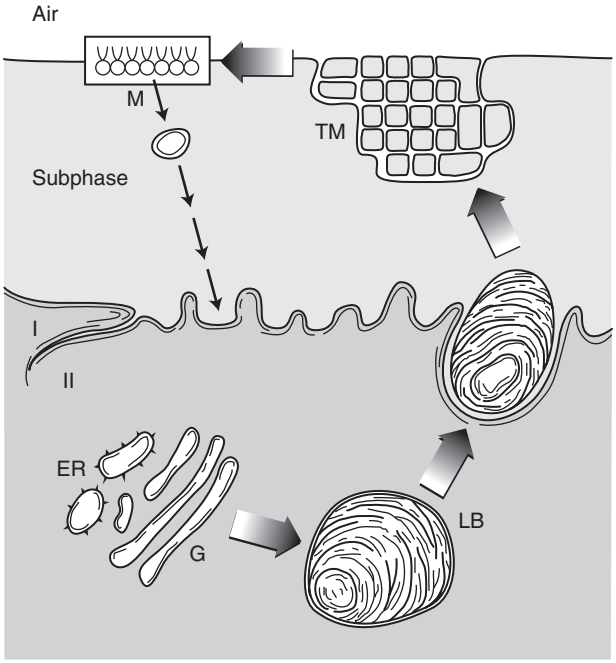


Figure 101-2 Diagrammatic representation of the biologic life cycle of pulmonary surfactant. Pulmonary surfactant is synthesized in type II alveolar cells and assembled into lamellar bodies *(LB)*. After exocytosis, lamellar bodies form tubular myelin *(TM)*, which is thought to be a major source of the surface film. TM consists of long rectangular hollow tubes composed of phospholipid bilayers that are fused together at the corners. A cross-sectional view with a lattice-like appearance is shown. TM is thought to be the major source for phospholipid adsorption to create a film at the air-water interface. Although shown as a single monolayer *(M)*, evidence of surfactant multilayers is seen. The surface monolayer reduces surface tension. Vesicles formed from the surface during repeated compression and expansion are taken up by the type II cells for degradation and recycling into lamellar bodies. *I,* Type I cell; *II,* type II cell; *ER,* endoplasmic reticulum; *G,* Golgi apparatus. *(From Possmayer F, Yu SH, Weber JM, Harding PG: Pulmonary surfactant,* Can J Biochem Cell Biol *62:1121, 1984; modified from Goerke J: Lung surfactant,* Biochim Biophys Acta *344:241, 1974.)*

SURFACE TENSION AND LUNG MECHANICS

Initial evidence of the importance of surface tension in pulmonary function was reported in 1929 by von Neergaard,[76] who observed that a greater pressure difference was required to keep lungs inflated with air than with fluid. Figure 101-4 shows data from a more recent pressure-volume relationship study on excised lungs.[77,78] When air is introduced stepwise into a lung, the initial increase in lung volume is modest, followed by a more rapid elevation to total lung capacity. As the pressure difference is lowered, considerably more air remains in the lungs at any given pressure than during inflation. This is known as *hysteresis* (literally, "lagging behind"). Furthermore, at zero pressure, a new initial volume is obtained that is larger than the volume observed during the initial inflation. Filling the lung with saline eliminates surface tension forces. Under these circumstances, the lung expands readily and uniformly, and little hysteresis is observed.

This simple experiment permits us to draw three important conclusions. First, the work done against surface tension forces constitutes a major part of the effort required to inflate the lung.[76] Assuming that the pressure required to inflate the lung

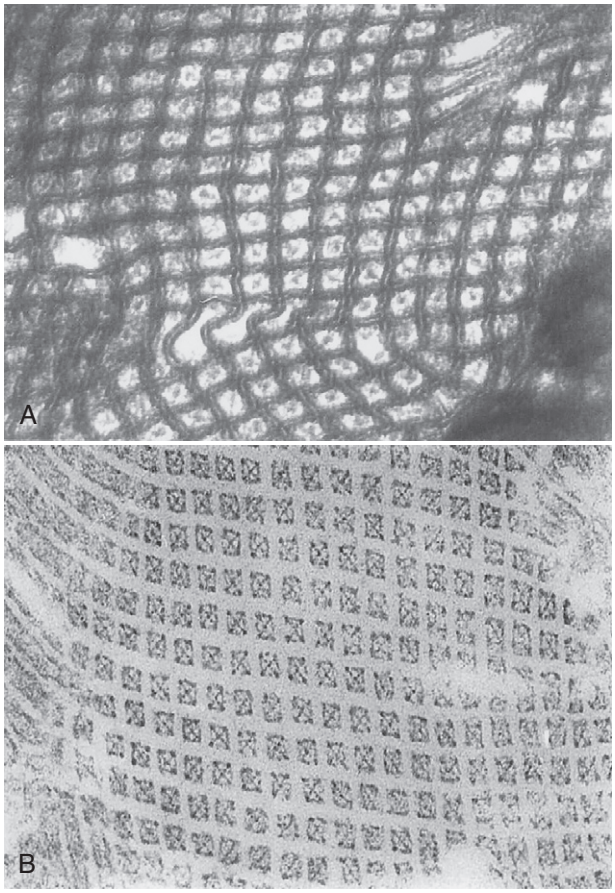


Figure 101-3 Tubular myelin. A, Electron micrograph showing cross-section of tubular myelin from bovine surfactant. Tubular myelin consists of fused tubes that, in this case, extend perpendicular to the plane of the image. The squares are approximately 50 nm across. **B,** Structures remaining after acetone washing to remove phospholipids. The X-shaped structures are thought to be surfactant-associated protein As that interact with the phospholipids via their carbohydrate recognition domains. *(From Nag K, Munro JG, Hearn SA, et al: Correlated atomic force and transmission electron microscopy of nanotubular structures in pulmonary surfactant,* J Struct Biol *126:1, 1999.)*

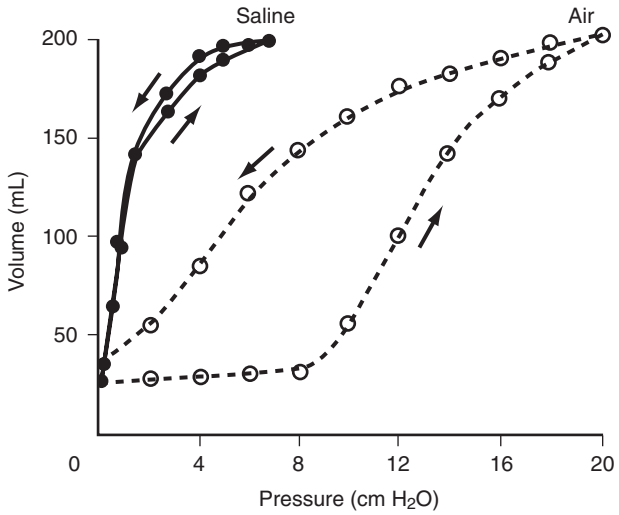


Figure 101-4 Pressure-volume relationships in cat lungs. Air filling *(open circles)*: A considerable pressure is required before the lungs begin to expand to maximum volume. On deflation, the lung remains more open than during inflation. This effect is known as hysteresis. Some air is left in the lung at zero pressure. Saline filling *(closed circles):* The lung inflates from zero pressure to a similar maximum volume as with air. Little hysteresis is present during deflation. *(From Clements JA: Surface phenomena in relation to pulmonary function,* Physiologist *5:11, 1962, and Radford EP Jr: Recent studies of mechanical properties of mammalian lungs. In Remington JW, editors:* Tissue Elasticity, *Bethesda, MD, American Physiological Society, 1957, pp 177-190.)*

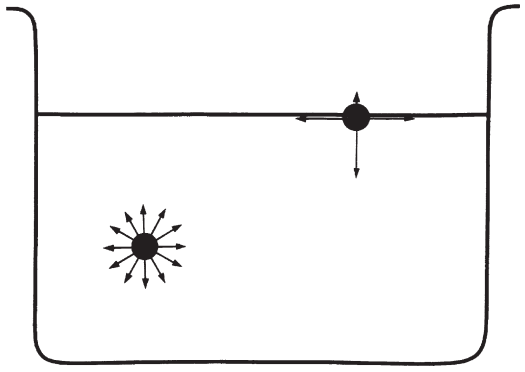


Figure 101-5 Diagrammatic representation of surface tension. A molecule of water within the bulk phase is attracted by all molecules surrounding it, but molecules of water at the surface experience a net attraction into the bulk phase *(From Possmayer F, Yu SH, Weber JM, Harding PG: Pulmonary surfactant,* Can J Biochem Cell Biol *62:1121, 1984.)*

to half-maximal volume with air represents both surface forces and tissue elasticity, whereas the pressure needed to achieve this volume with saline functions primarily against tissue elasticity, it can be seen that more than two thirds of the work can be accounted for by lung surface tension resistance. Second, the difference in the pressure-volume relationships during inflation and deflation indicates surface tension changes during this cycle. Finally, the observation that the air-filled lung retains some air on deflation suggests that surface tension must be low at low lung volumes.[77,79,80]

SURFACE TENSION

Surface tension arises from the attractive forces between molecules.[81-85] A molecule in the bulk phase is subject to attractive forces from the molecules around it, resulting in a net force of zero (Figure 101-5). Molecules at the surface are attracted by the molecules below (and to either side) but in the upward direction only by the weak attractive forces of water vapor and air. As a result, all the molecules at the surface experience a net attraction (downward) into the bulk phase. Work is required to move a molecule from its lower energy state in the bulk phase to the more energetic state at the surface. With water, the work or the energy required to move sufficient molecules to the surface to expand it by 1 cm^2 at 37° C is 70 mJ/cm^2 or 70 erg/cm^2. Because of the difference in energy of surface molecules, the most stable situation arises with a minimum surface area. This is why materials with high surface tension, such as water or mercury, form spherical droplets. Surface tension may also be thought of in terms of a thin elastic film of liquid at the surface that resists expansion (or works toward contraction) of the surface area.

This may be more readily understood by considering a bubble blown in water. The pressure difference across the surface of such a bubble is described by the well-known law of Young and Laplace:

$$\Delta P = 2\gamma / r$$

where P = pressure in millinewtons per meter squared (mN/m^2), r = the radius in meters, and γ = the surface tension in millinewtons per meter (mN/m). Because of the attractive forces on molecules at the surface, a force or a tension in the surface film resists expansion of the bubble and consequently acts to contract the surface area. This contractive force is known as the *surface tension* and has a value of 70 mN/m or 70 dyne/cm for water at 37° C (72 mN/m at 25° C).

Surface tension can be altered by the presence of a second substance in the liquid. Because of hydrogen bonding, water has a high γ. The γ of ethanol is less than that of water. With a water:ethanol solution, the water molecules interact less strongly with ethanol than with one another, and hydrogen bonding is reduced. Consequently, a molecule of ethanol at the surface of a water:ethanol solution will experience less attraction into the bulk phase than a water molecule. This replacement of some surface water with ethanol molecules results in a reduction in γ. The difference in attractive forces between surface molecules of water and ethanol results in a second effect. Because ethanol molecules at the surface experience less attraction into the bulk phase, a small but definite net accumulation of ethanol molecules at the surface will result. The resulting increase in the surface concentration of ethanol generates slightly lower γs than would be predicted from the bulk concentrations. Conversely, the presence of substances such as sodium chloride in water produces a slight increase in γ, probably because water molecules have a stronger association to the sodium and chloride ions in the bulk phase. Therefore water molecules require even more potential energy to attain the surface than with pure water.

The surface tension–reducing capacity of surfactant is the result of its phospholipid constituents. Phospholipids contain *hydrophilic* (water-attracting) polar head groups and *hydrophobic* (water-repelling) fatty acyl groups. Such molecules are known as *amphipathic* (literally, "feeling both ways"). Phospholipids are essentially insoluble, existing in aqueous dispersions as aggregates such as liposomes. Figure 101-6 shows a monomolecular film of PCs or lecithins spread at the air-liquid interface. Numerous different PC molecular species, with palmitate (16:0) at the *sn*-1 position but differing in the fatty acids at the *sn*-2 position of the glycerol moiety, are depicted (*sn* = stereospecific numbering). The polar phosphorylcholine groups interact with water, whereas the hydrophobic fatty acyl moieties extend toward the air. Interactions between these molecules are weaker than the attractive interactions between water molecules. As in the case of ethanol, these lipids reduce γ by displacing water from the surface.

PC monolayers can be formed by spreading the lipids at the air-water interface using an organic solvent such as chloroform. As more phospholipid is spread, γ falls until a surface tension of approximately 23 mN/m is attained. Application of additional phospholipids will not decrease γ further. Although PC dispersions adsorb to form a monolayer relatively slowly, under appropriate conditions and with long time periods, γs of approximately 23 mN/m can be obtained. Because of the presence of the SPs, surfactant adsorbs quite rapidly, again to a final γ of approximately 23 mN/m. This value of approximately 23 mN/m is known as the *equilibrium surface tension* for phospholipid monolayers. At equilibrium surface tension the air-liquid interface can no longer spontaneously accept more phospholipid molecules (i.e., the surface is "saturated" with phospholipid). The precise equilibrium surface tension depends on the composition of the film and the temperature, but 23 mN/m is used for discussion purposes.

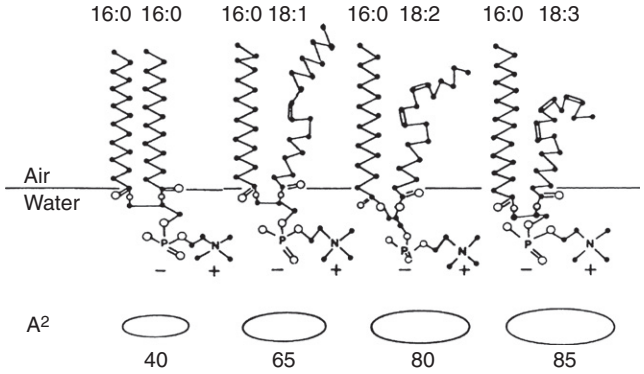


Figure 101-6 Orientation of various molecular species of phosphatidylcholine *(PC)* at the air-water interface. The hydrophobic fatty acyl groups extend into the air, whereas the hydrophilic polar phosphorylcholine groups interact with water via their positive and negative charges. Because of kinks at the double bonds, PCs with unsaturated fatty acids occupy a larger average molecular size, as indicated by *ovals* below the molecules. Unsaturated PCs cannot pack as well as dipalmitoylphosphatidylcholine, the molecule on the *extreme left*. *(From Possmayer F, Yu SH, Weber JM, Harding PG: Pulmonary surfactant,* Can J Biochem Cell Biol *62:1121, 1984.)*

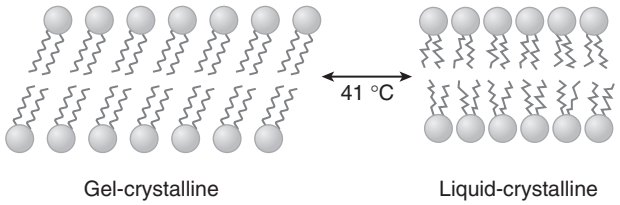


Figure 101-7 Diagrammatic representation of the phase transition from the gel-crystalline to the liquid-crystalline state of a bilayer of dipalmitoylphosphatidylcholine *(DPPC).* The hydrophobic palmitates interact together, and the polar phosphorylcholine groups interact with the water. At 41° C, the acyl groups gain sufficient thermal energy to twist, rotate, and move laterally. This "fluidity" permits the individual DPPC molecules to diffuse relative to one another while remaining in the same leaflet.

Dry or partially hydrated DPPC crystals placed on a water surface or mixed as a dispersion will spread to form a monolayer, again to a final equilibrium surface tension of 23 mN/m. It appears that as the DPPC head groups become fully hydrated, they can either remain at the surface, where they spread to form a monolayer, or they can form vesicles with other hydrated DPPC molecules. The rate of monolayer formation from DPPC increases many-fold at 41° C.[86-89] This temperature dependence of monolayer formation can be understood by considering bilayer structure, which is the molecular basis of biologic membranes (Figure 101-7). As hydrated DPPC bilayers are heated, the palmitic acids change from a relatively ordered state in which all carbon-carbon bonds are extended in the all-*trans* configuration to a disordered fluid state in which some of the carbon bonds are in the *gauche* configuration. Below the critical transition temperature (T_c) of 41° C, the acyl groups are packed together in a regular pattern, and the phospholipid molecules are relatively motionless, although they may rotate in an axis roughly perpendicular to the bilayer. At T_c, the fatty acyl groups gain sufficient thermal energy, so the ordered acyl

chains start to move and become disordered and fluid (i.e., the chains "melt"). The individual phospholipid molecules become more mobile, allowing them to diffuse rapidly relative to one another in their plane of the bilayer.

It is clear from cell biology studies that membrane fluidity is essential for life. Membrane lipids possess high proportions of phospholipids with unsaturated phospholipids at their *sn*-2 position. As shown in Figure 101-6, double bonds introduce a kink in the fatty acid. This reduces the ability of the phospholipid to pack closely in monolayers or bilayers. Consequently, T_c for phospholipids containing unsaturated fatty acids is much lower than for saturated phospholipids with fatty acids of similar length. Reducing the chain length of saturated fatty acids also lowers T_c. The concept of ordered and fluid fatty acyl chains is important for understanding the principles involved in alveolar stabilization as explained in the next section.

In summary, surface tension arises from the difference in attractive forces on molecules at an interface. The term *surface tension* is applied to air-liquid interfaces; *interfacial tension* is used for other interfaces. Molecules at a surface possess excess potential energy relative to molecules in the bulk phase. All the molecules at the surface experience an attraction into the bulk phase. This force is perpendicular to the surface. Clearly, the most stable situation arises with a minimum surface area. This is why water and mercury form droplets. One can think of γ in terms of the work required to expand the surface area. One can also consider γ in terms of an elastic film of surface molecules, in which the attraction of molecules into the bulk phase resists expansion and works toward minimizing surface area. This contractile force produces a pressure difference across bubbles or alveoli, which is equal to twice the surface tension divided by the radius (law of Young and Laplace; see Equation 1). The surface tension of water at 37° C is 70 mN/m.

A monolayer of phospholipids at the air-liquid interface (see Figure 101-6) lowers γ by replacing water at the surface. Phospholipids are amphipathic molecules possessing hydrophilic polar head groups and hydrophobic fatty acyl chains. Phospholipids such as PC are essentially insoluble in water and readily form lipid bilayers, which are the structural basis of biologic membranes. The gel-to-liquid crystalline T_c marks the temperature at which the fatty acyl groups in a bilayer change from an ordered state to a disordered fluid state (see Figure 101-7). Membrane fluidity is essential for membrane processes compatible with life. Below T_c, PC forms monolayers either from crystals placed at the surface or from dispersions in the bulk phase at an extremely slow rate. T_c is lowered by reducing the acyl chain length or introducing double bonds. Mixing DPPC, which has a T_c of 41° C, with unsaturated phospholipids reduces T_c and allows increased adsorption at 37° C.[90-93] However, such adsorption is still insufficient to stabilize the alveoli.

The equilibrium surface tension of 23 mN/m is attained when all available sites at the air-water interface are "fully" occupied by PCs. Equilibrium surface tension is the lowest γ that can be achieved by spreading sufficient phospholipid on the surface with an organic solvent. A similar γ is achieved when natural surfactant, which adsorbs rapidly, is injected below the subphase. Under equilibrium conditions, phospholipids cannot spontaneously enter the monolayer unless a similar amount of phospholipid leaves the surface.

REDUCTION OF SURFACE TENSION BY PULMONARY SURFACTANT

An important physicochemical difference exists between soluble and insoluble surface films. The γ of a bubble blown in a 50% ethanol solution is 30 mN/m. (The surface concentration of ethanol is slightly higher than the bulk concentration.) Increasing the bubble size does not affect γ because ethanol molecules are soluble and move into the surface film almost instantaneously. Likewise, reducing the bubble surface area does not affect the surface concentration of ethanol or γ. However, phospholipid films are insoluble, and reducing the size of the bubble leads to lateral compression of the monolayer. Slow *dynamic* (in the sense of continuous) *lateral compression* of the PC film depicted in Figure 101-6 results in closer packing of the surface phospholipids and a fall in γ because the phospholipid molecules are forced closer to one another and more water is excluded from the surface. However, phospholipid films such as that in Figure 101-6, which contain a high proportion of unsaturated fatty acids, are not very stable at 37° C. Because of the fluid nature of the fatty acids, such films can withstand limited dynamic compression only to a γ of approximately 20 mN/m.[85,94-96] Further reduction in surface area does not produce a concomitant reduction in γ, but it results in the expulsion of PCs from the two-dimensional monolayer (into a collapse phase of uncertain structure). With dynamic compression, γ can be driven slightly below equilibrium, but when dynamic compression ceases (i.e., surface area is held constant), γ returns to its equilibrium state. This increase in γ is thought to arise from loss of unsaturated PC molecules from the surface, thereby relieving the strain resulting from film overcompression. Such molecules can form bilayer structures such as vesicles, which are lost from the surface. However, as discussed in a later section, it is clear that, especially with surfactant, some of the lipid material can remain associated with the surface monolayer.

In contrast to unsaturated PCs, films of DPPC are stable at 37° C and can be compressed until γ approaches 0 mN/m. This film stability is attributed to the ability of the saturated palmitic acids to pack closely, thus allowing the DPPC molecules to organize into a highly ordered, two-dimensional surface gel at this temperature. Experimental evidence of the differences in the physicochemical properties of gel and fluid phospholipid monolayers is outlined in Figure 101-8, which depicts the effects of compressing DPPC (see Figure 101-8, *A*) or 1-stearoyl,2-oleoyl-PC (SOPC) (see Figure 101-8, *B*) monolayers on a Langmuir-Wilhelmy surface balance (see Figure 101-8, *C*). The Langmuir-Wilhelmy surface balance introduced by John Clements[79] provided some of the first direct experimental evidence of pulmonary surfactant. Figure 101-8, *A,* shows a so-called *compression isotherm* (i.e., compression at constant temperature) for DPPC monolayers spread at low surface concentration with organic solvent, which is then allowed to evaporate. Compressing the film from "lift off," the point on the right side of the graph at which a significant decrease in γ is first noted, results in a slow progressive decrease in γ. At a certain point (γ of approximately 60 mN/m [70% surface area] for the curve at 25° C), the decrease in slope is sharp. This leads to a slowly increasing plateau, which then curves upward as surface area is reduced further. As the monolayer is compressed still further, surface tension falls to approximately 0 mN/m, at which the DPPC monolayer collapses. At 37° C, because of increased mobility of the acyl groups, greater compression is required to reach the initial plateau, but the curves become identical as γ declines.

This DPPC isotherm contrasts with that for SOPC, which has a T_c of 6° C. With this unsaturated PC at 37° C, the gradual plateau at low γ is not observed (see Figure 101-8, *B*). Furthermore, it is apparent that SOPC monolayers cannot withstand sufficient lateral pressure to lower the surface tension to very low levels. At approximately 20 mN/m, further compression squeezes SOPC molecules out of the monolayer into a collapse phase. The lowest surface tension attained (approximately 20 mN/m) is only slightly below the equilibrium surface tension of 23 mN/m. Electron microscopic studies indicate that compressed DPPC monolayers near 0 mN/m are relatively solid and collapse as structures resembling small ice floe sheets as

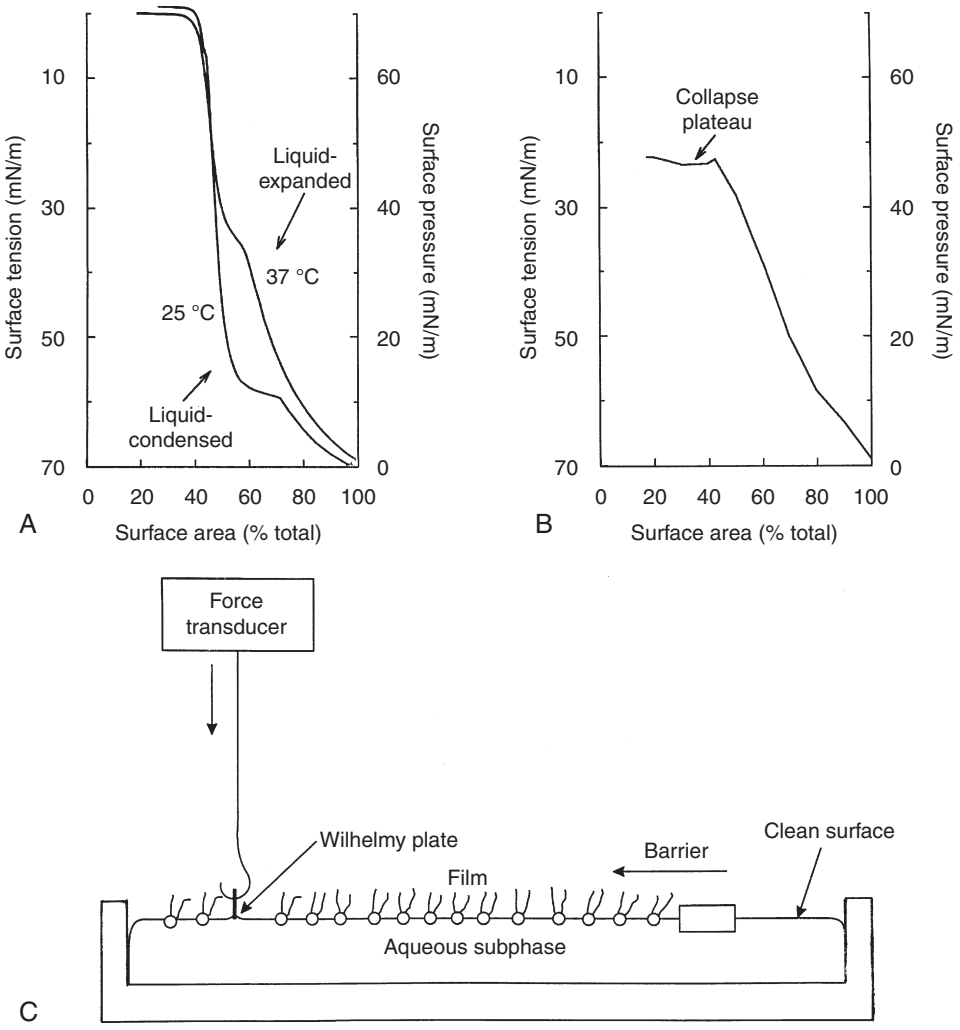


Figure 101-8 Effect of compression on surface tension of a dipalmitoylphosphatidylcholine *(DPPC)* monolayer or a monolayer of 1-stearoyl, 2-oleoylphosphatidylcholine *(SOPC)* on a Langmuir-Wilhelmy surface balance. A, Surface tension/surface pressure relationship for DPPC monolayers at 25° and 37° C. The liquid-expanded (LE) and liquid-condensed (LC) phase regions are indicated. Because of thermal agitation at 37° C, the liquid-expanded region extends to a lower γ (higher surface pressure). Surface tensions of approximately 0 mN/m are attained with both monolayers at approximately 50% original area. **B,** Surface tension/surface pressure versus area curve for SOPC at 37° C. Only the liquid-expanded state is observed, before the collapse point is attained at approximately 45% surface area reduction. Further surface area reduction results in a collapse plateau at which surface tension is not lowered further. **C,** Diagrammatic representation of a Langmuir-Wilhelmy surface balance. The barrier is used to compress the film. The Wilhelmy plate is used to monitor surface tension. *(**A** and **C,** Modified from Goerke J, Clements JA: Alveolar surface tension and lung surfactant. In Fishman AP, editors:* Handbook of Physiology, Section 3: The Respiratory System, *vol. III, part 1. Bethesda, MD, American Physiological Society, 1986, pp 247-261. **B**, Modified from Hawco MW, Davis PJ, Keough KM: Lipid fluidity in lung surfactant: Monolayers of saturated and unsaturated lecithins,* J Appl Physiol *51:509, 1981.)*

the barrier progresses near 0 mN/m. SOPC monolayers collapsing at γ approximately 20 mN/m appear more like fluid pools.[97]

The behavior of DPPC and SOPC monolayers can be readily understood in terms of the generalized phase diagram for phospholipids below T_c depicted in Figure 101-9. The interpretation of such curves is based on extensive work with fatty alcohols and fatty acids, as well as phospholipids.[89,98-102] At low surface concentrations, DPPC molecules act as a two-dimensional gas and have no discernible effect on γ. As surface area decreases to approximately 120 Å^2/molecule, the DPPC molecules interact with each other such that they become more erect. This marks the *liquid-expanded* (LE) state, which continues to approximately 80 Å^2, at which some of the molecules become compressed into the *liquid-condensed* (LC) state, giving rise to an LE/LC coexistence plateau. As more of the LE molecules become tightly packed and assume the LC state, γ decreases more rapidly with surface area reduction. This continues until γ approaches 0 mN/m at approximately 40 Å^2, at which the monolayer acts like a solid LC gel. Because SOPC at 37° C is far above T_c, only the LE phase is observed during monolayer compression, and the monolayer collapses near equilibrium surface tension. Below its T_c of 6° C, SOPC can be compressed to low γs.[95] It has been suggested that, at high compression, DPPC monolayers can be transformed to a more tightly packed LC solid state by altering head group orientation to create less tilt in the molecules, but this is controversial.[99,100,103]

Our appreciation of the molecular interactions occurring during monolayer compression has been greatly improved by the development of fluorescence imaging microscopy, Brewster angle microscopy, and atomic force microscopy. DPPC films in

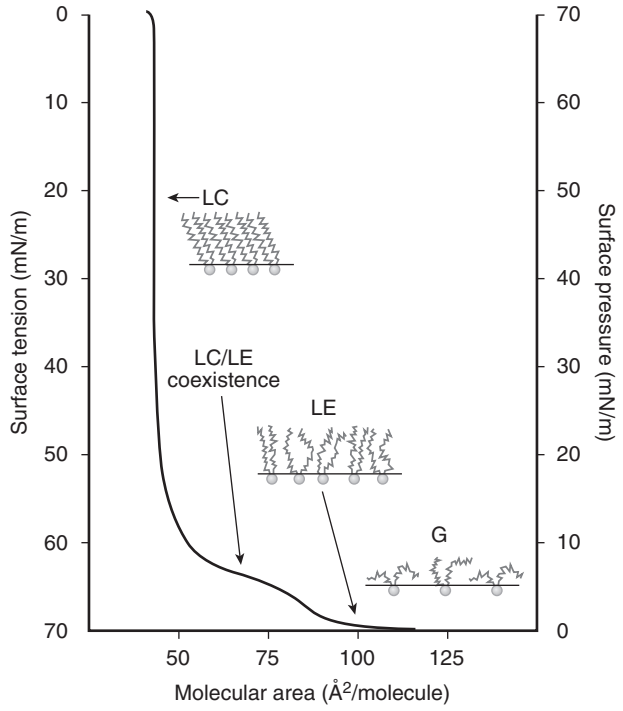


Figure 101-9 Phase diagram for dipalmitoylphosphatidylcholine showing a diagrammatic representation of the gaseous phase *(G),* the liquid-expanded *(LE)* phase, the LE:LC coexistence plateau, and the liquid-condensed *(LC)* phase. *(Modified from Kaganer VM, Möhwald H, Dutta P: Structure and phase transitions in Langmuir monolayers,* Rev Mod Phys *71:779, 1999.)*

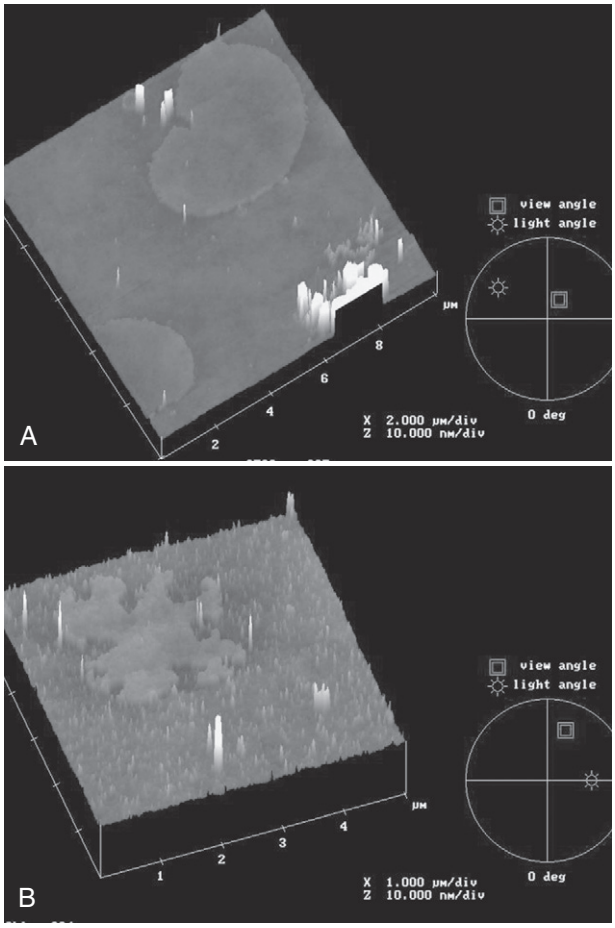


Figure 101-10 Atomic force microscopy images of dipalmitoylphosphatidylcholine (DPPC) (A) and bovine lipid extract surfactant (BLES) **(B)** monolayers in the liquid-expanded (LE): liquid-condensed (LC) coexistence region. The kidney bean-shaped LC structure in **A** is approximately 6 µm long and extends approximately 1.2 nm above the plane of the LE phase. The BLES LC structure in **B** is approximately 7 nm long and is floral shaped. It extends approximately 1.7 nm above the LE phase. The nature of the small spikes in the *insert* is not known. *SP-A,* Surfactant-associated protein A. *(Unpublished data from the work of Nag K, Peterson N, Possmayer F.)*

the LE/LC coexistence plateau can be examined directly on the monolayer to reveal kidney bean-shaped LC condensed structures as dark probe-excluding regions with fluorescence imaging microscopy and thickened monolayer regions with Brewster angle microscopy. Such structures can also be observed on *Langmuir-Blodgett films* deposited from the monolayer as elevated regions using atomic force microscopy (Figure 101-10). Although the kidney bean structure in Figure 101-10, *A* is obviously an LC gel-phase structure, some explanation is needed for the BLES floral-shaped structure in Figure 101-10, *B.* This is also an LC gel-phase structure that arises through segregation (i.e., phase separation) of the gel-phase phospholipids DPPC and DPPG. Fluid constituents remain in the LE phase.[104-106] The kidney bean shape of the DPPC LC structures (see Figure 101-10, *A*) and the floral shape of the BLES LC structures (see Figure 101-10, *B*) observed using atomic force microscopy arise through line tension, which is the two-dimensional equivalent of surface tension. Water and mercury form spherical droplets because of high surface tension (large interfacial tension between air and water). A high line tension would cause the shape of the domain to be spherical.[102] In fact this occurs with CLSE, which has cholesterol. DPPC and BLES domains have low line tensions between LE and LC, and as a result, their domains take up a longer perimeter.

The manner in which surfactant films benefit the lung can be considered in terms of both high lateral surface pressure and low surface tension. High surface pressure resists decreases in alveolar surface area by acting as a monomolecular surface "splint." Low surface tensions stabilize the lung by reducing the pressure gradient across the alveolar lining layer. This may also function to reduce transudation of interstitial fluid into alveolar spaces.[107,108] Surface pressure is related to surface tension by the followingequation, which states that the surface pressure, π, is equal to the surface tension of a clean surface (γ_o) minus the surface tension of the surface containing the film (γ):

$$\pi = \gamma_o - \gamma$$

It should be evident from this equation that applying a lateral pressure on a monolayer produces a corresponding decrease in surface tension. For historical reasons, surfactant specialists normally express this kind of information as surface tension (left axis in Figure 101-8), whereas physical chemists normally use surface pressure (right axis). As indicated earlier, films compressed to surface tensions below equilibrium[82,84,109,110] (23 mN/m for phospholipids) are no longer in thermodynamic equilibrium and are theoretically capable of returning to their equilibrium surface tension by shedding molecules from the compressed monolayer. However, although inherently metastable, films containing a high proportion of DPPC can remain at surface tensions near 0 mN/m for long periods (i.e., many minutes). This is more than sufficient to stabilize the alveoli during breathing.

As discussed further later on, it is clear that the surface tension of the alveolar lining layer in vivo falls to values very near 0 mN/m during expiration. This low surface tension is important for stabilizing the alveoli and preventing their collapse. Such low surface tensions could not be achieved experimentally through compression of fluid phospholipid films such as those with compositions similar to that of pulmonary surfactant on Langmuir-Wilhelmy balances (see Figure 101-8, *C*). This led to the concept that the alveolar surface lining must be covered by a film highly enriched in DPPC, which is the major tenet of the classical model mentioned at the beginning of this chapter.[48,49,107,109-113] Because removing surfactant from the lung disrupts the surface film, this enrichment could not be directly measured by chemical analysis but was inferred from physical measurements. It is generally held that the fluid lipid components could contribute to the surfactant adsorption, which is very slow at 37° C for DPPC. However, DPPC:unsaturated phospholipid mixtures adsorb relatively slowly in the absence of surfactant apoproteins. The fluid lipid constituents could also be important for the transport of surfactant components within the type II cells and for secretion of lamellar bodies.

In summary, phospholipids are amphipathic molecules that contain hydrophilic polar head groups and hydrophobic acyl chains. Amphipathic molecules form insoluble monolayers in which surface concentration can be increased by dynamic lateral compression. Dynamic compression can be exerted by a barrier, as with the Langmuir-Wilhelmy surface balance (see Figure 101-8, *C*). Lateral compression also increases when the surface area of a bubble (or an alveolus) is reduced by decreasing the volume. In either case, the resulting increase in the surface concentration of the amphipathic molecules produces a decrease in γ.

Dynamic compression of DPPC monolayers results in highly ordered, closely packed, gel-like LC films. Compressed DPPC monolayers can reduce γs to near 0 mN/m. Furthermore, such monolayers can remain at low γs for long periods (i.e., these films are very stable). In contrast, monolayers composed of phospholipids with bilayer gel to liquid-crystalline T_cs significantly lower than 37° C cannot be compressed to low surface tensions with conventional balances at biologic temperatures. Such fluid films collapse at surface tensions only slightly below their equilibrium tension of approximately 23 mN/m and tend to return to equilibrium once dynamic compression ceases.[114-117]

It can be shown that the surface pressure of a monolayer is equal to the surface tension of the clean surface minus the surface tension of the surface containing the film (see Equation 2). For water, which has a surface tension of 70 mN/m at 37° C, surface pressure can vary, ranging from 0 mN/m (for a clean surface) to near 70 mN/m (corresponding to a surface tension of 0 mN/m).

ROLE OF SURFACTANT IN ALVEOLAR STABILITY

Theoretical calculations based on measurements of transpulmonary pressure and morphologic considerations with normal and saline-filled lungs indicate that surface tension varies during lung inflation and deflation and falls to very low values during expiration.[77,79,118-120] Studies in detergent-rinsed and fluorocarbon-filled lungs, in which surface tension is altered by the remaining detergent or fluorocarbon, support the view of low alveolar surface tension.

More direct evidence was obtained by Schürch and coworkers[119,121-124] by monitoring the spreading properties of fluorocarbon or silicone oil test droplets deposited on the lung's alveolar surfaces in situ.[119,121-124] Such droplets spread to form a thin lens when surface tension of the droplet is similar to that of the underlying phase. When surface tension of the underlying substrate is lower than the test droplet, the droplet adopts a more nearly spherical shape (this is the reason water forms droplets on Teflon-coated materials). Calibration of the test droplets with DPPC monolayers on the Langmuir-Wilhelmy surface balance allowed precise estimation of the surface tension of the alveolar lining layer in situ. These microdroplet studies revealed that lung volume alterations corresponding to quiet breathing (e.g., between 40% and 50% total lung capacity) were associated with only modest increases in γ from 1 to 5 mN/m. Large inflations to total lung capacity resulted in alveolar surface tensions of approximately 30 mN/m, slightly higher than the equilibrium value of 23 mN/m. When lung volume was reduced to functional residual capacity, which is 40% of total lung capacity, γ fell to less than 1 mN/m. Furthermore, when the lung was held at this volume, surface tension remained at this low value for several minutes before increasing gradually. Even after 1 hour, γ at functional residual capacity remained at less than 10 mN/m. These measurements showed that the surface film covering the alveolar lining layer is very stable in the sense that it returns to equilibrium surface tension only very slowly. This property would minimize pressure differences between small and large alveoli and would prevent collapse of the smaller units. These observations reinforced the view that the endogenous surface monolayer must be highly enriched with DPPC and were considered consistent with the classical model of surfactant function.

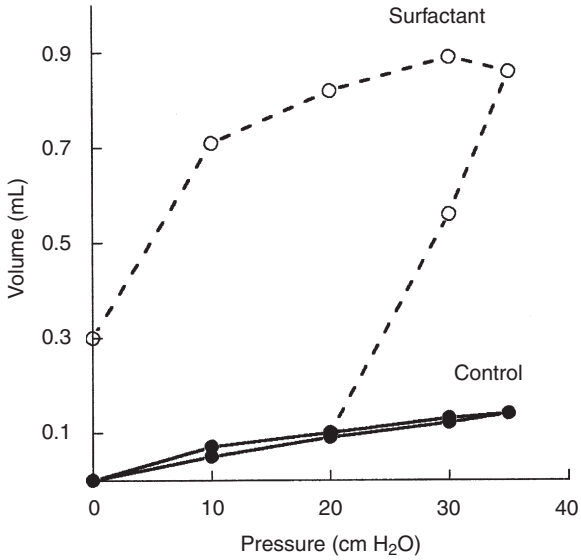


Figure 101-11 Pressure-volume maneuvers with prematurely delivered rabbit fetuses of 27 days gestation (term, 31 days). Control inflation (*closed circles*) shows little increase in lung volume or hysteresis. Lipid extract surfactant administration (*open circles*) results in a marked increase in lung volume and prominent hysteresis. *(Data from Metcalfe IL, Pototschnik R, Burgoyne R, Enhorning G: Lung expansion and survival in rabbit neonates treated with surfactant extract,* J Appl Physiol *53:838, 1982.)*

The functional significance of the presence of a lipid-protein complex capable of generating low surface tensions within the lungs can be readily demonstrated through surfactant supplementation of surfactant-deficient lungs. Treatment of prematurely delivered rabbit fetuses of 27 days gestation (term, 31 days) with natural or lipid extract surfactant increases survival. Surfactant administration results in a four- to five-fold increase in inflation during pressure-volume loops[125-127] (Figure 101-11) (see Chapter 106). Similar observations have been made with prematurely delivered fetuses of other species and with adult lungs made surfactant deficient through lavage. Administration of lipid mixtures similar to those present in surfactant has little immediate effect. This finding emphasizes the role of the SPs in surfactant function.[1,49,51,128]

Studies in which surfactant-deficient animals or prematurely delivered infants are treated with natural or lipid extract surfactants in vivo prove more difficult to interpret but clearly indicate increased compliance that results in an increase in lung volume.[1,125,129] Clinically, this can readily be monitored by a rapid increase in arterial oxygen tension, a finding indicating enhanced gaseous exchange.[130] Studies by Bachofen and Schürch[131] indicate that, in addition to promoting lung expansion, surface tension reduction of the alveolar lining layer influences lung alveolar morphology. At low lung volumes, alveolar septa are elongated, thereby maximizing alveolar surface area. At high surface tensions of 20 to 30 mN/m, these septa are contracted through extensive folding, resulting in 30% to 50% decreases in alveolar surface area. The combined abilities of surfactant to increase compliance and optimize alveolar surface area would appear to explain the rapid increases (within 30 minutes) in gaseous exchange often noted on treatment of surfactant-deficient infants and would have important implications for acute RDS.

TECHNIQUES FOR INVESTIGATING SURFACTANT FUNCTION

Discussion of the mechanisms involved in surfactant function requires a brief introduction into the technical approaches used and their limitations.

Langmuir-Wilhelmy Balance

The Langmuir-Wilhelmy surface balance illustrated earlier (see Figure 101-8, *C*) is an excellent tool for examining spread films because film composition and the amount of material deposited can be controlled accurately.[49,132] This approach is readily combined with other techniques, such as fluorescence imaging, Brewster angle microscopy, and grazing angle x-ray diffraction. By drawing mica sheets, glass slides, or cover slips perpendicular to the plane of the film, one can deposit *Langmuir-Blodgett films,* which are useful for atomic force microscopy, time-of-flight secondary ion mass spectroscopy, or autoradiography. Because of the large subphase, the Langmuir-Wilhelmy approach is not convenient for studying adsorbed films. Temperature control at 37° C is difficult. Compression rates can be altered, but experimentally productive compression and expansion rates are limited. In addition, Langmuir-Wilhelmy studies require considerable expertise and scrupulous cleanliness, and they take a long time to conduct. However, the major limitation of the Langmuir-Wilhelmy balance studies is that it has become apparent that, at the high surface pressures required to generate γs approaching 0 mN/m (values critical for surfactant function in vivo), film material "creeps" onto the Teflon barriers and walls.[115,133] This difficulty is particularly problematic with spread monolayers containing cholesterol and becomes more significant at physiologic temperatures. This situation is often referred to as "leakage" because frank film leakage did occur with early balance studies before Teflon tapes were introduced.

Pulsating Bubble Surfactometer

The pulsating bubble surfactometer (PBS), introduced by Enhorning,[134,135] is an artificial alveolus model. The key component is a small cuvette in which a bubble communicating with the atmosphere is drawn into the surfactant suspension to be tested.[49,85,132,136] This device can be thought of as a bubble on a tube model. With the commercial version of this apparatus, the pulled bubble diameter is maintained for 10 seconds to measure adsorption and then is pulsated at 20 cycles per minute with 50% surface area change. Bubble radius and the pressure difference across the bubble are recorded. Using the law of Young and Laplace (see Equation 1), a microchip controls bubble radius and generates γ at a minimum and maximum radius.

An important feature of the PBS is that it monitors surface activity at compression rates and ratios comparable to those in the alveolus in vivo. In practice, the Enhorning PBS is an easily learned, rapid method for testing small (approximately 25 μL) surfactant samples at 37° C for physiologically important parameters. It is therefore superb as a screening device, especially for animal or clinical samples. An apparent disadvantage is that, at γs approximating zero, the bubble tends to flatten, thus losing its spherical shape. This can affect calculation of γ, although the error appears to be small. In any case, γ is clearly low.[136] The major difficulty is that it has become apparent that some fluid can remain in the capillary when the bubble is created. This produces a variable increase in effective surface area.[137] This effect would be particularly serious for samples containing hydrophilic nonsurfactant proteins, for example in inhibitory studies. As with the Langmuir-Wilhelmy balance, leakage can occur in which material creeps up the polyacrylamide capillary. This leakage limits the usefulness of the PBS for mechanistic studies, although it is useful for monitoring surfactant effectiveness.

Captive Bubble Tensiometer

The captive bubble tensiometer (CBT) or surfactometer combines many of the advantages of the Langmuir-Wilhelmy balance with those of the PBS. With this device, introduced by Schürch,[119,133,138] an air bubble is introduced into the surfactant sample in an air-tight chamber where it floats up to a hydrophilic agar ceiling. The agar gel is highly hydrated and so cannot accept phospholipid material from the adsorbed film. Film leakage appears to be negligible. The CBT can be used to study adsorption, after which quasistatic or dynamic compression and expansion curves can be generated by increasing and decreasing pressure in the chamber. Surface area and surface tension are obtained from bubble shape, which is recorded with a video camera.[139]

The original CBT designed by Schürch used an air-tight syringe. More recent versions using gas-type plastic cuvettes have been introduced that are convenient for film spreading experiments. With such studies, it is important to change the subphase numerous times to remove the organic spreading solvent. Although both experiments and analysis with the CBT are slow, the availability of an apparently leak-proof system for studying surfactant at any temperature is an important advantage. This device has proved pivotal in studies challenging the classical model for surfactant function.

ROLE OF SURFACTANT-ASSOCIATED PROTEINS IN THE PHYSICOCHEMICAL FUNCTIONS OF PULMONARY SURFACTANT

As indicated by the law of Young and Laplace (see Equation 1) the pressure difference across an alveolus is inversely related to the radius. If the surface tension in the alveolus were 70 mN/m, the surface tension of water at 37° C, the pressure difference across the alveolus would be considerable, promoting alveolar instability. The mechanical properties of the lung suggest that the alveoli are covered with a phospholipid monolayer highly enriched in DPPC. However, at less than the bilayer T_c of 41° C, DPPC adsorbs and spreads only slowly at the air-liquid interface.[87-91,109,140,141] Thus, as demonstrated by the classical model, surfactant must contain fluid lipids and other substances (i.e., apoproteins) that facilitate rapid adsorption and spreading of DPPC without interfering with the ability of this disaturated phospholipid to reduce the surface tension to near 0 mN/m when the surface film is under dynamic compression during expiration.

In vitro studies indicate that surfactant apoproteins augment the surface activity of surfactant lipids in three distinct ways. First, they promote adsorption and spreading of surfactant lipids at the air-water interface. This forms a surface-active film that lowers γ toward equilibrium. Second, SPs modify the properties of the surface film and facilitate its ability to attain low surface tensions near 0 mN/m during film compression. Third, SPs enhance respreading or reinsertion of phospholipids from collapse phase.

Adsorption

The processes by which phospholipids adsorb (i.e., form surface-active films) at the air-water interface are poorly understood. Long chain molecules such as phospholipids have low critical micellar concentrations. The critical micellar concentration for DPPC of approximately 10^{-10} mol/L[109] means that DPPC in excess of this low concentration forms aggregates. The tendency of phospholipids to form aggregates arises from the so-called hydrophobic effect, which states that the high energetic cost of water:hydrocarbon interactions drives lipids to adopt specific aggregate forms in which water–fatty acid contact is minimized. Phospholipids such as saturated PC and unsaturated PC are shaped like cylinders with similar diameters for the polar head group and the hydrophobic diacylglycerol moieties.[142] Hydration of phospholipid films dried on the walls of a test tube with water or saline greater than T_c results in the formation of planar bilayer sheets that minimize water:hydrocarbon contact. If these sheets had ends (i.e., if the structures remained *open*), water:hydrocarbon interactions would still occur. However, such sheets curve to produce vesicles (also known as liposomes), which are *closed* structures. Depending on the preparative conditions, multilamellar vesicles of various sizes are generated, but in each case the structures are closed, engulfing some of the aqueous phase. Below T_c, hydration proceeds very slowly. This is why it is often stated that DPPC alone cannot be an effective pulmonary surfactant. Introducing short-chain phospholipids, unsaturated phospholipids (which have low T_cs), or cholesterol will decrease T_c of the mixture. In each case, the resulting vesicles are closed structures. Applying energy in the form of ultrasound using bath or probe sonicators will convert multilamellar vesicles into smaller unilamellar vesicles. This results in an increase in the surface area of the vesicle available for adsorptive processes, as reviewed by Hope and colleagues.[142]

In view of the foregoing considerations, it should not be surprising that surfactant film formation is thought to proceed not through individual phospholipid molecules, but through mechanisms whereby vesicles interacting with the surface rapidly donate many of their phospholipids to the interface. Observations by Schürch and co-workers[143] indicate that the porcine lipid extract surfactant Curosurf adsorbs in discrete, sudden bursts, corresponding to approximately 10^{14} phospholipid molecules.[143] This finding suggests a cooperative mechanism whereby film formation involves the collective transport of subphase material into the surface film. The collective transport of large amounts of lipid into the surface monolayer does not imply that all the lipids in a particular surface vesicle will become incorporated into the monolayer. As elaborated later, it has become evident that considerable amounts of lipid can remain associated with the monolayer as functional multilayers.

Phospholipid Adsorption

Studies of surfactant phospholipid adsorption are considered both in the absence and in the presence of surfactant apoproteins. Phospholipid adsorption characteristics change dramatically at the bilayer transition temperature T_c. Below T_c, dried phospholipid films hydrate extremely slowly, and, as a result, DPPC alone fails to generate surface-active films at physiologic temperatures lower than the T_c of 41° C. In addition, vesicles of saturated PCs prepared at temperatures higher than their T_c adsorb very slowly at temperatures less than their T_c. This rate increases slowly as a function of temperature and increases markedly once T_c is attained. For example, DPPC vesicles (prepared at greater than 41° C) at 1.0 mmol/L do not attain equilibrium in 15 minutes at less than 41° C, but they achieve equilibrium in approximately 1.5 minutes at all temperatures greater than T_c.[140] Likewise, dimyristoyl PC vesicles prepared at greater than the T_c of 23.5° C attain equilibrium at significant rates only at this temperature or greater. DPPC adsorption at 37° C can be enhanced by addition of fluid-phase phospholipids, such as unsaturated PC, unsaturated phosphatidylethanolamine, unsaturated phosphatidylinositol, or unsaturated PG, which lowers T_c of the mixture to less than 41° C.[84,144,145] PG is particularly effective in increasing DPPC adsorption.[146] Dialkyl phosphono analogues of DPPC, which are diethers rather than diacyl phospholipids, adsorb more readily than DPPC.[147]

It has long been thought that cholesterol present in mammalian surfactant at 2% to 10% weight/weight (which corresponds to 8% to 20 mol%) could play a critical role in surfactant phospholipid adsorption. In this context, it has been known for some time that cholesterol interacts with saturated PC, sphingomyelin, and sphingoid-based glycolipids to form a liquid-ordered L_o phase, which possesses properties between LE and LC.[148,149] This distinct liquid-ordered phase is the physicochemical basis of membrane rafts and caveolae, plasma membrane (and likely other cellular membrane) structures with numerous important roles, including cell signaling.[150,151] As mentioned earlier, cholesterol is removed from Curosurf and BLES and is not included in any of the current artificial synthetic surfactants. At present, no distinct physiologic functions or advantages can be attributed to this sterol in replacement surfactants. Adding (returning) cholesterol to BLES enhances adsorption and surface activity on the PBS at 25° C, but this difference is negated at 37° C.[93] The cholesterol content of rat and human surfactant is changed during vigorous exercise, although the physiologic significance is unknown.[20,152,153] Cholesterol levels also are altered in animals in torpor (induced by temporary programmed decreases in body temperature), but again, the significance is not clear.[20,154]

Addition of certain other lipids such as palmitic acid, hexadecanol, or dipalmitoylglycerol to DPPC markedly enhances adsorption in vitro.[155,156] As indicated earlier, palmitic acid is added to Surfactant-TA and Survanta, whereas hexadecanol is used as a spreading agent in Exosurf. However, as explained later, because of nonsurfactant protein inhibition, the in vivo efficacy of monolayer formation is dubious.

In addition to bilayers, certain phospholipids and phospholipid mixtures can form nonbilayer phases.[142] It has been suggested that a hexagonal II phase could be involved in surfactant adsorption. DPPC:phosphatidylethanolamine mixtures that can form a hexagonal II phase do adsorb rapidly.[157,158] However, evidence of a role for a hexagonal II phase in surfactant adsorption is lacking.[159]

Phospholipid Adsorption With Surfactant Proteins

The increased fluidity arising from introduction of non–gel-phase phospholipids and cholesterol can explain, in part, the rapid adsorption of pulmonary surfactant. However, the overall effects are relatively small. This has led to the conclusion that the ability of natural and modified pulmonary surfactant to adsorb sufficiently rapidly to stabilize the terminal air spaces can be attributed to the low-molecular-weight hydrophobic proteins SP-B and SP-C. Early studies demonstrating organic solvent extracts adsorbed rapidly were instrumental in the development of the modified natural surfactant used clinically.[129,145,156,159-163] Such preparations contain SP-B and SP-C, but not SP-A. SP-B and SP-C, alone or together, greatly enhance

adsorption of DPPC-containing mixtures to the equilibrium γ of 23 mN/m.[156,164-170] Whether SP-B or SP-C is more effective depends on the experimental conditions, but SP-B appears to have a greater effect with phospholipid mixtures closely mimicking natural surfactant.[168,169,171,172] SP-A has only a minor effect on adsorption of DPPC or DPPC-containing lipid mixtures but greatly enhances film formation of mixtures containing SP-B in a calcium-dependent manner.[166,173-175] The carbohydrate recognition domain of SP-A is important for this function.[27] SP-A does not influence phospholipid adsorption rate in the presence of SP-C.

The manner by which either SP-B or SP-C promotes adsorption of surfactant phospholipids is not known. These low-molecular-weight hydrophobic proteins can be thought of as bilayer breakers, which disrupt the bilayer structure in such a way that they permit cooperative flow of bilayer phospholipids to the surface monolayer. It is likely separate mechanisms are involved. The 79–amino acid SP-B has three intrapeptide and one interpeptide disulfide bonds. Each monomer has four or five amphipathic helices with hydrophobic amino acids on one face of the protein and hydrophilic amino acids on the other side. SP-B thus acts as a membrane surface protein, with the hydrophobic face buried in the membrane, whereas the hydrophilic residues can interact with phospholipid head groups or with the surrounding water. In addition to the intermolecular disulfide bridge at Cys 48 of the homodimer, SP-B (a dimer) is stabilized by salt bridges (Glu 51 of one SP-B monomer and Arg 52 of the other chain, and vice versa).[176,177] The dimeric nature of SP-B is important for its surface activity.

Several properties of SP-B could contribute to its bilayer-disrupting propensity. SP-B surfactant lipid mixtures can form discoidal disks approximately 50 nm in diameter,[178] similar to those observed in serum high density lipoprotein fractions.[179] These disks are thought to represent bilayer sheets with SP-B dimers associating with the fatty acyl perimeters to minimize aqueous contact. Discoidal fractions have been reported in natural surfactant and in reconstituted systems. Whether these discoidal particles are directly involved in phospholipid adsorption is not known.

SP-B possesses *fusogenic properties* that could contribute to its ability to enhance phospholipid adsorption. Phospholipid vesicles containing SP-B aggregate through membrane:membrane interactions and allow the phospholipids in the outer bilayer leaflets to flow onto the corresponding leaflet of the adjacent vesicle.[35,180] Lipid mixing should be distinguished from true fusion, in which the surface bilayers of the two vesicles combine to become a single membrane and the intravesicular aqueous compartments form a common pool.[142,181] Thus SP-B-mediated lipid mixing is correctly referred to as *hemifusion.* Because air is considerably less polar than water, vesicular hemifusion with the air-water interface could generate a monolayer. However, the molecular details are still vague. SP-B enhancement of DPPC adsorption is greater in the presence of acidic phospholipids such as PG. SP-B contains eight conserved positive residues, but only one conserved negative residue and so could interact with PG. Calcium also promotes adsorption of systems containing SP-B.[92,169,180]

SP-A, SP-B, DPPC, and calcium can combine to reconstitute tubular myelin, a natural surfactant form consisting of stacked square tubules hemi-fused at the corners[179,182] (see Figure 101-3). Tubular myelin may represent the most active form for surfactant adsorption because the rate at which natural surfactant preparations adsorb varies with tubular myelin content.[69] However, because it is not possible to purify this surfactant form, this conclusion cannot be validated.

PL adsorption is also enhanced in the presence of SP-C. SP-C is a 4.2-kDa monomeric protein with a short, 12–amino acid N terminus, ending in Lys-Arg, which marks initiation of a 23–amino acid α-helical segment, terminating in Met 35.[42] It has been reported that removal of the two palmitates esterified to Cys 4 and Cys 5 hampers the ability of SP-C to promote phospholipid adsorption to variable extents.[183-185] Depalmitoylated SP-C can dimerize readily and forms large β-sheet aggregates that could account, in part, for the difference in activity.[186-188] Neutralizing the membrane surface lysine initiating the α-helix greatly diminishes SP-C surface activity.[189,190]

To this point, the adsorption of surfactant phospholipids to a clean surface has been considered. Phospholipid adsorption to a partially occupied surface can occur by a different mechanism. In these experiments, DPPC:PG mixtures containing SP-B or SP-C were spread to a surface tension of 50 mN/m.[191,192] This surface film accelerated subsequent incorporation of lipids from DPPC:PG unilamellar vesicles and resulted in rapid lowering of surface tension to equilibrium. Lipid insertion was triggered by injecting calcium ions into the subphase. This triggering effect arose because calcium ions neutralize the negative charge on PG and thus minimize charge repulsion between surface and subphase phospholipids. SP-B is considerably more effective than SP-C in promoting the "pulling up" of lipids into the surface film. This mechanism could involve interaction of surface lipids with the nonlamellar structures discussed earlier. Such a mechanism could be important in lipid insertion during inspiration when surface tension rises to more than the equilibrium value. The dimeric nature of SP-B enhances surface properties.[193] Removal of the palmitates from SP-C has little effect on SP-C-mediated incorporation of lipids from DPPC:PG into the monolayer. Surprisingly, lipid insertion from neutral DPPC:PC unilamellar vesicles into DPPC:PG:SP-C–containing monolayers was slower with palmitoylated SP-C.[194]

Surface Tension During Film Compression

Surfactant apoproteins modify the properties of model surfactant films and facilitate their ability to attain low γ during film compression. In this context, the term *surface activity* is defined as the ability to reduce surface tension, and the same term is used for γ reduction through adsorption (i.e., 70 mN/m to equilibrium 23 mN/m) and for γ reduction during film compression (i.e., to values lower than equilibrium). The term *compressibility* can be used to describe the degree to which surfactant films lower γ during surface area reduction. Unsaturated phospholipids at equilibrium surface tension are highly compressible: Little reduction in γ occurs with large decreases in surface area. Thus compressibility is inversely related to the slope of the γ versus surface area reduction curve. Good surfactants have low compressibility: γ falls markedly during surface area reduction. The low compressibility of good surfactant films resembles DPPC. This is why the classical model concluded that surfactant monolayers were enriched in DPPC. Addition of the low-molecular-weight apoprotein SP-B or SP-C leads to decreased compressibility, so smaller surface area reductions are required to lower γ from equilibrium to near zero.[171,175,195-197] As in the case of adsorption, SP-A decreases compressibility of surfactant films containing SP-B but not films with SP-C. The manner in which SPs contribute to decreased surfactant compressibility is not understood.

The classical model of surfactant function argues that, for a film to achieve the low γs near 0 mN/m required to stabilize the terminal air spaces, the surface monolayer must be highly enriched with DPPC. Two mechanisms have been proposed for producing this purported DPPC enrichment: (1) the squeeze-out of non-DPPC fluid phospholipids during film compression and (2) selective DPPC adsorption.

Squeeze-Out

The squeeze-out model proposes that, during surface area reduction, gel-phase phospholipids are retained, whereas fluid-phase lipids are selectively expelled from surface monolayers.

This hypothesis arose primarily from Langmuir-Wilhelmy balance studies showing that DPPC is the only major lipid component in surfactant that could sustain sufficient surface pressure to generate γ near zero with surface balances. DPPC monolayers at equilibrium require approximately 12% surface area reduction to lower γ to near zero.[133] Both spread and adsorbed surfactant model systems lacking SP-B and SP-C exhibit high compressibility and require high compression ratios (surface area reduction) to attain low γ. As indicated earlier, compressibility is markedly reduced by the presence of the hydrophobic apoproteins.

Selective DPPC Adsorption

Selective DPPC adsorption means that the initial adsorbed surfactant monolayer contains a higher proportion of DPPC than the bulk phase surfactant. The basis of this proposed phenomenon can be best understood by comparing theoretical and experimental surface area reductions required to compress surfactant monolayers from equilibrium surface tension to γs less than 2.0 mN/m during the initial compression. DPPC monolayers require approximately 12% surface area reduction to lower γ from 23 mN/m to less than 2 mN/m.[129,133] Theoretically, DPPC:PG (70:30) mixtures with 1% SP-B or SP-C at equilibrium would require 30% surface area reduction for squeeze-out of the fluid PG (assumes perfect squeeze-out), resulting in a pure DPPC film (Figure 101-12). A further 12% surface area reduction of the remaining DPPC (70%) in the monolayer would require an 8.4% (0.12 × 70) area reduction to approach zero, resulting in a total area reduction of 38.4% (30 + 8.4). Applying the same reasoning to BLES, which contains approximately 35% DPPC, it would require at least 65% (100% total phospholipid, 35% DPPC) surface area reduction to expel the non-DPPC components from the monolayer. To achieve γ of zero, the remaining DPPC would have to be compressed 4.2% (35 × 0.12) of the surface area. Thus a minimum total surface area reduction of 69.2% (65 + 4.2) would be required to achieve low γ. Table 101-2 shows that the surface area reductions observed during the first quasistatic compression of films adsorbed on a CBT are considerably lower than theoretical.[129] Interpreted in terms of the classical theory, these studies suggest that SP-B and SP-C promote selective DPPC adsorption. This corresponds to a change in surface monolayer composition. However, these inferences are indirect, and the results could indicate a change in surface film organization.

Surface Tension During Film Expansion

Compression of spread DPPC monolayers at equilibrium results in a reduction in γ to near zero. However, with repeated compression and expansion cycles, greater compression ratios are required, particularly if compression is allowed to continue once low γ is attained. This results from low reinsertion of DPPC molecules into the monolayer after collapse. Addition of non-DPPC phospholipids improves respreading, although larger compression ratios are required for the squeeze-out of the non-DPPC components. However, in the absence of the low-molecular-weight hydrophobic proteins, respreading remains poor.[132,171,196-199] These observations demonstrate that SP-B and SP-C promote reinsertion of surfactant lipids. In addition, in the presence of these apoproteins, compressibility decreases during cycling. For example, CBT studies show that Curosurf or BLES films adsorb at low concentrations, requiring 50% to 60% surface area reduction to attain γ near zero during the first quasistatic compression, but approximately 20% during the fifth compression.[129,175,200] The ability of SP-B and SP-C to augment surface activity during cycling can be observed with spread monolayers, but it is more pronounced with surfactant material in excess of a single monolayer. These observations are best explained by the presence of surfactant multilayers, as discussed in the next section.

Evidence Counter to the Classical Model for Surfactant Function

The classical model argues that surfactant adsorption results in monolayers containing DPPC and non-DPPC lipids. This model suggests that repeated compression and expansion of the monolayer result in refining because of squeeze-out of fluid non-DPPC lipids, resulting in enrichment in DPPC.[107,113,201] This process would require incorporation of fresh surfactant during cycling to replace material lost by squeeze-out. The classical model readily explained observations with the Langmuir-Wilhelmy surface balance and the PBS in which repeated compression and expansion cycles led to lowering of γ to low values. The squeeze-out hypothesis was proposed to explain the original classical model when it was introduced.[107,113,201] The selective adsorption model, conversely, arose only because more recent observations with the CBT indicated that the surface activity of adsorbed films during the first compression could not be explained by the DPPC content of natural surfactant or its extracts[112,116,119,129] (see Table 101-2).

Although the classical model considers surfactant from the perspective of a single monolayer, considerable more recent experimental evidence is most readily explained in terms of functional multilayers, which can provide surfactant phospholipids to the surface monolayer.[132,159,200,202] Multilayers can be characterized as *adsorption reservoirs*, generated during film formation, and *compression reservoirs*, generated during film surface area reduction.

Adsorption Reservoirs

Figure 101-13 presents a CBT washout experiment that presents evidence for the formation of functional multilayers during surfactant adsorption.[200] A bubble was introduced into a BLES-1% SP-A (1.0 mg/mL) dispersion in saline 1.5 mmol/L calcium chloride. After adsorption to equilibrium, the CBT chamber was sealed, and the subphase was washed repeatedly to remove surfactant material not associated with the surface film. An initial quasistatic compression (see Figure 101-13, point 1) was then performed to γ approximately 1.0 mN/m. Quasistatic expansion of the bubble led to increases in γ to just above equilibrium. This γ was maintained even though the surface area was expanded well beyond the original value. Because the subphase had been depleted of surfactant, the excess material required to maintain γ near equilibrium must originate from a *surface-associated surfactant reservoir.* After relaxation, a second quasistatic compression (see Figure 101-13, point 2) was performed, followed by a second expansion. Again, γ remained near equilibrium. This also occurred with a third compression, but after the fourth maneuver, γ increased above equilibrium, indicating that the surface-associated reservoir was being depleted. Surface area reduction to attain γ near zero for each compression was 20% to 25%, although BLES contains approximately 35% DPPC. The final compressed monolayer was approximately five-fold larger than that obtained with the initial compression, a finding indicating the reservoir formed during adsorption contained functional surfactant corresponding to at least four monolayers.

Further evidence of the existence of the surface-associated multilayers that can act as surfactant reservoirs has been obtained through electron microscopy of surfactant systems in rabbit and guinea pig lung in situ[138,200] and in vitro.[203] The presence of a surface-associated reservoir has also been deduced from autoradiographic examination of Langmuir-Blodgett films from model and lipid extract surfactants and from radioactive studies on monolayers spread from adsorbed BLES films using filter paper-supported wet bridges.[204,205] These studies indicate the presence of surface-associated lipid aggregate complexes that are generated during adsorption.[206,207] Existence of a surfactant reservoir created during adsorption would serve to explain the remarkable bulk concentration dependence of surfactant observed in numerous studies.[173,175,200] For example, whereas

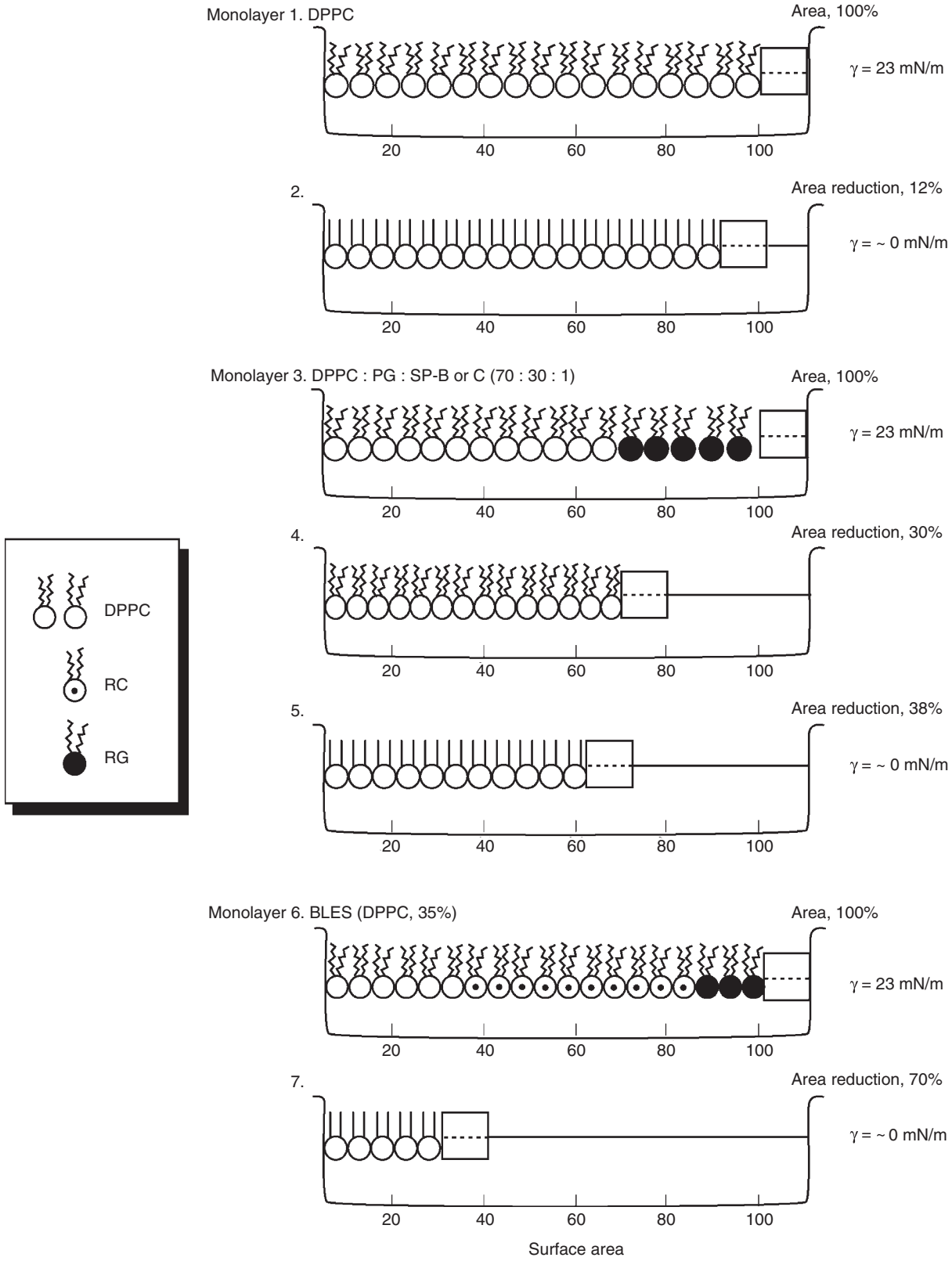


Figure 101-12 Representation of the theoretical surface area reductions required for various monolayers to reduce surface tension from approximately 23 mN/m (equilibrium) to near 0 mN/m. See text for explanation of calculations. *Monolayer 1*, Dipalmitoylphosphatidylcholine *(DPPC)* alone at equilibrium, 23 mN/m. Surface area reduction of approximately 12% leads to *monolayer 2*, which shows DPPC alone after compression to near 0 mN/m. *Monolayer 3,* DPPC:phosphatidylglycerol *(PG)* (70:30) with 1% surfactant-associated protein B *(SP-B)* or SP-C at 23 mN/m. Surface area reduction of 30% leads to *monolayer 4*, after squeeze-out of PG. Only DPPC remains. Compressing monolayer 4, an additional 12% leads to *monolayer 5*, which contains DPPC alone after squeeze-out of PG and compression to near 0 mN/m. *Monolayer 6*, Bovine lipid extract surfactant *(BLES)* at 23 mN/m. Compression of 70% leads to *monolayer 7*, showing BLES after squeeze-out of unsaturated phosphatidylcholine *(PC)* and PG and compression to near 0 mN/m. *(Modified from Possmayer F, Nag K, Capote KR, et al: Surface activity in vitro: role of surfactant proteins,* Comp Biochem Physiol A Mol Integr Physiol *129:209, 2001.)*

TABLE 101-2

Theoretical and Observed Surface Area Reductions Required to Reduce Surface Tension from Equilibrium (23 mN/m) to <2 mN/m

	SURFACE AREA REDUCTION REQUIRED (%)	
Film Composition	***Theoretical***	***Observed***
DPPC	—	12
DPPC/PG/SP-B (70:30:1)	38	20
DPPC/PG/SP-C	38	30
BLES	69	30
BLES plus SP-A	69	25

BLES, Bovine lipid extract surfactant; *DPPC,* dipalmitoylphosphatidylcholine; *PG,* phosphatidylglycerol; *SP,* surfactant-associated protein.

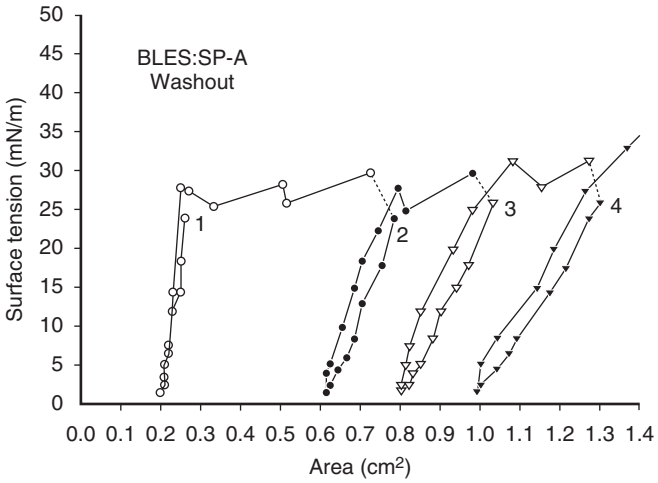


Figure 101-13 Physicochemical evidence for the adsorption surface-associated reservoir. After washing the subphase *(1),* the film *(open circles)* compressed to low tension.The bubble surface tension increases as surface area is increased but remains close to equilibrium.After a brief relaxation period *(2),* the film *(closed circles)* is compressed to low tension a second time.The bubble is again expanded, allowed to relax, and expanded a third time *(3, open triangles).* After the fourth compression, surface area expansion *(4, closed triangles)* leads to high surface tension, indicating that the surface-associated reservoir has been depleted (see text for further details). *BLES:SP-A,* Bovine lipid extract surfactant:surfactant-associated protein A. *(From Schürch S, Qanbar R, Bachofen H, Possmayer F:The surface-associated surfactant reservoir in the alveolar lining,* Biol Neonate *67:61, 1995.)*

50% to 60% surface area reduction is required to reduce γ from equilibrium to near zero during the first compression with BLES at 50 μg/mL, only 22.5 to 25% surface area reduction is required to reduce γ to near zero at bulk concentrations of 250 to 300 μg/mL. Addition of SP-A to BLES at low concentration greatly improves surface activity.[175,200,208]

Compression Reservoirs

The existence of surface-associated reservoirs, formed during film compression, that could supply phospholipid to the surface monolayer during film expansion would provide an efficient means for surfactant respreading. Evidence of such reservoirs is available from fluorescence imaging and atomic force microscopy. For example, fluorescence imaging studies reveal that compression of model systems such as DPPC:DPPG, DPPG:POPG, and DPPC:POPC:POPG results in loss of material from the surface into the bulk phase during collapse. Inclusion of either SP-B or SP-C (or synthetic analogues thereof) results in film buckling and formation of large folds or buds that extend into the subphase.[197,209-217] As the surface is expanded again, the folds are incorporated into the surface monolayer with minimal loss of material over many compression and expansion cycles. Examination of Langmuir-Blodgett films deposited under compression reveals large irregular plates divided by thin spaces with phospholipid multilayers containing protein.[189,197,212,215-218] In addition to the folds noted earlier, buds that grew into stacked bilayers have been noted, particularly with lipid extract surfactants and similar systems.[104-106,219]

Compression reservoirs that function in phospholipid reinsertion into the monolayer during film expansion not only provide a plausible function for SP-B and SP-C, but also explain why adsorbed films and films spread in excess of a single monolayer remain more surface active during cycling. SP-B and SP-C also promote uptake of phospholipid vesicles from the bulk phase.[192,193] Such uptake can occur only when γ rises above equilibrium.

Phase Transitions and the Classical Model

Further evidence incompatible with the classical model arises from fluorescence imaging studies. Dark probe-excluding regions, apparently LC domains, are observed during compression of DPPC:1% fluorescent probe on Langmuir-Wilhelmy balances (see previous section). Probe-excluding LC domains can also be observed with natural surfactant, lipid extract surfactants, and the phospholipid fraction from the calf surfactant CLSE. The classical model would imply that compression to high surface pressures (low γ) would result in nearly complete elimination of fluid probe-containing LE phase, leaving only dark LC phase in the monolayer. Fluorescence imaging studies with CLSE and porcine surfactant lipid extracts reveal probe-excluding domains that appear at γ near 60 mN/m and attain 33% to 40% total surface area at γ near 40 mN/m. However, these LC domains diminish and disappear at γ near 30 to 40 mN/m.[104,106,159,219] This behavior suggests that lateral phase separation occurs at high γs (low surface pressures), but it is followed by a loss of LE and LC phases resulting from phospholipid remixing at lower γs (higher surface pressures). This phase remixing (codissolving) can be attributed to the cholesterol present in these extracts. Cholesterol appears to serve to maintain the monolayer in a more fluid phase capable of reaching relatively (but not very) low γ. These studies provided no evidence for generalized exclusion of non-DPPC lipids leading to monolayer enrichment in DPPC as required by the classical model.

Further evidence contrary to the classical model was obtained with fluorescence studies employing BLES or the phospholipid fraction from CLSE (no cholesterol). Such films readily attain γ near zero during compression while containing less than 40% surface area probe-excluding regions.[159,220] This level of LC phase is comparable to the DPPC mole fraction. The ability of LE:LC-containing phospholipid monolayers to achieve γ of approximately zero in the presence of more than 60% LE phase is incompatible with the classical model, which would predict that only LC phase would be present at low γs.

The classical model evolved from the observations that DPPC was the only major surfactant component that could achieve and maintain surface tensions below equilibrium during monolayer compression. Such studies were normally conducted using the Langmuir-Wilhelmy balance introduced by Clements.[79] Studies by Hall's group have demonstrated that the ability to achieve γ near zero depends not only on monolayer composition but also on the rate at which the film is compressed. As stated earlier, phospholipid monolayers can be compressed dynamically to surface tensions below their equilibrium surface tensions, but above T_c, surface tension will rapidly return to equilibrium.

However, compressing POPC (T_c = −6° C) or dimyristoyl PC (DMPC) (T_c = 24.5° C) at rates of 32% of initial area/second permits such films to reduce to approximately 2 mN/m.[221,222] Furthermore, such rapidly compressed films can maintain γ below equilibrium for many minutes. Once compressed rapidly across the equilibrium γ of approximately 23 mN/m threshold, monolayers can be compressed or expanded more slowly without losing this remarkable property. Such rates are inaccessible on the Langmuir trough, which is normally compressed at approximately 3% total surface area/minute, but are readily attainable with the pulsating and captive bubble surfactometers and correspond to those in the lung. As emphasized in a later discussion, films below γ equilibrium are metastable (also true for DPPC) but remain at low tensions for periods much longer than required to stabilize the terminal air spaces during breathing or with high-frequency (fixed-volume) ventilation.

The mechanism by which spread monolayers of fluid-phase phospholipid attains this remarkable metastable state is not understood. Piknova and co-workers[159] suggested that as fluid LE monolayers are compressed beyond equilibrium, they can either collapse to three-dimensional multilayers or form a disordered phase that, although thermodynamically metastable, is kinetically trapped for long periods. A possible analogy for this kinetically trapped metastable phase may be glass, which is an amorphous (nonordered) solid. The observation that single-component fluid phospholipid monolayers can be compressed into viscous metastable phases raises the question of whether either the gel-phase lipid DPPC or surfactant apoproteins may be required in the lung. A possible explanation is that these surfactant components facilitate transformation of LE phase to the viscous state. Such an interpretation would be consistent with evidence from compression and expansion cycles as described. Regardless of the interpretation, it should be clear that the rapid compression studies provide further strong evidence against the absolute requirement for a monolayer highly enriched in gel-phase phospholipids such as DPPC, as argued by the classical model. (After tornadoes, straws are sometimes reported to be found stuck through barn boards. It thus appears that rapid motion can alter physical stiffness.)

In summary, it is evident that surfactant apoproteins play essential roles in the biophysical functions of pulmonary surfactant. These low-molecular-weight hydrophobic proteins enhance surfactant lipid adsorption so surfactant can form phospholipid films at the rates required by the lung. Observations have suggested that surfactant films can act as functional multilayers. Evidence has been obtained for both adsorption reservoirs and compression reservoirs that can provide surfactant phospholipids to the surface monolayer during film expansion. SP-B, SP-C, and SP-A plus SP-B contribute to the formation of these reservoirs, but the mechanisms involved are not clear. The classical model for surfactant function implies that, during film compression, LC regions consisting primarily of DPPC increase and coalesce as a single gel-phase monolayer at γ near zero. Fluorescence studies on porcine and calf surfactant extracts do not observe a large increase in LC phase. Rather, at a critical surface pressure, LC phase lipids appear to dissolve again (remix) with the fluid components. This behavior is not predicted by the classical model.

Other studies have demonstrated that monolayers consisting of the phospholipid fraction from calf surfactant can be compressed to low surface tensions without the anticipated large increase in LC gel phase. The observation that low surface tensions (high surface pressures) can be achieved in the presence of LE and LC phases is counter to the classical theory.

Finally, studies using CBT have shown that monolayers of fluid lipids such as DMPC and POPC can achieve γs near zero at 37° C when compressed at rapid rates that are similar to those occurring in the lung. It is suggested that when fluid monolayers are compressed slowly, they collapse. However, when compressed rapidly enough, fluid monolayers can be kinetically trapped as an amorphous solid that collapses very slowly. Such monolayers, although metastable by definition (they are below equilibrium γ), could stabilize the alveoli. The existence of such metastable monolayers is opposite to the requirement for the DPPC-enriched monolayer proposed by the classical model.

SURFACTANT INACTIVATION

In addition to surfactant deficiency, such as occurs in neonatal RDS, alveolar stability can also be compromised through surfactant inactivation. Numerous natural and synthetic materials including meconium (fetal feces), lysophospholipids (produced by phospholipase A_2), pulmonary edema fluid, and nonsurfactant proteins can inhibit surfactant function in vitro.[11,49,155,223-230] Although the importance of surfactant inhibition in acute RDS is recognized, the mechanisms are not well understood. Considerable effort has been devoted toward examining the effects of serum proteins on surfactant function. Acute lung injury arising from certain insults results in disruption of the capillary-endothelial integrity, leading to increased protein influx into alveolar spaces.[11,71,230] The enhanced permeability and increased protein oncotic pressure antagonize the efforts of the alveolar epithelium to remove fluid from the alveoli. In addition, by inhibiting surfactant function, serum proteins increase the hydrostatic pressure gradient across the alveolar surface, possibly further promoting pulmonary edema. Perhaps not surprisingly, wholly artificial surfactants lacking surfactant apoproteins are highly susceptible to protein inhibition.[155,231,232] The ability of several clinical preparations to resist protein inhibition correlates with their SP-B content.[233-235] SP-C–based surfactant preparations show resistance to meconium.[224]

SP-A is not present in the lipid extract surfactants used clinically. However, addition of SP-A to lipid extract surfactant effectively reverses in vitro inhibition resulting from serum proteins.[174,236] SP-A does not block surfactant inhibition resulting from nonprotein inhibitors such as lysophospholipids. Lysophospholipids can sensitize lipid extract surfactant to inhibition by proteins.[228] SP-A deletion knockout studies demonstrate that SP-A is not essential for normal lung function. Evidence indicates that this collectin can play a role in reversing surfactant inhibition by proteins in vivo[237-240] as well as in vitro, but this remains controversial.

Surfactant inactivation varies with both the particular serum protein examined and the surfactant preparation employed. This finding indicates that protein inactivation could involve several mechanisms. Some proteins— albumin, for example— appear to inhibit surfactant by adsorbing rapidly, thereby monopolizing the air-water interface. Surfactant inhibition is readily reversed by increasing the concentration of lipid extract surfactant. This reversal of protein inhibition is nonstoichiometric and cannot be readily explained by competition.[230,241] Albumin binds free fatty acids, but whether this property is involved in surfactant inhibition is not known. Studies indicate albumin interferes with surfactant respreading from compression reservoirs.[213] However, this interference and the ability of albumin to compete with surfactant for the air-water interface only occur at surface tensions higher than those observed in the lung.[121,124,133] A possible explanation is that several inhibiting mechanisms occur during acute lung injury and acute RDS. For example, lyso-PC at low concentration does not inhibit surfactant but can sensitize it to protein inhibition.[228,242] The C-reactive peptide, an acute phase protein, can bind the phosphorylcholine-head groups of PC and DPPC.[243] Inhibition by C-reactive protein can be reversed by phosphorylcholine, which is water soluble. The reason that C-reactive protein can inhibit surfactant, whereas SP-A, which also binds DPPC, blocks protein inhibition, is not understood.

Fibrinogen and fibrin monomers are particularly effective in inactivating surfactant.[11,229] These proteins appear to inactivate surfactant by sequestering surfactant constituents, such as surfactant apoproteins, in the hyaline membranes characteristic of respiratory distress.[244-246] Procoagulant activity increases in RDS and acute RDS. The ability of the lung to remove fibrin-based clots is also compromised in acute RDS. These observations further emphasize the complexity of the relationship between lung injury and surfactant inactivation. Studies in vitro have reported surfactant inhibition by proteins and meconium can be reversed by high-molecular-weight dextran[247] or polyethylene glycol.[248] Attempts to overcome surfactant inactivation in vivo have been variable.[249,250] Surfactant is also inactivated by oxidative stress. Superoxide and hydroxyl radicals likely react with surfactant lipids and SPs.[251-253] SPs can also be affected by nitration.[254,255] SP-A (and SP-D) has been reported to block lipid oxidation by free radicals.[252] In addition, SP-A can reverse the inhibitory effects of surfactant oxidation.[256]

Artificial surfactant preparations lacking surfactant apoproteins are susceptible to inactivation by serum proteins. Addition of the low-molecular-weight hydrophobic proteins, particularly SP-B, results in resistance to inhibition by serum proteins. SP-A is capable of resisting surfactant inactivation by serum proteins in vitro, and this may be important in vivo. SP-A may also be important in preventing and reversing surfactant inactivation during oxidative stress.

CONCLUSION

The physicochemical principles governing surfactant function reviewed in this chapter provide insight into the design of exogenous surfactants for clinical use. It is apparent that different optimizing strategies will be required to develop exogenous surfactants for treating neonatal and adult respiratory distress. However, the precise requirements for acute lung injury and acute RDS must still be defined.

It is clear an effective exogenous surfactant must adsorb rapidly at 37° C and spread quickly to form a surface film with an equilibrium surface tension of approximately 23 mN/m. Excellent spreading within the terminal air spaces is important for exogenous surfactant to ensure adequate distribution.[257] In addition to rapid adsorption and good spreading, exogenous surfactants should achieve low surface tensions with the dynamic compressions produced during normal breathing or mechanical ventilation. The highest γs that can be attained during inspiration and expiration while still compatible with adequate lung function are not known.[121] Effective exogenous surfactants must resist inactivation by serum proteins, other inhibitors, and oxidative stress. This is particularly true for acute lung injury and acute RDS. Therapeutic surfactants should also be resistant to film overcompression at low surface tensions. In addition, considerable benefit would accrue if the exogenous preparations could resist conversion from the highly active LA subtype to the poorly active SA form. Exogenous surfactants could also contribute by modulating conversion of endogenous surfactant, thereby maintaining a high proportion in the active LA form.

The artificial synthetic surfactants used clinically can become "activated" in the lung through interaction with endogenous surfactant apoproteins.[1,54] This interaction should be optimized in the design of exogenous preparations. The ability of exogenous surfactant to act as "substrate" for recycling into lamellar bodies and secretion as "new" surfactant must also be considered. It is thought that recycling of artificial surfactant components and mixing with endogenous surfactant apoproteins explain their effectiveness in RDS. Their inability to act directly may explain why artificial surfactants have not proven effective with acute RDS. The use of synthetic lipid molecules that resist degradation by secreted phospholipids or cellular catabolic systems could prove useful.[147] However, such lipid analogues must be recycled appropriately and should not accumulate within cells.

It has become evident that peptides or peptoids that can mimic the activities of specific surfactant apoproteins are required for exogenous treatment, especially for the treatment of acute RDS.[39,258-261] Therefore this chapter closes with a recapitulation of the nature of the surfactant apoproteins and emphasizes their proposed roles in surfactant function. SP-A by itself has little effect on phospholipid adsorption. However, SP-A markedly enhances the phospholipid adsorption observed in the presence of SP-B and calcium.[92,166,208,236] In the presence of SP-B, SP-A counteracts serum protein inhibition of surfactant function. SP-A preferentially binds gel-phase lipids demonstrating a very high preference for DPPC.[27,28] Although only small amounts are present in lamellar bodies, SP-A is an important constituent of tubular myelin. Tubular myelin structures can be formed in vitro with DPPC, PG, SP-A, SP-B, and calcium.[179,183] Its ability to interact with DPPC would explain the role of SP-A in maintaining the integrity of LAs. SP-A also could contribute to formation of a surface-associated surfactant reservoir.[200]

SP-A and SP-D are members of the collectin superfamily and have important roles in the innate host defense system.[21,22,29,31,32] In vitro studies indicate that SP-A can play an important role in regulating alveolar levels of surfactant lipids, but this is controversial because SP-A–deficient knockout mice have normal surfactant levels and turnover.

SP-B is a low-molecular-weight hydrophobic protein that is an important constituent of the lipid extract surfactant used clinically.[24,35,132] SP-B deficiencies resulting from gene disruption in mice or human SP-B mutations are associated with neonatal pulmonary complications and death.[10,40] SP-B enhances the adsorption of surfactant lipids. In addition to promoting adsorption to clean surfaces, SP-B present in partially filled monolayers can assist in the "incorporation" of phospholipid from vesicles in the subphase. SP-B can stabilize surface monolayers at low surface tensions hindering the return to the equilibrium surface tension of 23 mN/m. With DPPC-PG, SP-A, and calcium, SP-B is involved in the formation of tubular myelin in vitro. The ability of SP-B to promote hemifusion of phospholipid vesicles indicates that SP-B may be responsible for forming the lattice corners of tubular myelin. SP-B contributes to the formation of adsorption and compression reservoirs.

Like SP-B, SP-C is present in the lipid extract surfactants used clinically.[42] Recombinant SP-C is the only apoprotein present in the synthetic SP-C-containing surfactant Ventacute. SP-C enhances phospholipid adsorption. Surface SP-C can also promote the uptake of phospholipid vesicles into the surface film until equilibrium is attained. SP-C contributes to the mechanical stability of surface films compressed to low surface tensions. SP-C also promotes the uptake of phospholipids from a surface-associated surfactant reservoir during monolayer expansion. This property appears to resist the decrease in surface activity of surfactant films subjected to repeated overcompression at low surface tensions. The palmitoyl groups esterified to N-terminal cysteines of SP-C appear to be important for these latter functions.

This chapter reviews the physicochemical functions of pulmonary surfactant with emphasis on the SPs. Although a great deal has been learned about the nature and properties of these apoproteins, further experimentation is required to confirm some of the suggested mechanisms proposed in this chapter. A more complete understanding of these mechanisms is clearly essential for producing optimal surfactants for clinical application.

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102

Scott H. Randell and Stephen L. Young

Structure of Alveolar Epithelial Cells and the Surface Layer During Development

This chapter describes the developmental anatomy of the alveolar epithelium and the alveolar interfacial (surfactant) layer. When possible, it includes data from human studies, but the fundamental process of alveolar epithelial maturation appears to be common to most mammals, and the chapter also draws freely from animal data. Several developmental anomalies affect in utero development of the pulmonary epithelium, but comprehensive discussion of them is beyond the scope of this chapter (see Stocker[1] and Chapter 78).

HISTORICAL PERSPECTIVE

In 1953, Low resolved a riddle of the lung: Are alveolar capillaries exposed directly to alveolar air? With the new technology of electron microscopy, he conclusively demonstrated the presence of a thin alveolar membrane consisting of capillary endothelium, lung interstitium, and a continuous covering of (type I) epithelium (Figure 102-1).[2] Even before Low's anatomic work, physiologic considerations predicted the presence of a surface-active alveolar lining layer. Terry[3] had proposed, in 1926, that a thin film overlaid the alveolar epithelium. By 1929, von Neergaard's experiments[4] had proved that surface forces had a profound influence on the mechanical properties of lungs, and evidence mounted during the next 30 years that surface tension at the alveolar air-liquid interface must be extraordinarily low,[5] especially at low lung volumes. Macklin[6] hypothesized that the alveolar lining film functioned to remove particulates, maintain a favorable surface tension, protect the alveolar septa from desiccation, and suppress invading microorganisms. Early histologists had recognized the granular pneumonocyte (now known as the type II cell) as a lung secretory cell, and Macklin correctly proposed that it was the source of an alveolar lining layer. He speculated that ". . . [the film's] mechanism of production insured a constancy of volume, thickness, weight and solid content. . . ."[6] Despite the soundness of the physiologic experiments, the morphology of the surfactant layer remained controversial because it could not be demonstrated microscopically. Light microscopy was unable to resolve whether the observed carbohydrate-rich lining of alveoli was due to a mucoid surface layer or to cell membranes. Electron microscopy initially failed because aqueous solutions and organic solvents used for tissue processing washed away the lipid-rich lining layer. By using perfusion fixation methods, Weibel and Gil[7] were finally able to demonstrate remnants of the interfacial film in situ. Later refinements[8] showed details of an unbroken superficial alveolar lining, probably a pure phospholipid monolayer, on top of an aqueous hypophase containing reserves of newly secreted material and pools of surfactant desorbed from the air-liquid interface (Figure 102-2).

IN UTERO DEVELOPMENT OF THE ALVEOLAR EPITHELIUM

The mammalian lung begins as a tiny foregut pouch formed at the level of the pharynx. During early organogenesis, the lung anlage consists of clumps of endodermally derived, multipotent, columnar to cuboidal epithelial cells, which are surrounded by mesenchyme. Important developmental signals are exchanged between the mesenchyme and the epithelium. During the lung bud stage, direct contact of cardiac mesoderm with foregut ventral endoderm is required to induce

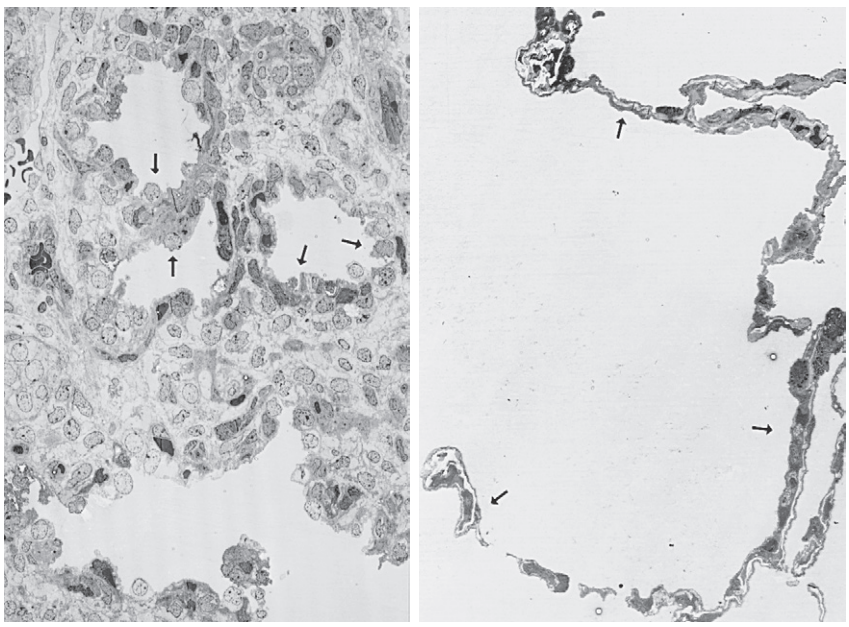


Figure 102-1 Electron micrographs of fetal (week 25, *left panel*) and adult human lungs *(right panel)* at equal magnification. The dramatic expansion of the air spaces, thinning of the interstitium, and the extreme thinning of the epithelial cells *(arrows)* are some of the critical developmental changes enabling efficient gas exchange.

lung-specific genes.[9] Tissue recombination experiments using slightly later embryos have shown that branching morphogenesis of the bronchial tree is dependent on surrounding mesenchyme and that regional-specific epithelial cytodifferentiation is also dependent on intimate contact with its corresponding mesenchyme[10,11] (see Chapter 78).

The stages of human lung development[12] (see Chapter 78) serve as a useful framework to describe important steps in epithelial cell development (Figure 102-3). The *glandular* (or pseudoglandular) stage of human lung development ends at 16 weeks gestation. At this time, conducting airways to the level of the terminal bronchioles are surrounded by a loose investment of mesenchyme, which includes blood vessels. The airways are lined with multipotent, glycogen-rich, and morphologically undifferentiated epithelial cells that have a columnar to cuboidal shape. Sixteen weeks of gestation marks the onset of the *canalicular* period, during which the respiratory bronchioles and alveolar ducts of the gas exchange region of the lung are formed. The mesenchyme becomes more condensed, blood vessels proliferate, and the epithelium overlying capillaries thins, which is the hallmark of type I epithelial cell differentiation.

After 24 to 28 weeks, the *saccular* period begins. Primitive forms of the future alveoli are present (hence the term *saccular*) and are lined by squamous type I cells and cuboidal type II cells. At approximately 25 weeks gestation, the human lung has the potential to support gas exchange, although successful unassisted ventilation is unlikely because the lung and its surfactant system are still underdeveloped.

Significant species differences exist in the timing of alveolar epithelial cell differentiation. In laboratory rodents and in lambs, the first appearance of type II cell lamellar bodies occurs at approximately 80% of gestation, whereas in humans their appearance at 24 weeks marks 60% of gestation. The most remarkable maturational timing belongs to the marsupials, in which type II cell lamellar bodies appear in time for the marsupial's precocious transition to air breathing, often occurring at less than 20% of gestation when lung development has not progressed beyond the canalicular stage.[13] Lamellar bodies appear in female offspring slightly earlier than they appear in males, partly because androgens exert an inhibitory influence on alveolar epithelial maturation.[14] Molecular mechanisms that govern epithelial phenotype have been identified (reviewed by Mendelson[15] and Warburton and colleagues,[16] and see Chapter 81). Human type II cells at 24 weeks gestation or rat type II cells at 19 days of gestation (term = 21 days) can be recognized morphologically by their cuboidal shape, apical intercellular junctions, microvilli, and a few lamellar bodies. They have a large and dispersed cytoplasmic pool of glycogen that displaces organelles toward the cell periphery. Glycogen likely serves as a precursor for the production of surfactant phospholipids[17] (also see Chapter 103). The cells are rich in the organelles of synthesis, such as granular endoplasmic reticulum, polyribosomes, and the Golgi apparatus with its associated vesicles. Impressive changes in type II cell morphology occur during late gestation, with a rapid loss

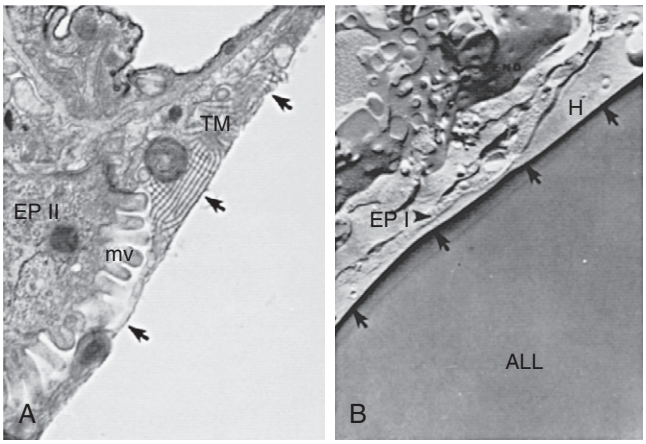


Figure 102-2 A, Electron micrograph of a perfusion-fixed adult rat lung. A type II cell *(EP II)* can be seen with microvilli *(mv),* and the alveolar lining layer is visible *(arrows)*. Lamellar forms *(L)* are present in the hypophase fluid, and tubular myelin *(TM)* is in contact with the surface. **B,** Freeze fracture micrograph, en face view, of the lining layer *(ALL),* hypophase fluid *(H),* and a type I cell *(EP I). (**A** courtesy Robert Mercer, PhD; **B** from Manabe T: Freeze-fracture study of alveolar lining layer in adult rat lungs,* J Ultrastruct Res *69:86, 1979.)*

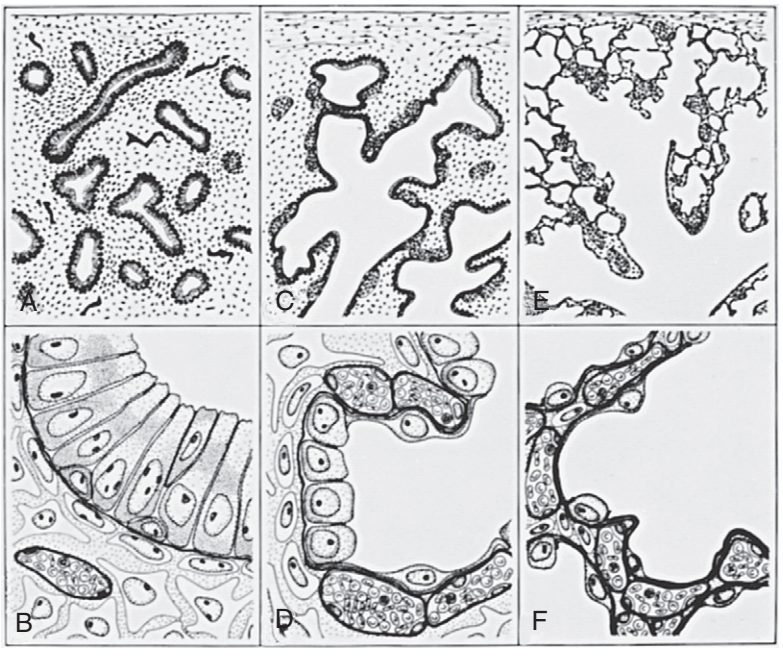


Figure 102-3 A highly schematized illustration of the light microscopic appearance of human lung during the glandular **(A, B)**, canalicular **(C, D)**, and saccular **(E, F)** stages of in utero development. Undifferentiated columnar cells of the early bronchi **(B)** rest on a basal lamina, which has been emphasized. Mesenchymal cells and capillaries are present below the basal lamina. **D** shows that the epithelium overlying proliferating blood vessels thins to a squamous type I morphology and that the most distal cells retain a cuboidal shape. At the saccular stage **(E, F)**, thinner septa are lined by differentiated type I and type II cells. The low- and high-magnification drawings are shown at approximately the same magnification in each panel.

of the glycogen pool and proliferation of the cellular structures used for surfactant production and secretion (Figure 102-4).

Biochemical markers are valuable in the study of lung development and have been used to study cellular parental lineage and the timing of cellular differentiation of the lung's distal epithelium. Wuenschell and co-workers[18] demonstrated epithelial coexpression of markers characteristic of neuroendocrine cells, Clara cells, and type II cells during the glandular stage of lung development when only conducting airways were present. Later, these markers were restricted to their respective cell lineages. The early expression of the Clara cell marker secretoglobin 1A1 (SCGB1A1, also known as Clara cell-specific protein and sometimes abbreviated CCSP or CC10) or the type II cell marker surfactant protein C (SFTPC) enables the generation of triple transgenic mice with inducible genetic modifications of specific populations of lung epithelial cells.[19,20] These mice have revealed cell lineage patterns, and this approach has general applicability as a research tool for inducible, lung epithelial-specific genetic manipulation in mice.

Williams and co-workers[21] described a monoclonal antibody directed against a surface marker of type I cells, T1α. Their studies demonstrated expression of T1α on distal bronchial epithelial cells early during development, when no squamous cells and only cuboidal cells were present. This finding suggests a direct lineage of the early saccular epithelium to type I cells in addition to the more accepted transformation of type II to type I epithelial cells. Genetic deletion of T1α inhibited type I cell differentiation and enhanced epithelial and mesenchymal cell proliferation resulting in a neonatal lethal phenotype.[22] Additional type I cell markers (Table 102-1 and reviewed by Williams[23]) include the water channel aquaporin 5 and the epithelial sodium channel, indicating a likely role for type I cells in alveolar fluid homeostasis (see Chapter 82).

Type II cells are considered the usual precursors of type I cells both during development and during repair of the injured epithelium in adults.[24,25] Before the identification of specific surface antigens, morphology was the principal method for classifying type I cells, which cover more than 90% of the adult lung alveolar surface area.[26] The cytoplasm of type I cells is so thin that most organelles appear to be clustered within the perinuclear region. The volume of a type I cell is approximately five times greater than that of a type II cell, consisting mainly of cytoplasm. Although mitochondria or rough endoplasmic reticulum content is reduced per unit volume of type I cell cytoplasm, the absolute volume of organelles per cell is equivalent to that of type II cells.[27]

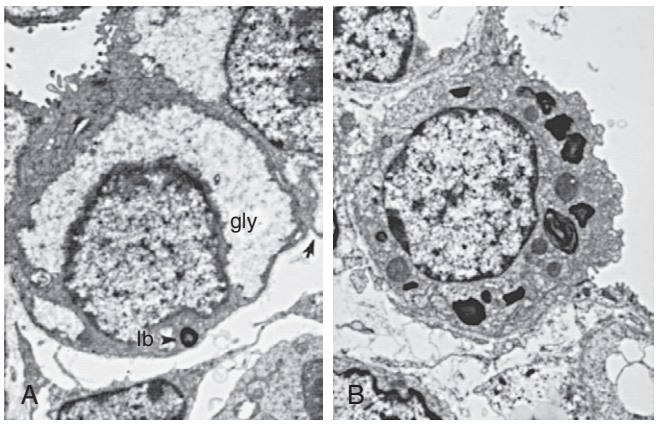


Figure 102-4 Electron micrographs of rat lung illustrating type II cells during development. The earliest morphologically recognizable type II cell **(A)** has large pools of glycogen *(gly)* and small lamellar bodies *(lb)*. A foot process *(arrow)* can be seen perforating the basement membrane. At the time of birth **(B)**, the type II cell undergoes a rapid depletion of glycogen and acquires many lamellar bodies.

INTRACELLULAR METABOLISM OF SURFACTANT

Details of the biochemistry and biosynthesis of surfactant lipids and the four surfactant-related proteins are given in Chapters 100, 101, and 103. De novo lipid biosynthesis occurring in the endoplasmic reticulum of type II cells has been demonstrated by in vivo injection of tritiated choline. After surfactant lipids are synthesized in the endoplasmic reticulum, they are transported to lamellar bodies. Early autoradiographic studies demonstrated that [^{3}H]choline radiolabel appears in multivesicular bodies after the endoplasmic reticulum but before it is found in lamellar bodies.[28] Thus morphologic and biochemical evidence places the multivesicular body in the pathway leading to lamellar body genesis, yet its role remains poorly understood.[29] Two morphologic types of multivesicular bodies are visible in type II cells: one with vesicles plus an electron-lucent background and another composed of vesicles within an electron-dense matrix (Figure 102-5). An intermediate, possibly immature form of lamellar bodies, termed the composite body, contains both lamellae and vesicles. Mature lamellar bodies do not contain vesicles but have a limiting membrane, a thin rim of granular material, many phospholipid-rich lamellae, and often a central core of granular material (Figure 102-6). (see also Figure 102-4.)

Lamellar bodies belong to a family of lysosome-related organelles, also including platelet dense granules and melanosomes, that are affected in Hermansky-Pudlak syndrome, a genetically heterogeneous condition, the diathesis of which includes lamellar body abnormalities and interstitial lung disease (see Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/omim/203300). Mutations in Hermansky-Pudlak syndrome, and in its animal models,[30] alter biogenesis, trafficking, secretion, or reuptake pathways regulating homeostasis of lysosome-related organelles.

Pathways of protein biosynthesis and cellular packaging by type II cells have been determined and appear to be similar to those noted in other cell types, with the exception of a possible synthetic, rather than degradation, role for the multivesicular body.[28] Proteins for export, including surfactant apoproteins, are synthesized in the endoplasmic reticulum, and several studies indicate extensive posttranslational protein modifications in the *trans*-Golgi.[28,31,32]

Biochemical studies indicate extensive uptake and recycling of surfactant lipid and protein by type II cells.[33,34] Morphologic descriptions of recycling pathways suggest that endocytosed components are directed into lamellar bodies, presumably for resecretion. Multivesicular bodies appear to be part of the endocytic pathway and may function as a nexus between synthetic, recycling, and degradation systems.[35] Electron-lucent multivesicular bodies are the first structures to receive freshly

TABLE 102-1

Selected Alveolar Epithelial Cell Markers, Their Function, and Knockout Phenotype

	Marker	Function	Knockout
Type I cell	T1α	Unknown	Dysmorphic alveoli
	ENaC	Na^+ transport	Alveolar flooding
	AQP5	H_2O transport	No phenotype
Type II cell	SP-A	Surfactant	Innate immunity
	SP-B	Surfactant	Lethal at birth
	SP-C	Surfactant	Interstitial fibrosis
	SP-D	Surfactant	Lipid storage, emphysema

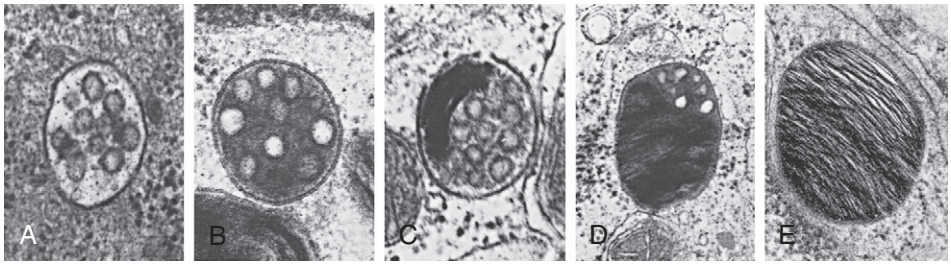


Figure 102-5 Electron micrographs of multivesicular bodies **(A, B)** and composite forms **(C, D)** from rat lung type II cells. The mature lamellar body **(E)** contains no vesicles. The magnifications vary, multivesicular bodies are approximately 0.2 μm, composite forms are approximately 0.5 μm, and lamellar bodies average approximately 1 μm in diameter. *(From Young SL, Fram EK, Craig BL: Three-dimensional reconstruction and quantitative analysis of rat lung type II cells: a computer-based study,* Am J Anat *174:1-14, 1985.)*

endocytosed membrane markers such as cationic ferritin or lectins.[29] About 30 minutes later, the markers are found within lamellar bodies, the mature secretory and storage granules of the type II cells. Autoradiographic studies[35,36] of radiolabeled lipid and surfactant protein instilled into rat lungs revealed recycling of both components, although lipid and protein were differently localized to electron-dense (protein) and electron-lucent (lipid) multivesicular bodies, and lamellar bodies later received both labels.

ORGANIZATION OF THE TYPE II CELL DURING DEVELOPMENT

Three-dimensional views of type II cells obtained from rats from gestational day 20 to adulthood demonstrate dynamic spatial reorganization of organelles during differentiation and maturation. At day 20, composite bodies, which may represent immature lamellar bodies, and a few mature lamellar bodies were localized to the basal side of the cell. Later in life, lamellar bodies increased and were distributed throughout the cell.[37] Composite forms were polarized toward the basal aspect, away from the alveolar air border. Composite body number peaked at the time of the rapid appearance of lamellar bodies, around the time of birth in rats, consistent with their putative role as lamellar body precursors.[28]

GESTATIONAL CHANGES IN ALVEOLAR BASAL LAMINA

The basal lamina influences overlying epithelial cells and is likely an important mediator of epithelial-mesenchymal interactions. On gestational day 19 in rats, the basal lamina is continuous, but, as cellular maturation proceeds, type II cells extend cytoplasmic foot processes, which become closely apposed to interstitial lipofibroblasts, through interruptions of the basal lamina (see Figure 102-4).[38-40] Foot processes increase during late gestation and in the early postnatal period, in parallel with increasing cellular lipid synthesis and cytodifferentiation. Steroids accelerate foot process appearance and enhance type II cell differentiation and surfactant lipid and apoprotein synthesis. Foot processes rapidly decrease after birth but reappear during repair of the injured alveolar epithelium in human adults.[41] These observations imply that foot processes are a platform for epithelial-mesenchymal communication.

The basal lamina underlying the alveolar epithelium in adults is polyanionic.[39] The anionicity of the basal lamina decreases with increasing gestational age in rats, and a shift of anionic sites takes place from the interstitial side of the basement membrane to the epithelial side of the basement membrane.[39] The basal lamina is more anionic beneath type I than type II cells. The biochemical composition of the alveolar basal lamina varies between species and as a function of development.[42] The causes and molecular details of alveolar basal lamina variation are largely unknown but are likely important in view of the substantial effects of different substrates on type II cell differentiation in vitro.[42-44]



Figure 102-6 Freeze fracture of rat lung type II cell lamellar body. The folding of the lamellae is seen at the *arrows. (From Williams MC: Ultrastructure of tubular myelin and lamellar bodies in fast-frozen adult rat lung,* Exp Lung Res *4:37, 1982.)*

BIRTH AND THE TRANSITION TO AIR BREATHING

Secretion of surfactant begins in utero, and serial analysis of amniotic fluid reflects increasing production of surfactant lipids and apoproteins, which are released into fetal alveoli (saccules) and are carried out of the lung by evolving lung liquid.

Immediately after birth, the lung must function as the organ of gas exchange, and dramatic events convert the newborn lung from fluid filled to gas filled in moments. Fluid adsorption and surfactant production are two major tasks for the alveolar, and possibly the bronchiolar, epithelium, and both must occur to achieve normal gas exchange after birth. Fluid adsorption from the fetal alveolar space begins in early labor and is augmented by adrenergic hormones.[45] Pathways of solute and fluid transport are being clarified by transporter expression studies and testing of their function in vitro (Kemp and colleagues[46] and see Chapter 82). There are few morphologic correlates of fluid transport across the alveolar epithelium, although ultrastructural studies suggest the possibility of a paracellular route through cell junctions, a transcellular route via the endocytic vesicles of type I cells, or a basolateral exit in which lung fluid is transported by alveolar epithelial cells or by Clara cells.[47]

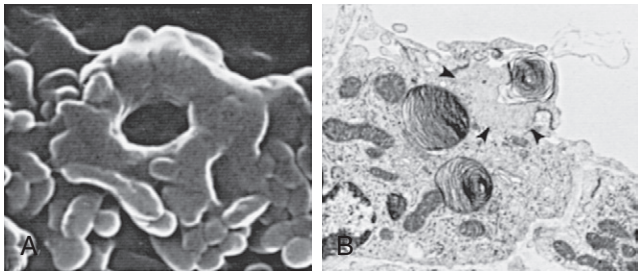


Figure 102-7 A, Scanning electron micrograph showing the plasma membrane pit, which probably corresponds to the secretory pore formed during lamellar body extrusion. **B,** Transmission electron micrograph showing a secretory event and the broad base of F-actin *(arrowheads)* beneath the secretory granule. *(From Kliewer M, Fram EK, Brody AR, Young SL: Secretion of surfactant by rat alveolar type II cells: morphometric analysis and three-dimensional reconstruction,* Exp Lung Res *9:351, 1985.)*

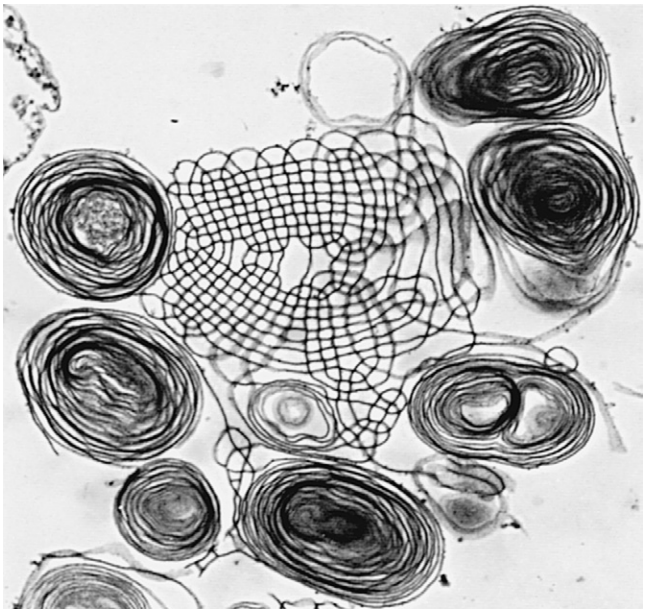


Figure 102-8 Transmission electron micrograph of lamellar bodies forming tubular myelin in the (fluid-filled) air space of a fetal rat.

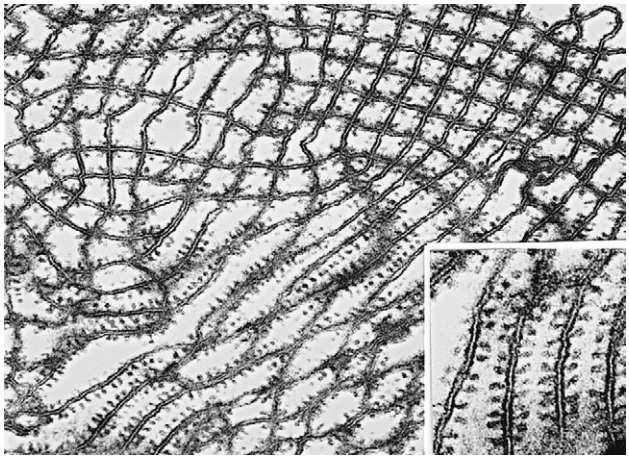


Figure 102-9 Transmission electron micrograph of tubular myelin. The regularly spaced globular profiles on short stalks that decorate the lipid bilayers are probably SP-A (*inset* shows a section parallel to the tubular structure at higher magnification). *(Courtesy C. Kuhn III, MD.)*

At term, the newborn rat lung has two times as many type II cells per gram of dry tissue as the adult lung, and each type II cell contains approximately half the adult volume of lamellar bodies. At birth, or at any time during the postnatal period in normal animals, intracellular lamellar body pools do not become depleted.[48,49] However, alveolar surfactant lipid pools increase substantially, representing accumulation of newly synthesized surfactant.[49] Thus flux through the system must be dramatically increased.[50]

Surfactant exists in several recognizable morphologic forms, although technical concerns over lipid preservation create potential artifacts that make quantitation challenging. The best-recognized form of surfactant is the intracellular lamellar body. (See also Figures 102-4 and 102-5.) The characteristic lamellae likely represent a large phospholipid bilayer sheet, draped and folded to appear almost circular in some profiles. Freeze fracture, which does not fix or extract lipids, demonstrates complete filling of the lamellar body with lamellae, suggesting that large globular proteins are not embedded in the lipid membranes within the granule (Figure 102-6). Lamellar bodies have a limiting outer bilayer membrane, the components of which are becoming better defined. The lipid-transporting adenosine triphosphate binding cassette (ABC) superfamily member ABCA3 is localized to the lamellar body outer membrane, and mutation of ABCA3 leads to fatal neonatal respiratory distress.[51] Although there are some variations, lamellar bodies contain a thin rim of granular material beneath the bounding membrane plus pools of granular material within a central core. A comprehensive view of lamellar body subcompartment biochemical composition does not yet exist, although the lamellae probably represent lipid and the core material contains protein and enzyme activities, such as acid phosphatase. The presence of acid phosphatase is consistent with the lamellar body being in the family of lysosome-related organelles. Surfactant proteins are localized to lamellar body granular cores and to type II cell cytoplasmic vesicles.[52]

THE SECRETORY EVENT

During exocytosis of lung surfactant (reviewed by Dietl and colleagues[53]), the limiting membrane of the lamellar body fuses with the apical plasma membrane of the type II cell, and a dense band of F-actin forms at the cytoplasmic face of the lamellar body being secreted[54] (Figure 102-7). Between 15 and 50 lamellar bodies are secreted per rat type II cell per hour, although the cell is capable of much faster secretion after β-adrenergic stimulation.[55,56] The freshly secreted lamellar body, stripped of its surrounding membrane, enters the acidic, calcium-rich alveolar hypophase liquid.[57] A series of morphologic transformations occurs within a few hundred milliseconds by unknown, but presumably spontaneous, mechanisms. Several lamellar bodies (from a few to at least 24) contribute closed tubular sheets of membranous material to the lung-specific structure known as tubular myelin (Figure 102-8). This unique and highly ordered array has an absolute requirement for calcium ions and is reversibly disassembled by ethylene glycol tetraacetic acid.[58] Electron micrographs of tubular myelin reveal regularly spaced small globular profiles on short stalks ("lollipops") decorating the lipid bilayers (Figure 102-9). These images are consistent with rotary

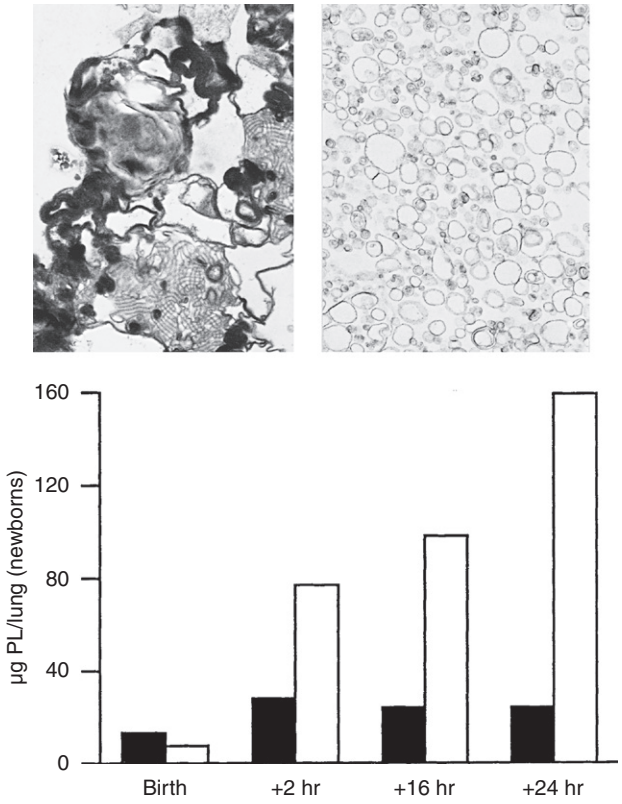


Figure 102-10 The onset of air breathing at birth produces large changes in the alveolar pool of phospholipids.The electron micrographs show the highly aggregated forms of newly secreted surfactant *(upper left panel)* and the vesicular forms that are probably spent surfactant *(upper right panel)*.The amount of phospholipid in each fraction is shown in the bar graph with an equal amount of aggregated *(dark bars)* and vesicular forms *(open bars)* at birth to a marked increase in the vesicular forms after a few hours of air breathing. *(From Spain CL, Silbajoris R, Young SL:Alterations of surfactant pools in fetal and newborn rat lungs,* Pediatr Res *21:5, 1987.)*

shadowing images of pure surfactant protein A (SP-A) oligomers,[59] and immunolocalization suggests that SP-A is localized at the "corners" of tubular myelin.[60] Reconstitution experiments also reveal lollipop decorations of phospholipid membranes when SP-A is added to phospholipid mixtures. Fully developed tubes with 90-µm intersecting membranes can be generated by addition of SP-A plus SP-B to phospholipid mixtures.[61]

ESTABLISHMENT OF THE AIR-LIQUID INTERFACE

Freeze fracture and transmission electron micrographic images are in agreement about the structure of tubular myelin, and serial section reconstructions indicate that tubular myelin collections may contain uninterrupted tubes greater than 12 µm in length.[62] Large tubular myelin aggregates are easily sedimented at low centrifugal force. Tubular myelin is an unstable form of surfactant that may be specialized to enhance lipid insertion into the alveolar interfacial film. Rapid adsorption rates of lipids from tubular myelin likely permit insertion of saturated phospholipids into the interfacial film within a single breath cycle. Lipid insertion into the surfactant film uses a mechanism involving direct contact of the tubules with the surface layer, and at least one of the apoproteins, SP-A, is dissociated from the lipid during adsorption.[63,64]

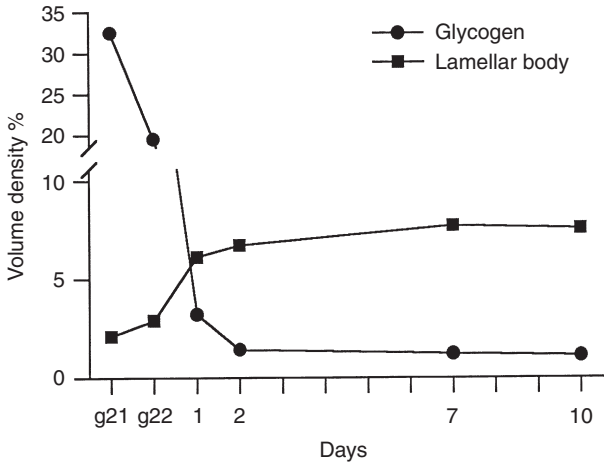


Figure 102-11 Changes in the percent composition of glycogen and lamellar bodies in type II cells. *(Modified from Massaro GD, Clerch L, Massaro D:Perinatal anatomic development of alveolar type II cells in rats,* Am J Physiol *251:R470-R475, 1986.)*

DESORPTION AND RECYCLING OF SURFACTANT

The interfacial film of surfactant is subject to high pressures (low surface tension) at low lung volumes, promoting desorption of surfactant lipid. The morphologic forms representing desorbed surfactant are poorly understood, but there is evidence that surfactant material for recycling is shaped like collapsed disks or liposomal vesicles.[33,65] Studies in adult rats demonstrated an increase in small vesicular surfactant forms in the alveolar pool after a period of large tidal volume ventilation.[66] The morphologic forms of desorbed surfactant that are taken up and recycled by type II cells are not well understood. In addition to uptake by type II cells, extracellular cycling of surfactant may include reincorporation into tubular myelin and other aggregated forms that are then reinserted in the interfacial monomolecular film.[33]

CHANGES IN ALVEOLAR SURFACTANT AT THE START OF AIR BREATHING

The in utero, fluid-filled lung has no air-liquid interface, and the initiation of air breathing at birth must be accompanied by formation and maintenance of an adequate interfacial surfactant layer. Experiments with newborn rats and rabbits demonstrated 10-fold increases in the amount of phospholipid recoverable from bronchial lavage during the first postnatal day (Figure 102-10).[49,50] The separation of lavageable phospholipids into classes by differential centrifugation showed that aggregated forms, including tubular myelin, represented a relatively stable amount of lipid but that the class of lavageable lipid in nonaggregated liposomal forms increased greatly until a stable adult ratio was achieved by the end of the first postnatal day (see Figure 102-10). These observations are consistent with the hypothesis that newly secreted surfactant is highly aggregated and that spent surfactant produced by respiratory cycling appears as small vesicles.

POSTNATAL MATURATION

At the end of normal gestation in rats, the glycogen content of maturing type II cells decreases rapidly, and lamellar body and mitochondrial volumes increase. The type II cell at birth generates a higher surfactant content per gram of dry lung weight than at any other time of life, but the type II cell is still immature, and in rats the volume of lamellar bodies nearly doubles from birth to adulthood[67] (Figure 102-11). Clara cells undergo

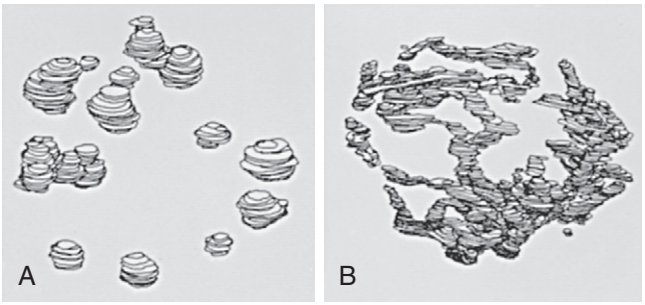


Figure 102-12 Three-dimensional reconstructions of the mitochondria of single type II cells from a rat on the day of birth **(A)** and an adult rat **(B)**. Both panels are approximately the same magnification.

a differentiation process similar to that of the type II cell but somewhat later.[68] Nuclear size, glycogen content, and granular endoplasmic reticulum content decrease, and agranular endoplasmic reticulum, mitochondria, and secretory granule content increase; most of the changes occur after birth.

An interesting postnatal change occurs in type II cell mitochondria. As noted in morphometric studies, mitochondrial volume density increases after birth, but the changing shapes and configuration of mitochondria have not been readily appreciated. Three-dimensional studies reveal dramatic conversion of type II cell mitochondria from isolated spherical organelles at gestational day 22 to a branched, interconnected morphology in adults (Figure 102-12).[37] These changes are similar to those found in mitochondria from widely divergent cell types in which transitions between metabolically quiescent and active states occur, such as exponentially growing algae[69] or housefly flight muscle during metamorphosis.[70]

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103

Seamus A. Rooney

Regulation of Surfactant-Associated Phospholipid Synthesis and Secretion

Lung surfactant is composed largely of lipids, of which phospholipids account for at least 80% of the total. Phosphatidylcholine (PC) is by far the most abundant phospholipid in surfactant and accounts for approximately 80% of its total phospholipid. At least 50% and often as much as 85% of the PC in surfactant preparations consist of the disaturated, surface-active species.[1] Phosphatidylglycerol is the second most abundant phospholipid in surfactant. It represents approximately 13% of the total phospholipid. In some situations, phosphatidylglycerol is replaced by phosphatidylinositol, and the relationship between the amounts of these two acidic phospholipids in surfactant appears to be reciprocal. The focus in this chapter is on the biosynthesis of the major surfactant phospholipids—PC and disaturated PC. Biosynthesis of the acidic phospholipids—phosphatidylglycerol and phosphatidylinositol—is also discussed. The biosynthesis of other typical membrane phospholipids found in low amounts in surfactant—phosphatidylethanolamine, sphingomyelin, phosphatidylserine, and cardiolipin—is beyond the scope of this chapter but is discussed elsewhere.[2]

Even though surfactant has a characteristic phospholipid composition, it contains no unique phospholipid component. Although present in relatively large amounts in surfactant, disaturated PC and phosphatidylglycerol are also found in nonsurfactant fractions of the lung[1] and, indeed, in other organs of the body.[3] Thus it is difficult to study the synthesis of surfactant-associated phospholipids specifically. Of course, rates of substrate incorporation into phospholipids in surfactant-enriched fractions (e.g., lamellar bodies) can be measured, but this is of little help when enzyme activities are to be determined. Studies can be carried out in isolated type II pneumocytes, which are the cellular sites of surfactant phospholipid biosynthesis, but even the type II cell synthesizes phospholipid for membranes as well as for surfactant. Therefore in most studies it has not been possible to distinguish between synthesis of membrane phospholipids and synthesis of those associated with surfactant, and the data must be interpreted accordingly. In contrast, secretion of specific surfactant-associated phospholipids can be studied relatively easily because membrane phospholipids are not secreted to any appreciable extent. Thus the lipids secreted by cultured type II cells have the typical profile of surfactant-associated phospholipids.[1] This is also true of lipids recovered in lung lavage.[1]

PATHWAYS IN THE BIOSYNTHESIS OF PHOSPHOLIPIDS AND FATTY ACIDS

The pathways in the biosynthesis of PC, phosphatidylglycerol, and phosphatidylinositol are illustrated in Figure 103-1. Phospholipid biosynthesis begins with the biosynthesis of phosphatidic acid (PA). This phospholipid occupies a central position in glycerolipid biosynthesis; it is the common lipid precursor of all other glycerophosphatides as well as acylglycerols. The glycerophosphate backbone of PA originates from the triose phosphate, dihydroxyacetone phosphate, an intermediate in glycolysis. Dihydroxyacetone phosphate is metabolized to 1-acylglycerol 3-phosphate by two pathways: acylation followed

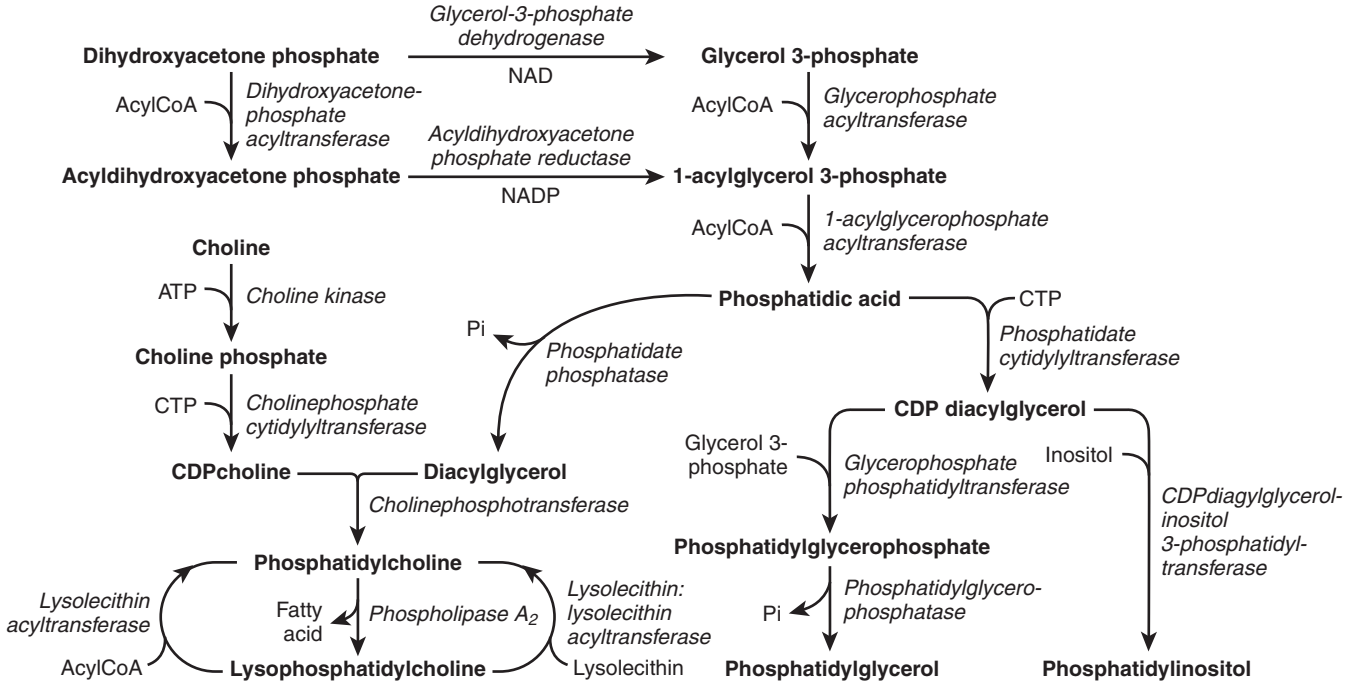


Figure 103-1 Pathways in the biosynthesis of phosphatidylcholine, phosphatidylglycerol, and phosphatidylinositol. *(From Rooney SA: The surfactant system and lung phospholipid biochemistry,* Am Rev Respir Dis *131:439, 1985.*

by reduction or reduction followed by acylation. Evidence shows that both pathways exist in the lung and are involved in the synthesis of PC and phosphatidylglycerol in type II cells.[4] PA is subsequently synthesized by further acylation of 1-acylglycerol 3-phosphate.

Numerous nonlipid precursors can be incorporated into phospholipids during the biosynthesis of PA.[1] Glucose, derived from dietary sources through the blood or from glycogen degradation in the lung, can be incorporated into phospholipids through the glycolytic pathway and dihydroxyacetone phosphate. Glycerol can be incorporated after phosphorylation to glycerol 3-phosphate by the action of glycerol kinase. Fatty acids are incorporated into phospholipids during the previously described acylation steps, as well as in subsequent reacylation reactions.

PC and the acidic phospholipids, phosphatidylglycerol and phosphatidylinositol, are synthesized from PA by different mechanisms. In the case of PC biosynthesis, PA is first dephosphorylated, and the resulting diacylglycerol (DAG) is combined with cytidine diphosphocholine (CDPcholine) to form PC. CDPcholine arises from choline after initial phosphorylation and subsequent transfer to cytidine triphosphate in reactions catalyzed by choline kinase and choline-phosphate cytidylyltransferase (CCT), respectively. Choline is essentially a vitamin in that it is not synthesized in the body but is obtained from dietary sources, often in the form of PC.[5,6]

Although disaturated PC can be synthesized by the foregoing de novo mechanism, evidence shows that it also can be synthesized by remodeling of the 1-saturated-2-unsaturated molecular species. At least half of lung and type II cell disaturated PC is synthesized by remodeling.[4,7] As shown in Figure 103-1, at least two remodeling mechanisms have been described. Both involve initial deacylation of de novo synthesized 1-saturated-2-unsaturated PC. The resulting 1-acyl-2-lysophosphatidylcholine (lysolecithin) is then either reacylated by reaction with a saturated acyl-coenzyme A or transacylated in a reaction in which two molecules of lysophosphatidylcholine react to form one molecule each of PC and glycerophosphocholine. Of the two

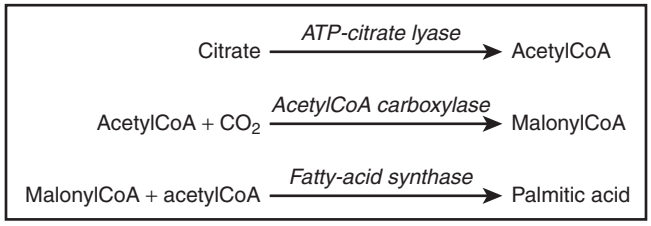


Figure 103-2 Enzymes involved in fatty acid biosynthesis.

remodeling mechanisms, only the reacylation pathway is of any quantitative importance.[4,7,8]

In the synthesis of phosphatidylglycerol and phosphatidylinositol, PA reacts with cytidine triphosphate to form CDPdiacylglycerol. This intermediate then reacts with either inositol or glycerol 3-phosphate to form phosphatidylinositol or phosphatidylglycerophosphate, respectively. The last substance does not accumulate but is immediately dephosphorylated to phosphatidylglycerol.

Fatty acids are clearly integral components of phospholipids and consequently of surfactant. Fatty acids may be synthesized de novo in the lung or supplied from extrapulmonary sources by the blood.[9,10] Although fetal and adult lungs can synthesize fatty acids de novo, the relative contributions of de novo synthesis and exogenous sources to the supply of fatty acids for surfactant synthesis are not known.[7,9] In view of the poor blood supply to the lungs during fetal life, it is likely that de novo synthesis is of quantitative importance at that stage of development. The fatty acid biosynthesis pathway is illustrated in Figure 103-2. Starting from citrate, an intermediate in the Krebs cycle, long-chain fatty acid biosynthesis is accomplished by just three enzymes: adenosine triphosphate (ATP)-citrate lyase, acetyl-coenzyme A carboxylase, and fatty acid synthase (FAS). FAS catalyzes seven distinct biochemical reactions, four of which are repeated for the addition of each two-carbon unit. In bacteria and plants, the seven reactions are carried out by separate proteins, but

TABLE 103-1

Developmental Changes in Phospholipid Content and Composition of Rabbit Lung Lavage

	PHOSPHOLIPID CONTENT (μg PHOSPHORUS/g LUNG DRY Wt)		
Gestational Age (days)	Total Phosphatidylcholine	Disaturated Phosphatidylcholine	L/S Ratio*
27	2.6	1.3	0.8
28	5.4	3.0	1.9
29	7.4	3.4	5.1
30	8.4	4.2	4.3
31	25.4	13.4	9.9
+1†	274.0	147.0	31.1
+2†	389.0	208.0	39.2
Adult	264.0	143.0	<50.0

Data from References 274-276.

L, Phosphatidylcholine (lecithin) as percentage of total phospholipid; *S,* sphingomyelin as percentage of total phospholipid.

†Newborn animals at 1 and 2 days of age.

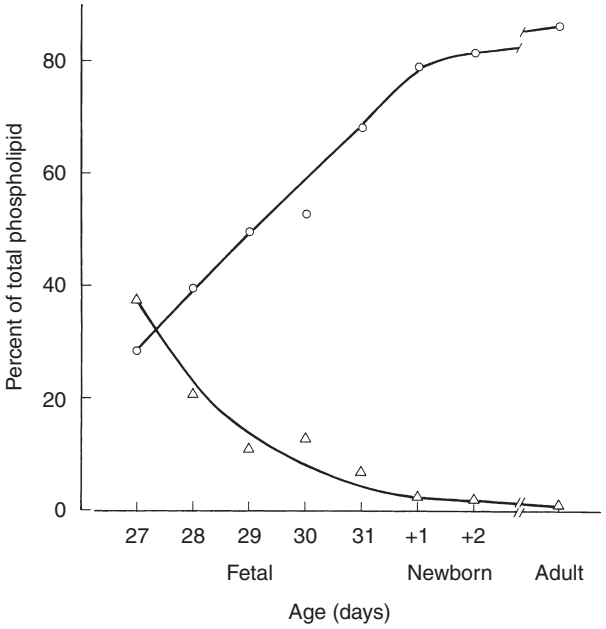


Figure 103-3 Developmental changes in the amounts of phosphatidylcholine *(circles)* and sphingomyelin *(triangles)* in rabbit lung lavage. *(From Rooney SA: In* The Surfactant System and the Neonatal Lung. *Mead Johnson Symposium on Perinatal and Developmental Medicine No. 14. Evansville, IN, Mead Johnson, 1979, pp 19-24.)*

in animals and yeasts, they are carried out by single multifunctional proteins.[11,12] The 16-carbon fatty acid, palmitic acid, is the usual product of de novo fatty acid biosynthesis in mammals. All the reactions illustrated in Figures 103-1 and 103-2 have been shown to occur in the lung. Most of the enzymes listed have been assayed in isolated type II cells.[1]

DEVELOPMENTAL AND HORMONE-INDUCED CHANGES IN LUNG PHOSPHOLIPID CONTENT AND BIOSYNTHESIS

Normal Development

Surfactant production increases toward the end of gestation as the fetus prepares for extrauterine life.[1] The amount of total and disaturated PC in lung lavage from fetal rabbits increases 10-fold between days 27 and 31 (full term) of gestation, and a further increase of similar magnitude occurs immediately after birth (Table 103-1). At the same time, the composition of the phospholipids recovered in lung lavage changes substantially. As shown in Figure 103-3, PC increases from less than 30% of the total phospholipid at 27 days gestation to approximately 70% at term and to 80% or more after birth. During this period, sphingomyelin decreases from approximately 40% of the total at 27 days gestation to less than 10% at term and to 1% to 2% after birth. As shown in Table 103-1, this results in a dramatic increase in the ratio of PC (lecithin) to sphingomyelin (L/S ratio). Because fetal lung fluid contributes to amniotic fluid, measurement of the L/S ratio in human amniotic fluid, obtained by amniocentesis, is widely used to predict the degree of fetal lung maturity and hence the optimal time for elective delivery.[13,14] A developmental increase occurs in the amount of phosphatidylglycerol in late-gestation fetal lung lavage,[15] and measurement of this phospholipid in human amniotic fluid can provide an additional assessment of the extent of fetal lung maturity.[13]

The prenatal increase in surfactant PC appears to be largely the result of increased synthesis, whereas the massive increase immediately after birth is caused by increased secretion in response to labor and ventilation (see later discussion). The rate of incorporation of radiolabeled choline into total or disaturated PC in fetal lung has been used extensively to assess the rate of synthesis of these phospholipids. A major problem in the interpretation of such data is that unless the intracellular pool sizes of choline, choline phosphate, and CDPcholine are known, rates of radiolabeled choline incorporation are not necessarily synonymous with rates of synthesis. Despite this caveat, rates of choline incorporation into PC in fetal lung have correlated well with other parameters of synthesis, such as increased mass of PC and increased enzyme activities.[8] As shown in Figure 103-4, a developmental increase in the rate of choline incorporation into PC occurs in fetal rat lung, and this continues into newborn life. Similar developmental increases have been reported in other species.[1,8] CCT is a major rateregulatory enzyme in the biosynthesis of PC,[2,16] and a developmental increase in its activity in the lung occurs either at the end of gestation or immediately after birth.[8] A developmental increase in CCT activity has also been reported in isolated type II cells,[17] and the increase in activity is accompanied by increased enzyme mass, mRNA content, and mRNA stability,[18,19] a finding indicating increased expression of the CCT gene.

A developmental increase in lung fatty acid biosynthesis occurs during late fetal life. The rate of de novo fatty acid biosynthesis can be assessed by measuring the rate of ^3H_2O incorporation into fatty acids. The problem of pool size does not arise in this situation because intracellular and extracellular water become equally labeled. A developmental increase in the rate of fatty acid synthesis in fetal rat lung has been reported using this method.[20,21] As shown in Figure 103-4, the increase occurs between days 19 and 21 of gestation and is followed by a decrease after birth. The increase in fatty acid biosynthesis coincides with the increase in PC biosynthesis (see Figure 103-4). The increase in de novo fatty acid synthesis in fetal rat lung is accompanied by a similar developmental increase in FAS activity.[22,23] The increase in FAS activity results from increased gene expression because a corresponding developmental increase occurs in the level of FAS mRNA.[24,25] A similar developmental increase in FAS expression occurs in late-gestation fetal rabbit lung.[26] In contrast to FAS, no changes or minor and inconsistent developmental increases in the activities and mRNA levels of ATP-citrate lyase and acetyl-coenzyme A carboxylase have

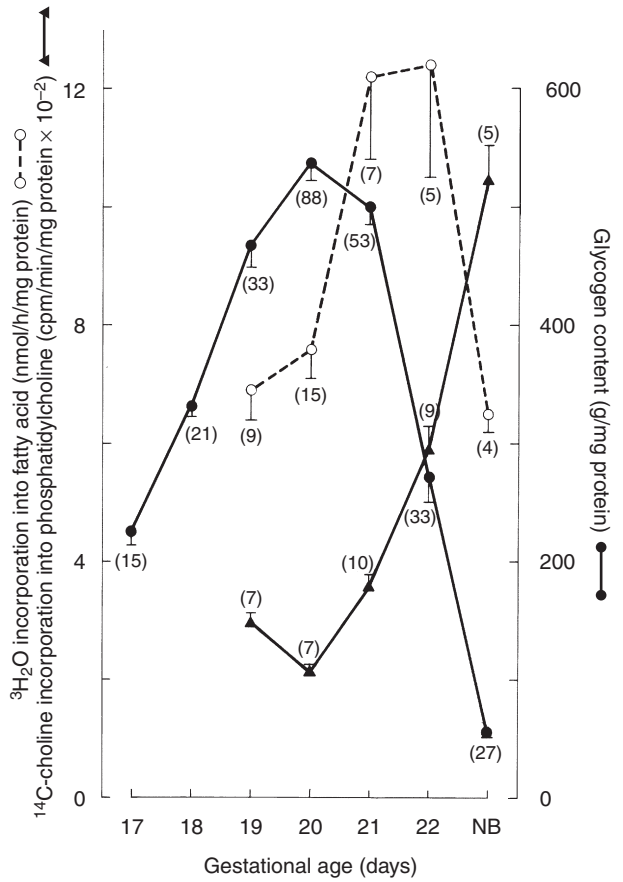


Figure 103-4 Developmental changes in glycogen content and in rates of ^3H_2O incorporation into fatty acids and of [^{14}C] choline incorporation into phosphatidylcholine in developing fetal rat lung. *(From Rooney SA, Gobran LI, Chu AJ: Thyroid hormone opposes some glucocorticoid effects on glycogen content and lipid synthesis in developing fetal rat lung,* Pediatr Res *20:545, 1986.)*

been reported.[20,22-25] Therefore the developmental increase in de novo fatty acid biosynthesis is mediated by the increase in FAS expression.

As is also shown in Figure 103-4, a developmental increase is followed by a decrease in lung glycogen content of fetal rat lung. Similar developmental changes in lung glycogen have been reported in other species.[1] Parallel developmental changes in the enzymes controlling glycogen synthesis and degradation have also been reported.[22,27,28] The temporal relationship between glycogen depletion and increased PC synthesis, which has also been reported in isolated fetal type II cells,[29] prompted speculation that glycogen provides substrate or energy for surfactant phospholipid biosynthesis in the fetal lung.[27,29,30] However, a direct relationship between glycogen depletion and increased phospholipid synthesis has not been proven. The reciprocal relationship that exists between fetal lung glycogen content and PC biosynthesis also exists between glycogen and fatty acid biosynthesis (see Figure 103-4). Glycogen is therefore just as likely to provide substrate or energy for the biosynthesis of fatty acids as for that of phospholipids.

Influence of Hormones

Lung maturation and surfactant production in the fetus can be accelerated by certain hormones and growth factors.

Glucocorticoids

In 1969, Liggins[31] reported that administration of dexamethasone to fetal lambs resulted in delivery of live animals as early as 117 to 123 days of gestation, although term is approximately 147 days. Because such premature fetuses were born alive and had partly expanded lungs, he suggested that the hormone "accelerated appearance of pulmonary surfactant, possibly as a result of premature activity of enzymes involved in a biosynthetic pathway." That the glucocorticoid did increase the amount of surfactant was soon demonstrated by deLemos and colleagues,[32] who measured lung pressure-volume relationships and the surface activity of lung extracts in the same species. Shortly thereafter, similar findings were reported in the rabbit,[33] a species in which accelerated morphologic maturation of the fetal lung was also observed.[34] Within 3 years of the initial observation, Liggins and Howie[35] reported the first successful use of glucocorticoids in the prevention of respiratory distress syndrome (RDS) in human infants. Since that time, investigation has been intense in many laboratories into the mechanism by which glucocorticoids accelerate fetal lung maturation and stimulate surfactant production.

The influence of glucocorticoids on surfactant phospholipid synthesis and the related biochemical parameters of fetal lung maturation were studied in several species. Experimental models included intact animals and explants of fetal lung in organ culture. Direct injection of fetal rabbits with cortisol or administration of betamethasone to pregnant rabbits results in an increase in the amount of surfactant phospholipid in fetal lung lavage. An increase occurs in the amount of total phospholipid and PC, an increase in PC as a percentage of the total phospholipid, and an increase in the L/S ratio.[36,37] Similarly, glucocorticoid administration in vivo increases the rate of PC biosynthesis in fetal rabbit,[37] rat,[21] mouse,[38] baboon,[39] and human[40] lung; culture of fetal rat,[41] rabbit,[42] baboon,[43] and human[44] lung explants in the presence of the hormone results in the same effect and also increases the amounts of total PC,[44,45] disaturated PC,[45,46] and phosphatidylglycerol.[45] Glucocorticoids also stimulate fatty acid biosynthesis in fetal lung. They increase the rate of de novo fatty acid biosynthesis in fetal rat,[21] rabbit,[47] and human[48] lung. In addition the hormone has been reported to accelerate the glycogen developmental profile (see Figure 103-4) in fetal lung. Thus glucocorticoids increase lung glycogen content at the time of the normal developmental increase in this parameter[21] and decrease it later in gestation at the time of the normal developmental decrease.[21,37,38]

Despite the early prediction by Liggins[31] that glucocorticoids accelerate enzyme activity, initially considerable controversy and confusion occurred with respect to the effects of glucocorticoids on enzymes of lipid biosynthesis in the fetal lung. Eventually it was established that the hormones increase the activity of two lipogenic enzymes in fetal lung in late gestation-CCT and FAS. CCT activity increases in fetal rabbit,[36,37,49] rat,[46,50,51] and mouse[38] lung tissue and in fetal rat type II cells[52] after injection of the mother with glucocorticoids. It also increases in fetal rabbit,[53] rat,[54] and human[55] lung explants treated with glucocorticoids in culture. Similarly, FAS activity increases in fetal rat lung tissue[22] and in isolated type II cells[56] after maternal injection with dexamethasone and in response to the same hormone in cultured explants of fetal rat[25] and human[57] lung. However, unlike in rats and humans, it has been reported that betamethasone does not increase FAS activity in fetal lamb lung.[58] Whether that is the result of a species difference or a function of gestational age needs to be investigated. Glucocorticoids do not increase the activities of ATP-citrate lyase[25] or acetyl-coenzyme A carboxylase.[22] Thus, as is the case in normal development, the glucocorticoid-induced increase in fetal lung fatty acid biosynthesis is entirely the result of increased FAS activity.

The situation with respect to other enzymes of phospholipid synthesis is less clear. Fetal lung phosphatidate phosphatase activity has been reported to increase with glucocorticoid administration in the rabbit and mouse but not in the rat.[8,59] Glucocorticoids were also shown to increase cholinephosphotransferase activity in a few studies, but it was not a consistent finding.[8,59] Similarly, lysolecithin acyltransferase, lysolecithin:lysolecithin acyltransferase, and glycerophosphate phosphatidyltransferase activities have been reported to increase in some studies, but not in others.[59]

The finding that glucocorticoid effects occur in cultured explants indicates that the hormone acts directly on the lung. Glucocorticoid receptors have been demonstrated in fetal and adult lung,[60] and data indicate that the effects of glucocorticoids on PC synthesis and CCT and FAS activities are directly mediated by the receptor. Stimulation of PC synthesis in the rat,[61] rabbit,[42] and human[44] and of CCT[54,55] and FAS[48,62] activities in the rat and human depends on glucocorticoid concentration. The EC_{50} values (concentration at which 50% of the maximal stimulation is achieved) are of the same order of magnitude,[42,44,54,63] or even lower,[62] than the dissociation constant (K_d) values for specific binding of the glucocorticoid to the nuclear receptor. Furthermore, the order of potency of various steroids in producing the previously described effects is the same as that of their binding to the receptor.[42,54,62] Glucocorticoid stimulation of PC synthesis[42] and of CCT[64] and FAS[62,64] activities in fetal lung explants is not apparent until after exposure to the hormone for approximately 12 hours. Maximal stimulation is achieved in 20 to 36 hours. This time course is consistent with induction of new protein synthesis. The stimulatory effects of the hormone are antagonized by actinomycin D[42,54,62] and cycloheximide,[42,65] and this finding again shows that the action of the hormone involves increased transcription and induction of new protein synthesis.

Glucocorticoid stimulation of CCT activity (a direct effect on the lung) is clearly receptor mediated and dependent on mRNA and protein synthesis. However, quantification by immunotitration[66] and Western blotting[51] revealed that the amount of the CCT protein is not increased by the hormone. Similarly glucocorticoids have no effect on CCT mRNA content,[19] although small increases have been reported in some studies.[43,67] As discussed in detail later, the preponderance of the evidence suggests that the effect of the hormone results from the activation of existing enzyme, rather than increased expression of the CCT gene.

Conversely the glucocorticoid-induced increase in FAS activity in fetal lung is clearly the result of increased gene expression. The increase in activity is accompanied by an increase in the amounts of FAS protein[56,57,62,68] and mRNA[25,56,57,69] in the rat and human, whereas lung FAS mRNA content is decreased in glucocorticoid-insufficient fetal mice.[70] The glucocorticoid-induced increase in FAS mRNA results from both increased transcription[56,57,68] and mRNA stability.[57,71] Increased transcription is the predominant mechanism in the rat, whereas increased mRNA stabilization predominates in the human.[57,68,71] Glucocorticoid-induced transcription of the FAS gene has been reported to be mediated by an 89–base pair fragment in the promoter region.[72] It is therefore clear that the increase in FAS activity in fetal lung is the result of increased expression of the FAS gene. FAS is the only enzyme involved in lipid synthesis that is induced by glucocorticoids in fetal lung. Consistent with a role for FAS in surfactant production, the glucocorticoid-induced increase in its expression is more pronounced in type II cells than in other lung cells.[56,57] As discussed later, evidence exists that increased FAS activity accounts for the stimulatory effects of glucocorticoids on PC synthesis and CCT activity.

Although maternal injection with glucocorticoid results in increased CCT[52] and FAS[56] activities in fetal type II cells (the site of surfactant synthesis within the lung), this action of the hormone is not the result of a direct effect on the type II cell. Adult type II cells contain glucocorticoid receptors,[73] and glucocorticoids have stimulatory effects on some parameters in type II cells,[74-76] including stimulation of surfactant secretion (see later discussion). However, glucocorticoids have little effect on parameters of PC synthesis when either adult[77] or fetal[67,78] type II cells are cultured with the hormone. Data indicate that the effects of glucocorticoids on surfactant lipid synthesis are mediated by mesenchymal factors. The maximum effect of cortisol on FAS expression in fetal rat type II cells requires a fibroblast-conditioned medium.[67] In early studies,[79] a factor released from fibroblasts, fibroblast-pneumonocyte factor, was proposed to mediate the effect of glucocorticoids, but fibroblast-pneumonocyte factor has never been isolated or characterized. However, other factors are known to be released from fibroblasts, and one or more of those may be the active ingredient in a fibroblast-conditioned medium. Indeed, keratinocyte growth factor (KGF), also called fibroblast growth factor-7, stimulates surfactant phospholipid synthesis, CCT activity, and FAS expression in fetal rat type II cells.[80] Administration of KGF in vivo also increases the amount of disaturated PC in the lungs of preterm rabbits.[81] Dexamethasone increases KGF expression in fetal lung fibroblasts, and an anti-KGF antibody blocks the glucocorticoid-induced increase in PC synthesis in fetal rat type II cells cultured with fibroblast-conditioned medium.[82] Although involvement of other factors is also possible,[82] such data suggest that KGF may have a major role in mediating the effects of glucocorticoids on stimulation of surfactant synthesis.

Thyroid Hormone

Thyroid hormone has been reported to accelerate surfactant phospholipid production in several species. Direct injection of fetal rabbits with thyroxine (T_4) results in accelerated morphologic lung maturation, as well as increased amounts of surfactant in lung lavage.[8,83] T_4 also increases the L/S ratio in fetal lamb tracheal fluid.[84] Administration of triiodothyronine (T_3) to pregnant rats results in increased biosynthesis of total and disaturated PC as well as increased CCT activity in the fetal lung.[46] The effect of thyroid hormone is a direct effect on the lung because both PC synthesis and CCT activity are increased in fetal rat lung explants exposed to T_3 or T_4 in culture.[41,46,61] T_3 also increases PC synthesis in explants of fetal rabbit[85] and human[44] lung. Fetal lungs of genetically hypothyroid mice are less mature and contain less surfactant than normal.[86,87]

Thyroid hormone receptors are present in adult and fetal lung and in type II cells.[88] Half-maximal stimulation of PC synthesis in fetal rabbit and human lung explants is achieved at a concentration of T_3 that is almost identical to the Kd for binding of the hormone to the nuclear receptor.[44, 85] The relative potencies of various thyroid hormone analogues in stimulating PC biosynthesis and in binding to the receptor are also the same,[44,85] and the stimulatory effect of T_3 is abolished by actinomycin D and cycloheximide.[85] These data indicate that the stimulatory effect of thyroid hormone in fetal lung is receptor mediated and involves increased gene expression. The gene or genes induced by thyroid hormone remain to be identified.

Several of the stimulatory effects of thyroid hormone in fetal lung are similar to those of glucocorticoids. However, the effects of the two hormones on certain parameters of surfactant phospholipid synthesis have been reported to be additive,[44,61,85,89] a finding suggesting that glucocorticoids and thyroid hormone act through different mechanisms. Furthermore, these two hormone types have opposite effects on other developmental parameters in fetal lung. Thyroid hormone and glucocorticoids both accelerate the normal developmental decrease in fetal lung glycogen content; however, the normal developmental increase in glycogen (which occurs earlier in gestation [see Figure 103-4]) is accelerated by glucocorticoids

but is prevented by thyroid hormone in the fetal rat.[21] Furthermore the glucocorticoid-accelerated increase is antagonized by thyroid hormone.[21] Similarly, although dexamethasone accelerates the normal developmental increase in de novo fatty acid biosynthesis in fetal rat lung, both the normal increase and the stimulatory effect of the glucocorticoid on this parameter are antagonized by thyroid hormone.[21] T_3 does not stimulate de novo fatty acid biosynthesis or FAS activity in fetal rat or human lung explants.[22,48,62] Furthermore it diminishes the stimulatory effect of glucocorticoid on FAS activity in fetal rat[22,62] but not human[48] lung.

A major difficulty in studying the effects of thyroid hormone on the fetal lung in vivo is that, in most species, T_4 does not easily cross the placenta. In early studies, therefore, T_4 was administered directly to the fetus.[83] However, directly injecting the fetus, even with saline, causes stress, which itself stimulates surfactant production,[36] so in that model some effects of thyroid hormone may have been masked. Administration of a relatively high dose of T_3 to pregnant rats results in elevated levels of the hormone in the fetal serum, and this model has been employed to examine effects of thyroid hormone on surfactant synthesis in vivo.[46] In the same in vivo model, antagonism between the effects of glucocorticoids and thyroid hormone on fatty acid biosynthesis, FAS activity, and glycogen accumulation has been observed[21,22] In contrast, although the stimulatory effect of T_3 on PC synthesis in cultured fetal lung[44,61,85] occurs at a concentration consistent with receptor mediation, the inhibitory effect of thyroid hormone on FAS in the same model requires a much higher hormone concentration.[62] The physiologic relevance of the inhibitory effects of thyroid hormone is therefore not clear.

Thyrotropin-Releasing Hormone

In an effort to overcome the problem of poor placental transfer of thyroid hormone, thyrotropin-releasing hormone, a tripeptide that readily crosses the placenta, was administered to pregnant rabbits and was found to increase the amount of surfactant phospholipid in lung lavage from the fetuses.[90] Thyrotropin-releasing hormone has been reported to have a similar effect in the fetal lamb,[91] and it has also been noted to accelerate functional and morphologic lung maturation in the fetal rabbit.[92] A combination of thyrotropin-releasing hormone and cortisol is more effective than either hormone alone in the fetal rabbit[93] and lamb.[91,94] However, a combination of thyrotropin-releasing hormone and betamethasone is no more effective than betamethasone alone in prevention of RDS in premature human newborns[95-97] (see Chapter 108). Although data are contradictory on its effect on PC biosynthesis,[43,90] thyrotropin-releasing hormone was reported to increase CCT mRNA content markedly in fetal baboon lung explants.[43]

Estrogen

Clinical data suggest that low estrogen levels are associated with an increase in the incidence of RDS,[8,98,99] and in one early study, administration of estrogen to pregnant women resulted in an increase in the amniotic fluid L/S ratio.[100] In that study, estrogen was just as effective as dexamethasone.[100] In animal studies, estrogen has been reported to stimulate surfactant production in the late-gestation fetal rabbit[101-104] and rat[105,106] but not in the lamb.[107] The most dramatic changes are found in the fetal rabbit when the hormone is administered to the pregnant doe. In this model, the hormone increases the amount of surfactant phospholipid in lung lavage[101] and the rate of PC synthesis[102,104] and CCT activity in the lung.[102,104,108] In addition, it decreases lung glycogen content.[53,103] in vivo administration of estrogen also accelerates morphologic maturation of the fetal rabbit[103] and rat[106] lung.

Estrogen increases PC synthesis[53,105,109] and CCT activity[53] and decreases glycogen[105] in organ cultures of fetal rat and rabbit lung. However, the effects in organ culture are less pronounced than those occurring in vivo, they are apparent only at relatively high hormone concentrations,[53,105] and they do not appear to be mediated by an estrogen receptor.[53] It is therefore not clear that the stimulatory effects of estrogen occur directly on the lung. Thus, although estrogen clearly stimulates fetal lung surfactant synthesis, particularly in the rabbit, the mechanism by which this occurs and its physiologic significance are not known.

Prolactin

A developmental increase occurs in blood prolactin levels, and infants with RDS have lower levels of this hormone than controls at the same gestational age.[8,110] Although a temporal relationship does not prove cause and effect, such clinical observations led to the hypothesis that prolactin has a role in fetal lung maturation and surfactant production. Support for this theory comes from the observation that administration of prolactin to fetal rabbits led to an increase in the amounts of total phospholipid, total PC, and disaturated PC in fetal lung tissue.[111] Lung lavage was not examined, so surfactant-associated phospholipids were not specifically measured.[111] In other studies, prolactin had no effect on lung PC content or synthesis in the fetal rabbit[112] or on surfactant content in the fetal rabbit[113] or lamb.[112] The lung has prolactin receptors,[114,115] and prolactin was reported to stimulate PC synthesis in cultured explants of fetal rat[116] but not fetal rabbit[117] or human[118] lung. In the rat and human systems, it enhanced the stimulatory effect of glucocorticoids.[116,118] In view of these conflicting data in both in vivo and cultured lung systems, a role for prolactin in regulation of surfactant production has not been established.

Other Hormones and Growth Factors

In addition to KGF,[80-82] other hormones and growth factors that have been reported to stimulate surfactant phospholipid production in fetal lung include corticotropin,[8] corticotropin-releasing hormone,[43] epidermal growth factor,[119,120] parathyroid hormone,[121] leptin,[122] insulin-like growth factor,[123] and gastrin-releasing peptide.[123] Platelet-activating factor has been reported to decrease lung glycogen in the fetal rabbit in vivo,[124] and a temporal relationship exists between the decrease in glycogen content and increased platelet-activating factor concentration in cultured human fetal lung explants.[125] However, a role for platelet-activating factor in regulation of surfactant phospholipid synthesis has not been established. Cyclic adenosine monophosphate (cAMP), which is clearly involved in regulation of surfactant secretion (see later discussion), may also have a regulatory role in its synthesis.[8] cAMP increases FAS expression in human[126] but not in rat[68] fetal lung explants. Vitamin D was reported to stimulate surfactant synthesis in fetal rat type II cells[127] but it did not increase FAS expression.[68]

Numerous agents have been reported to inhibit surfactant phospholipid synthesis in the late-gestation fetal lung. Infants of mothers with diabetes are particularly prone to RDS.[128] In animal models, fetuses of rats and rabbits made diabetic by administration of streptozotocin and alloxan exhibit delayed lung maturation and decreased surfactant, as determined by several criteria.[129-132] However, although the infants of mothers with diabetes are hyperinsulinemic and hyperglycemic, in the animal models the fetuses generally have high glucose levels, but insulin levels are often normal. It is therefore not clear whether the delayed lung maturation is the result of the increased insulin or the high glucose concentration. Infusion of insulin into fetal rhesus monkeys leads to hyperinsulinemia without elevated glucose levels, but no evidence of delayed lung maturation in such animals is available.[133] Conversely hyperinsulinemia with hypoglycemia in the fetal rat[134] and lamb[135] does reduce surfactant production. In cultured fetal

lung systems, insulin does not decrease PC synthesis,[136,137] but it diminishes the stimulatory effect of glucocorticoids on both PC synthesis[137] and CCT activity.[138] A high glucose concentration has also been reported to decrease surfactant production and morphologic maturation in cultured fetal rat lung explants.[139,140]

It is known that premature male infants are more prone to RDS than female infants.[141] The lungs of female fetuses tend to be approximately 1 week more mature than those of males as determined by the amniotic fluid L/S ratio and disaturated PC content.[142] Evidence is available of this female advantage in fetal lung maturation and surfactant synthesis in rats[143-145] and, to a lesser extent, in rabbits,[146,147] but not in monkeys.[148,149] This maturational difference may result from inhibition of surfactant production in male fetuses by androgens.[150,151] More recent studies suggest that the androgen effect is caused by enhancement of lung morphogenesis and branching earlier in gestation,[152] and it may be mediated by changes in the relative amounts of epidermal growth factor and transforming growth factor-β receptors in fibroblasts.[153]

Transforming growth factor-β_1 was reported to antagonize the dexamethasone-induced increase in FAS expression in human fetal lung explants,[154] an effect that appears to be mediated at the transcription level.[72] Retinoic acid was reported to antagonize the stimulatory effect of glucocorticoids on FAS expression in fetal rat lung explants, but retinoic acid on its own had no effect.[68] In contrast, retinoic acid was reported to increase the amount of fetal lung surfactant phospholipid and rate of PC synthesis.[155] Vitamin A–deficient fetal rats were reported to have a reduced amount of surfactant phospholipid, an effect accompanied by decreased FAS expression but normal CCT activity.[156]

MECHANISM OF REGULATION OF FETAL LUNG PHOSPHATIDYLCHOLINE BIOSYNTHESIS

Numerous studies in the developing fetal lung, in isolated type II cells, and in other organs established that CCT is a major rate-regulatory enzyme in de novo PC biosynthesis.[2,4,16] As shown in Figure 103-1, CCT catalyzes the conversion of choline phosphate to CDPcholine. The size of the choline phosphate pool is larger than that of choline or CDPcholine in both whole lung and type II cells.[4] As discussed earlier, a developmental increase in CCT activity occurs in association with increased PC biosynthesis in the developing lung. Glucocorticoids, thyroid hormone, and estrogen, which stimulate PC synthesis and increase PC content in fetal lung, also increase CCT activity.

The developmental increase in fetal lung CCT activity is largely the result of increased expression of the CCT gene,[18,19] although activation of existing enzyme may also be involved.[157,158] In contrast, although the stimulatory effect of glucocorticoids on fetal lung CCT activity is dependent on new protein and mRNA synthesis, CCT is not the protein that is induced by the hormone; CCT enzyme mass[51,66] and mRNA content[19] are not increased by glucocorticoids in the fetal lung. The effect of glucocorticoids on CCT activity is caused by activation of existing enzyme, rather than by synthesis of new CCT. How is CCT activated?

CCT belongs to a group of proteins called amphitropic[159] or ambiquitous,[160] the activities of which are regulated by interconversion between a soluble and a membrane-bound form. Considerable evidence shows that CCT activity is regulated by reversible membrane association; the membrane-bound form is active, whereas the soluble form is inactive and requires lipid for activation.[2,161] The curvature and other physical properties of the membrane are critical in the interaction with CCT.[162,163] Physiologic activation of CCT in many systems is thought to involve translocation of the enzyme from the cytosol to membranes.[164] Translocation from cytosol to microsomes has been reported in association with increased PC synthesis in choline-depleted type II cells.[165] However, translocation is not always involved in CCT activation because activation without translocation has been reported.[2,166] Little evidence is available that translocation has a role in hormonal stimulation of CCT activity in the fetal lung. The stimulatory effects of dexamethasone on CCT in the fetal rat[54] and of estrogen in the fetal rabbit[108] are clearly not the result of translocation. The bulk of the enzyme is in the cytosolic fraction, and the subcellular distribution is not altered by the hormones. Estrogen increases only the CCT activity in the cytosol and has no effect on activities in the microsomal or mitochondrial fractions.[108] Similarly dexamethasone does not alter the subcellular distribution of CCT in fetal lung type II cells, although in that study, most of the activity was in the microsomal fraction.[52]

It is clear, however, that hormonal activation of CCT is mediated by endogenous lipids. Fetal lung CCT activity can be increased several-fold by inclusion of fatty acids,[167] as well as phospholipids,[108,168,169] in the assay mixture, and when the enzyme is assayed in the presence of sufficient lipid to achieve maximal activation in vitro, the stimulatory effects of glucocorticoids,[46,51,54,64,66] T_3,[46] and estrogen[102,104,108] are considerably reduced or completely abolished. Because the lipids would be expected to activate any newly synthesized CCT just as much as existing enzyme, these data are consistent with the concept that it is the catalytic activity, rather than the amount of CCT, that is increased by the hormones. CCT activity in fetal lung cytosol is markedly decreased by extraction of lipids with solvents that do not cause protein denaturation.[51,108,157] Lipid extraction under the same conditions completely abolishes the stimulatory effects of betamethasone in the rat[51] and of estrogen in the rabbit.[108] CCT activity and the stimulatory effects of the hormones are fully restored on re-addition of the appropriate lipid extract.[51,108] Furthermore the stimulatory effects of the hormones can be switched from the treated to the control groups by interchanging the added lipids.[51,108] Such data establish that the stimulatory effects of those hormones are mediated by a lipid factor. Indeed betamethasone elevates the levels of some phospholipids and fatty acids in fetal rat lung.[51] Estrogen also increases phospholipid levels in fetal rabbit lung, but its effect on fatty acids has not been examined.[108]

As discussed earlier, glucocorticoids enhance fatty acid biosynthesis and increase expression of the FAS gene in late-gestation fetal lung. Indeed FAS is the only gene for a lipogenic enzyme that is known to be induced by glucocorticoid in fetal lung. Inhibition of fatty biosynthesis in fetal rat lung explants completely abolishes the stimulatory effect of dexamethasone on CCT activity.[64] This finding suggests that the stimulatory effect of glucocorticoids on CCT activity in fetal lung is mediated by induction of the FAS gene. Whether FAS induction also accounts for the estrogen- and thyroid hormone–induced increases in CCT activity is a question that remains to be investigated.

It is not clear at present whether the glucocorticoid effect on CCT activity is mediated by free fatty acids, by the lipids into which they become incorporated, or possibly by other fatty acid metabolites. The observations that linoleic acid was the fatty acid that was increased to the greatest extent by betamethasone in fetal rat lung and the same fatty acid was the most effective in activating CCT in vitro led to the suggestion that glucocorticoid stimulation of fetal lung CCT activity is mediated by linoleic acid.[170] However, linoleic acid is an essential fatty acid that is not synthesized by mammals. It is consequently difficult to reconcile the notion that glucocorticoid activation of CCT is mediated by induction of FAS with the concept that it is also mediated by linoleic acid. In summary, although evidence is compelling that the stimulatory effect of glucocorticoids on CCT activity results from increased FAS expression and

is mediated by endogenous lipids, the precise identity of the activator(s) remains to be established.

Three CCT isoforms exist: α, β_1, and β_2. The β isoforms are splice variants of the same gene, but CCTα is encoded by a different gene.[166,171] Some sequence differences exist among the three isoforms, but the catalytic and membrane-binding domains are highly conserved.[166] All three isoforms are enzymatically active and are regulated by reversible membrane association.[166] The membrane-bound form of CCTα has been reported to be largely associated with the nucleus in some systems,[2,16,166] but it is also found in association with endoplasmic reticulum,[171] especially in the lung, where it is entirely extranuclear.[172,173] In contrast, CCTβ_1 and CCTβ_2 are associated with the endoplasmic reticulum.[16,171] CCTα is widely expressed,[171] and it is the most extensively investigated of the three isoforms. The three CCT isomers are expressed in fetal lung, but only CCTα is expressed in adults.[171] Therefore an interesting question that has not yet been addressed is whether one or both of the CCTβ isoforms may have a role in regulation of PC synthesis during fetal lung development.

Although the glucocorticoid-induced increase in CCT activity appears to be primarily the result of enzyme activation, the possibility of increased synthesis cannot be discounted entirely. In contrast to the effect of estrogen in fetal rabbit lung, in which stimulation of CCT is completely abolished by inclusion of phosphatidylglycerol in the in vitro assay mixture,[102,104,108] glucocorticoid stimulation of CCT activity in rat and mouse lung is not completely abolished by inclusion of activating lipids.* Indeed, although markedly reduced, the stimulatory effect of dexamethasone remained statistically significant even in the presence of phosphatidylglycerol.[38,54] In human lung explants, increased CCT activity in response to dexamethasone was even more pronounced and became statistically significant only when phosphatidylglycerol was included in the assay mixture.[55] That the stimulatory effect of the hormone was apparent when the enzyme was maximally activated in vitro suggests an increase in enzyme mass. As discussed earlier, CCT mRNA was reported to be increased in some studies,[43,67] although that was not a consistent finding.[19]

Apart from CCT, it is possible that other enzymes have regulatory roles in phospholipid biosynthesis, at least under some circumstances. In one study,[174] mice overexpressing CCT in the lungs had only modest increases in PC synthesis and content, a finding suggesting that other steps in the PC biosynthetic pathway may also be important in its regulation. As discussed earlier, activities of fetal lung phosphatidate phosphatase, cholinephosphotransferase, lysolecithin acyltransferase, lysolecithin:lysolecithin acyltransferase, and glycerophosphate phosphatidyltransferase have been reported to be increased in some studies either during normal development or after hormone treatment. Data suggest that glycerophosphate acyltransferase, an enzyme catalyzing an early step in glycerophospholipid synthesis (see Figure 103-1), has a regulatory role in type II cells isolated from adult rats[175] and rabbits.[176] Evidence also exists that choline kinase and phosphatidate phosphatase are regulatory enzymes in other organs.[2] However, rate-regulatory roles for these and other enzymes have not been established in the fetal lung.

SECRETION OF SURFACTANT-ASSOCIATED PHOSPHOLIPIDS

Lamellar inclusion bodies are the secretory organelles of the type II pneumocyte. The phospholipid composition of isolated lamellar bodies is virtually identical to that of surfactant.[1] After their biosynthesis, surfactant phospholipids are stored in lamellar bodies and are finally secreted into the alveolar lumen by the process of regulated exocytosis.[177-181] A lamellar body in the process of exocytosis is shown in Figure 103-5. It is possible that there is also some constitutive secretion of surfactant lipids; the basal secretion of phospholipids that is observed in isolated type II cells cultured without secretagogues may well be a constitutive process. However, secretion of surfactant appears to be a slow process, with little difference between regulated and constitutive mechanisms.[182]

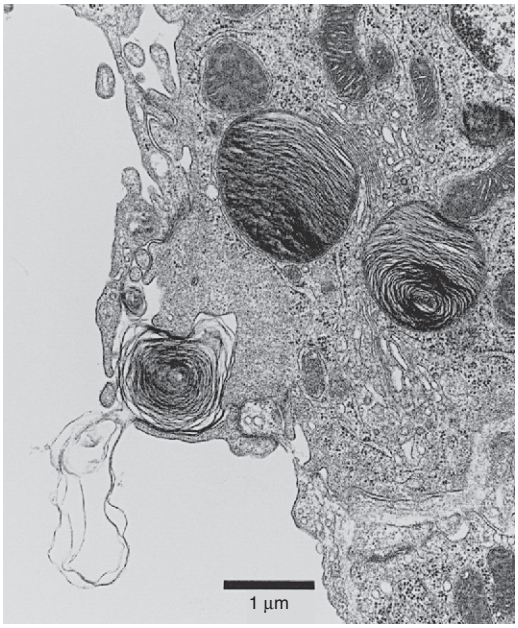


Figure 103-5 Exocytosis of a lamellar body by an adult rat type II cell. This electron micrograph was generated by Dr. Stephen L. Young at Duke University. *(From Rooney SA, Young SL, Mendelson CR: Molecular and cellular processing of lung surfactant,* FASEB J *8:957, 1994.)*

Surfactant secretion has been measured in several systems ranging from intact animals in vivo to isolated type II cells in culture.[177-180] Isolated type II cells have been the model of choice for most studies on the regulation of surfactant secretion. Most such studies have been carried out on type II cells isolated from adult animals, but surfactant secretion in fetal and newborn type II cells[76,183-185] has also been investigated. In most studies, secretion of surfactant phospholipid has been assessed biochemically by labeling the intracellular PC pool with [^{3}H] choline during overnight culture of the cells and then measuring the amount of [^{3}H]PC or disaturated PC released into the medium. However, cell physiologic and fluorescent techniques have been used to study surfactant secretion and membrane fusion kinetics in single type II cells.[182,186-189]

Various physiologic and pharmacologic agents stimulate surfactant secretion in isolated type II cells, and some agents inhibit it.[177-180] Well-established surfactant secretagogues in cultured type II cells include the following: β-adrenergic, adenosine A_{2B}, and $P2Y_2$ receptor agonists; agents such as tetradecanoylphorbol acetate (TPA); cell-permeable DAGs; forskolin and cholera toxin, which bypass receptors and activate downstream signaling steps; and the ionophores ionomycin and A23187, which promote calcium (Ca^{2+}) influx into the cell (Figure 103-6).

A working model of the signal-transduction mechanisms that mediate surfactant secretion is shown in Figure 103-6. The signaling mechanisms consist of three distinct pathways, although overlap and interactions exist among them.[177,190] The first mechanism involves activation of adenylate cyclase

*References 38, 46, 51, 54, 64, 66

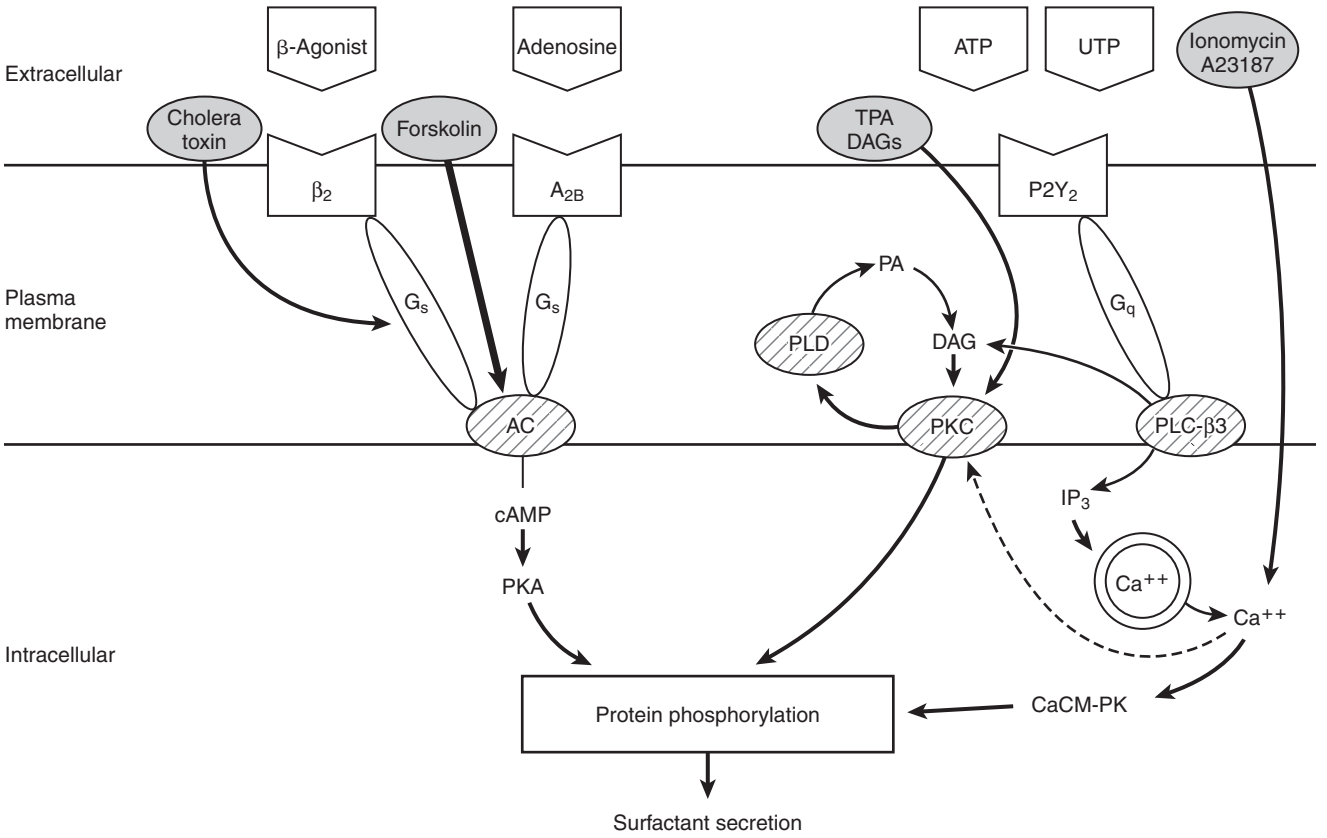


Figure 103-6 Schematic representation of signal transduction mechanisms mediating surfactant phospholipid secretion in type II cells. See text for explanation and abbreviations. *(Modified from Rooney SA, Young SL, Mendelson CR: Molecular and cellular processing of lung surfactant,* FASEB J *8:957, 1994.)*

(AC), generation of cAMP and subsequent activation of cAMP-dependent protein kinase (protein kinase A). This pathway is activated by agonists such as terbutaline and the adenosine analogue *N*-ethylcarboxyamidoadenosine that bind to β-adrenergic and adenosine A_{2B} receptors, respectively, both of which are coupled to AC through the G-protein G_s. The AC pathway is also activated by cholera toxin, which permanently activates G_s, and by forskolin, which directly activates AC.

The second mechanism involves direct or indirect activation of protein kinase C (PKC). TPA and cell-permeable DAGs directly activate PKC. ATP and uridine triphosphate (UTP) activate PKC by binding to $P2Y_2$ receptors that are coupled to phospholipase C-β_3 (PLC-β_3) through G_q. Activation of PLC-β_3 results in hydrolysis of phosphatidylinositol bisphosphate and formation of DAG, which then activates PKC. PKC, in turn, activates phospholipase D (PLD),[191] which hydrolyzes PC to form choline and PA. Phosphatidate phosphatase converts PA to DAG (see Figure 103-1), which further activates PKC. The PLD loop may provide a mechanism whereby surfactant secretion is sustained for a prolonged period. PA itself may also have a signaling role,[192] although that possibility has not been investigated in the type II cell. In addition to acting through $P2Y_2$ receptors, ATP also stimulates surfactant secretion by the cAMP pathway; ATP, but not UTP, promotes cAMP formation in the type II cell.[193] This effect of ATP is not the result of direct action at the adenosine A2B receptor.[177] Whether it is caused by catabolism to adenosine with consequent activation of A_{2B} receptors[194] or by activation of an unidentified AC-coupled receptor[177,195] has not been established.

The third mechanism involves elevation of intracellular Ca^{2+} levels. This can be accomplished by increasing the level of inositol triphosphate, the other product of the action of PLC-β_3; inositol triphosphate promotes mobilization of Ca^{2+} from intracellular stores.[196] It can also be accomplished by ionophores that promote Ca^{2+} influx into the cell from the medium. Ca^{2+} activates a Ca^{2+}/calmodulin-dependent protein kinase (CaCM-PK) and may also act synergistically with DAG to activate some PKC isoforms. Protein phosphorylation by protein kinase A, PKC, or CaCM-PK ultimately leads to surfactant secretion.

Secretion is an indispensable step in the overall homeostasis of surfactant.[179,197] Failure of secretion would likely lead to a drastic deficiency in surfactant. Because severe surfactant deficiency is probably incompatible with life, it is likely that there is sufficient redundancy so a fatal defect in surfactant secretion does not occur. This would account for the number of potential physiologic agonists that can stimulate surfactant secretion and would also explain the multiple signaling mechanisms.

There is a developmental increase in agonist-induced surfactant secretion in fetal and neonatal rat Type II cells.[183,185] The response to the $P2Y_2$ agonist UTP is negligible in early newborn cells, but it increases with age,[185] and the response to ATP, terbutaline, *N*-ethylcarboxyamidoadenosine, and TPA also increases with age.[183] Dexamethasone significantly increases the response to terbutaline, *N*-ethylcarboxyamidoadenosine, ATP, and UTP in early newborn rat type II cells while causing a small decrease in the basal rate of surfactant secretion.[76] This effect is mediated by the glucocorticoid receptor and is probably the result of induction of a signaling step downstream of the receptors that is common to the different signaling pathways.[76] Thus, in addition to stimulation of surfactant synthesis, regulation of its secretion may be an additional function of glucocorticoids in the developing lung.

There are many subtypes and isomers of the receptors and other signaling proteins shown in Figure 103-6, and there is increasing information on the identity of those involved in surfactant secretion. Of the three β-receptors, β_3 is not expressed in the lung,[198] but the β_1 and β_2 genes are both expressed in the type II cell.[199,200] However, pharmacologic data suggest that surfactant secretion is regulated by the β_2 receptor.[201,202] There are four subtypes of adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3,[203] and all four are expressed in the type II cell.[199] Agonist and antagonist potency data and the observation that adenosine agonists that stimulate surfactant secretion also enhance cAMP formation make it clear that it is the adenosine A_{2B} receptor that regulates surfactant secretion.[195] P2 receptors are divided into two major groups: P2X receptors are ion channels, whereas P2Y receptors are metabotropic and are coupled to G-proteins.[204] To date, seven mammalian P2X and seven P2Y receptors have been cloned.[205,206] Evidence is good that the P2 purinoceptor responsible for stimulation of surfactant secretion is $P2Y_2$,[177,195] which was termed P_{2u} in an older nomenclature system.[204] The $P2Y_2$ receptor is expressed in the type II cell,[193,199,207] ATP and UTP are equally potent at $P2Y_2$ receptors,[204] and both nucleotides stimulate surfactant secretion in type II cells with similar EC_{50} values.[193] Additional agonist potency order data are also consistent with involvement of the $P2Y_2$ receptor in surfactant secretion.[193,208,209]

Mammalian PLC is divided into four subfamilies of: β, γ, δ, and ε.[210] $P2Y_2$ receptors are coupled to PLC-β,[211] of which there are four isoforms: β_1, β_2, β_3, and β_4.[212] PLC-β_3 is the only PLC-β isomer expressed in the type II cell[199] and therefore the one involved in regulation of surfactant secretion. The $P2Y_2$ receptor is also coupled to PLC-β_3 in other systems.[211] Twelve PKC isoforms are currently known to exist: α, βI, βII, δ, ε, γ, η, θ, ζ, λ/i, μ, and ν.[213] PKCμ and PKCν are structurally different from the other PKCs[214,215] and have been designated as a new class of protein kinases, protein kinase D (PKD), of which there are currently three members.[216] In this system, PKCμ becomes PKD1. The α, βI, βII, δ, η, θ, and ζ isoforms of PKC as well as PKD1 are expressed in the type II cell.[199,217] PKD1 is activated by the $P2Y_2$ agonists ATP and UTP as well as by TPA and a cell-permeable DAG, dioctanoylglycerol, in the type II cell.[218] This finding suggests a physiologic role for PKD1 in regulation of surfactant secretion. TPA and dioctanoylglycerol also activate PKCs α, βI, βII, δ, and η[217,218]; however, because both agonists are nonphysiologic, the significance of this in terms of regulation of surfactant secretion is not clear. PKD is involved in several signaling pathways and has been reported to be downstream of PKC.[216] However, in type II cells, no evidence is available that any PKC isomers other than PKD1 are activated by $P2Y_2$ agonists.[218]

The specific isomers of other signaling proteins (see Figure 103-6) involved in regulation of surfactant secretion have not been identified. Two mammalian PLDs exist, PLD1 and PLD2,[219] and both are expressed in the type II cell.[181] However, the PLD isomers involved in surfactant secretion are not known. Eight of the nine membrane-associated mammalian AC isoforms are expressed in the lung,[220,221] and AC-II and AC-IV, but not AC-I, are expressed in the type II cell.[222] Whether other ACs are also expressed in the type II cell is not known, and the AC isoforms involved in surfactant secretion have not been identified. Currently no information is available about the subunits or isomers of protein kinase A[223] and CaCM-PK[224, 225] that are expressed in the type II cells or are involved in surfactant secretion.

Other signaling mechanisms may also be involved in surfactant secretion.[181] For instance, data suggest involvement of phospholipase A_2 as well as a PC-specific PLC in ATP- and TPA-mediated secretion.[177] High- and low-density serum lipoproteins stimulate PC secretion by activation of receptors coupled to PKC through G_i.[226,227] Mastoparan, an agent that activates several signaling mechanisms, is a powerful surfactant secretagogue, and there are indications that its effect is also mediated in part by G_i.[228] Mitogen-activated protein (MAP) kinase activation is not involved in surfactant secretion, although this enzyme is often downstream of PKC activation,[229] and it is activated by $P2Y_2$ agonists in numerous systems.[181,230,231] Although ATP and TPA activate the MAP kinase cascade in type II cells,[232] MAP kinase inhibitors do not antagonize agonist-stimulated PC secretion.[181,232,233] Involvement of the MAP kinase cascade in surfactant-associated protein (SP)-A inhibition of surfactant secretion has also been discounted.[180,233] Experiments with inhibitors similarly suggest that tyrosine kinase signaling does not have a role in regulation of surfactant secretion,[181] although it is involved in P2 receptor signaling in other systems.[231]

Distal steps in the surfactant secretory pathway have been incompletely studied. As shown in Figure 103-6, all the signal-transduction pathways are believed to result in phosphorylation of proteins after activation of specific protein kinases. Virtually no information is available on the identity of the proteins phosphorylated in response to surfactant secretagogues, although TPA[234] and terbutaline[235] were reported to promote phosphorylation of certain proteins in type II cells. Relatively little is known of the steps beyond protein phosphorylation that ultimately result in exocytosis of lamellar bodies[177]; essentially no information is available about whether the SNARE (soluble NSF [*N*-ethylmaleimide–sensitive fusion protein] attachment protein receptor) paradigm of vesicle (lamellar body) docking and membrane fusion[236,237] applies to surfactant secretion.[177] However, some of the proteins involved in the SNARE exocytosis mechanism are phosphorylated,[236,237] and it is possible that such proteins are the site of action of the protein kinases involved in surfactant secretion. Also evidence exists of a role for synexin in fusion of lamellar bodies with the cell membrane in type II cells,[238,239] and it has been suggested that both synexin and the SNARE mechanism may be independently involved in mediating surfactant secretion.[239]

Little is known about the physiologic regulation of surfactant secretion.[177,181] Two physiologic factors are known to stimulate surfactant secretion in vivo: ventilation/exercise, which stimulates secretion in both adults[240-243] and newborns,[244,245] and labor, which stimulates secretion in newborns.[246] Ventilation-induced surfactant secretion is thought to result from a direct mechanical effect on the lung,[247] and in vitro experiments have shown that stretch increases surfactant secretion in type II cells.[248] Ventilation causes stretch of type I cells, and the type I cell then acts as a mechanotransducer to stimulate surfactant secretion by the type II cell.[189] The amount of literature is extensive on the mechanisms by which the effects of physical forces are transduced.[247] Evidence shows the involvement of at least three signaling pathways: activation of AC, PLC, and MAP kinase. Although, as discussed earlier, MAP kinase is not involved in regulation of surfactant secretion,[181,232,233] both AC and PLC are involved (see Figure 103-6).

There is direct evidence of a physiologic role for the AC signaling mechanism in regulation of surfactant secretion. A physiologic role for β-receptor activation is suggested by the finding that the stimulatory effects of ventilation[241] and labor[249] are antagonized by β-antagonists. A similar role for the adenosine A_{2B} receptor is suggested by the finding that the ventilation-induced increase in surfactant secretion in newborn rabbits is attenuated by an adenosine receptor antagonist.[244] Finally, although atropine blocks the effect of ventilation in vivo,[240,244,245] a finding suggesting cholinergic regulation, evidence is substantial that cholinergic regulation of surfactant secretion is mediated by activation of β-receptors on the type II cell in response to catecholamines released from the adrenal

medulla.[177] β-agonists,[241,250] cholinergic agonists,[240,244,250] and adenosine[251] all stimulate surfactant secretion in vivo.

No direct evidence is currently available that PLC activation has a physiologic role. As shown in Figure 103-6, PLC-β_3 is central to both the PKC and CaCM-PK signaling pathways. Apart from ATP, UTP, and possibly lipoproteins,[227] the only well-established surfactant secretagogues that activate the PKC signaling pathway are the nonphysiologic agonists TPA and cell-permeable DAGs, whereas those that activate the CaCM-PK pathway are the equally unphysiologic ionophores ionomycin and A23187. It is highly likely that some physiologic agonist activates those signaling mechanisms, and it is tempting to speculate that ATP or UTP may be such an agonist. Currently, no information is available on whether the $P2Y_2$ receptor on the type II cell has a physiologic role in regulation of surfactant secretion, but evidence is good that the $P2Y_2$ receptor has such a role in many other systems.[204,205,252,253] Activation of P2Y receptors is one of the mechanisms by which the effect of mechanical force is transduced[254]; a mechanical stimulus promotes release of ATP and UTP in airway epithelial and other cells.[254-256] The nucleotides then activate P2Y receptors on either the same or adjacent cells in an autocrine or paracrine manner.[253,254,256] Mechanical stimulation activates PLC in airway epithelial and other cells,[257,258] and the stretch-induced increase in surfactant secretion in type II cells is accompanied by elevated intracellular Ca^{2+} levels.[248] Thus possibility is reasonable that the stimulatory effect of ventilation on surfactant secretion is mediated by the type II cell $P2Y_2$ receptor and therefore by the PKC or CaCM-PK signaling pathways. Based on tissue culture data, ATP levels in bronchoalveolar lavage fluid[259] are sufficient to stimulate surfactant secretion.

SECRETION OF SURFACTANT PROTEINS

Secretion of the protein components of surfactant has been less extensively investigated than that of the phospholipids. Lamellar bodies are enriched in the hydrophobic proteins, SP-B and SP-C,[260] and these organelles have a central role in the overall processing of both proteins.[261] Secretion of SP-B and SP-C in rat type II cells is stimulated by the same agonists that stimulate PC secretion: terbutaline, *N*-ethylcarboxyamidoadenosine, ATP, TPA, and ionomycin.[262] A combination of agonists and a PKC inhibitor have the same effects on secretion of SP-B and SP-C as on that of PC.[262] In addition evidence shows that in vivo secretion of SP-B and PC have similar kinetics.[263] Therefore secretion of surfactant phospholipids and of hydrophobic proteins is similarly regulated, and secretion of both involves exocytosis of lamellar bodies.

In contrast secretion of SP-A and SP-D occurs by a different mechanism. Although data are conflicting with respect to the distribution of SP-A between lamellar bodies and other compartments of the type II cell,[180,264-266] it is general agreed that secretion of SP-A occurs independently of lamellar bodies. SP-A secretion in isolated type II cells is usually not stimulated by agonists that stimulate PC secretion,[184,262,265,267] although in two studies it was stimulated by TPA.[268,269] Furthermore, both in vivo[270,271] and tissue culture[266] experiments suggest that newly synthesized SP-A is secreted independently of lamellar bodies. Lamellar bodies are devoid of SP-D,[272,273] and secretion of SP-D is not stimulated by surfactant phospholipid secretagogues in isolated type II cells.[262,267,268]

In summary, secretion of surfactant phospholipids, SP-B, and SP-C occurs largely by regulated exocytosis of lamellar bodies, whereas secretion of SP-A and SP-D occurs independently of lamellar bodies and is either not regulated or is regulated by a different mechanism from that of the phospholipids and hydrophobic proteins.

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Alan H. Jobe and Machiko Ikegami

Pathophysiology of Respiratory Distress Syndrome and Surfactant Metabolism

The pathophysiology of the respiratory distress syndrome (RDS) and the surfactant system were linked by the recognition of abnormal surfactant in RDS by Avery and Mead in 1959.[1] This chapter focuses on the pathophysiology of RDS from the perspective of surfactant metabolism and function. The effects of surfactant treatment on the initial pathophysiologic events and on surfactant metabolism also will be reviewed. Clinical aspects of surfactant treatment for RDS and its resolution will be covered in another chapter.

LUNG STRUCTURE OF THE PRETERM WITH RESPIRATORY DISTRESS SYNDROME

Lung structure is the substrate on which surfactant functions. The preterm lung changes structure as it matures, and the structural maturation of the lung affects the responses of the lung to surfactant and the lung complications that may occur after preterm birth. The lung of the term infant contains approximately 30% of the 500 million alveoli that are present in the adult lung[2,3] (Figure 104-1). The fetal lung at the margin of viability at 24 weeks gestation has just matured beyond the canalicular stage to be in the early saccular stage of lung development.[4] The saccular lung has undifferentiated distal air saccules with a poorly developed capillary microvasculature. The potential surface area for gas exchange of the developing human lung does not increase much before 30 weeks gestation, and alveolar septation of the distal saccules does not begin until after 32 weeks. However, the rate of septation to form alveoli then is high until term. After birth, the rate of alveolar septation decreases until alveolar development ceases at 18 to 24 months of age. A slow process of alveolar septation and lung remodeling may continue throughout life. The early lung maturation that commonly occurs in preterm infants with maternal glucocorticoid treatment or with exposure to chorioamnionitis will accelerate mesenchymal involution and increase surfactant but may delay alveolar septation.[5,6] A major factor determining how the preterm lung will respond to treatment and injury is the structural maturation of the lung at delivery.

OVERVIEW OF SURFACTANT METABOLISM

The general scheme of surfactant metabolism as it is understood in the adult lung is the basis for the discussion of surfactant metabolism in the preterm lung with RDS[7] (Figure 104-2). More extensive descriptions of the surfactant lipids and proteins, the biophysics of surfactant function, and regulation of synthesis and secretion are found in other chapters in this section. Surfactant lipids are synthesized from precursors, such as palmitic acid, that are made by type II cells or are transported to the type II cells from the circulation. The lipids are processed via the Golgi to multivesicular bodies that associate with the surfactant-associated protein (SP)-B and SP-C and are stored in the form of membrane-enclosed lipid and protein structures called lamellar bodies. Lamellar bodies are localized to the apical (air) surface of the type II cell and are secreted by exocytosis either constitutively or when type II cells are stimulated by secretogogues such as β-agonists and purines or by mechanical stretch. The other surfactant proteins SP-A and SP-D are secreted primarily independently of the lamellar bodies.

Surfactant also has an extracellular "life cycle" once secreted to the thin fluid hypophase of the healthy lung. The SP-A associates in the hypophase with the lipids plus SP-B and SP-C secreted as lamellar bodies to form tubular myelin and other loose lipid arrays that are macroaggregated lipoprotein forms.[8] These large lipoprotein aggregates are the forms of surfactant that facilitate adsorption to the air-water interface. The surfactant film is multilayered and continuously replenished from surfactant in the hypo-phase.[9] Lipids are cleared from the airspaces as small liposomal vesicles that contain the phospholipids, but essentially no surfactant proteins. The surfactant proteins also are cleared by macrophages and type II cells, but their forms for clearance have not been characterized. Approximately half of the surfactant components are catabolized by alveolar macrophages in the adult mouse lung and the other half are catabolized by type II cells.[10] However, the type II cells also take up phospholipids, SP-B, SP-C, and SP-A and recycle them via multivesicular bodies back to lamellar bodies for resecretion.

The surfactant lipids and proteins are dynamically cycling from type II cells to the surfactant layer and back to type II cells. In the adult rabbit lung, the rate of synthesis of surfactant phosphatidylcholine is 1.5 μmol/kg • hr, 0.5 μmol/kg • hr is recycled back from the airspace, and approximately 2 μmol/kg • hr is secreted into an alveolar pool of approximately 10 μmol/kg.[11] Approximately 25% of the phospholipids are recycled in the adult. At steady state, the alveolar surfactant pool is replaced every 5 hours. Thus surfactant pool is very metabolically active. The airway clearance rates and efficiencies of recycling for SP-A, -B, and -C are similar to the lipids in the adult lung.[12-14] As will be apparent, the recycling pathways are important for surfactant treatment strategies.

CHARACTERISTICS OF SURFACTANT IN THE PRETERM

Composition

The composition of surfactant and the characteristics and function of the surfactant proteins are discussed extensively in the chapters in this section. We focus on the composition of surfactant in the preterm. The surfactant recovered from the air spaces of preterm animals with RDS has less saturated phosphatidylcholine relative to total phospholipids.[15] The lower amount of saturated phosphatidylcholines will result in a surfactant with decreased surface activity. The surfactant of a mature animal or man contains approximately 8% phosphatidylglycerol and little phosphatidylinositol. In contrast surfactant from the preterm contains much more phosphatidylinositol than phosphatidylglycerol.[16] These two acidic phospholipids are interchangeable in terms of surfactant function, but the lack of phosphatidylglycerol in surfactant indicates lung immaturity or injury to the type II cells. The changes in the lecithin/sphingomyelin (L/S) ratio in amniotic fluid with advancing gestation result primarily from the contributions of fetal lung fluid to amniotic fluid. The L/S ratio increases after at approximately 34 weeks gestation

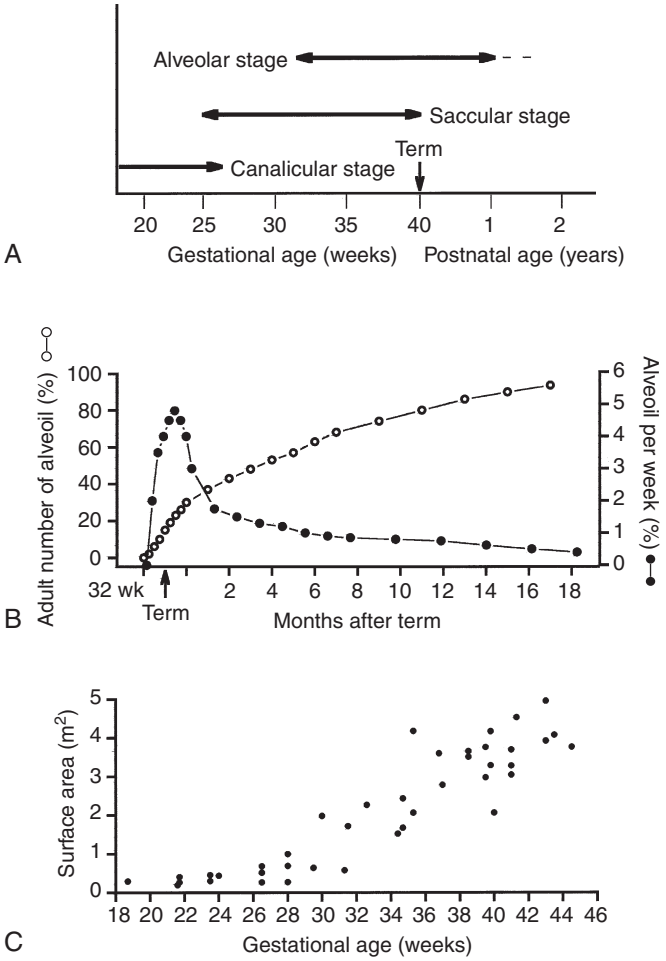


Figure 104-1 Lung structural maturation. A, The stages of lung development from 20 weeks gestation to 2 years. **B,** Alveolar number and weekly rate of accumulation of alveoli are expressed as percentage of the adult number of alveoli. The curves assume that the term infant has 30% of the adult number of alveoli. **C,** The large increase in lung surface area does not occur until after the saccular lung begins to alveolarize. *(**A,** Modified from Burri PH: Structural aspects of prenatal and postnatal development and growth of the lung. In McDonald JA, editor:* Lung Growth and Development, *New York, 1997, Marcel Dekker, pp 1-35;* **B,** *Modified from Hislop AA, Wigglesworth JS, Desai R: Alveolar development in the human fetus and infant,* Early Hum Dev *13:1, 1986;* **C,** *Modified from Langston C, Kida D, Reed M, et al: Human lung growth in late gestation and in the neonate,* Am Rev Respir Dis *129:607, 1984.)*

in normal pregnancies, indicating progressive lung maturation.[17] The L/S ratio measures primarily saturated phosphatidylcholine relative to sphingomyelin, a lipid not specific to the fetal lung surfactant. The phosphatidylinositol content of amniotic fluid increases after 28 weeks gestation to peak at 35 weeks gestation, and phosphatidylglycerol does not begin to increase until after 34 weeks gestation.[16] Infants with early lung maturation caused by fetal stress or fetal exposure to inflammation can increase the amounts of saturated phosphatidylcholine, decrease phosphatidylinositol, and increase phosphatidylglycerol in amniotic fluid to have a surfactant composition comparable to a mature lung. Infants recovering from RDS have increasing amounts of phosphatidylglycerol in the surfactant. In contrast RDS that progresses to bronchopulmonary dysplasia (BPD) results in surfactant with a delayed appearance of phosphatidylglycerol, presumably because of injury to type II cells.[18]

The surfactant specific protein content of surfactant from the preterm lung is low relative to the amount of lipid. Type II cells with lamellar bodies appear in the human lung after approximately 22 weeks, but very little surfactant protein mRNA is expressed until later in gestation. The timing of gene expression and protein secretion in the normal human preterm fetus can be inferred from the increases in the proteins in amniotic fluid.[19] SP-A increases after 32 weeks gestation; SP-B increases after 34 weeks gestation. In animal models SP-A and SP-B mRNA expression is low until late in gestation and then increases rapidly to term. SP-B protein must be extensively processed before it enters lamellar bodies. With induced lung maturation in fetal sheep, both the mRNA and the protein processing of SP-B increase in parallel.[20] SP-D mRNA in the lung also is low until late gestation, and amniotic fluid levels of SP-D do not change much with advancing gestation.[21] SP-A and SP-D in amniotic fluid may also come from nonpulmonary sources because these innate host defense proteins are made in locations other than the lung.

In contrast to other surfactant proteins, SP-C mRNA is highly expressed at the tips of branching airways during early lung development.[22] SP-C mRNA also is expressed in the developing type II cells before SP-C is found in the fetal airways. Incompletely processed SP-C is in tissue before mature SP-C is measurable in the secreted surfactant in the fetal rabbit, and the ratio of mature SP-C to saturated phosphatidylcholine increases 10-fold over 2 days gestation in the rabbit.[23] SP-C has not been measured in amniotic fluid from humans.

Surfactant Pool Size

During normal gestation, the lungs store progressively larger amounts of surfactant in the maturing type II cells. The appearance of surfactant in fetal airspaces lags behind the accumulation of surfactant in fetal lung tissue. After approximately 34 weeks, surfactant is secreted by the fetus and large amounts of surfactant can be isolated from amniotic fluid at term. The term infant has a large excess of surfactant that facilitates rapid pulmonary adaptation to air breathing. The amount of surfactant in the lung saccules in infants younger than 32 weeks and in the developing alveoli and small airways after 32 weeks depends on the physiologic events experienced by the fetus or newborn. With labor and the stress of delivery, some of the lamellar bodies are secreted into the fetal lung fluid, and surfactant concentration increases as fetal lung fluid volume decreases.[24] More surfactant is secreted after the initiation of ventilation in response to stretch, increased catecholamines, and purinoceptor agonists. In the preterm sheep, the fetal surfactant stores in the type II cells are secreted within approximately 30 minutes of ventilation after birth.[25]

The only direct measurements of surfactant pool sizes of infants were made by alveolar lavage of lungs of infants who died of RDS soon after birth and in the era before mechanical ventilation was available.[26] The lavages contained approximately 5 mg/kg surfactant. Measurements by bronchoalveolar lavage in preterm rabbits and lambs with severe RDS yield values less than 3 mg/kg. Preterm lambs can be supported with continuous positive airway pressure if their surfactant pool size is approximately 3 mg/kg.[27] Smaller pool sizes result in severe respiratory failure. The differences in phospholipid composition between exogenous surfactant given as treatment and the endogenous surfactant have been used to estimate the pool size in infants with RDS. By measuring the change in phosphatidylglycerol composition, Hallman and colleagues[28] estimated a surfactant pool size of approximately 9 mg/kg for infants with RDS.

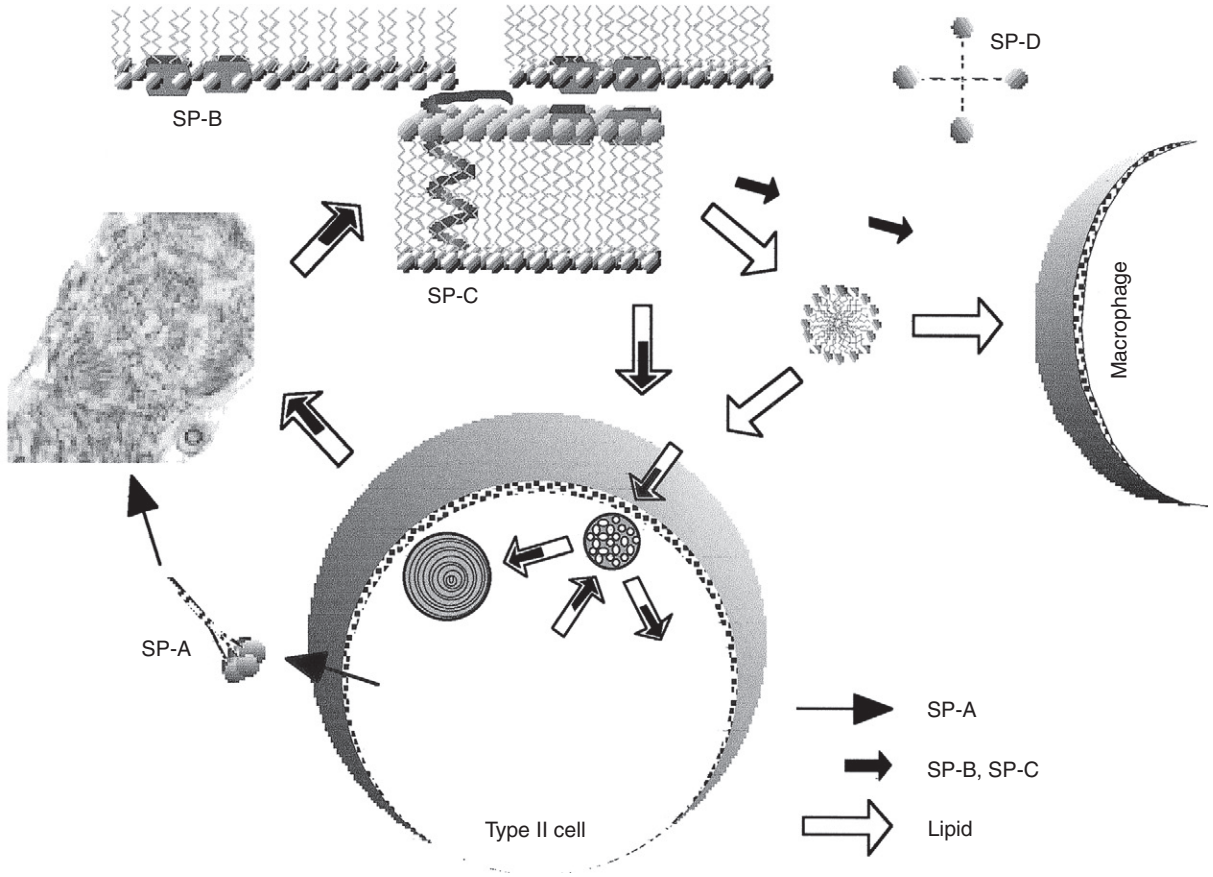


Figure 104-2 Major metabolic pathways of surfactant. Surfactant components are synthesized and packaged into lamellar bodies in type II cells.The lamellar bodies are secreted to the hypophase and surfactant is adsorbed to the air fluid interface. Surfactant components are cleared from the airspaces primarily by type II cells and macrophages. *SP,* Surfactant-associated protein.

Similar measurements by Griese and colleagues[29] yielded a surfactant pool size estimate of 20 mg/kg. A more recent measurement using stable isotope-labeled dipalmitoylphosphatidylcholine yielded a value of 5.6 mg/kg.[30] These techniques assume good mixing of the treatment surfactant with the endogenous pool, no loss of the treatment surfactant from the alveolar pool, and that airway aspirates represent what is in the distal lung. These assumptions are not strictly valid, and the techniques used clinically will overestimate the endogenous pool size by at least two-fold.[25]

The endogenous pool size of surfactant is the major determinant of lung compliance in preterm animals[31,32] (Figure 104-3). Very preterm infants with severe RDS probably have surfactant pools of less than 5 mg/kg. Although not measured in humans, term newborn animals have surfactant pools of approximately 100 mg/kg. In contrast the surfactant pool size in the adult human is only approximately 4 mg/kg.[33] Therefore preterm infants have perhaps 5% of the amount of surfactant in the term newborn lung, but they have amounts comparable to the healthy adult human. This issue will be addressed relative to surfactant function later in this chapter.

Changes in Surfactant Pool Size After Birth

The pool sizes of surfactant that can be recovered by alveolar lavage increase slowly after birth in preterm ventilated monkeys and sheep. In monkeys recovering from RDS, the surfactant increases from approximately 5 mg/kg toward term values by 3 to 4 days of age[34,35] (Figure 104-4). No direct measurements of surfactant pool sizes as infants recover from RDS are available. The changes in surfactant concentration in airway samples will, in part, reflect pool sizes. The concentration of surfactant was similar for infants without RDS and for infants with RDS who were treated with surfactant.[36] Infants with RDS had a progressive increase in surfactant concentration during approximately 4 days.

Surfactant treatments with the commonly used dose of 100 mg/kg do not result in large or sustained increases in alveolar surfactant pool size. Soon after administration, the majority of the lipids can no longer be recovered by lavage. For example, in sheep only approximately 25% of the treatment dose is recovered 5 hours after treatment.[37] In preterm ventilated baboons, approximately 20% of the treatment amount of surfactant was recovered 24 hours later.[38] Infants with RDS had a mean surfactant pool size of 17 mg/kg 33 hours after an initial 100 mg/kg surfactant treatment, as estimated with stable isotopes.[30] The increase in alveolar surfactant that accompanies the resolution of RDS does not occur in preterm ventilated baboons that are developing BPD.[39] In these animals, the alveolar pool size after surfactant treatment at birth and 6 days of ventilation was approximately 30 mg/kg, which had not increased after a second surfactant treatment and a further 8 days of ventilation. The alveolar content of the surfactant proteins SP-A and SP-D also were low in these preterm baboons.[40] Nevertheless, the lung tissue content of saturated phosphatidylcholine increased approximately four-fold, and the tissue content of SP-A and SP-D increased, indicating a defect in surfactant processing and secretion in the early phases of BPD.

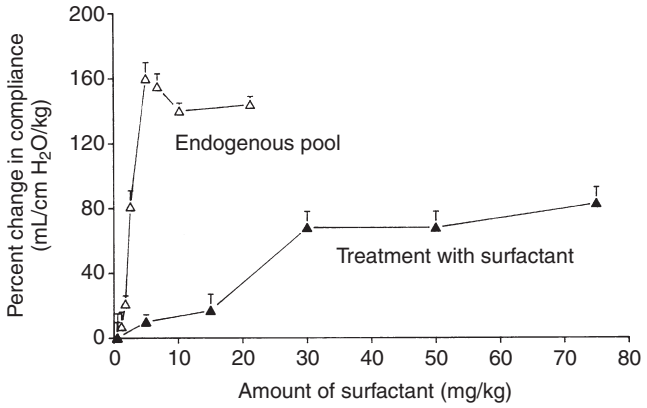


Figure 104-3 Compliance changes in relation to surfactant pool sizes. The curve for the endogenous pool was measured for alveolar washes of ventilated preterm rabbits that were 27 to 29 days gestational age at delivery.The increase in compliance includes a surfactant effect as well as spontaneous lung structural maturation that occurred in the 2-day interval.The curve for treatment with surfactant gives the responses of 27-day gestation rabbits to different treatment doses of surfactant. *(Data from Ikegami M, Jobe AH, Yamada T, et al: Relationship between alveolar saturated phosphatidylcholine pool sizes and compliance of preterm rabbit lungs, the effect of maternal corticosteroid treatment,* Am Rev Respir Dis *139:367, 1989 and Seidner S, Pettenazzo A, Ikegami M, et al: Corticosteroid potentiation of surfactant dose response in preterm rabbits,* J Appl Physiol *64:2366, 1988.)*

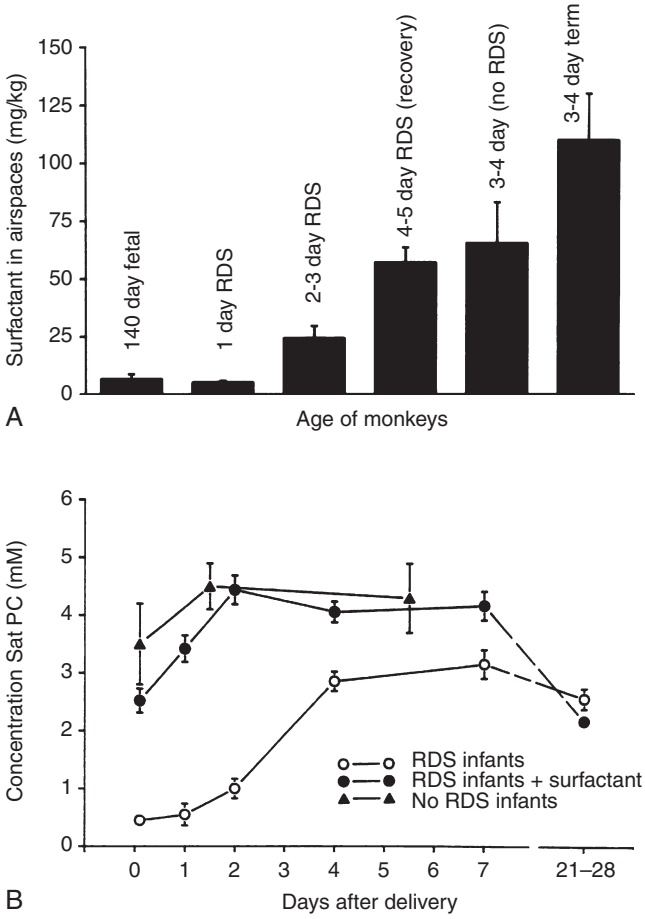


Figure 104-4 Surfactant pool sizes with resolution of respiratory distress syndrome (RDS). A, The amount of surfactant recovered by alveolar lavage from mechanically ventilated preterm monkeys with RDS is shown relative to age and stage of the disease. **B,** The concentrations of saturated phosphatidylcholine *(Sat PC)* in airway samples from infants with RDS, infants with RDS treated with surfactant, and infants without RDS are graphed relative to age from birth.The concentrations of Sat PC approached values for healthy preterm infants by 4 to 7 days. *(**A,** Data from Jackson JC, Palmer S, Truog WE, et al: Surfactant quantity and composition during recovery from hyaline membrane disease,* Pediatr Res *20:1243, 1986, and Jackson JC, Palmer S, Flandaert T: Developmental changes of surface active material in newborn non-human primates,* Am Rev Respir Dis *129:A204, 1984.* **B,** *Data from Hallman M, Merritt TA, Akino T, et al: Surfactant protein-A, phosphatidylcholine, and surfactant inhibitors in epithelial lining fluid: correlation with surface activity, severity of respiratory distress syndrome, and outcome in small premature infants,* Am Rev Respir Dis *144:1376, 1991.)*

Surfactant Metabolism in the Preterm

To understand how the alveolar surfactant pool is maintained in the lung, it is helpful to divide metabolism into the anabolic components of synthesis and secretion and the catabolic activities of uptake, degradation, and recycling. Surfactant components labeled with stable isotopes were used to evaluate surfactant metabolism in infants. Although these measurements have a number of limitations, they provide information that corroborates the more complete measurements that have been made in term and preterm animals. Most metabolic studies have focused on saturated phosphatidylcholine because it is the major component of surfactant and is primarily responsible for the biophysical properties of surfactant.

Measurements of synthesis of surfactant phospholipids generally involve the intravascular injection of labeled precursors. The labeled precursors are taken up by type II cells and incorporated into phosphatidylcholine, which is transacylated to increase the amount of saturated phosphatidylcholine. The surfactant lipids together with SP-B and SP-C are processed and packaged via multivesicular bodies to become the storage-secretion granule, the lamellar body[41] (see Figure 104-2). Secretion can be measured by recovering the labeled surfactant components from the airspaces, which evaluates the overall kinetics of synthesis and secretion. The absolute amount of a surfactant component that is synthesized cannot be easily determined because the radiolabeled precursor is diluted into the plasma pool and is further diluted by the precursor pool in the type II cell. However, the net kinetics of synthesis and secretion provide critical information for understanding why surfactant deficiency requires days to resolve in infants with RDS.

After term delivery and ventilation of lambs, an intravascular injection of radiolabeled palmitic acid is incorporated into lung phosphatidylcholine within minutes.[42] However, radiolabeled surfactant is not detected in the air spaces for approximately 5 hours, and the amount of radiolabeled surfactant continues to accumulate in the airspaces for 30 to 40 hours[43] (Figure 104-5). A similar curve for accumulation of de novo synthesized surfactant was measured for ventilated preterm lambs with mild RDS. Subsequent surfactant treatment of these preterm lambs caused a large dilution of the endogenous surfactant. Surfactant lipids labeled with ^{13}C-glucose could not be detected in airway samples of surfactant-treated very preterm baboons until 24 hours of age, after a ^{13}C-glucose infusion for the first 24 hours after birth.[44] The peak accumulation of ^{13}C-glucose in phosphatidylcholine was at approximately 100 hours. The curve for the preterm baboon has a longer delay in detection than the curve for the preterm sheep because the radiolabel was not given as

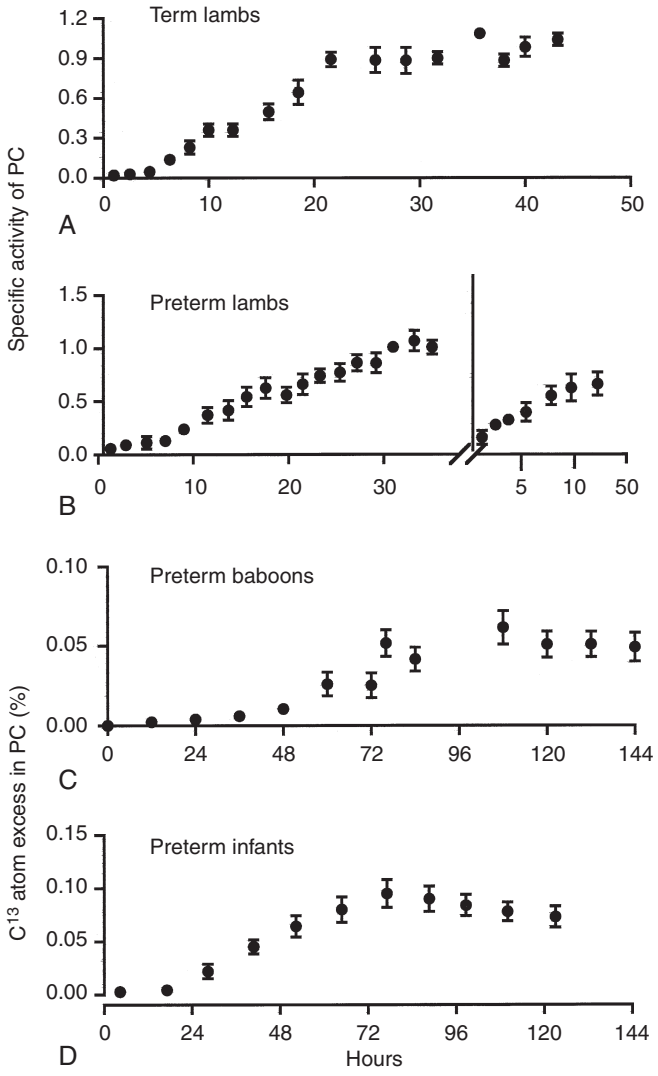


Figure 104-5 Time course of appearance of de novo synthesized surfactant phosphatidylcholine (PC) in airway samples of term lambs, preterm lambs, preterm baboons, and preterm infants. **A,** Labeling of saturated PC in airway samples of ventilated term lambs given ^{3}H-palmitic acid as a bolus intravascular injection at birth. Data are means ± SE of specific activities (counts per minute/μmol PC) normalized to the maximal values for each animal. **B,** Labeling of saturated PC in airway samples of ventilated preterm lambs given a single intravascular injection of ^{3}H-palmitic acid at birth and not treated with surfactant until approximately 38 hours of age.The discontinuity in the curve demonstrates the fall in the specific activity after treatment with surfactant (dilution of surfactant pool with treatment dose of surfactant). **C,** Labeling of PC in airway samples of preterm surfactant-treated baboons that were ventilated for 6 days. The animals received ^{13}C-glucose by intravascular infusion for the first 24 hours of age and the ^{13}C in the palmitate of PC is expressed as ^{13}C-atom percent excess. **D,** Labeling of PC in airway samples of preterm surfactant treated and mechanically ventilated infants with RDS following an intravascular infusion of ^{13}C-glucose for the first 24 hours of life.The label in the PC is expressed as atom percent excess. *(***A** *and* **B,** *Data from Jobe AH, Ikegami M, Glatz T, et al: Saturated phosphatidylcholine secretion and the effect of natural surfactant on premature and term lambs ventilated for 2 days,* Exp Lung Res *4:259, 1983.* **C,** *Data from Bunt JE, Carnielli VP, Seidner SR: Metabolism of endogenous surfactant in premature baboons and effect of prenatal corticosteroids,* Am J Respir Crit Care Med *160:1481, 1999.* **D,** *Data from Bunt JE, Carnielli VP, Darcos Wattimena JL, et al: The effect in premature infants of prenatal corticosteroids on endogenous surfactant synthesis as measured with stable isotopes,* Am J Respir Crit Care Med *162:844, 2000.)*

a pulse label and time was required to get enough label into the airspace to be detected. Preterm surfactant-treated infants given infusions of ^{13}C-palmitic acid and ^{13}C-glucose have curves similar to the baboons for the detection of surfactant lipids in airway samples[45] (see Figure 104-5). The conclusion is that secretion is delayed for hours after synthesis, and surfactant made shortly after birth is secreted gradually over a prolonged period of time. The curves are not altered much by surfactant treatment or by age after birth when the labeled precursor is given.[44] Antenatal glucocorticoids may increase lipid synthesis, but this conclusion is not unequivocal because precursor pools may be altered by the glucocorticoids.[45] Surfactant treatment does not inhibit de novo synthesis of lipids or the surfactant proteins in the preterm lungs. Surfactant treatment also does not alter the ultrastructure of type II cells or the volume fraction of lamellar bodies in type II cells.[46] The timing of phosphatidylcholine secretion measured with stable isotopes was similar for infants with RDS ventilated with conventional or high frequency oscillatory ventilation.[47]

The other half of the metabolic equation is the loss of surfactant from the lung. This measurement has three components that include uptake and catabolism of surfactant components from the airspace by type II cells and macrophages and recycling by type II cells. Little surfactant is lost from adult lungs to the lymph or vascular space unless the lung is injured, and minimal amounts of surfactant are lost by suctioning the airways unless edema fluid is present. In the term lamb, a trace dose of surfactant given at birth had a half-life of approximately 6 days, which is longer than values of approximately 10 hours for adult animals.[48] Almost all of a trace dose of surfactant given to preterm lambs with mild RDS was recovered in the lungs after 24 hours of ventilation, demonstrating minimal catabolism[49] (Figure 104-6). However, only 20% of the labeled surfactant was still in the airspaces, and 80% was associated with the lung tissue. Similar measurements in preterm lambs with more severe RDS who were treated with surfactant demonstrated a 30% loss of the surfactant used for treatment by 24 hours and only 14% of the surfactant recovered by alveolar lavage.[50] This rate of loss of surfactant from the lungs yielded a biological half-life of approximately 48 hours. The percent of a treatment dose of surfactant that remained in the airspaces was decreased with high tidal volume ventilation or with high frequency oscillatory ventilation in preterm lambs.[51,52] The amount of surfactant lost from the lungs was increased by ventilator-induced lung injury. Surfactant-treated and ventilated preterm baboons that were developing BPD retained only 4% of the treatment in the airspaces, and approximately 80% of the surfactant was lost from the lungs by 6 days, yielding a half-life of approximately 60 hours.[38] These results indicate that the term and preterm lung degrades surfactant lipids slowly and that injury can increase the loss of surfactant from the lungs.

No information is available in the preterm about where degradation of surfactant occurs. The normal preterm lung at birth contains few macrophages and essentially no granulocytes. However, inflammation and injury will rapidly recruit inflammatory cells to the lung, which may then increase the loss of surfactant. A significant amount of a treatment dose of surfactant that becomes associated with the lung tissue probably is in type II cells. In the term newborn rabbit, more than 90% of

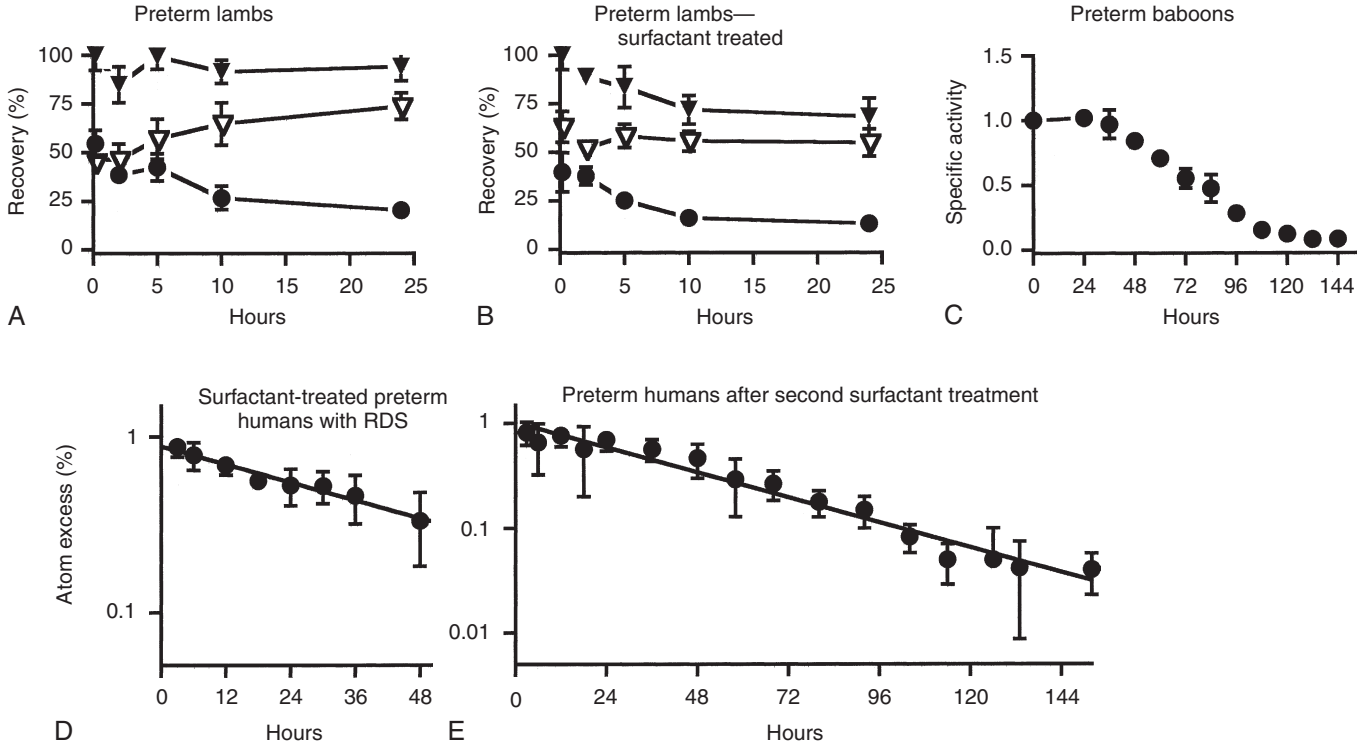


Figure 104-6 Loss of labeled phosphatidylcholine (PC) given into the airways of (A) preterm lambs not treated with surfactant, (B) preterm lambs treated with surfactant, (C) preterm surfactant-treated baboons, and (D and E) surfactant-treated preterm infants. All animals and infants were mechanically ventilated and had respiratory distress syndrome. A, The preterm lambs received a trace dose of natural sheep surfactant labeled with ^{3}H-dipalmitoylphosphatidylcholine. Recoveries in alveolar washes, lung tissue and the total lungs *(alveolar + tissue)* were measured 2 hours, 5 hours, 10 hours, and 24 hours after birth, **B,** Preterm lambs were treated at birth with 100 mg/kg of natural sheep surfactant, containing radiolabeled phosphatidylcholine and the lambs were ventilated for periods to 24 hours for measurements of the percent recovery of the phosphatidylcholine from the surfactant used for treatment. **C,** Curve for specific activity of phosphatidylcholine in airway samples of preterm baboons treated at birth with ^{14}C-dipalmitoylphosphatidylcholine–labeled surfactant.The specific activities were normalized to the values for the surfactant used to treat the baboons. **D,** Curve for atom percent excess in airway samples for ^{13}C-dipalmitoylphosphatidylcholine–labeled surfactant used to treat preterm ventilated infants with RDS.The atom percent excess in the initial surfactant dose given after delivery fell exponentially for 48 hours. **E,** A second dose given at approximately 2 days of age resulted in a similar curve. *(***A,** *Data from Jobe AH, Ikegami M, Seidner SR, et al: Surfactant phosphatidylcholine metabolism and surfactant function in preterm, ventilated lambs,* Am Rev Respir Dis *139:352, 1989.* **B,** *Data from Ikegami M, Jobe A, Yamada T, et al: Surfactant metabolism in surfactant-treated preterm ventilated lambs,* J Appl Physiol *67:429, 1989.* **C,** *Data from Seidner SR, Jobe AH, Coalson JJ, et al: Abnormal surfactant metabolism and function in preterm ventilated baboons,* Am J Respir Crit Care Med *158:1982, 1998.* **D** and **E,** *Data from Torresin M, Zimmermann LJ, Cogo PE, et al: Exogenous surfactant kinetics in infant respiratory distress syndrome: a novel method with stable isotopes,* Am J Respir Crit Care Med *161:1584, 2000.)*

the surfactant phospholipids are recycled back into the type II cells for resecretion.[53] In the only estimate available, ventilated preterm lambs with mild RDS had a turnover time for surfactant phosphatidylcholine of approximately 13 hours.[49] These animals had no measurable surfactant catabolism, indicating efficient recycling.

Measurements of surfactant clearance and biological half-life in animals were estimated from the amount of labeled surfactant remained in the lung tissue and in the airspace after an interval of time. The only practical measurement in the human is to give a trace or treatment dose of surfactant containing a stable isotope and to follow the change in concentration of the label in the surfactant sampled by aspiration of the airspaces. This measurement gives an integrated assessment of the mixing of the exogenous label with the endogenous airspace surfactant and tissue pools of surfactant. A decrease in the concentration of the label in the airway sample represents dilution of the label with surfactant from the lung, but it does not necessarily measure catabolism. Such measurements have been made in surfactant-treated preterm ventilated baboons and infants with RDS[30,38] (see Figure 104-6). In baboons the concentration of labeled lipid (specific activity) did not change for approximately 24 hours, indicating that little endogenous surfactant was present. However, independent information from preterm lambs and baboons indicate that only approximately 20% of the surfactant would have been in the air spaces at 24 hours, and 25% of the surfactant would have been lost from the lung compartment.[49,50] Subsequently the specific activity decreased exponentially, yielding a biological half-life of approximately 30 hours. The biological half-life values for infants with RDS measured with ^{13}C-dipalmitoylphosphatidylcholine were 34 ± 9 hours after surfactant treatment at a mean of 4.6 hours of age and were similar after a second dose of surfactant given at a mean age of 37 hours. The consistency of the indirect measurements in humans and the animals indicates that surfactant catabolism is slow in the preterm with RDS. The treatment dose of surfactant does not remain in the airspace but becomes part of the overall

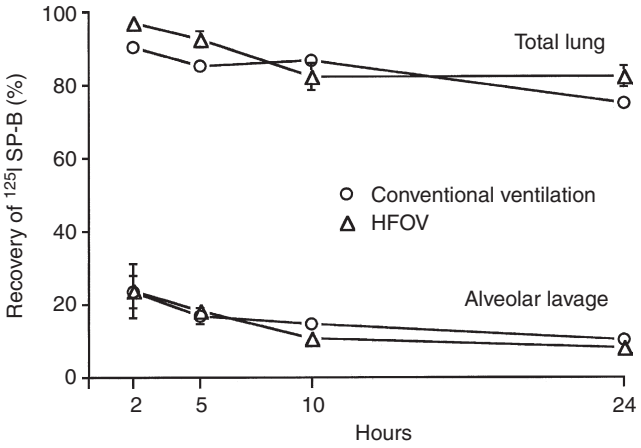


Figure 104-7 Recoveries of ^{125}I-labeled SP-B from the alveolar lavages and total lungs *(alveolar + tissue)* of preterm surfactant treated and ventilated lambs. The lambs were conventionally ventilated or ventilated with high frequency oscillatory ventilation *(HFOV).* Although loss of SP-B from the lungs was minimal, the amount of SP-B recovered by alveolar lavage was low. *(Data from Ikegami M, Wada K, Emerson GA, et al: Effects of ventilation style on surfactant metabolism and treatment response in preterm lambs,* Am J Respir Crit Care Med *157:638, 1998.)*

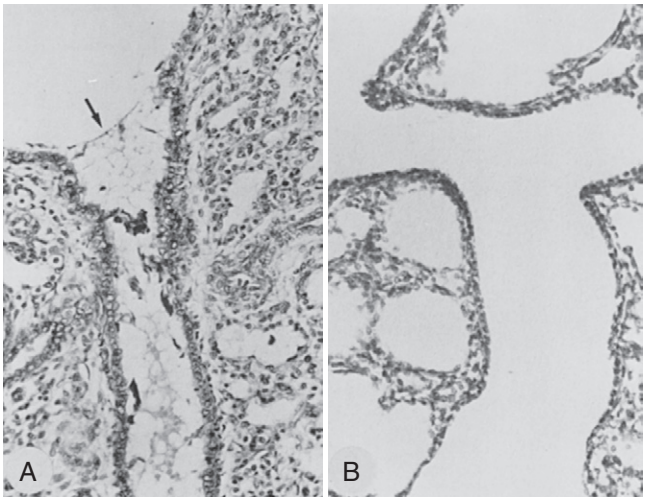


Figure 104-8 Bronchiolar-epithelial injury. A, Lungs from preterm ventilated rabbits had fluid-filled airways with epithelial tears in the absence of surfactant treatment. **B,** With surfactant treatments, the bronchiolar epithelium remained intact, and fluid was cleared from the airways and alveoli. *(From Lachmann B, Berggren P, Curstedt T, et al: Combined effects of surfactant substitution and prolongation of inspiration phase in artificially ventilated premature newborn rabbits,* Pediatr Res *16:921, 1982.)*

metabolic pool of surfactant. Measurements with stable isotopes in preterm infants also indicate that surfactant catabolism is increased and recycling efficiency is decreased with lung injury and with the development of BPD.[54,55]

The surfactant proteins SP-A, SP-B, and SP-C are cleared from the airspaces and the lung compartment of adult animals in parallel with the surfactant lipids.[13] The proteins are recycled with an efficiency slightly lower than the lipids, and degradation of SP-A occurs approximately equally in macrophages and type II cells.[10] The preterm ventilated lamb lung clears SP-A from the airspaces more rapidly than the lipids.[56] The lipophilic proteins SP-B and SP-C are cleared from the airspaces and from the lung compartment similar to saturated phosphatidylcholine in ventilated preterm lamb lungs[37,52] (Figure 104-7). SP-B clearance is similar in lambs ventilated with a conventional style of ventilation or with high frequency oscillatory ventilation. In the only measurement in humans, SP-B secretion paralleled and clearance was somewhat faster than saturated phosphatidylcholine.[57]

Lung Injury With Mechanical Ventilation

The lungs of infants who died from RDS have alveolar atelectasis, alveolar and interstitial edema, and hyaline membranes, primarily in distorted small airways. Hyaline membranes are a coagulum of cell debris, surfactant, and serum proteins that result from epithelial injury and large amounts of soluble and insoluble proteins in the air spaces. The earliest anatomic lesion identified in the lungs of infants who died of RDS shortly after birth was epithelial disruption in the small airways.[58] In preterm surfactant-deficient rabbits, bronchiolar epithelial injury developed within minutes of delivery, and ventilation and this injury was prevented with surfactant treatment[59] (Figure 104-8). The epithelial damage occurs because the small airways of the immature lung are compliant and distort during the ventilation. Lung fluid clearance is not complete and small airways are fluid filled at end expiration. Ventilation requires high peak pressures for the noncompliant lung to achieve relatively normal tidal volumes and carbon dioxide removal.

The ventilated preterm lung has both endothelial and epithelial abnormalities that lead to proteinaceous pulmonary edema after delivery and ventilation.[60] In preterm lambs ventilated for the first 3 hours of life, 11.5% per hour of the labeled albumin mixed with the fetal lung fluid at birth left the lung, and 1.7% per hour of the labeled albumin given intravascularly at birth was recovered from the air spaces, demonstrating the bidirectional movement of albumin across an injured alveolar epithelium.[61] The protein in the alveolar washes increased more than four-fold, indicating that the net protein movement was from the intravascular compartment to the air spaces. The rate of protein accumulation in the air spaces was striking in preterm ventilated rabbits. Approximately 2% to 3% of the intravascularly injected and labeled albumin was recovered from the air spaces within 20 minutes of birth. The leakage was not homogeneous.[62] Leakage involved an increasing number of saccules with time and appeared to occur directly into the alveoli rather than at the bronchiolar level.

Lung gas volumes of infants with RDS can be low because the lung has not yet developed sufficiently to hold much gas or because distal air spaces are uninflated. Another cause of the volume loss in the lungs of infants with RDS is alveolar edema. After 3 hours of ventilation, the total lung capacity was 48 mL/kg in preterm monkeys without RDS and 19 mL/kg for monkeys with RDS.[63] The lungs were flash frozen and evaluated by light and scanning electron microscopy. The lungs of the animals with RDS showed overexpansion of the distal airways and underexpanded and fluid-filled alveolar spaces (Figure 104-9). As seen by scanning electron microscopy, the alveoli of the animals with RDS were filled with proteinaceous liquid, and the interstitium was swollen by edema fluid (Figure 104-10). The monkeys with RDS had alveolar and interstitial edema because of the combination of slow clearance of fetal lung fluid after birth and the entrance of proteinaceous edema into the air spaces.

Four clinical variables are important determinants of the amount of edema formation: gestational age, tidal volume, positive end-expiratory pressure, and surfactant treatment. In preterm animal models, the protein leaks from the vascular space to the alveolar space and from the alveolar space to the vascular space

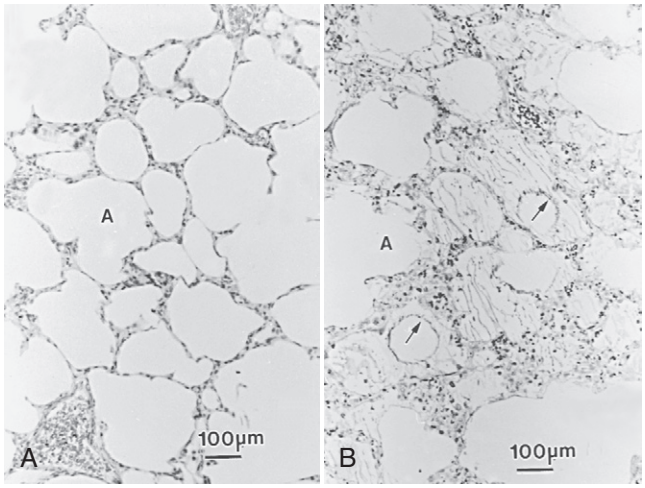


Figure 104-9 Alveolar filling with respiratory distress syndrome. **A,** Light micrograph of lung tissue from a preterm monkey lung without respiratory distress syndrome (RDS). The alveoli and alveolar ducts (indicated by *A*) are air-filled. **B,** In contrast, lung tissue from a monkey with RDS has some air-filled alveoli, and other alveoli are completely or partially filled with proteinaceous material. The *arrows* indicate partially filled alveoli. *(From Jackson JC, Truog WE, Standaert TA, et al: Effect of high-frequency ventilation on the development of alveolar edema in premature monkeys at risk for hyaline membrane disease,* Am Rev Respir Dis *143:865, 1991.)*

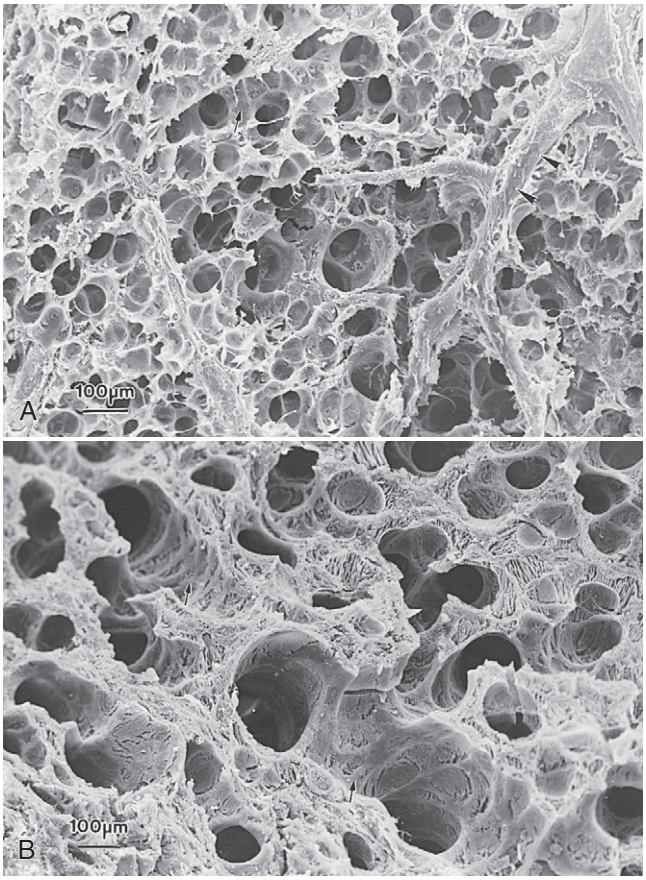


Figure 104-10 Scanning electron micrographs from **(A)** a preterm monkey without respiratory distress syndrome (RDS) and **(B)** a preterm monkey at the same gestational age with RDS. The lung from the RDS animal has dilated alveolar ducts and interstitial and alveolar edema. *(From Jackson JC, Truog WE, Standaert TA, et al: Effect of high-frequency ventilation on the development of alveolar edema in premature monkeys at risk for hyaline membrane disease,* Am Rev Respir Dis *143:865, 1991.)*

increased as gestation decreased[64] (Figure 104-11). The pressure needed to achieve an adequate tidal volume and gas exchange decreased as gestational age increased. Therefore an explanation of the increased leak was the barotrauma caused by the ventilatory requirements needed to support the more immature lung. In the mature lung, pulmonary edema occurred after lung overdistention using high pressures (volutrauma). High pressures were not required to injure the immature surfactant-deficient lung because nonuniform inflation resulted in focal overdistention. Without surfactant treatment, pulmonary edema occurred with relatively low tidal volume ventilation.[51] The end-expiratory volume of the preterm lung also was an important injury variable[65] (Figure 104-12). Ventilation of preterm lambs with the same tidal volume resulted in different amounts of protein recovery from the airways depending on the positive end-expiratory pressure (PEEP) used to support end-expiratory lung volume.[66]

The severity of the surfactant deficiency also influenced the development of pulmonary edema. Surfactant-sufficient preterm lambs and goats did not lose large molecular weight substances from the air spaces, whereas surfactant-deficient animals did.[67] Treatment of premature lambs with surfactant decreased the protein leakage from the vascular space to the airspaces. Surfactant treatment also decreased the endothelial leak of plasma proteins. Surfactant protected the lung from injury caused by high tidal volumes that would otherwise injure the surfactant-deficient lung.[51] A major effect of surfactant is probably by the increased uniformity of lung expansion.[68]

SURFACTANT FUNCTION IN THE ALVEOLUS

Surfactant Forms

Surfactant is present in the alveolus in different structural forms that have different characteristics and functions.[7] After secretion the lamellar bodies unravel to form the elegant structure called *tubular myelin*. This lipoprotein array has SP-A at the corners of the lattice and requires at least SP-A, SP-B, and the phospholipids for its unique structure. Tubular myelin and other loose surfactant lipoprotein arrays in the hypophase generate the surface film within the alveolus and small airways. New surfactant enters the surface film and "used" surfactant leaves as small vesicles, which then are cleared from the air spaces. The major differences in composition between the surface-active tubular myelin and the biophysically inactive small vesicles are that the small forms contain little SP-A, SP-B, or SP-C.

Surfactant is secreted to yield an alveolar pool that is primarily lamellar bodies and tubular myelin. During neonatal transition to air breathing, the percent of surface-active forms falls as the small vesicular forms increase[69] (Figure 104-13). At steady state, approximately 50% of the surfactant in the air spaces is in a surface-active form, and 50% is in the inactive vesicular form. The total surfactant pool size is not equivalent to the amount of active surfactant. The maintenance of surfactant function depends on the preservation of the active forms of surfactant in the air space.

Surfactant Inactivation

Surfactant inactivation or inhibition is a complex concept because it is the integrated result of multiple factors that can alter the alveolar forms and biophysical properties of the surfactant

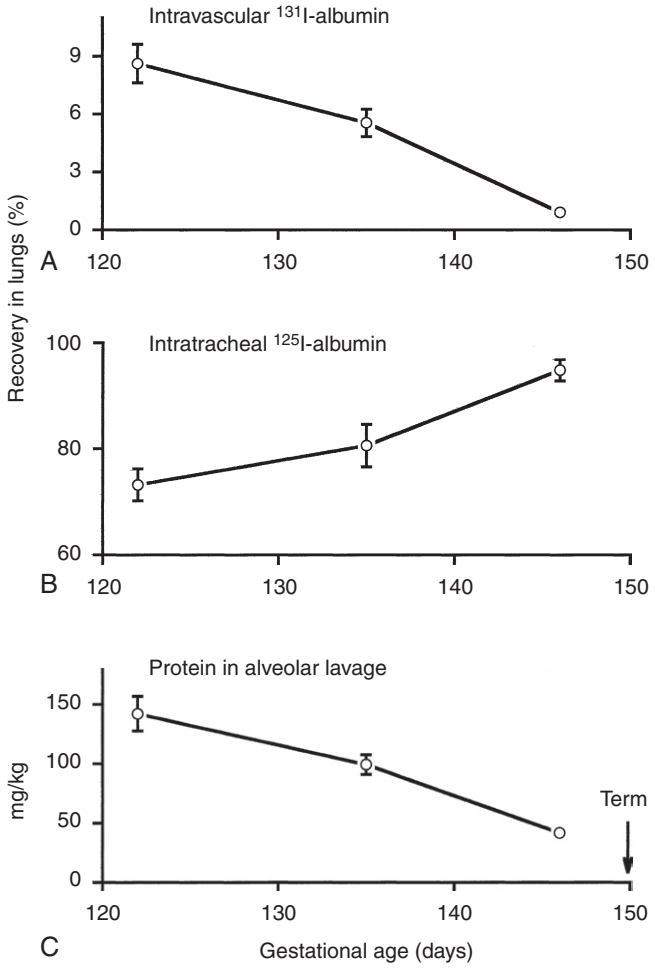


Figure 104-11 Effect of gestational age on the movement of labeled albumin and protein in and out of the air spaces of preterm lambs. Peak ventilatory pressures were held constant at the three gestational ages for 3 hours of ventilation. The more preterm lambs were treated with surfactant to achieve similar ventilatory pressures. **A,** ^{131}I-albumin was given into the vascular space, and the net recovery in alveolar washes and in the total lung (sum of parenchyma plus alveolar wash) decreased as gestational age increased toward term. **B,** ^{125}I-albumin was given into the airways at birth and the amount that was lost from the lungs decreased as gestation increased. **C,** The total amount of protein in alveolar washes decreased as gestation increased. *(Data from Jobe A, Jacobs H, Ikegami M, et al: Lung protein leaks in ventilated lambs: Effects of gestational age,* J Appl Physiol *58:1246, 1985.)*

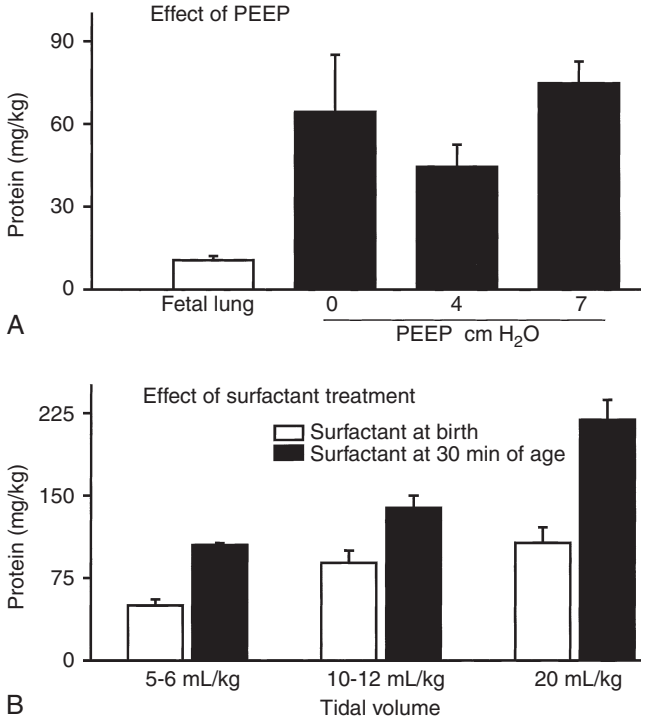


Figure 104-12 Effect of positive end-expiratory pressure (PEEP), tidal volumes, and surfactant on the amount of protein recovered in alveolar lavages of ventilated preterm lambs. **A,** Lambs were ventilated for 7 hours to similar P_{CO_2} values after surfactant treatment with PEEP values of 0, 4 or 7 cm H_2O. In comparison to the protein in the alveolar lavages of unventilated fetal animals, all ventilated animals had increased protein in alveolar lavages. However, the protein was higher with the use of 0 or 7 cm H_2O PEEP than 4 cm H_2O PEEP. **B,** Preterm lambs were ventilated for 30 minutes after birth with different tidal volumes and were then ventilated with a tidal volume of 10 mL/kg until the lambs were 6 hours old. Some lambs were treated with surfactant at birth and others were treated at 30 minutes of age. The high tidal volume of 20 mL/kg increased the protein recovered by alveolar lavage, and surfactant treatment decreased protein recovery at all tidal volumes. *(**A,** Data abstracted from Naik AS, Kallapur SG, Bachurski CJ, et al: Effects of ventilation with different positive end-expiratory pressures on cytokine expression in the preterm lamb lung,* Am J Respir Crit Care Med *164:494, 2001.* **B,** *Data abstracted from Wada K, Jobe AH, Ikegami M: Tidal volume effects on surfactant treatment responses with the initiation of ventilation in preterm lambs,* J Appl Physiol *83:1054, 1997.)*

in the air spaces[70] (Box 104-1). The net effects can be a decrease in the effective pool size, a degradation of the surface tension lowering properties of the surfactant or both. Injury to the alveolar epithelium and edema change the environment where surfactant acts. The mechanisms that contribute to surfactant inactivation will vary with the type of lung injury. The biophysically active surfactant pool can be depleted by an increased rate of conversion to the inactive vesicular forms. This conversion is accelerated by proteinaceous edema and inflammatory products, probably because proteases degrade surfactant proteins.[71] Ventilation styles that use large tidal volumes and no PEEP can deplete the active surfactant pool and high-frequency oscillatory ventilation can preserve the surfactant in adult animal models.[72] However, in surfactant-treated preterm lambs, ventilation with high-frequency oscillation did not have an advantage over conventional ventilation.[52] Meconium and bilirubin can accelerate the conversion to inactive surfactant forms, and surfactant with less surfactant protein will be inactivated more quickly.

Another mechanism of inactivation is the removal of the surfactant by sequestration into clots or hyaline membranes. Surfactant is a thromboplastin and will activate clotting, and in vitro the clot will capture most of the pulmonary surfactant that is in the plasma.[70] Although most soluble proteins can interfere with surface adsorption and film formation, the products of clot lysis are particularly inhibitory.[73] Albumin

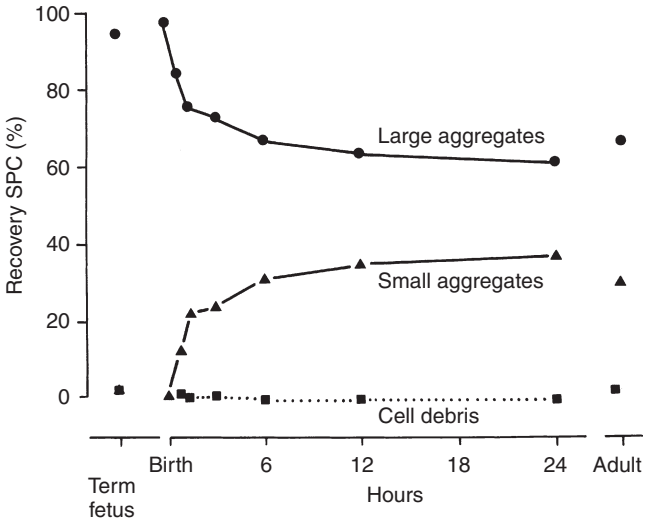


Figure 104-13 Changes in surfactant aggregate size distribution with time after birth. Surfactant suspensions recovered by alveolar wash from term rabbit fetuses and at various ages after birth were fractionated by centrifugation. The large aggregates were recovered by centrifugation, and the small aggregates were the fraction that did not pellet. The distribution of saturated phosphatidylcholine *(SPC)* in fractions is also indicated for alveolar washes from adult rabbits. *(Modified from Bruni R, Baritussio A, Quaglino D, et al: Postnatal transformations of alveolar surfactant in the rabbit: changes in pool size, pool morphology and isoforms of the 32-38 kDa apolipoprotein,* Biochim Biophys Acta *958:255, 1988.)*

BOX 104-1

Mechanisms of Surfactant Inactivation and Substances That Contribute to Inactivation

Form conversions in airspaces

Normal process of alveolar metabolism
Increased conversion with proteinaceous edema (proteases?)
Ventilation, meconium, decreased surfactant protein content

Removal of surfactant from alveolar pool

- Clots and hyaline membranes

Inhibitors of adsorption and film stabilization

- Proteins
  - Edema fluid
  - Plasma
  - Fibrinogen, monomers
  - Albumin
  - Laminin
  - Hemoglobin
- Lipids
  - Lysophosphatidylcholine
  - Cholesterol
  - Red cell membranes
- Other inactivators
  - Meconium
  - Bilirubin
  - Oxidizing agents
  - Amino acids

is not as inhibitory as fibrinogen, although it is the protein that is in the highest concentration in edema and inflammatory fluid. Hemoglobin and other protein products of lung injury also are inhibitors. The phenomenon of interference with film formation by soluble proteins is dependent on both the relative and absolute concentrations of the surfactant and the inhibiting proteins.[74] If surfactant concentrations are high, potent inhibitors at high concentration have little adverse effect when tested in vitro. However, when surfactant concentrations are low, low concentrations of inhibitors can severely degrade surfactant function. Surfactants that contain low amounts of surfactant proteins also are more sensitive to inactivation by soluble proteins.

Lung function will deteriorate when inactivation interferes with enough surfactant to alter the function of the surfactant at the air-fluid interface. Therefore inactivation can be a severe problem when the surfactant pool size is small, as in RDS. Airway samples taken at the time of intubation from infants with RDS had high minimal surface tensions[75] (Figure 104-14). Inhibition of surfactant function contributed to the problem because the airway samples contained surfactant with good function that could be isolated by centrifugation. The soluble proteins from these airway samples inhibited natural surfactant more than did proteins from airway samples from infants without RDS. The importance of surfactant inactivation can be demonstrated using a preterm ventilated lamb model of RDS[76] (Figure 104-15). Lambs at less than 125 days gestation (term is 150 days) have a surfactant pool of less than 1 mg/kg and thus are severely surfactant deficient. Ventilation for 30 minutes caused severe lung injury, and surfactant treatment then resulted in improved oxygenation and a fall in minimal surface tensions of the surfactant in airway samples. However, decreased oxygenation recurred because these animals had progressive lung injury. Most of these adverse effects were avoided by giving the surfactant before ventilating animals with low tidal volumes and a gentle style of ventilation.[51]

These inhibitory effects explain the progressive deterioration in lung function after birth in infants with severe RDS. The infants with RDS have low surfactant pool sizes and with ventilation they develop edema, inflammation, and lung injury. The combination of clot formation (hyaline membranes) and increased soluble inhibitory proteins deplete the functional surfactant pool, which results in progressive respiratory failure.

Inactivation phenomena depend on the type of surfactant being tested. The animal source surfactants in clinical use have in common organic solvent extraction steps that remove nonspecific contaminating proteins, SP-A and SP-D. SP-B and SP-C are retained in the surfactant extract in variable amounts with the phospholipids and neutral lipids. The only functional abnormality in surfactant from mice that lack SP-A is increased sensitivity to inhibition.[77] The addition of SP-A to an organic solvent–extracted surfactant made that surfactant less sensitive to inactivation by albumin and fibrinogen.[78] The potent inactivation of surfactant by fibrinogen was reversed by 0.5% SP-A. Addition of SP-A to a SP-B and SP-C–containing surfactant preserved the in vivo function of the surfactant in the presence of plasma when the mixture was used to treat preterm rabbits.[79,80] These observations may have direct clinical relevance because Hallman and colleagues[28] reported that soluble proteins in airway samples from infants with RDS were much more inhibitory to surfactant samples with low SP-A to saturated phosphatidylcholine ratios than were surfactant samples with higher ratios.

SP-B and SP-C also influence the sensitivity of lipid mixtures to inactivation.[81] Synthetic surfactants that lack these surfactant proteins are sensitive to inactivation by albumin or fibrinogen. Addition of native SP-B and SP-C improved resistance to inactivation.[82] Recombinant SP-C and lipids and mixtures of SP-B and

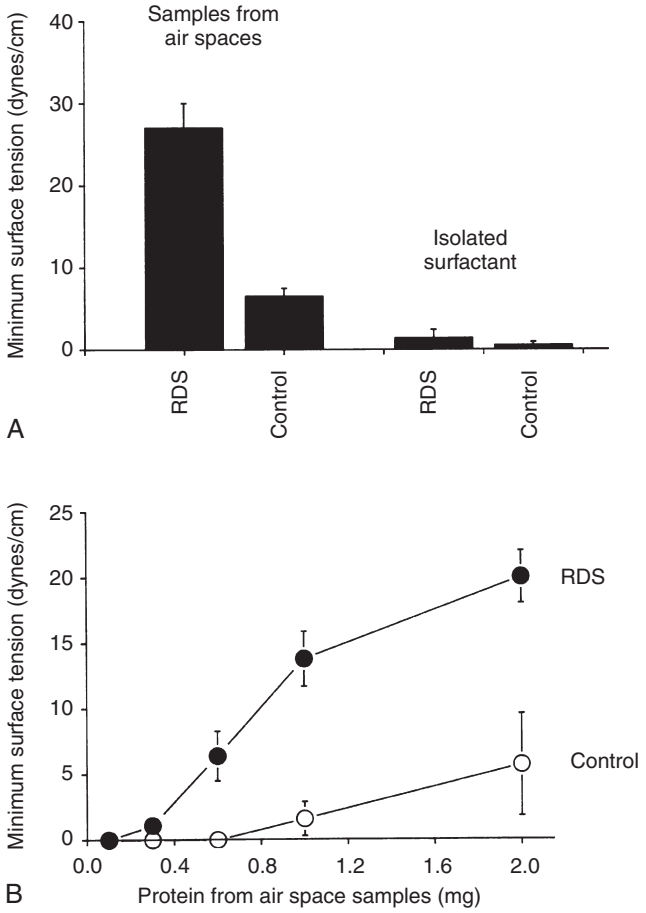


Figure 104-14 Minimum surface tensions of airway samples from (A) infants with respiratory distress syndrome (RDS) and control infants (B) and inhibition of surfactant by proteins. A, Samples from infants with RDS had high minimum surface tensions, whereas control samples had low minimum surface tensions. After isolation of surfactant by centrifugation, the surfactants from RDS and control infants had low minimum surface tensions. **B,** Supernatant protein fractions from airway samples were added to a constant amount of natural sheep surfactant. Protein fractions from infants with RDS had higher inhibitory activity than proteins from the control infants. *(From Ikegami M, Jacobs H, Jobe AH: Surfactant function in the respiratory distress syndrome,* J Pediatr *102:443, 1983.)*

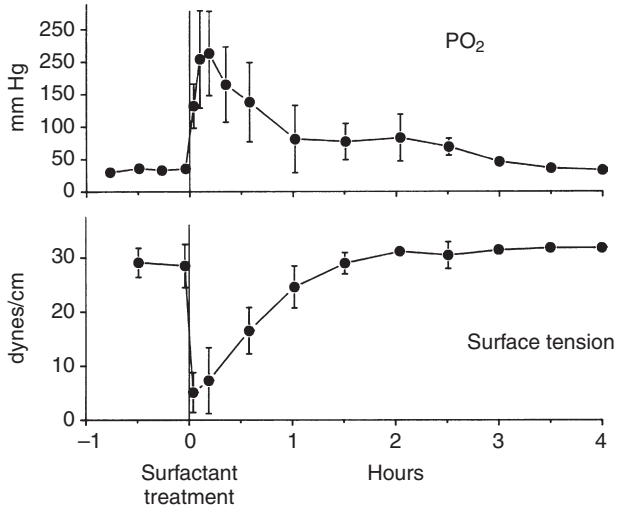


Figure 104-15 Surface tension and clinical response to surfactant treatment. Immature lambs had severe respiratory failure despite ventilatory support and 100% O_2. With surfactant treatment at approximately 45 minutes of age, PO_2 values increased and surface tensions of airway samples decreased. Subsequently the respiratory function of the lambs deteriorated as minimum surface tension values increased in the airway samples. *(From Ikegami M, Jobe A, Jacobs H, et al: A protein from airways of premature lambs that inhibits surfactant function,* J Appl Physiol *57:1134, 1984.)*

SP-C and lipids are less sensitive to protein inactivation than are lipid mixtures alone.

Quality of Surfactant in the Preterm Infant

The preterm infant with RDS has surfactant with properties that are inferior to surfactant from the mature lung, which further compounds the problem of a small pool size of surfactant.[15] Surfactant from the preterm with RDS has a different composition from surfactant from the mature or adult lung. The phosphatidylglycerol content is lower, the phosphatidylinositol content is higher, and the percent saturated phosphatidylcholine is lower. Surfactant from the preterm is less dense than surfactant from term or adult lungs because it contains less SP-A relative to saturated phosphatidylcholine. The alveolar pool from preterm lambs with RDS has a higher percentage of the surfactant in inactive forms than more mature lambs, and in vitro conversion rates from active to inactive forms are more rapid. The surfactant from the preterm also is more sensitive to inactivation in vitro by soluble proteins, most likely because of a lower surfactant protein content. Furthermore, when surfactants from preterm lambs or baboons were tested in preterm surfactant-deficient rabbit lungs, they were less effective at improving compliance than is surfactant from term animals.[15,38] Therefore surfactant from the preterm is intrinsically less effective and more susceptible to inactivation than is surfactant from the mature lung.

Surfactant Function With Surfactant Treatment

The surfactants used clinically are organic solvent extracts of alveolar washes or lung tissue. These surfactants contain no SP-A, the amount of SP-B and SP-C is variable, and processing and sterilization disrupt the lipoprotein structure. The clinical surfactants are more sensitive to inactivation by plasma proteins than is natural surfactant. However, surfactant can have improved function after exposure to the preterm lung. When surfactant is recovered by alveolar wash after surfactant treatment of preterm lambs with RDS, the surfactant has enhanced function.[83] This improved function can be demonstrated by comparing the surfactant used to treat the lambs with the surfactant recovered from the lambs for effects on surfactant-deficient rabbit lungs (Figure 104-16). The very preterm lung cannot improve the function of surfactant used for treatment, whereas the more mature lung can. The mechanism for the enhanced function probably is the mixing of the surfactant used for treatment with small amounts of endogenous surfactant lipids and proteins. Many hours after treatment, the enhanced function of surfactant can occur by recycling of components from the surfactant used for treatment through the type II cells. The sensitivity of the surfactants used clinically to inactivation also can be modulated after exposure to the preterm lung.[84] The surfactants become less susceptible to

inactivation if mixed with 5% or 10% by weight natural surfactant. Therefore small endogenous surfactant pools can interact with the large doses of surfactant used clinically to result in an improved surfactant. This activation phenomenon will not occur if the lung is injured.

Surfactant and Innate Host Defenses

The lung of the infant with RDS is not inflamed at birth unless chorioamnionitis and infection was present before birth. However, the initiation of ventilation in the preterm lung initiates inflammation.[65] The initiation of ventilation results in nonuniform inflation of the surfactant-deficient lung and stretch-mediated injury. If the tidal volume is high or the functional residual capacity is too high or too low, further lung injury will occur. The use of oxygen also may initiate an inflammatory response. Surfactant treatment decreases the severity of this initial inflammation, in part by its mechanical effects on the lungs to make lung inflation more uniform at lower pressures. However, surfactant also can have effects on the inflammatory response because of its multiple innate host defense functions.[85] The preterm lung is severely deficient in SP-A and SP-D. These proteins opsonize a variety of organisms and promote phagocytosis and suppress inflammation in the adult lung. Addition of recombinant SP-D to surfactant prevented the systemic shock caused by intratracheal endotoxin in preterm ventilated sheep.[86] The commercial surfactants do not contain SP-A or SP-D. The hydrophobic proteins SP-B and SP-C also have antiinflammatory properties and can mitigate oxidant-induced injury. The potential for surfactant components to modulate the inflammation that accompanies RDS have not been tested clinically.

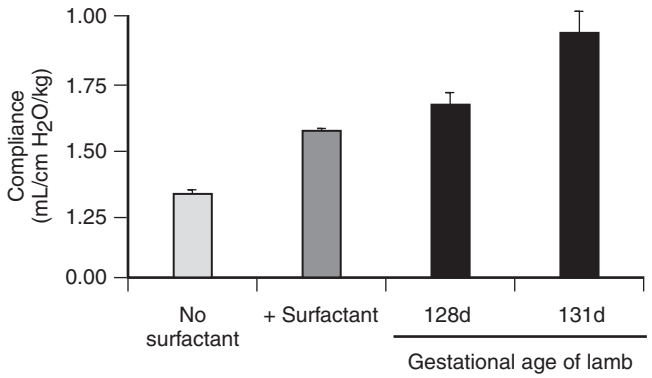


Figure 104-16 Surfactant function after surfactant treatment. Preterm lambs were treated with 100 mg/kg surfactant and ventilated for several hours. Surfactant was recovered by alveolar wash and used to treat preterm surfactant-deficient rabbits. The surfactant from the more mature lambs at 131 days gestation had enhanced function relative to the surfactant used to treat the lambs. *(Modified from Ikegami M, Ueda T, Absolom D, et al: Changes in exogenous surfactant in ventilated preterm lamb lungs,* Am Rev Respir Dis *148:837, 1993.)*

A Model for the Initial Events in Respiratory Distress Syndrome

The preterm infant born at less than 32 weeks gestation and disposed to develop RDS has a saccular lung and a small surfactant pool (Figure 104-17). Antenatal glucocorticoid treatment can increase the surfactant pool and increase the structural potential of the lung for gas exchange.[87] The techniques used for resuscitation and initiating ventilation will influence the initial epithelial and endothelial injury and subsequent ventilation may further aggravate the injury.[88] The small surfactant pool can be inhibited by the products of the injury and initial inflammation that enter the airspaces. Surfactant treatment can decrease the initial lung injury and can increase the surfactant pool size. The surfactant used for treatment becomes substrate for the recycling metabolic pathways. The initial severity of RDS then is a reflection of the reciprocal interactions of surfactant function altered by surfactant inhibition acting on the lung tissue. Surfactant function can be improved with surfactant treatment or by decreasing lung injury. Lung tissue function will be determined by the stage of development and the amount of lung injury. These effects occur at various degrees in different lung regions because the injury that occurs with RDS is not uniformly distributed throughout the lung.

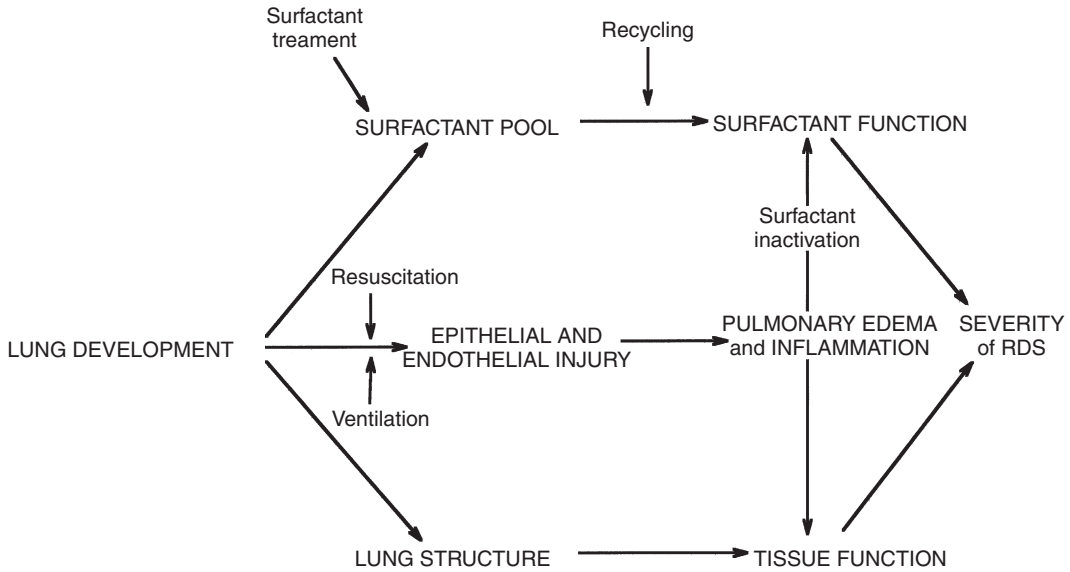


Figure 104-17 Flow diagram of the major variables influencing the clinical course of respiratory distress syndrome (RDS).

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Ian Gross and Philip L. Ballard

Hormonal Therapy for Prevention of Respiratory Distress Syndrome

Over the past 20 years, a multifaceted approach to the prevention of respiratory distress syndrome (RDS) of the newborn has evolved. This approach involves the antenatal prediction of fetal lung maturation by examination of the amniotic fluid, the use of hormones antenatally to accelerate fetal lung maturation, and postnatal surfactant replacement therapy. In this chapter, the clinical evidence that glucocorticoids accelerate fetal lung maturation and act synergistically with postnatal surfactant therapy to decrease the incidence of RDS and other neonatal disorders is reviewed. Guidelines for the use of prenatal glucocorticoid therapy are provided.

BIOLOGIC CONSIDERATIONS

A number of hormones have been shown to influence aspects of fetal lung maturation both in vivo and in vitro[1,2] (Box 105-1).[1,2] Glucocorticoids are the most important of the stimulatory agents; they increase synthesis of all components of surfactant and accelerate morphologic development. Endogenous fetal corticosteroids have a physiologic role in modulating the rate of lung development and likely cause precocious maturation under conditions of in utero stress. Corticoid levels increase in newborn infants who have respiratory disease or other illnesses, and this response may contribute to the resolution of RDS that normally occurs after a few days. Prenatal corticosteroid therapy for prevention of RDS achieves levels of circulating glucocorticoid activity comparable to those during the postnatal surge, and thus antenatal corticosteroid therapy is felt to mimic a physiologic stress response.[3] In the absence of endogenous fetal and maternal corticosteroids (as occurs in homozygous mice genetically engineered with a knockout of either the corticotropin-releasing hormone or glucocorticoid receptor), the lung is structurally and biochemically immature at birth, and the animals die of respiratory failure.[4,5] Effects in the developing lung represent one of many examples of the role of glucocorticoids in modulating tissue differentiation and helping to prepare the fetus for birth.

Glucocorticoids act by binding to specific receptors in lung cells. The receptor-glucocorticoid complex binds to regulatory elements in the promoter of target genes and increases (or in some cases decreases) synthesis of mRNA and protein.[6] Because effects are receptor mediated, responses occur at physiologic levels of glucocorticoid (e.g., 10 to 100 nM cortisol), and higher doses of steroid evoke little additional response. The peak fetal plasma concentration of glucocorticoid after maternal betamethasone treatment (12 mg) is estimated to result in 75% or greater occupancy of receptors.[3] From in vitro studies using both high-resolution gel electrophoresis and DNA microarray profiling of gene expression, it is estimated that 2% to 6% of the proteins in human fetal lung are regulated by glucocorticoids.[7] Glucocorticoids are known to induce the four surfactant-associated proteins, a number of lipogenic enzymes, ion and water transport proteins (Na^+, K^+-ATPase, Na^+ channel, aquaporins), elastin, and a limited number of other proteins; however, the majority of the glucocorticoid-responsive proteins of fetal lung have not been confirmed or characterized.[7,8]

BOX 105-1

Hormones That Influence Fetal Lung Maturation in Animal and Culture Models

Stimulation
- Glucocorticoids
- Cyclic adenosine monophosphate
- Thyroid hormones/thyrotropin-releasing hormone
- Epidermal growth factor
- Retinoic acid
- Bombesin-related peptides
- Parathyroid hormone-related peptide
- Lipopolysaccharide
- ? Estrogen

Inhibition
- Insulin/hyperglycemia/?butyrate
- Tumor-necrosis factor alpha
- Transforming growth factor-β
- Interleukin-1
- Phorbol esters
- Androgen

Although an increase in transcription of responsive genes in cultured lung occurs in 1 to 2 hours of corticosteroid exposure, accumulation of new proteins usually requires 12 to 24 hours. The earliest effect on pulmonary function in the fetal lamb occurred between 8 and 15 hours after treatment; optimal responses were observed at 48 hours and persisted for at least 7 days.[9] Thus a benefit of glucocorticoid administration may not be observable in those situations in which the infant is delivered shortly after therapy is initiated.[10,11] It is known that receptor-bound glucocorticoid is necessary for continuing hormone action[1,2] and that glucocorticoid action in vitro is often reversible on removal of hormone (e.g., induction of surfactant proteins in cultured lung and in fetal sheep).[12,13] Therefore some glucocorticoid responses may be of limited duration in utero. Clinically, reduction of RDS appears to diminish 7 to 10 days after hormone administration is completed.[11] For this reason, retreatment has been used for women with recurring premature labor, and, as will be discussed later, the efficacy and safety of this practice is currently being investigated.

Glucocorticoids also regulate gene expression by an indirect process termed transrepression in which the corticosteroid-receptor complex interacts with transcription factors such as activator protein-1 (jun/fos) and NFκB, regulators of cytokine production. This interaction blocks up-regulation of the cytokine/chemokine cascade in response to various stimuli and is the major mechanism for the antiinflammatory effects of glucocorticoids.[14] Possible involvement of the transrepression mechanism in glucocorticoid effects on fetal lung development are not known but are possible because several cytokines are known to inhibit surfactant production.

The physiologic role and mechanism of other hormones that stimulate lung development (see Box 105-1) have been less extensively studied. An increased endogenous level of the second messenger cyclic adenosine monophosphate stimulates type II cell differentiation and induces surfactant lipids and surfactant-associated proteins (SP)-A and SP-B in cultured fetal lung.[12] Some evidence suggests that treatment of fetal animals with terbutaline or other β-agonists, particularly in combination with glucocorticoid, enhances lung maturation. It is possible that cyclic adenosine monophosphate–mediated effects occur clinically after treatment with β-agonists, prostaglandins or their inhibitors, or agents that inhibit phosphodiesterases (e.g., xanthine derivatives), but possible clinical benefit or harm is not documented. Thyroid hormones stimulate surfactant lipid synthesis in cultured tissue and in fetal lambs, acting in a synergistic manner with glucocorticoids (and prolactin in vivo).[15-17] Nuclear receptors for triiodothyronine (T_3) are present in fetal lung cells, and the properties of thyroid hormone stimulatory effects (e.g., response with physiologic concentrations of hormone) are consistent with a receptor-mediated process.[16] Target proteins for thyroid hormones in fetal lung, except for one lipogenic enzyme (cholinephosphate cytidylyltransferase), are unknown. Clinically, injection of thyroid hormone into amniotic fluid has been reported to cause a slow increase in the lecithin/sphingomyelin ratio, but clinical benefit and safety have not been investigated.[17] Combined glucocorticoid and thyrotropin-releasing hormone (TRH) treatment has been investigated as a potentially useful clinical approach for prevention of newborn lung disease (see later discussion).

Several agents have an inhibitory effect on levels of surfactant components in vitro (see Box 105-1). Tumor necrosis factor-α, transforming growth factor β (TGF-β) and activators of protein kinase C such as phorbol esters and lipopolysaccharide (endotoxin) are of particular interest because they are involved in the etiology of lung inflammation and infection. A negative effect of these agents on levels of SP-A and SP-B has been observed in cultured lung, even when there is combined treatment with glucocorticoids. In cultured fetal lung cells, treatment with glucocorticoid reduces levels of endogenous TGF-β, which likely contributes to type II cell differentiation by relieving suppressed expression of a subset of regulated genes.[18] Thus antenatal glucocorticoid treatment may act both directly (gene induction) and indirectly (up-regulation of TGF-β–suppressed genes) to accelerate lung maturation.

Dysfunction of surfactant occurs in both infants and adults with specific lung diseases. Ventilated premature infants experience episodes of respiratory decompensations that are associated with dysfunctional surfactant and low surfactant content of SP-B.[19] Down-regulation of SP-B may occur secondarily to infection and inflammation with increased production of cytokines. It is possible that down-regulation of surfactant components contributes to the pathobiology of newborn lung diseases such as meconium aspiration, bronchopulmonary dysplasia, and pneumonia.

CLINICAL EVIDENCE FOR THE EFFECTIVENESS OF ANTENATAL GLUCOCORTICOID THERAPY

The effects of glucocorticoids on lung maturation and perinatal outcome have been extensively studied. Liggins and Howie[20] reported in 1972 that a significant reduction in the incidence of RDS and mortality in premature infants occurred after maternal treatment with glucocorticoids. However, despite the positive effects noted in this and many other studies, some obstetricians continued to question the effectiveness and safety of the use of prenatal glucocorticoids for the prevention of RDS. In 1993 it was estimated that only 15% of preterm infants between 500- and 1500-g birth weight were receiving prenatal glucocorticoid therapy in the United States.[21] In 1994, the National Institutes of Health (NIH) convened a Consensus Conference[23] to examine the data and make recommendations relating to glucocorticoid therapy, fetal maturation, and perinatal outcome. The clinical information used was primarily the comprehensive meta-analysis of published randomized trials of Crowley[22,23] and several observational databases. The latter consisted of treatment and outcome information that was prospectively collected from five registries, including multicenter surfactant trials, and involved more than 30,000 low-birth-weight infants. The major findings of these two sources of information will be reviewed

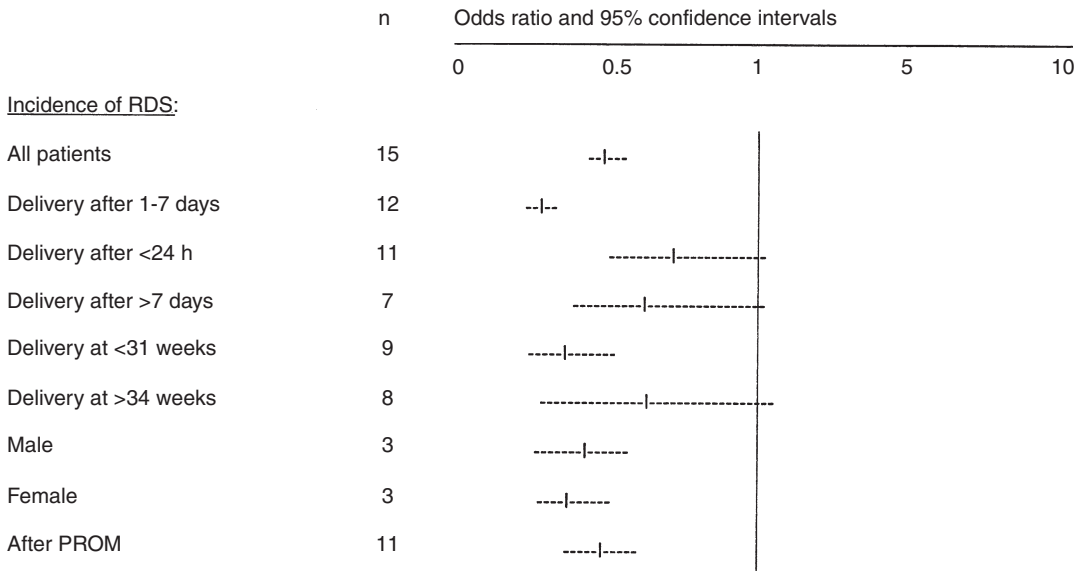


Figure 105-1 Meta-analysis of randomized clinical trials of antenatal glucocorticoid therapy for prevention of respiratory distress syndrome. A significant reduction in the incidence of respiratory distress syndrome is indicated if the mean and 95% confidence interval are less than 1.0 (*n* = number of trials evaluated). *(Data from Crowley P: Promoting pulmonary maturity. In Chalmers IM, Keirse MJNC, editors:* Effective Care in Pregnancy, *vol 1. Oxford, UK, 1989, Oxford University Press, p 746.)*

here, as well as the recommendations of this NIH Consensus Conference.

Impact of Antenatal Glucocorticoids on the Incidence of Respiratory Distress Syndrome

The meta-analysis[22] (Figure 105-1) revealed that glucocorticoid therapy significantly reduced the overall incidence of RDS by approximately 50%. The effect was greatest in those infants who were delivered from 1 to 7 days after the initiation of maternal treatment. A trend toward decreased RDS was observed in babies born less than 24 hours or more than 7 days after the initiation of therapy, but the differences were not statistically significant.

Steroids were effective over a wide gestational age range. A reduction in the incidence of RDS in babies born before 31 weeks of gestation was significant. Babies born after 34 weeks are a heterogeneous group and some have lungs that are almost mature. A trend toward decreased RDS was seen in this age-group. The effectiveness of glucocorticoids in infants born before 28 weeks gestation has been a source of controversy. Although it has been questioned whether the incidence of RDS is decreased,[24] benefit is seen in terms of diminished severity of RDS, decreased mortality, and a lower incidence of intraventricular hemorrhage (IVH).

The U.S. Collaborative Antenatal Steroid Therapy trial[10] found that male fetuses were not responsive to glucocorticoid therapy. However, these data were not appropriately analyzed and this finding has not been replicated in other studies, and the meta-analysis reveals that decrease in RDS is significant in both male and female infants.

Effect of Antenatal Glucocorticoids on Complications Associated With Premature Birth

According to the meta-analysis, as is shown in Figure 105-2, antenatal glucocorticoid therapy results in decreased early neonatal mortality as well as a reduction in two major complications of prematurity, IVH, and necrotizing enterocolitis. The observational database did not support the decrease in necrotizing enterocolitis, but it did find a decreased incidence of IVH, even in infants born less than 24 hours after the first dose of steroid. The decrease in these two complications of prematurity may relate to the fact that steroid-treated babies have a more benign clinical course, but specific maturational effects of glucocorticoids on the gut and brain may also be factors. The intestine, like the lung, arises from the embryonic endodermal tube, and in animals glucocorticoids induce specific enzymes in the developing gut and cause precocious maturation of closure (restricted passage of intact proteins).[3]

When glucocorticoid therapy for the prevention of RDS was first proposed, major concerns arose regarding potential toxicity, particularly to the developing brain. Studies with newborn rats, whose brains are at approximately the same stage of development as a third trimester human, had shown that administration of large doses of glucocorticoid results in a reduction in numbers of brain cells, particularly in the cerebellum, where cells are undergoing rapid mitosis. The doses and relative exposure time in these animal studies, however, far exceeded those used in the clinical trials. Long-term follow-up studies of infants exposed to antenatal glucocorticoids have revealed no adverse effects on developmental outcome. At 12 years of age, no deficits in motor skills, language, cognition, memory, or scholastic achievement were observed, despite greater survival of smaller infants in the steroid-treated group.[25,26] In fact, meta-analysis of three published studies reveals a trend toward decreased neurologic abnormalities at follow-up. There also appear to be no long-term adverse effects on physical growth or health. It should be noted that these studies did not involve repeated weekly courses of glucocorticoids. As will be discussed later, concerns exist relating to fetal toxicity when repeated courses of steroids are administered to pregnant women.

Effects of Antenatal Glucocorticoids on Neonatal and Maternal Infection

One of the concerns regarding glucocorticoid therapy has been its impact on infection. Meta-analysis indicates that a small increase in maternal infection (odds ratio 1.15) and a slight reduction in neonatal infection (odds ratio 0.80) occurs, but

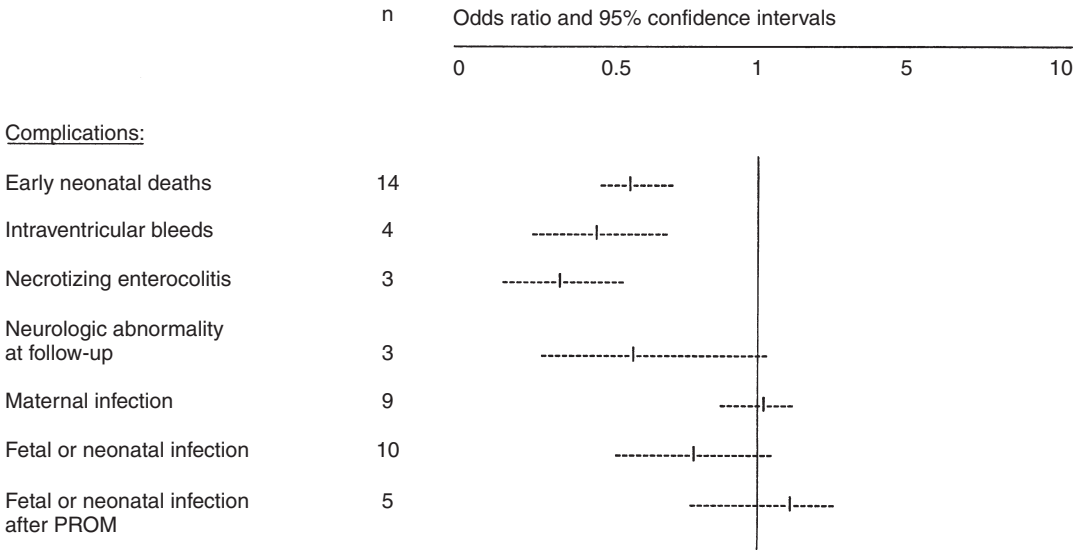


Figure 105-2 Meta-analysis of clinical trials of prenatal glucocorticoids: effects on perinatal complications. Therapy is associated with a significant reduction in deaths, intraventricular hemorrhage, and necrotizing enterocolitis (n = number of trials evaluated). *(Data from Crowley P: Promoting pulmonary maturity. In Chalmers IM, Keirse MJNC, editors:* Effective Care in Pregnancy, *vol 1. Oxford, UK, 1989, Oxford University Press, p 746.)*

neither of these effects is statistically significant. When steroids are administered in the presence of premature rupture of the membranes (PROM), a trend toward an increase in fetal and neonatal infection (odds ratio 1.46) is seen, but this effect is also not statistically significant.

Effects of Antenatal Glucocorticoids in the Presence of Premature Rupture of the Membranes

Although the risk of fetal or neonatal infection may be somewhat increased, analysis of the randomized trials reveals that glucocorticoids decrease the incidence of RDS in the presence of PROM. Evidence from the observational database indicates that glucocorticoid therapy in patients with PROM results in a decrease in IVH and overall mortality. Thus the overall maternal-infant benefit/risk ratio for steroid therapy is also favorable with PROM.

Use of Glucocorticoids in Complicated Pregnancies

Liggins and Howie[22] reported that fetal mortality increased when steroids were administered to women with hypertension and proteinuria. However, this observation was not confirmed in two subsequent trials, and apparently the result arose from mismatched groups in terms of severity of preeclampsia.[10,27]

Glucocorticoid administration may complicate the management of diabetes in pregnancy and the decision to use steroids in this situation is a matter of clinical judgment.

Little information is available on the effectiveness of glucocorticoid therapy in pregnancies complicated by multiple gestation, intrauterine growth restriction, or hydrops fetalis. In these circumstances, fetuses should probably receive routine steroid therapy.

Recommendations of the 1994 NIH Consensus Conference

Antenatal glucocorticoid therapy clearly has several significant benefits for the premature infant. These are summarized in Box 105-2. The recommendations of the 1994 NIH Consensus Conference are listed in Box 105-3. These recommendations were drafted after review and discussion of the relevant data and provide authoritative guidelines. With the publication and dissemination of these guidelines, there appears to be no reason to withhold steroid therapy in patients who meet the recommended criteria.

BOX 105-2

Benefits of Antenatal Glucocorticoid Therapy

From meta-analysis of published studies
- Decreased respiratory distress syndrome
- Decreased incidence of intraventricular hemorrhage
- Decreased incidence of necrotizing enterocolitis
- Decreased mortality
- A trend toward better neurologic and intellectual function at follow-up

From observational database
- Decreased respiratory distress syndrome
- Decreased incidence of intraventricular hemorrhage, even if delivery occurs less than 24 hours after initiation of therapy
- Decreased mortality

From individual studies
- Decreased incidence of clinically significant patent ductus arteriosus
- Decreased duration (and cost) of hospitalization

Since the 1994 NIH Consensus Conference, attention has focused on the question of dexamethasone-specific toxicity, the impact of repeated courses of antenatal glucocorticoids, and the effects of combinations of hormones. A review of these topics follows.

REPEATED COURSES OF ANTENATAL GLUCOCORTICOIDS

Both experimental[12] and clinical studies (see Figure 105-1) suggest that some of the effects of antenatal glucocorticoids on fetal lung development diminish after approximately a week.

BOX 105-3

Summary of Recommendations of the National Institutes of Health (NIH) Consensus Development Conference: The Effect of Corticosteroids for Fetal Maturation on Perinatal Outcomes

- All fetuses between 24 and 34 weeks gestation that are at risk for preterm delivery are candidates for treatment.
- The decision should not be altered by fetal race, sex, or availability of surfactant therapy.
- Prenatal corticosteroids should be given unless immediate delivery is anticipated.
- Tocolytic therapy is not a contraindication.
- *In the absence of clinical chorioamnionitis,* antenatal corticosteroids are recommended in patients with premature rupture of membranes at less than 32 weeks gestation.
- Treatment is recommended in complicated pregnancies, unless evidence suggests that it will have an adverse effect on the mother.

NOTE: Although the Consensus Conference recommended either dexamethasone or betamethasone, the currently recommended regimen is betamethasone, 12 mg q24h × 2 doses intramuscularly.

The question has arisen of what to do if a pregnant mother is given a course of steroids because she is at risk for premature delivery, but the pregnancy then continues without delivery having occurred. It was the practice of some obstetricians to give repeated, even weekly, courses of steroid in this situation until the pregnancy progressed beyond the stage where the infant would have been born prematurely.

Studies in a variety of animal species have shown that multiple courses of antenatal glucocorticoids do result in greater enhancement of lung maturation than a single course, but that associated toxicity, particularly growth restriction, is a risk.[28] A retrospective analysis of the data derived from a multicenter randomized clinical trial of antenatal TRH plus glucocorticoid compared with glucocorticoid alone (both dexamethasone and betamethasone were used)[29,30] revealed that compared with infants who received one course of glucocorticoid, those who were exposed to two courses had lower birth weights, and administration of three courses was associated with increased risk for death and evidence of adrenal suppression. Furthermore, administration of two or three courses did not result in improved outcome in terms of RDS, IVH, or chronic lung disease.

The NIH Consensus Conference in 2000[31] revisited the issue of antenatal steroids and recommended that repeat courses should not be used routinely. The Cochrane review of this subject, which was updated in 2003,[32] was limited by the fact that only three studies were included and many of the analyses involved only one or two trials. This review reported that no decrease in the incidence of RDS was seen after multiple courses of glucocorticoids compared with a single course. One study found that the severity of lung disease was less in the repeated course group and in two studies fewer infants in the repeated dose group received surfactant.

Two larger, multicenter, randomized clinical trials have now examined this issue. The National Institute of Child Health and Human Development (NICHD) Maternal Fetal Units Network enrolled pregnant women with intact membranes between 23 weeks and 32 weeks gestation who had received one course of glucocorticoid.[33] They were then randomized to weekly courses of betamethasone or placebo. The primary end point was the presence of any of the following neonatal conditions: severe RDS, IVH of grade III or IV, periventricular leukomalacia, chronic lung disease, or stillbirth or neonatal death. The study was terminated early because of a tendency to decreased birth weight in the repeat steroid group without a significant reduction in the primary end point. However, decreases were seen in specific neonatal outcomes, including the number of infants who required surfactant therapy, ventilator support, and continuous positive airway pressure (CPAP), and in the incidence of pneumothoraces. The authors commented that a major problem with repeated dose therapy is that the majority (72%) of women who received repeated doses of steroids ended up delivering after 32 weeks gestation, so their newborn infants were not at high risk for RDS. However, a benefit was seen for infants delivering at less than 32 weeks gestation.

The Australasian Collaborative Trial of Repeat Doses of Steroids[34] reported a more positive short-term outcome in infants exposed to repeated courses of antenatal betamethasone. In this trial, women who remained at risk for preterm birth at less than 32 weeks gestation were randomized to placebo or weekly courses of betamethasone. The repeat doses group demonstrated a significant decrease in the primary endpoints, RDS, severe lung disease, need for oxygen therapy, and duration of mechanical ventilation. Mean weight, length, and head circumference at birth and discharge did not differ between the groups. (Z-scores for weight and head circumference at birth, but not at discharge, were lower in the repeated treatment group.) Long-term follow-up of these infants has not been reported yet.

Given the current state of knowledge in this area, we recommend that an initial course of antenatal betamethasone should be administered if concern exists that premature delivery will occur. If the pregnancy continues, one additional course of betamethasone may be considered if threatened premature delivery recurs 10 or more days later.

DEXAMETHASONE-SPECIFIC TOXICITY

In one study antenatal dexamethasone therapy was recently found to result in a higher incidence of periventricular leukomalacia (PVL) than antenatal betamethasone. In a retrospective analysis, Baud and colleagues[35] examined the effects of dexamethasone, betamethasone, or no antenatal steroid therapy on the incidence of RDS, IVH, PVL, and necrotizing enterocolitis. Administration of either of the two glucocorticoids resulted in a decrease in all these complications of prematurity, except PVL. Whereas betamethasone-treated infants had less PVL than non–steroid-treated infants, those that had been exposed to antenatal dexamethasone had more. It was suggested that the sulfites in dexamethasone were responsible for this effect and in vitro studies support this possibility.[36] However, sulfites are present in many medications that are routinely given to neonates. An alternative explanation relates to the method of administration of these two glucocorticoids. Dexamethasone is usually given intravenously, whereas betamethasone is administered intramuscularly as a slowly released mixture of phosphate and acetate forms. The higher peak blood levels of dexamethasone may be responsible for the toxicity, perhaps mediated by a mechanism that does not involve receptor binding to DNA.[37]

The apparent increased central nervous system-related risk associated with antenatal dexamethasone therapy is also supported by recent follow-up observations related to early postnatal use of this corticosteroid. Treatment of infants with dexamethasone to prevent bronchopulmonary dysplasia in the first few days after birth, but not after 7 days, is associated with increased cerebral palsy and other abnormal neuromotor

outcomes. This adverse effect of postnatal dexamethasone may reflect the relatively high doses used, intravenous administration, delayed clearance due to liver immaturity, and prolonged treatment regimens.

SYNERGISM BETWEEN ANTENATAL GLUCOCORTICOID AND POSTNATAL SURFACTANT THERAPIES

Several animal studies have demonstrated the benefit of antenatal glucocorticoid combined with postnatal surfactant therapy. Work by Fiascone and colleagues[38] and Ikegami and colleagues,[39] in which prematurely delivered fetal rabbits were ventilated and lung compliance was measured, found that both prenatal glucocorticoid treatment and postnatal natural surfactant treatment resulted in a significant improvement in lung compliance. However, the combination of steroid and surfactant produced a greater effect than did either therapy alone. These results are consistent with the observations that glucocorticoids increase nonsurfactant components of lung compliance, such as tissue elasticity, in addition to enhancing surfactant production. Glucocorticoids also accelerate the development of lung parenchymal structure, decreasing mesenchymal volume and septal thickness, and these changes per se can increase tissue compliance.[40] It is also possible that the enhanced alveolarization resulting from glucocorticoid treatment provides a better substrate for surfactant therapy.

Clinical data also support the positive interaction between antenatal glucocorticoid and postnatal surfactant therapy. Kari and associates[41] randomized patients with threatened preterm delivery at less than 32 weeks gestation to antenatal glucocorticoid therapy or placebo. All babies born before 33 weeks who required ventilation with greater than 40% oxygen also received postnatal surfactant. As listed in Table 105-1, the infants who were exposed to antenatal steroids had significantly less respiratory disease as well as a lower incidence of IVH and PVL.

COMBINED HORMONAL TREATMENT FOR THE PREVENTION OF RESPIRATORY DISTRESS SYNDROME

Even with optimal glucocorticoid treatment some infants develop RDS. In an effort to further enhance lung maturation, the effects of combinations of hormones have been studied in experimental models.[15-17] Evidence suggests that thyroid hormones stimulate lung development and may act synergistically with glucocorticoid. Triiodothyronine (T_3) is the most active thyroid hormone in terms of surfactant phospholipid production, but it does not cross the placenta well, whereas TRH administered to the mother is transferred to the fetus and increases TSH, T_3, and thyroxine (T_4) levels in cord blood.[42,43] The combination of glucocorticoid and TRH has been evaluated in a number of randomized clinical trials.

A series of trials was completed before the widespread availability of surfactant. These studies generally found that there was a decreased incidence of RDS, chronic lung disease, and mortality in babies who delivered from 1 to 10 days after the initiation of maternal therapy with TRH and glucocorticoid compared with glucocorticoid alone.[44] However, three large trials, which also incorporated postnatal surfactant therapy, were subsequently published.[45-46] These trials, which included an "intention to treat" analysis, indicated that no additional benefit was observed when TRH was added to glucocorticoid therapy. Collectively these findings suggest that a combination of prenatal glucocorticoid and postnatal surfactant is an effective therapy, which is not enhanced by the addition of TRH.[47]

TABLE 105-1

Prenatal Glucocorticoid Effects in the Era of Postnatal Surfactant Therapy

	Dexamethasone (n = 41)	Placebo (n = 38)
Gestational age (wk)	29.9 + 2.0	29.8 + 2.3
Respiratory distress syndrome, incidence	44%	79%*
Received surfactant	22%	53%*
Days ventilated	2.0	5.3*
Days in oxygen	2.0	7.0*
Intraventricular hemorrhage or periventricular leukomalacia	10%	46%*

Data from Kari MA, Hallman M, Eronen M, et al: Prenatal dexamethasone treatment in conjunction with human surfactant therapy—a randomized placebo-controlled multicenter study, *Pediatrics* 93:730, 1994.

*$p < .05$.

Infants born 1 to 14 days after glucocorticoid treatment were analyzed.

CONCLUSION

Development of the fetal lung is influenced by a number of hormones and agents that either accelerate or inhibit differentiation. Endogenous and administered corticosteroids modulate the developmental process, acting through glucocorticoid receptors to induce a network of gene products involved in cellular differentiation and production of surfactant. Animal and clinical data demonstrate that the advent of surfactant therapy has further enhanced the benefits of antenatal glucocorticoid administration for lung maturation. Optimal management of premature infants currently consists of prenatal hormone administration followed by postnatal surfactant therapy, if indicated. Betamethasone is the glucocorticoid of choice. The 2000 NIH Consensus Conference[33] recommended that repeat courses should not be used routinely. This approach has had a significant impact on the prevention or amelioration of RDS and other major complications in prematurely born infants.

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Alan H. Jobe

Surfactant Treatment

A brief history of the development of surfactant for the treatment of respiratory distress syndrome (RDS) provides perspective on how this major therapeutic advance occurred. In 1959, not long after surfactant had been identified as critical to maintaining lung inflation at low transpulmonary pressures,[1,2] Avery and Mead[3] reported that saline extracts from the lungs of preterm infants with RDS lacked the low surface tensions characteristic of pulmonary surfactant. After unsuccessful attempts to treat infants with RDS with aerosolized phospholipids,[4] intratracheal administration of surfactant recovered from the air spaces of mature animal lungs was found to improve lung expansion and ventilation in preterm animals.[5,6] The clinical potential of surfactant treatment for RDS was demonstrated by Fujiwara and colleagues[7] in a nonrandomized study in 1980, using a surfactant prepared from an organic solvent extract of bovine lung. Small, investigator-initiated, randomized, controlled trials testing surfactants prepared from bovine alveolar lavage or human amniotic fluid demonstrated significant decreases in pneumothorax and death by 1985.[8-10] Subsequent multicenter trials demonstrated decreased death rates and complications of RDS.[11] A synthetic surfactant was approved by the Federal Drug Administration (FDA) for the treatment of the syndrome in the United States in 1990, and surfactant from bovine lung was approved in 1991. Surfactants were a new class of drug developed specifically for preterm infants. Current research interests are the optimal timing of surfactant treatments and ventilatory support strategies and the development of synthetic surfactants that contain recombinant surfactant proteins.

SURFACTANTS FOR CLINICAL USE

Surfactants for clinical use were developed empirically by testing surface properties of mixtures of lipids and proteins with the goal to emulate the properties of natural surfactant.[12] Although dipalmitoylphosphatidylcholine is the principal surface active component of surfactant, it is not an effective surfactant because it is a solid at 37° C and does not rapidly spread or adsorb to a surface.[13] Dipalmitoylphosphatidylcholine forms a stable film with an equilibrium surface tension similar to that of natural surfactant, and when spread from an organic solvent, surface tension values approach 0 dynes/cm with surface compression. Surface adsorption can be enhanced by adding other components, such as dioleoylphosphatidylcholine, phosphatidylglycerol, or cholesterol. Simple mixtures of dipalmitoylphosphatidylcholine with other lipids can have surface properties similar to those of a natural surfactant.[14]

Two synthetic surfactants were extensively evaluated in clinical trials and were in clinical use until around the year 2000. A 7:3 weight ratio of dipalmitoylphosphatidylcholine and unsaturated phosphatidylglycerol was used in England.[15] The other synthetic surfactant to be used clinically was a mixture of dipalmitoylphosphatidylcholine, hexadecanol, and tyloxapol. These synthetic surfactants were not as effective as natural surfactants and are no longer used clinically.[16]

Highly surface active surfactants recovered from animal lungs were effective surfactant treatments for preterm surfactant-deficient lungs, demonstrating that formulations for clinical use could come from animal sources.[17] The surfactants from natural sources are of two general types: surfactants recovered from alveolar lavages and surfactants recovered from minced lungs. The surfactant first used clinically by Fujiwara and colleagues[7] was prepared by saline extraction of minced lungs. The saline extract was then further extracted with organic solvents to yield phospholipids, neutral lipids, and small amounts of the hydrophobic surfactant-associated proteins (SP)-B, and SP-C. This lipid extract contains the components of natural surfactant except SP-A. However, it also has increased amounts of unsaturated phospholipids and neutral lipids that interfere with surface properties. Synthetic lipids were added to improve surface properties[12] and enhance clinical effects. Another surfactant for pig lung was developed using the strategy to remove the neutral lipids from the lipid extract that interfere with function by liquid-gel chromatography.[18] This surfactant contains 99% polar lipids, mainly phospholipids, and 1% SP-B plus SP-C, with the neutral lipids removed.

Another approach to making an animal source surfactant is to recover surfactant from alveolar lavages and prepare organic solvent extracts of that material. Several commercial surfactants made from large bovine lungs are in clinical use. The organic solvent extraction step removes nonessential proteins that might be sensitizing. Peptides that mimic the biophysical functions of surfactant proteins also can be combined with lipids to make effective surfactants.[19]

PHYSIOLOGIC EFFECTS OF SURFACTANT TREATMENT ON PRESSURE-VOLUME CURVES

The pressure-volume curve defines the static effects of surfactant on the preterm lung[20] (Figure 106-1). When comparing the lungs of control preterm animals with surfactant-treated animals, surfactant decreased the *opening pressure*, defined as the pressure at which the lung begins to fill above dead space volume. The opening pressure of preterm surfactant deficient rabbit lungs decreased from approximately 20 to 15 cm H_2O with surfactant treatment in this example. The explanation for this effect is that the surfactant-deficient immature lung resists inflation because of the high surface tensions at the interfaces of gas with fluid in small airways. Surfactant decreases the resistance

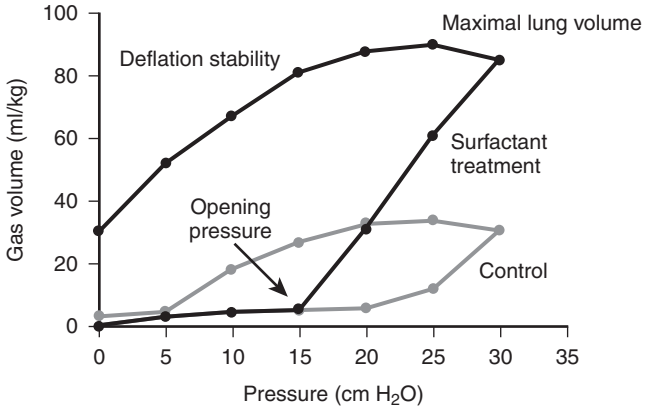


Figure 106-1 Representative pressure-volume curves for 27-day preterm rabbit lungs treated with surfactant in comparison to control lungs.The curves were measured after a 30-minute period of ventilation. Surfactant has pronounced effects on opening pressure, maximal lung volume, and deflation stability relative to the control lungs. *(Modified from Rider ED, Jobe AH, Ikegami M, et al: Different ventilation strategies alter surfactant responses in preterm rabbits,* J Appl Physiol *73:2089, 1992.)*

to fluid movement in the airways and expansion of lung units is facilitated. The major effect of surfactant treatment is the large increase in lung volume at 30 cm H_2O. Because dead space volume will not change with surfactant treatment, this increase in volume represents the recruitment of more surface area for gas exchange. No maximal lung volume (V_{max}) is distinct for the preterm lung. In the mature lung, V_{max} is defined by the limits of expansion of the lung matrix. In contrast the preterm lung will continue to expand with increasing pressure until rupture occurs.[21] For the surfactant-deficient preterm lung, the potential gas volume cannot be achieved because inflation pressures needed to recruit that volume will exceed the rupture pressure. Surfactant also stabilizes the lung during deflation and maintains a lung volume at 5 cm H_2O pressure in excess of the V_{max} achieved in control lungs. The dynamic equivalent to static deflation stability is the functional residual capacity, which will increase with surfactant treatment.

Evaluation of degree and uniformity of inflation is another way to assess surfactant effects on the lung. Based on the simplest model of two alveoli as interconnecting spheres, surfactant deficiency will cause alveolar (or small airway) instability characterized by collapse of units with small radii and dilation of units with large radii. This instability results from Laplace's law: pressure at the surface of an alveolus = 2 × surface tension/radius. Because the alveolus is open to atmospheric pressure, the pressure outside must be balanced by the pressure on the inner surface or the alveolus will collapse. The forces acting on the alveolus are chest wall elasticity, lung tissue elasticity, and surface tensions at the alveolar surface. All alveoli are not the same size. Therefore Laplace's law predicts that the retractive force of surface tension will be greater on the small alveoli than on the large alveoli, with the result being collapse of the small alveoli with further filling of the large alveoli. Surfactant, however, has the unique property of variable surface tension depending on the rate and amount of surface area compression. As alveolar radius decreases, surface tension falls, preventing collapse of the small alveolus. This stabilizing influence is lost in the absence of surfactant.

The concept of interdependence results from the scaling up of the alveolar model. Each alveolus depends on the position and elasticity of the neighboring alveolar walls to maintain

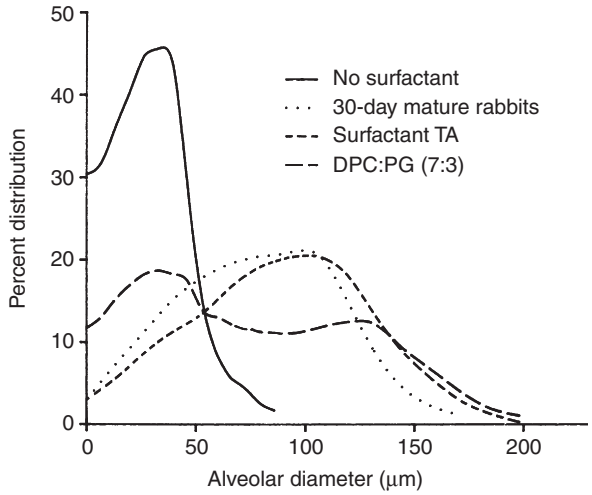


Figure 106-2 Alveolar size distributions for 30-day-gestation mature rabbits, 27-day preterm rabbits receiving no surfactant, and preterm rabbits treated with surfactant TA and a dipalmitoy lphosphatidylcholine:phosphatidylglycerol synthetic surfactant (DPG:PG).The lungs were immersed in formalin at a deflation pressure of 10 cm H_2O for fixation. Surfactant treatment normalized alveolar diameters toward those measured for the mature newborn rabbits. *(From Fujiwara T: Surfactant replacement in neonatal respiratory distress syndrome. In Robertson B, van Golde LM, Battenberg JJ (eds):* Pulmonary Surfactant. *Amsterdam, Elsevier Science Publishers, 1984, pp 480-503.)*

normal shape and volume. If adjacent alveoli collapse, the alveolus either tends to overexpand and distort or collapse. In surfactant-deficient states, the more normal alveoli tend to overexpand as other regional alveoli collapse, generating a nonhomogeneously inflated lung. Surfactant treatment improves uniformity of inflation, minimizes small airway dilation and promotes alveolar inflation. Surfactant treatment of 27-day preterm rabbits resulted in a distribution of alveolar sizes comparable to those of term rabbits[12] (Figure 106-2). Surfactant treatment also resulted in more normal lung morphometry in preterm lambs receiving continuous mechanical ventilation 24 hours after delivery.[22] The alveoli of the lambs not treated with surfactant were shallow, and alveolar ducts were dilated. Most of these abnormalities were prevented with surfactant treatment (Figure 106-3). This surfactant effect on uniformity of lung expansion may be the mechanism that decreases lung injury.

EFFECTS OF SURFACTANT TREATMENT ON DYNAMIC LUNG FUNCTION

If surfactant treatment were to completely normalize lung function, functional residual capacity (FRC) should increase, the time constant for inflation should decrease, and the time constant for expiration should increase. These effects occur to variable degrees, probably depending on factors related to the disease process in each infant, the style of ventilatory management, and the surfactant used to treat the infant. FRC in infants with RDS increased by approximately 150% to approach a normal value after surfactant treatment in one report.[23] In another report, FRC increased from 7.6 mL/kg to 15.4 mL/kg 1 hour after surfactant treatment.[24] The effect of surfactant treatment on FRC depends on the positive end-expiratory pressure (PEEP) used to ventilate the infant. FRC can be too high after surfactant treatment, resulting in CO_2 retention.[25]

Compliance uniformly increased in animal models of surfactant treatment.[26] However, compliance did not increase in infants soon after surfactant treatment in most clinical reports. For example, Davis and associates[27] reported no change in compliance for mechanical breaths with surfactant treatments, although spontaneous breaths after surfactant treatment demonstrated improved compliance. There are several explanations for this inconsistency. The animal models of RDS are ventilated in a standard fashion often with control of tidal volume. In the clinical evaluations of surfactant, tidal volumes were seldom measured. Large tidal volumes or high FRC may mask a "compliance effect" of surfactant treatment. Kelly and associates[28] found no improvement in dynamic compliance after treatment of infants with surfactant, whereas static compliance decreased as a result of lung volume recruitment. However, when the ventilator pressures were lowered, consistent improvements in both static and dynamic compliance occurred. Figure 106-4 illustrates what probably is occurring in infants. Surfactant treatment increases lung volumes at both low and high transpulmonary pressures. Over a range of pressures, the curves are parallel, and the calculated compliance does not change with surfactant treatment. By lowering pressures, mechanical ventilation becomes more efficient, and compliance improves. If peak inspiratory pressures and/or PEEPs are too high, compliance will decrease after surfactant treatment because the lung is overinflated.

Another variable in the compliance response is the type of surfactant used for treatment. A synthetic surfactant without surfactant proteins may not demonstrate improved compliance in the first few hours after treatment. In contrast treatment with animal source surfactants can result in rapid improvements in compliance. These different responses between synthetic surfactants and surfactant from animal lungs have been noted in preterm animal models[29] and in clinical practice.[30] Differences in acute physiologic responses between the surfactants from animal lungs that are now used clinically are not large, and differences in longer term outcomes have not been convincingly demonstrated.

Surfactant treatments also will have effects on the time constants for lung inflation and deflation. The stiff, atelectatic lung of the infant with RDS does not inflate easily, and deflation to low lung volumes is rapid because the elastic recoil properties of the lungs are not counterbalanced by surfactant. With mechanical ventilation, inspiratory times and peak pressures are used empirically to adjust tidal volumes. The time constant for expiration (defined as the time required for the lung to empty two thirds of its volume calculated from the peak tidal volume to FRC) is a passive property of the lung that is not controlled by the clinician. Although measurements of expiratory time constants have not been reported for surfactant-treated infants, the mean expiratory time constant increased from 25 +/− 6 ms for controls to 50 +/− 9 ms for surfactant-treated preterm rabbits.[31] The expiratory time constant increased as the SP-B content of the surfactant increased in preterm rabbits.[32] Increases in expiratory time constants in surfactant-treated infants ventilated at rapid rates could significantly increase expiratory lung volumes to the point of overdistention, resulting in decreased compliance and carbon dioxide retention. The combination of surfactant treatment and a ventilatory style that promotes air trapping may mask surfactant treatment effects.

OXYGENATION RESPONSE TO SURFACTANT

The most consistent response to surfactant treatment is an improvement in oxygenation,[11] which occurs within seconds to minutes after surfactant treatment of preterm animals[26] (Figure 106-5). The rapid improvement in oxygenation results from the acute increase in lung volume and increases in the surface area for gas exchange with surfactant instillation. Surfactant treatments also decrease pulmonary vascular resistance

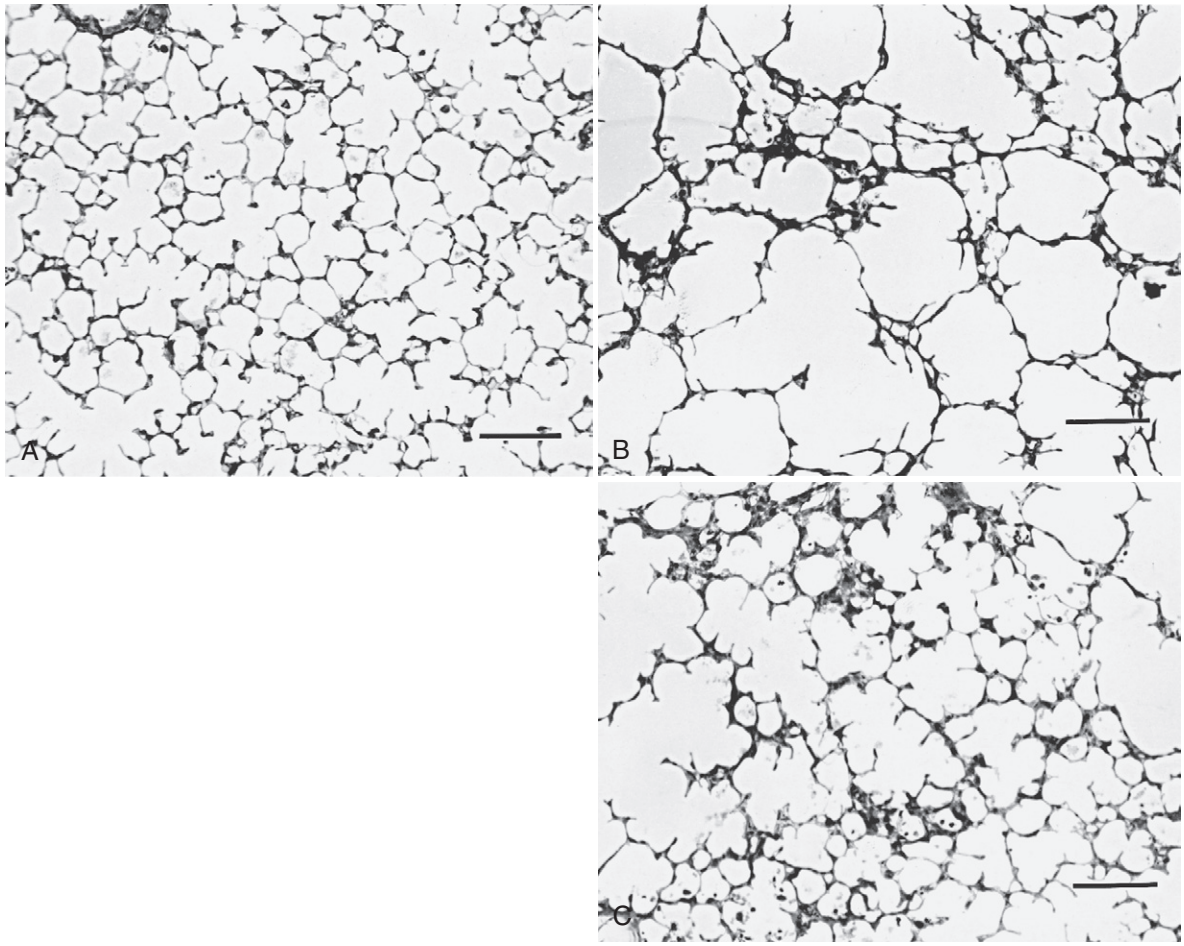


Figure 106-3 Effect of surfactant on uniformity of alveolar expansion. **A,** Light micrograph from a preterm lamb lung fixed at delivery without mechanical ventilation demonstrates uniform alveolar sizes. **B,** After ventilation for 24 hours without surfactant treatment, some alveoli are shallow and alveolar ducts are distended, and other alveoli are collapsed. **C,** After surfactant treatment at birth and ventilation for 24 hours, alveolar sizes are more uniform. Bar is 100 μm in each figure. *(From Pinkerton KE, Lewis JF, Rider ED, et al: Lung parenchyma and type II cell morphometrics: Effect of surfactant treatment on preterm ventilated lamb lungs,* J Appl Physiol *77:1953, 1994.)*

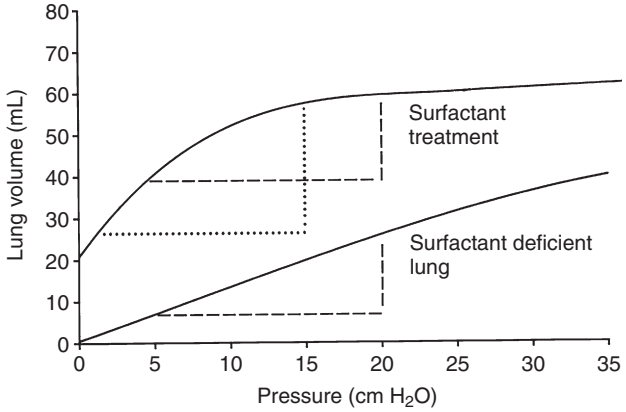


Figure 106-4 Idealized deflation limbs of static pressure-volume curves for a surfactant-deficient lung and surfactant-treated lung. The surfactant treatment has increased lung volume and deflation stability. Compliances calculated over the pressure range of 5 to 20 cm H_2O as indicated by the *dashed lines* are similar despite surfactant treatment. By lowering ventilatory pressures to 15 over 2 cm H_2O, as indicated by the *dotted lines,* compliance has improved with surfactant treatment.

in infants with RDS.[33] Although the fall in pulmonary vascular resistance may reverse the ductal shunt (from right-to-left to left-to-right) and decrease hypoxic shunt,[34] this effect is probably delayed in most clinical situations until after the initial oxygenation response has occurred.[35]

CLINICAL TRIALS OF SURFACTANT FOR RESPIRATORY DISTRESS SYNDROME

The use of surfactant in preterm infants is the most thoroughly studied therapy in neonatal care. The clinical trials have been compiled by Soll[36] in a meta-analysis (Figure 106-6). Two strategies for the use of surfactant have been evaluated: One involves the treatment of infants at high risk of RDS in the delivery room concurrently with the initiation of breathing and resuscitation. The other involves the treatment of infants at 2 to 24 hours of age after a diagnosis of RDS has been made. Treatment in the delivery room is referred to as *prevention* or *prophylactic treatment* because the goal is to prevent both RDS and any injury to the preterm surfactant-deficient lung that might result from mechanical ventilation. The odds ratio for death by 28 days of age was approximately 0.6 for either treatment strategy.[11] With surfactant treatment, the absolute decrease in death from any cause was 30% to 40%.

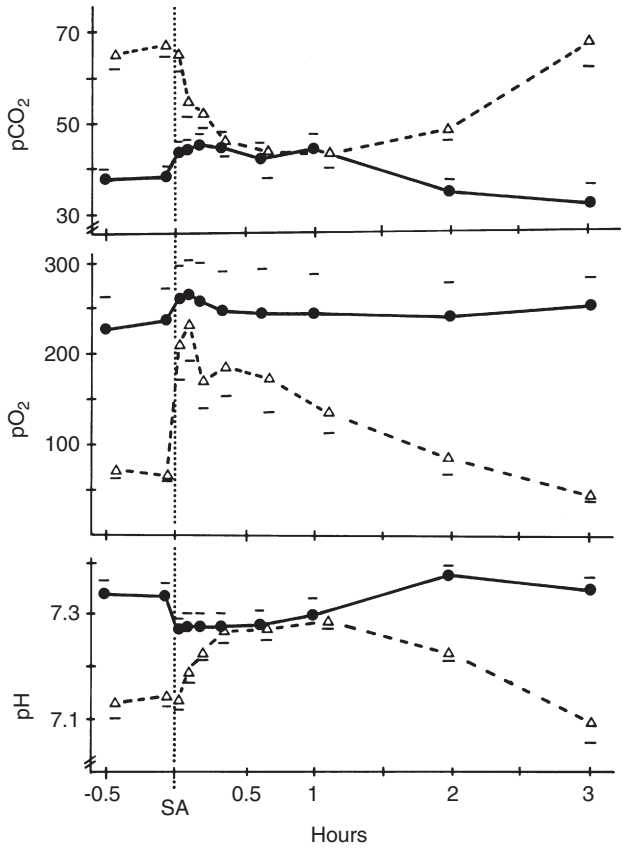


Figure 106-5 Blood gas and pH responses of very preterm lambs to treatment with 100 mg/kg natural sheep surfactant. Preterm lambs were delivered at approximately 121 days gestation (term is 150 days) and treated with surfactant either at birth (•) or after ventilation for approximately 30 minutes (Δ). Oxygenation was good and P_{CO_2} values were normal with surfactant treatment at birth. Oxygenation improved very rapidly after surfactant treatment at 30 minutes of age. *(From Jobe A, Ikegami M, Glatz T, et al: Duration and characteristics of treatment of premature lambs with natural surfactant,* J Clin Invest *67:370, 1981, by copyright permission of The American Society for Clinical Investigation.)*

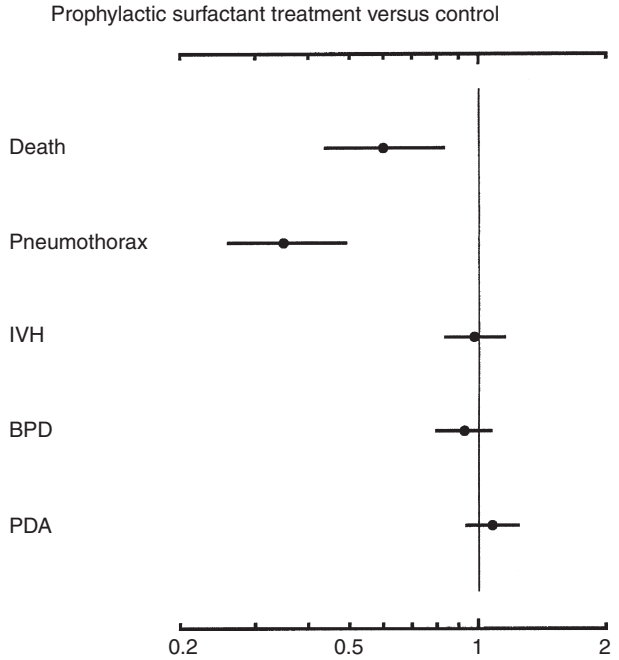


Figure 106-6 Odds ratios and 95% confidence intervals from meta-analysis of clinical trials of natural surfactant treatments given at birth for respiratory distress syndrome (RDS) in comparison to untreated controls. *BPD,* Bronchopulmonary dysplasia; *IVH,* intraventricular hemorrhage; *PDA,* patent ductus arteriosus. *(Data from Soll RF: Prophylactic natural surfactant extract for preventing morbidity and mortality in preterm infants,* Cochrane Database Syst Rev *(2):CD000511, 2000.*

The major pulmonary complications of RDS are acute respiratory failure, air leaks (pneumothorax and pulmonary interstitial emphysema), and the chronic lung disease of infants called *bronchopulmonary dysplasia*. Surfactant treatment results in improved lung function and in a striking decrease in pneumothorax and other air leaks. The incidence of bronchopulmonary dysplasia was not consistently lower, although it was reduced in individual studies. A problem with the reported incidence of bronchopulmonary dysplasia was that the severity of the lung disease was not quantified. Many tiny preterm infants have a supplemental oxygenation requirement and some radiographic abnormalities at 28 days of age, but they do not need oxygen or other treatments for lung disease at discharge.[37] The most frequently used definition for bronchopulmonary dysplasia is the use of oxygen at 36 weeks. Some infants receive oxygen who do not need oxygen when evaluated by objective oxygen saturation criteria.[38] Overall the severity of bronchopulmonary dysplasia has decreased in the very-low-birth-weight population, and surfactant treatments must be contributing to this improved outcome.[39] Surfactant treatment increases survival and these survivors may have the highest risk of bronchopulmonary dysplasia. Egberts and de Winter[40] point out that the incidence of bronchopulmonary dysplasia decreased in the clinical trials if the calculation was based on survivors rather than on infants randomized.

An initial hope was that the improved cardiopulmonary stability and oxygenation resulting from surfactant treatment would protect preterm infants from the nonpulmonary complications of prematurity, such as patent ductus arteriosus, severe intraventricular hemorrhage, and necrotizing enterocolitis. Unfortunately, treatment has not consistently decreased the frequency of patent ductus arteriosus or severe intraventricular hemorrhage. Large decreases in intraventricular hemorrhage in association with surfactant treatment have been reported in individual trials,[41] suggesting that other aspects of neonatal care may be interacting with surfactant therapy to influence the incidence of this complication. A thorough analysis of the available data on the possible association of intraventricular hemorrhage with surfactant treatment did not lead to any direct links.[42] Necrotizing enterocolitis and retinopathy of prematurity were not tabulated for the meta-analyses, but no trends were reported in the individual trials. One study found a decrease in retinopathy of prematurity in the infants treated with surfactant.[43] Infants treated with surfactant generally perform as well as or better than control infants when assessed at long-term follow-up.[44-46] Surfactant-treated infants needed less supplemental oxygen at 6 months and had less wheezing at 12 and 24 months of age relative to control infants.[47] In this study, less cerebral palsy also was diagnosed in surfactant-treated infants.

TIMING OF SURFACTANT TREATMENT

In the initial trials, surfactant was administered 6 to 24 hours after birth when a diagnosis of severe RDS could be made more accurately. In contrast delivery room treatment was considered

to be optimal only if given before the infant breathed or received positive-pressure ventilation.[48] This delivery room strategy was based on information from surfactant treatment of preterm animals that demonstrated airway epithelial damage after very short-term periods of ventilation of the surfactant-deficient lung.[49] The two treatment strategies were compared in eight trials using different surfactants.[50] No differences in the incidences of intraventricular hemorrhage, bronchopulmonary dysplasia, or patent ductus arteriosus were seen. Delivery room treatment decreased pneumothorax, and this outcome would be expected because early treatment protected the infant from pneumothorax for the period before treatment. The important difference was a decrease in death with prophylactic surfactant relative to treatment of RDS.

In the more recent trials, delivery room treatment was delayed until after the infant had stabilized for a few minutes and the treatment of RDS was not delayed for 6 hours. Therefore the two treatment strategies approached each other in time. Treatment of infants at birth has two disadvantages: A number of the infants will be treated unnecessarily because they would not have developed RDS, and instillation of a large volume of surfactant can interfere with normal resuscitative efforts. Kendig and co-workers[48] demonstrated that surfactant treatment within the first 15 minutes after birth was equivalent and perhaps preferable to treatments before the infant was allowed to breathe. Based on the meta-analyses of the initial trials comparing delivery room treatment with treatment of RDS, Horbar and colleagues[51] argued that the standard of care should be delivery room treatment for very-low-birth-weight infants. However, in practice, the majority of infants were not treated in the delivery room. They then performed a collaborative quality improvement initiative and demonstrated that the age at treatment could be reduced from a mean of 78 minutes to 21 minutes, but the intervention had no effect on clinical outcomes.[52] A reasonable approach is to treat most infants as soon as clinical signs of RDS appear. Waiting for the disease to progress to establish the diagnosis more firmly before treatment will minimize the efficacy of the therapy and increase complications. Delivery room treatment is most appropriate for the smallest infants who are at the highest risk of RDS. However, delivery room treatment should be given by a person experienced in neonatal resuscitation.

Decisions about the timing of surfactant treatment are further complicated by the use of continuous positive airway pressure (CPAP) soon after birth to stabilize respiratory function and to avoid intubation and mechanical ventilation. With clinician experience with the use of CPAP, many infants can initially transition from the delivery room and do well on CPAP despite some RDS.[53] The success rate for CPAP management increases as birth weight and gestational age increases, but some infants at gestations of 25 weeks or less do not require surfactant treatment or mechanical ventilation. A management strategy to emphasize CPAP will result in a striking decrease in surfactant use, and reports of clinical experiences suggest a decreased rate of bronchopulmonary dysplasia without increased death or other complications of prematurity.[54] The benefits of avoiding mechanical ventilation for some infants may put other infants at risk for complications that result from delays in surfactant treatment. The compromise to intubate very-low-birth-weight infants at delivery, give surfactant, and then attempt early extubation to CPAP still requires intubation, which may result in injury. Clinical trials for the initial management of very-low-birth-weight infants with surfactant or CPAP are in progress. However, the different management strategies depend on procedural skills, experience, and multiple aspects of the subsequent care, which may confound clear answers to when infants are best treated with surfactant.

REPETITION OF TREATMENT

Multiple doses of surfactant were given in most trials because the response to an individual dose was often transient. In preterm animals, exogenously administered surfactant is not lost from the lungs,[55] but its function can be inhibited by soluble proteins and other factors. Multiple doses of surfactant are thought to be useful because they can overcome this functional inactivation of surfactant. Multiple doses of surfactant have been compared with single-dose treatments. In one trial, second and third doses given 12 and 24 hours after an initial treatment at approximately 6 hours of age reduced the frequency of pneumothorax from 18% with a single dose to 9% with three doses ($p < .01$), and the death rate at 28 days decreased from 21 to 13% ($p < .05$).[56] In another study of 75 large infants with RDS (mean birth weight, 1900 g), those who received multiple doses had better oxygenation during the first several days of life.[57] An unresolved question is whether all infants treated with surfactant would benefit from subsequent treatments. All recent trials have used retreatments. Although most infants respond favorably to treatment, approximately 20% of infants thought to have RDS will have little or no response. These infants may have other diseases, such as pneumonia, pulmonary hypoplasia, or congenital heart disease. Structural immaturity of the lungs and birth asphyxia with decreased cardiovascular performance can also blunt the response to surfactant. It is reasonable to re-treat with surfactant if the initial response to treatment was poor and other causes of respiratory failure have been excluded. Many infants will have such a good response to the first dose that they have minimal residual lung disease. In clinical practice, re-treatment should be individualized and considered for infants with enough residual lung disease to put them at risk for complications such as pneumothorax. The major reason for re-treatment is to overcome inhibition of surfactant resulting from lung injury. If treatment is given early in the clinical course and a gentle approach to ventilation is used, significant lung injury can be avoided. The dose of 100 mg/kg surfactant that is normally used is large relative to the endogenous pool in a normal lung, and it is metabolized slowly.[55] Therefore retreatment with surfactant will not be for inadequate amounts of surfactant, but for inadequate surfactant function. On average, infants with RDS receive less than 2 doses of surfactant. More than two (or perhaps three) doses of surfactant is excessive and is unlikely to improve outcomes.

COMPLICATIONS OF SURFACTANT TREATMENT

The complications associated with the administration of surfactant are listed in Table 106-1. This documentation of treatment complications resulted from an extensive data collection required for the initial clinical use of surfactant in 1990 and 1991.[58] Similar problems associated with surfactant treatments were reported by Horbar and colleagues.[59] The treatment procedures for each surfactant approved for clinical use differ in detail but generally combine positioning of the infant, manipulation of the endotracheal tube, and instillation of suspensions of surfactant in a 3- to 5-mL/kg vehicle. The acute physiologic effects are related to the handling of the infant and the acute effects of the surfactant on the lungs. Manipulation of the head, neck, and endotracheal tube can cause vagal responses resulting in bradycardia and cyanosis. The bolus of fluid can acutely obstruct airways leading to cyanosis, bradycardia, and carbon dioxide retention. If surfactant is rapidly distributed to the distal lung, lung compliance can improve, the Po_2 can increase, and the Pco_2 can decrease. Reflux of some of the surfactant up the endotracheal tube is frequent. These acute effects of surfactant are easily dealt with by adjustments in the ventilator and probably have no long-term effect on outcome. Of more

TABLE 106-1

Problems Associated With Administering Surfactant: Percentage of 17,641 Infants Treated

Problem	Percentage
Cyanosis	3.7
Bradycardia	4.2
Tachycardia	0
Increased O_2	3.3
Decreased O_2	16.6
Increased Pco_2	0.5
Decreased Pco_2	0.1
Reflux	14.6
Others	2.7
At least 1 event	30.4

Data from Zola EM, Overbach AM, Gunkel JH, et al:Treatment investigational new drug experience with Survanta (beractant). *Pediatrics* 91:546, 1993.

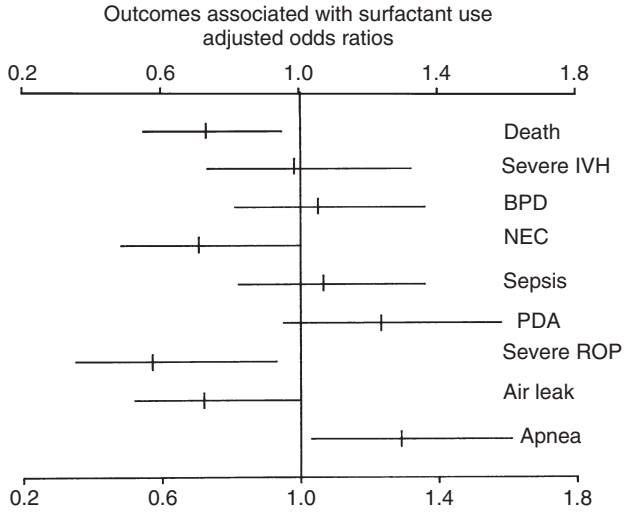


Figure 106-7 Adjusted odds ratios for infants before and after the introduction of surfactant for the treatment of RDS. The odds ratios and 95% confidence limits are from Horbar and colleagues.[62] *BPD,* Bronchopulmonary dysplasia; *IVH,* intraventricular hemorrhage; *NEC,* necrotizing enterocolitis; *PDA,* patent ductus arteriosus; *ROP,* retinopathy of prematurity.

concern are the reports of transient changes in blood pressure, cerebral blood flow velocities, and electrocortical depression after surfactant treatments.[60-62] The mechanisms and importance of these effects are not known.

The only severe complication consistently associated with surfactant treatment is pulmonary hemorrhage. The overall relative risk for pulmonary hemorrhage after surfactant therapy was 1.47 (95% confidence interval 1.05 to 2.07) in the clinical trails.[63] Incidences ranged from approximately 1% to 5% of treated infants. The presence of a patent ductus arteriosus with a left-to-right shunt resulting in elevated pulmonary vascular pressures was linked to pulmonary hemorrhage in several trials. Although this association has not been identified in other trials, increased pulmonary vascular pressures leading to stress failure of alveolar capillaries is the likely explanation for the pulmonary hemorrhage.[64] The pulmonary hemorrhage after surfactant treatment does not often occur at the time of treatment. Hemorrhage can occur in the smallest infants a number of hours after surfactant treatment has improved lung function. Pulmonary hemorrhage is frequent in autopsies of infants who have died of RDS. The association of pulmonary hemorrhage with surfactant treatment may result from the decreased death rate in tiny preterm infants receiving surfactant, which allows pulmonary hemorrhage to become apparent clinically. Surfactant treatment after pulmonary hemorrhage can improve subsequent lung function.[65] This result is consistent with the concept that pulmonary hemorrhage inactivates surfactant. Although not well documented, pulmonary hemorrhage seems to be less frequent in recent years. Perhaps changes in ventilation style toward the maintenance of better lung inflation, lower tidal volumes, and acceptance of higher carbon dioxide levels have contributed to the decrease in pulmonary hemorrhage.

ROUTINE CLINICAL USE OF SURFACTANT

The randomized, controlled trials demonstrated that surfactant decreased death when used to treat infants with RDS. No assurance, however, is available that general clinical use would result in similar effects. Several reports of the probable effect of surfactant introduction on outcomes for preterm infants have been published. Incidences of death and other major neonatal complications were analyzed for the period before surfactant introduction (before 1989) and after general clinical availability. Horbar and colleagues[66] reported outcomes for 2870 infants weighing 601 to 1300 g before surfactant and 1413 infants after surfactant was available. Mortality decreased from 28% before surfactant to 20% after surfactant was introduced. This decrease in mortality was associated with an increase in the duration of ventilation and length of hospitalization. Of perhaps more importance is the information on secondary outcomes (Figure 106-7). Surfactant treatments were not associated with increased incidences of intraventricular hemorrhage, bronchopulmonary dysplasia, sepsis, or patent ductus arteriosus. Beneficial secondary effects were decreased necrotizing enterocolitis, air leak, and severe retinopathy of prematurity. The increase in apnea probably resulted from the survival of more small infants. This favorable clinical experience was not replicated in a compilation of data for 5629 infants weighing 500 to 1500 g from 14 centers.[67] Although death decreased by 30% after the introduction of surfactant, intraventricular hemorrhage increased from 17% to 23% (adjusted risk ratio, 1.4; 95% confidence interval, 1.2 to 1.6). Nevertheless, the effect of surfactant on death can explain 80% of the decline in infant mortality between 1989 and 1990. The use of surfactant decreased overall costs for both survivors and the infants that died. Palta and co-workers[68] also noted a decrease in death with the introduction of surfactant. Intraventricular hemorrhage also decreased, but this outcome was associated primarily with increased use of antenatal corticosteroids. The incidence of bronchopulmonary dysplasia increased, perhaps because more small infants were surviving. The epidemiology is important for understanding how the introduction of a new therapy affects overall outcomes.

ADMINISTRATION OF SURFACTANT

The instillation procedures used in the trials to treat RDS were selected empirically based on the techniques of treatment for preterm animal models of RDS. Surfactant can be given to infants at risk of developing RDS in the delivery room soon after delivery and intubation as a single bolus into the endotracheal tube followed by mechanical ventilation.[48] For infants with RDS, surfactant has been given by a variety of bolus injection schemes that include positioning the chest of the infant in an effort to optimize distribution. These treatment procedures were standardized for each clinical trial such that each surfactant in current use was given by a somewhat different technique. The essence of each treatment technique is the instillation of surfactant into the proximal or distal trachea with positioning of the chest. The only clinical trial comparing

treatment techniques found that there were no differences in short-term efficacy when the surfactant dose was divided into two or four aliquots for administration or when two or four chest positioning maneuvers were used.[69]

The goal of the treatment technique is to get the surfactant into an infant's lungs with as little physiologic disturbance as possible. This clinical treatment goal may conflict with the goal of an optimal clinical outcome. At the level of the alveolus, the primary goal is to achieve as uniform a distribution of surfactant as possible. If distribution were perfectly uniform, expansion of the lung should be uniform. The physiologic result should be decreased oxygen and pressure needs and less lung injury. In contrast, if the distribution of surfactant were primarily to one lobe or one lung, that lung volume would expand if pressures were not decreased. The inflated lung would tend to overexpand and potentially be injured.[12] If the pressures were decreased, the untreated lung would lose whatever gas volume it was receiving and become more atelectatic. At the alveolar level, alveoli are interdependent in that if a group of alveoli collapse, the adjacent alveoli may overinflate and distort[22] (see Figures 106-2 and 106-3). The frequent occurrence of bronchopulmonary dysplasia after surfactant treatment may result in part from the nonhomogeneous distribution of surfactant, contributing to focal lung injury.

Most experimental studies of surfactant distribution have used radiolabeled surfactant components (primarily dipalmitoylphosphatidylcholine) or other easily measured substances mixed with surfactant.[70] The distribution of surfactant was measured by external scanning of radioactivity,[71] autoradiograms of lung slices,[72] or the content of surfactant in lung pieces of various sizes.[73] Each technique has different strengths and weaknesses, giving only partial information about surfactant distribution. Charon and colleagues[71] measured surfactant distribution in infants. They mixed technetium-sulfur colloid with surfactant and treated eight infants using a four-bolus, four-position treatment technique. They found relatively homogeneous distributions in all infants that did not correlate with clinical response. Subsequently van der Bleek and colleagues[74] reported that microaggregated technetium-albumin mixed with surfactant appeared to be uniformly distributed by external scanning of the lungs of saline lavaged rabbits. When the lungs were divided into 200 pieces and distribution per piece was measured, however, the distribution was not homogeneous. Scanning of whole lungs does not accurately reflect distribution. Analysis of lung pieces also does not provide information about alveolar distribution. For example, if the preterm human lung at 32 weeks gestational age were cut into 200 pieces for a distribution measurement, each piece would contain approximately 200,000 saccules. Therefore, even if distribution to lung pieces were homogeneous, significant inhomogeneity must exist at the alveolar level.

VARIABLES THAT INFLUENCE SURFACTANT DISTRIBUTION

The biophysical properties of surfactant facilitate adsorption to an air-fluid interface and rapid spreading over a surface. Therefore it is logical to anticipate that surfactant will distribute by this spreading behavior. Davis and associates[75] used external scanning to measure the kinetics of surfactant or saline distribution to the lungs of saline lavaged piglets. They found that surfactant began to distribute within 5 seconds of dosing, with substantial distribution to the lung fields within 20 seconds. In contrast, saline did not distribute as rapidly, and multiple filling defects were noted on the scans. These results demonstrate rapid spreading of surfactant with a more uniform ultimate distribution than for saline alone. Surfactant given into single lobes in rabbits did not spread to other lobes even after 10 hours of spontaneous ventilation.[76] Therefore surfactant treatments given into one lung or lobe will not treat other parts of the lung.

The surfactants used clinically are given as suspensions in volumes that range from 2.5 to 5 mL/kg. Intuitively, the larger the volume administered, the more likely multiple bronchi will be filled, and the better the distribution is likely to be. Large volumes, however, are more likely to cause airway obstruction, respiratory distress, cyanosis, and other undesirable side effects. Gilliard and co-workers[77] found that instillation of 1.5-mL/kg surfactant into ventilated rabbits with normal lungs resulted in a nonuniform distribution. Increasing surfactant concentration did not improve the distribution. The same amount of surfactant suspended in 15 mL/kg resulted in a homogeneous distribution. However, with lung injury, the distribution was volume independent and reasonably uniform. This result was interpreted to mean that with severe lung injury, the alveoli were flooded and provided a fluid volume to facilitate surfactant distribution. The residual fetal lung fluid and alveolar edema associated with RDS may help distribute surfactant. Large treatment volumes are not realistic for clinical practice, and some nonhomogeneity of surfactant distribution is an inevitable consequence of present treatment techniques.

Given that surfactant distribution can be improved by increasing the treatment volume, and the administration of large volumes over short treatment intervals results in respiratory complications, it might seem logical to deliver surfactant to the lungs more slowly. This approach has been evaluated in two animal models of RDS. Segerer and associates[78] reported that 4 mL/kg of a surfactant suspension given as a bolus via the endotracheal tube in 10 seconds to saline-lavaged rabbits resulted in a reasonably uniform distribution. In contrast infusion of the same volume of surfactant over 45 minutes through the pressure-monitoring channel in an endotracheal tube resulted in an extremely nonhomogeneous distribution. Ueda and associates[79] compared the four aliquot, four-position technique used clinically with a slow infusion in preterm lambs with RDS. The infusion treated group received 2 mL/kg surfactant in the right lateral position in 15 minutes followed by 2 mL/kg in the left lateral position in 15 minutes. Both groups of lambs had improvements in PO_2 and lung mechanics, although the responses were not as large with surfactant infusion. The bolus treatments delivered surfactant quite uniformly to the different lobes of the sheep lung, whereas infusion resulted primarily in upper lobe localization (Figure 106-8). The animals received a second treatment 2 hours after the initial treatment, and the second treatment localized to the same lung pieces that received the majority of the surfactant with the initial treatments. Therefore the first treatment determined primarily which lung volumes will open, and the second treatment tended to treat the same lung volumes that were initially treated. Pulmonary blood flow was relatively uniform across the lung pieces of the bolus-treated animals. In contrast blood flow decreased to the lung pieces that received the most surfactant by infusion, probably because of overinflation of those lung regions. In the injured lung, instilled surfactant goes preferentially to lung regions that are partially inflated and not to atelectatic or overinflated areas.[80]

Surfactant distributes to the lungs by bulk movement of the suspension down the airways and by spreading. The style of ventilation immediately after treatment could affect the distribution. In clinical practice, conventional volume ventilation is routinely used after surfactant treatments. Walther and colleagues[81] reported that surfactant instilled into the airways of preterm lambs distributed similarly with conventional or high-frequency ventilation. In contrast Heldt and co-workers[82] found that high-frequency ventilation seemed to delay surfactant delivery to distal airspaces of preterm rabbits. The efficacy of surfactant treatment techniques and distribution may be influenced

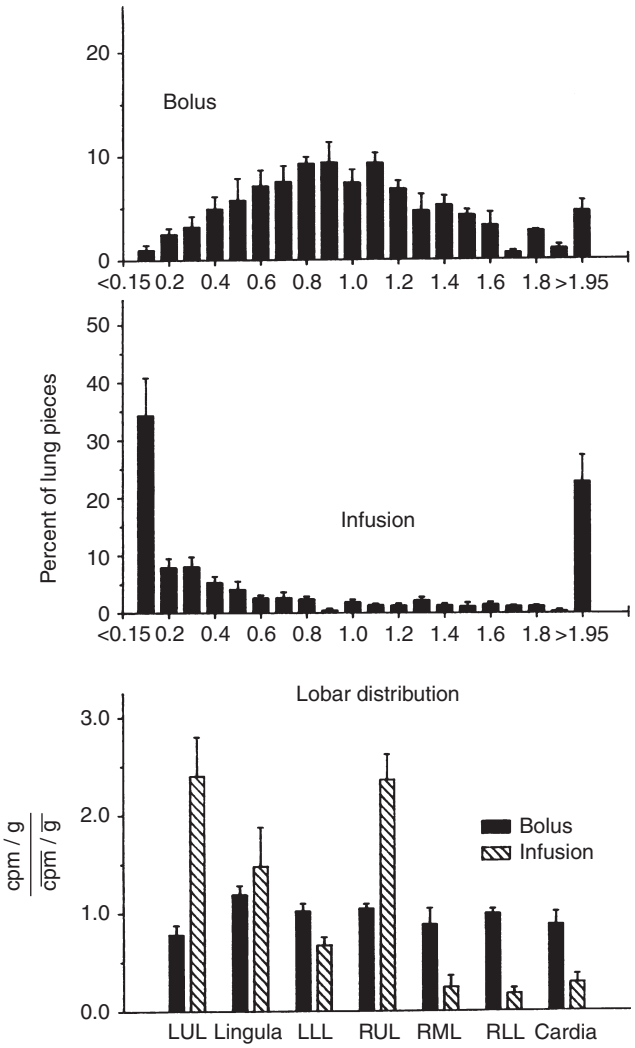


Figure 106-8 Distribution of surfactant by a bolus technique in comparison with a 30-minute infusion to preterm ventilated lamb lungs. The 100 mg/kg dose of surfactant was radiolabeled, and the amounts of surfactant recovered in lung pieces or by lobe were measured. Bolus treatment resulted in approximately 40% of the lung pieces containing within +/− 25% of the mean amount of surfactant. In contrast infusion resulted in 7% of the pieces of lung receiving within +/− 25% of the mean. The majority of the infused surfactant was found in the upper lobes. *(Redrawn from Ueda T, Ikegami M, Rider ED, et al: Distribution of surfactant and ventilation in surfactant-treated preterm lambs,* J Appl Physiol *76:45, 1994.)*

by different ventilation strategies. Surfactant can be delivered to the lungs by aerosol, but the surfactant goes primarily to the ventilated lung regions resulting in a nonuniform distribution.[83] The surfactant dose that can be delivered by aerosol also is low.

VENTILATION EFFECTS ON SURFACTANT TREATMENT RESPONSES

Although mechanical ventilation and the use of PEEP is routine in infants with RDS, multiple ventilatory techniques are in common use. The interaction between ventilatory management and surfactant treatment has not been studied extensively, although in experimental animals the ventilation technique is an important variable in the response of the preterm lung to surfactant treatment.[20] In clinical practice, surfactant does not have equivalent effects in different neonatal units, and the differences may be explained by ventilatory management.[84] Lung injury is induced by ventilation of the normal lung at volumes close to maximal lung volume.[85] With surfactant deficiency, ventilation at low lung volumes (below the normal FRC) also causes progressive injury and pulmonary edema.[86] If alveolar expansion is optimized in the saline-lavaged rabbit model of surfactant deficiency, the effectiveness of surfactant treatments is enhanced. The responses also depend on the type of surfactant used for treatment. Surfactants that contain only lipids are not as effective at improving compliances or pressure volume curves of preterm rabbits.[87] Addition of the surfactant proteins SP-B and SP-C improves function, but the enhanced function is evident only if PEEP is used to ventilate the animals. The use of PEEP in the preterm rabbit model resulted in striking decreases in the leak of albumin from the vasculature to the lungs, indicating less injury.

The equipment used for the mechanical ventilation of preterm infants and the ventilatory goals have changed since surfactant was tested clinically in the 1980s. Most trials of ventilation now focus on very-low-birth-weight infants at highest risk of bronchopulmonary dysplasia. These infants usually have RDS and have been treated with surfactant before trial enrollment. Examples are the trials comparing high-frequency oscillation with conventional ventilation.[88,89] The newer management approaches to keep the FRC relatively high (the open lung strategy) while ventilating with low tidal volumes (4 to 6 mL/kg) to achieve carbon dioxide values higher than the normal range (greater than 50 mm Hg) depend on good surfactant function. If surfactant is deficient, FRC cannot be maintained without excessive PEEP. Surfactant treatment before the initiation of high-frequency oscillation improves respiratory stabilization and allows oscillation at lower mean airway pressures.[90,91] CPAP is also now used commonly as a way to decrease the need for mechanical ventilation and perhaps to decrease the incidence and the severity of bronchopulmonary dysplasia.[53] An advantage of CPAP is the avoidance of intubation, but surfactant cannot be given without intubation. Elective intubation followed surfactant treatment and extubation to CPAP can decrease the need for mechanical ventilation in infants with RDS.[92] The biophysical properties of surfactant will improve lung function in the preterm optimally only when the ventilator support adequately maintains FRC and minimizes injury. Unfortunately no consensus has been achieved on how ventilatory strategies can best be matched to the severity of lung disease in individual infants.

SURFACTANT-MATERNAL CORTICOSTEROID INTERACTIONS

Prenatal corticosteroid therapy decreases the incidence of death and the incidence and severity of RDS as well as other complications of prematurity.[93] Prenatal corticosteroids and postnatal surfactant each decrease RDS or death by similar amounts. The question of clinical relevance is how these two effective therapies interact. The two therapies have additive effects on postnatal lung function in animal models (Figure 106-9). After fetal cortisol exposure, preterm lambs treated with surfactant have improved lung compliance, increased lung volumes, and decreased alveolar edema relative to lambs receiving either treatment.[94] Prenatal corticosteroid treatment of preterm rabbits also resulted in an enhanced postnatal response to surfactant. Corticosteroid-exposed rabbits had larger compliance responses to lower doses of surfactant than did control animals.[95] Prenatal corticosteroid treatments also decreased vascular to alveolar protein leak in the preterm lung and changed the surfactant protein content of surfactant. The net effect was to decrease surfactant inhibition. If the only effects of prenatal corticosteroids on the developing lung were to increase

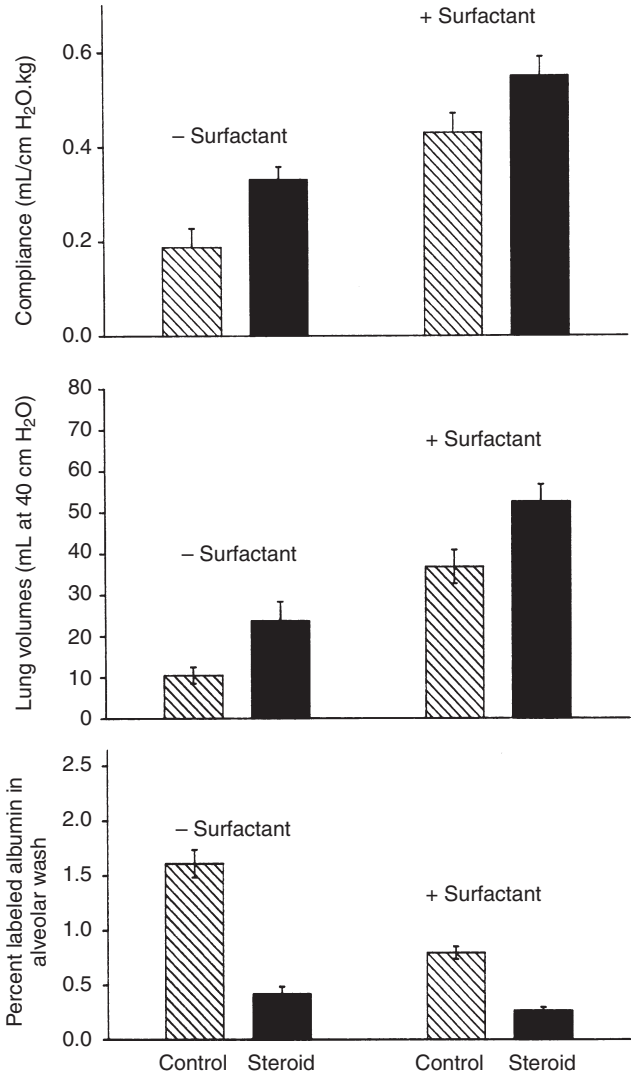


Figure 106-9 Postnatal lung function in preterm lambs randomized to prenatal cortisol and postnatal surfactant treatments. Each treatment increased postnatal compliances, increased lung volumes, and decreased pulmonary edema as measured by the amount of intravascular ^{125}I albumin that was recovered by alveolar wash.The lambs that received both treatments had responses that were approximately the sum of the surfactant and cortisol responses. *(Data from Ikegami M, Polk D, Tabor B, et al: Corticosteroid and thyrotropin-releasing hormone effects on preterm sheep lung function,* J Appl Physiol *70:2268, 1991.)*

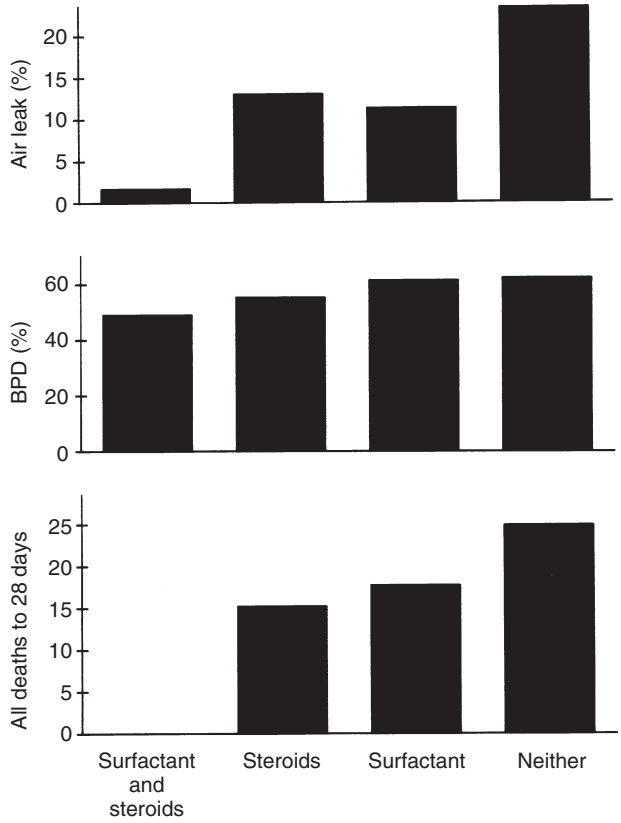


Figure 106-10 Combined effects of prenatal corticosteroids and postnatal surfactant on death, air leaks, and bronchopulmonary dysplasia (BPD) for infants randomized to surfactant or placebo treatment. *(Data from Jobe AH, Mitchell BR, Gunkel JH: Beneficial effects of the combined use of prenatal corticosteroids and postnatal surfactant on preterm infants,* Am J Obstet Gynecol *168:508, 1993.)*

surfactant, prenatal corticosteroids and postnatal surfactant treatments would be acting by the same mechanism. Experiments in developing animals indicate that the primary effects of prenatal corticosteroid treatments are on lung structure, causing increased lung gas volumes that resulted in improved ventilation and better responses to surfactant treatment.

Randomized, controlled trials of the interactions between postnatal surfactant and prenatal corticosteroids are not available because these therapies were developed in different eras. It is no longer ethical to randomize infants to either surfactant or prenatal corticosteroids. Retrospective evaluations of the surfactant trial databases demonstrate a beneficial interaction between prenatal corticosteroids and postnatal surfactant. In one report, treatment with surfactant or prenatal corticosteroids decreased air leak and death similarly (Figure 106-10). However, those infants that received both treatments had the best outcome.[96] Kari and associates[97] also found that infants with RDS randomized to receive maternal corticosteroids had a better response to postnatal surfactant than did infants with RDS not receiving prenatal corticosteroids. The combined use of antenatal corticosteroids and postnatal surfactant are major contributions to the improved outcomes of preterm infants.

THE CHOICE OF SURFACTANTS FOR THE TREATMENT OF RDS

The initial surfactants approved for clinical use by the FDA in 1990 to 1991 were a synthetic surfactant that did not contain surfactant proteins and a surfactant made from bovine lungs. Subsequently other animal lung source surfactants have been approved for clinical use. The clinician who selects a surfactant for clinical use for infants with RDS must sort through claims of product superiority from the pharmaceutical companies. Surfactants have been directly compared in randomized controlled trials, and in general the synthetic surfactants that do not contain surfactant proteins are less effective and are seldom used currently.[16,98] The animal source surfactants do have different characteristics; they are suspended in different volumes, different doses per kilogram are recommended, and treatment techniques vary somewhat. In general, surfactants that contain more of the surfactant proteins SP-B and SP-C have a more rapid onset of action in experimental models. However, in clinical

TABLE 106-2

Comparison of Two Surfactant for Early Treatment of Respiratory Distress Syndrome (BPD)*

	EARLY TREATMENT*	
	Surfactant 1	**Surfactant 2**
Characteristics of Populations		
Number of infants randomized	375	374
Gestational age (weeks, mean)	26.6	26.5
Birth weight (g, mean)	907	910
Age at treatment (minutes)	8	9
Days intubated (median)	3	4
Outcomes		
Died (%)	12	13
BPD; oxygen at 36 weeks (%)	34	33
Severe BPD; ventilator at 36 weeks (%)	4	6
Interventricular hemorrhage; grades III or IV (%)	10	14
Pulmonary interstitial emphysema (%)	5	8
Pneumothorax	7	5

*Data from Bloom BT, Clark RH: Comparison of Infasurf (calfactant) and Survanta (beractant) in the prevention and treatment of respiratory distress syndrome. *Pediatrics* 116:392, 2005.

practice, premature infants with a diagnosis of RDS are much more diverse clinically than experimental animals. The reasons for the preterm delivery (antenatal infection, pre-eclampsia, multiple births, etc.) and prenatal corticosteroids will modulate the severity of RDS and clinical responses to surfactant. Furthermore the ventilation-induced lung injury will change surfactant responses and outcomes. With current management approaches, outcomes other than mild to moderate bronchopulmonary dysplasia are infrequent. In the example listed in Table 106-2, early treatment with two different surfactants resulted in similar and low incidences of complications.[99] Although claims of superiority of one surfactant over another are made, seldom are differences in death, severe bronchopulmonary dysplasia, or neurodevelopmental outcomes compelling. Any differences may result more from management styles than from the different surfactants. Synthetic surfactants that contain the surfactant proteins or peptides that mimic surfactant protein function can be effective in animal models and clinically.[19] New surfactants will need to have cost, ease of use, and safety benefits to replace the effective surfactants that are presently available.

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107 Genetics and Physiology of Surfactant Protein Deficiencies

Lawrence M. Nogee

The principal cause of the respiratory distress syndrome (RDS) in prematurely born infants is an inability to produce sufficient amounts of pulmonary surfactant because of immaturity.[1] Surfactant phospholipids in combination with specific proteins help reduce alveolar surface tension at the air-liquid interface and prevent end-expiratory collapse. The risk for RDS is related to gestational age, with more-immature infants being at highest risk, although RDS may also develop in term infants.[2,3] In recent years it has been recognized that RDS in some term infants may result from inherited abnormalities in the genes encoding proteins important in surfactant metabolism. Genetic deficiencies of surfactant protein (SB) B (SP-B) and member A3 of the adenosine triphosphate–binding cassette family (ABCA3) are autosomal recessive causes of neonatal respiratory failure resulting from loss-of-function mutations in the genes encoding these proteins. Mutations in the surfactant protein C (SP-C) gene have been associated with lung disease of more variable onset and severity. Although these conditions are rare, they are associated with significant morbidity and mortality, and it is important that they be recognized in a timely fashion to appropriately counsel families of affected infants. Surfactant protein deficiencies provide insights into the roles of these proteins in normal lung function and surfactant homeostasis and demonstrate how genetic mechanisms may contribute to the development of more common forms of lung disease.

PULMONARY SURFACTANT PROTEINS

The hydrophobic surfactant proteins SP-B and SP-C have important roles in enhancing the surface tension–lowering properties of surfactant phospholipids.[4,5] Both SP-B and SP-C are found in varying amounts in the mammalian-derived surfactant preparations used to treat premature infants with RDS.[4] SP-B and SP-C are both derived from proteolytic processing of precursor proteins that are encoded by single genes on chromosomes 2 and 8 respectively, with the genetic loci referred to as *SFTPB* and *SFTPC*.[6,7] The mature forms of SP-B and SP-C are routed to lamellar bodies, the storage organelles for surfactant in the alveolar type II cell, and secreted along with surfactant phospholipids.[8]

Pulmonary surfactant also contains two larger glycoproteins, surfactant proteins A (SP-A) and D (SP-D), which are members of the collectin family, with both collagenous and carbohydrate-binding lectinlike domains.[9] The principal roles of SP-A and SP-D in the lung are likely related to local host cell defense.[10,11] Both SP-A and SP-D bind to a wide array of microorganisms, facilitate their uptake and/or killing by alveolar macrophages, and also have immunomodulatory functions.[12-15] Genetically engineered SP-A– and SP-D–deficient mice do not develop neonatal respiratory disease but do appear to have an increased susceptibility to infection with a variety of organisms that are relevant to human neonates, including group B streptococcus and respiratory syncytial virus.[16-18] SP-D–deficient mice also develop lung disease as they age, with both emphysema and fibrosis suggesting a role in regulating chronic inflammation, as well as lipoproteinosis suggesting a possible role in surfactant homeostasis.[19-21] Two genes and a pseudogene for SP-A and a single gene for SP-D are located on chromosome 10.[22] Mutations in the SP-A and SP-D genes associated with human lung disease have not yet been reported.

SURFACTANT PROTEIN B DEFICIENCY

Hereditary SP-B deficiency was the first recognized inborn error of surfactant metabolism.[23] The typical presentation is a term infant with symptoms and signs of respiratory distress consistent with surfactant deficiency but without other risk factors for lung disease. The radiographic findings are usually similar to those observed in premature infants with RDS, although this has not been examined systematically.[24,25] Air leak is not uncommon, and affected infants may also have persistent pulmonary hypertension. The lung disease in SP-B–deficient infants is often very severe, and affected infants may require support with extracorporeal membrane oxygenation. However, the initial severity of respiratory disease can be quite variable, and some affected infants have not required mechanical ventilation until well after birth. The factors responsible for the variability in the severity of the initial lung disease in affected infants are unknown, but the lung disease in SP-B deficiency is invariably progressive with escalating need for respiratory support and persistent and worsening alveolar and interstitial infiltrates seen on radiography. An initial positive response to surfactant replacement therapy may be observed but with diminished response to subsequent doses. Glucocorticoids may improve the lung disease in some affected infants, perhaps because they have mutations that allow for some SP-B production.[26] The disease is rapidly fatal, with more than 80% of affected infants dying within 3 months despite aggressive supportive measures.[27,28]

MOLECULAR GENETICS AND EPIDEMIOLOGY

The SP-B gene *(SFTPB)* contains 11 exons, of which the last is untranslated. The gene is transcribed into a 2-kilobase (kb) messenger RNA (mRNA), which directs the synthesis of a 381–amino acid pre-proprotein. The first 23 amino acids comprise a signal peptide that is removed cotranslationally. Mature SP-B corresponds to codons 201 (encoding phenylalanine) to 279 (encoding methionine) of the mRNA and is encoded in exons 6 and 7 of the gene[29] (Figure 107-1). The first and most common mutation identified involves a substitution of three bases (GAA) for one (C) in codon 121 of the SP-B mRNA, located in exon 4 of the SP-B gene, leading to a net insertion of two bases, and is termed 121ins2. The 121ins2 mutation causes a frameshift and introduces a premature codon for the termination of translation in exon 6, thus completely precluding production of mature SP-B; the resulting transcript is also unstable, accounting for the lack of SP-B mRNA.[30]

Many other disease-causing mutations and a sizable deletion in *SFTPB* have subsequently been identified[26,27,31-37] (see Figure 107-1). All are loss-of-function mutations that result in markedly

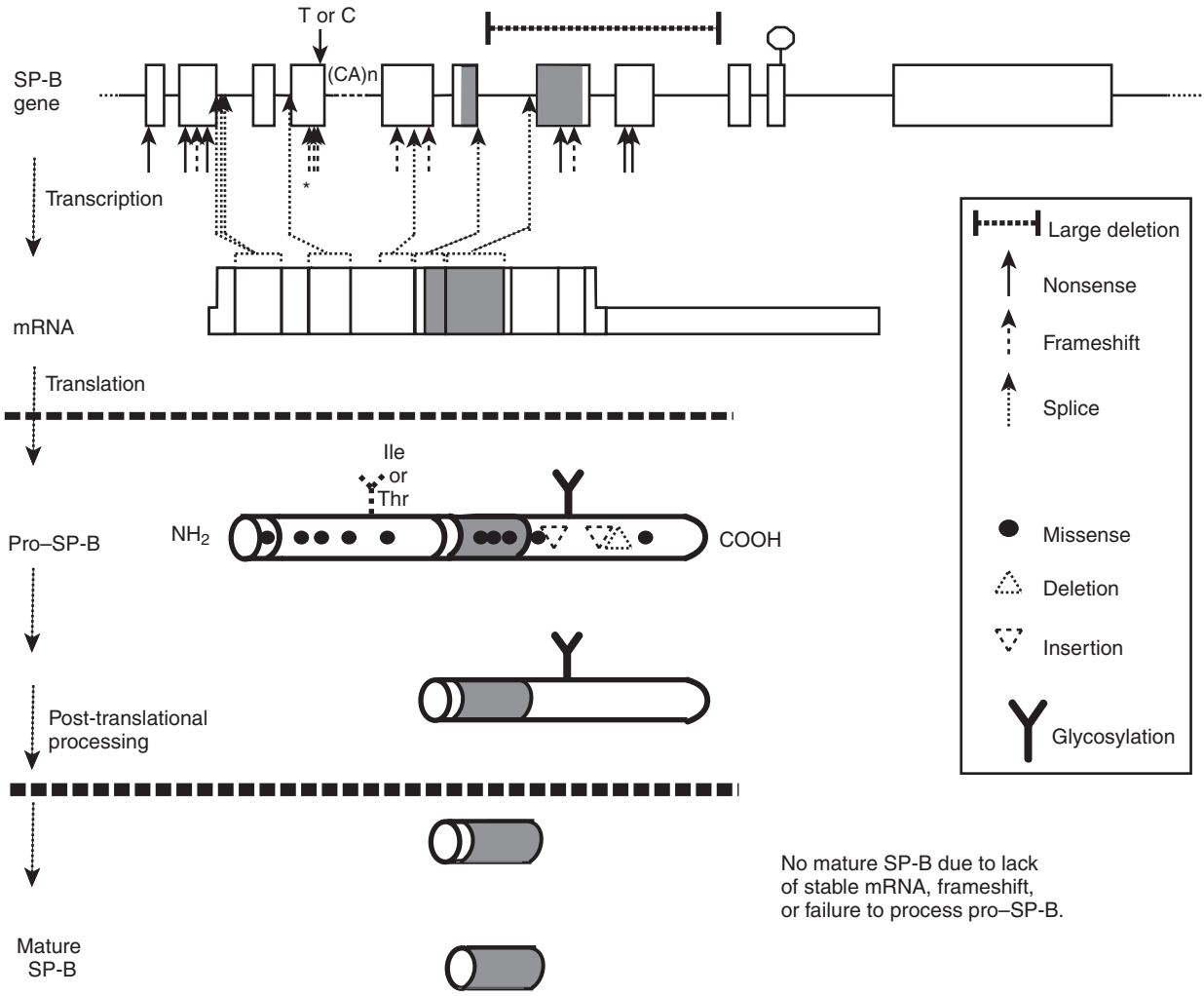


Figure 107-1 SP-B gene, protein synthesis, and postranslational processing of SP-B and mutations in the SP-B gene. The normal transcription, translation, and processing of SP-B are outlined with the SP-B gene at the top. The 11 exons are represented by *boxes,* and the introns are represented by *lines*. The locations of a coding polymorphism resulting in either isoleucine *(Ile)* or threonine *(Thr)* in exon 4 and variable nucleotide repeat sequences (CA_n) in intron IV are indicated. Untranslated regions of the mRNA are indicated by the *narrower rectangles*. Regions corresponding to mature SP-B are *shaded. Arrows* indicate the locations of known nonsense and frameshift mutations in the SP-B gene, which result in absent SP-B mRNA and protein. The location of the 121ins2 mutation is indicated with an *asterisk*. Nonsense and frameshift mutations often result in an unstable mRNA, interfering with SP-B production at a pretranslational level *(thin dashed line).* Mutations that interfere with normal SP-B mRNA splicing are indicated by *arrows* pointing to the location in the gene and indicating the affected exon on the mRNA. Missense mutations or in-frame deletions or insertions, which are indicated by their location in the SP-B proprotein, interfere with the processing of pro–SP-B to mature SP-B as indicated by the *thick dashed line.*

reduced or absent amounts of mature SP-B. Some mutations allow production of pro–SP-B, but its processing to mature SP-B is hindered, resulting in the lack of mature SP-B in lung tissue and tracheal secretions of affected infants.[27] Mutations on both *SFTPB* alleles in affected infants are consistent with the autosomal recessive inheritance pattern.

Pro–SP-B contains three tandem domains with structural homology to the saposins, lysosomal proteins that bind lipids and activate lysosomal hydrolases.[38] Mature SP-B corresponds to the middle domain of the proprotein,[39] and mutations in this region could theoretically result in the production of an SP-B protein with abnormal surface properties. However, a synthetic peptide containing one such mutation (R236C) was able to augment surface tension lowering normally in an in vitro system,[40] suggesting that the disease in infants with this mutation also resulted from impaired processing of pro–SP-B containing the mutation. Mature SP-B forms higher-order oligomers dependent on sulfhydryl-bond formation and ionic interactions.[41,42] Mutations that prevent oligomerization of SP-B could also in theory result in an SP-B with decreased activity, and possibly even act in a dominant negative fashion to interfere with SP-B from a normal allele. Such a mechanism for SP-B deficiency has not been recognized to date.

The exact incidence of SP-B deficiency is unknown. The number of reported cases is relatively small,* and population-based studies to determine the incidence of disease have not been performed. A carrier frequency of approximately 1 in 1000 individuals has been reported for the 121ins2 mutation.[49,50] Because this mutation has accounted for approximately two thirds of the mutant alleles identified, this implies that the frequency of

*References 26, 27, 31, 33-36, 43-48.

any SP-B gene mutation in the population is approximately 1 in 600. Because SP-B deficiency is an autosomal recessive disorder, if both parents have a mutation they have a 25% chance of having an affected child. The predicted incidence of disease would thus be approximately 1 in 1.4 million (600 × 600 × 4), indicating that SP-B deficiency is a very rare disease. This is probably an underestimate, because most studies to date have focused on children with either the 121ins2 mutation and/or very severe lung disease, whereas children with mutations resulting in milder disease may have been underrecognized.

The 121ins2 mutation has been found mainly in individuals of Northern European descent and likely results from a common ancestral origin ("founder effect").[51] Two other SP-B gene mutations (122delC, 122delT) have been identified within five nucleotides of the site of the common mutation, and a single nucleotide polymorphism is located within 30 base pairs (bp) of the site of the 121ins2 mutation, suggesting that this region of the SP-B gene could also be a "hot spot" for mutation.[33,47] Clusters of mutations have also been identified in exons 2 and 9. Other mutations that have been observed in more than one unrelated individual include the 122delT mutation in individuals of Middle Eastern descent, the 1043ins3 mutation in Asians, the R295X mutation in individuals of Hispanic descent, and a splicing mutation (c.479G>T) in French-Canadians. The frequencies of these mutations and incidence of the disease in these populations have not been determined.

Rare children with *SFTPB* mutations and a relatively milder phenotype have been identified. The mutations in these children allow for the production of some SP-B and thus result in "partial deficiency."[26,31] Whether the small amount of mature SP-B (approximately 8%-10% of control levels) or some retained function of pro–SP-B accounts for the milder phenotype is unclear. These observations suggest that there is a critical level of SP-B needed for normal lung function. This hypothesis is further supported by studies in which mice genetically engineered so that their production of SP-B could be shut off developed respiratory failure when their SP-B levels fell to 20% to 30% of that of SP-B levels observed in controls.[52]

SP-B production increases with advancing gestational age and is down-regulated with inflammation.[53-55] Lung disease could develop in individuals with loss-of-function mutations on one *SFTPB* allele (haploinsufficiency) if other factors, such as prematurity or infection, further delay or reduce SP-B expression below a critical level. Severe neonatal lung disease was observed in a child who had undetectable amounts of SP-B in bronchoalveolar lavage fluid (BALF) during his acute illness and an *SFTPB* mutation on only one allele with an apparently normal second *SFTPB* allele. The child's lung disease gradually improved in association with increased amounts of SP-B detected in subsequent BALF samples, suggesting that the mutation on one allele contributed to transient SP-B deficiency.[32] In addition, genetically engineered mice heterozygous for a null *sftpb* allele have half normal levels of SP-B protein and do not have neonatal respiratory disease but were more susceptible to pulmonary oxygen toxicity than their wild-type littermates.[56-58] In contrast to these observations, pulmonary function test results in seven human adult carriers for the 121ins2 mutations did not differ from those in controls, nor do the parents of affected infants generally have a history of neonatal lung disease.[59] Larger, population-based studies will be necessary to determine whether carrying one abnormal *SFTPB* allele is a risk factor for lung disease.

OTHER SURFACTANT PROTEIN B GENE VARIANTS AND LUNG DISEASE

Although rare, SP-B deficiency demonstrates the essential role of SP-B in normal surfactant metabolism. Given this critical role, *SFTPB* is a reasonable candidate gene to account for possible genetic variation observed in more common lung diseases such as RDS. A number of variants in the *SFTPB* have been identified that could potentially affect SP-B expression. A single nucleotide polymorphism has been identified in codon 131, which encodes either threonine (Thr) or isoleucine (Ile).[27,29,47,60,61] The 131Thr allele, but not 131Ile, has a potential additional recognition site for N-linked glycosylation.[62] Whether glycosylation at this site significantly affects pro–SP-B structure or its routing through the cell is unknown. However, the 131Thr allele was associated with an increased risk for acute RDS in adults and an increased risk for neonatal RDS in premature infants.[63-65] *SFTPB* variants that result in small differences in SP-B function or metabolism could thus be important in the pathogenesis of other lung diseases. Multiple other single nucleotide polymorphisms have also been identified in both coding and noncoding regions of *SFTPB*, including one in the promoter region that affects gene transcription and protein levels.[47,63,66-69] Exon 8 contains an alternative splice site, the utilization of which would result in an SP-B proprotein with four amino acids deleted from the carboxyl terminus domain.[48,70] This splice variant has been detected in normal human lung tissue and in increased in amounts in lung tissue from patients with various lung diseases.[70] It is not known whether this alternative transcript is translated and whether the resulting proprotein would be processed normally. Further studies are needed to address these questions, as well as whether the increase in the use of the alternative splice site is a consequence of or contributes to lung disease.

The fourth intron of *SFTPB* also contains a variable nucleotide tandem repeat sequence, and allelic variants containing different numbers of repeats have been characterized.[71,72] An increased risk for RDS in infants with alleles that differ from the predominant allele alone or in association with polymorphic variants in the SP-A gene loci has been observed in some studies.[64,71,73-76] The mechanisms by which these intron IV allelic variants would affect RDS risk are unclear because these variants have not been shown to directly affect SP-B expression or function, although they may be related to alterations in mRNA splicing or alterations in transcription factor binding.[77,78] They could also be in linkage with other variations in the SP-B gene with direct functional importance.[79]

Surfactant isolated from BALF of SP-B–deficient infants does not lower surface tension effectively, which is not surprising given the apparent importance of SP-B in surfactant function.[30,80-82] Tubular myelin, the latticelike extracellular form of surfactant the formation of which requires SP-B (as well as SP-A and calcium), is also not found in lung tissue of affected infants.[83,84] However, additional disturbances in surfactant metabolism also contribute to the pathophysiology of the lung disease in SP-B–deficient infants. These include abnormal surfactant phospholipid composition, particularly a marked reduction in phosphatidylglycerol content, abnormal lamellar body formation, and an apparent block in posttranslation processing of the SP-C precursor protein (pro–SP-C).[30,85,86] These latter changes result primarily from the loss of SP-B and not from treatments used to sustain SP-B–deficient infants, because abnormal lamellar body formation and impaired pro–SP-C processing were also observed in the lungs of genetically engineered mice that were unable to produce SP-B and that died within minutes of birth because of respiratory insufficiency.[87,88] These observations indicate a fundamental intracellular role for SP-B (or pro–SP-B) in surfactant metabolism in addition to its extracellular role in lowering surface tension.

Lung tissue from SP-B–deficient infants contains proteins that are immunoreactive with antisera directed against the amino-terminal domain of pro–SP-C, intermediate in size between pro–SP-C and mature SP-C, and which contain amino-terminal epitopes by protein sequence analysis.[86,89] These observations are consistent with a block in the final processing steps of pro–SP-C to

mature SP-C. This would result in a relative deficiency of mature SP-C, further contributing to surfactant dysfunction.[90] These peptides are found in BALF from affected infants, and intense immunostaining of intraalveolar material using antisera directed against pro–SP-C is observed in lung tissue from SP-B–deficient infants, indicating that they are secreted. However, they are not very surface active and likely to inhibit surfactant function. The precise mechanisms underlying this block in SP-C processing are unknown. Ultrastructural examination of the lungs of SP-B–deficient infants reveals a lack of normally formed lamellar bodies, the intracellular storage organelles of surfactant, with abundant, abnormal-appearing lipid vesicles, indicating that intracellular expression of SP-B (or pro–SPB) is needed for normal lamellar body biogenesis. The final steps in processing of pro–SP-C to mature SP-C occur late in the secretory pathway, either in lamellar bodies or in their immediate precursors.[91-94] Thus the inability to form these organelles because of the lack of SP-B results in incompletely processed pro–SP-C, as well as inadequate packaging of surfactant components for secretion. SP-B also facilitates membrane fusion, and an explanation for these observations is that without SP-B, transport vesicles containing incompletely processed pro–SP-C are unable to fuse with developing lamellar bodies, thus exposing the amino-terminal epitopes of pro–SP-C to the necessary processing enzymes.[92,95]

Other mechanisms that impair SP-B production, such as mutations in genes for the enzymes needed for the proper processing of pro–SP-B to mature SP-B, or accelerate SP-B catabolism, could result in deficiency of SP-B and similar lung disease. Fatal neonatal lung disease inherited in an autosomal recessive fashion, associated with reduced or undetectable amounts of SP-B in the BALF or lung tissue of affected children, but without *SFTPB* mutations identified to account for the lack of SP-B, has been reported.[67] Many, but not all, of these cases were due to mutations in the gene encoding ABCA3, which may also interfere with SP-B metabolism (discussed in more detail later). A comprehensive approach to the evaluation of children with severe neonatal lung disease of unclear etiology, including ultrastructural examination and collection of frozen, as well as fixed, tissue from biopsy and autopsy studies, will be essential to elucidating novel mechanisms of lung disease.

ADENOSINE TRIPHOSPHATE–BINDING CASSETTE FAMILY A3 DEFICIENCY

ABCA3 is a 1704–amino acid transmembrane protein that is highly expressed in alveolar type II cells where it is localized to the limiting membrane of lamellar bodies.[96-99] It is a member of the adenosine triphosphate–binding cassette family of proteins, which hydrolyze adenosine triphosphate to translocate a wide variety of substrates across biologic membranes.[100] The gene encoding ABCA3 *(ABCA3)* is large, spanning some 80,000 bases on the short arm of chromosome 16, and contains 33 exons.[101] ABCA3 contains 12 membrane-spanning domains and two nucleotide-binding domains (Figure 107-2). Although the function of ABCA3 is unknown, its localization in type II cells and the findings that other members of the ABCA

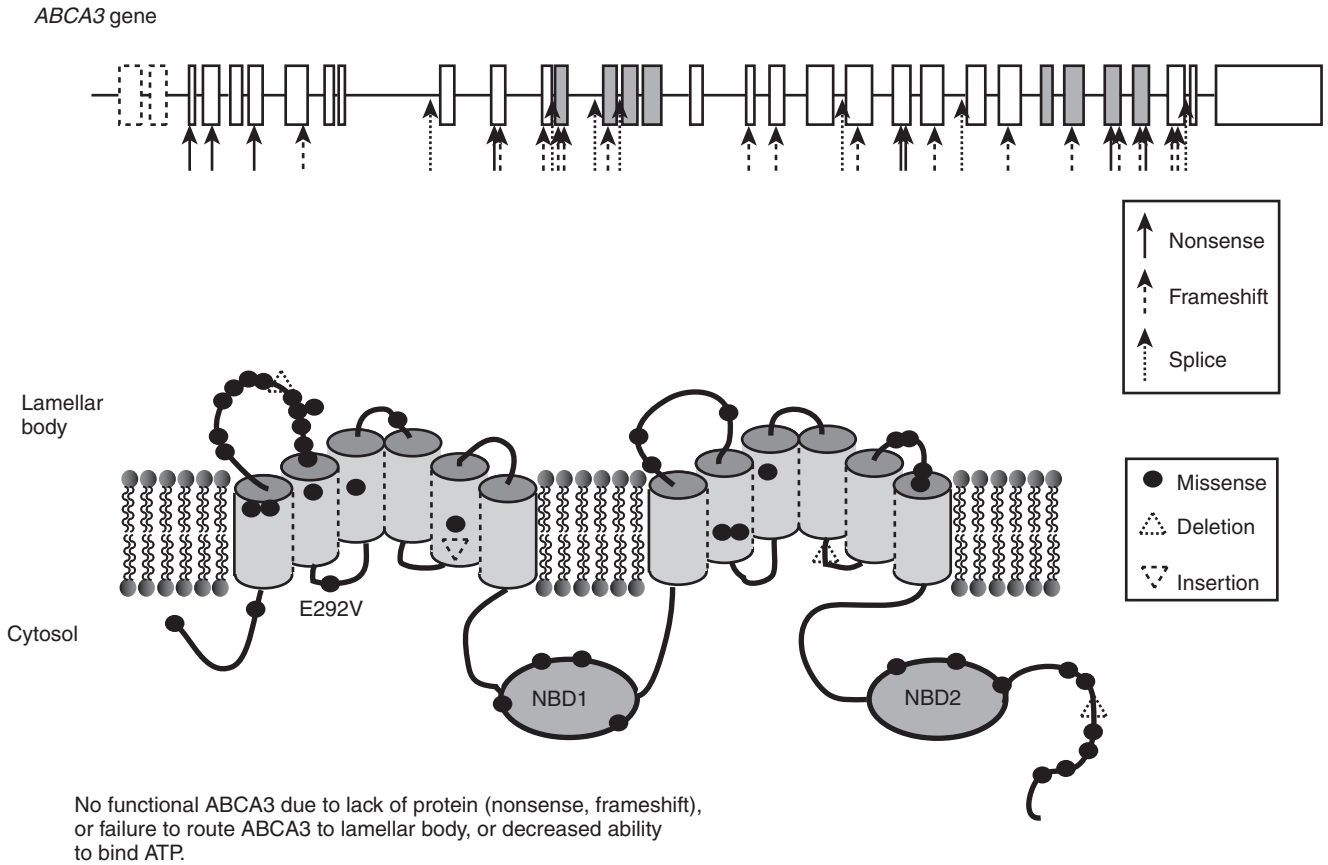


Figure 107-2 ABCA3 gene, predicted protein structure, and location of mutations. The 30 coding exons of the *ABCA3* gene are indicated by *solid boxes* and two 5′ untranslated exons indicated by *dashed boxes.* The locations of known nonsense, frameshift, and splice-site mutations in the gene are indicated by *arrows.* The structure of the ABCA3 protein is predicted by homology with other ABCA proteins, with 12 membrane-spanning domains *(cylinders)* and two nucleotide-binding domains *(NBD)* indicated by *ellipses.* The locations of known missense mutations or small in-frame insertions or deletions are indicated on the protein structure.

subfamily transport lipids are consistent with a role for ABCA3 in importing lipids needed for surfactant function into lamellar bodies.[96-98] These properties also suggested ABCA3 as a possible candidate gene for genetic surfactant deficiency.

ABCA3 deficiency was recognized as a cause of lung disease when *ABCA3* mutations were found in 16 of 21 term newborns with severe lung disease who all had clinical and radiographic features of surfactant deficiency and no other cause for their lung disease identified.[102] Both the clinical and lung pathologic features of these infants were similar to those of infants with SP-B deficiency. Ultrastructural examination of lung tissue from infants with *ABCA3* mutations demonstrated absence of normal lamellar bodies in their alveolar type II cells, which instead contained many small dense organelles with very tightly packed membranes and eccentrically placed electron-dense inclusions, giving them a "fried egg" appearance.[103] These findings thus supported a role for ABCA3 in both surfactant metabolism and lamellar body biogenesis.

Subsequent studies confirmed and extended these findings. *ABCA3* mutations were identified in 8 of 12 infants who had undergone lung transplantation within the first year of life for respiratory failure of unknown cause that had its onset in the neonatal period. Surfactant isolated from BALF of these children had significantly reduced phosphatidylcholine content and a markedly reduced ability to lower surface tension.[80] In another series of infants with fatal neonatal lung disease of unknown etiology, *ABCA3* mutations were found in 8 of 14 patients studied, and decreased or absent ABCA3 protein was demonstrated in lung tissue of patients with mutations. SP-B and SP-C were also decreased in amount in BALF from some patients, which may have further contributed to impaired surfactant function.[104] In vitro studies support both a role for ABCA3 in surfactant lipid metabolism and that the mutations observed in patients with fatal lung disease were functionally significant. ABCA3 containing missense mutations that had been identified in patients with lung disease were either abnormally trafficked when expressed in cells in tissue culture, had reduced binding of adenosine triphosphate, or resulted in reduced uptake of lipids into lysosomes compared with wild-type ABCA3.[105,106] Genetically engineered mice homozygous for null ABCA3 alleles have been generated, and these animals have a phenotype identical to that of human infants with loss-of-function *ABCA3* mutations, with perinatal lethal respiratory disease, abnormal lamellar body formation, and abnormal lung phospholipid profiles.[107-110] Collectively these observations indicate that loss-of-function mutations in *ABCA3* result in altered surfactant lipid metabolism and impaired lamellar body biogenesis, although the exact substrates transported by ABCA3 remain to be determined.

ABCA3-deficient infants often present with severe neonatal lung disease similar to that observed in SP-B–deficient infants.[80,102,104,111,112] However, infants with *ABCA3* mutations on both alleles and either transient or even no apparent neonatal lung disease have been observed, and long-term survival with chronic interstitial lung disease (ILD) is possible.[113-116] No predominant *ABCA3* mutation has been identified, and a wide range of different mutations, including nonsense, frameshift, splice-site, and missense mutations scattered throughout the gene have been reported, indicating considerable allelic heterogeneity.[80,102,104,112-118] The most frequently identified *ABCA3* mutation consists of a substitution of valine for glutamine in codon 292 (E292V), and the population frequency of this mutation was approximately 1 in 300 individuals depending on ethnic background.[119] Because *ABCA3* E292V has been found mainly in patients with relatively milder disease, genotype may be important in predicting phenotype, although additional studies are needed to evaluate this possibility.[113]

The overall frequency of *ABCA3* mutations in the general population and the incidence and prevalence of lung disease caused by ABCA3 deficiency are currently unknown. It is likely that the disease is rare, because death resulting from unexplained neonatal respiratory failure is unusual, and the types of pediatric ILD associated with *ABCA3* mutations are also unusual.[120,121] The high ascertainment rate for *ABCA3* mutations in studies that have examined the cause of unexplained severe lung disease in neonates or young infants suggests that *ABCA3* mutations are a more frequent cause of this phenotype than SP-B deficiency.[112] As with SP-B deficiency, the finding of patients with a milder phenotype suggests that there is a critical level of ABCA3 expression needed for normal lung function, and *ABCA3* genetic variants could thus contribute to the pathogenesis of other lung diseases, including RDS in premature infants.[119,122]

SFTPC MUTATIONS AND LUNG DISEASE

SP-C is a 35–amino acid extremely hydrophobic protein that also enhances the surface tension–lowering properties of surfactant phospholipids. SP-C is encoded by a small (<3.5 kb) gene *(SFTPC)* on human chromosome 8 that contains six exons, the last of which is untranslated. The gene directs the synthesis of a proprotein (pro–SP-C) of either 191 or 197 amino acids, depending on alternative splicing at the beginning of the fifth exon. SP-C is extensively posttranslationally modified, including the palmitoylation of cysteine residues at positions 5 and 6 of mature SP-C, such that it is a proteolipid.[6,123] Pro–SP-C is proteolytically processed at both the amino and carboxyl termini to yield the 35–amino acid mature SP-C that is secreted into the airspaces. Pro–SP-C does not contain a signal peptide, and the domain corresponding to mature SP-C is thought to anchor it in the membrane such that pro–SP-C is an integral membrane protein (Figure 107-3). Because of its importance in surfactant function, it was anticipated that SP-C deficiency caused by loss-of-function mutations could also result in neonatal lung disease resembling RDS. However, no patients with such mutations and RDS have been reported to date. Moreover, genetically engineered mice with an inability to produce SP-C do not develop neonatal lung disease but instead develop ILD and emphysema with aging (depending on their genetic background).[124,125] Thus SP-C does not appear to be essential for normal neonatal lung function.

Mutations in *SFTPC* cause human lung disease of variable severity and onset of symptoms, with some patients remaining free of symptoms well into adulthood.[90,126-133] Severe and even fatal neonatal disease has been observed, although the majority of reported patients with *SFTPC* mutations have not had neonatal lung disease, and *SFTPC* mutations are an unusual cause of neonatal respiratory failure.[102,134] The most commonly reported clinical presentation is that of ILD in young infants. Clinical symptoms have included tachypnea, retractions, and hypoxemia in room air; failure to thrive and digital clubbing have also been observed in affected infants. The mechanisms responsible for the variability in disease severity remain largely unknown, although heterozygosity for an *ABCA3* mutation may exacerbate the severity of the pulmonary symptoms.[135]

As opposed to SP-B and ABCA3 deficiencies where loss-of-function mutations on both alleles result in loss of protein expression or function and lung disease is inherited in a recessive fashion, the lung disease caused by *SFTPC* mutations generally results from a mutation on only one *SFTPC* allele; lung disease is transmitted as an autosomal dominant with a high degree of penetrance in familial cases, or sporadically caused by a de novo mutation.[127,128,130,132] All identified mutations are predicted to result in an abnormal form of pro–SP-C, including missense mutations, in-frame insertions or deletions, or distal frameshift mutations that are likely associated with stable transcripts. Nonsense mutations, early frameshifts (likely to be associated with

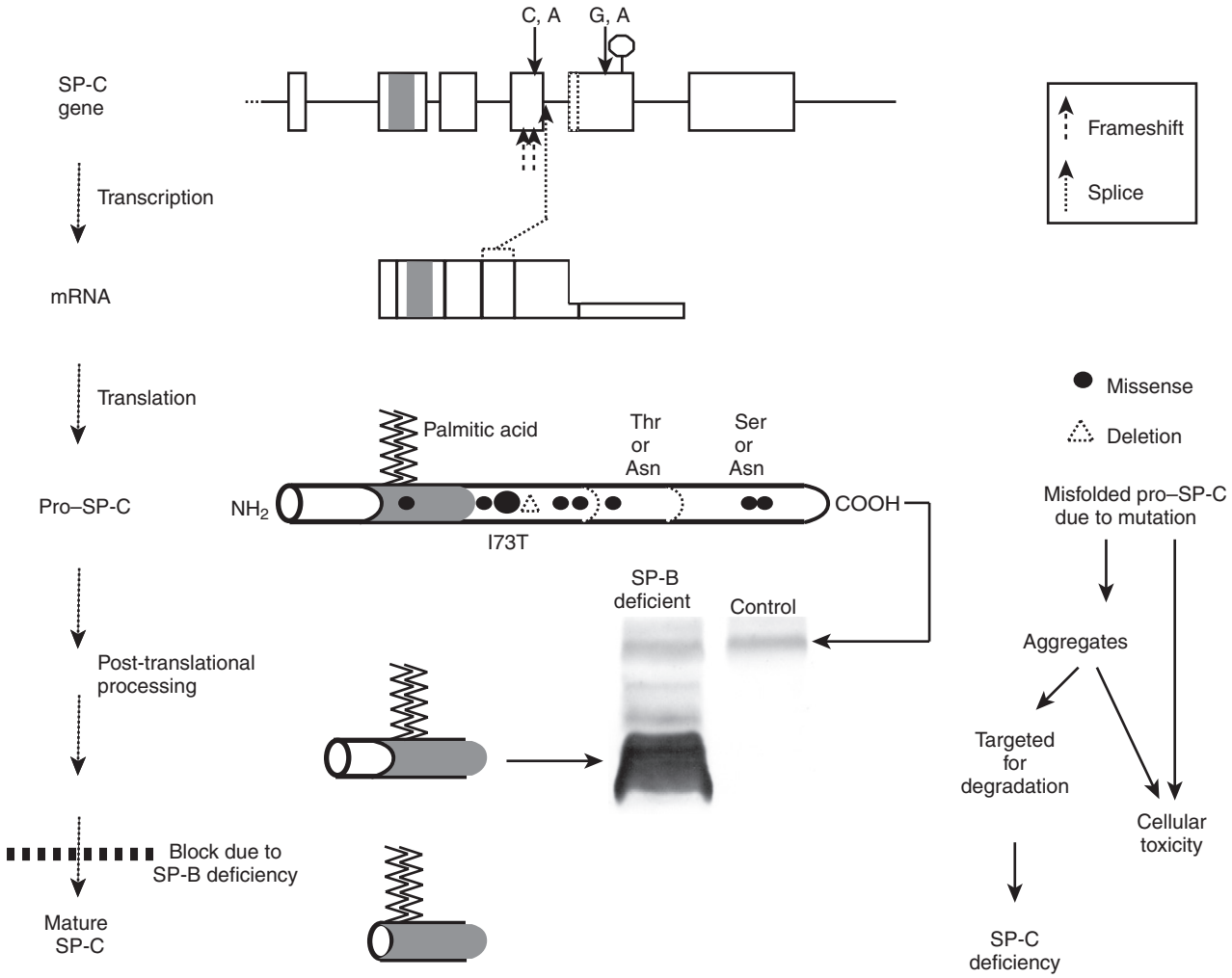


Figure 107-3 SP-C gene, processing, and effects of mutations. The normal transcription, translation, and processing of SP-C are outlined with the SP-C gene at the top. The six exons are represented by *boxes,* and the introns are represented by *lines.* The locations of two coding polymorphisms resulting in either threonine *(Thr)* or asparagine *(Asn)* in exon 4 and serine *(Ser)* or asparagine *(Asn)* in exon 5 are indicated. The normal processing of pro–SP-C to mature SP-C is shown on the *left,* with the portion corresponding to mature SP-C *shaded.* A *dashed line* indicates the site of the block in pro–SP-C processing that occurs in the absence of SP-B. The accumulation of immunoreactive peptides in the lung tissue from an SP-B–deficient infant are shown in the Western blot *(inset)* and are not present in lung tissue from a control infant. *Arrows* indicate the locations of frameshift or splicing mutations in the SP-C gene, and the locations of missense mutations or an in-frame deletion are indicated on the proprotein. SP-C gene mutations result in an abnormal form of pro–SP-C, with SP-C deficiency and/or toxicity resulting from misfolded pro–SP-C caused by the mutations.

unstable SP-C transcripts), or major deletions that would preclude SP-C production have not been reported, suggesting that haploinsufficiency is unlikely to be the mechanism for lung disease. One specific *SFTPC* mutation resulting in the substitution of threonine for isoleucine in codon 73 (I73T) has been found in multiple unrelated families and associated with both familial and sporadic lung disease.[126,128,130] The finding of a common mutation may be useful in population-based studies to determine the incidence and prevalence of lung disease associated with *SFTPC* mutations, which are currently unknown.

SFTPC mutations could result in lung disease either by causing SP-C deficiency because of a dominant-negative mechanism, or through a toxic gain-of-function mechanism because of the presence of abnormal pro–SP-C within alveolar type II cells. Pro–SP-C containing a mutation (Δexon4) identified in human subjects with ILD was rapidly degraded in vitro in the absence of inhibitors of proteosome-mediated degradation.[136] Pro–SP-C self-associates in the secretory pathway, and mutated pro–SP-C that is targeted for degradation could interact with normal pro–SP-C, leading to its degradation as well, and thus SP-C deficiency.[137,138] Decreased expression of both pro–SP-C and mature SP-C were observed in patients with the *SFTPC* Δexon4 mutation, supporting such a mechanism.[90] Mature SP-C was also markedly reduced in a patient with a different *SFTPC* mutation who had normal to increased pro–SP-C expression by immunohistochemical analysis[132] but was present in BALF from a child with yet a different *SFTPC* mutation,[129] indicating that different mutations may cause lung disease through different mechanisms. Precisely how deficiency of mature SP-C eventually results in ILD is unknown.

The carboxyl-terminal domain of pro–SP-C, in which the majority of known pathogenic mutations are located, has homology with other proteins in what has been termed the Brichos family (membrane proteins in which abnormal aggregates have been implicated in the pathophysiology of familial dementia and neoplasia).[139] The Brichos domain of pro–SP-C

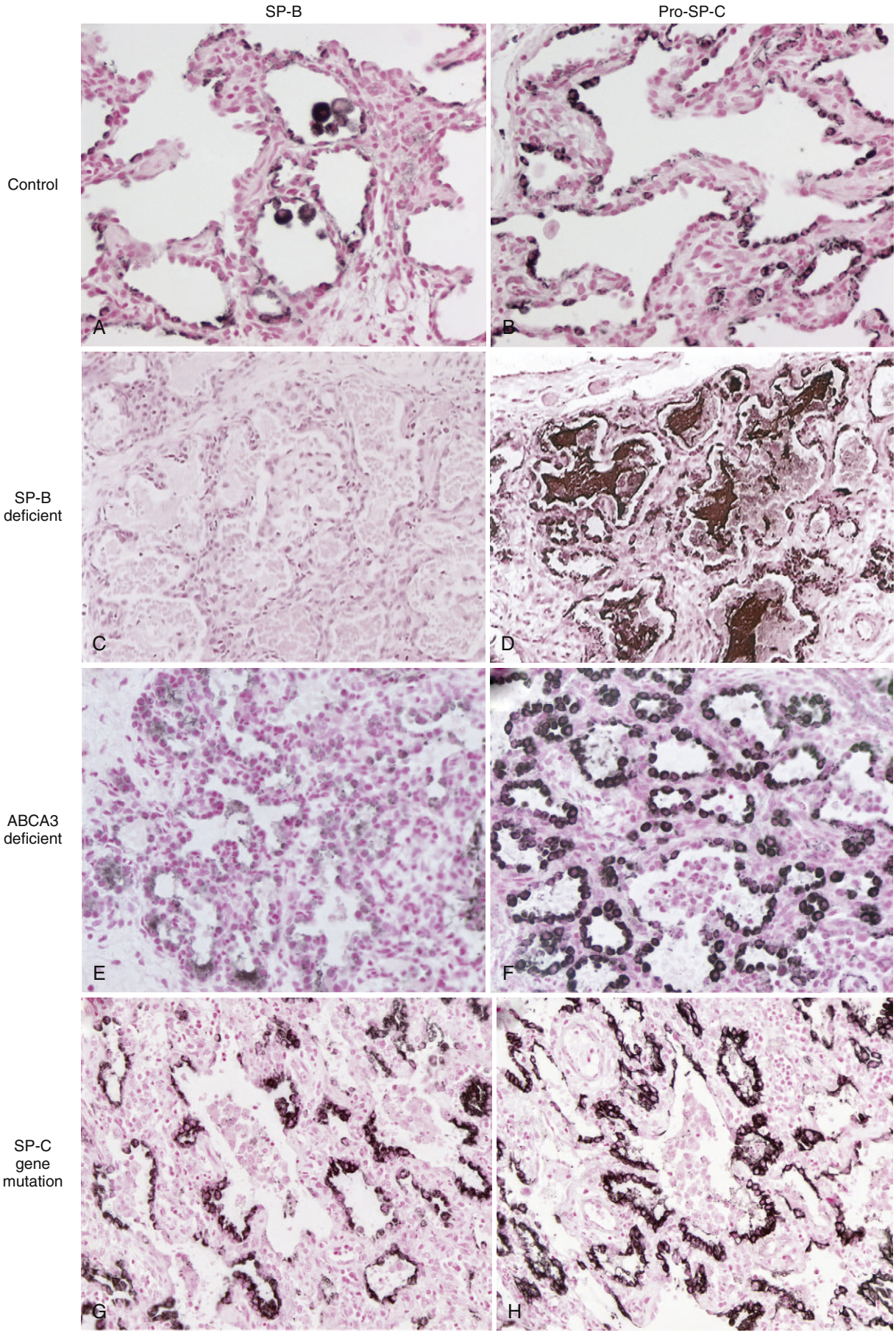
may be important in binding to and stabilizing the mature SP-C domain in a conformation that is less prone to forming amyloid-like aggregates.[140,141] *SFTPC* mutations may induce misfolding of pro–SP-C with exposure of hydrophobic epitopes, resulting in accumulation of misfolded protein aggregates early in the secretory pathway. Therefore lung disease caused by *SFTPC* mutations could represent a conformational disease.[142] The presence of misfolded protein in the endoplasmic reticulum could result in endoplasmic reticulum stress, triggering the unfolded protein response, resulting in up-regulation of chaperone proteins and other downstream pathways, eventually leading to apoptosis and/or inflammation. In support of such a toxic gain-of-function mechanism, robust rather than reduced or absent staining for pro–SP-C has been observed in the lung tissue of some patients with *SFTPC* mutations,[127-130,132] and aggregates of pro–SP-C caused by mutations have been observed both in vitro and in lung tissue from an infants with *SFTPC* mutations.[129,132,143] The potential toxicity of mutated SP-C to lung epithelial cells has been demonstrated in vitro[127,144,145] and by the observations that transgenic animals that overexpressed either mature SP-C without the flanking domains of the proprotein or the *SFTPC* exon 4 mutation died of a marked disruption of lung development.[136,146] This impairment of normal lung development associated with high levels of SP-C expression in utero also suggests a potential mechanism whereby some *SFTPC* mutations could result in severe neonatal lung disease. Finally, another mechanism for disease has been proposed in which abnormal routing of mutated pro–SP-C leads to accumulation within the endosomal pathway, inhibiting recycling of surfactant components.[129]

LUNG PATHOLOGIC FINDINGS OF INBORN ERRORS OF SURFACTANT METABOLISM

The histopathologic findings in the lungs of infants with mutations in *SFTPB, ABCA3,* or *SFTPC* are similar and overlapping. Prominent findings in all three disorders include alveolar type II cell hyperplasia, prominent collections of foamy macrophages in the airspaces, and interstitial thickening. Cholesterol clefts may be found in airspaces or within cells; nonspecific findings including diffuse alveolar damage and interstitial fibrosis may also be present. Variable amounts of granular eosinophilic material within entrapped foamy macrophages may be seen in distal airspaces. This finding may be so prominent that the pathologic findings are labeled as pulmonary alveolar proteinosis because of the similarity to the findings in adults with that disorder, where there is accumulation of surfactant phospholipids and proteins in the airspaces. Other diagnostic terms that were also originally used to describe lung pathologic conditions in adults have been applied to children with these disorders, including desquamative interstitial pneumonitis and nonspecific interstitial pneumonia. Although these pathologic features were similar to those seen in adults, the clinical features of children with these lung diseases differed from those in adults, often with the onset of disease in infancy and a more severe course, associated with a positive family history.[147,148] The term "congenital alveolar proteinosis" was used to distinguish neonatal onset and fatal disease in infants from the adult disorder in which the onset and progression of disease are more gradual.[149-152] SP-B deficiency was the cause for lung disease in one of the kindreds reported in one of these studies and ABCA3 deficiency in the other (Nogee and colleagues,[48] Moulton and colleagues,[151] and unpublished data). Findings of pulmonary alveolar proteinosis have also been reported in association with *SFTPC* mutations.[129,130] Chronic pneumonitis of infancy is a more recently described condition specific to young infants that encompasses the pathologic findings observed in children with mutations in *SFTPB*, *ABCA3,* or *SFTPC* but is also nonspecific because mutations in any one of the three genes have been reported in children with this disease.[27,102,128,153] Recently the term "surfactant dysfunction mutation" has been proposed to encompass the pathologic findings related to all of these conditions,[154,155] reflecting the nonspecific nature of the pathology at the level of light microscopy.

Specific immunostaining for surfactant proteins may be helpful in distinguishing between SP-B deficiency and other genetic mechanisms, although such analyses are currently confined to research laboratories (Figure 107-4). Immunostaining can be performed on archived lung specimens, allowing for retrospective diagnosis in children who died years earlier. In SP-B deficiency, staining for mature SP-B is usually absent or markedly reduced; staining for pro–SP-B may be also absent depending on the responsible *SFTPB* mutation.[27] In contrast, robust staining for pro–SP-C is observed in both the alveolar epithelium and extracellular material. The extracellular pro–SP-C–positive staining is likely due to the secreted, incompletely processed pro–SP-C and has correlated very well with the finding of mutations in *SFTPB*.[27] Surfactant protein immunostaining patterns associated with *SFTPC* or *ABCA3* mutations are less specific. Reduced epithelial staining for pro–SP-C has been seen in some patients with *SFTPC* mutations but may be robust in others, with pro–SP-C staining confined to the alveolar epithelium.[90,128] Reduced staining for mature SP-B had also been observed in some, but not all, children with *ABCA3* mutations.[104,113] Pro–SP-B staining in these children is generally robust, as is staining for pro–SP-C, which is confined to the alveolar epithelium, thereby distinguishing it from SP-B deficiency. Antibodies to ABCA3 have recently been generated, and in one study ABCA3 mutations were identified in all children with neonatal respiratory failure in whom ABCA3 expression was not detected or was markedly reduced compared with controls, which included children with *SFTPB* or *SFTPC* mutations.[104] These findings suggest that specific immunostaining for ABCA3 will be helpful in discriminating between these disorders. However, because some *ABCA3* missense mutations result in protein that is appropriately routed but not functional,[105,106] it is likely that positive staining for ABCA3 will not be able to exclude the disease.

Figure 107-4 Immunohistochemical staining of lung tissue. Lung tissue was immunostained for mature SP-B *(left)* and pro–SP-C *(right).* Lung tissue from a control infant who died of chronic lung disease demonstrates positive staining for both mature SP-B **(A)** and pro–SP-C **(B)** observed in alveolar epithelial cells *(top row).* In contrast, absent staining for mature SP-B **(C)** but intense staining for pro–SP-C in the alveolar epithelium and extracellular proteinaceous material **(D)** is observed in lung tissue from an SP-B–deficient infant who was homozygous for the 121ins2 mutation *(second row).* Staining for mature SP-B is also markedly reduced **(E)** in lung tissue from a patient who was a compound heterozygote for two ABCA3 mutations, with robust staining for pro–SP-C that is confined to the alveolar epithelium **(F).** Staining for mature SP-B **(G)** and for pro–SP-C **(H)** is robust in tissue from a patient with the most frequently found SP-C gene mutation (I73T) on one allele *(bottom). (Photographs courtesy Susan Wert, MD, Children's Hospital Medical Center, Cincinnati, Ohio.)*


SP-B
Pro-SP-C
Control
SP-B deficient
ABCA3 deficient
SP-C gene mutation
A
B
C
D
E
F
G
H

EVALUATION OF INFANTS WITH SUSPECTED INBORN ERRORS OF SURFACTANT METABOLISM

Clinical features that distinguish SP-B or ABCA3 deficiency from reversible causes of neonatal respiratory failure have not been identified. Term infants with respiratory distress that is not explained by history and does not improve after the first week of life, or infants who died of lung disease of unclear etiology should be suspected of having one of these disorders. Although a family history of lung disease may prompt suspicion for a genetic disorder, a positive family history is often absent given the autosomal recessive inheritance patterns. The differential diagnosis includes RDS, transient tachypnea of the newborn, pneumonia (both bacterial and viral), persistent pulmonary hypertension, pulmonary hypoplasia, unrecognized cardiac malformations, and developmental anomalies of the lung such as alveolar capillary dysplasia. Because the initial lung disease in some children with *ABCA3* mutations may be very mild, these children may be discharged from the neonatal intensive care unit and be seen later with signs and symptoms of persistent diffuse lung disease without a clear cause.

Routine laboratory findings in SP-B– and ABCA3–deficient infants are nonspecific. Tracheal aspirate samples for lecithin-sphingomyelin ratios have been reported as low and within the normal range but have not been systematically evaluated because such testing is not routinely done in term infants.[46,151] Analysis of tracheal aspirate or BALF samples for surfactant protein levels remains a research tool but is unlikely to provide a definitive result. SP-B levels are usually undetectable in SP-B–deficient infants but may also be undetectable or very low in children with ABCA3 deficiency.[27,104] Children with diffuse alveolar injury and more global surfactant deficiency not caused by genetic mechanisms may also have undetectable or very low levels of SP-B. Conversely, the presence of normal levels of SP-B in tracheal aspirate or BALF does not exclude the possibility of ABCA3 deficiency.[80]

A definitive diagnosis may be made through genetic testing. Such testing is noninvasive, and sequenced-based analysis of all of the coding exons of *SFTPB*, *ABCA3*, and *SFTPC* is available through certified diagnostic laboratories. A finding of likely disease-causing mutations on both alleles of either *SFTPB* or *ABCA3* is diagnostic for SP-B or ABCA3 deficiency, respectively. The interpretation of genetic testing may not always be straightforward. It may be difficult or impossible to distinguish whether a newly identified missense mutation represents a functionally significant or benign variant, because functional assays for such mutations are not readily available. Such testing is not 100% sensitive, because functionally significant mutations in the unexamined regions of the gene will be missed, as might be major deletions or rearrangements. Children with only one identifiable *ABCA3* mutation yet who have a clinical phenotype and lung pathologic findings consistent with ABCA3 deficiency have been reported, indicating that some *ABCA3* mutations have gone undetected.[102,112,113] Other limitations to genetic testing include cost and the length of time needed for results to be reported, particularly for a child with rapidly progressive disease.

Tissue examination may be helpful if lung biopsy is deemed necessary for proper diagnosis or on autopsy tissue when no suitable material is available for genetic studies. Lung histologic findings may be suggestive of an inborn error of surfactant metabolism, although not specific for a given genetic disorder. Because there are characteristic ultrastructural findings associated with both ABCA3 and SP-B deficiency, tissue should be prepared for electron microscopy with proper fixatives if an inborn error of surfactant metabolism is a possibility.[156,157] Strong consideration should also be given to snap freezing tissue in liquid nitrogen because such studies may facilitate future investigations.

Term infants with the nonspecific phenotype of severe RDS and without any identifiable mutations in *SFTPB*, *ABCA3,* or *SFTPC* have been reported, and it is thus likely that other inherited disorders resulting in abnormal surfactant production remain to be discovered.[102,104,112] Candidate genes could include those encoding other important enzymes and factors needed for the proper production, posttranslational processing, intracellular routing and packaging, or secretion of surfactant components. For example, haploinsufficiency for thyroid transcription factor 1 (TTF-1, also known as NKX2.1) because of loss-of-function mutations on one allele or a gene deletion has been associated with neonatal respiratory disease in term infants.[158-160] Similar to the nonspecific appearance of a sepsis-like illness and metabolic disturbances in children with inborn errors of intermediary metabolism, children with inborn errors of lung cell metabolism may present with a nonspecific picture of neonatal respiratory distress and radiographically diffuse lung disease. Elucidation of these inborn errors should enhance our understanding of normal surfactant metabolism and may provide clues to the pathogenesis of lung diseases outside the neonatal period.

TREATMENT

There are no specific therapies for inborn errors of surfactant metabolism. Whole lung lavage was ineffective in treating neonates with findings of alveolar proteinosis now known to have resulted from SP-B or ABCA3 deficiency.[151] SP-B deficiency is generally a fatal disease with the majority of reported infants having died within the first 6 months of life despite maximal medical therapy. Purified preparations of SP-B are not available for replacement therapy. Replacement therapy with exogenous surfactants containing SP-B has been attempted but did not alter the course in an infant in whom diagnosis was made before birth and treatment given from birth with more than 80 doses of surfactant.[46] Because exogenous replacement surfactants contain variable amounts of SP-B, as well as other surfactant components, the amount of SP-B administered may have been insufficient or repeated treatment with surfactant lipids and SP-C may have been toxic. Even if a preparation of purified SP-B were available, exogenous replacement therapy might be ineffective because exogenously administered SP-B might not reach the intracellular compartments needed to correct the intracellular deficits, or endogenous pro–SP-B synthesis might be necessary. Viral vectors capable of directing SP-B expression have been generated and tested in vitro and in animal systems, although multiple obstacles need to be overcome before gene therapy may be considered for human infants.[161-163]

The clinical course of infants with ABCA3 deficiency is more variable. Although most affected infants reported to date have had a course similar to that of infants with SP-B deficiency and died within the first 6 months of life, an increasing number of children with milder disease have been recognized. These children are usually treated with agents commonly used to treat forms of ILD, including corticosteroids and hydroxychloroquine, although data supporting the efficacy of these therapies in this condition are lacking. Infants with severe disease have received exogenous surfactant, with anecdotal reports of transient responses, but this has not been examined critically. Even if infants were to respond, the practical considerations of dosing and route of delivery for long-term therapy are considerable. Because many children with *ABCA3* mutations have missense mutations that result in inappropriate intracellular routing of ABCA3,[105,106] chemical chaperones to facilitate routing that may eventually be useful in this condition.[164-166]

Patients with *SFTPC* mutations are also often treated with agents such as steroids and hydroxychloroquine, but there are only anecdotal reports to support their efficacy.[167] Because of

the findings of pulmonary alveolar proteinosis in some of these patients, whole lung lavage has been attempted, but because this will not correct the underlying genetic defect it seems unlikely to result in sustained improvement. Anecdotal reports of clinical improvement in patients with *SFTPC* mutations after either whole lung lavage or treatment with other therapeutic agents must be interpreted in light of the natural variable history of the disorder.[130,167,168]

Lung transplantation has been performed for all three disorders with outcomes that are similar to those in infants with other pulmonary conditions.[80,85,132,169] Lung transplantation requires long-term immunosuppressive therapy with continued risks of infection and rejection. The procedure is costly and available only at a limited number of medical centers, potentially requiring a long-distance transport of a critically ill child in unstable condition. The availability of donor lungs is also limited, and death may occur before donor lungs become available. Compassionate care is an appropriate option for infants with SP-B deficiency given its bleak prognosis. The more variable courses associated with ABCA3 deficiency and especially with *SFTPC* mutations make decisions regarding lung transplantation or cessation of care more difficult for patients with these disorders. Finally, the parents of affected children should be referred for formal genetic counseling, because there is a 25% recurrence risk with SP-B and ABCA3 deficiency for subsequent pregnancies, and prenatal diagnosis may be an option if the mutation(s) responsible for disease in a family is identified.[170] Counseling of families of patients with *SFTPC* mutations is more difficult given the dominant inheritance and variable penetrance, because parents or other family members may be asymptomatic carriers of the mutation found in the proband, yet be at risk for development of lung disease.

SUMMARY

Hereditary SP-B deficiency is a rare disorder that causes fatal lung disease in term infants and demonstrates an essential role for SP-B in normal lung metabolism. Children with ABCA3 deficiency may have a clinical picture similar to that of infants with SP-B deficiency or may have milder, chronic lung disease. Mutations in the SP-C gene are associated with sporadic and familial lung disease of variable severity and age of onset. The pathophysiology of the lung diseases resulting from the lack of SP-B, ABCA3, and SP-C or from abnormally folded pro–SP-C is incompletely understood. Environmental factors and other genes that may have roles in modifying the course of these diseases need to be identified, and more effective treatments are needed.

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Fetal and Neonatal PHYSIOLOGY

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ELSEVIER
SAUNDERS

SECTION XIV

Physiology of Gastrointestinal Tract in the Fetus and Neonate

108

Douglas G. Burrin

Trophic Factors and Regulation of Gastrointestinal Tract and Liver Development

FETAL AND NEONATAL GASTROINTESTINAL AND LIVER GROWTH

Nature of Growth

To understand the role of trophic factors in fetal and neonatal gastrointestinal (GI) and liver growth it is important to first consider the nature of growth. The fetal and neonatal period is the most dynamic period of postconceptual growth and includes critical developmental milestones, such as gastrulation, organogenesis, morphogenesis, cellular differentiation, and functional maturation, all of which are described in detail in other chapters in this section. In the case of the intestine, this includes formation of the gut tube, the appearance of villi and digestive enzymes, and the development of swallowing and mature motility patterns.[1] Growth at the tissue and cellular level is characterized by increased cell numbers, that is, hyperplasia, and increased cellular size, that is, hypertrophy. Intestinal growth also involves expansion of the number and size of crypt and villus units.[2] Moreover, the timing and characteristics of fetal and neonatal GI and liver growth are exquisitely coordinated with the events of birth and weaning to ensure survival of the organism. The regulation of the timing and nature of GI and liver growth is complex and involves multiple and often redundant factors. Among these factors are intrinsic cell programs or signals arising from gene expression, as well as extracellular signals, such as peptide growth factors, hormones, nutrients, and microbes, which originate from surrounding cells, the blood, and the gut lumen.

The application of molecular biologic techniques in model organisms such as the mouse, zebrafish, fruit fly, and nematode have revealed that genetic regulatory factors have a critical influence on early organogenesis and morphologic development of the gut, pancreas, and liver.[3-6] These types of studies have identified a group of key homeodomain transcription factor genes, including *box, pdx1, cdx2,* and *GATA*, and signaling pathways, including Wnt/β-Catenin, Hedgehog, and Notch.[7,8] Another important aspect of growth in the gut is the continual proliferation, migration, and loss of epithelial cells along the mucosal surface. In the small intestine, this process involves four cell lineages (absorptive enterocyte, goblet, Paneth, endocrine) that differentiate from one pluripotent stem cell located in the crypt[9] (Figure 108-1). Recent studies have identified several genes that play a key role in the determination of lineage cell commitment in the intestine, including *atoh1, notch, hes1,* and *neurogenin3*.[10-12] The homeodomain transcription factors are also involved in the anterior-posterior pattern formation of demarcations in muscular sphincters and regional epithelial morphology along the length of the gut.[1,5] Genetic regulation via homeodomain factors also influences function maturation of the intestinal tract that occurs during the late gestation and neonatal period. For example, the expression of lactase-phlorizin hydrolase, a key enzyme involved in digestion of milk lactose, is up-regulated by a synergistic interaction between Cdx2 and HNF1 transcription factors.[13]

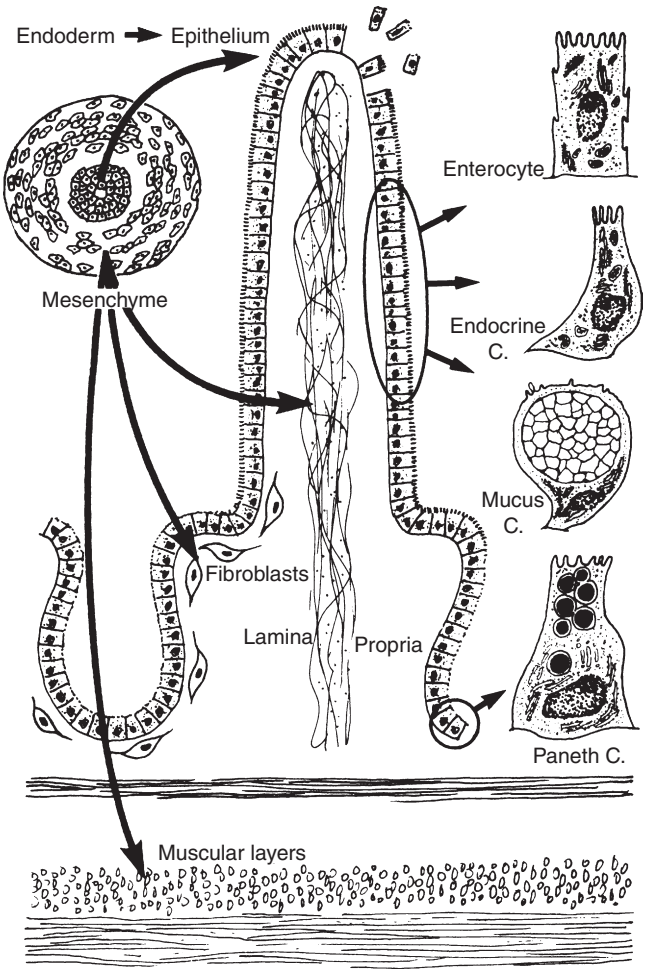


Figure 108-1 Description and localization of the four intestinal epithelial cell lineages within the crypt-villus region. The stem cells located in the lower crypt area differentiate into absorptive enterocytes, mucin-secreting goblet cells, endocrine cells, and Paneth cells. *(From Haffen K, Kedinger M, Simon-Assmann P: Cell contact dependent regulation of enterocytic differentiation. In Lebenthal E [ed]:* Human Gastrointestinal Development, *New York, 1989, Raven Press, p. 20.)*

Environmental Influences

Normal GI growth and development during fetal life are critical to facilitate the successful adaptation from nutritional support via the umbilical circulation to that of oral ingestion of breast milk by the neonate. An increase in circulating fetal glucocorticoid concentration just before and during vaginal birth is an important trigger of gut functional development.[14] In the neonatal period, growth of the GI tract is influenced by multiple physiologic factors that serve to prepare the developing neonate for separation from maternal nutritional support (i.e., weaning). In addition, a number of important environmental cues signal adaptive changes in GI function to facilitate postweaning survival. For example, the microbial colonization of the gut may serve to prime intestinal lymphoid cell development for normal innate immune function.[15-17] During these processes, extracellular signals, such as peptide growth factors, are often considered to be the major trophic factors that influence growth. However, the term *trophic* actually means "of or pertaining to nutrition," and, in the case of the gut, nutrients present in amniotic fluid and breast milk are a major trophic influence. There are numerous extracellular trophic signals including foods, nutrients, peptide growth factors, gut peptide hormones, steroid and thyroid hormones, microbes, and neural inputs. The cells in the fetal and neonatal GI tract and liver are influenced by extracellular signals from multiple sources including (1) blood-borne factors in the circulation such as hormones that act via endocrine mechanisms; (2) luminal factors derived from amniotic fluid, mammary secretions, or microbes; and (3) local factors secreted via autocrine or paracrine mechanisms from surrounding cells (Figure 108-2).

Recent studies have revealed novel insights into the role of mesenchymal cells, especially myofibroblasts,[18-20] and luminal microbes on intestinal epithelial growth and differentiation. Mesenchymal cell interactions are mediated via local secretion of tissue growth factors (e.g., fibroblast growth factor [FGF], hepatocyte growth factor [HGF], keratocyte growth factor [KGF], insulin-like growth factor [IGF]) and basement membrane proteins (laminins, collagens, proteoglycans).[21] The extracellular matrix protein, laminin, may be particularly critical for expression of *cdx2* and induction of epithelial differentiation genes, such as lactase-phlorizin hydrolase. Interaction between the integrin receptors on the basal surface of epithelial cells with basement membrane proteins can also affect differentiation and cell death. Studies have shown that disruption of the integrin-receptor binding via protease degradation leads to detachment of epithelial cells from the basement membrane and activation of anoikis-mediated apoptotic signaling pathways.[22-24] Symbiotic relationships between commensal microorganisms and their mammalian host, such as ruminants, are well known. Yet there is a rapidly growing appreciation for how these environmental influences also affect the health and disease of the human gut. Studies in conventional and germ-free animals demonstrated that the presence of intestinal microbes exerts a trophic effect on the gut, as evidenced by increased epithelial cell proliferation, mucosal thickness, and lymphoid cell density. Studies with germ-free mice monoassociated with specific bacterial species (e.g., *Bacteroides thetaiotaomicron*) have shown how microbes can alter particular pathways of intestinal differentiation.[3,25] The neonatal gut is sterile, but colonization occurs rapidly during early postnatal life and plays a critical role in development of mucosal immune function. Microbes also produce bioactive molecules and substrates, including toxins and short-chain fatty acids (SCFAs) that influence the proliferation and function of mucosal epithelial and immune cells. The aim of this chapter is to provide a brief overview of some of the major trophic factors in these categories and discuss their relevance to the growth and development of the fetal and neonatal gut and liver. Most of the references cited in this chapter are review articles, and the number of original articles listed is limited because of space.

MAJOR TROPHIC FACTORS

Nutrients

Enteral Versus Parenteral

Nutrition is perhaps the most potent trophic stimulus of GI tract growth. The diet acts directly by supplying nutrients for growth and oxidative metabolism of the mucosal epithelial cells, but it also acts indirectly by triggering the release of local growth factors, gut hormones, and activating neural pathways. It has been shown that the trophic actions of several nutrients are mediated by specialized enteroendocrine cells within the mucosal epithelium that literally sense the amount and chemical composition

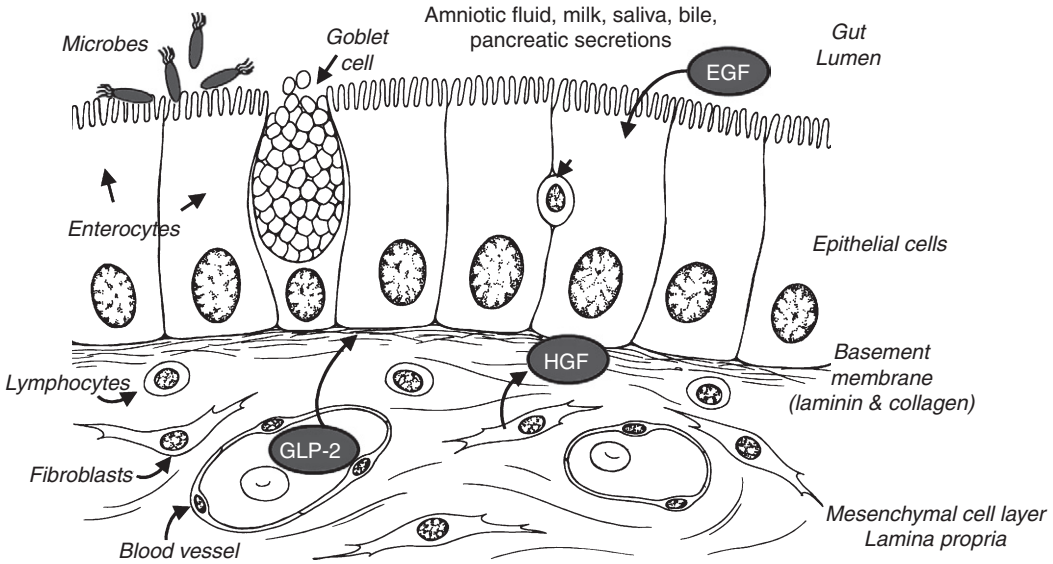


Figure 108-2 Spatial organization of various cell types and structural architecture of intestinal mucosa. Also shown are the sites of input and expression of selected extracellular factors within the intestinal mucosa. *EGF,* Epidermal growth factor; *GLP-2,* glucagon-like peptide 2; *HGF,* hepatocyte growth factor; *IEL,* intraepithelial lymphocyte.

of luminal nutrients.[26] The molecular mechanisms for these responses are emerging and show that membrane receptors and transport proteins act as chemical sensors that signal the presence of nutrients via enteric nerves to mediate local GI function but also peripheral responses via the extrinsic nervous system. Maternal malnutrition and neonatal starvation cause reduced gut tissue mass, shortened villi, and generalized increased catabolism and decreased protein synthesis.[27,28] However, the route of nutrient input, either enteral or parenteral, has a critical impact on the trophic response. In the late-gestation fetus, the onset of amniotic fluid swallowing coincides with increased intestinal growth and development. Studies in fetal sheep and pigs have shown that preventing this process by esophageal ligation suppresses intestinal growth.[14,29] In the neonate, total parenteral nutrition (TPN) leads to significantly reduced growth and atrophy of the intestinal mucosa.[30] The TPN-induced intestinal atrophy is associated with reduced gut DNA and protein mass, cell proliferation, villus height, and protein synthesis and increased apoptosis and proteolysis. Studies with neonatal animals and human infants show that the lack of enteral nutrition is also associated with reduced secretion of many gut peptide hormones and growth factors.[30] Animal studies have shown that several growth factors and gut peptides can prevent intestinal atrophy when coinfused with TPN, yet few of these studies have been done with neonatal animals. The practice of feeding small volumes of enteral nutrition, known as minimal enteral feeding or trophic feeding, has been shown to enhance GI motility and other functions.[31,32] The precise relationship between enteral feeding level and gut function has not been established; however, studies in neonatal piglets suggest that an enteral intake of between 20% and 40% of the total nutrient intake is necessary to maintain normal growth.[33] An additional consideration is whether to provide enteral nutrition orally, intragastrically, intraduodenally, as a bolus, or continuously. Studies in piglets suggest that, in comparison with continuous feeding, bolus feeding resulted in increased gut growth; however, this does not appear to be linked to secretion of trophic gut peptides.[30]

Breast Milk Versus Formula

The relative significance of milk-borne trophic factors has been one of the most intensely studied areas of pediatric nutrition and gastroenterology.[17,34-36] Many of the trophic peptide growth factors discussed in this chapter are present in breast milk, but not infant formulas, and have been implicated in the beneficial outcomes of breast-fed infants. Studies have assessed the relative trophic effect of colostrum and mature milk compared with formula in neonatal animal studies in which macronutrient intake was controlled. These studies have largely confirmed the idea that breast milk has a greater trophic effect on the GI tract than formula, as measured by typical indices of structural and cellular growth. However, the most significant advantage of breast milk in the neonatal intestine may be related not to growth but rather to mucosal barrier and immune function. Considerable evidence suggests that immunoprotective factors in breast milk (e.g., secretory immunoglobulin A, lactoferrin, oligosaccharides) act to modulate mucosal immune function and bacterial colonization, thereby limiting the incidence of infection, sepsis, and necrotizing enterocolitis.[34,37-40] Many of the trophic factors in milk are polypeptides that survive digestion, retain their biologic activity, and interact with specific receptors present on the mucosal epithelium of neonates. A number of studies have shown that these milk-borne growth factors stimulate neonatal intestinal growth when given in purified and recombinant forms either orally or systemically. Moreover, in preterm neonates the presence of increased intestinal permeability could facilitate the intestinal absorption of milk-borne peptide growth factors; however, there are limited instances where this process has been found to be physiologically significant.

Macronutrients

The chemical form and nutrient composition can also influence the impact of enteral nutrition on GI growth and function.[27,28] Some studies indicate that enteral nutrition in a complex, polymeric form is more trophic to the small intestine than in a simpler, elemental form. The dietary restriction of protein and energy generally suppresses gut growth and mucosal immune function. The enteral infusion of individual nutrients, by themselves, can have a trophic stimulus on the gut if administered in a sufficient large amount. However, a number of specific nutrients have trophic actions when supplemented to a complete diet; among those are glutamine, arginine, threonine, leucine, nucleotides, SCFAs, long-chain polyunsaturated fatty acids (LC-PUFAs), and retinoic acid.

Glutamine is a key intestinal oxidative fuel that is extensively metabolized and oxidized to CO_2 by intestinal tissues when fed either enterally or parenterally.[41] However, several studies have shown that enteral and parenteral glutamine also stimulates intestinal growth and enhances function in healthy and diseased conditions.[42,43] Studies in cultured intestinal epithelial cells indicate that glutamine, arginine, and leucine directly stimulate cell proliferation, activate mitogenic intracellular signaling pathways,[44] and may be a critical precursor for glucosamine and arginine synthesis.[45-47] Arginine is an essential amino acid for neonates and may be an especially important substrate for maintenance of intestinal nitric oxide synthesis, blood flow, and immune function. Some studies have shown that enteral arginine supplementation can reduce the incidence of necrotizing enterocolitis in neonates.[48,49] Other nonessential amino acids, including glutamate, proline, and ornithine have been shown to have stimulatory actions on the gut, perhaps because they are precursors for glutamine and arginine synthesis. Threonine is also a key nutrient for the intestine, because it is used for the synthesis of threonine-rich mucins by goblet cells; the intestinal threonine use by goblet cells appears to be dependent on the enteral rather than parenteral route.[50,51]

Nucleotides are ubiquitous, low-molecular-weight, intracellular compounds that are integral to numerous biochemical processes and are especially important as precursors for nucleic acid synthesis in rapidly dividing cells, such as epithelial and lymphoid cells in the mucosa.[52,53] Nucleotides consist of a purine or pyrimidine base, which can be synthesized within cells de novo from glutamine, aspartic acid, glycine, formate, and carbon dioxide as precursors, or they can be salvaged from the degradation of nucleic acids and nucleotides. Human milk is an excellent source of dietary nucleotides for infants during the first months of life, and its nucleotide content is markedly higher than that of cow's milk and most infant formulas, although several commercial formulas now include nucleotides. Numerous reports show that dietary supplementation with nucleosides, nucleotides, or nucleic acids supports small intestinal mucosal function, growth, and morphology.

SCFAs, largely in the form of acetate, propionate, and butyrate, are produced by microbial fermentation of carbohydrates in the large bowel.[16,38,54] Colonic epithelial cells derive most (60% to 70%) of their energy from SCFAs, and butyrate is the preferred oxidative fuel compared with glucose, glutamine, or ketone bodies. The diet of the human neonate is largely devoid of fiber, yet the production of SCFAs from large-bowel microbial fermentation increases with the degree of microbial colonization and postnatal age. Normal substrates for colonic SCFA production in neonates include endogenous secretions and malabsorbed dietary carbohydrates, such as lactose and oligosaccharides.[55] Oligosaccharides are the second most abundant carbohydrate in human milk, but cow's milk and infant formulas are substantially devoid of them.[34,56] Considerable evidence exists for the specific intestinal trophic effects of SCFAs. Intraluminal and systemic infusions of SCFAs have a stimulatory effect on intestinal mucosal proliferation, gene expression, blood flow, and gut hormone

secretion, yet studies with cultured colonic tumor cell lines indicate that butyrate induces differentiation and apoptosis, thereby suppressing neoplasia.[57-59]

There is considerable interest in dietary long-chain fatty acids, because breast milk generally contains higher concentrations of *n*-3 LC-PUFAs than do formulas. As a result, many infant formulas are now formulated with these fatty acids. Interest in the *n*-3 LC-PUFAs or omega-3 fatty acids, particularly docosahexanoic acid, eicosapentaenoic acid, and arachidonic acid, has been heightened by recent studies showing that supplementing these fatty acids can lower the incidence and inflammatory effects of necrotizing enterocolitis in neonatal infants and rats.[60-62] Information is limited regarding the intestinal trophic effects of either *n*-3 LC-PUFAs or other long-chain fatty acids in developing animals. However, a series of studies have demonstrated that *n*-3 LC-PUFAs enhance intestinal adaptation after small-bowel resection, and their effects were greater than those of less-saturated oils; they also found that medium-chain triglycerides are less trophic than long-chain triglycerides. In contrast, studies with neonatal piglets indicated that the long-chain fatty acid, oleic acid, can cause significant mucosal injury and increased permeability and that this effect is more severe in newborn than in 1-month-old piglets.[63,64]

Gastrointestinal Hormones

The gut is one of the largest endocrine organs in body and secretes numerous peptide hormones primarily in response to nutrient ingestion but also by stage of development and disease. Many of these gut hormones discussed in this section have been implicated in the stimulation of gut and liver growth in response to enteral nutrition. Gastrin is secreted from the G cells within the antrum of the stomach and acts primarily to stimulate proliferation of parietal and enterochromaffin-like cells within the gastric mucosa.[65] Cholecystokinin (CCK) is expressed in endocrine cells of the gut and in neurons within the gut and brain, and its primary target tissues are the pancreas and gallbladder. Structural homology exists between gastrin and CCK with respect to both the peptide sequence and receptor function. Studies have shown that hypogastrinemia, produced by antrectomy and targeted disruption of the gastrin gene, leads to atrophy of the gastric mucosal cells. CCK stimulates pancreatic growth and cell proliferation, and these trophic effects have been attributed exclusively to interaction via the CCK-A receptor. The neonate exhibits hypergastrinemia and comparatively high gastric pH, yet gastrin secretion is induced by feeding.[31] The development of pentagastrin-responsive gastric acid secretion occurs within 1 week in neonatal piglets and can be prematurely induced with glucocorticoids, consistent with up-regulation of gastrin receptor expression with age.[66] The trophic effects of gastrin are most evident in the stomach and are mediated by increased ornithine decarboxylase activity and cell proliferation. Gastrin-releasing peptide is a 27–amino acid peptide with a carboxyl-terminus region that is structurally related to bombesin, a 14–amino acid neuropeptide found in amphibian skin.[67] Gastrin-releasing peptide is secreted from neurons located throughout the GI tract in response to vagal stimulation. Bombesin is also found in milk, and both oral and systemic administration to suckling animals stimulates intestinal growth.[68]

Glucagon-like peptide 2 (GLP-2) is a product of the intestinal proglucagon gene expressed in the enteroendocrine L cells located predominantly in the distal intestine.[69,70] GLP-2 is secreted in response to feeding, especially carbohydrate, and studies in fetal and neonatal piglets suggest an ontogenic increase in GLP-2 secretion. GLP-2 has significant trophic effects on the neonatal and adult gut that are mediated by increased cell proliferation, protein synthesis, blood flow, and glucose transport. The trophic actions of GLP-2 are mediated by paracrine signals that are believed to originate from enteric neurons and subepithelial myofibroblasts.[69] GLP-2 has also been shown to possess antiinflammatory effects in conditions of bowel injury, resulting from colitis and chemotherapy.[71] Peptide YY is secreted from the same enteroendocrine cell as the glucagon-like peptides, GLP-1 and GLP-2, and together these hormones have been implicated as factors responsible for the "ileal-brake" phenomenon. Studies indicate that peptide YY stimulates gut growth in some, but not all, cases and does not stimulate proliferation of cultured epithelial cells. Neurotensin is a 13–amino acid peptide secreted from the enteroendocrine N cells located exclusively in the gut within the distal small intestine.[72,73] Neurotensin expression is markedly increased during the neonatal period, and secretion is stimulated specifically by ingestion of fat. Administration of neurotensin stimulates gut growth in animals after small-bowel resection even in the absence of enteral nutrients.

Tissue Growth Factors

Tissue growth factors can be generally categorized as polypeptides that are secreted locally and act via paracrine or autocrine mechanism to affect cellular growth and function.[74] However, a number of the tissue growth factors are also present in the blood and GI secretions and thus may act via endocrine mechanism, for example, insulin-like growth factor I. Moreover, many of these growth factors are present in breast milk and thought to influence neonatal gut growth.[36,39,68,75] Among the best known of these is epidermal growth factor (EGF), a member of a family of peptides that includes transforming growth factor-α (TGF-α), heparin-binding EGF, amphiregulin, epiregulin, betacellulin, neuregulin, and neuregulin 2. These factors act as ligands for membrane-bound EGF receptor and other EGF-related (ErbB family) receptors that become activated via phosphorylation on binding. EGF is widely distributed in most body fluids (notably mammary, salivary, biliary, and pancreatic secretions) but is not produced in the epithelial cells, in contrast to its homologue, TGF-α. Most of the EGF family peptides are trophic to the gut, stimulating cell proliferation and suppressing apoptosis; however, they also modulate a number of other physiologic functions including enhanced tooth eruption, decreased gastric acid secretion, increased mucus secretion and gastric blood flow, reduced gastric emptying, and increased sodium and glucose transport.[76-79] EGF also has been found to increase mucosal growth and functional adaptation after intestinal resection, diarrhea, and TPN and to prevent the incidence of necrotizing enterocolitis.[80-83] Enteral nutrition stimulates GI secretion, resulting in the release of EGF into the gut lumen where it is postulated to play a protective role, whereas TGF-α functions in the maintenance of epithelial cell proliferation and migration in the healthy gut. EGF appears to be more trophic to the gut when it is given intravenously than when given enterally, perhaps because the EGF receptor is localized to the basolateral membrane. However, many neonatal animal studies have shown that oral EGF administration does indeed augment gut growth and functional development. These findings combined with recent evidence from transgenic mice support the idea that both local expression and milk-borne ingestion of EGF and TGF-α play a physiologic role in neonatal gut growth and development.

The IGF family of peptides includes insulin, IGF-I, and IGF-II.[36,74,84-87] The biologic actions of insulin are mediated via the insulin receptor, whereas the actions of both IGF-I and IGF-II are largely mediated through the type I IGF receptor. The insulin and type I IGF receptors are present in epithelial cells; they are more abundant on the basolateral than the apical membrane and more abundant in proliferating crypt cells than in differentiated enterocytes. Although insulin secretion is confined to the pancreas, both IGF-I and IGF-II are expressed throughout the body, including the gut. However, within the intestinal mucosa,

expression of both IGF-I and II appears to be localized to mesenchymal cells in the lamina propria, although epithelial cells may also produce IGF-II. The expression of IGF-I and II in the gut is highest in the fetal and neonatal period and declines with age. In addition, insulin, IGF-I, and IGF-II are present in milk, and IGF-I is found in salivary, biliary, and pancreatic secretions and amniotic fluid. Numerous studies have shown that either administering IGF systemically or increasing its expression locally (as in transgenic mice) stimulates intestinal growth and function in normal animals and under conditions of TPN, gut resection, dexamethasone treatment, sepsis, and radiation therapy.[88-90] The expression of IGF-binding proteins has been shown to inhibit IGF action in gut tissues. Some studies with fetal and neonatal animals given pharmacologic oral doses of insulin and IGF-I have demonstrated a stimulation of gut growth, disaccharidase activity, and glucose transport.[85,86] Yet others have shown only limited effects of oral IGF on the neonatal gut suggesting that the IGFs may not have a physiologic role in the neonate. Growth hormone (GH) has a major influence on IGF-I expression during postnatal growth, and studies in postweanling rodents indicate that hypophysectomy results in gut atrophy, whereas transgenic overexpression of GH in mice increases gut growth.[30,87,91-93] However, the significance of GH in neonatal gut and liver growth may be limited by the abundance and responsiveness of the GH receptor. Studies with hypophysectomized neonatal rats suggest some degree of pituitary-dependent intestinal growth and development. Other studies in rats demonstrate that GH treatment does not prevent TPN-induced intestinal atrophy but may augment intestinal growth after massive small-bowel resection.

Transforming growth factor-β (TGF-β) is structurally unrelated to TGF-α and is found in three major forms (TGF-β-1 through 3) in mammalian tissues.[74,94] TGF-β expression has been found throughout the small intestine in both lamina propria and epithelial cells; the levels are low in neonates and increase with age. In contrast, the levels of TGF-β-2 are high in early milk and decline as lactation progresses. At least five receptors (types I-V) have been found to bind one or more of the various TGF-β ligands, although the type I and II receptors appear to mediate the effects on cell proliferation. TGF-β is a potent inhibitor of epithelial cell proliferation and may also induce differentiation. TGF-β has been implicated as an intermediate signal whereby butyrate suppresses proliferation and induces differentiation of colonic epithelial cells. TGF-β also stimulates epithelial cell migration and production of extracellular matrix proteins such as collagen via induction of connective tissue growth factor, thus making it a critical factor in the process of restitution of the epithelium after mucosal damage.[95] This latter function of TGF-β may play an important role in the intestinal inflammatory response, as evidenced by the fact that its expression is up-regulated in inflammatory bowel disease and that TGF-β–deficient transgenic mice develop inflammatory disease.

HGF, vascular endothelial growth factor (VEGF), FGF, and KGF are expressed in the gut tissues and may play a role in mucosal growth and repair.[30] HGF is expressed by mesenchymal, but not epithelial cells, whereas the HGF receptor *(c-met)* is found in epithelial cells; the c-*met* receptor is localized on the basolateral membrane. Studies with cultured intestinal epithelial cells demonstrate that HGF stimulates cell proliferation and wound closure proliferation but decreases transepithelial resistance.[77,96,97] Hepatocyte growth factor is found in human milk mononuclear cells and partially accounts for the stimulatory effect of human milk on intestinal cell proliferation. Studies in rats have shown that HGF, given either systemically or orally, increased gut growth and nutrient transport after massive small-bowel resection and colitis.[98,99]

VEGF is expressed in the small intestine, predominantly in the lamina propria mast cells.[30,77,100,101] A recent report indicates that the receptor (flt-1) for the VEGF 165–amino acid isoform is present in intestinal epithelial cells; however, VEGF did not stimulate cell proliferation of these cells. A recent report in mice demonstrated that VEGF and FGF-2 reduce the rate of crypt cell apoptosis after total body irradiation treatment. FGF and the FGF receptors have been found in intestinal tissues, yet their function remains poorly understood. Both VEGF and FGF-2 have been implicated in angiogenesis during intestinal repair.[102] Increased local expression of KGF has been found in patients with inflammatory bowel disease, and administration of KGF enhanced mucosal healing in rats after induction of colitis.[103] The trefoil factors (TFFs) are a family of peptides (TFF1-TFF3) that play an important protective role in the gut.[104] TFF3 is expressed and secreted from goblet cells of the small and large intestine and interacts with mucins on the apical cell surface. Trefoil peptides have been shown to promote cell migration and suppress apoptosis via activation of intracellular signaling pathways linked to mitogen-activated kinase and the EGF receptor; however, TFF receptors have not been identified.

Glucocorticoids

The role of glucocorticoids in neonatal intestinal development has been studied extensively, especially in rodents.[105] The impact of glucocorticoids, particularly dexamethasone, on human intestinal growth and development has received considerable attention in the past decade because of their use in treatment of pulmonary function in premature infants.[106] Results of studies in fetal animals suggest that increased endogenous glucocorticoid levels are critical signals that stimulate GI development and growth.[23] The prenatal cortisol surge is an important signal for development of the neonate, and premature birth precludes the exposure to this key intestinal maturation signal. This idea is supported by studies in infants and piglets showing that premature birth results in insufficient intestinal maturation of intestinal lactase and lactose digestive capacity.[14,29] Numerous studies demonstrate how glucocorticoids stimulate neonatal intestinal development and maturation, especially with regard to disaccharidase expression. However, their effects on neonatal mucosal growth, per se, as indicated by cell proliferation, cell cycle, protein turnover, and apoptosis are not completely understood. Studies in vivo and in vitro suggest that glucocorticoids inhibit intestinal mucosal growth by suppressing cell proliferation and increasing protein catabolism.[30]

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ESOPHAGUS

Organogenesis

At 22 to 23 days gestation, a median ventral diverticulum is first noted within the region of the posterior confines of the developing foregut. This diverticulum, which is destined to become the cranial end of the fetus's esophagus and trachea, is termed the *tracheal diverticulum*. The diverticulum becomes groovelike as it elongates concurrently with the proliferation of endodermal cells at its lateral aspect. This phenomenon forms lateral ridges, which themselves ultimately create a division between the trachea and the esophagus occurring at 34 to 36 days gestation. Muscular and submucosal layers are present at this time point, and it is at this stage in development that tracheoesophageal anomalies such as the relatively common tracheoesophageal fistula occur. As these tracheoesophageal folds continue to develop, a spindle-shaped dilation occurs along the caudal aspect of the foregut—this structure ultimately becomes the stomach. The foregut between this dilation and the tracheal diverticulum is the *developing esophagus.* Elongation of the developing esophagus occurs first craniad and then caudad so that it is ascent of the pharynx rather than descent of the stomach that accounts for most esophageal growth. By 7 weeks gestation, the final, relative length of esophagus is achieved. In addition, any tracheoesophageal anomalies are present and, theoretically, identifiable at this stage of development.[1]

Anomalies of Embryogenesis

Esophageal atresia and *tracheoesophageal fistula* are commonly found in association. In the most common form of this anomaly, there is a blind proximal esophageal pouch associated with a distal tracheoesophageal fistula. As shown in Figure 109-1, other less-common configurations can be observed. Interruption of the events responsible for elongation and separation of the esophageal and tracheal tubes during the fourth fetal week is thought to lead to the development of this anomaly. The most full-blown manifestation of this disease of embryogenesis is a complete communication between the esophagus and trachea, or *laryngotracheoesophageal cleft.*

Most presentations of *esophageal stenosis* in children and adults result from an acquired anomaly rather than a congenital defect. However, a narrowing of the esophagus is occasionally found in the middle to distal third of the esophagus and presents as either a web or a long segment of narrowed esophagus. This anomaly likely results from incomplete recanalization of the esophagus. Another form of esophageal stenosis is one created by the presence of abnormal tissue rests within the esophageal wall. Often called an *esophageal ring,* this is commonly respiratory tissue and often contains cartilage, as well as ciliated epithelium.[2] This lesion is generally found within the lower third of the esophagus.

Esophageal duplications may be in direct communication with the esophagus or they may lie within the mediastinum completely separate from the esophagus. It is generally thought that intramural duplications or those that communicate directly with the esophagus are examples of faulty recanalization of the esophageal lumen (as suggested by the Bremer theory of duplications), whereas the cause of those duplications lying free within the mediastinum-thorax is not so easily understood.[3] Results of separate studies by Bentley and Smith[4] and by McLetchie and partners[5] have suggested that the developmental error underlying the formation of these cysts occurs during the formation of the notochord (at 18 to 19 days gestation) before development of the foregut itself has begun. Attachment of endodermal cells to the developing notochord permits formation of such enterogenous cysts.

As already noted, during the development of the esophagus the lining is initially columnar epithelium, which later becomes ciliated as the transition is made to the final stratified squamous epithelium. As this differentiation proceeds, if glandular columnar epithelium develops instead of the stratified squamous epithelium, gastric-type epithelium can occur within the esophagus. Termed *heterotopic mucosa,* this is a particularly common finding, and as many as 70% of clinically normal people are known to have such ectopic mucosa at some point within their esophagus.[6] Intraesophageal thyroid tissue has also been described[7]; the embryogenesis of this phenomenon is obscure.

Because it is known that the elongation of the esophagus is a phenomenon that creates the "descent" of the stomach, one can conceptualize that cessation of the elongation before the time that the stomach has reached the appropriate level would result in a situation in which a portion of the stomach remains in the thorax. This is a true *congenital short esophagus,* and the fact that a portion of the stomach remains supradiaphragmatic needs to be distinguished from a similar condition in which the supradiaphragmatic stomach is the result of a hiatal hernia.

STOMACH

Organogenesis

At 4 to 5 weeks gestation, a fusiform dilation at the caudal region of the foregut is first noted. Disagreement within the literature remains about whether the initial fusiform dilation destined to become the stomach occurs before or after the initial appearance of the tracheal diverticulum; however, it is known that the initial enlargement of the stomach is in a ventrodorsal direction.[1] The stomach begins its development at the C3 to C5 level. Because of the marked cephalic growth of the foregut, "descent" of the stomach occurs so that its eventual final location is in the region between T10 and L3. Although still unproved, it is generally thought that, as the stomach develops, its dorsal border grows more rapidly than the ventral border does so that a 90-degree clockwise rotation occurs on its

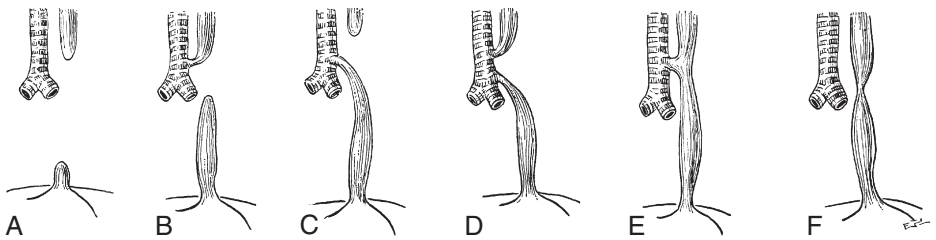


Figure 109-1 A-F, Types of esophageal atresia and tracheoesophageal fistula. Type **C,** with a blind proximal esophageal pouch and distal tracheoesophageal fistula, is the most common presentation (85%) of this anomaly. Type **A,** isolated esophageal atresia, and type **E,** isolated (H-type) tracheoesophageal fistula, constitute about 10% of this entity. *(From Gross RE:* The Surgery of Infancy and Childhood, *Philadelphia, 1953, WB Saunders, p 76.)*

longitudinal axis. As a result of this leftward rotation, the dorsal border of the stomach becomes the greater curvature of the organ, whereas the ventral border rotates toward the right and becomes the lesser curvature. The end result is that the original left side of the stomach becomes its ventral surface and the original right side of the stomach becomes its dorsal surface; the pylorus rotates upward and to the right and the cardia rotates downward and to the left. Studies have challenged this classic hypothesis, and the suggestion has been made that the left side of the stomach becomes the greater curvature (this anatomic orientation could result from accelerated growth only, with no actual rotation).[1] Although both hypotheses have solid foundations, it seems that the rotational hypothesis better explains why the left vagus supplies the anterior portion of the stomach and the right vagus innervates the posterior wall. Regardless, it is known that the final position of the longitudinal axis of the stomach is transverse to the longitudinal axis of the body itself and that this process is completed by approximately 8 weeks gestation.

Anomalies of Embryogenesis

Total agenesis of the stomach is an anomaly not encountered in otherwise viable infants. *Microgastria* is not uncommonly encountered and is thought to be the result of a failure or arrest of appropriate development of the caudal portion of the embryologic foregut. It is almost always associated with an enlarged esophagus and incomplete gastric rotation.[8] Although the mucosa of the gastric wall is normal, there may be other associated gastrointestinal anomalies—most frequently, incomplete rotation. Asplenia is a common concurrent finding, which is not unexpected because embryologically the spleen is derived from the dorsal mesogastrium, which has an intimate relationship with the developing stomach. *Gastric atresias,* almost always partial and in the form of membranous diaphragms, have been encountered, as have been complete, solid atresias of a portion of the stomach. Discontinuity of the stomach may occur in cases of such solid atresias. These atresias tend to be located within the antral and pyloric regions, and infants present as newborns with complete gastric outlet obstruction. Incomplete prepyloric membranes also occur. The embryologic characteristics of gastric atresias are considered to be different from those encountered within the duodenum, because there is no solid phase of epithelial occlusion of the stomach during development. It has been suggested that areas of endodermal redundancy may be the origin of small, weblike membranous diaphragms, whereas the larger zones of atresia are thought to be due to local attenuation of the developing endodermal tube during the formation of the pylorus. In this instance, endodermal proliferation fails to keep up with the elongating foregut.[1] Others have suggested that an in utero vascular accident (as occurs with other small and large intestinal atresias) is the cause of the solid atresias found within the gastric region; however, most clinicians favor the former theory.[9]

Pyloric stenosis results from hypertrophy of the muscularis of the pyloric channel, especially the circular muscle. It was for a long period considered to be a developmental defect of the pyloric musculature and thus congenital. Because this anomaly generally occurs at about 3 weeks of life and has not been known to occur earlier than the 4th or 5th day of life, it is no longer considered to be a true congenital defect. Its pathogenesis remains, essentially, unexplained.

Most *gastric duplications* occur along the greater curvature or on the anteroposterior walls of the stomach. Communications to the stomach are not always present, and the mucosal lining is generally gastric. Several embryologic theories have been suggested, which include the persistence of vacuoles within the primitive foregut epithelium[3] and the persistence of embryonic diverticula.[10] However, as with the esophagus, larger duplications, as well as those that lie completely outside the normal gastric wall, are likely the result of faulty separation of the notochord and endoderm.[5]

Acute gastric *volvulus* in children is a rare condition. Chronic gastric volvulus is encountered more frequently. Gastric volvulus is the result of errors in gastric rotation that lead to laxity or lack of the normal attachments of the stomach to the body. These attachments include the gastrophrenic ligaments, the gastrocolic ligament, the short gastric vessels, and the retroperitoneal fixation of the duodenum. The volvulus itself is classified according to the plane of rotation. If the stomach rotates on its long axis, it is called an *organoaxial volvulus*. In this situation, the greater curvature generally passes anteriorly but can be displaced posteriorly. A less common volvulus is mesenteroaxial volvulus, and rotation here is on an axis from the greater to the lesser curvature. In this instance, the pylorus and cardia commonly rotate anteriorly, although rotation in the opposite direction is also possible.

The most common *heterotopic mucosal tissue* found within the stomach is pancreatic; it is most frequently found within the antral region. Heterotopic tissue often presents as a 1- to 2-cm–diameter mass within the submucosal layers. Both translocation of the embryonic pancreatic cells and metaplasia in situ have been postulated as the cause of this anomaly. On rare occasions, heterotopic intestinal epithelium has been observed. Some have suggested that the presence of such epithelium (generally columnar epithelial cells with a striated border and interspersed goblet cells) is due to metaplasia induced by gastritis.[11] Conversely, there is clearly some embryologic basis for this phenomenon, because such cells are found in abundance within the pylorus and cardia of fetuses and newborn infants.[12] It is thought that this heterotopic tissue results from the persistence of a primitive form of gut epithelium.

DUODENUM

Organogenesis

Although the histogenesis of the duodenum is not significantly different from that of the rest of the small bowel, the organogenesis is quite different in many regards. The duodenum's development begins early in the fourth week of gestation, at which time it is the most caudal portion of the foregut and the most cranial portion of the midgut. This junction of the foregut and midgut is at a region just distal to the entry of the bile duct. The duodenal loop, which is convex throughout organogenesis and later life, is originally oriented in an anterior fashion. However, when gastric rotation takes place, the duodenal loop also rotates to the right so that the duodenum ends up lying with its loop convex to the right. The duodenum's rotation results in movement of the duodenum and pancreas to the right upper quadrant, at which point they are pressed against the posterior abdominal wall so that fusion of peritoneal structures with the posterior abdominal wall results in the duodenum becoming retroperitoneal. Because it represents a watershed between the foregut and the midgut, its blood supply is derived from both the celiac and superior mesenteric arterial trees. Unlike the rest of the midgut, the duodenum is not herniated into the extracoelomic cavity because of mesenteric-retroperitoneal attachments that anchor it in the right upper quadrant.

Duodenal mucosal proliferation begins around the 4th week of gestation, and an exuberant amount of duodenal epithelial proliferation occurs through the 10th week of gestation. The cellular proliferation is so abundant that at 5 to 6 weeks gestation complete duodenal occlusion routinely occurs. This occlusion is an epithelial proliferative formation rather than a stage of anatomic "narrowing" during the gut's development. Epithelial proliferation is the basis for most current theories

of duodenal atresia-stenosis, as well as the basis for the Bremer theory of duplications.[3] Furthermore, at this time, the proliferating epithelia bulge into the antimesenteric mesenchyme so that budlike diverticula appear in the embryo. These diverticula also appear in other regions of the small bowel but to a lesser extent. Such diverticula resorb into the gut wall at a later time during development, but they may play a role in the pathogenesis of duplications (the Lewis and Thyng theory of gastrointestinal duplications).[10]

Anomalies of Embryogenesis

The duodenum may be involved in many different manifestations of disordered embryogenesis. *Duodenal atresia* results from a failure of recanalization during the solid stage of duodenal development. If the lumen does not re-form as a result of revacuolization of the epithelial plug, an atresia will result. Atresias commonly occur in the second or third portion of the duodenum, with the site of atresia located just distal to the opening of the bile duct. The duodenum may or may not be in continuity (Figure 109-2). Similarly, if the vacuolization process has been incomplete, a *duodenal stenosis* may exist. Although duodenal atresia may often be a solid phenomenon, webs, either partial or complete, may also create obstruction within the duodenal region. Duodenal webs are also thought to result from a failure of recanalization. Duodenal obstruction of a partial or complete nature may occur because of an *annular pancreas* or *incomplete intestinal rotation.* In this anomaly, the cecum overlies the second portion of the duodenum, and obstruction results from peritoneal attachments (Ladd bands), which run from the cecum to the lateral body wall. Obstruction of the duodenum can also be created by a *preduodenal portal vein* that arises from abnormal development of vitelline vein anastomoses. This anomaly is commonly associated with other serious malformations that are often of a rotational variety.

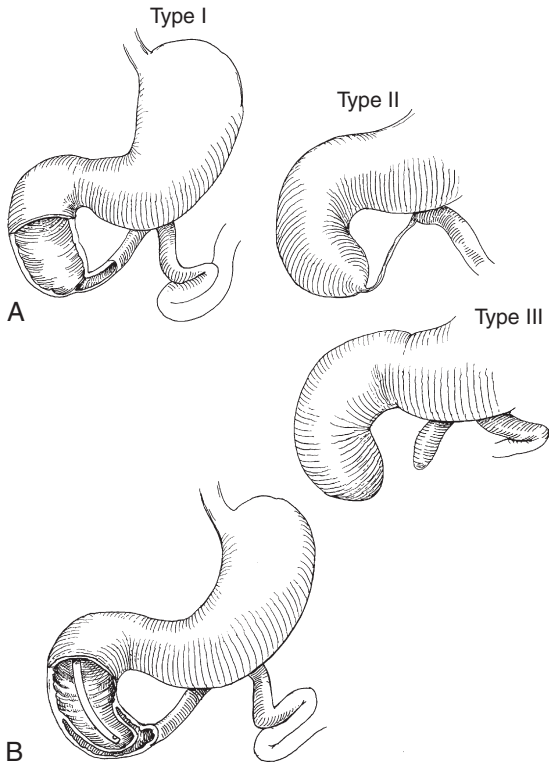


Figure 109-2 A and **B,** Duodenal atresia-stenosis, an anomaly of failure of duodenal recanalization, may be seen in several ways. *Type I* may be either a complete or an incomplete membrane, and the membrane may be more proximally based (so-called windsock anomaly) as seen in **B.** *(From Ross AJ III: Intestinal atresia. In Nora PF (editor):* Operative Surgery, *ed 3, Philadelphia, 1990, WB Saunders, p 1042.)*

PANCREAS

Organogenesis

Organogenesis of the pancreas is very closely related to that of the duodenum. During the 5th week of gestation, two buds appear as diverticula of endodermal cells emanating from the more caudal region of the foregut. The two endodermal buds are termed the *dorsal* and *ventral pancreatic anlagen*, and their eventual fusion creates the pancreas. The ventral anlage normally develops at the site of entry of the bile duct into the duodenum, and during the duodenum's rotation to the right this ventral anlage is carried dorsally along with the bile duct. Although initially this ventral anlage lies on the same side and below the dorsal primordium, this rotation permits the ventral anlage to fuse with the proximal portion of the dorsal anlage by the 7th week of gestation. The course of the ventral pancreatic anlage may be widely variable; errors in this rotation commonly lead to pancreatic anomalies.

Most of the pancreas is derived from the dorsal bud; the ventral bud is responsible for forming only the uncinate process in the inferior portion of the head of the pancreas. The pancreatic ducts normally anastomose as the two anlagen fuse. The main pancreatic duct (duct of Wirsung) is derived from the former duct of the ventral bud and the distal portion of the dorsal bud. The portion of the duct more proximal to the dorsal bud eventually becomes the accessory pancreatic duct (duct of Santorini), lying approximately 2 cm cranial to the main duct (Figure 109-3).

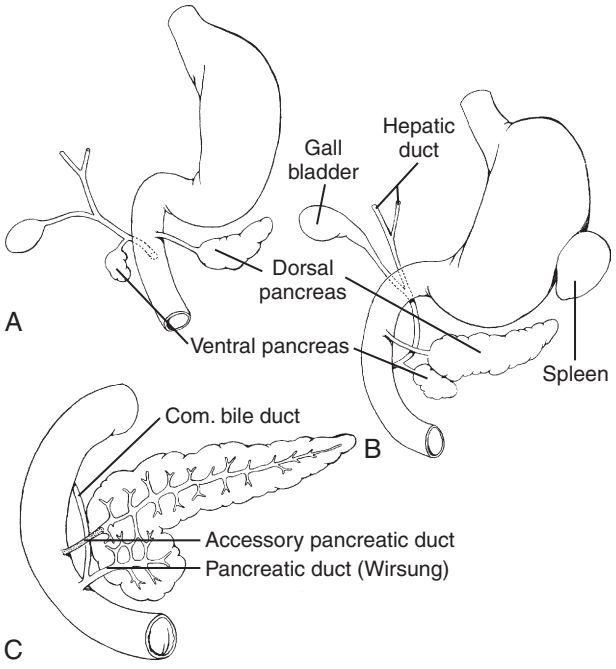


Figure 109-3 A-C, The migration of the ventral pancreatic anlage and subsequent relationship of the dorsal and ventral anlagen and pancreatic ducts are depicted. *(From Allan FD:* Essentials of Human Embryology, *New York, 1969, Oxford University Press.)*

Anomalies of Embryogenesis

Annular pancreas is a well-known cause of duodenal obstruction and a commonly encountered error in development (Figure 109-4). One finds a thin, flat band of tissue surrounding the second portion of the duodenum. The tissue of this band is histologically normal, and it is noted to be continuous with the head of the pancreas. The annular pancreas need not be complete and, as such, may manifest as a duodenal stenosis rather than a complete duodenal obstruction. Furthermore, intrinsic duodenal stenosis or atresia, or both, have been noted to occur in addition to the annular pancreas itself. A number of theories regarding the embryogenesis of this anomaly have been proposed. Although none completely explains all the intricacies involved in the embryogenesis of annular pancreas, there is no question that an aberration in the rotation of the ventral anlage has a key role.

Another common pancreatic anomaly is that of *variations of the pancreatic ducts.* Normally, the main duct (duct of Wirsung) and accessory duct (duct of Santorini) open into the duodenum and are interconnected. Obliteration of one or the other duct or the complete separation of one from the other is not at all uncommon so that the main drainage occurs through the accessory duct. *Complete agenesis* of the pancreas has not been reported in otherwise viable newborns, and *partial agenesis* of the pancreas is an exceedingly rare anomaly.

MIDGUT

Organogenesis

The midgut consists of small bowel beginning with the duodenum distal to the point of entry of the ampulla of Vater. It also includes the cecum and appendix, as well as the ascending colon and right one half to two thirds of the transverse colon. The midgut is supplied by the superior mesenteric artery and is thus also defined as that portion of bowel supplied by the superior mesenteric artery. The midgut is also defined from an embryologic standpoint as that portion of gut that opens ventrally into the yolk sac. The organogenesis of the midgut is one of the most fascinating aspects of development and was first described in 1923 by Dott, whose sketches remain the standard index for understanding the development of the appropriate intraabdominal rotational anatomy.[13]

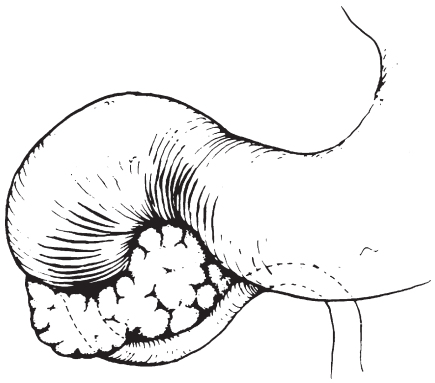


Figure 109-4 Failure of appropriate migration of the ventral pancreatic anlage leads to anomalies such as annular pancreas with resultant duodenal obstruction. *(From Schnaufer L: Duodenal atresia, stenosis, and annular pancreas. In Welch KJ, Randolph JG, Ravitch MM, et al, editors:* Pediatric Surgery, *ed 4, Chicago, 1986, Year Book Medical Publishers.)*

By the beginning of the 6th week of development, elongation of the midgut occurs at a rate faster than that of the elongation of the embryonic body itself. This is seen especially at the cranial end of the midgut. The result is a U-shaped ventral loop of gut (the midgut loop), which projects anteriorly and enters the extraembryonic coelom of the developing body stalk. Conceptually, one could consider this a huge physiologic umbilical hernia. The apex of this loop entering the extraembryonic coelom (marked by the yolk stalk) is directly in line with the superior mesenteric arterial axis and is termed the *omphalomesenteric duct*. The midgut proximal to this point is termed the *prearterial segment* (signifying that it is proximal to the superior mesenteric artery), whereas bowel distal to this point is the *postarterial segment.* Developmentally, the prearterial segment of bowel is small bowel, whereas the postarterial segment is mostly colon. After the appearance of the U-shaped ventral loop of the midgut, a counterclockwise (approximately 90 degree) rotation begins. During the time that the bowel is herniated into the extraembryonic coelom, most growth occurs within the prearterial segment so that jejunum and ileum develop at a rate proportionately greater than that of the colon. Within the postarterial segment, a cecal bud forms at 6 to 7 weeks gestation. Beginning at approximately 10 weeks development, the bowel begins to return to the embryonic abdominal cavity. The return of the bowel to the abdomen occurs rather suddenly and rapidly; the forces that dictate this phenomenon remain unknown. It has been postulated that external pressure forces the mass of bowel back through the umbilical ring, but this has not been substantiated. Both the prearterial and postarterial bowel segments rotate a total of 270 degrees counterclockwise before their rotation is completed. Although much of the rotation of the postarterial segment occurs during the time of its return into the body cavity, it is thought that the prearterial segment rotates 180 degrees while it remains herniated. It is the prearterial segment that enters first, passing posteriorly to the superior mesenteric artery during its return. This segment is followed by the cecum and colon (postarterial), which come to lie in front of the superior mesenteric artery. The terminal prearterial segment enters last (Figure 109-5).

Formal fixation begins after the midgut has returned to the abdominal cavity (approximately the 12th gestational week). The cecum and developing appendix lie in the subhepatic region at this time point, but further growth of the ascending colon forces the cecum down into the right lower quadrant. The force of the mesenteries of the ascending and descending colons against the posterior abdominal wall fuses these surfaces. As a result, the ascending and descending colons come to lie in the retroperitoneal space. The attachments of the fascial fusion planes of the right and left colon are termed the *fascial fusion planes of Toldt*.

The apex of the cecum does not grow as rapidly as the rest of the cecal pouch, which ultimately results in the development of the appendix. The appendix generally lies on the medial aspect of the cecum. During rotation and fixation of the cecum, however, the appendix can become retrocecal or retrocolic. A similar form of fusion occurs in the midgut portion of the duodenum, which attaches to the retroperitoneum in the left upper quadrant, termed the *ligament of Treitz*. As a result of the attachments of the right and left colon and the midgut duodenum, the small bowel mesentery becomes fan shaped with a very broad oblique line of attachment from the left upper quadrant to the right lower quadrant (Figure 109-6). This broad-based attachment limits the mobility of the mesentery and prevents midgut volvulus from occurring around the superior mesenteric artery. When the intestinal rotation is incomplete (i.e., malrotated), mesenteric attachment may not be sufficient to prevent the development of midgut volvulus. Errors in rotational development need operative correction to avoid this potentially lethal problem (Figure 109-7).

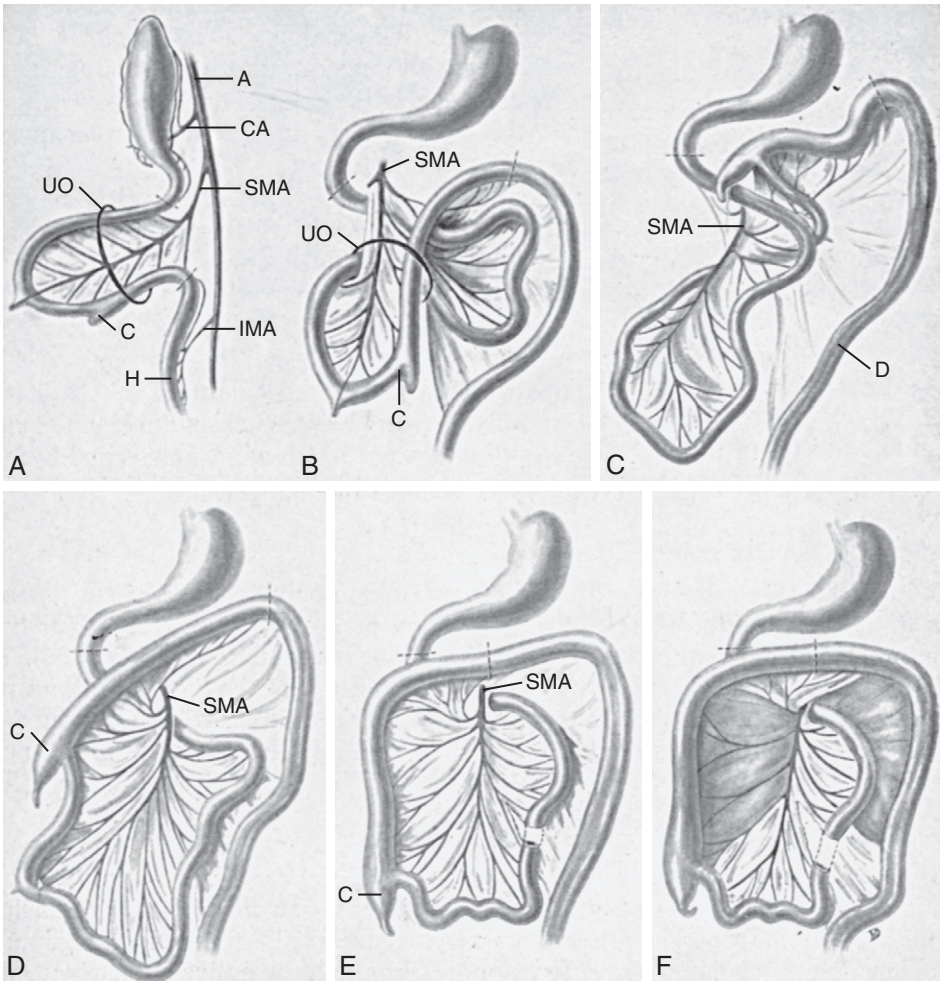


Figure 109-5 The normal rotation and fixation of the developing midgut. A, Herniation into the extraembryonic coelom at week 5. **B,** At week 10, initial rotation has taken place, and the midgut begins its return into the abdominal cavity. **C-E,** Completion of the 270-degree counterclockwise rotation of the prearterial and postarterial segments. **F,** Final fixation. *(From Gross RE:* The Surgery of Infancy and Childhood, *Philadelphia, 1953, WB Saunders, p 131.)*

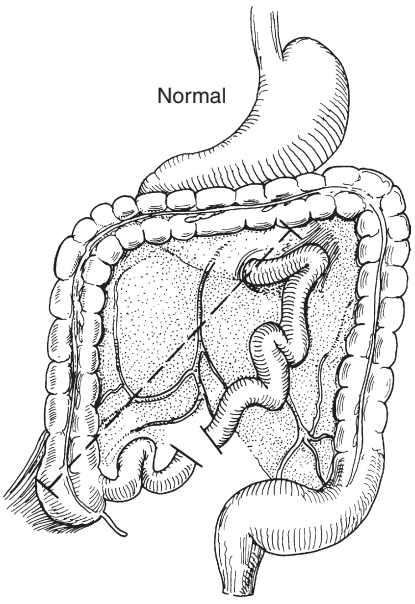


Figure 109-6 Normal rotation and fixation of the midgut. Note the broad-based fixation between the left upper and right lower quadrants, which fixes the superior mesenteric artery such that it cannot be the "root" of a volvulus. *(From Ross AJ III: Malrotation of the intestine. In Nora PF, editor:* Operative Surgery, *ed 3, Philadelphia, 1990, WB Saunders, p 1048.)*

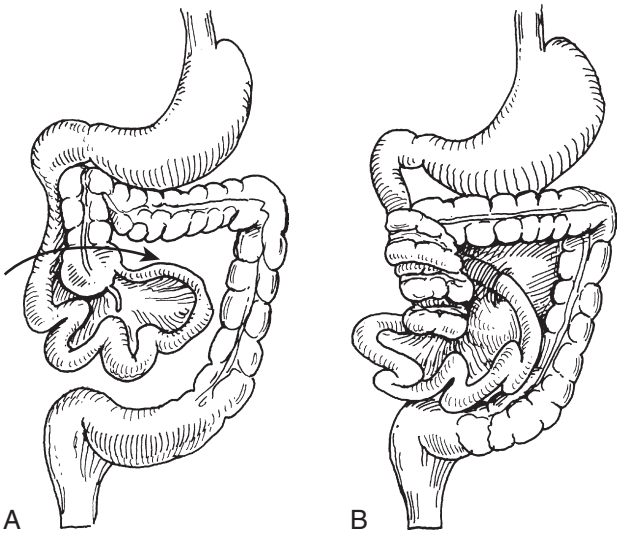


Figure 109-7 A, Malrotated bowel with the cecum overlying the duodenum. Bands from the cecum to the right upper quadrant could obstruct the duodenum. **B,** The lack of fixation in **A** permits the bowel to volvulize; note how the entire axis of the superior mesenteric artery is compromised. *(From Ross AJ III: Malrotation of the intestine. In Nora PF, editor:* Operative Surgery, *ed 3, Philadelphia, 1990, WB Saunders, p 1048.)*

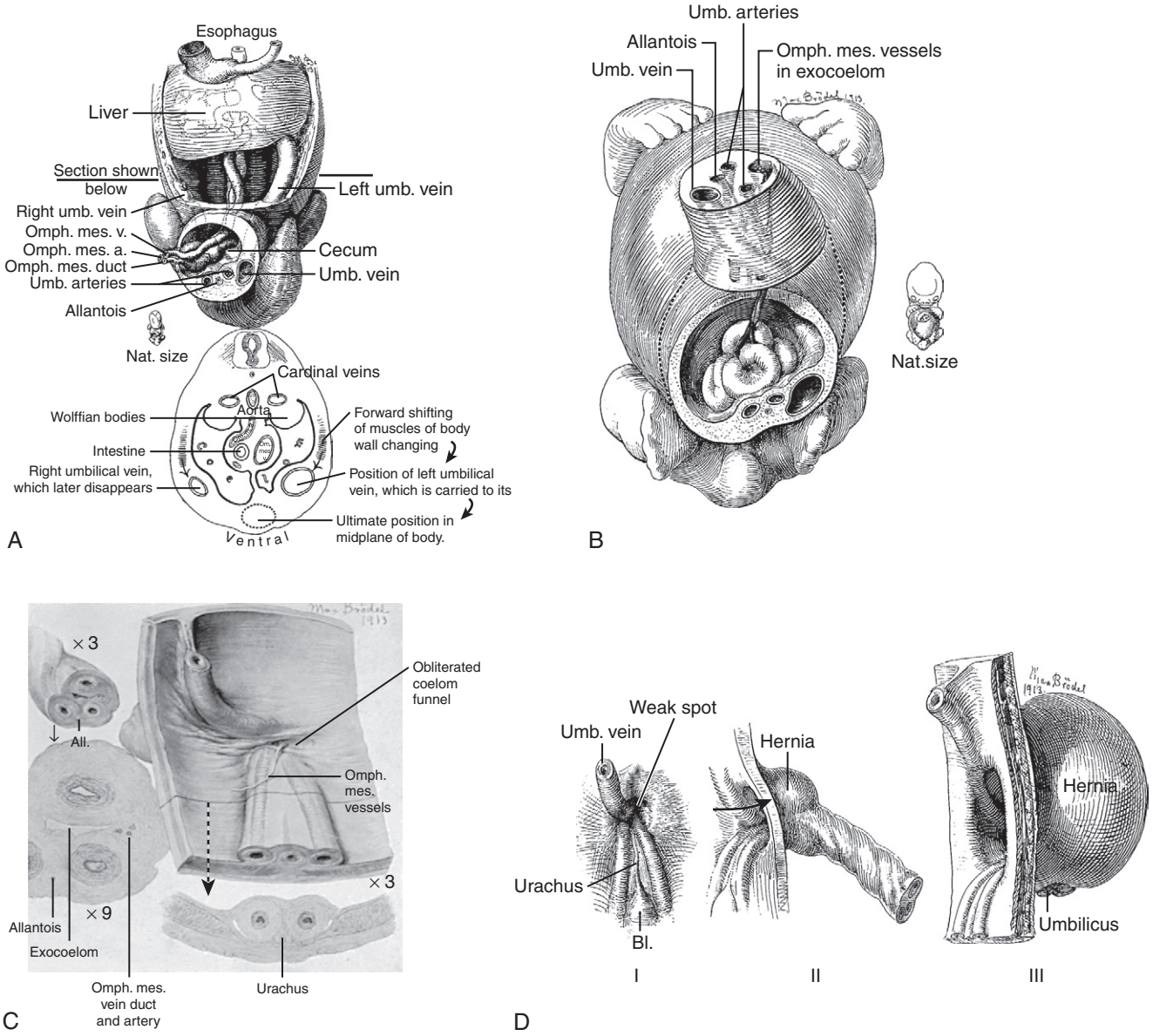


Figure 109-8 A-D, Max Brodel's drawings depicting the embryology of the umbilicus. Note the disappearance of the right umbilical vein and the resultant weak spot to the right side of the umbilicus. It is proposed that rupture at this weak spot is the cause of gas troschisis, thus explaining the usual finding of the defect occurring to the right of an infant's umbilical cord. *(From Cullen TS:* The Umbilicus and its Diseases, *Philadelphia, 1916, WB Saunders, p 745.)*

Anomalies of Embryogenesis

Failure of the midgut to return to the abdominal cavity is a frequently encountered congenital anomaly known as *omphalocele.* This developmental arrest is associated with a small abdominal cavity. It is unclear whether the small abdominal cavity is due to failure of the development of the body wall by appropriate migration of the lateral body folds medially or to failure of the return of the intestinal contents, or both. The amount of intestine herniated varies from a small amount that is barely distinguishable from an umbilical hernia to a massive anomaly containing the entire midgut and liver. Unless ruptured, the omphalocele is covered by a sac consisting of both peritoneum and amnion, and the umbilical cord is seen to enter the large exomphalos. In this anomaly, because midgut rotation does not occur, all children with omphalocele have an incompletely rotated intestine. Omphalocele needs to be distinguished from *gastroschisis,* in which there is an abdominal wall defect lateral (usually to the right) to the intact umbilical cord. Gastroschisis is thought to be due to a rupture of the lateral umbilical ring at the site of regression of the right umbilical vein during development (Figure 109-8). In this situation, in addition to the intact umbilical cord, there is no membrane covering the herniated bowel, which is generally thick, matted, and leathery. Rotation in this anomaly is similarly incomplete (Figure 109-9). Meckel diverticulum is another commonly noted embryologic anomaly that arises from the remains of the vitelline *(omphalomesenteric)* duct or embryonic yolk stalk. This anomaly is a fingerlike pouch approximately 5 cm long arising from the antimesenteric border of the ileum some 40 to 50 cm proximal to the ileocecal junction. It is found in approximately 2% to 3% of the normal population. As many as 24 variations of persistent vitelline duct anomalies have been

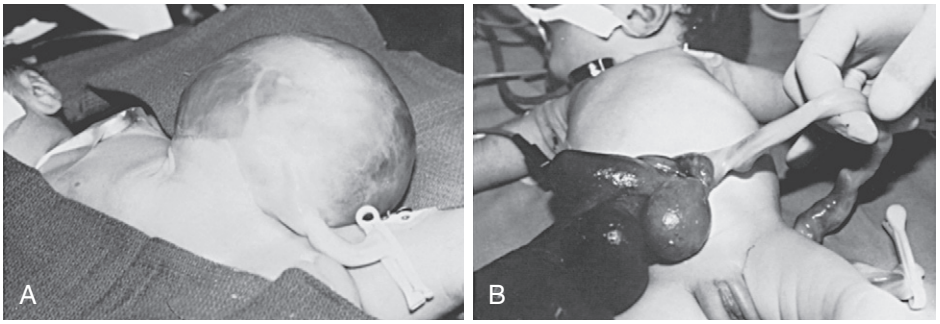


Figure 109-9 A, An infant with a large hepatoomphalocele. Note that the defect includes an intact sac and that the umbilical cord enters the sac. **B,** An infant with gastroschisis. Note that the defect is to the right of an intact umbilical cord normally situated on the abdominal wall. Also note the absence of a sac and the thick, matted bowel.

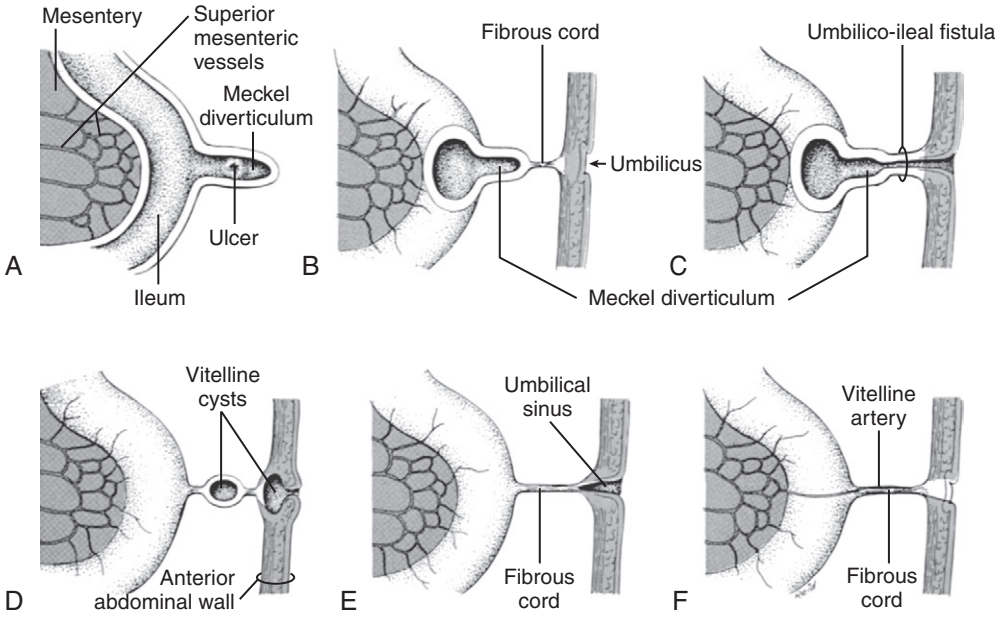


Figure 109-10 A-F, Meckel diverticulum and other anomalies of a persistent omphalomesenteric duct are shown. It is easily appreciated how **B** through **F** can be the focus of either volvulus or obstruction from an adhesive band. *(From Moore KL:* The Developing Human*, ed 4, Philadelphia, 1988, WB Saunders, p 236.)*

described. In addition to the uncomplicated variety of Meckel diverticulum, the tip of the vitelline duct remnant can be attached to the body wall or another intraabdominal structure. Volvulus or obstruction, or both, can occur at this site. In addition, this diverticulum may be patent, creating an *omphaloileal fistula* (connecting the ileum with the umbilicus). A mucosal remnant may persist, creating either an *umbilical sinus* or *polyp.* Furthermore, cystic remnants may persist along the length of the vitelline duct lying within the body wall or within the abdominal cavity itself (Figure 109-10).

Anomalies of midgut rotation are generally referred to as *malrotations.* If the extent of midgut rotation is less than the full 270 degrees, the mesentery may be incompletely based, allowing the bowel to rotate on the superior mesenteric artery axis, creating a volvulus-type picture. The number of possible anomalies of rotation is bewildering; however, all intestinal malrotations have the risk of volvulus.

Congenital intestinal atresia and *stenosis* are common anomalies. The observations of Louw and Barnard[14] have clearly demonstrated that such atresias are due to late intrauterine mesenteric vascular accidents. On rare occasions, jejunoileal atresia may be due to a failure of recanalization of the intestinal lumen occluded by epithelial cells. Atresia of the colon is far less common than atresia of the ileum or jejunum. Its cause is generally accepted as being similar to that of jejunoileal stenosis-atresia.

Duplications of the intestinal tract have been broadly categorized as cystic duplications and tubular duplications.[15] Cystic duplications are far more common and generally do not communicate with the intestinal tract, whereas tubular duplications communicate with the intestinal lumen. Duplications are almost invariably located on the mesenteric side of the bowel. When duplications enlarge, they can compress (and possibly obstruct) adjacent bowel or create a mass effect, or both. Most duplications are located within the small intestine, most commonly within the ileum. Most have mucosa resembling that of the adjoining gut. Duplications are generally thought to arise from (1) a failure of normal regression of the known embryonic diverticula with persistence of the transitory diverticula noted during embryogenesis (Lewis and Thyng theory); (2) traction between endoderm and overlying structures during early embryogenesis (Bentley and McLetchie theory; Figure 109-11); (3) errors of recanalization of epithelial plugs within the small intestine (Bremer theory); or

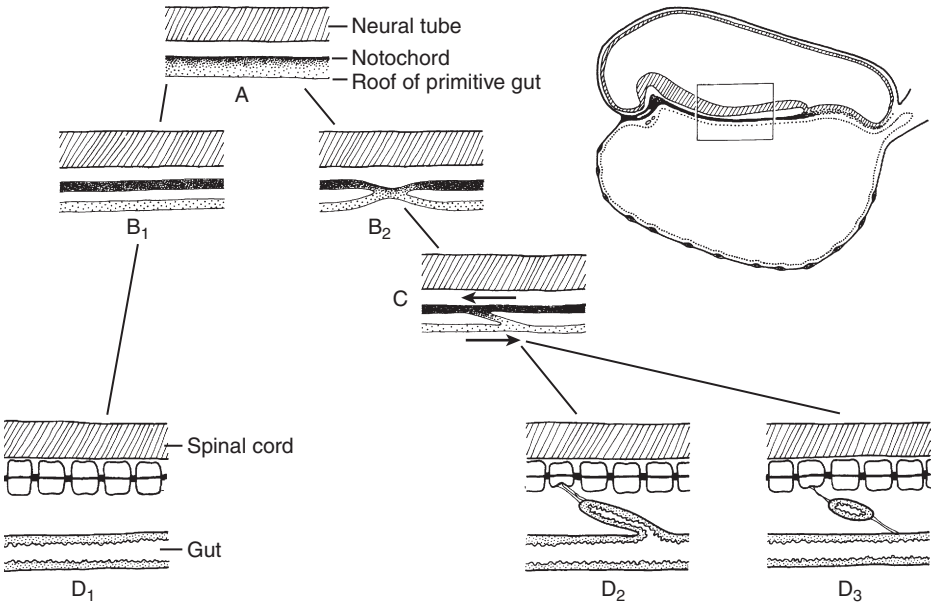


Figure 109-11 Abnormal separations of the notochord from the endoderm at week 4 *(A, B_2, C)* can result in the formation of an enteric duplication, either cystic *(D_3)* or diverticular *(D_2).* Normal is depicted in *A, B_1, D_1.* It can be appreciated why a vertebral malformation is often seen concurrent to the enteric duplication. *(From Gray SW, Skandalakis JE:* Embryology for Surgeons, *Philadelphia, 1972, WB Saunders, p 173.)*

(4) adherence of the endodermal walls lining the developing gut to create a double lumen.[3-5,10]

HINDGUT

Organogenesis

The hindgut comprises that region of the digestive tube that is supplied by the inferior mesenteric artery. The watershed region between the superior and inferior mesenteric arteries is located in the mid to distal transverse colon. This region, as well as the descending colon, sigmoid colon, rectum, and superior portion of the anal canal, is a derivative of the hindgut. The epithelium of the urinary bladder and most of the urethra are derivatives of the hindgut as well.

It is well known that the growth of the postarterial limb lags behind that of the prearterial limb so that at 10 weeks gestation, when the intestines return to the abdominal cavity, the caliber of the large intestine is much smaller than that of the small intestine. Transient occlusion of the colon by a solid epithelial plug probably occurs between 5 and 8 weeks gestation.[1]

Developmentally, the most complex region of the hindgut is its most terminal portion, the cloaca. At approximately day 13 of development, a ventral diverticulum, the *allantois*, forms. The future cloacal region is located at the junction of the allantoic stalk and the hindgut. The gut posterior to the allantois (tailgut) remains small and disappears by about the 6th week of development. Although the cloaca is lined with endoderm, it is in direct contact with an ectodermally lined structure termed the *proctodeum* (anal pit). The area of contact between the ectodermal and endodermal surfaces is termed the *cloacal membrane*. Thus the cloaca proper has the allantois ventrally and the cloacal membrane, an ectodermal-endodermal interface, posteriorly.

As organogenesis proceeds, the cloaca becomes partitioned by a mesenchymal-mesodermal wedge termed the *urorectal septum*. This structure begins development in the angle between the allantois and the hindgut and proceeds in a cephalic to caudal direction moving toward the cloacal membrane (Figure 109-12). The septum stimulates lateral cloacal ridge development and fusion, and by week 7 of development it reaches and fuses with the cloacal membrane. This results in the formation of a perineal body, which divides the cloacal membrane into a caudal anal membrane and a larger ventral urogenital membrane.

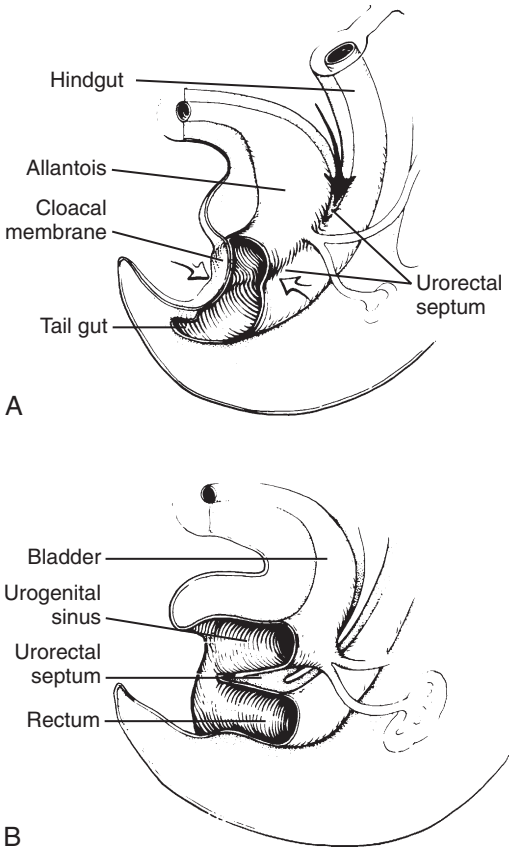


Figure 109-12 **A** and **B,** Descent of the urorectal septum is demonstrated. This results in the partitioning of the distal hindgut, or cloaca, into a urogenital sinus and rectum. It can be appreciated how deviations in the descent of this septum can lead to anorectal malformations, which often include urogenital anomalies as well. *(From Ziegler MM, Duckett JW, Howell CG, et al: Cloacal exstrophy. In Welch KJ, Randolph JG, Ravitch MM, et al, editors:* Pediatric Surgery, *ed 4, Chicago, 1986, Year Book Medical Publishers.)*

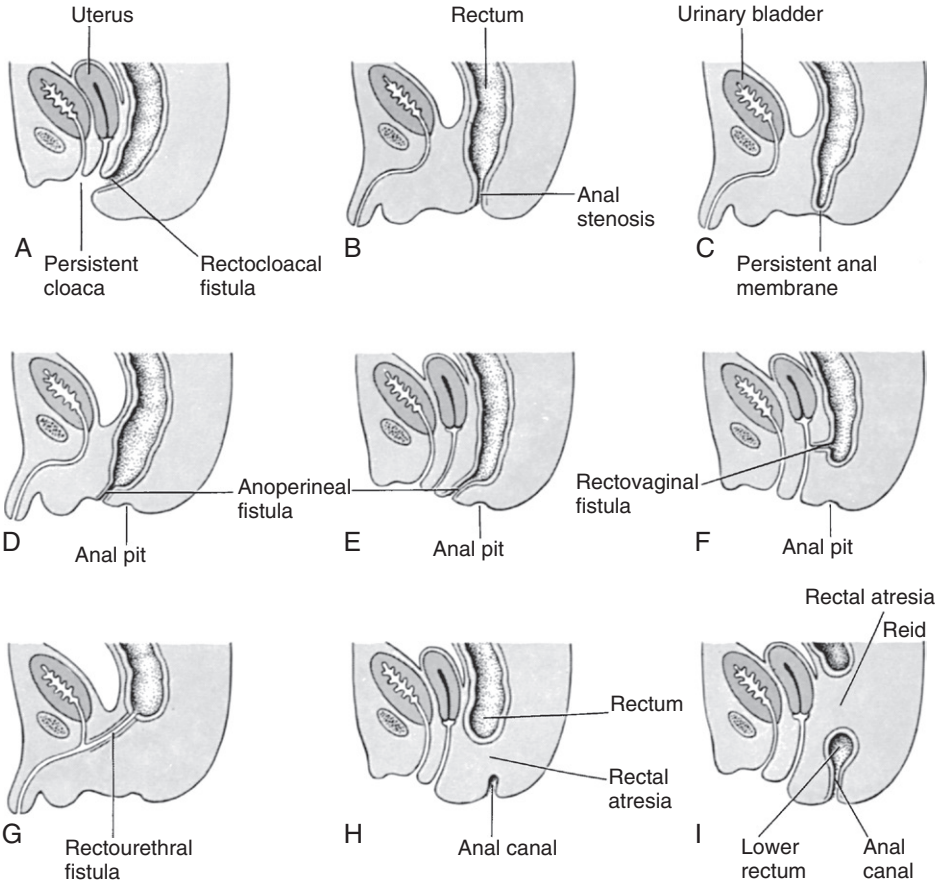


Figure 109-13 Various anorectal anomalies. A, Persistent cloaca, a common confluence of the intestinal, urinary, and reproductive tracts, results from failure of descent of the urorectal septum. **B-E,** Manifestations of low-type imperforate anus. **F-H,** Manifestations of high-type imperforate anus. **I,** Rectal atresia (rare). *(From Moore KL:* The Developing Human, *ed 4, Philadelphia, 1988, WB Saunders, p 241.)*

Anatomically, this creates a rectum and upper anal canal dorsally and a urogenital sinus ventrally. At approximately 8 weeks of development, the anal membrane (proctodeum) ruptures. The caudal portion of the digestive tract (the anal canal) is now in communication with the amniotic cavity. The superior two thirds of the anal canal is derived from the hindgut and the inferior third from the proctodeum. The junction is noted by the pectinate line. This pectinate line represents not only the junction of the proctodeum's ectoderm and the hindgut's endoderm but also the region of vascular watershed between these two structures. The superior rectal artery supplies the upper portion of the anal canal (from the inferior mesenteric artery), and the inferior rectal artery supplies the lower third (from the internal pudendal artery). The urorectal septum descent also defines a cloacal sphincter, the posterior aspect of which develops into the external anal sphincter, and the anterior aspect becomes part of the transversus perinei superficialis, the bulbospongiosus, the ischiocavernosus, and the urogenital diaphragm. In females, the fusion of the müllerian ducts (to form the uterus and vagina) moves down the urorectal septum and reaches the urogenital sinus at week 16 of development. In the male, the urogenital diaphragm is eventually obliterated by the fusion of the genital folds, and the sinus itself becomes incorporated into the urethra.

The obvious complex development of the cloacal region makes it easy to understand why anorectal malformations are common. Most arise from abnormal partitioning of the cloaca by the urorectal septum and its descent to create anorectal and urogenital structures. More often than not, it would seem that many of the anomalies can be explained by a dorsal (posterior) deviation of this septum, which leads to anomalies such as imperforate anus with rectourethral fistula. These anomalies would develop at approximately 6 to 8 weeks gestation (Figure 109-13).

Anomalies of Embryogenesis

The most common disorder of embryogenesis involving the hindgut is generally referred to as *imperforate anus.* Found more commonly in males, anorectal malformations result from the abnormal development of the urorectal septum so that there is incomplete separation of the cloaca into its urogenital and anorectal components. As a result, the anal canal may end blindly or there may be an ectopic opening on the perineum, almost always directed anteriorly toward or into the vulva in females or into the scrotum or urethra in males (see Figure 109-13). The anorectal malformations are commonly categorized as either low or high, determined by whether the blind end of the rectum is above or below, respectively, the level of the levator musculature. The high type of lesion is more common than the low and, generally, more complex. In its most complete form (usually encountered in females) one is left with a *persistent cloaca.* In this anomaly, only a single perineal hole is present, which functions as a common outlet for the intestinal, urinary, and reproductive tracts.

The congenital lack of ganglion cells within the myenteric plexus of the bowel is termed *congenital aganglionic megacolon*, or *Hirschsprung disease.* Congenital megacolon results from the failure of neuromyenteric cells to migrate normally into the wall of the colon and, in most cases, the rectosigmoid is the only bowel involved. This anomaly probably arises between the 6th and 8th weeks of gestation. The "normal" bowel is the proximal dilated bowel that results from the aganglionic bowel's inability to undergo parasympathetic-mediated relaxation. Total colonic and total intestinal aganglionosis have been reported. The aberrant developmental stimulus leading to the lack of the parasympathetic ganglion cells is unclear.

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Delma L. Broussard and Steven M. Altschuler

Development of the Enteric Nervous System

Multiple maturational milestones, including coordination of sucking and swallowing, effective gastric emptying, propagation of small intestinal contents, and colonic elimination, are necessary for successful enteral feeding of the infant. Symptoms consistent with poor gastrointestinal (GI) motility, such as vomiting, abdominal distention, and constipation, are common in the preterm infant. Developmental patterns of GI motility have been described in both animals and humans. This chapter reviews the physiology of GI movements, the extrinsic innervation of the gut, and the development of the intrinsic neural tissue of the gut, the enteric nervous system (ENS).

PHYSIOLOGY OF GASTROINTESTINAL CONTRACTIONS

Myogenic Control

Neurochemical, humoral, and mechanical stimuli control smooth muscle contractile activity. Smooth muscle cells generate spontaneous electrical activity through a fluctuation in resting membrane potential. These periodic depolarizations are below the membrane potential necessary to initiate a contraction and are known as *slow waves, electrical control activity, basic electrical rhythm*, or *pacesetter potentials*. Contractions occur only when a slow wave, after neural or chemical stimulation, exceeds the excitation threshold necessary for an action potential. After depolarization of a smooth muscle cell, an increase occurs in intracellular calcium. Calcium binds to calmodulin, a regulatory protein, and permits binding of the contractile proteins actin and myosin, resulting in a contraction. Therefore the action potential, referred to as *electrical response activity* or *spike potential*, occurs against the background of the slow wave (Figure 110-1).[1] Slow waves control the timing, speed, and direction of intestinal contractions.[2]

Neural Control

Central Nervous System

The central nervous system (CNS) and neural tissue within the intestinal wall, known as the ENS, control GI motor, secretory, and vascular activities. The CNS and ENS consist of afferent (sensory) neurons, efferent (motor) neurons that synapse directly with effector cells (muscle secretory cells, blood vessels), and interneurons, which integrate messages from the afferent neurons and program the efferent neurons. The sensory fibers travel closely with parasympathetic preganglionic fibers in the vagus nerve and sympathetic postganglionic fibers in the splanchnic nerves. Their neuronal cell bodies are located in the nodose and dorsal root ganglia, respectively. Vagal afferents terminate in the mucosal and muscular layers of the GI tract from the soft palate to the descending colon, as well as the liver, gallbladder, and pancreas.[3-5] Splanchnic afferents innervate the serosal surface and the bowel mesentery, as well as its mucosal and muscular layers.[6]

GI motor innervation is provided by the sympathetic and parasympathetic divisions of the autonomic nervous system (Figure 110-2). Postganglionic neurons of the sympathetic division are located in the prevertebral position on either side of the spinal cord, and the ganglia of the parasympathetic division are within the GI tract. Preganglionic sympathetic and parasympathetic neurons are located in spinal cord segments T2 through L2 (sympathetic) and in the medulla and sacral spinal cord (parasympathetic).

Most parasympathetic innervation of the gut is supplied by the vagus; the sacral nerves innervate only from the middle of the transverse colon to the rectum. Vagal efferents terminate primarily within the enteric ganglia.[7,8] Because the vagus nerve innervates most of the gut, organization of its projections within the brain stem has been studied extensively. Neural tracer studies have demonstrated a viscerotropic organization of afferent projections within the vagal sensory nucleus and the nucleus tractus solitarii (NTS), as well as efferent fibers within the motor nuclei, the dorsal motor nucleus of the vagus nerve, and the nucleus ambiguus (NA) of the rat.[3,4,9-13]

The NTS is the first synaptic site for vagal afferent fibers of the alimentary tract through cranial nerves IX and X. Within the NTS are interneurons involved in the coordination of afferent and efferent information to control esophageal and GI motor activity. Afferent fibers, depending on their site of origin with the rat GI tract, project to the distinct subnuclei within the NTS. For example, the central subnucleus (NTS_{cen}) has been implicated in

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the coordination of esophageal peristalsis with use of organ-specific injections of neural tracers.[3,14-16] In the rat, esophageal vagal afferents terminate in the NTS_{cen}, and its neurons (esophageal premotor neurons) project directly to esophageal motoneurons located in the compact formation of the NA (NA_c).[17,18] The efferent neurons for the striated muscle of the esophagus are located in the NA, and the smooth muscle efferents originate in the dorsal motor nucleus of the vagus nerve in the rat.

Identification of anatomic circuits that project to specific bowel segments has enabled investigation of neurotransmitters, which may be involved in the control of esophageal and other GI motor activities in animal models. Pseudorabies virus, a swine neurotropic herpesvirus that undergoes retrograde axonal transport, has been instrumental in the colocalization of neurotransmitters within the brain stem neurons involved in esophageal peristalsis.[16] After injection of the virus into an end organ, active infection develops within a neuronal cell body following retrograde transport. The virus replicates and subsequently undergoes retrograde transport to the synapsing afferent terminals. (Specific transsynaptic retrograde transport to first-, second-, and third-order neurons is a function of postinjection time.) The virus can be localized immunohistochemically by using specific pseudorabies virus antisera.

Several neurotransmitters have been implicated as mediators of synaptic transmission in the brain stem–esophageal swallowing circuit, including the excitory amino acid glutamate (GLU).[19-21] GLU binds to a family of receptors that includes the *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) class. Pharmacologic studies demonstrate that the application of GLU-agonist NMDA to the rat NTS_{cen}[20-22] and NA_c[23] stimulates esophageal motor activity. NMDA is a heteromeric protein composed of several subunits, of which NMDAR1 is a subunit necessary for a functional receptor. The demonstration of the colocalization of NMDAR1 messenger RNA and pseudorabies virus immunoreactive neurons in the esophageal premotor neurons (NTS_{cen}) and esophageal motoneurons (NA_c)[24] adds further support to this hypothesis.

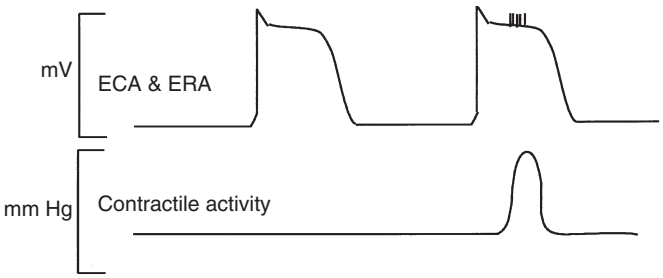


Figure 110-1 Schematic of pacemaker electrical control activity *(ECA)* with electrical response activity *(ERA)* in the second cycle. The ERA results from depolarization of the ECA beyond a threshold potential and produces a smooth muscle contraction. *(Modified from Sarna SK, Otterson ME: Small intestinal physiology and pathophysiology,* Gastroenterol Clin North Am *18:383, 1989.)*

Enteric Nervous System

Although the CNS plays a significant role in the control of GI movements, central innervation of the GI tract is relatively sparse when compared with the large number of ENS neuronal fibers. The ENS is unique, compared with other peripheral nervous system ganglia, because of its ability to mediate reflex activity independent of the CNS. The ENS is a complex system of neurons, which integrates extrinsic neuronal and humoral stimuli to produce stereotypic patterns that regulate motility, secretion, and vascular flow. For example, contraction of an orad segment of bowel to a food bolus is coordinated with relaxation of a caudad segment to attain peristalsis. Consequently, the ENS has been referred to as the "little brain" in the gut (Figure 110-3).[25]

The ENS consists of two major plexuses—the myenteric (Auerbach), located between the external circular and longitudinal muscle layers, and the submucosal (Meissner); it also includes six relatively minor plexuses, occupying different layers of the bowel wall (Figure 110-4).[26] The density of each plexus varies according to location in the GI tract and by animal species.[27-29]

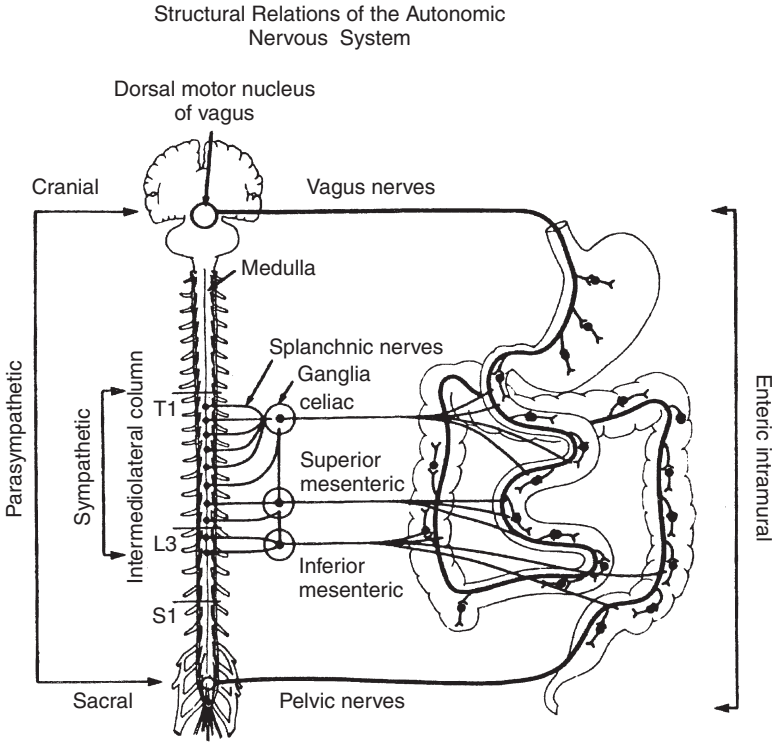


Figure 110-2 Organization of the projections of the autonomic nervous system to the gut. The enteric nervous system is within the bowel wall. *(Modified from Wood JD, Wingate DL: Gastrointestinal neurobiology: the little brain in the gut. In* American Gastroenterological Association Undergraduate Teaching Project in Gastroenterology and Liver Disease, *Timonium, Md, 1988, Milner-Fenwich, p 1.)*

The importance of the ENS is supported by abnormal myoelectrical recordings from the small intestine after chemical ablation of the myenteric neurons.[30] In addition, extrinsically denervated bowel can still mix and propel foodstuffs, and an autotransplanted small-bowel segment does not cause obstruction.[31]

The ENS and CNS ganglia share ultrastructural and neurochemical similarities. Neither the enteric ganglia nor the CNS ganglia contain connective tissue or blood vessels.[32,33] The enteric glia, support cells of enteric neurons, have processes that abut the basal lamina surrounding the myenteric plexus, analogous to the pia-astroglial border of the brain.[34] The internal structure of the enteric glia consists of high concentrations of cytoskeletal protein glial fibrillary acidic protein, which is also a marker for astroglia.[35] The functions of enteric glia are not clear; however, selective destruction of enteric glia in niacin-deficient mice results in diarrhea.[36] The anatomic, structural, and chemical similarities of enteric glia and astroglia support the hypothesis that their roles are analogous.[34]

Neurotransmitters

Many CNS neurotransmitters are also found in the ENS. Acetylcholine is the most abundant excitatory neurotransmitter mediating smooth muscle contractions in the ENS. Although many other enteric neurotransmitters have been suggested by immunocytochemical, pharmacologic, and electrophysiologic studies, not all have met the criteria for a neurotransmitter. These criteria are (1) a pharmacologic response to exogenous application of the potential neurotransmitter, (2) a mechanism for transmitter uptake and synthesis, (3) storage in presynaptic vesicles, and (4) a physiologic action following the neurotransmitter release by nerve stimulation.

Nitric oxide (NO), which is a gas, has been shown to mediate nonadrenergic noncholinergic relaxation in GI smooth muscle. There are several proposed mechanisms for the effects of NO. NO mediates relaxation through an increase in 5′-cyclic guanosine monophosphate.[37,38] Other studies suggest that NO does not act alone but may interact with other inhibitory neurotransmitters such as vasoactive intestinal peptide to mediate its effects.[39] NO synthase, the enzyme responsible for NO synthesis, has been demonstrated in the ENS of several animal species.[40-44] There is also electrophysiologic evidence for NO in the human GI tract,[45-47] as well as immunocytochemical labeling of NO-containing enteric neurons in the developing human intestine.[48]

Pyloric stenosis is the most common cause of gastroduodenal obstruction in infancy. The pathophysiology of antropyloric muscle hypertrophy and decreased neuronal fibers is not known. Animal and human studies have suggested that NO may play a role in pyloric stenosis. NO,[49] vasoactive intestinal peptide,[50] and substance P[51] fibers have been found to be decreased in the circular muscle layer of infants with hypertrophic pyloric stenosis. Although several neurotransmitters are decreased in the circular muscle layer of infants with pyloric stenosis, a transgenic neuronal NO synthase gene–deficient mouse phenotypically expresses hypertrophy of the circular muscle of the pylorus and marked enlargement of the stomach as its only phenotypic abnormalities.[52] Unlike the ubiquitous distribution of NO in wild-type mice, homozygous mutant mice do not have NO-positive fibers

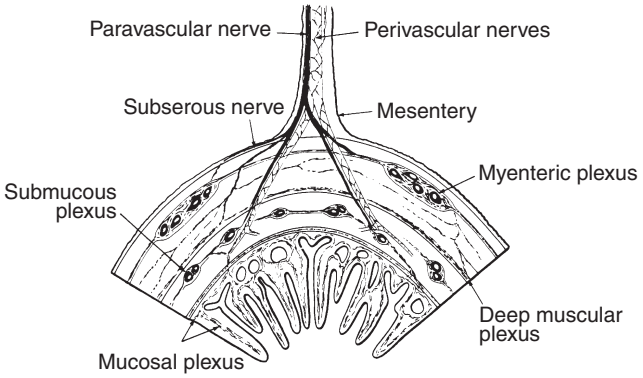


Figure 110-4 Arrangement of enteric plexuses in whole mounts of intestine. In addition to the large myenteric and submucosal plexuses, several smaller plexuses are shown. *(From Furness JB, Costa M: Arrangement of enteric plexuses. In Furness JB, Costa M, editors:* The Enteric Nervous System, *New York, 1987, Churchill Livingstone.)*

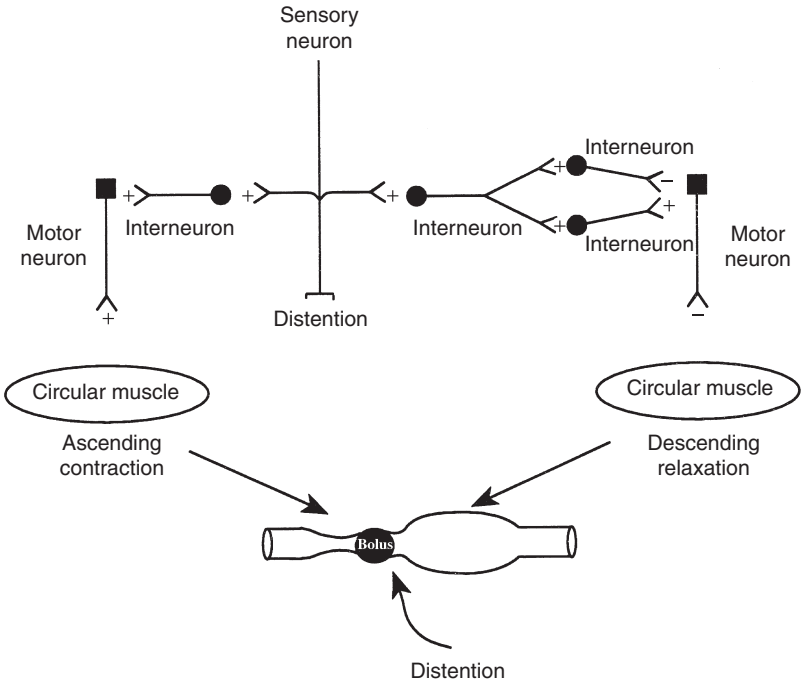


Figure 110-3 Schematic of intestinal peristalsis of a food bolus, illustrating the local coordination of sensory and motor information within the enteric nervous system. *(Modified from Makhlouf GM: Regulation of muscle function by neuropeptides. In Fisher R, et al, directors: American Gastroenterological Association Postgraduate Course 1992:* Gastrointestinal Motility Disorders: What's New and What to Do! *Philadelphia, 1992, WB Saunders.)*

in the myenteric plexus or circular muscle of the stomach or pylorus. NO deficiency has also been described in aganglionic segments of patients with Hirschsprung disease.[53]

Intestinal guinea pig muscle tension studies have implicated GLU as a potential excitatory neurotransmitter in the gut. Longitudinal muscle strips associated with the myenteric plexus contract in the presence of exogenous GLU, an abundant excitatory amino acid neurotransmitter in the CNS.[54-57] This contraction can be inhibited by agents that antagonize the CNS GLU receptor NMDA and by drugs that block cholinergic receptors. GLU can be synthesized from exogenous L-glutamine in myenteric neurons and released from myenteric neurons after neuronal depolarization in vitro.[58] These data support an excitatory GLU neural pathway in the myenteric plexus through NMDA-type receptors and suggest that glutamatergic neurons are interneurons initiating contraction through cholinergic motoneurons. In situ hybridization studies have localized the NMDAR gene to the neurons of the guinea pig taenia coli myenteric plexus[59] and rat intestine.[60] The NMDAR has also been implicated in colonic peristalsis.[61] These studies support the concept that GLU is a neurotransmitter in the ENS that mediates its effects through NMDARs, but further studies are necessary to confirm a functional NMDAR. CNS astroglia take up neuronally released GLU and convert it to glutamine, through the enzyme glutamine synthetase.[62] Our laboratory[63] and that of Jessen and Mirsky[35] have demonstrated the presence of glutamine synthetase in enteric glia, the support cells of enteric neurons. However, further studies are needed to confirm the uptake of GLU by enteric glia.

Development of the Enteric Nervous System

Enteric neurons and glial elements are derived from the migration of neural crest cells.[64-66] Early investigations of the origin of the ENS, using quail-chick chimeras, demonstrated that vagally derived neural crest cells migrated throughout the entire bowel, but sacral émigrés were found only in the postumbilical hindgut.[67] Because the neural crest émigrés are not recognized as neurons or glia as they first enter the bowel, novel labeling methods have been developed to more specifically trace the migration of neural crest cells. Fluorescent dye 1,1-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate (DiI) or retrovirus LZ10, constructed with *lac Z* reporter gene and detected with β-galactosidase immunoreactivity, was injected by Pomeranz and colleagues[68] into the neural tube of chick embryos before neural crest migration. Injections into the vagal crest level resulted in labeled cells in the avian stomach and duodenum. Injections into the sacral crest resulted in labeled cells in the postumbilical bowel; however, injections into the truncal crest localized to the sympathetic ganglia and not to the bowel wall. DiI tracer experiments have also demonstrated that the murine gut is colonized by the vagal and sacral regions of the neural crest.[69,70]

The premigratory neural crest cells are multipotent; therefore, factors that determine their differentiation have been of interest.[71] Premigratory chick crest cells replaced with those of quails (or the reverse) in younger embryos demonstrate that the phenotypic expression of the grafted crest cells is independent of the site of origin and determined by the migration pathway of the host embryo.[66,67,72] Phenotypic expression of the neural crest cells is dictated by the enteric microenvironment at the site of terminal differentiation. This is illustrated by the transient expression of the catecholaminergic neuronal phenotype by crest-derived cells that colonize the rodent bowel.[73] Transiently catecholaminergic cells are found in the murine and rat foregut at embryo days 9.5 and 11, respectively. During their migration through the vagal nerve pathway, transiently catecholaminergic cells express tyrosine hydroxylase, aromatic L-amino acid decarboxylase, dopamine β-hydroxylase, catecholamine storage, and norepinephrine transport as is seen in other sympathetic neurons. When these cells reach the bowel, they no longer express the catecholaminergic phenotype. Differentiated neurons express serotonergic and peptidergic neurotransmitters (e.g., serotonin, substance P, and neuropeptide Y).

Hirschsprung Disease

Hirschsprung disease is a GI disorder manifested by intestinal obstruction, as a result of the absence of neural crest–derived enteric ganglia in the terminal hindgut. It is a common disease occurring in 1 in 5000 live births. Patients with Hirschsprung disease commonly present with partial or complete obstruction during the first year of life. The cellular mechanisms for this disease are not known, but animal models such as the lethal spotted (*ls/ls*) mouse, in which congenital aganglionosis is an autosomal recessive trait, have suggested possible mechanisms.[74] As is seen in association with Hirschsprung disease, the colon of the *ls/ls* mouse is dilated proximal to the aganglionic segment, which acts as a functional obstruction. The aganglionosis develops as a result of failure of precursors of enteric neurons[75] and enteric glia[76] to colonize the bowel wall. Neurotropins[77] and laminin,[78] in addition to other molecules, may play a role in the control of enteric neuron migration and differentiation. After reaching the gut, neural crest cells express a 110-kDa cell-surface laminin-binding protein, which is not detected in ENS precursor cells.[78] It has been hypothesized that laminin interacts with the laminin-binding protein to cause neural crest cells to terminate migration. Neural crest cells from the *ls/ls* mouse can colonize the colonic walls of normal mice but not the distal colon of *ls/ls* mice.[79] Examination of the aganglionic segment of *ls/ls* bowel reveals that there is an overabundance of laminin and other components of the extracellular matrix in the gut wall.[80] It has been hypothesized that the accumulation of laminin could result in premature cessation of migration of crest-derived cells and, therefore, the absence of ganglionic cell bodies in the distal colonic segments of *ls/ls* mice. The 110-kDa laminin-binding protein may promote neurite extension.[78] The inhibition of cranial-to-caudal migration of vagal enteric neuroblasts to the large intestine of *ls/ls* mice suggests that a defect in the mesenchyme of the large intestine prevents colonization.[81] In vitro studies of the effects of neuronal development on smooth muscle cells from normal and aganglionic human bowel have suggested that the aganglionic colon is a less-favorable environment for neuronal growth because of a membrane-linked factor.[82]

Genetic and molecular studies in both animals and humans have proposed that specific genes may be responsible for regulating the migration of enteric neurons. In the mouse, the homeobox-containing gene, *Hoxa-4*, is expressed in various developing tissues, including the mesodermal layer of the gut. Exploration of the function of *Hoxa-4* has used a transgenic mouse expressing multiple copies of the *Hoxa-4* gene, which demonstrates congenital megacolon as its phenotype.[83] However, the pathogenesis of the bowel lesions of transgenic and lethal spotted mice is different. In this transgenic mouse model, crest-derived cells can enter the terminal colon, but their development is abnormal, resulting in hypoganglionosis. The *ls/ls* mouse has an abnormality of the extracellular matrix that prevents normal migration into the terminal bowel.

Pedigree studies have demonstrated that Hirschsprung disease is a heterogenous genetic disorder with autosomal dominant, autosomal recessive, and polygenic forms, as well as a few cases that result from environmental factors.[84] One autosomal dominant form has been mapped to human chromosome 10q11.1.[85] This region contains the *RET* proto-oncogene, a protein tyrosine kinase gene expressed in the cells derived from the neural crest. Mutations in the *RET* gene of Hirschsprung disease in both sporadic and familial cases have now been identified.[86,87] These data suggest that *RET* plays a critical role in the development

of the mammalian ENS. However, the existence of families with Hirschsprung disease without linkage to the *RET* gene suggests that there are additional genes affected in patients with Hirschsprung disease.[88]

Another susceptible chromosome in an inbred Mennonite kindred with Hirschsprung disease is chromosome 13q22.[89] Extensive molecular and genetic studies have implicated the endothelin-B (ET_B) receptor gene *(EDNRB)* as a gene candidate for Hirschsprung disease susceptibility within a subset of this family. Endothelins are a group of peptides that bind to two receptors, including ET_B. The ET_B receptor is expressed in the human colon, particularly in the myenteric plexus, mucosa, ganglia, and blood vessels of the submucosa,[90] areas that may be abnormal in Hirschsprung disease. Targeted disruption of the *EDNRB* gene in the mouse results in aganglionic megacolon.[91] After the transfection of a mutant *EDNRB* haplotype (identified in this pedigree) into a cell line, the resulting mutant did not demonstrate a normal ET_B-induced intracellular Ca^{2+} response, further supporting the susceptibility of this gene in Hirschsprung disease. These data suggest that *EDNRB* is important in the normal development of enteric ganglion in some patients with Hirschsprung disease.

Intestinal Motor Activity

The stomach and small intestine exhibit periodic motor activity during fasting. A migrating band of contractions of maximum spiking frequency and pressures is observed throughout the fasting small intestine. This is known as the migrating motor complex (MMC) or interdigestive motor complex (Figure 110-5).[92] The MMC is a recognizable pattern of cyclic groups of caudally migrating contractions or myoelectric activity of the antrum and small intestine that occurs in mammals in the fasted state.[92,93] The MMC consists of four phases: phase I, a period of quiescence, characterized by an absence of action potentials; phase II, a period of irregular contractile and myoelectric activity; phase III, maximal-amplitude regular contractions occurring at the frequency of the slow wave; and phase IV, a brief period of contractile activity before the start of phase I.[93-95] The MMC is thought to sweep residual products of digestion toward the colon, serving as a housekeeper.[96]

The MMC is primarily controlled by local enteric mechanisms.[2] Denervation of the extrinsic nerves to the gut does not stop initiation of the MMC cycle,[97-101] but the MMC cycle's duration becomes more variable, suggesting that the extrinsic nerves modulate periodicity. It is hypothesized that the MMC is under local control by the ENS, which has a neuronal circuit that periodically activates smooth muscle cells to contract at their maximal frequency.[99,102] Intraluminal calories disrupt MMC activity at all levels of the bowel and initiate generalized irregular contractions—the fed pattern—in the stomach and small intestine (Figure 110-6).[93] Introduction of nutrients into the bowel lumen results in a fed pattern in all areas of the bowel, including a noncontiguous bowel loop. The fed pattern does not occur, however, if the noncontiguous bowel is extrinsically denervated.[103] This suggests that initiation of the fed motor pattern requires regulation by extrinsic nerves or humoral factors. The caloric content of the ingested nutrient influences GI motility. Fats reduce spiking amplitude and intestinal transit[104] to a greater extent than do carbohydrates and protein.[105]

Development of Intestinal Motility

Animal studies of the fetal development of small intestinal motility, using long-term implanted electrodes, have demonstrated a progressive increase in the duration of spiking activity in dogs and sheep to a well-defined propulsive motor pattern.[106] Three stages of development of intestinal myoelectrical activity in the fetuses of several species have been identified. Stage 1 is characterized by unorganized spiking activity. In stage 1, there is a high percentage of retrograde propagation of low-amplitude spike bursts, suggesting that its main motor function is to mix intestinal contents. Stage 2, the fetal pattern, exhibits occasional cyclic spike bursts superimposed on slow waves that propagate aborally or orally. When stage 2 is associated with distally propagated phases of spiking activity recurring at short intervals,

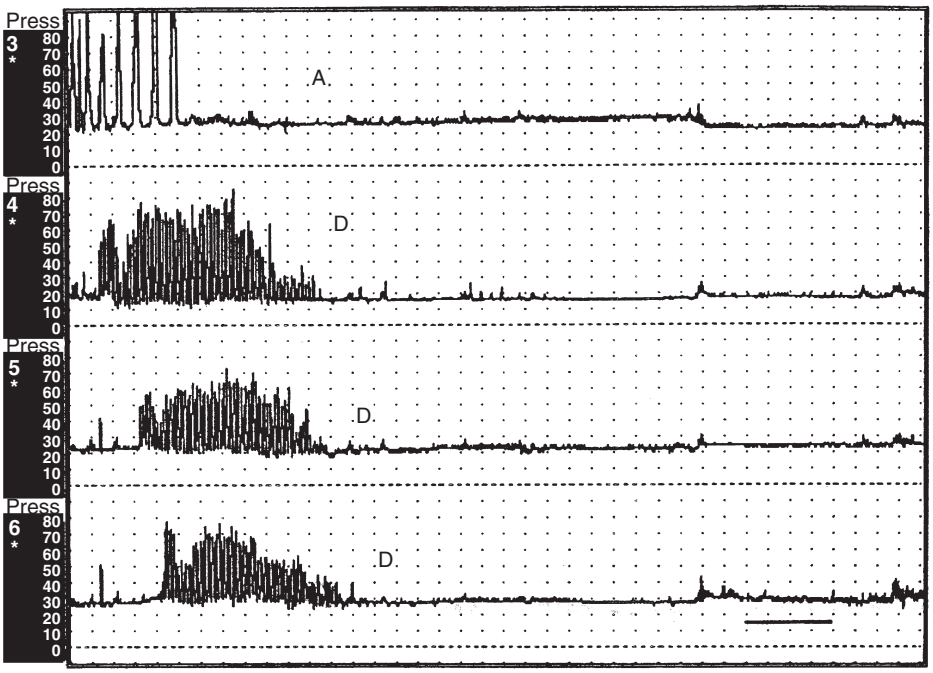


Figure 110-5 A normal MMC during fasting in a human subject. The recording was obtained with intraluminal miniature pressure transducers spaced 8 cm apart in the antrum *(A)* and duodenum *(D)*. High-amplitude contractions are distally propagated. (Scale bar = 2 min.) *(From Broussard DL: Gastrointestinal motility in the neonate,* Clin Perinatol *22:39, 1995.)*

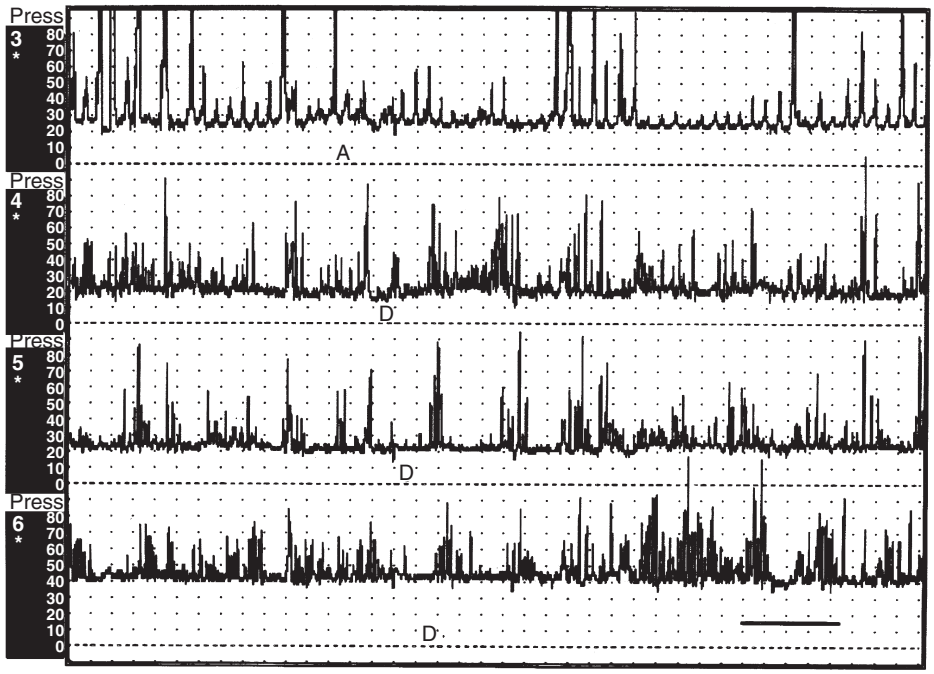


Figure 110-6 Antroduodenal motility pattern after a meal. Generalized spiking activity in the antrum *(A)* and duodenum *(D)* observed with miniature pressure transducer catheter. (Scale bar = 2 min.) *(From Broussard DL: Gastrointestinal motility in the neonate,* Clin Perinatol *22:39, 1995.)*

intraluminal contents may be propelled slowly for storage in the ileum and colon. Stage 3 resembles the adult MMC pattern.

Comparison of small-bowel motility patterns of ungulates (the GI motility patterns of which mature prenatally) and species with small intestinal motility patterns that develop after birth demonstrate parallel development of the ENS and CNS. Ungulates have full CNS maturity at the time of birth, which allows them to run with the herd soon after birth. In dog and rat pups that are born blind, deaf, and poikilothermic, maturation of the CNS and ENS is mainly postnatal.[107] Intestinal motor activity develops according to a species-specific, gestation-dependent pattern and is independent of birth.

The first studies in human fetuses to investigate intestinal transit used amniography. McLain[108] showed there was no transit of contrast down the intestine until 30 weeks gestation. Thereafter, an increasing aboral transit and propagation rate was seen as the pregnancy progressed. Subsequent manometric studies in infants illustrated a gestationally dependent pattern of development of small-intestinal motility, with developmental stages similar to those of dogs and sheep.[109,110] Increasing gestational age resulted in an increase in both duration and propagation of phase II contractions before the development of the MMC pattern.[110,111] The amplitude of contractions is also increased with gestational age.[110-112] The cycle time of the MMC is shorter in the term infant than in the adult,[111,113] suggesting that further maturation of the MMC occurs in infancy.

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Chris J. Dickinson

Development of Gastric Secretory Function*

The development of the human stomach is complex because of the many roles it plays in the digestive process. The stomach serves as a reservoir for food and regulates the flow of nutrients into the small bowel. In addition, the stomach is a secretory organ that continues digestive processes that were initiated in the mouth. Although hydrochloric acid is the major gastric secretory product, the stomach also secretes mucus, pepsinogen, bicarbonate, intrinsic factor, prostaglandins, and regulatory peptides. This chapter outlines gastric organogenesis and gives an overview of the regulation of gastric secretion, with emphasis on developmental studies in humans and animal models.

In interpreting the data currently available regarding the development of gastric secretory capability, several points should be kept in mind. First, although the mechanisms regulating gastric acid secretion are consistent across species, the relative contribution of individual components is quite variable. Second, considerable gaps remain in understanding the development of gastric secretory processes in humans. Third, although there have been numerous advances in understanding the molecular mechanisms that regulate the development of the small intestine and the digestive enzymes (e.g., sucrase-isomaltase and lactase), much less is known regarding the development of gastric secretory function.

ORGANOGENESIS AND CELL DIFFERENTIATION

The human stomach derives from the endoderm of the foregut at about 4 weeks gestation. As the gastric pouch continues to grow, the dorsal portion of the stomach enlarges at a more rapid rate than does the ventral border, producing the greater curvature. As this disproportionate growth continues, the stomach eventually undergoes a 90-degree rotation clockwise to assume the adult position by 8 weeks. The final J shape of the human stomach is not seen until 22 weeks.[1] Fetal ultrasound studies have greatly refined this process and can be used to detect abnormalities in gut function very early in gestation.[2] Histologically, the stomach is initially lined with stratified or pseudostratified epithelium.[3,4] At 6 to 9 weeks, the first gastric pits appear,[5,6] followed by the development of glands at approximately 12 weeks gestation, when the first primitive parietal cells can be recognized. The antral glands develop slightly later than those of the fundus but are present by 13 weeks gestation. At 12 to 13 weeks, the glands contain virtually no parietal cells but an abundance of mucous and endocrine cells. The gastric glands then expand toward the fully developed mature structures. In the hamster, the glands

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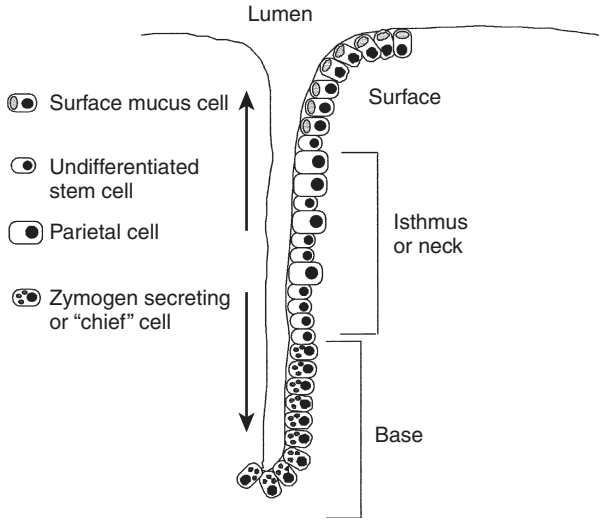


Figure 111-1 Representation of a gastric gland. The gastric gland is divided into three contiguous and overlapping areas: surface, isthmus or neck, and base. Unlike in the small intestine, gastric stem cells are located in the middle of the gland. As differentiation progresses, the three main cell types migrate to different areas.

appear to multiply by dichotomous branching.[7,8] In humans, the gastric glands appear to mature in a similar fashion and are very convoluted during this process, which is complete by 5 to 6 months of gestation.[6]

For many years it was unclear whether the various secretory cells of the stomach (mucous, acid-secreting parietal, pepsin-secreting chief, and endocrine cells) arose from a common progenitor cell or whether there were multiple stem cells. It is now known that a multipotent stem cell (known as the isthmus) is located in the middle region of the gastric pit.[9-14] Investigators also know that neck cells of neonatal mice can be grafted into the subcutaneous tissue of nude mice and it will differentiate into all four cell types.[15] This is consistent with a multipotent stem cell model analogous to that of the small intestinal crypt (Figure 111-1). It is interesting that grafts from embryonic day 14 mice maintain the axial patterning of gastric glands despite being in a different endocrine environment. Although these grafts do develop parietal and chief cells, there is a delay in the appearance of differentiated endocrine cells, suggesting that extragastric factors may be required for their development.

These data have led investigators to search for genes that might regulate gastric differentiation. Unfortunately, no such gene has yet been identified, but several things are known. First, the *N-myc* gene is necessary for development of the stomach, because *n-myc* knockout mice have a poorly developed gut.[16] Second, GATA-4 is known to be expressed first in primitive endoderm and then in definitive gastric endoderm. GATA-4–null blastocysts develop an abnormal gastric epithelium that has a squamous morphology.[17] Third, sonic hedgehog, an endoderm-derived signaling molecule, is overexpressed in the GATA-4–deficient cells.[17] Fourth, the nuclear transcription factor SP-1 is expressed in large quantities in the developing gut and is responsible for basal and gastrin-mediated transcription of some important genes, such as the proton pump H^+/K^+–adenosine triphosphatase (ATPase).[18,19] It is likely that over the coming years other important transcription factors necessary for gastric development will be identified in mouse models.

The regulation of gastric development took a leap forward when the human fetal stomach was studied in organ culture.[20] Overall, cell division decreased after 12 to 17 weeks gestation and became concentrated in the neck region. Unlike the mature human stomach, the fetal stomach can grow in serum-free media, suggesting that the fetal stomach contains all the growth factors necessary for growth.

Other studies have confirmed that all the epithelial cells of the gastric gland (mucous, parietal, chief or zymogenic, and so on) arise from stem cells in the neck region. A stem cell in the neck gives rise to prepit or premucous, preparietal, and prezymogen cells.[11-13] These premucous cells then migrate up the gland to the surface, where they eventually are shed into the lumen. The prezymogen cells migrate down into the base of the gland, where they differentiate into pepsin-secreting chief cells and eventually undergo apoptosis. These cells also secrete intrinsic factor in rodents but not humans. Preparietal cells differentiate in the neck and migrate only a very short distance into the large, acid-secreting mature parietal cell. Transgenic experiments confirm that all three cell types are monoclonal and thus arise from a common precursor.[21] Parietal cell ablation experiments demonstrate a block in the differentiation of the other two cell types, suggesting that a balance between the cell types is maintained by interactions among the cells.[22]

REGULATION OF ACID SECRETION IN ADULTS

Since the discovery of the acidic nature of gastric secretions by Beaumont in 1826, investigators have sought to determine the regulation of their release in humans.[23] It is now known that the parietal cell uses H^+/K^+-ATPase to secrete hydrochloric acid into the lumen of the stomach. Furthermore, several stimuli regulate acid secretion in mammals. These include neurogenic input (primarily via the vagus) and endocrine or paracrine effects from somatostatin-producing D cells, gastrin-producing G cells, histamine-containing enterochromaffin-like (ECL) cells, and other peptide hormones. An overview of the regulation of gastric acid secretion is necessary to understand the developmental changes seen in humans and animal models (Figure 111-2). It should be noted that although these regulatory mechanisms are seen in a wide variety of mammalian species, the relative contribution of individual components has intraspecies variation. Thus animal models may not entirely reflect human physiology. Moreover, it has also been shown that the contribution of these inputs can vary during development.

As shown in Figure 111-2, the regulation of gastric acid secretion is quite complex. Excellent reviews of this topic have been published elsewhere, but an overview is provided here.[24] As noted by Beaumont and Pavlov,[23,25] the central nervous system during the cephalic phase of gastric acid secretion can stimulate acid secretion via specific sites in the medulla and the vagus nerve.[26,27] The predominant vagal effect is mediated by acetylcholine released from postganglionic fibers and muscarinic receptors. Acetylcholine has multiple effects, including a direct stimulatory effect on the parietal cell. Acetylcholine also stimulates the release of somatostatin from D cells, which provides a brake on acid secretion via multiple pathways. In addition to acetylcholine, gastrin-releasing peptide (GRP) is secreted from postganglionic vagal nerve fibers and directly stimulates gastrin release[28] via GRP receptors on G cells.[29,30] Vasoactive intestinal polypeptide (VIP) is also released from vagal postganglionic fibers, but, unlike GRP, VIP does not have a direct effect on the G cell. VIP stimulates somatostatin release[31] from D cells, which, in turn, inhibits gastrin secretion. Other candidate peptides regulating the cephalic phase of acid secretion include calcitonin gene–related peptide and corticotropin-releasing hormone.

During the gastric phase of a meal, antral distention evokes low levels of gastrin release that is inhibited by low doses of the muscarinic antagonist atropine but enhanced by atropine at higher doses.[32] Other studies show that low-dose atropine depresses gastrin release in response to feeding in both intact and vagotomized dogs, suggesting that this effect is mediated by local neural reflexes.[33] Gastrin is also released by the presence of food in

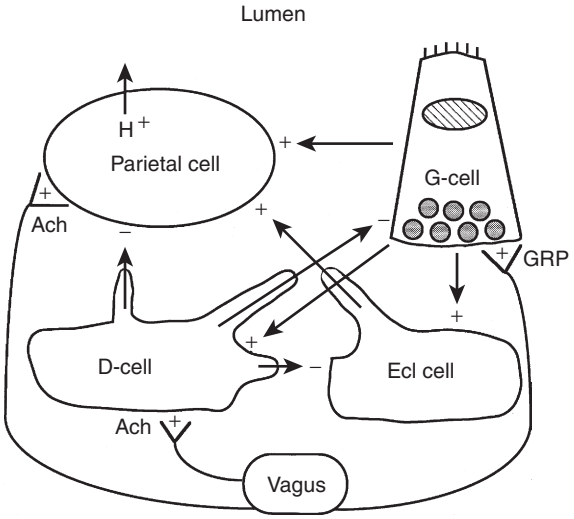


Figure 111-2 Regulation of gastric acid secretion. See also text. The acid-secreting parietal cell is regulated by inputs from the vagus nerve and three main cell types found in gastric mucosa: gastrin-secreting G cells, somatostatin-secreting D cells, and ECL cells that secrete histamine. Vagal postganglionic fibers release acetylcholine *(ACh)* that directly stimulates acid secretion from parietal cells and somatostatin release from D cells. Other vagal GRP-containing fibers stimulate gastrin secretion from G cells. Gastrin is posttranslationally processed to its biologically active forms within secretory granules that release their contents into the circulation. Increases in plasma gastrin enhance acid secretion from parietal cells, histamine release from ECL cells, and somatostatin release from D cells. Unlike gastrin, which acts in an endocrine fashion, somatostatin and histamine are released from D and ECL cells in a paracrine manner. Histamine stimulates acid secretion from parietal cells, whereas somatostatin inhibits secretion of acid from parietal cells and inhibits the release of gastrin from G cells and histamine from ECL cells.

the stomach. Neurotoxins, such as tetrodotoxin, GRP antagonists, and atropine, abolish both stimulation of gastrin release and inhibition of somatostatin release, indicating that peptone-induced gastrin release is mediated, in part, by both cholinergic and GRP neurons. However, other groups have shown that amino acids and amines also stimulate gastrin release from isolated canine G cells.[34] Circulating gastrin is the principal mediator of postprandial gastric acid secretion in adult humans.[35] Gastrin stimulates acid secretion by a direct effect on the parietal cell or by stimulating the release of histamine from ECL cells, which, in turn, enhances acid secretion.

The entry of chyme into the small intestine initiates the intestinal phase of the acid secretory process. The primary stimulatory factors in the small bowel are distention and the products of protein digestion, and these contribute relatively little to overall gastric acid secretion. The presence of food, especially fat, does initiate several inhibitory mechanisms. Duodenal fat releases intestinal cholecystokinin (CCK), which stimulates the release of somatostatin from gastric D cells.[36] Somatostatin has several inhibitory effects on gastric acid secretion, including suppression of release of gastrin from G cells, histamine release from ECL cells, and acid secretion from parietal cells. Although many other peptides released during the intestinal phase of digestion can inhibit gastric acid secretion, it appears that many of their effects are mediated by gastric D cells.[37] As the stomach empties, the continued secretion of acid is no longer buffered by the presence of food, and luminal pH drops. This lower pH has no direct effects on the G cell, parietal cell, or ECL cell but does cause a release of somatostatin from D cells to provide an inhibitory feedback loop for acid secretion.

NEONATAL PARIETAL CELL SECRETION

At birth, the gastric pH is greater than 2, probably because of the alkaline nature of swallowed amniotic fluid.[38,39] However, by several hours of age the stomach begins to secrete acid, and the pH falls. Unfortunately, the amount of acid produced in the human neonatal stomach has been difficult to determine because many studies have not included important parameters to quantify acid secretion accurately, such as body weight.[38,39] Most studies agree that acid secretion increases from birth to day 10 and then declines again over the next 20 days.[40,41] Acid production then slowly increases toward adult levels by about 3 months of age.[42] To examine gastric acid secretion more thoroughly, investigators have examined acid secretion in the absence (basal acid output [BAO]) or presence of secretagogues such as gastrin (maximal acid output [MAO]). As with previous studies, Euler and associates[43,44] demonstrated that the BAO of term infants was very low during the first 5 hours of life when compared with basal values of infants 4 to 14 hours of age. Basal acid secretion then increased in the infants from 6 to 8 hours of life. It is interesting that administration of pentagastrin did not increase acid production over basal levels. The investigators concluded that either acid secretion was already at maximal levels or the acid secretory apparatus was insensitive to gastrin at this stage of development. Euler and associates found that infants had elevated levels of serum gastrin that did not fall despite the increase in acid secretion. This suggested that the normal inhibitory effect of acid on gastrin release mediated by somatostatin was also not fully functional. Recent studies in neonates demonstrate that the proton pump H^+,K^+-ATPase is expressed at low levels as early as 25 weeks gestation. H^+,K^+-ATPase gene expression increases in concert with gastric acid secretion, gestational age, and postnatal age.[45]

Hyman and others investigated acid secretion in 34 healthy 7-day-old preterm infants of less than 37 weeks gestation and found that BAO was proportional to body weight but less than that of term infants.[46,47] BAO then gradually increased from 1 to 4 weeks of age and varied with postgestational but not postconceptual age, suggesting that extrauterine factors were involved. Hyman has suggested that feeding may play a primary role in this effect, but this has not been experimentally confirmed. Although there is no acid secretory response to pentagastrin in term infants at birth, preterm infants demonstrate this effect at 1 week of age. Unlike the effect of age on BAO, MAO in preterm infants increases with postgestational age and postconceptual age but remains far below that seen in older children and adults.[48-50]

To investigate the acid secretory capacity of the neonatal stomach in response to other stimuli, investigators examined MAO in response to the histamine analogue betazole.[51] They noted that BAO was low within the first 24 hours of life but that there was a twofold to threefold increase in acid output in response to betazole, which is consistent with findings in other studies. Betazole-stimulated MAO, however, was much lower than that seen in older infants, suggesting that the acid secretory apparatus was not functionally mature at this early stage in development.

MECHANISMS FOR DIMINISHED PARIETAL CELL FUNCTION IN THE NEONATE: ANIMAL MODELS

To explore the mechanisms that regulate the development of gastric acid secretory capacity, investigators have turned to animal models to examine each of the individual components in the

acid secretory apparatus. In human neonates, BAO is quite low at birth but rises shortly thereafter. Moreover, the acid secretory response is sensitive to meals but not to exogenous gastrin stimulation despite high plasma gastrin levels. Johnson[52] has extensively studied the development of acid secretion in the rat and noted a similar pattern in the ontogeny of gastric acid secretion. One theme common to all animal models is that the development of gastric acid secretion depends on the maturation of the parietal cell and those cells important for the regulation of acid secretion (outlined earlier).

PARIETAL CELL

In the mouse, gastric glands are first seen on fetal day 18, at which time there is no ultrastructural evidence of distinct parietal cells or alpha or beta H^+,K^+-ATPase subunit expression.[53,54] On fetal day 19 in the mouse, parietal cells can be identified, and both subunits of H^+,K^+-ATPase are now associated with apical membranes. Marino and colleagues[18] examined H^+,K^+-ATPase expression as well as expression of a second enzyme, carbonic anhydrase II, that generates a H^+ within the parietal cell in the rat. They noted that H^+,K^+-ATPase expression was present in 1-week-old pups, slowly increased over the next 7 weeks of development, and was limited to parietal cells. Conversely, carbonic anhydrase II was expressed in many cell types within the gastric mucosa at high levels shortly after birth. Carbonic anhydrase expression then declined slowly over the next several weeks but maintained high levels of expression in parietal cells. Hervatin and colleagues[55] examined H^+,K^+-ATPase activity in the developing rat and detected enzymatic activity on fetal day 19 although most of the enzyme was in the inactive state. H^+,K^+-ATPase activity rose from fetal day 19 to birth and then fell to almost zero by day 12 of life. Activity then rose over the next several weeks to adult levels, and the enzyme was found primarily in the active state. Other studies in the mouse[53,54] and rabbit[56] also detect the presence of functional H^+,K^+-ATPase early in gestation. Unlike in the rat, basal acid secretion in pigs is present at birth, but there is little response to secretagogues until 1 week of age.[57] In summary, it appears that the parietal cells are present at birth and contain the machinery necessary to secrete acid, albeit at diminished levels when compared with mature parietal cells.

Other investigators have examined the ability of the parietal cell to respond to gastrin, histamine, and acetylcholine. The receptors and postreceptor mechanisms for these secretagogues appear to develop with age; however, their patterns of development are both cell and species specific.[58-62] Despite the presence of parietal cells and functional H^+,K^+-ATPase at birth, basal acid secretion is very low at birth.[58,59] In the adult, basal acid secretion is controlled largely by vagal cholinergic inputs. Thus the low level of basal acid secretion suggests that these animals lack muscarinic receptors shortly after birth. However, muscarinic receptors are found at 70% to 80% of adult levels from birth onward and are functional, in that they can stimulate the release of pepsin from chief cells.[58] Investigators have hypothesized that this may reflect a low level of receptors on parietal cells or a failure of the parietal cell to develop postreceptor mechanisms that mediate the response. It is interesting that histamine and gastrin do not stimulate acid or pepsin secretion until day 20 of life.[59] Nevertheless, in neonates, protein hydrolysate formulas can stimulate gastric acid secretion twofold via unknown mechanisms.[63] Injection of corticosterone into 8-day-old pups before the natural steroid burst seen at weaning around day 21 prematurely induces basal acid secretion and the acid secretory response to all three secretagogues.

In other studies in the developing rat, investigators measured the binding of gastrin to its receptor and found no specific binding until day 20 of life; the receptor number was increased by treatment with corticosteroids.[64-66] The parietal cell response to histamine is more variable in its time of presentation but is documented to occur between 14 and 20 days of life in rats.[51,59] Although investigators can describe the presence of receptors in developing gastric mucosa and note the effects of secretagogues on acid secretion, it has been very difficult to examine postreceptor mechanisms in the developing animal. To determine that the changes in second messengers reflect changes in a particular cell type requires the use of isolated cell systems and large quantities of tissue. Thus this important area remains relatively unexplored.

INTRINSIC FACTOR SECRETION FROM PARIETAL CELLS

In humans, the parietal cell secretes intrinsic factor,[67] and investigators have examined its ontogeny to gain insight into the development of the parietal cell. Marino and associates[47] found that basal intrinsic factor production was similar in term and preterm infants. Conversely, term infants had much higher gastric acid secretion than preterm infants, suggesting that the development of these processes in human parietal cells was not linked. Other investigators have examined histamine-stimulated intrinsic factor secretion and noted low levels at birth that increase to normal adult values by 2 weeks of age.[51] Again, a dissociation was seen between the maturation of intrinsic factor secretion and gastric acid secretion—histamine-stimulated acid secretion did not reach adult levels even at 2 to 3 months of age. In the rat, intrinsic factor is a product of the chief cell, and thus it is difficult to use the rat as a suitable model for human development.[67,68]

G-CELL FUNCTION

Gastrin-producing G cells can be detected in the human fetus as early as 12 weeks of gestation.[69] It is interesting that most of these early G cells also express thyroid-releasing hormone (TRH), and the number of TRH-positive G cells increases to reach a maximum between 26 and 36 weeks gestation.[69] Thereafter, the number of TRH-positive G cells decreases, and none are found in the mature human stomach. Moreover, there is no known physiologic role for TRH in the developing stomach, and TRH is not found in G cells of other developing animals. In the rat, gastrin immunoreactivity is detected in the pancreatic islets before it is seen in the gastric antrum.[70] As fetal development proceeds, gastrin expression increases in the antrum and decreases in the islets. It appears that fetal pancreatic gastrin, in combination with transforming growth factor α, plays an important role in islet neogenesis.[71]

Human plasma gastrin levels in the neonate are approximately three times higher than those found in adults and pregnant women.[42] Unfortunately, the physiologic significance of neonatal hypergastrinemia is not known. It has been suggested that these elevated levels may play a role in the growth and development of the gastrointestinal tract.[72] To explore the role of gastrin in gut development, two groups have developed gastrin-deficient mice.[73-75] These mice are viable and develop normally with normal weight gain. This suggests that gastrin is not a vital hormone. However, both basal and stimulated gastric acid secretions are severely impaired in homozygous animals. Furthermore, there is a reduction in gastric mucosal thickness, with reductions in all three cell types. Although CCK can bind to the gastrin receptor, CCK does not substitute for gastrin because both gastrin-deficient and gastrin receptor–deficient mice have a similar gastric phenotype.[76]

Antral gastrin content increases in the developing rat until weaning. Early weaning or administration of corticosteroids or thyroid hormone increases antral gastrin content and promotes

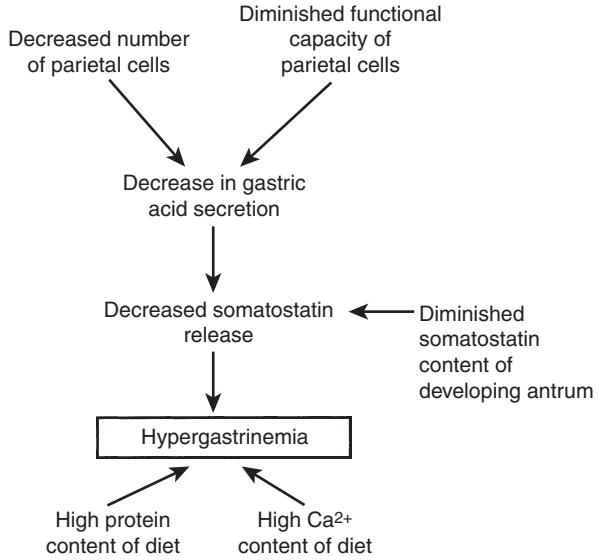


Figure 111-3 Mechanism(s) of neonatal hypergastrinemia.

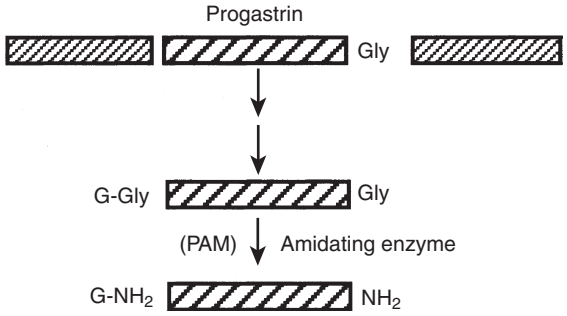


Figure 111-4 Progastrin posttranslational processing. Progastrin *(top)* is sequentially processed to a glycine-extended form of gastrin *(G-Gly).* G-Gly then serves as a substrate for the amidation enzyme *(PAM),* which catalyzes the formation of amidated gastrin *(G-NH_2).* The C-terminal amide moiety is necessary for G-NH_2 binding to the gastrin-CCK_B receptor. Developing tissues often contain more G-Gly than G-NH_2 before maturation of PAM.

the maturation of the gastric mucosal gastrin receptors.[52,55,65] It is unknown whether a similar phenomenon also occurs in the human stomach with a change in diet, but human plasma gastrin levels do remain elevated for several months after birth while infants are primarily milk fed.[77] The cause of neonatal hypergastrinemia is multifactorial, and the rat seems to provide a suitable model for study (Figure 111-3). A milk-based diet that is high in known gastrin secretagogues such as amino acids and calcium[78] may account for some of the hypergastrinemia. In both rats and humans there is diminished gastric acid secretion because of a diminished number of functional parietal cells. In mature humans this inhibits somatostatin secretion, which increases gastrin secretion. Consistent with this is the finding that the somatostatin content of the rat gastric antrum is low at birth and increases gradually until weaning at around day 21.[79] Moreover, infusion of somatostatin does not inhibit gastrin release in newborn rat pups until day 18 of life, suggesting that somatostatin receptor or postreceptor mechanisms are functionally mature until weaning.[80] The combined effect of low somatostatin content and diminished response to exogenous somatostatin before weaning suggests that somatostatin plays a major role in neonatal hypergastrinemia.

The exact nature of the role that gastrin plays in neonatal gastric acid secretion also is not fully elucidated. For example, gastrin is the primary mediator of gastric acid secretion during a meal in mature humans.[35] However, it has been unclear whether this is a direct effect on the parietal cell, because H_2 receptor antagonists block much of this response. This suggests that gastrin's effects on acid secretion are mediated via histamine release from ECL cells. In other species, it appears that gastrin has a more direct effect on the parietal cell. In the developing rat, gastrin-stimulated acid secretion is not blocked by H_2 receptor antagonists but is partially attenuated in mature animals.[81] It is currently unknown whether gastrin exerts its effects directly on the parietal cell of human infants or whether its effects are mediated by histamine release.

Gastrin, as is the case with other regulatory neuropeptides, is initially synthesized as a large, biologically inactive precursor termed *progastrin* (Figure 111-4). Endoproteolytic processing of progastrin within G cells results in the formation of a glycine-extended form of gastrin (G-Gly) that is found in high concentrations in the developing rat gastric antrum.[82] G-Gly serves as a substrate for the amidation enzyme, peptidylglycine α-amidating monooxygenase, that catalyzes the formation of fully processed, mature, amidated gastrin.[83] Gastrin (G-NH_2) requires its carboxyl-terminal amide moiety for full biologic activity mediated by gastrin-CCK_B receptors. Indeed, removal of the carboxyl-terminal amide in gastrin completely abolishes its acid stimulatory effects mediated by standard gastrin-CCK_B receptors, and the immediate precursor of amidated gastrins, G-Gly, is at least four orders of magnitude less potent than G-NH_2 in stimulating acid secretion from gastric parietal cells.[84] Nevertheless, interest in the physiologic effects of G-Gly has been fueled by the observations that G-Gly is stored in brain and gut tissues,[85] is secreted with G-NH_2 from antral G cells into the circulation, and achieves concentrations in plasma roughly equivalent to those of G-NH_2.[86] Moreover, G-Gly is seen in greater concentrations than G-NH_2 during development and in some malignant tissues that express gastrin, such as Zollinger-Ellison tumors and colon cancers.[87,88] Finally, biosynthetic studies suggested that G-Gly may be a distinct end-product of progastrin processing.[89] Thus the evidence pointed to G-Gly's role as a growth factor but not as a direct acid secretagogue.

To further characterize the potential trophic effects of G-Gly, we examined whether G-Gly might function as a growth factor in a fashion that could be distinguished from its relatively weak effects on the standard gastrin-CCK_B receptor.[84] We compared the abilities of G-NH_2 and G-Gly to stimulate DNA synthesis.[90] Both G-NH_2 and G-Gly stimulated [^{3}H]thymidine incorporation in a dose-dependent fashion. As expected, the stimulation induced by G-NH_2 was completely reversed by selective gastrin-CCK_B receptor antagonists. In further studies, we characterized a distinct receptor and signaling cascade for G-Gly.[91] However, the inability of investigators to isolate a complementary DNA encoding the putative G-Gly receptor has hampered further study in this area. Other data help confirm the role that G-Gly may play in gastrointestinal physiology. G-Gly can stimulate growth in an autocrine fashion.[92] Although acute administration of G-Gly has no effect on gastric acid secretion,[84,93] chronic administration of G-Gly markedly enhances stimulated but not basal acid secretion from isolated parietal cells and in vivo. This action of G-Gly is mediated via an increase in the expression of H^+,K^+-ATPase within gastric parietal cells.[94] G-Gly may play a role in the growth of the colon.[95,96] Indeed, gastrin-deficient mice have a diminished colonic proliferative index, and transgenic mice overexpressing G-Gly have enhanced colonic proliferation.[97] These observations suggest that growth-related receptors for G-Gly work in concert with G-NH_2 to enhance the functional development of the gut (Figure 111-5).

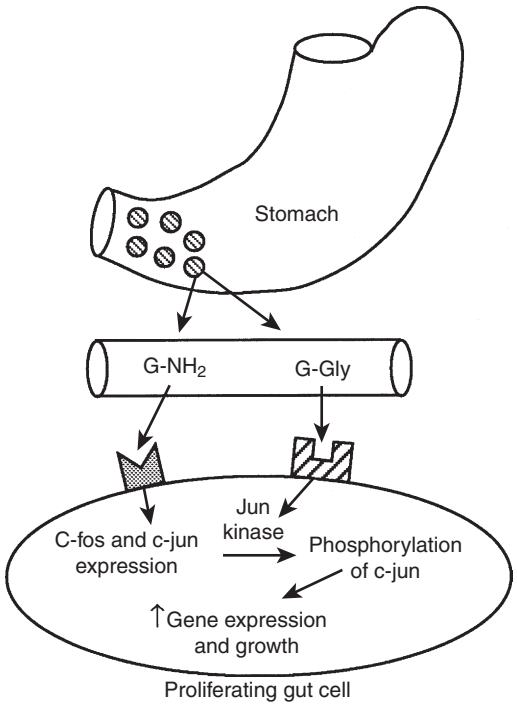


Figure 111-5 Mechanisms of G-NH_2 and G-Gly trophic effects. Amidated gastrin *(G-NH_2)* and its glycine-extended precursor *(G-Gly)* are released from G cells in the gastric antrum into the circulation. G-NH_2 acts through gastrin-CCK_B receptors to enhance the expression of c-fos and c-jun. G-Gly, via another distinct receptor and jun kinase, phosphorylates and thus bioactivates c-jun. Then c-fos and phosphorylated c-jun induce the expression of other genes necessary for the proliferation of gut cells.

D-CELL FUNCTION

In the fetal human, somatostatin has been found as early as 8 weeks gestation.[98,99] However, unlike gastrin, somatostatin exerts its effects in a paracrine manner, and therefore the release of gastric somatostatin may not result in an increase in plasma levels. Thus it has been difficult to describe carefully the development of gastric D-cell function in humans.

Gastric D-cell development, however, has been studied in animal models. In the rat, antral G cells can be detected at day 19 of gestation, but D cells are not clearly identified until day 4 of life.[79,100,101] Gastric somatostatin concentrations increase slowly from birth to weaning at day 21.[79] At day 21, there is a marked increase in gastric acid secretion, which is followed by an abrupt increase in gastric somatostatin content on day 24 that is more marked in the antrum than in the fundus.[79] Others have noted similar changes in somatostatin gene expression,[102] suggesting that peptide content is closely linked to gene expression. As is the case with the development of G-cell function, diet and glucocorticoids play a similar role in the development of D-cell content.[100,103] Rabbits and sheep are similar to humans in that immunoreactive somatostatin can be detected during fetal development although at levels much lower than that seen in mature animals.[104,105] The rabbit stomach also demonstrates a marked reduction in somatostatin and an increase in gastrin content in the third trimester that coincides with the development of acid secretion.[104]

To explore the effect of somatostatin on gastric development, investigators created a mouse deficient in the somatostatin receptor subtype found in the stomach (SSTR-2).[106] SSTR-2 mice developed and gained weight normally but have a marked increase in gastric acid secretion that was abolished by an antigastrin antibody. This is similar to the effect seen in humans overexpressing gastrin and confirms the inhibitory role of somatostatin on gastrin release and gastric acid secretion.

ENTEROCHROMAFFIN-LIKE CELLS

Few data exist concerning histamine-secreting ECL cells during development. Histamine is synthesized from histidine via the action of histidine decarboxylase (HDC). HDC activity is first seen in the developing rat at day 10 of life.[59] Gastrin stimulation of HDC activity is first noted on day 16 of life.[59] However, other investigators can detect the presence of immunoreactive histamine on embryonic day 16 in the rat gastric muscular layer and on embryonic day 18 within the mucosa.[107] From embryonic day 18 the number of histamine-positive cells increases, and at birth many of these have the paracrine-like processes that are characteristic of mature ECL cells. The number of mature ECL cells increases from birth to weaning, when their numbers and distribution are similar to those seen in a mature animal.[107] Because mature ECL cells express gastrin-CCK_B receptors, it is likely that the diminished number of gastrin receptors found in the developing rat reflect a loss of receptors not only on the parietal cell but also on ECL cells.[52,55,65] This has not been tested experimentally, but, because gastrin is an important regulator of histamine release from ECL cells, it suggests that histamine secretion might also be diminished.

Histamine's effects on gastric acid secretion are mediated via histamine2 receptors. As was the case for gastrin- and SSTR-2–deficient mice, histamine2 receptor–deficient mice are viable and grow normally.[106] However, unlike the gastrin-deficient mice, these mice have unexplained normal gastric acid secretion. They do have an elevated serum gastrin level and an increase in gastric mucosal thickness that contains increased numbers of small parietal cells.

SUMMARY OF REGULATION OF GASTRIC ACID SECRETION IN DEVELOPMENT

From the various human and animal studies, it appears that at birth gastric acid secretion from parietal cells is diminished (see Figure 111-3). The reasons for this include a decrease in the number of cell surface receptors and a diminished number of fully functional parietal cells.[52] This lack of acid production results in a decrease in somatostatin release that further increases gastrin secretion. One clinical implication of these observations is that, because gastric acid secretion is requisite to the formation of duodenal ulcers, one would expect to see a low incidence of this condition in neonates. Moreover, the lack of acid production contributes to the relatively low incidence of erosive esophagitis in infants with gastroesophageal reflux.

CHIEF CELL FUNCTION

In humans, the primary function of the chief cell is to secrete the proteolytic enzyme pepsin. Pepsin is packaged and stored in the chief cell within secretory granules in an inactive or zymogen (pepsinogen) form. Immunoreactive pepsinogen can be detected in the human fetus by 8 weeks of gestation,[108] and chief cells can be identified by the end of the 13th week.[3] Peptic activity can be seen in the fetal stomach by 16 weeks of gestation.[4] Peptic activity increases thereafter, and thus premature infants weighing 1 kg have approximately half the gastric peptic activity of term infants.[109] There are several different forms of pepsinogen in humans, but the primary type is pepsinogen IV.[108] Pepsin is the primary enzyme responsible for gastric proteolytic activity in humans; however, other species secrete proteolytic enzymes, such as chymosin, that are not found in humans.[110] Thus it is difficult to use animal models to examine the total gastric proteolytic activity for insight into human development.

Like gastric acid secretion, the secretion of pepsinogen appears to be diminished in the rat pup before weaning.[111] The rat chief cell response to carbachol is present at birth, but the amount of pepsinogen secreted is very low.[111] As is the case with the development of gastric acid secretion, the development of pepsinogen secretion is sensitive to corticosteroids.[112] Although thyroxine has a similar effect, this effect is mediated by an increase in endogenous glucocorticoid levels.[111]

GASTRIC LIPASE

Although not generally thought of as an organ responsible for fat absorption, the neonatal stomach secretes a lipase distinct from that found in the pancreas. It is not surprising that gastric lipase has a lower pH optimum than does pancreatic lipase.[113-115] Moreover, gastric lipase is somewhat more important in the neonate than in the adult because of the relative pancreatic insufficiency seen in newborn infants.[116] Although lingual lipase is the major source of gastric lipolytic activity in the rat,[117] it appears that there is little lingual lipase in humans.[118] Human gastric lipase is synthesized in gastric fundic chief cells and cosecreted with pepsinogen.[119,120] In humans, gastric lipase appears as early as 10 to 13 weeks after conception, consistent with the development of early gastric glands.[121] In preterm infants, gastric lipolytic activity is low at 24 to 26 weeks gestation but increases to reach a peak around 32 weeks gestation.[122] Thereafter, gastric lipase activity slowly declines until term and remains level throughout infancy.[122,123] In adults, lipase secretion is stimulated by agents such as gastrin that promote pepsin secretion from chief cells, but this has not been studied in human neonates.

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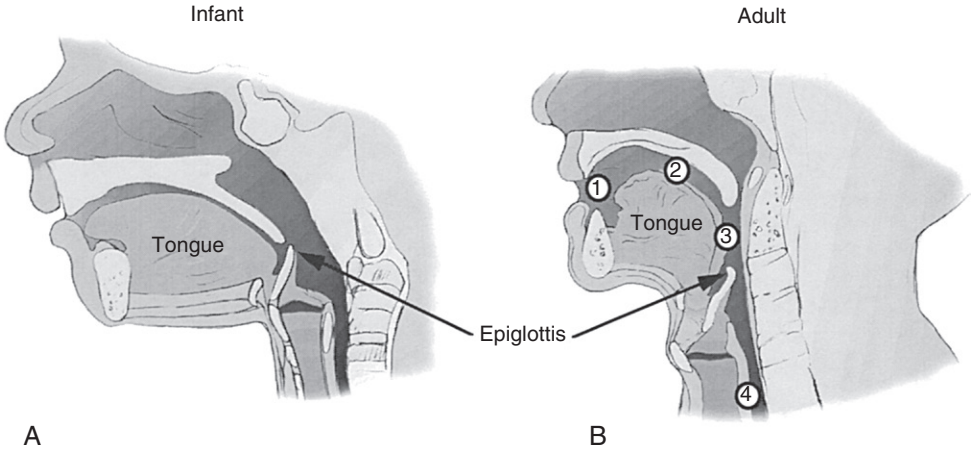


Figure 112-1 Comparative diagrams of the anatomy of the mouth and pharynx of neonates (A) and adults (B). Note the reduction in the relative size of the pharyngeal chamber and overlap between the soft palate and the epiglottis. The numbers on the figure refer to the phases of swallowing in the adult. *1,* Preparatory; *2,* oral; *3,* pharyngeal; *4,* esophageal. *(From Rudolph CD: Diagnosis and management of children with feeding disorders. In Hyman PE, Di Lorenzo C, editors:* Pediatric Gastrointestinal Motility Disorders. *New York, Academy Professional Information Services, 1994, p 37.)*



Figure 112-2 Mechanism of sucking from a nipple. Note that the lips seal the anterior oral cavity and the tongue seals against the palate to close the posterior opening of the oral cavity. The tongue and jaw descend with sucking, creating a negative intraoral pressure. *(From Bu'Lock F, Woolridge MW, Baum JD: Development of coordination of sucking, swallowing and breathing,* Dev Med Clin Neurol *32:669, 1990.)*

tongue movements develop that move a food bolus from the front of the tongue back toward the pharynx. At 6 months, infants will occlude their lips to remove pureed foods from a spoon and will move the food toward the midline with their tongue for chewing. As alveolar ridges and teeth develop over the next year, the infant develops increased tongue mobility that allows lateralization of solid foods for mature mastication.[10,11]

Pharyngeal Swallowing

The exquisite pattern of muscular contractions that produce the swallowing action is centrally integrated by two regionally distinct groups of medullary interneurons, the nucleus tractus solitarius (ventrally located) and the nucleus ambiguus (dorsally located), which form the "swallowing center." The nucleus tractus solitarius receives inputs from cortical fibers and peripheral sensory fibers originating in the pharynx, larynx, and esophagus. The nucleus tractus solitarius acts as a "pattern generator" and precisely regulates the sequence of motor nuclei excitation that leads to pharyngeal and esophageal contraction. The nucleus ambiguus receives some sensory input but predominantly acts as a relay station for the sequential activation of motor neurons responsible for pharyngeal and esophageal contraction and UES and LES relaxation.[12,13]

Swallowing reflexes are triggered when the contents of the oral cavity are propelled posteriorly toward the pharynx. This is achieved via highly complex contractions of the longitudinal and transverse intrinsic lingual musculature of the tongue.[1] Swallowing is initiated by distal elevation of the tongue followed by a posteriorly propagated peristaltic-like wave along the medial portion of the tongue.[3,14] Abnormal tongue movements such as incomplete peristaltic contractions and tremulous contractions are frequent in premature infants and diminish as they approach term.[3] If these poorly coordinated lingual movements fail to clear the oral cavity of its contents, the residue may overflow into the oropharynx when the airway is not protected, leading to aspiration.

As the oral contents enter the pharynx, a pharyngeal swallow is triggered. In term infants the time delay between initial suck and pharyngeal contraction is 500 milliseconds,[15] suck-oral transit time is 900 milliseconds, and pharyngeal transit time is 600 milliseconds.[4] The phasic relationship between sucking and swallowing is not constant; preterm and newborn infants demonstrate one to four sucks per swallow[3,5,15] whereas older term infants demonstrate one to two sucks per swallow.[3,4] During pharyngeal swallowing the velum and laryngeal musculature elevate, closing the nasopharynx and epiglottis and forming a conduit for passage of the milk bolus through the pharyngoesophageal junction. In adults, contraction of pharyngeal musculature does not actively propel the food bolus. Instead, the pressure wave associated with pharyngeal contraction occurs after the bolus has passed and functions predominantly to clear the pharynx of food residues.[16] Uncoordinated or low-amplitude pharyngeal contraction may lead to the retention of feeding bolus residues in the pharynx.

Coordination of Swallowing With Breathing

In utero, the pharynx is a conduit for fluid. The human fetus ingests up to 750 mL of amniotic fluid per day.[17] Swallowing of amniotic fluid contributes to amniotic fluid homeostasis,[18,19] and

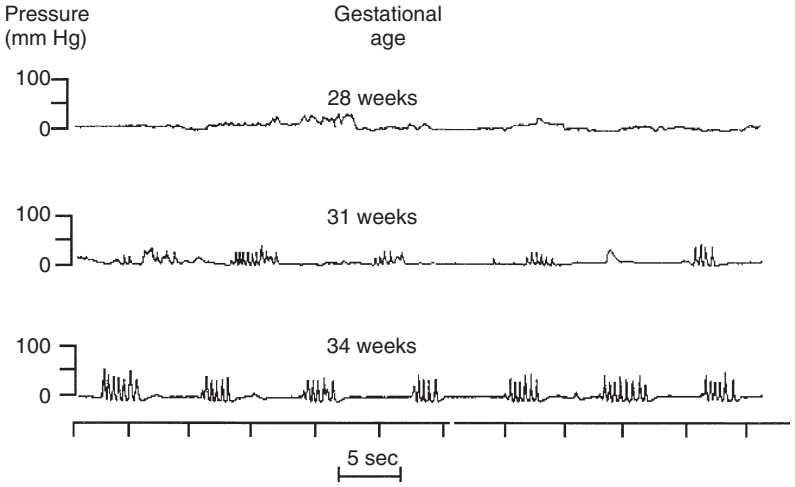


Figure 112-3 Intraoral pressure recordings of sucking patterns in premature infants during active sleep. Between 28 and 34 weeks post-menstrual age sucking develops from poorly coordinated low-amplitude sucks to higher-amplitude burst sucking. *(From Hack M, Estabrook MM, Robertson SS: Development of sucking rhythm in preterm infants,* Early Hum Dev *11:133-40, 1985).*

Stage	Sample Tracing	Suction/Expression Amplitude (mm Hg)	Description
1A	Suction Expression	Absent +0.5 to +1.0	No suction Arrhythmic expression
1B	Suction Expression	–2.5 to –12.5 +0.5 to +1.0	Arrhythmic alternation Suction/expression
2A	Suction Expression	Absent +0.2 to +4.0	No suction Rhythmic expression
2B	Suction Expression	–7.5 to –150 +0.2	Arrhythmic alternation Suction/expression
3A	Suction Expression	Absent +0.8 to +1.0	No suction Rhythmic expression • Suction amplitude increase • Wide amplitude range • Prolonged sucking bursts
3B	Suction Expression	–15 to –75 +0.5 to +0.7	Rhythmic suction/expression
4	Suction Expression	–50 to –75 +0.4 to 1.0	Rhythmic suction/expression • Suction well defined • Decrease amplitude range
5	Suction Expression	–110 to –160 +0.6 to +0.75	Rhythmic/well defined suction/expression • Suction amplitude increase • Sucking pattern similar to that of full term infant

A

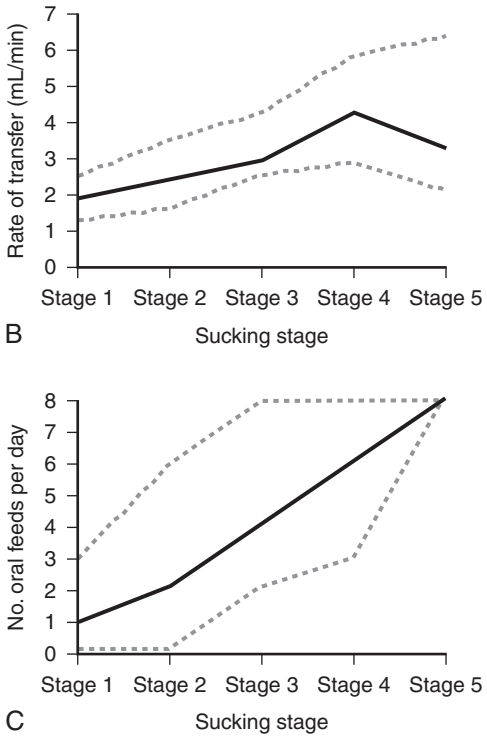


Figure 112-4 A, Stages of sucking characterized in preterm infants. **B,** Rate of milk transfer during feeding in infants at the different stages of sucking. **C,** Number of feedings per day at different stages of sucking. Data shown as median with *dotted lines* indicating the interquartile range. *(Modified from Lau C, Alagugurusamy R, Schanler RJ, et al: Characterization of the developmental stages of sucking in preterm infants during bottle feeding,* Acta Paediatr *89:846-52, 2000.)*

the swallowed fluid appears to be trophic for mucosal development of the GI tract.[20] After birth, however, the pharynx performs a dual function regulating the passage of food (into the esophagus) and air (into the larynx). As discussed above, anatomic differences between the newborn and adult larynx provide some protection of the airway in the neonate.[21] The laryngeal inlet is located high in the pharynx in the newborn at the level of cervical vertebrae C1-3. The tip of the epiglottis often extends into the nasopharynx, separating the respiratory passages and digestive conduit. During the 3rd year of life the larynx descends to the adult C3-5 location, creating a longer common passageway for the airway and digestive tracts in the oropharynx. This anatomic arrangement facilitates phonation but increases the risk of aspiration during swallowing. Although swallowing and respiration must be precisely controlled in the newborn, the prevention of aspiration becomes more difficult with growth.

Neonates possess an airway inhibitory mechanism that is triggered 200 milliseconds before pharyngeal swallowing and lasts approximately 600 milliseconds.[15] In mature term infants the ratio of swallowing to breathing is usually 1:1.[3] In contrast, newborn and preterm infants rarely synchronize breathing and swallowing. In the newborn, unstable breathing, prolonged inhibition, and depressed respiratory rate and minute volume occur frequently during periods of rapid swallowing.[5,15,22] The premature infant exhibits bursts of rapid swallowing interpolated with periods of rapid breathing.[3,23] Episodes of apnea, bradycardia, and oxygen desaturation are therefore common during burst swallowing.[23] The poor coordination of breathing and swallowing seen in premature and some newborn infants indicates that the interneuronal pathways between the medullary swallow and respiratory centers that synchronize respiratory inhibition are not fully functional.

ESOPHAGEAL MOTILITY

The motility of the esophagus, comprising the UES, LES, and the esophageal body, has now been well characterized in premature infants from 26 weeks gestation to term. The major features of esophageal motility are summarized in Figure 112-5.

Upper Esophageal Sphincter

UES pressure is generated predominantly by tonic contraction of the cricopharyngeus muscle. With swallowing, the cricopharyngeus muscle is inhibited, producing relaxation. Approximately 100 milliseconds after relaxation the UES is opened by the intrabolus pressure and the superior excursion of the hyoid and larynx.[24,25] Studies of term infants and children (2 months to 7 years) have demonstrated UES pressures comparable with those of adults (40-50 mm Hg) and UES relaxation in response to swallowing.[26-28] UES function has been investigated in premature infants at gestational ages as young as 33 weeks. In these infants, UES resting tone ranged from 2 to 28 mm Hg, and the UES was found to relax appropriately in response to dry swallow.[29] In addition, the magnitude of UES resting pressure is dependent greatly on the behavioral state with periods of apparent "comfort" associated with significantly lower UES pressures than periods of activity and apparent "discomfort" or abdominal straining.[29] These findings are consistent with effect of behavior and arousal reported in older children and adults.[26,30,31] Premature infants demonstrate a UES contractile reflex in response to esophageal distension with air or liquids.[32-35]

Esophageal Body

Swallowing initiates primary peristaltic esophageal contractions, which can be measured as pressure wave sequences propagated in an aborad direction along the length of the esophageal body. Primary esophageal peristalsis has been recorded in the human premature infant down to 26 weeks gestation[34-36] and term infants.[37] This peristalsis is essentially *normal;* however, the rate of propagation of the peristaltic wave is slower in younger premature infants.[34] Infusion of fluids and air into the esophageal lumen triggers reflex secondary peristalsis and reflex swallowing in the premature infants that is volume dependent.[32-35] Premature infants also exhibit esophageal body contractions that occur spontaneously and that are not triggered by the usual mechanisms (i.e., swallowing or esophageal distension). These spontaneous contractions are usually propagated in a nonperistaltic fashion (i.e., retrograde, synchronous, or incomplete propagation).[36-38] Similar spontaneous esophageal motor activity has been observed in adults, but these events are far less common and are usually associated with occurrence of the migrating motor complex of the small intestine.[39] It would appear that these motor patterns occur as a background to what appears to be normal, mature, primary and secondary peristalsis and therefore do not appear to impair esophageal function at least in the healthy infant. An example tracing illustrating swallow-related peristaltic and spontaneous nonperistaltic contractions is shown in Figure 112-5.

Lower Esophageal Sphincter

The LES functions as a physical esophagogastric antireflux barrier and comprises two sphincter mechanisms, the intrinsic smooth muscle sphincter and the crural diaphragm, which provides extrinsic support and squeeze. Both sphincter components work together and contribute to LES pressure. Early reports concluded that premature infants have poor LES tone because of immaturity of sphincter control mechanisms.[40-42] However, more recently it has been shown that in premature infants the LES generates tonic pressures that are sufficiently higher than intragastric pressure to maintain effective esophagogastric competence (5-10 mm Hg).[34-36,40,43] Contrary to early reports indicating that LES pressure increases with maturation,[43] more recent evaluations have shown no such pattern of maturation.[44] The LES relaxes with swallowing to allow passage of a food bolus (see Figure 112-5). This "swallow-related" relaxation lasts for 3 to 6 seconds during which the LES pressure drops to within 2 to 4 mm Hg of intragastric pressure. Swallow-related relaxation is well developed in the premature infant.[36-38] In addition to swallow-related relaxation, the LES exhibits transient LES relaxation (TLESR) (see later).

Sphincteric Mechanisms of Gastroesophageal Reflux

Gastroesophageal reflux (GER) results when stomach contents (liquid, gas, or liquid and gas in combination) are expelled into the esophageal lumen; this is a normal physiologic phenomenon that often occurs during or after feeding. Abnormally frequent GER, poor clearance of GER from the esophageal lumen, or exposure of GER to airway structures may lead to the manifestation of GER disease, which has a number of clinical presentations including feeding problems with or without weight loss, irritability, and obstructive apnea. GER is common in premature infants, and it was originally thought that this was related to poor LES competence resulting from immaturity of neural control mechanisms. As described previously, more recent studies in these infants have shown that mechanisms of sphincter competence are well developed and that GER usually occurs in association with complete sphincter relaxation. A number of motor mechanisms of GER triggering have been identified; these include predominantly swallow-related LES relaxation in association with failed peristalsis, prolonged inhibition of LES tone and esophageal contraction by multiple swallowing, and TLESR, which are triggered in the absence of swallowing[33] (Figure 112-6). TLESR is by far the most common mechanism of GER triggering (see Figure 112-6) and has been described in all age-groups from 26-week premature infants[45] through to adults.[46] Compared with normal swallow-related LES relaxations, TLESRs occur independently of pharyngeal swallowing, are prolonged in duration (>10 seconds), relax more completely (lower nadir), and are associated with inhibition of the esophageal body and crural diaphragm. Physiologically, TLESRs serve to vent gases from the stomach during belching to prevent GI bloating.[46,47] TLESRs are also stimulated in response to gastric distension after a meal, being most common in the early postprandial period and reducing in frequency after the first postprandial hour[48]; this is also the case in infants[49] (see Figure 112-6).

Neurophysiologically, TLESRs occur via a vagovagal reflex initiated by stretch-sensitive receptors located in the smooth muscle of the stomach wall, particularly the cardia of the stomach, which

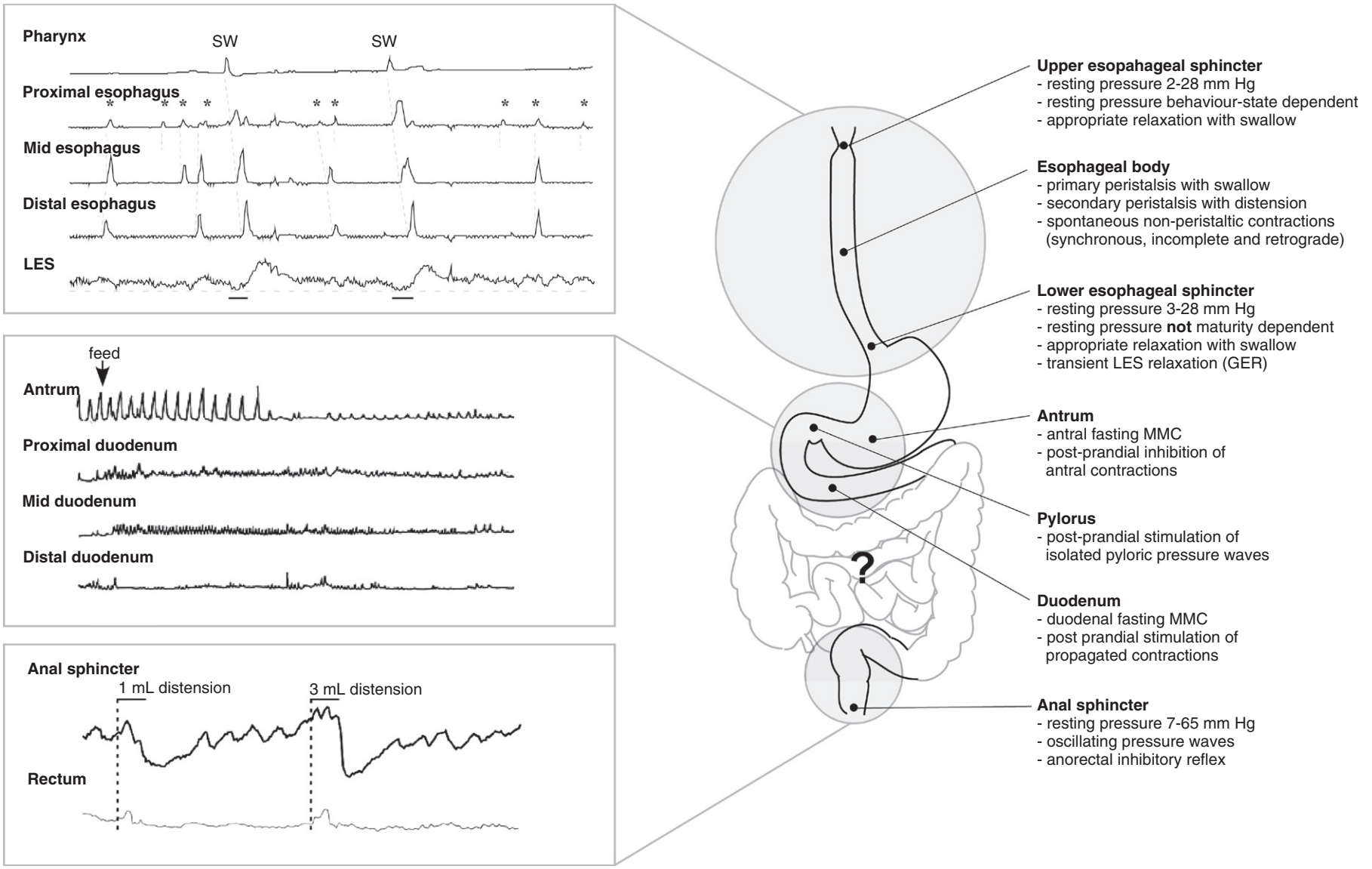
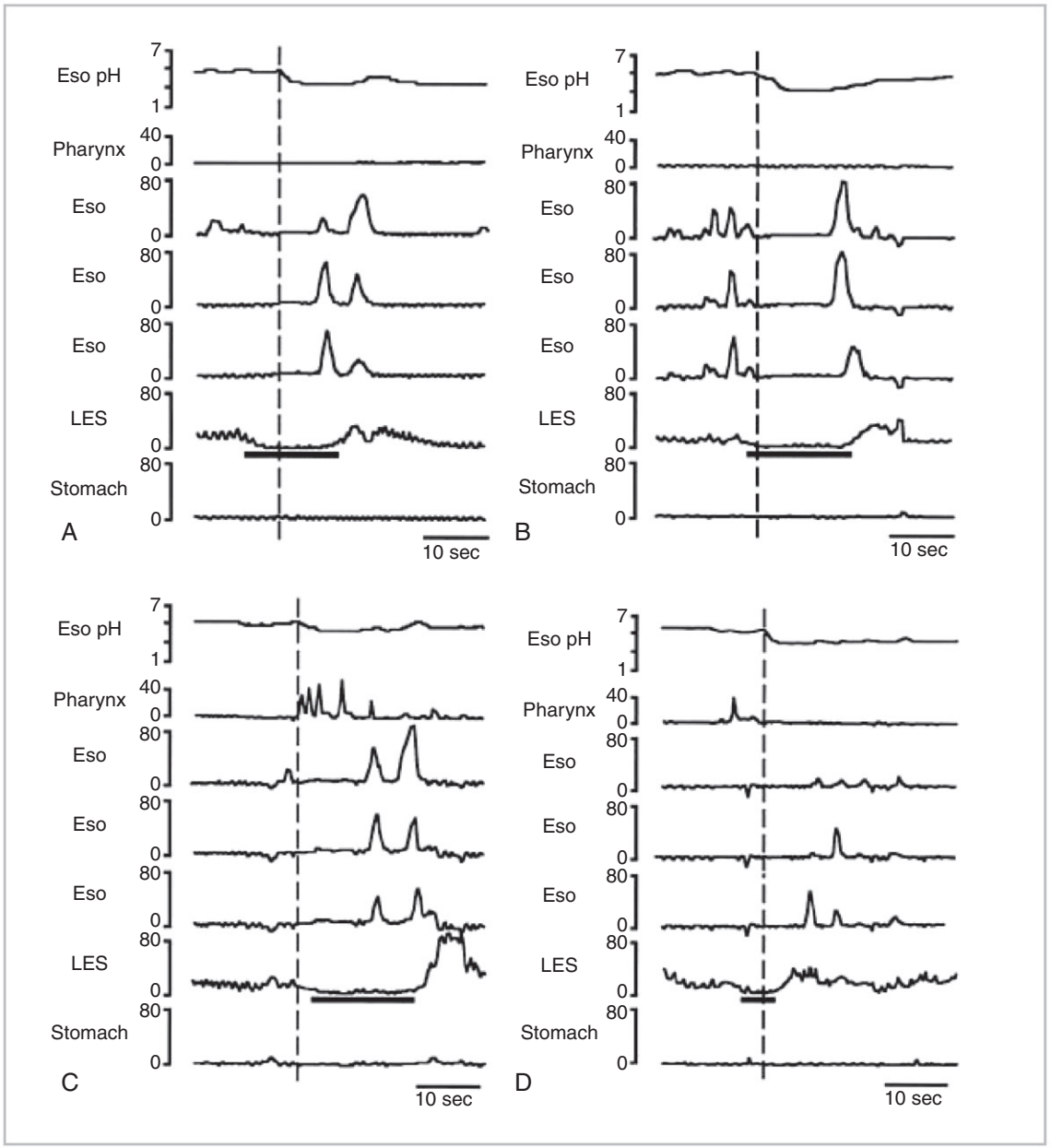
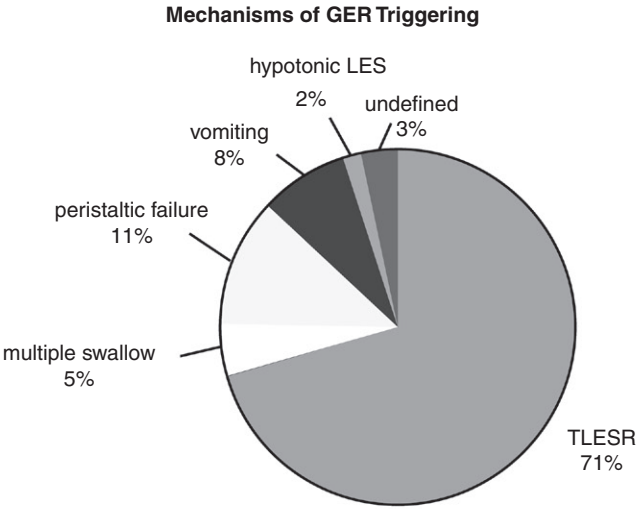


Figure 112-5 Patterns of GI motility described in the premature neonate and example tracings *(left)* of esophageal, antroduodenal, and anorectal motility recorded with perfusion manometry. *Top tracing,* Esophageal motility; two pharyngeal swallows *(sw)* trigger primary peristalsis, which is propagated down the length of the esophagus. The swallows also trigger LES relaxation *(black bars).* Spontaneous nonperistaltic contractions (incomplete and synchronous) are also shown *(asterisks). Middle tracing,* Antroduodenal response to feeding; feeding inhibits antral contraction and stimulates duodenal contraction. *Bottom tracing,* Anorectal motility; rectal balloon distension initiates reflex relaxation of the anal sphincter.





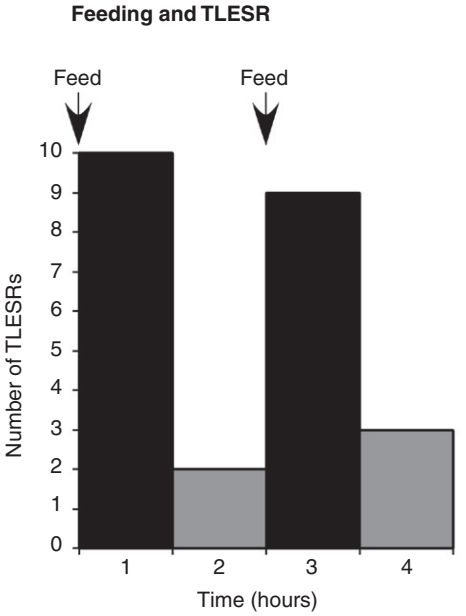


Figure 112-6 Mechanisms of acid GER in premature infants. Manometric tracings,TLESR (**A** and **B**), prolonged LES relaxation during multiple swallowing **(C),** and failed peristalsis during normal swallow-related LES relaxation **(D).** In all cases LES relaxation initiates acid GER, which is indicated by the drop in esophageal pH (onset marked by *dotted line*). *Pie chart,* The proportion of acid GER triggered by different mechanisms;TLESR is the predominant reflux mechanism and LES hypotonicity is the least common mechanism. *Graph,* The relationship between feeding and TLESRs; gastric distension by feeding resulting in an increased rate of TLESR via vagovagal mechanisms. Data from two hourly fed premature infants.

is the region most sensitive to TLESR triggering.[50] The stretch-sensitive sensory nerve fibers of the afferent arm of the reflex pathway terminate in the brain stem (nucleus tractus solitarius) and ultimately synapse with vagal motor neurones (dorsal motor nucleus of the vagus nerve and nucleus ambiguus) projecting to the LES, esophagus, pharynx, and crural diaphragm.[47] In premature infants TLESRs have the same characteristics (duration, nadir pressure) as those described in adults,[38] indicating that the basic integrative mechanisms underlying TLESR are well developed in these infants. Infants with reflux disease may have functional differences that pathologically increase the likelihood of liquid (rather than gas) reflux being triggered during a TLESR.[49] The mechanisms that precisely regulate the rate of triggering of TLESR, as well as the GER "type" triggered during TLESR, are complex and poorly understood.

GASTRIC MOTILITY

The motility of the stomach is less completely characterized in infants than that of the esophagus. Gastric emptying, gastric pacemaker activity (by electrogastrography), and antropyloric motility have been evaluated in infants down to 27 to 30 weeks. The predominating patterns of gastric motility are summarized in Figure 112-5.

The stomach is divided into two myoelectrically distinct regions: the proximal stomach, consisting of the fundus and orad third of the corpus, and the distal stomach, comprising the remaining corpus, antrum, and pylorus. Each of these regions exhibits a distinct pattern of contractile activity that contributes to the storage and mechanical breakdown of ingested foods and facilitates the selective emptying of solids and liquids into the duodenum. The frequency of gastric contractions is regulated by gastric slow-wave activity that can be measured by surface electrogastrography (EGG). The predominant frequency of the gastric slow wave is approximately 3 cycles/minute. EGG activity is absent at the time of birth in both preterm and term infants.[51] In the younger infant, regular 3 cycles/minute gastric slow waves are largely absent, with fewer than 30% of gastric slow waves occurring at the normal frequency of 2 to 4 cycles/minute, but this increases steadily over the first 6 months of life[52] to the range of 70% to 90% observed in older infants, children, and adults. As described later, premature and term infants can empty stomach contents normally in early life, and therefore the functional significance of these EEG-based findings is currently unclear. However, because EGG electrical patterns reflect an electrical activity generated by contractile waves that are responsible for mechanical digestion in the older infant and adult, it is not surprising that their ontogeny coincides with the timing of introduction of pureed or solid feedings in the normal infant.

Control of Gastric Emptying

Ingestion of a meal inhibits the tone of the fundus and contraction of the antrum; this mechanism enables the stomach to function as a food reservoir undergoing substantial changes in volume without significantly altering intragastric pressure. Gastric tone is best measured by a barostat, a device that records the volume of air contained within a bag placed in the proximal stomach and maintained at a constant pressure. In adults gastric volume can increase by more than 400% in response to a meal.[53] In the term neonate the mechanisms responsible for proximal gastric relaxation function poorly, but compliance quickly doubles in the first 3 postnatal days.[54] Gastric inhibition will normally be triggered by swallowing; this is known as receptive relaxation and aids the passage of the food bolus into the stomach.[55,56] Esophageal or gastric distension also triggers gastric inhibition; this is known as adaptive relaxation and is mediated via a vagovagal reflex initiated by excitation of highly sensitive gastric mechanoreceptors. In the cat esophageal and/or gastric distension will produce long-lasting inhibition of gastric tone that is absent in the presence of vagal cooling.[57] Mechanoreceptor excitation occurring with distension of the proximal stomach will also trigger local or vagal reflexes that stimulate motility of the antrum.[58,59]

Intraduodenal infusion of nutrients decreases fundic tone, suppresses antral contraction, and stimulates the pylorus to contract phasically and tonically (isolated pyloric pressure waves).[60,61] These changes in the pattern of motility act to slow gastric emptying and are regulated by neural feedback. Changes in the physical or chemical nature of intraluminal contents are detected by mucosal receptors located in or immediately below the gastric, duodenal, and small-intestinal epithelium. Although mucosal receptors with specificity for carbohydrates, pH, amino acids, short- or long-chain fatty acids, temperature, and touch have been described (mostly in the cat), the majority of mucosal receptors in most species are multimodal and are excited by both mechanical and chemical stimuli. Afferent signals originating at these receptors are transmitted to the brain stem (nucleus tractus solitarius) principally via unmyelinated vagal afferent fibers.The liver also contains receptors to detect osmotic, ionic, nutrient (glucose, amino acids, and sugars), pressure-volume, and temperature changes in the portal circulation and possesses a rich vagal afferent innervation that may alter gastric contraction via vagovagal pathways.[62-65] A variety of hormones also alter gastric emptying. For example, the products of protein or fat digestion stimulate the release of cholecystokinin from the small-intestinal mucosa. Cholecystokinin increases satiety, inhibits gastric motility (possibly via excitation of gastroduodenal vagal mucosal mechanoreceptors), and stimulates duodenal contraction.

Liquid Emptying

Liquids empty from the stomach faster than solids; fluids pass around solids whereas solids must be reduced into smaller-sized particles.[66] During initial filling the greater curvature of the gastric sinus bulges downward, a posture that facilitates the separation of liquids and solids and favors selective liquid emptying across the pylorus.[66] Liquid emptying is regulated by the pressure gradient between the stomach and the duodenum generated by tonic contraction of the proximal stomach[67]; the pylorus also has a profound effect on gastric emptying through the action of isolated pyloric pressure waves leading to pulsatile transpyloric flow.[68-70]

Solid Emptying

Solid food is processed by the distal stomach. After initial liquid emptying the bulge of the greater curvature of the gastric sinus is reduced, and the antrum assumes a tubular posture, bringing solids into line with the pylorus.[66] Movement of gastric contents from the corpus into the antrum is regulated via vagal mechanoreceptors in the antrum that reflexly inhibit corpus tone.[57] Distally propagating antral contractions occur at a frequency of two or three per minute, and these serve to break up digestible solids and sweep particles toward the gastric opening. The passage of solid particles across the pylorus is exquisitely controlled. Particles greater than 1 mm in diameter are usually blocked by pyloric contractions, some of which can be followed by retrograde flow from the duodenum, which returns particles to the antrum to be broken down further.[66,67]

Gastric Emptying in Neonates

Gastric emptying in neonates is generally considered a function of fluid (milk) flow across the pylorus; however, on acidification milk does separate into a semisolid curd and liquid whey fraction. This may account for differences in gastric emptying of formulas containing different ratios of casein and whey. The presence of

milk in the fundus stimulates gastric contraction that empties the milk into the duodenum. Gastric emptying rate of term and preterm infants has been extensively evaluated with use of ultrasonographic, scintigraphic, marker dilution, and breath test techniques. Gastric emptying is biphasic; that is, a rapid linear phase is then followed by a slower exponential phase. Measurements of 50% emptying times in premature infants fed breast milk range from 25 to 72 minutes.[71-74]

Feedback regulation of gastric emptying rate in response to the duodenal nutrient infusion is exhibited by infants as young as 32 weeks gestation.[75] As in adults,[76] increased caloric density of feedings slows gastric emptying,[77,78] and long-chain triglycerides produce greater inhibition than medium-chain triglycerides.[77] In term infants, starch solutions do not inhibit gastric emptying as effectively as isocaloric glucose solutions because of the lack of pancreatic amylase responsible for hydrolyzing starch into glucose.[79] Nonnutritive influences such as feeding temperature, nonnutritive sucking, or phototherapy do not influence gastric emptying rate.[80-82] The effect of gestational age on gastric emptying rate is not clear. Newell and colleagues[71] reported that infants ranging from 25 to 36 weeks gestation exhibit similar rates of emptying whereas Gupta and Brans[83] found that gastric emptying in term infants is faster than in preterm infants in the first 12 hours of life. Studies in premature infants have shown that nasogastric feeding or intraduodenal feeding infusion inhibits antral motility (see Figure 112-5) and stimulates isolated pyloric pressure waves.[84] As previously mentioned isolated pyloric pressure waves serve to regulate fluid flow across through the gastric outlet.

A profound but as yet unexplained difference exists between gastric emptying of expressed breast milk and infant formula. Despite the two being isocaloric, emptying of breast milk can occur at double the rate of infant formula.[71-73] Breast milk contains various regulator peptides such as gastrin, growth factors, and cytokines. In addition, feedings of breast milk alter the postprandial expression of gut peptides in the plasma compared with formula feedings.[85] The effects of these factors on gastric motility have not been evaluated. In contrast to the emptying rate, feeding type does not alter gastric slow-wave activity based on EGG,[52] which further questions the functional significance of EGG recordings as a marker of gastric function.

SMALL-INTESTINAL MOTILITY

Direct measurement of small-intestinal motility in infants has been limited to studies of the duodenum. The major motility patterns characterized in premature infants are shown in Figure 112-5.

The small intestine is a long tubular structure lined with absorptive epithelium, surrounded by layers of smooth muscle. The innermost layer is a thin layer underlying the mucosa, the muscularis mucosae. The middle and thickest layer, the circular muscle layer is arrayed circumferentially so that contraction narrows the lumen, whereas contraction of the thinner, outermost longitudinal layer foreshortens the intestine. Between the mucosa and each layer of muscle lies a meshwork of nerve fibers and ganglia that integrate varied contractile responses depending on the input of sensory signals from the lumen and mechanoreceptors in the muscles, as well as extrinsic input from vagal and sympathetic nerves. During the 5th to 8th week of gestation, circular muscle layers develop in the small intestine, with the longitudinal muscle not being present until 10 weeks gestation. The neurons migrate to the bowel as neural crest cells and differentiate through gestation with mature phenotypes being achieved in the third trimester. During fetal morphogenesis, the small intestine elongates, reaching a length of approximately four times the heel-to-crown length at birth (200-250 cm) and subsequently elongates to approximately 6 m in the adult.[86,87] The small intestine is segmented into a relatively short proximal segment, the retroperitoneal duodenum; a midregion, the jejunum; and distal portion, the ileum. Mucosal functions clearly vary in each region of the small intestine; however, regional differences in the organization of the muscle and nerve in each region are not well characterized. Most studies of small intestinal patterns of contraction have focused on the duodenum and jejunum, but limited studies of the ileum demonstrate somewhat different organization and control of patterns of contraction.[88,89]

Small-Intestinal Contraction

The smooth muscle cells of the small intestine are connected by gap junctions to form a syncytium. Between the muscle layers, specialized cells known as the interstitial cells of Cajal generate "slow-wave" oscillations of membrane potential that are transmitted through the syncytium (Figure 112-7). The slow wave is propagated rapidly around the circumference of a ring of bowel but not longitudinally, creating the conditions for the simultaneous contraction of a localized ring of bowel around the lumen. However, the slow-wave depolarizations do not cause muscle contraction. Further depolarization to a "threshold potential" is stimulated by the release of acetylcholine from nerves, triggering a rapid influx of calcium into the cells, generating a "spike" potential associated with contraction of the muscle cells. Thus the slow wave determines the maximal rate of contraction for each segment of the intestine, but the linkage of slow-wave depolarization with the actual occurrence of contractions depends on neural activity. In humans, the slow-wave frequency decreases from approximately 12 per minute in the duodenum to 8 per minute in the ileum. Slow waves propagate longitudinally down the bowel at a rate of 60 to 100 cm/minute.

As a ring of muscle contracts around the lumen, it generates force that is measured as a change in pressure in the lumen. The intrinsic neural plexus integrates excitatory and inhibitory input from mucosal sensory receptors, mechanoreceptors, and extrinsic vagal and sympathetic nerves to determine whether a segment of the intestine contracts. Sequential, coordinated contraction of rings of bowel along the longitudinal axis of the small intestine results in regions of high lumen pressure and low lumen pressure that mix and move the contents of the small intestine. In humans, most contractions involve only 1 to 4 cm of bowel at a time and last approximately 5 seconds.[90]

Coordination of Small-Intestinal Contraction

During fasting, small-intestinal contractions are grouped into characteristic patterns (Figure 112-8). The migrating motor complex (MMC) consists of a pattern of contractions that migrates

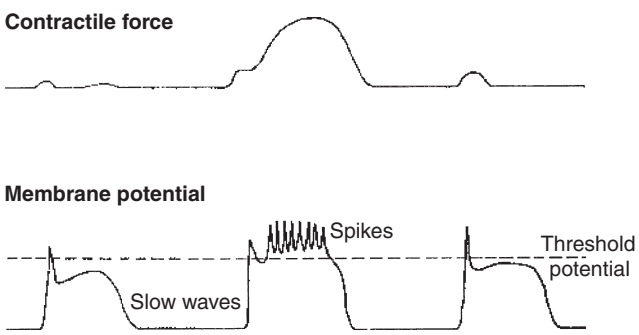


Figure 112-7 Diagram of the electrical and mechanical activity of smooth muscle showing the relationship between fluctuations in membrane potential and contractile force. *(Modified from Read N: Small intestinal motility physiology. In McCallum R, Champion M, editors:* Gastrointestinal Motility Disorders: Diagnosis and Treatment. *Baltimore, 1990, Williams & Wilkins, pp 23-36, with permission.)*

aborally from the antrum to the cecum.[91,92] In each region of the bowel, a period of no contractile activity (phase 1) is followed by irregular activity (phase 2) and then strong, regular contractions at the slow-wave frequency (phase 3).The bowel then again becomes quiescent. In the adult human, this pattern recurs every 45 to 180 minutes. Phase 3 activity lasts from 6 to 10 minutes and occupies 5% to 10% of the total MMC cycle whereas phase 2 activity occupies 20% to 30% of the MMC cycle.The migration velocity of the MMC along the small intestine is 4 to 6 cm/minute in the proximal intestine and decreases to 1 to 2 cm/minute in the ileum. The absence of this MMC pattern is associated with the development of bacterial overgrowth of the small intestine,[93] probably because the entire luminal contents, including bacteria, are swept out of the bowel during the migration of the strong phase 3 contractions down the bowel.

Feeding, bowel distension, and a variety of other stimuli disrupt the fasting MMC pattern with a more irregular pattern of contractions (see Figure 112-8). During this irregular pattern of contractile activity intestinal chyme is mixed and churned, but the net effect is to propel the nonabsorbed contents aborally from the stomach to the cecum within 2 to 12 hours after feeding.[94] The period of disruption by a meal varies with the size and content of a meal. Meals containing fat disrupt the MMC for a longer period of time than meals that contain equivalent amounts of energy in the form of protein or carbohydrate.[94] Vagotomy inhibits the conversion from a fasting to fed pattern suggesting that sensory input from the vagus initiates a reflex alteration of intestinal contractile patterns or suppresses the interdigestive patterns. Local factors are also important because the infusion of glucose into an isolated denervated loop of the small intestine can disrupt the fasting motor pattern.[95] The MMC pattern returns after the meal is conveyed down the small intestine. In most mammals, the MMC pattern is present only during fasting, but in ruminants, after weaning, the MMC persists after the meal.[96] In the pig, the MMC persists after fasting when food is taken without limit, but if pigs are fed only once per day the large meals disrupt the MMC cycle.[96]

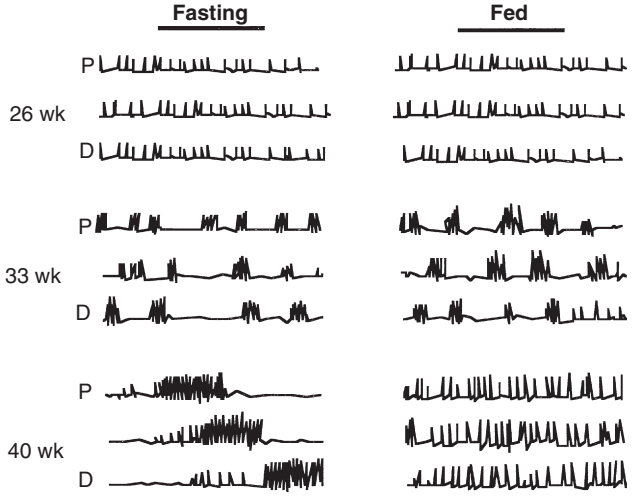


Figure 112-8 Examples of fasted and fed intraluminal pressure recordings from the upper small intestine at increasing gestational age in human infants. At 26 weeks, a random disorganized pattern is present with low-amplitude contractile waves. No change in pattern occurs with feeding. At 33 weeks, clusters of contractions are prolonged with intervening quiescence. Cluster activity persists after feeding, with a decrease in the periods of quiescence. At 40 weeks gestation, an organized MMC exists. Feeding results in an irregular, disorganized contractile pattern. In normal newborn infants, cluster patterns can persist after feeding. *P,* Proximal; *D,* distal.

Several other patterns of small-intestinal contractile activity have been described. Migrating clusters of contractions that last less than 2 minutes and migrate approximately 20 to 30 cm are observed after the ingestion of indigestible substrates such as cellulose, laxatives, or enterotoxins or partial obstruction. In the ileum, high-amplitude contractions lasting up to 15 seconds occur on exposure of mucosa to short-chain fatty acids, bile acids, and laxatives.[88,89] These contractions appear to clear the ileum of refluxed fecal contents and therefore may also be important for the prevention of bacterial overgrowth of the small intestine. Vomiting is often preceded by vagal nerve–mediated, retrograde giant migrating contractions that are two to three times larger in amplitude and four to six times longer in duration than the usual short-duration phasic contraction. These contractions usually begin in mid–small intestine and rapidly propagate to the antrum at a velocity of 10 to 12 cm/second, clearing intestinal contents into the stomach.[97]

Ontogeny of Coordinated Small-Bowel Contraction

The ontogeny of organized patterns of contraction of the small intestine was first studied in fetal and newborn lambs and dogs[98] (see Figures 112-8 and 112-9). Electrodes implanted on the intestine revealed "slow-wave" electrical rhythms at the earliest time recorded, the beginning of the third trimester. Episodic "spike" contractions occurred throughout the bowel, but no organized contractions were observed. As gestation progressed, this pattern of unorganized activity was replaced by a pattern described as the *fetal burst* pattern. Clusters of spike activity progressed for distances along the intestine in a pattern similar to the migrating cluster pattern observed in adult intestine. In the older fetus, the clusters became more organized and tended to migrate aborally for longer distances. In the lamb, an MMC pattern emerged before birth, whereas in the dog this pattern was not observed until 10 days after birth.

Since these early fetal studies, the ontogeny of small-bowel contractile activity in the human premature infant has been well studied with use of intraluminal perfused catheter techniques.[99-103] A similar sequence of development of increasingly coordinated activity begins with disorganized, low-amplitude, irregular contractile activity at 25 to 30 weeks gestation. Between 30 and 33 weeks rhythmic clusters of activity are observed that rarely migrate over large distances. From 33 to 36 weeks, migrating clusters with higher amplitudes of contraction are present. Increasing periods of time are seen with no contractile activity. By 36 weeks gestation, the mature MMC pattern is detected with quiescent periods (phase 1), irregular (phase 2), and regular (phase 3) contractions sequentially occurring in an aborally migrating pattern along the intestine. The length of time between episodes of phase 3 activity increases from 25 to 45 minutes in newborns[100,102,103] to 60 minutes at approximately age 2 years,[104] and approximately 100 to 150 minutes in adolescents and adults.[105] The duration of phase 3 does not change significantly after birth. Migration velocities of 2 to 4 cm/minute and mean amplitudes of contraction of 20 mm Hg in newborns increase to nearly double in adulthood.[93] Cluster activity persists after birth but becomes less common through childhood.

As patterns of coordinated contraction mature, the bowel also appears to undergo maturation in the responses to various factors that alter motility. In the term infant, feeding abruptly interrupts the MMC with the onset of irregular contractions,[106,107] although some cluster activity may persist. In older premature infants, an irregular pattern of activity replaces the fetal burst pattern, but, in infants younger than 30 weeks gestation, feeding has little effect on intestinal motor patterns. As is observed in adults, in preterm infants of approximately 33 weeks gestation, the length and degree of interruption of the fasting patterns vary with the number of calories delivered to the bowel.[108] Alterations in response to pharmacologic agents also mature with

age. Erythromycin initiates an almost immediate MMC pattern in adults and newborn infants[109] but not in premature infants before 31 weeks gestation (Figure 112-10).[109,110]

The factors controlling the ontogeny of small-intestinal motility remain rather unclear. The maturation of the enteric nervous system is likely an important factor. Hormonal changes may also play a role. Morriss and colleagues[111] noted an increase in duodenal contraction in those infants exposed to prenatal maternal corticosteroids. Some evidence exists that early feeding of preterm infants promotes postnatal maturation of intestinal motor activity.[112] The nutrient exposure in the feedings may replace exposure to the nutrients and growth factors in swallowed amniotic fluid. The effects of a lack of swallowed amniotic fluid on fetal development of small-bowel motility have not been studied. Assessment of the maturity of small-bowel motility may be useful to predict feeding readiness of preterm infants. Those infants with less-mature patterns of small-intestinal motility and without conversion to other motility patterns after feeding appear to be less tolerant of feeding challenges.[113] Perhaps monitoring of intestinal motility will provide a rational approach for determining the stage of gut neuromaturation and the relative risk of feeding for each preterm infant. Technical improvements in the methods of monitoring small-intestinal motor activity will be required before this approach can be adopted for routine clinical use.

COLONIC AND ANORECTAL MOTILITY

Colonic motility has not been directly measured in infants because of the technical difficulty of performing such measurements. Understanding of the development of colonic motility is

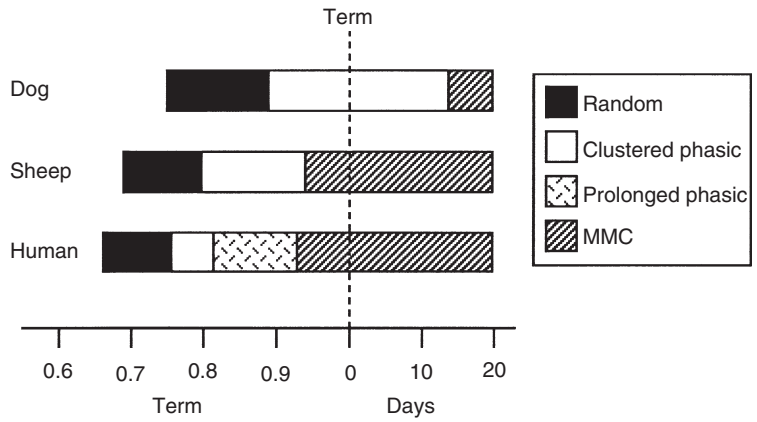


Figure 112-9 Qualitative maturation of motility patterns in the dog, sheep, and human. *(From Bisset W, Watt J, Rivers R, Milla P: Ontogeny of fasting small intestinal motor activity in the human infant,* Gut *29:483-8, 1988.)*

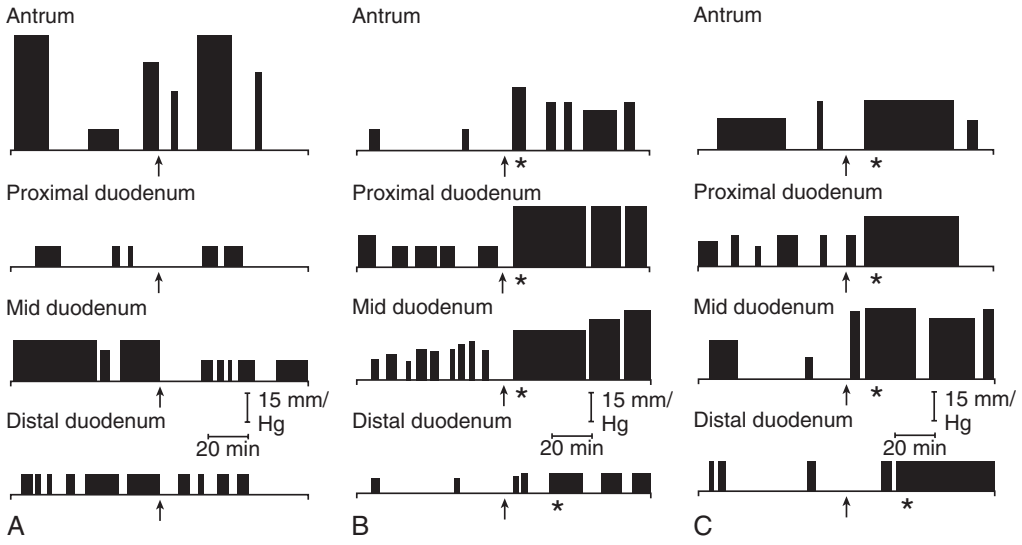


Figure 112-10 Schematic representation of motor activity recorded in a very preterm **(A),** a preterm **(B),** and a term **(C)** infant. In all three illustrations, motor activity recorded from the most proximal port is shown in the *top line;* motor patterns in each *lower line* were recorded from sites 2.5 cm distal to the one above it. Where the line is flat, motor quiescence was present. The *dark bars* show the duration and amplitude of contractions. In the very preterm infant **(A),** motor activity occurs independently in each lead before and after the administration of erythromycin *(arrows).* In the preterm infant **(B),** the administration of erythromycin *(arrows)* results in an increase in the amplitude of contractions in the antrum and the duodenum and in an increased motor activity that migrates distally, as indicated by the *asterisks.* In the term infant **(C),** erythromycin *(arrows)* also causes the appearance of migrating activity identified by the *asterisks;* however, the overall duration of each episode is longer than that which is seen in the preterm infant. *(From Jadcherla SR, Berseth CL: Effect of erythromycin on gastroduodenal contractile activity in developing neonates,* J Pediatr Gastroenterol Nutr *34:16-22, 2002.)*

therefore limited and based on measurements performed in older children and animal models. Anorectal motility has, however, been well characterized, and the major features are described in Figure 112-5.

Colon

The colon is a region of the digestive tract that is largely inaccessible and difficult to investigate. The placement of probes via anal intubation and endoscopic techniques is widely used; however, this requires bowel preparation that cleanses the colon of its normal fecal and bacterial environment, having unpredictable effects on motility. Nevertheless, investigations of colonic transit and contractile activity in children and adults have provided some insight into colonic function in health and disease.[114] Colonic transit is usually very slow, constituting two thirds of total mouth-to-anus transit (34-55 hours in normal adults).[115] This allows the colon sufficient time to carry out its primary function, the facilitation of transmural exchange of water and electrolytes.

Unless extreme circumstances prevail the colon is in a continuous digestive state and does not exhibit interdigestive motility patterns such as the MMC. Instead the colon exhibits specific patterns of motility that result in either mass movement or mixing of ileal effluent. Mass movement is achieved via high-amplitude propagated contractions (HAPCs). HAPCs are seen in the entire colon, exhibit contraction amplitudes of greater than 80 mm Hg, have durations greater than 10 seconds, and are propagated in an aboral direction over at least 30 cm at approximately 1 cm/second (Figure 112-11). Although highly effective in moving colonic intraluminal contents, HAPCs occur infrequently in adults, usually six times a day in the cleansed colon[116,117] and two times a day in the normal unprepared colon.[112] In children, HAPCs occur more often and are more likely to be associated with defecation.[118]

Other types of motility that are more frequently seen include independent contractions of individual or multiple colonic segments (Figure 112-12). These contractions move the colonic contents over short distances both aborally and orally to facilitate mixing. These segmental or nonpropagating contractions are not normally seen in infants but are expressed in older children.[118] The expression of colonic motility follows a circadian rhythm occurring after awakening and then cyclically during the day. Both adults and children demonstrate an immediate postprandial colonic response that manifests as an increase in colonic motility in direct response to nutrient (predominantly fats and carbohydrates) contact with the duodenojejunal mucosa and perhaps gastric distension. The mechanism for this response is unclear but may involve both vagal and endocrine systems. The duration of this response increases with age, lasting 30 minutes in infants[118,119] and 2 hours in adults.[120] In children younger than 4 years of age the postprandial response consists solely of HAPCs whereas children older than 4 years exhibit 60% HAPCs and 40% segmental contractions.[118,119]

Very little information is available regarding the prenatal development of colonic motility. In rhesus primates, the postprandial increase in colonic motility observed in the term infant is similar to the human and adult response. In the preterm rhesus primate a lesser response to meals occurs, suggesting that the colon response to meals matures late in gestation.[121] This finding may explain why the first meconium bowel movement is delayed by up to 3 days in premature infants.[122-124] Fetal hypoxemia frequently provokes passage of meconium by the term infant in utero, with resultant risks of meconium aspiration.[125] In contrast, even severe hypoxia rarely causes in utero meconium passage in the premature infant,[126] which further supports the contention that colonic motility is deficient in the preterm infant.

Anorectum

The anorectum is an anatomically complex region. Fecal continence is dependent on the ability to perceive rectal filling and then to retain its contents until the appropriate moment for defecation arises. Neonates usually defecate immediately on entry of stool into the rectum. The ability to delay defecation until a socially appropriate time requires coordination of the muscles of the sigmoid colon, rectum, levator ani, puborectalis (U-shaped striated muscle passing around the distal rectum), and anal canal. This is usually learned with toilet training. Normal term neonates pass their first meconium stool within 48 hours of birth whereas premature infants may experience a delay of more than a week before their initial defecation.[122-124]

The rectum acts as receptacle for fecal matter that arrives as a consequence of colonic HAPCs. In adults the rectum has a resting

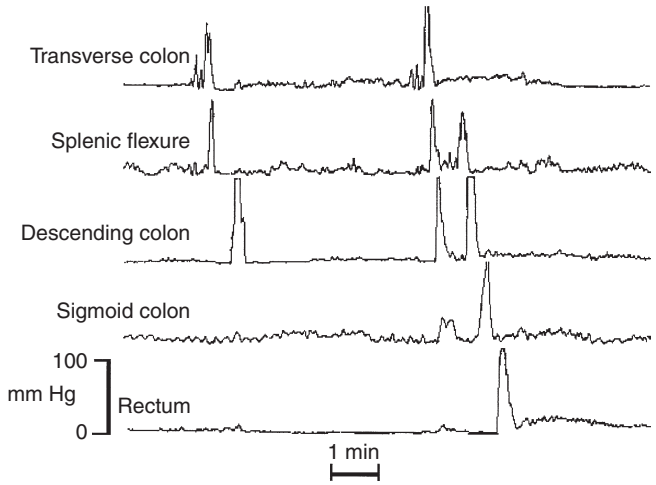


Figure 112-11 Colonic nonpropagating contractions in a child. Nonpropagating contractions occur in the transverse colon and a rectal motor complex in the rectum lasting 6 minutes. *(From Di Lorenzo C, Flores AF, Reddy SN, et al: Colonic manometry in children with chronic intestinal pseudo-obstruction,* Gut *34:803-7, 1993.)*

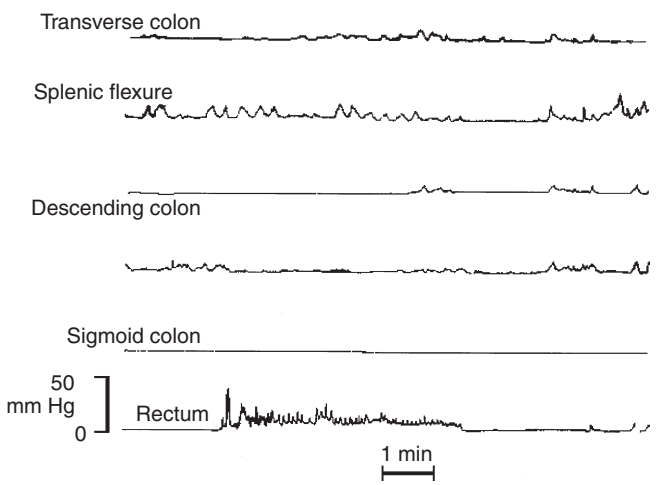


Figure 112-12 Postprandial HAPCs observed in a child. Pressures are recorded in the transverse colon *(T),* splenic flexure *(SF),* descending colon *(D),* sigmoid colon *(S),* and rectum *(R). (From Di Lorenzo C, Flores AF, Reddy SN, et al: Colonic manometry in children with chronic intestinal pseudo-obstruction,* Gut *34:803-7, 1993.)*

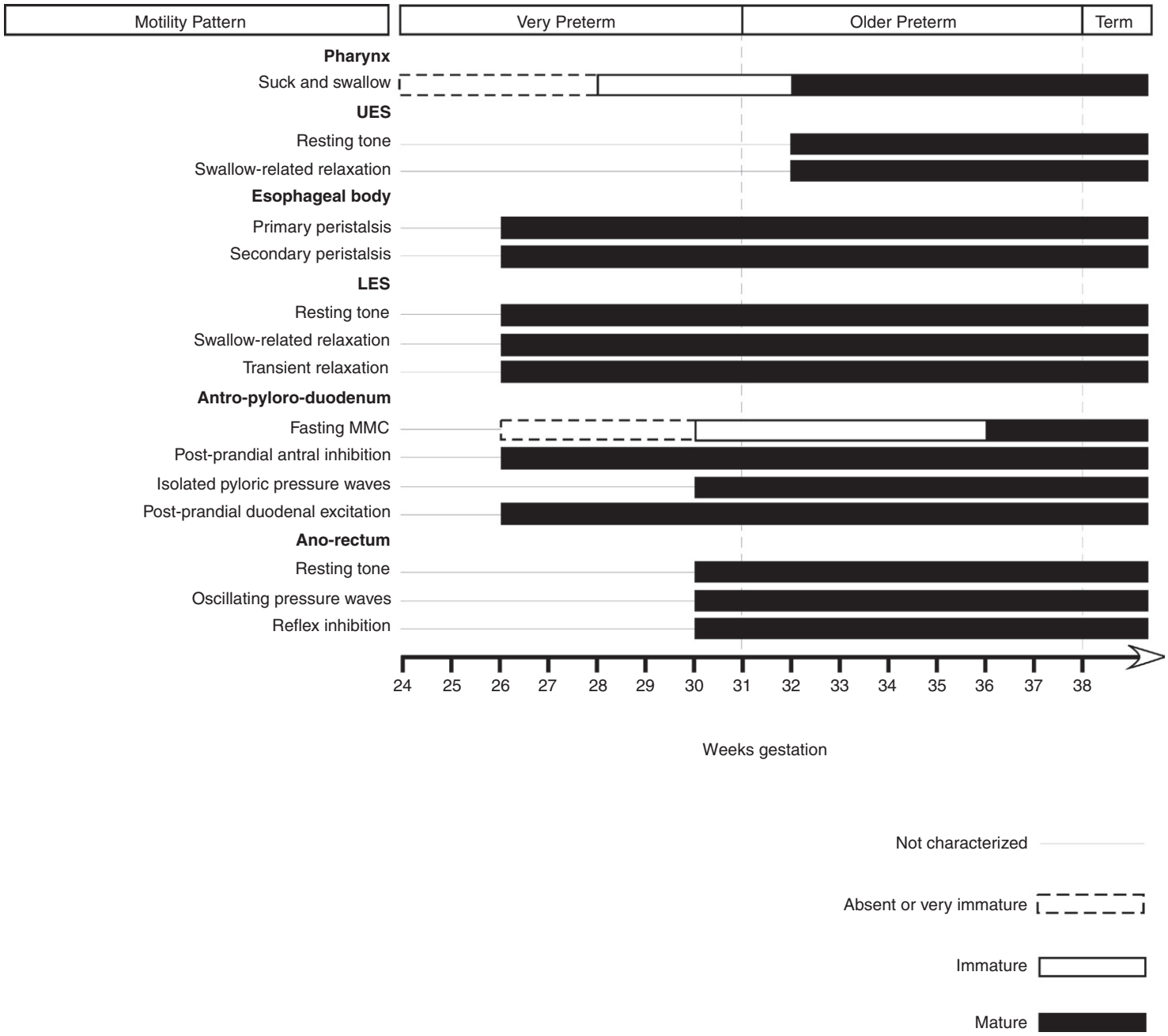


Figure 112-13 Pictorial summary of the understanding of the relative maturity of predominant GI motility patterns in very premature, older premature, and term infants.

pressure of 6 mm Hg and demonstrates three different patterns of phasic contraction: (1) isolated prolonged contractions (10 to 20 seconds' duration), (2) cluster contractions lasting 1 to 2 minutes and occurring every 20 to 30 minutes (10 to 12 seconds' duration), and (3) the rectal motor complex, a series of phasic contractions at the rectosigmoid junction lasting 10 minutes and occurring in a cyclical nature every 50 to 300 minutes dependent on the time of day (see Figure 112-11).[127] During defecation, rectal contraction serves to position fecal material into the proximal anal canal. Extrusion of the feces is achieved via relaxation of the anal canal in combination with straining (contraction of diaphragmatic and abdominal muscles) and propagated colorectal contractions. In addition, the puborectalis muscle relaxes and the levator ani muscles contract, causing the pelvic floor to descend, opening the anorectal angle, and assisting fecal passage.[128-131]

The anal canal measures 3 to 4 cm in adults and 1.25 to 2 cm in neonates. It consists of two sphincteric muscle groups, the external striated sphincter and the thicker internal smooth muscle sphincter. The external sphincter is under voluntary control via the pudendal nerve, and the internal sphincter is under both parasympathetic and sympathetic autonomic control. Resting anal sphincter tone is predominantly (85%) generated by the internal smooth muscle sphincter. The external striated muscle sphincter plays only a partial role in generating resting tone but can generate squeeze pressures that double the pressure in the anal canal. The ability to develop adequate squeeze pressure is essential in the maintenance of fecal continence because it overrides the effect of involuntary internal sphincter relaxation. It is thought that contraction of the external sphincter during internal sphincter relaxation allows the rectal contents to be sampled by receptors at the border of the anal canal that allow discrimination among solid, liquid, and gas.[132]

Anorectal Motility in Neonates

Basal pressure of the anal sphincter is 0 to 74 mm Hg in newborn infants and 15 to 98 mm Hg in older children and adults.[133,134] The internal sphincter contracts rhythmically 8 to 14 times per minute (oscillating pressure waves) and relaxes via a local reflex in response to balloon distension of the rectal wall, the rectoanal reflex (see Figure 112-5).[133] The threshold volume of air required to produce rectoanal reflex relaxation is 2 to 3.5 mL in infants,[133,134] 11 mL in older children,[130] and 20 mL in adults.[126] The anorectal relaxation reflex is present[135-137] and can be elicited in infants as young as 28 to 30 weeks gestation.[137,138] The anorectal reflex is normal in most infants, even those who experience delay in meconium passage,[139] but is absent in infants with Hirschsprung disease because of the lack of intrinsic inhibitory innervation of the rectum.[140]

OVERVIEW OF THE DEVELOPMENT OF MOTILITY IN THE INFANT

Numerous investigations performed over the last 10 to 20 years have characterized patterns of GI motility in preterm and term infants, in older preterm and term infants (>31 weeks gestation), and in some instances in infants as young as 26 weeks gestation. As shown in Figure 112-13 an almost complete picture now exists of the relative maturation of the motor patterns and reflexes of greatest importance to appropriate functioning of the GI tract (with the exception of the colon). The GI tract itself exhibits "mature" motility patterns in most regions with some notable exceptions. These are impairment of lingual function, ability to generate sucking pressures, and patterns and coordination of suck and swallow. This immaturity is the most likely cause of delays and difficulties in establishing full oral feeding; however, this deficiency is easily overcome in most infants with use of nasogastric tube feeding. Once nutrients enter the stomach it would appear that an appropriate fed antroduodenal motility response ensures regulated delivery of nutrients to the duodenum and mixing of luminal contents with GI secretions. Despite these normal fed patterns, however, a delay occurs in development of the fasting MMC that may lead to bacterial overgrowth and/or may contribute to feeding intolerance associated with slow intestinal transit. Well-developed esophageal motility allows for effective esophageal volume clearance in response to GER; however, if materials (feedings, oral secretions, or refluxate) come into contact with airway structures (made more likely by poor coordination of swallowing and breathing) then this may lead to significant complications including apnea and aspiration.

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changing dietary requirements during the first year of life are accompanied by basic changes in gastric physiology during that same period of time. The primary responsibility of the small intestine is to deliver nutrients close to the intestinal mucosa, which has the regional specialization necessary to absorb various intestinal contents. The colon, particularly in early life, has a limited role in delivering nutrients to the body. It primarily stores the nutrients that are not absorbed in the small intestine and packages them into a form that is suitable for defecation.

ESOPHAGUS

Sucking consists of organized movements of skeletal muscular origin. The primary purpose of sucking is to create a fluid bolus that can be transported into the esophagus where a monophasic peristaltic smooth muscle contraction transports the bolus into the stomach. A coordinated suck and swallow mechanism does not usually begin until the 30th week of gestation or later. Many indications certainly exist from in utero studies that the fetus is able to swallow amniotic fluid as early as 15 to 20 weeks gestation.[1,2] The mouthing and poorly coordinated sucking performed by the midgestational fetus and the early preterm infant reflect the inability of this age group to coordinate the oral, pharyngeal, and esophageal phases of swallowing. As the newborn reaches 34 to 36 weeks gestational age, he or she is able to undertake bursts of at least 30 sucks. At this stage, the infant generates sufficient intraoral pressure to form a bolus and is better able to coordinate the suck and swallow mechanism with breathing. Initiating oral feedings in premature infants before effective sucking and coordinated swallowing are developed places the neonate at risk for aspiration. The degree of sophistication of the neural processes responsible for the skeletal muscle involved in sucking and swallowing often develops later than the integration required for satisfactory performance of the more distal smooth muscle aspects of the intestinal tract. This often results in infants' requiring nasogastric tube feedings to receive adequate nutrition.

After a bolus has passed to the hypopharynx, relaxation of the upper esophageal sphincter is required. Inability to relax the upper esophageal sphincter at the appropriate time can result in reflux of food into the oral or nasal pharynx. As a bolus passes the upper esophageal sphincter, it enters the smooth muscle portion of the esophagus, which in the human infant is the distal one half to two thirds of the esophagus. Here a peristaltic wave transports (or pushes) the ingested nutrient into the stomach.

In the distal esophagus is found a tonic region of muscle called the *lower esophageal sphincter.* This sphincter inhibits the reflux of gastric contents into the esophagus. The lower esophageal sphincter must relax at the appropriate time to allow ingested foodstuffs to enter the stomach. Great interest has been shown in the ability of the lower esophageal sphincter to inhibit gastroesophageal reflux in the neonate. It appears from animal studies that the muscle in this area is able to generate stress similar to muscle from this region in the adult; however, it may be at a mechanical disadvantage in generating lower esophageal sphincter pressures.[3] It is also possible that much of the gastroesophageal reflux seen in the infant may be caused by inappropriate relaxation.[4-6]

GASTRIC MOTOR FUNCTION

The stomach acts as a reservoir for ingested food, begins mechanical trituration of ingested food, and empties the nutrients into the small intestine. Various regions of the stomach are responsible for these different physiologic functions. The proximal region of the stomach, known as the *fundus*, through a process of receptive relaxation or gastric accommodation, is able to receive relatively large volumes of ingested nutrients with only small increases in pressure. This allows the fundus of the stomach to serve as a reservoir for the ingested nutrients. This function is mediated to a large extent by the central nervous system through the action of the vagus nerve, which mediates inhibitory neurotransmitters during gastric distension. Receptive relaxation during the newborn period has received little study, although it is likely that this area may be important in the pathophysiology of certain events such as gastroesophageal reflux during infancy. It has also been proposed that the fundus can facilitate emptying of liquids from the stomach by developing low-grade tonic contractions that enhance gastric emptying effects, which are primarily due to coordinated antral-pyloric peristalsis. One study has shown that the neuroregulatory mechanisms that result in the coordination of antral-pyloric motility and gastric emptying are developed by 30 weeks postgestational age.[7]

The most distal portion of the stomach is known as the *antrum* and is responsible for the mechanical trituration or mixing and grinding of ingested foodstuffs, which occur before gastric emptying. Although newborns rarely ingest solids, curds formed from ingested milk products may require some trituration before they are emptied from the stomach. The antrum participates in a peristaltic sequence with the pylorus and duodenum to promote emptying of food. This implies that the antrum and pylorus do more than simply act as a filter to prohibit large food particles from leaving the stomach. Data from newborn animal models suggest that newborn antral smooth muscle does not have the ability to use the same calcium stores for contraction as in the adult.[8] It is possible that this contributes to the newborn antral smooth muscle's relative inability to contract with short, transient contractions and makes it more likely that contractions will occur in a prolonged tonic fashion.[8]

To attempt to compare the rates of gastric emptying in the preterm infant with those of the term infant and the adult is in many respects similar to comparing apples and oranges because the composition of diet and method of feeding are so different. In adults, the most important factor in determining the rate of gastric emptying of a liquid is the liquid caloric density. In addition, the rate of gastric emptying is controlled by feedback from the small intestine. Stimulation of duodenal receptors by acid, fat, carbohydrate, tryptophan, or increasing osmolarity decreases rates of gastric emptying. Similarly, the stimulation of ileal receptors by acid, fat, and carbohydrate may decrease gastric emptying, apparently by humoral mechanisms.[9,10]

Similar to findings in these adult studies, evidence in studies of term infants suggests that increasing caloric density is associated with slower rates of gastric emptying.[11,12] In that regard, Siegel and colleagues performed a series of careful studies evaluating the effects of caloric density[13] and the nutrient content of formula[14] on gastric emptying in infants ranging in gestational age from 32 to 39 weeks. Incremental increases of caloric density from 0.2 to 0.66 cal/mL decreased the rate of gastric emptying, indicating that feedback control mechanisms were comparable with those in the adult.[15] In term neonates, starch-containing solutions empty at a rate similar to that of water.[11,12] This finding is most likely explained by the slower rates of starch hydrolysis caused by the low amylase concentrations in the proximal intestine of the infant,[16] resulting in glucose's not being released in the duodenum; therefore gastric emptying is not reduced accordingly. A similar lack of effect of complex carbohydrates on gastric emptying is seen in adults with pancreatic insufficiency.[17]

Clinical experience suggests that gastric emptying is delayed in preterm infants. It is oftentimes not clear whether this is caused by a primary problem with gastric motor function or whether it reflects the inability of the duodenum to receive the gastric contents. Studies comparing gastric emptying rates in preterm and term infants are difficult to evaluate, but this suggests that gastric emptying is slower in preterm infants than in term infants.[18,19] One study evaluated gastric electrical activity and gastric emptying in preterm and term infants and showed that

gastric electrical activity and gastric emptying show an intrinsic maturation depending on gestational age.[20]

It is unclear whether formulas of different composition and roughly the same caloric content are emptied at different rates by the newborn. In adults, it has been suggested that triglycerides of various chain lengths exert variable effects on gastric emptying, with medium-chain triglycerides being less inhibitory than long-chain triglycerides. Similar findings have been demonstrated in infants of 30 weeks gestation[13] or longer; however, other studies have not confirmed these differences. One study showed no gastric electrical activity or gastric emptying in preterm infants fed either a standard preterm formula or protein hydrolysate formula.[21]

Nonnutritive sucking does not appear to have much influence on gastric emptying times in the infant.[22] Various other nonnutritive factors, such as formula temperature,[23,24] phototherapy,[23] and position,[18] have been shown to have no effect on gastric emptying times in infants who are examined within reasonable limits of physiologic variation. However, it has been shown that elevated bilirubin levels can decrease rates of gastric emptying.[25] Systemic illness can also decrease rates in newborn infants, as is demonstrated by dilution markers in infants with respiratory distress syndrome.[26]

The use of pharmacologic agents to accelerate gastric emptying in infants should be reserved for those instances when one is certain that no other underlying abnormality exists. Although metoclopramide has been shown to increase the gastric emptying rate in preterm and term infants,[27,28] it has also been shown to cross the blood-brain barrier. Its very high incidence of central nervous system side effects in older children and its relatively low therapeutic efficacy have largely led to its abandonment as a front-line therapeutic agent. Similarly, cisapride[29] and low-dose erythromycin[30] may increase emptying rates in preterm infants, although clinical success attributable to these agents has not been consistently observed.[31] One study showed that low-dose erythromycin accelerated gastric emptying and improved feeding intolerance in preterm infants.[32] Nasojejunal feedings bypass the stomach, allowing enteral feeding even if gastric emptying is delayed; however, because intestinal motility also is immature, the nasojejunal feeding may provide only limited improvement in feeding tolerance.[33,34]

SMALL INTESTINE

In contradistinction to the esophagus and stomach, both of which tend to have more regionalized motor function, the small intestine can be viewed as a hollow tube consisting of supporting tissue, three muscle layers, and mucosa that absorbs nutrients. The three layers of muscle are identified as the muscularis mucosae, the internal circular muscle layer, and the external longitudinal muscle layer. Most evaluations of small-intestinal motility center on the role of the internal circular muscle layer in the generation of intraluminal pressure, which results from contraction of this muscle layer.

These muscle layers are closely regulated and have their motor activity coordinated by the enteric nervous system, which is modulated by the central nervous system and a variety of neurohumoral agents. In the normal adult it has become clear that there are at least two different types of small-intestinal motor activity. One occurs during the fasting state. This type of motor activity consists of clusters of phasic activity that migrate down the intestinal tract at periodic intervals. These migrating clusters of phasic motor activity normally occur every 1 to 2 hours and are known as migratory motor complexes (MMCs). One of the primary functions of these MMCs is to act as an "intestinal housekeeper" to move forward intestinal contents that have been left behind and to stop bacterial counts from increasing to high levels in the proximal small intestine. In the postprandial period after food is ingested, this periodicity of MMCs changes into what appears to be a continuous series of seemingly sporadic contractions that occur throughout the intestine. It is thought that this activity is responsible for the mixing and churning of ingested nutrients as they leave the stomach. It is also likely that the somewhat random-appearing nature of these contractions is actually a rather sophisticated series of peristaltic cascades that results in propulsion of nutrients through the small intestine.

When one examines movement of foodstuffs through the small intestine, it is important to understand what is being described. Techniques exist to measure actual transit time of foodstuffs from the proximal to the distal small intestine, but the measurements of contractile activity in the small intestine do not necessarily equate with transit times. The actual motor activity that is responsible for the movement of foodstuffs in the postprandial period through the small intestine has not been well characterized and is poorly quantitated in most clinical settings. This is because the peristaltic sequences that are responsible for the movement of foodstuffs through the small intestines are not monophasic but consist of a multitude of contractions that coordinate peristalsis, retroperistalsis, and mixing in a manner that ultimately results in movement of food in an aboral direction. Most quantitative analysis of small intestinal motor activity consists of analysis of MMCs, most of which occur in the fasting period.[35]

It has been noted by Amarnath and colleagues[36] and by Berseth[37] that few infants display a pattern consistent with a true MMC. They tend to have episodes of motor inactivity interspersed with nonmigrating phasic motor activity. This nonmigrating motor activity has been demonstrated in up to 60% of the recording time in both term and preterm infants. It has been shown that these episodes of nonmigrating activity change with gestational age in that episodes lengthen in duration and increase in overall frequency.[37]

The sporadic contractions seen in the adult intestine in the postprandial state are not as predictable as in the preterm infant[38] in whom one can see no change, an increase, or a decrease in the intestine in response to feeding. Berseth and Nordyke[39] have noted that, as the infant increases in gestational age, the likelihood of a mature feeding pattern increases. They have suggested that the presence of such a pattern may predict which infants are more likely to tolerate enteral feedings.[38] It has also been suggested that infants who have difficulty tolerating feedings are more likely to show an immature feeding pattern after enteral feedings.[40] A more recent study showed that term infants have increased duodenal motor activity after a feeding like adults, whereas most preterm infants have decreased duodenal motor activity.[41]

In adults, initiation of an MMC is coincident with a rise in serum motilin levels. Intravenous motilin in an adult can cause the premature appearance of an MMC,[42,43] as can erythromycin, which competitively binds to the motilin receptor.[44] Because MMCs are rarely seen in extremely preterm infants, Jadcherla and Berseth studied the correlation of small-intestinal motor activity with serum motilin levels. They found that even when MMCs did occur in infants, no rise was seen in serum motilin levels.[45] However, it was demonstrated that the administration of erythromycin did initiate MMCs in infants of 32 weeks gestation, suggesting that motilin receptors are available at that age. More recently, Jadcherla and Berseth showed that erythromycin, a motilin-receptor agonist, increased antral and duodenal contractions in term and preterm infants whose gestational ages were 32 weeks and older.[46]

Initiation of enteral feedings tends to bring about a more rapid maturation of motor patterns in the preterm infant.[47,48] It is possible that, in preterm infants lacking MMCs during the fasting phase, a defect exists in the "housekeeping" function of small-intestinal motor activity, which may predispose to bacterial

overgrowth. Some clinicians have speculated that this may be a predisposing factor to necrotizing enterocolitis.

COLONIC MOTILITY

Colonic motility has not received as much study as the functions of upper areas of the gastrointestinal tract, although it is known that its embryologic development occurs in concert with that of the small intestine. Peristaltic waves in the colon can be seen early in gestation. Meissner and Auerbach plexuses are present by the 10th to 12th weeks. The distribution of ganglion cells has been studied quite fully in infants because of the pathophysiology associated with colonic aganglionosis. It is now believed that aside from the well-described area of hypoganglionosis present within the first 10 mm of the anal valve, a normal distribution of ganglion cells exists in the premature infant as young as 24 weeks gestation.[49] More than 98% of normal newborns pass meconium within the first 36 to 48 hours. However, preterm infants weighing less than 1 kg oftentimes do not pass stool in the first 3 days.[50] It has been noted that high-amplitude propagating contractions in the colon are a good indicator of normal bowel function and passage of fecal material. These high-amplitude propagating contractions have been noted to decrease in frequency from several an hour after a meal in toddlers to just a few per day in adults.[51,52] Very little is known about high-amplitude propagating contractions during the newborn period. Work done on colonic motility of newborn rhesus primates suggested that the term newborn colon exhibits responses to feeding similar to those seen in the adult colon, whereas the response in the preterm primate is less well defined. This suggests that the colonic response matures late in gestation.[53]

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Steven L. Werlin and Alan N. Mayer

Development of the Exocrine Pancreas

EMBRYOLOGY AND HISTOGENESIS OF THE HUMAN PANCREAS

The dorsal and ventral anlagen of the human pancreas develop as evaginations of the primitive foregut during the 5th week of gestation (Figure 114-1).[1] The larger dorsal anlage, which develops into the tail, body, and part of the head of the pancreas, grows directly from the duodenum. The ventral anlage develops from one or two buds from the primitive liver and eventually forms the major part of the head of the pancreas. At approximately 17 weeks gestation, the dorsal and ventral anlagen fuse as the buds develop and the gut rotates. The ventral duct forms the proximal portion of the major pancreatic duct of Wirsung. The dorsal duct forms the distal portion of the duct of Wirsung and the accessory duct of Santorini. Variations in fusion account for the variety of developmental abnormalities of the pancreas.

Histologic examination of the early pancreas reveals predominantly undifferentiated epithelial cells, which by 9 to 12 weeks development form a lobular-tubular pattern; zymogen granules are absent, but the Golgi apparatus is present.[2,3] Primitive acini containing rough endoplasmic reticulum and recognizable zymogen granules are present by 14 to 16 weeks. Golgi vesicles become prominent at this time. Activity of secretory enzymes is first detectable at this age. By 16 to 20 weeks development, large numbers of zymogen granules are present. As the pancreas matures, the luminal volume decreases and acinar cell volume increases. Connective tissue continues to decrease both throughout gestation and in the postnatal period. By 20 weeks gestation, acinar cells contain mature-appearing zymogen granules, well-developed endoplasmic reticulum, and highly developed basolateral membranes. The pancreas from the 6-day-old rat shows a similar increase in acinar volume and small lumina. Stroma continues to decrease, and acinar cells have a mature appearance. After birth, the volume of the exocrine pancreas continues to grow, nearly tripling in size during the first year of life from 5.5 g to 14.5 g.[4] The adult pancreas weighs 85 g. During the first 4 months, the ratio of acinar cells to connective tissue increases fourfold.

Centroacinar and duct cells (which are responsible for water, electrolyte, and bicarbonate secretion) are also found by 20 weeks. The ductal system contains less than 5% of the volume of the exocrine pancreas and only 0.5% of total pancreatic volume. In the postnatal period, luminal volume increases, along with the increase in acinar cell volume.

Islets are first identifiable at 12 to 16 weeks, at which time immunoreactive insulin is present in beta cells. Mixed cells, those with characteristics of both acinar and islet cells that are only rarely seen in the adult, may be found.[5] Endocrine cells of the fetus, but not the adult, may contain more than one hormone, and more than one hormone may also be found within a single granule. The histologic appearance of the pancreas in an infant born at term is similar to that in the adult.

ONTOGENY OF NONHUMAN PANCREAS

Many of our concepts concerning human pancreatic development are based on studies performed in rodents. Although the time span is different, the histologic, ultrastructural, and biochemical developmental stages of rat and human pancreas are thought to be similar. Several distinct prenatal developmental periods have been defined.[6-8] With use of a mouse model and a retroviral vector, it has now been shown that the endocrine pancreas and exocrine pancreas derive from a common cell origin.[9]

Using the combination of embryo microdissection and reverse transcriptase polymerase chain reaction to measure low-level gene expression, Gittes and Rutter[10] related the onset of cell-specific gene expression to morphogenesis within 2 to 3 hours. They found expression of pancreas-specific transcripts in the foregut before formation of the pancreas. Insulin and glucagon were detected 10 to 12 hours before evagination of the pancreatic bud but only in the area destined to become pancreas. Carboxypeptidase was first detected at 10.5 days and amylase at 12 days, both after the formation of the pancreas. Early expression of endocrine protein suggests a possible role in ontogenesis.

Human mucin gene expression *(Muc 1)* was evaluated in the fetal pancreas by Batra and colleagues,[11] who found that *Muc 1* messenger RNA (mRNA), a marker for differentiation of pancreatic cells, was not detectable until 18 to 19 weeks gestation. In adults, *Muc 1* expression correlates with the differentiation state of pancreatic tumors.

Hisaoka and colleagues[12] related pancreatic morphogenesis and organization of the extracellular matrix. Throughout fetal development, epithelial compartments are separated by the basement membrane components (laminin and type IV collagen) present at the epithelial mesenchyme interface. Spatial distribution of types I and III collagen changes with time. Laminin-1 expression is important in the induction of exocrine cell differentiation.[13] These extracellular matrix components are presumed to play a role in the regulation of differentiation. Organ culture studies have shown that local mesenchymal factors, but not nerves or hormones, are required for morphogenesis and cytodifferentiation, which themselves are separable events.

The proliferating cell nuclear antigen (PCNA), a protein associated with DNA polymerase Δ, is expressed during the S phase of the cell cycle. Activity of PCNA is related to [^{3}H]thymidine uptake.[14] With use of monoclonal antibody immunostaining, the percentage of PCNA-positive cells was high at 16 days gestation in the rat (60%), declined (to below 10%) at birth, increased to 30% at age 5 days, and then fell slowly until the pup weaned, at which time a precipitous fall to the adult level (below 5%) occurred (age 60 days).[14] This was highly correlated with [^{3}H] thymidine uptake.

The acinar cell population is predominantly mononuclear at birth but after weaning becomes progressively binucleate.[15] Before weaning, acinus formation is due to mononuclear acinar cell and duct cell proliferation. In Syrian hamsters primordial pancreatic cells most resemble centroacinar cells, and transitional cells are also found in the fetal pancreas.[16]

The shape of the mature acinar cell is pyramidal with a basal nucleus. The most prominent organelles in the fasted state are large numbers of zymogen granules, located apically. Abundant rough endoplasmic reticulum (the location of protein synthesis) and Golgi apparatus (the nidus of packaging proteins for export) are present. Junctional complexes join adjacent acinar cells.

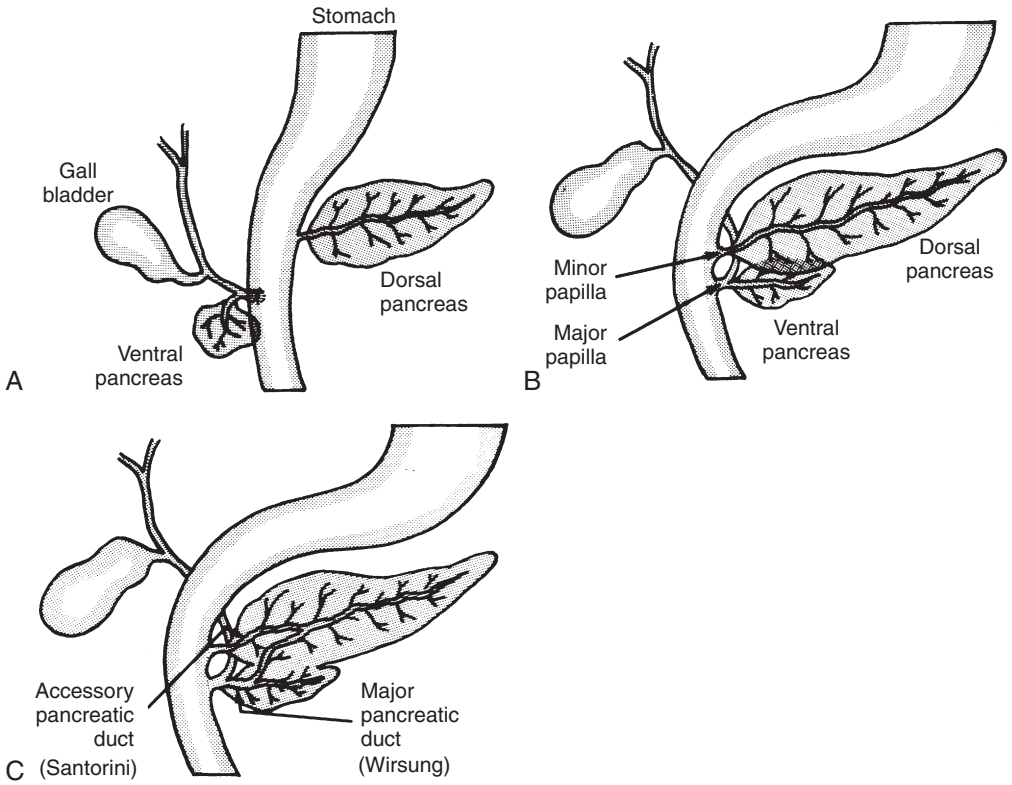


Figure 114-1 Development of the human pancreas. A, Gestational age, 6 weeks. **B,** Gestational age, 7 to 8 weeks. The ventral pancreas has rotated but has not yet fused with the dorsal pancreas. **C,** The ventral and dorsal pancreatic ductal systems have fused. *(From Kelley VC [ed]:* Practice of Pediatrics, *vol V, Philadelphia, 1987, Harper & Row.)*

The apical membrane contains abundant microvilli projecting into the lumen. The final three-dimensional structure of the exocrine pancreas consists of a complex series of branching ducts surrounded by grapelike clusters of acinar cells. The ontogeny of cell surface glycoproteins is critical for normal cell-cell interactions and morphogenesis.[17,18] Stage-specific polylactosamine (carbohydrate) antigens have been described in many organs, including the pancreas. These antigens are thought to be important for cell-to-cell communication and recognition.[19]

In the rat the expression of the mature phenotype is first detected at a gestational age of 15 days, the onset of the differentiated stage. Inhibition of glycoprotein synthesis (with tunicamycin therapy) in pancreatic rudiments between days 15 and 17 delays histogenesis, as well as the normal increase in amylase found at this age.[20] At 17 days, acinar lumina are first seen coincident with differentiation of the apical and basolateral domains of the acinar cell membrane. The adult pattern is fully developed at age 19 days.

Until day 14 of gestation, little or no secretory product, specific mRNA for secretory proteins, or zymogen granules are found. From days 14 to 18, synthesis of embryonic proteins (found in a wide variety of other fetal tissues, but not in zymogen granules) continues. A constant low level of a few pancreas-specific secretory proteins is found. From days 18 to 21, a rapid increase in specific mRNA transcripts occurs, followed by a nonparallel increase in pancreas-specific proteins and zymogen granules.[21,22] RNA synthesis peaks at day 18 followed by a peak in protein synthesis 1 day later. During this period, although total pancreatic protein synthesis increases only 25%, synthesis of some pancreas-specific proteins (e.g., amylase and chymotrypsinogen) increases by more than 100%. Amylase-specific mRNA increases 600-fold at a gestational age between 14 and 20 days, coincident with a 1000-fold increase in amylase-specific activity. During the final days of gestation and during early neonatal life, synthesis of secretory proteins accounts for more than 90% of all pancreatic protein synthesis. Synthesis of secretory proteins is regulated at the level of transcription.

The temporal profiles of secretory proteins in the prenatal pancreas have been defined in the rodent.[23-25] Because the appearance of the various secretory proteins is not parallel, independent regulation of gene expression is suggested.[23,24] The times of first appearance of digestive enzymes in the human fetal pancreas have been variably described. Trypsin, chymotrypsin, phospholipase A, and lipase are present in the 14- to 16-week fetus in low concentrations, and these steadily increase with gestation.[3,26,27] Chymotrypsin activity is low at birth, peaks at age 3 days, then declines slightly. Pancreatic secretory trypsin inhibitor is present in the fetal pancreas at 10 weeks gestation.[28] Pancreatic amylase is present in amniotic fluid at 14 weeks gestation.[29] A wide day-to-day variation of enzyme concentration is found. Thus amylase, lipase, trypsinogen, and chymotrypsinogen are present in the fetal pancreas by 16 weeks gestation, the time of rapid development of zymogen granules.

The postnatal developmental profile of mRNA transcripts coding for chymotrypsinogen, trypsinogen, lipase, and amylase and the expression of these proteins in the calf have been described.[30] Specific mRNA levels of amylase, lipase, and trypsin increased with age; chymotrypsin mRNA decreased from birth until day 28 and then increased at day 119 back to the level found in the newborn. Specific activities of the proteins did not necessarily parallel mRNA levels. At day 119, levels of trypsinogen, lipase, and amylase mRNAs were markedly increased; the level of chymotrypsinogen mRNA remained decreased when compared with the level found in the animal born at term. All four mRNAs were increased in weaned animals but to differing

degrees. Thus, in the cow, these genes are differently expressed during development.

The presence of at least three different lipases, one of which may be identical to the zymogen granule membrane protein GP3, has been described.[31] Each lipase appears to be under different regulatory control, both before and after birth. Yang and co-workers[32] have also shown dis-coordinate expression of pancreatic lipase and two related proteins in the human fetal pancreas.

Thus the timing of initiation of synthesis of individual proteins is different, and the final levels are also different. Although the curves for the appearance of each protein are quite similar, they are time shifted. The final adult level of each enzyme is unrelated to the onset of synthesis or to the peak fetal level. Concentrations of enzyme change considerably after birth.[23] The level of secretory protein in the mature pancreas represents the balance between synthesis and secretion. In addition, the presence of mRNA does not necessarily indicate an abundance of its translational product.

SECRETION FROM THE EXOCRINE PANCREAS

Although a continuous slow basal secretion of pancreatic enzymes occurs, physiologically significant secretion occurs only after stimulation by a secretagogue. A large number of agents have been described that are effective secretagogues in the experimental animal, but only acetylcholine, secretin, and cholecystokinin (CCK) are of significance in humans. Acetylcholine is released locally in the pancreas after vagal stimulation. CCK and secretin are synthesized and stored in the intestinal amine precursor uptake and decarboxylation cells and released after ingestion of a protein or fatty meal.

Each secretagogue then interacts with its specific receptor on the acinar cell membrane. Intracellular second and third messengers include Ca^{2+}, protein kinase C, diacylglycerol, and inositol phosphates.[33,34] This process eventually leads to fusion of zymogen granules and cell membranes and to exocytosis of pancreatic protein into the ductal system. Luminal factors, including the process of assembly and disassembly of a matrix (containing GP2 and proteoglycans), are thought to perform critical functions during storage and secretion of pancreatic secretory proteins.[35] Regulation by CCK and secretin is critical. Secretin acts directly on the pancreatic ducts. Human pancreatic acinar cells lack both CCK-A and CCK-B receptors. The primary target for CCK is now thought to be vagal receptors.[36] A portion of GP2 is cleaved by protein kinase C and released into the pancreatic duct during stimulated secretion.[37] Although nonparallel secretion of pancreatic proteins has also been described, at the present time, most authors consider that nonparallel secretion is probably the exception and not the norm.[38]

Fluid and electrolyte secretion from the centroacinar and ductal cells is controlled by secretin, which was the first hormone identified. Intracellular messengers in this system are cyclic adenosine monophosphate and protein kinase A. The concentrations of bicarbonate and chloride in pancreatic secretion depend on the flow rate. At lower flow rates, the concentration of bicarbonate is low and that of chloride is high.[38] As the rate increases, bicarbonate secretion increases and chloride secretion decreases, whereas sodium and potassium secretions remain constant.

The pancreas synthesizes more than 20 proteins specifically designed for export. After synthesis, it must segregate those proteins designated for export from those synthesized for internal use. Classic studies have demonstrated the synthetic pathway with use of the pulse-chase technique. Newly synthesized proteins travel from the endoplasmic reticulum to the Golgi apparatus, where they are "packaged" to the condensing vacuoles and then converted into mature zymogen granules in the apical portion of the cell. Little or no processing of individual proteins takes place from the time they leave the rough endoplasmic reticulum until the time they leave the cell. In rodents this entire process takes less than 1 hour. Molecular chaperones or chaperonins are involved in the transit process as they are in other cell types.[39]

ONTOGENY OF SECRETORY FUNCTION IN THE HUMAN

Because of both the technical difficulties and the ethical concerns about performing invasive procedures in healthy infants, few studies of pancreatic secretory function in the human infant have been published. Because direct collection of pancreatic juice is not possible in the infant and young child, pancreatic secretions must be collected in the duodenum in the fasted or basal state, after indirect stimulation with a meal, or after direct stimulation with secretagogues such as secretin or cholecystokinin, or both. Pancreatic function may be determined indirectly by the measurement of fecal elastase and by fat balance studies.

Many of the early studies of pancreatic function in infants are fraught with methodological difficulties including the qualitative nature of the collections and the lack of separation between the pancreatic and salivary amylase and lipase isoenzymes.[40-43] In a small group of older children, Zoppi and associates[41] demonstrated that amylase secretion continues to increase with age (the oldest child studied was 13 years old), whereas output of lipase, proteinases, fluid, and bicarbonate was independent of age. A good correlation was found between protein secretion and enzyme activity. Norman and co-workers[44] measured pancreatic secretion in eight healthy term infants aged 3 to 15 days after a test meal of breast milk. Although the trypsin-to-chymotrypsin ratio was relatively constant, the levels of the individual enzymes varied considerably between infants. Salivary but not pancreatic amylase was present in low levels.

Basal and stimulated pancreatic secretion in groups of well premature and term neonates, infants 1 month of age, and toddlers 2 years of age have been reported[45] (Figure 114-2). Results obtained from term and premature infants were similar, and data were pooled in the report. Although total protein content was similar in the pancreatic secretions collected from the duodenum of all three groups, each of the five individual secretory proteins studied had its own developmental profile. Trypsin was low at 1 day, but at 1 month it was similar to the level seen at 2 years. Chymotrypsin remained low at 1 month. Carboxypeptidase was low both at 1 day and at 1 month when compared with the level found at 2 years. Amylase was not detected until age 2 years. Similarly, lipase was undetectable at 1 day and at 1 month. Thus the ontogeny of the various enzymes is not parallel, a finding that has also been documented in rodents. Responses to secretin and CCK were poor in the newborn and infant. Responses at 1 month were similar, whether the infants were fed a cow's milk–based formula or a soy formula.

These data confirm previous reports concerning proteinases but are contradictory with respect to amylase and lipase. It has clearly been shown that pancreatic amylase is present early in gestation. Similarly, salivary amylase is secreted in high concentration in the newborn. Serum pancreatic isoamylase and isolipase are both low at birth and increase with age. These findings suggest that the newborn pancreas can synthesize but not secrete amylase. The failure to find measurable amylase may reflect the fasted state of the infants. A similar argument can be constructed to explain the extremely low levels of lipase.

Compared with secretion in the adult, enzyme secretion is decreased in the infant and young child, and secretion of water and bicarbonate is blunted as well. An increase in ductal cell function also occurs with increasing age. Secretion of water increases 10-fold by age 9 months.[43]

Despite this relative pancreatic insufficiency the human newborn infant and the premature infant absorb both fat and carbohydrate with seemingly little difficulty. Although an adult-type

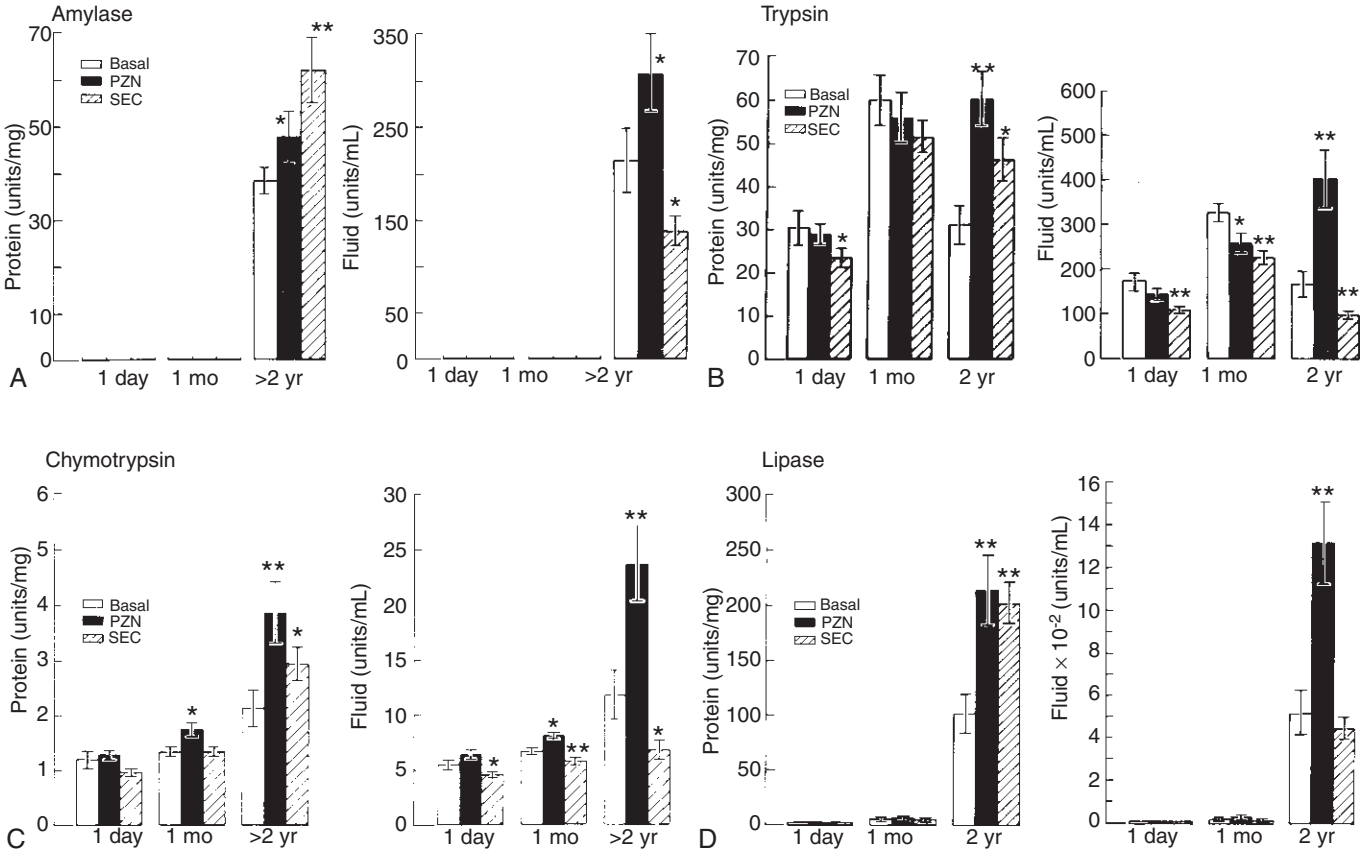


Figure 114-2 Effect of CCK and secretin on specific activities of pancreatic enzymes in duodenal fluid in infants and children. A, Amylase. **B,** Trypsin. **C,** Chymotrypsin. **D,** Lipase.

coefficient of fat absorption is not seen until age 4 to 6 months of life, "clinically" important malabsorption does not occur unless an additional pancreatic insult, such as cystic fibrosis, is present.[46] The lack of significant malabsorption is almost certainly a result of the presence of the nonpancreatic (lingual and gastric) lipases.

Although the ontogeny of complex carbohydrate digestion and absorption has not yet been as carefully examined as that of fat absorption, some information exists regarding the ability of the newborn to digest starches. Blood sugar rises only minimally after the ingestion of cooked starch. Intestinal hydrolysis of starch is rapid in the 1-year-old; at age 6 months, hydrolysis of amylopectin is incomplete and duodenal amylase activity is low. Despite the poor duodenal hydrolysis of starch, only minimal carbohydrate is found in the stool of infants fed formulas containing complex carbohydrates, because of salvage of colonic carbohydrate by bacterial fermentation and absorption of short-chain organic acid products.[47] The digestibility of starches, modified starches, and complex carbohydrates varies considerably. In adults the increase in breath hydrogen after ingestion of meals containing complex carbohydrates varies considerably, suggesting that even in the adult a large amount of fermentable material escapes small-bowel absorption.[48] Neonates also have glucoamylase, brush border enzyme, which aids in the digestion of starch and increases intraluminal glucose by removing glucose units from the nonreducing ends of starch molecules.

Most infants, even those who are premature, thrive on formulas that contain glucose polymers or corn syrup solids as their carbohydrate source. It has been our experience, which is supported by a small number of published case reports, that some infants have watery diarrhea when fed such formulas.[49-51] This diarrhea resolves when the formula is changed to one containing an alternative carbohydrate.

Diet modulates pancreatic enzyme secretion in the human as it does in the animal models discussed later in this chapter. Although these changes may occur at the level of synthesis, some changes reported to occur in animals clearly occur faster than can be explained by changes in synthesis. Premature infants fed a soy-based formula have greater stimulated secretion of lipase and trypsin compared with infants fed a cow's milk–based formula.[52] Total parenteral nutrition induces reversible pancreatic atrophy in animals and pancreatic hyposecretion in humans. Thus, as in the animal models described later in this chapter, some changes in pancreatic enzyme secretion are programmed, and others are inducible by diet.

ONTOGENY OF RODENT EXOCRINE PANCREATIC SECRETION

The pancreas of the newborn rat is both histologically and ultrastructurally fully developed; it is packed with secretory proteins. Although it appears poised to secrete, it is functionally immature. Immaturity has been documented at a number of steps along the stimulus-secretion chain. Responsiveness to cholinergic and peptidergic (e.g., CCK) secretagogues in a manner similar to, but not identical with, that of the adult gland does not occur until age 24 to 48 hours.[53-55] The newborn pancreas, although unresponsive to cholinergic agents and to CCK, responded with increased secretion of amylase to the calcium ionophore A23187 in some studies but not in others.[55-57] Responsiveness would suggest that the secretory mechanism distal to calcium mobilization is intact.

Muscarinic receptor density is low in the fetal and newborn periods and steadily increases with age, reaching maximal levels at age 1 month; it then decreases steadily until age 1 year.[58] A parallel increase occurs in secretory response and receptor density.

Binding of radiolabeled CCK to pancreatic acinar cells rises rapidly (from low levels at birth), reaching levels of the mature pancreas at age 3 weeks.[59,60] Others have found that the postnatal increase in secretory responsiveness was not associated with increased CCK binding.[57] These receptors are of the A or intestinal type that just after birth are already coupled to G proteins.[61] CCK-A glycoforms were different before birth. In the cow the CCK-A receptor predominates at birth, but later the CCK-B receptor predominates.[19] Results of these studies suggest that decreased receptor density cannot explain the lack of secretory responsiveness in the immature pancreas and that the immaturity is distal to calcium mobilization. The absence of the zymogen granule membrane protein GP2 in the fetus may contribute to secretory unresponsiveness.[11]

The ontogeny of the vasoactive intestinal polypeptide (VIP) receptor has been described in rats and calves. In the rat, VIP receptors are present at day 19 of gestation.[22,62] In the cow, a single class of receptors is present at birth; however, at 28 and 119 days after birth, two classes (high affinity and low affinity) of receptors are found.

Because protein phosphorylation appears to play an important role in the pancreatic response to secretagogues, the roles of protein kinases have been examined. Calcium-calmodulin–dependent protein kinase increases in parallel with responsiveness to secretagogues from the late fetal to the newborn period.[63] Similarly, protein kinase C was found to be low in the term fetus. Levels increased in the newborn period, reaching adult values by age 2 days.[64] Newborn rat pancreas was unresponsive to 12-0-tetradecanoylphorbol acetate (TPA), an activator of protein kinase C, but the 2-day-old pancreas responded to TPA with increased amylase secretion. These studies of protein kinases suggest immaturity of the secretory response to regulatory peptides at a number of levels.

Thus immaturity both of receptors to secretagogues and of protein kinases is found in the neonatal rat. At this time, the lack of responsiveness to secretagogues in the neonatal rat cannot be attributed to any one pharmacologic deficiency and may in fact be multifactorial.

EFFECTS OF DIET AND WEANING IN RODENTS

A protein-free, carbohydrate-rich diet has no effect on mRNA coding for elastase isoenzymes; however, trypsinogen isoenzyme mRNA levels increase slightly.[65] Because this same dietary manipulation decreases cationic trypsinogen synthesis, the trypsinogen genes must be under different translational and transcriptional control.

In the rat fed a high-fat diet, lipase synthesis increases within 24 hours, amylase synthesis does not increase, and peptidase synthesis increases by day 5.[66] Lipase-specific mRNA increased fourfold on day 1 followed by a second 6.5-fold increase on day 4. Amylase mRNA levels remained unchanged. Because of the biphasic increase in lipase and lipase-specific mRNA, these authors postulated that the first phase was a result of an increase in mRNA synthesis and the second phase was a result of increased translation, possibly caused by hormonal influences.

The pancreatic content of exportable proteins and zymogen granules falls dramatically after birth.[8,67] This fall is not preprogrammed but rather relates to feeding and stimulation of secretion.[68,69] Alterations in diet, time of weaning, and time of first feeding all affect the levels of secretory proteins in predictable ways.

Studies in which the diets of immature animals are altered have shown that the changes in concentrations of various pancreatic enzymes are not all preprogrammed. The molecular basis for many of these effects has been reviewed.[70] Changing the diet from high fat–low carbohydrate to high carbohydrate–low fat by early weaning increases chymotrypsin and lipase and decreases amylase. Delaying weaning, by prolonged nursing, postpones these changes in enzyme concentration. Animals weaned on a high-fat diet similar to mother's milk demonstrated changes in enzyme patterns similar to those found in animals prematurely weaned. The changes found with early weaning are similar to those induced with glucocorticoids. Premature weaning induces an increase in corticosteroid levels in the infant rat. Changes in diet after 21 days of age induce characteristic changes in pancreatic enzyme composition; thus increasing the dietary intake of starch increases amylase, increasing intake of fat increases lipase, and increasing protein intake increases trypsin. Thus glucocorticoids may modulate some effects of feeding; however, others seem to be preprogrammed.

REGULATORY FACTORS

Plasma CCK (CCK-8 + CCK-33 + CCK-39) levels were measured before and after breast-feeding in 4-day-old infants.[71] A significant increase was seen immediately after a meal, followed by a decrease to basal levels at 10 minutes and a secondary increase at 30 and 60 minutes after feeding. This biphasic rise is not seen in older children or adults. Plasma gastrin-34 levels increased by 50% 5 and 10 minutes after the onset of suckling and immediately after breast-feeding in 3-day-old infants. Postprandial levels of 11 regulatory hormones were measured by Salmenperä and colleagues[72] in 9-month-old infants who had been fed exclusively by either breast or bottle. The basal level and postprandial rise in CCK were lower in breast-fed than in bottle-fed infants.

In the rat colon, CCK mRNA is found in low amounts in the fetus, is absent at birth, and then increases steadily until adulthood.[73] Immediately after birth, the rat small intestine contains CCK-like bioactivity. In the pig, CCK immunoreactivity is found in the small intestine at 6 to 8 weeks gestation.[74] In the guinea pig, plasma CCK levels are low at birth but rise to near adult levels at day 15.[75] Thus CCK is present in the gut and is presumably available to participate in the regulation of pancreatic growth in the fetus.

Progastrin and gastrin are present in many species, including humans.[76] In the human fetal pancreas, the levels of gastrin and progastrin are low when compared with those in other species. In the mouse, the gastrin gene is first expressed at 9.5 to 10 days gestation, whereas immunoreactivity does not develop until day 20. The gastrin gene is unique among those expressed by the pancreas in that it is active in the fetus but inactive in the adult, except when the Zollinger-Ellison syndrome develops. Thus CCK and gastrin, both trophic agents for the pancreas, are present at birth when the rate of pancreatic growth is the greatest.

CCK controls gene expression at the translational level.[77] Long-term stimulation with CCK and cerulean (a CCK analogue) stimulates mRNA synthesis for genes coding for trypsinogen and chymotrypsinogen but not amylase.[78]

Pancreatic growth in the mature animal can be altered by a variety of agents, diets, and experimental procedures.[79] The trophic effects of CCK, gastrin, and CCK-like peptides have been well documented. In the adult rat, the postprandial CCK level regulates ornithine decarboxylase and secretory protein gene expression at the translational level. Treatment of rats with the CCK antagonist L364718 not only prevents the trophic effects of exogenous CCK but also causes pancreatic atrophy, suggesting that CCK is necessary for both growth and maintenance of the gland.[80] When given to the immature rat, this agent does not alter normal growth but does block the trophic effects of exogenous

CCK.[81] CCK-induced growth is mediated by a single-type variety of type A receptors with both high- and low-affinity states.[82-85]

Although published studies exist concerning the roles of many of the previously discussed agents and treatments in the immature pancreas, interpretation and comparison of these studies present numerous difficulties in data analysis, including that (1) the various agents tested were given in different doses, at different ages, and for different lengths of time and (2) the animals were evaluated for effects on growth in different ways. A major fact often overlooked is that the growth rate and the rates of synthesis of both DNA and protein vary considerably with developmental ages.[86]

The concentrations of DNA and protein in immature rat pancreas do not parallel the rates of their synthesis.[86] DNA concentration increases rapidly from a low level on the first day of life, peaks between ages 3 and 14 days, then slowly falls to a lower adult level. In contrast, DNA synthesis decreases in the first 24 hours, peaks at age 3 days at a rate 30 times that observed in the adult, then falls slowly to the adult value. Similarly, protein concentration, nearly as high at birth as in the adult, plummets at day 3 before it slowly begins to rise to adult levels. Protein synthesis, in contrast, is as low at birth (as it is in the adult) but is three to five times higher at all intermediate ages tested.

A great deal has been learned concerning the effects of many agents on the growth of the exocrine pancreas in the developing animal. CCK and CCK-like peptides have a variety of effects that seem to be age dependent. DNA synthesis is not increased by CCK before age 28 days, and variable effects on growth have been described depending on age, dosage, and CCK analogue studied.[87-89] The potent specific CCK antagonist L364718 blocks the effects of exogenous cerulean on the growth of the neonatal rat pancreas, but when given alone it has no effect. This suggests that endogenous CCK is not a controlling factor in the growth of the neonatal rat pancreas.[90]

CCK action is mediated by CCK receptors. Of the two types of CCK receptors (A and B), the adult rat pancreas has only type A, which mediates pancreatic growth in adult rats. Although the Otsuka Long-Evans Tokushima fatty rats lack the CCK-A receptor gene, pancreatic growth in the immediate postnatal life is normal although the pancreas is slightly smaller at 5 to 6 weeks of age.[91] However, in later growth at 24 to 25 weeks, Otsuka Long-Evans Tokushima fatty rats showed a significantly lower protein-DNA content than the wild-type strain, which suggests that the CCK-A receptor plays a small role in the early postnatal growth of the pancreas but is required for later cell growth. Thus CCK might not be important in regulating pancreatic growth in early postnatal life.

The role of gastrin is likewise questioned. Administration of a specific gastrin antagonist, CI-988, to neonates for 5 days reduced stomach growth but not pancreatic growth.[92] Thus although pharmacologic doses of gastrin and CCK are trophic for the neonatal exocrine pancreas, it is unclear whether they play a role in the early development of the exocrine pancreas.

Hydrocortisone decreases protein synthesis in the first week of life but has no effect on DNA synthesis in the immature animal.[87] Other studies have described hypertrophy and hyperplasia or hypertrophy alone,[88,93] depending on age. Cerulean and hydrocortisone potentiate each other's trophic effects. The lack of increase in DNA synthesis in the suckling animal after treatment with either CCK or hydrocortisone may be due to the finding that synthesis is already proceeding at the maximum rate possible.

A temporal relationship exists between corticosterone levels, cytoplasmic corticosteroid receptors, and increases in pancreatic secretory products in the developing rat pancreas.[93,94] Corticosterone levels and dexamethasone binding increase from birth, reach a peak at approximately age 25 days, then decrease to adult levels. Increases in circulating levels of steroids precede increases in receptor density, which increases sharply after age 15 days. These increases parallel those of both amylase and hydrolase activities, which can be induced by exogenous steroids. The pancreatic glucocorticoid receptor is under autologous control by glucocorticoid. Early weaning, which augments corticosterone levels, causes similar changes in the content of exportable proteins in the pancreas. The highest density of steroid receptors occurs at age 21 to 28 days, the period of peak responsiveness of the pancreas to hydrocortisone. Thus glucocorticoids clearly modulate postnatal pancreatic development.[95]

Thyroxine induces precocious increases of amylase, lipase, chymotrypsin, and trypsin in neonatal rats, and chemical thyroidectomy retards pancreatic development.[96] Endogenous thyroxine peaks between days 10 and 16, the time point when amylase levels are also increasing. Thyroxine induces maturation of secretory function by modulating the maximum binding capacity of high-affinity CCK receptors. In the immature animal, thyroid hormone acts both directly on the pancreatic acinar cell and indirectly through the adrenal system.[97]

Pancreatic duct cells have high levels of epidermal growth factor.[98] Parenteral but not oral epidermal growth factor increases pancreatic amylase in suckling rabbits.[99] Inhibition of ornithine decarboxylase (and thus polyamine metabolism) inhibits stimulated pancreatic growth.[100] The role of polyamines in normal pancreatic growth and development is not established. Secretin increases DNA, amylase, and chymotrypsin in 6-day-old rats.[101] The effects of peptide YY (PYY) and somatostatin, which are antitrophic to the pancreas in mature animals, have not been studied in developing animals.

Other regulatory factors have also been implicated in rodent pancreatic exocrine development. Acting directly on the pancreas, bombesin induces hypertrophy and hyperplasia in newborn rats by.[102] In adult animals bombesin regulates pancreatic gene expression at the level of mRNA. This effect is not mediated by CCK.[103,104] Transforming growth factor (TGF) alpha appears after birth and increases progressively with age in the exocrine pancreas.[105] Artificially mutated TGF-β receptor in mice leads to a pancreas with acinar hyperplasia and atypia.[106] Overexpression of TGF-β interferes with acinar differentiation.[107] TGF-β added to rat embryonic pancreatic rudiments resulted in differential gene activation.[108] Fibroblast growth factors (FGFs) and FGF receptors (FGFRs) are important mediators of epithelial-mesenchyme interactions. FGFs are expressed throughout rat pancreatic development. In mesenchyme-free culture of embryonic pancreatic epithelium, addition of FGFs promotes growth, morphogenesis, and cytodifferentiation of exocrine pancreatic cells.[109] In the presence of mesenchyme, growth and development of pancreatic epithelium progresses without the addition of FGFs. Abrogation of FGFR-2IIIb in the system attenuates both growth and development.

FETAL ANTIGENS

Fetoacinar protein (FAP) is a specific acinar cell antigen found only in the exocrine pancreas. In the human fetus, FAP is present at 9 to 10 weeks gestation[110] and peaks at 15 to 25 weeks when acinar cell proliferation is most intense. FAP synthesis then progressively decreases. The levels found in adults are lower than those in the fetus. FAP is found in high concentration in amniotic fluid. FAP can also be considered an oncodevelopmental antigen, because in pancreatic cancer and in some cases of chronic pancreatitis FAP is elevated. FAP has been shown to be a variant of bile salt–dependent lipase, differing only by a decrease in O-glycosylation.[111]

A second fetal antigen, fetal antigen 1 (FA1), is found in both the exocrine and endocrine fetal pancreas and hepatocytes.[112] FA1 is found in high concentration in the fetal venous blood and amniotic fluid during the second and third trimesters. At 7 weeks

gestation, FA1 is found in 94% of the ductal epithelial cells. By week 17, only 64% of the duct cells test positive for FA1 by immunoperoxidase staining, decreasing to only 11% in the 4-month-old infant. The role or roles and controls of FA1 in pancreatic development are not known.

MOLECULAR EMBRYOLOGY

The parenchyma of both endocrine and exocrine glands originate from the endodermal epithelium of the primitive gut tube[113] (Figure 114-3). Even before the pancreatic primordia evaginate from the primitive gut tube, many molecular programs that specify a pancreatic fate have been set in motion by the inductive influences of neighboring tissues. During gastrulation, the endoderm is subdivided into anterior and posterior domains by signals from the adjacent mesectoderm. Retinoic acid,[114] bone morphogenic proteins, and FGFs[115] play an important role during late gastrulation in localizing the prepancreatic domain, which will respond to subsequent instructive signals that induce pancreatic tissue.

The ventral and dorsal aspects of the endoderm that will give rise to the pancreas are exposed to different local contacts and therefore rely on distinct sources for their induction. The endoderm fated to become the ventral pancreas begins to express pancreatic genes first, induced by activin and bone morphogenic protein–family molecules derived from the adjacent lateral plate mesoderm.[116] The dorsal pancreas is induced by transient contact with the notochord, followed by interposition of the dorsal aorta. Contact with the notochord induces a localized exclusion of sonic hedgehog (shh) from the prepancreatic endoderm. This signaling event is necessary for dorsal pancreatic development, because ectopic expression of shh interferes with subsequent events.[117] The dorsal aorta also provides essential signals to promote pancreatic fate,[118] among them vascular endothelial growth factor.[119]

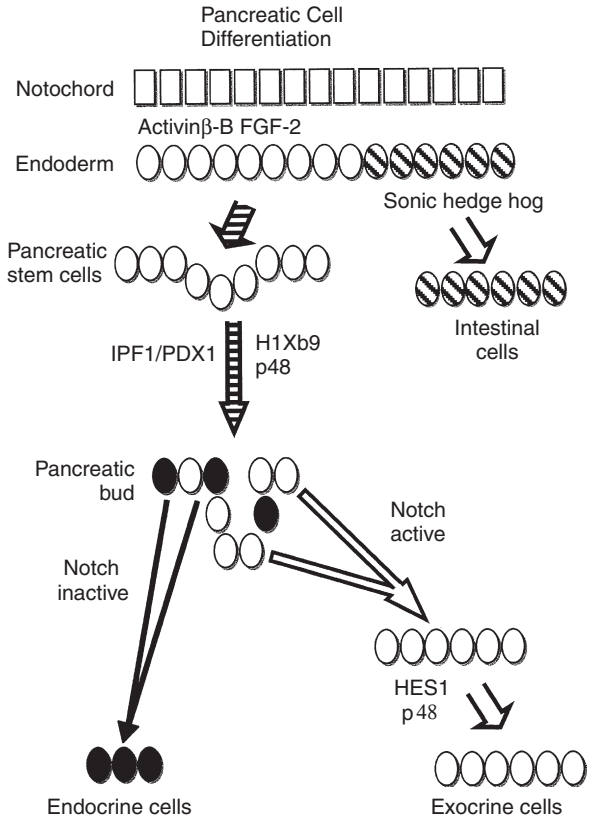


Figure 114-3 Origin of the exocrine and endocrine cell lineages in the developing pancreas. This model is based on descriptions from various reports (e.g., see Kim and Hebrok[143] and Crisera and colleagues[144]). *Arrows* in the diagram indicate the sequential activities based on the appearance of specific cell markers in the developing mouse pancreas. *(Modified from Edlund H: Pancreas: how to get there from the gut?* Curr Opin Cell Biol *11:663-8, 1999.)*

These early events render the prepancreatic endoderm competent to form pancreatic tissue. The next step involves expression of transcription factors in the endoderm and in the surrounding mesenchyme to further specify a pancreatic fate. One of the essential genes is Hlx9, encoding the homeobox protein HB9. Its genetic ablation results in selective absence of the dorsal pancreas.[120] Highlighting the role of the surrounding mesenchyme, the LIM homeodomain protein Isl1 is expressed in the dorsal pancreatic mesenchyme and is required for its elaboration. In the absence of Isl1, the dorsal pancreas does not form, but the ventral pancreas is unaffected.[121]

The pancreatic-duodenal homeobox gene 1(Pdx-1) gene is expressed in the prepancreatic endoderm at e8.5 in the mouse,[122] and its ablation results in arrest of pancreatic development shortly after formation of the pancreatic anlagen.[123] A mutation in the human Pdx-1 gene was found to cause congenital pancreatic agenesis in an infant.[124] The function of Pdx-1 is instructive, as evidenced by the ability to transform liver into pancreas by hepatic expression of the *Xenopus* Pdx-1 homologue fused to the VP16 transactivator.[125] Pdx-1 also has a later role in the maintenance of beta-cell identity and the transcription of the insulin gene.

The transcription factor PTF1a is expressed in the early pancreas around E9.5, after Pdx-1 and in a domain more narrowly approximating the developing pancreas. Genetic ablation of PTF1a has provided important insight into the early events of pancreatic specification. Normally, all the PTF1a-expressing cells will be found in both the endocrine and exocrine pancreas. By genetically tagging the mutant cells that would otherwise express PTF1a, cells lacking the gene were shown to be incorporated into the developing duodenum as full-fledged intestinal epithelial cells.[126] This illustrates the developmental plasticity of early pancreatic development and highlights the principle that multiple successive specification events are needed to guide cells from progenitor to a fully differentiated state.

After specification of pancreatic cell fate and initial budding of the pancreatic primordium, the pancreatic precursor cells become diversified into exocrine and endocrine lines. The molecular effectors that influence these events have been deciphered in recent years, revealing a striking conservation of mechanism for cell fate allocation between different organ systems. Recent data have demonstrated a role for retinoic acid in the exocrine-endocrine cell fate decision, possibly upstream of Notch signaling (see later).[127] Of interest, retinoic acid treatment seems to have divergent effects on the dorsal and ventral pancreas, promoting endocrine fate in the dorsal pancreas but exocrine fate in the ventral pancreas. These data stand as further evidence for distinct developmental programs in the dorsal and ventral primordia.

Taking a cue from earlier work in neuronal cell fate allocation,[128] researchers examined Notch signaling pathway mutant mice and discovered that Notch signaling is a key participant in the exocrine-endocrine cell fate allocation decision. Specifically, it was previously known that the basic helix-loop-helix (bHLH) transcription factor Neurogenin3 (Ngn3) is required for all endocrine cell lineages.[129] Ngn3 activates expression of Notch ligands Delta, Serrate, and Jagged, which then activate Notch signaling on adjacent cells. This signal results in expression of Hes-1, which in turn represses Ngn and other target genes. Thus

an undifferentiated pancreatic cell fated to become an islet cell will signal to its immediate neighbors to adopt an exocrine fate. By this mechanism, known as lateral inhibition, Notch signaling promotes the divergence of the pancreatic lineage into exocrine and endocrine fates.[130] Subsequent cell fate decisions further diversify the endocrine cells into the four lineages known to populate the islets: alpha cells (glucagon secreting), beta cells (insulin secreting), delta cells (somatostatin secreting), and PP cells (pancreatic polypeptide). Each of these is derived from a common Ngn3-expressing precursor by a combinatorial hierarchy of transcription factors that promote a lineage-specific program.

Whereas Notch is required for early commitment to the exocrine lineage, continuous activation of Notch signaling can inhibit exocrine development as well.[131,132] The reason for this seemingly contradictory result is that Notch maintains pancreatic progenitors in an undifferentiated state and prevents their terminal differentiation. Thus Notch signaling reserves a population of cells for subsequent formation of the exocrine gland, beginning around E13.5 in the mouse. Outgrowth of the pancreas is closely linked to exocrine morphogenesis and differentiation, but the essential factors that influence this step remain partially characterized. Factors secreted from the mesenchyme that stimulate exocrine growth include FGF 10 (Fgf10)[133] and epidermal growth factor (Egf).[134] Pbx[135] is a transcription factor the genetic ablation of which can be complemented by exogenous wild-type mesenchyme, thus implicating this factor as a mesenchymal mediator of pancreatic outgrowth. On the basis of the detection of dephospho-β-catenin, the Wnt signaling pathway was shown to be activated in the pancreatic anlage.[136] Using a conditional knockout strategy, Murtaugh and colleagues[136] showed that specification of Ptf1a-positive exocrine pancreatic progenitors was dependent on β-catenin. In contrast, the endocrine lineages were not affected by loss of β-catenin. Consistent with this result, attenuation of Wnt signaling by expressing a dominant-negative Wnt receptor affects pancreatic growth, but endocrine function remains normal.[137]

Compared with the islet cells, considerably less is known about the molecular determinants of exocrine pancreatic differentiation. As such, only two factors are known to be specifically required for exocrine differentiation: Ptf1a and Mist1. Ptf1 consists of a heterodimer comprising the ubiquitous transcriptional regulator E2A and a tissue-specific protein p48.[138] This complex binds to the promoter regions of exocrine-specific genes and activates their transcription. Genetic ablation of p48 in mice results in failure to form an exocrine pancreas, as well as morphologic deformities of the islets.[139] More recent studies using Ptf1a-Cre knock-in mice have broadened our understanding of the role for p48 to include a much earlier function in specifying pancreatic identity from uncommitted endodermal precursors.[126]

Mist1 is also a bHLH transcription factor expressed in the exocrine pancreas but also in salivary gland and stomach.[140] *Mist1*-null mice exhibit lesions in the exocrine pancreas, possibly resulting from defective cell-cell adhesion, but still retain exocrine-specific gene expression.[141] Mist1 has thus been proposed to function in cooperation with PTF1 to maintain a stable exocrine differentiated state. Mist1 is also required for granule organization in acinar and other exocrine cell types.[142]

In summary, the exocrine pancreas is a relatively late–developing organ in the scheme of embryonic development. Its formation is contingent on the successive execution of multiple earlier steps, from specification of pancreatic cell fate in the endoderm, to cell lineage allocation, induction of outgrowth, and then completion of the differentiation program. In the last several years genetic technology has helped to provide molecular annotation of these events, offering novel connections between genes and developmental mechanisms.

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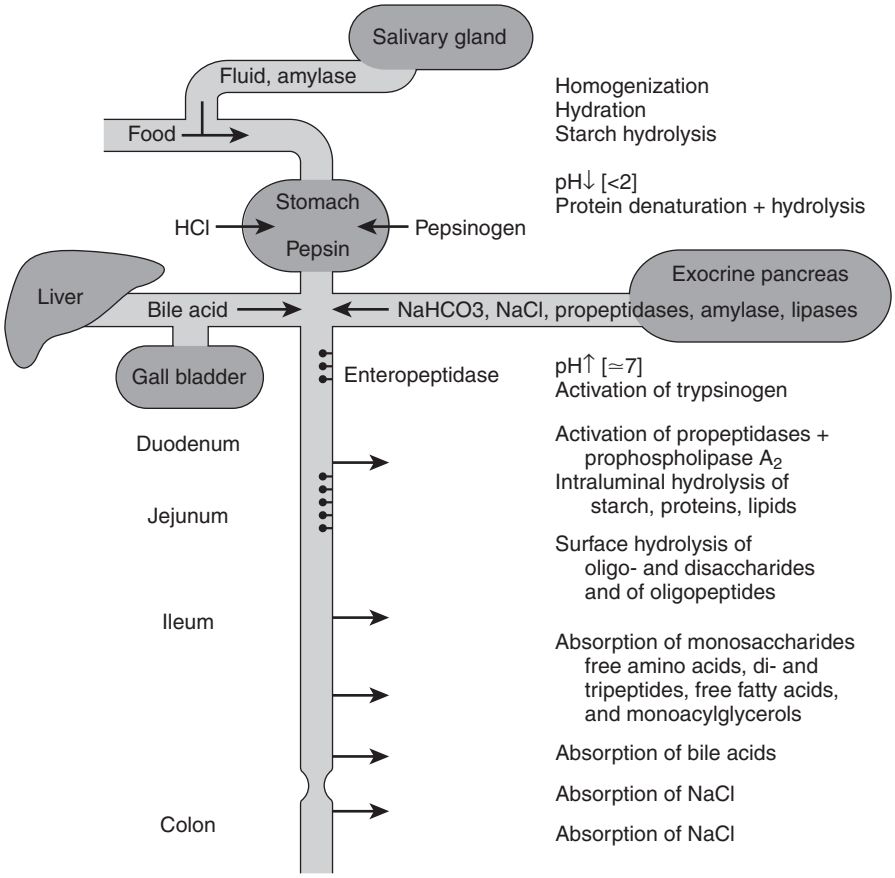


Figure 115-1 Overview of digestion and absorption. *(Modified from Johnson LR [ed]:* Physiology of the Gastrointestinal Tract. *New York, 1994, Raven Press, pp 1751-1772.)*

Developmental Aspects of Protein Digestion and Absorption

Digestion

Gastric Acidity

The first traces of gastric acidity appear in 4-month-old fetuses.[5] The human fetus has the potential to produce gastric acid and gastrin from the middle of the second trimester. Parietal cell activity is present in the body, antrum, and pyloric regions in the fetus from 13 to 28 weeks.[5-7] When term and premature infants were compared, hydrochloric acid secretion was found to be much lower in premature infants than in term infants.[8] No change was seen in basal and pentagastrin-stimulated output of acid in term neonates between days 1 and 2 of life; furthermore, the basal acid secretion was no different from the stimulated one.[9] Thus gastric acid secretion in the newborn infant is maximal under basal conditions. The other possibility is that newborn parietal cells are unresponsive to pentagastrin.

Gastric acid secretion is limited in very-low-birth-weight infants. However, both basal and pentagastrin-stimulated acid secretion doubles from the first to fourth week of postnatal life in preterm infants.[10] The actual pH of the stomach contents in infants is substantially influenced by food intake. The entry of milk into the infant's stomach causes a sharp increase in the pH of the gastric contents and a slower return to lower pH values than in older children and adults.[11]

Gastric Proteolytic Activity

Proteolytic activity is present in fetuses older than 16 weeks gestation.[12] Pepsinogen and cathepsin do not appear until 17 to 18 weeks gestation.[13] Peptic activity of newborn infants is low and is in proportion to the degree of maturity.[14] It increases in the fundus about four-fold after food intake on the second day of postnatal life.[15]

The output of pepsin is low in the newborn infant and increases until the third postnatal month. The range of values found in the second and third postnatal month is less than the range of adult values.[16] In contrast, pepsin activity in biopsy specimens from the stomachs of infants and children did not change between the ages of 6 months and 15 years.[17] Formula feeding evokes an increase of pepsin activity in the stomach content of 3- to 4-week-old, orogastrically fed, premature infants.[18]

Pancreatic Proteolytic Activity

The protease cascade in the small intestine is catalyzed by food-stimulated secretion of enterokinase from the upper small-intestinal epithelium (see Figure 115-1 and Box 115-1). This enzyme catalyzes the activation of trypsinogen to trypsin that, in turn, activates several other inactive zymogens into proteases, which are active in the intestinal lumen. However, even though enterokinase is detectable at 24 weeks gestation, its concentration is relatively low and reaches only 25% of adult activity at term.[19] This theoretically can be limiting to protein digestion and may be responsible for an increased capability of larger antigens

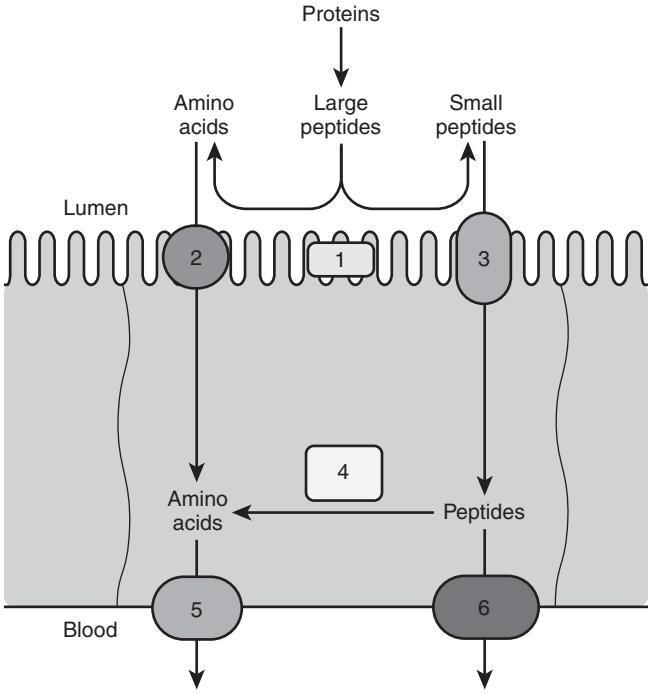


Figure 115-2 Protein absorption in the small intestine. *1,* Brush border membrane peptidases; *2,* brush border membrane amino acid transporters; *3,* brush border membrane dipeptide and tripeptide transporters; *4,* intracellular peptidases; *5,* basolateral membrane amino acid carriers; *6,* basolateral membrane dipeptide and tripeptide carriers. *(Modified from Johnson LR, editor:* Physiology of the Gastrointestinal Tract. *New York, 1994, Raven Press, pp 1751-1772.)*

BOX 115-1

Protein Digestive Processes

Stomach
Proteolytic enzymes contained in gastric juice
Requires acid environment of stomach to hydrolyze protein
Synthesized in the gastric chief cells as inactive pre-proenzymes (pepsinogen)

Pancreas
Enterokinase—an intestinal brush border enzyme that activates pancreatic proteases. Stimulated by trypsinogen contained in pancreatic juice.

Pancreatic endopeptidases
Trypsin: cleaves peptide bonds on the carboxyl side of basic amino acids (lysine and arginine)
Chymotrypsin: cleaves peptide bonds on the carboxyl side of aromatic amino acids (tyrosine, phenylalanine, and tryptophan)
Elastase: cleaves peptide bonds on the carboxyl side of aliphatic amino acids (alanine, leucine, glycine, valine, isoleucine)

Pancreatic exopeptidases
Carboxypeptidases A and B: zinc-containing metalloenzymes that remove single amino acids from the carboxyl-terminal ends of proteins and peptides
Carboxypeptidase A: polypeptides with free carboxyl groups are cleaved to lower peptides and aromatic amino acids
Carboxypeptidase B: polypeptides with free carboxyl groups are cleaved to lower peptides and dibasic amino acids

Modified from Johnson LR, editor: *Physiology of the Gastrointestinal Tract.* New York, 1994, Raven Press, pp 1751-1772.

or microorganisms to pass into the intestine without breakdown by luminal enzymes.

Pancreatic enzymes begin to form at about the third fetal month,[12] and pancreatic secretion starts at the beginning of the fifth month of gestation. Biochemical studies on the activity of trypsinogen and chymotrypsinogen at various gestational ages have been reported.[20] Immunoreactive trypsin 1 and immunoreactive chymotrypsin A are detectable in amniotic fluids at 17 to 18 weeks of pregnancy.[21] Levels of trypsin concentration encountered during the first 2 years of life are reached by the age of 3 months. Chymotryptic activity was low in the newborn infant (3 days of age) and increased gradually, approaching the levels of older children at approximately 6 months.[22] From birth onward, the concentration of chymotrypsin (after pancreozymin-secretin stimulation) increases approximately three-fold and reaches adult levels in 3-year-old children. Serial measurement of fecal chymotrypsin concentrations in preterm infants (23 to 32 weeks gestation) during the first 4 weeks of life demonstrated values generally similar to those found in term infants. Median fecal chymotrypsin concentrations calculated for each infant (with use of data from stools passed between day 2 and day 12 of life) have been shown to be lower in infants who were small for gestational age when compared with those who were appropriate for gestational age.[23] Premature infants fed soy-based formula for 1 month exhibited higher trypsin activity after cholecystokinin-pancreozymin stimulation than did those fed a milk-based formula.[24]

Absorption

For a very few days after birth, neonates have the ability to absorb intact proteins. This ability, which is rapidly lost, is of importance because it allows the newborn animal to acquire passive immunity by absorbing immunoglobulins in colostral milk. The small intestine rapidly loses the capacity to absorb intact proteins—a process called closure—and, consequently, animals that do not receive colostrum within the first few days after birth will likely die of opportunistic infections.

Otherwise, absorption of intact proteins occurs only in a few circumstances.[25] Preterm neonates, particularly those of less than 33 weeks gestation, have higher serum concentrations of β-lactoglobulin than term neonates given equivalent feeding. Thus the ability of the gastrointestinal tract to exclude antigenically intact food proteins increases with gestational age, and gut closure occurs normally before birth in humans.[25] With use of lactulose-to-mannitol ratios, in preterm infants (26-36 weeks gestation) intestinal permeability was not related to gestational age or birth weight but was higher during the first 2 days of life than 3 to 6 days later. It is higher in preterm infants than in healthy term infants only if measured within 2 days of birth. This suggests rapid postnatal adaptation of the small intestine in preterm infants.[26]

Proteolytic and Peptidase Activity

During fetal development, protease and aminopeptidase activity in the jejunum does not change markedly between weeks 8 and 17 of gestation, but in the ileum a pronounced increase occurs.[27] In the embryonal developmental period (to week 8), the activity of proteases was mainly detected on the luminal surface of the primitive pseudostratified columnar epithelium of the intestine anlage. The highest activity was displayed by dipeptyl peptidase IV (DPP IV). Beginning with week 8, villi are formed from the duodenum up to the ileum, and, after week 9, differentiation of crypts of Lieberkühn is observed. The activity of proteases was

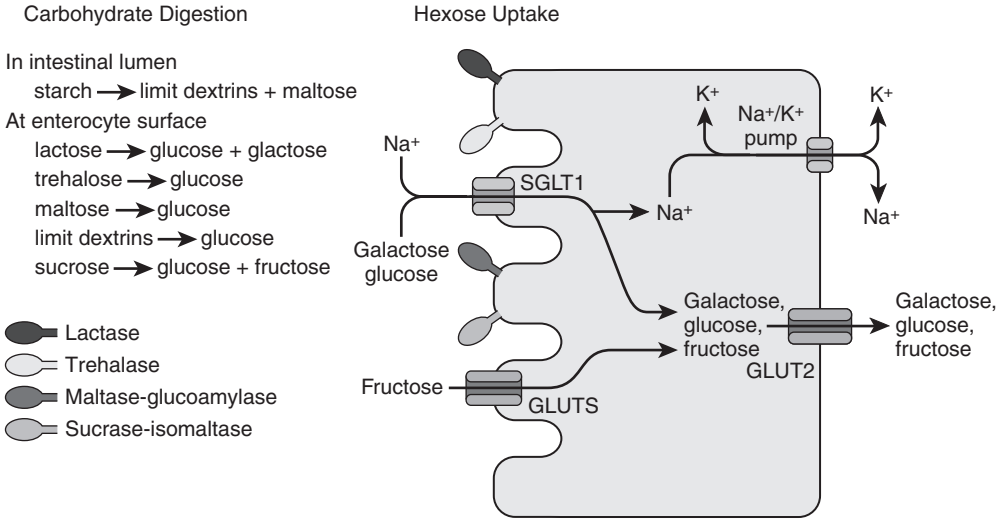


Figure 115-3 Digestion and absorption of carbohydrates.

high (especially DPP IV) in the differentiating microvillus zone of primitive enterocytes.The gradient of apex-base activity of the villus is maximal on the apex of the villi. In one study, brush border and intracellular proteolytic enzyme activities were measured in fetuses (8-22 weeks gestation), children (7 months–14 years), and adults. The peptidase activities in all three of the groups were comparable, suggesting that the small intestine of the term and preterm newborn should be able to efficiently digest peptides.[28]

Proteins are present in the amniotic fluid. Approximately 50% of its volume is swallowed by the fetus daily[29]; thus the protein amount obtainable at or near term from the amniotic fluid is approximately one fifth of the daily protein gain by the fetus.A fairly wide spectrum of protein is transferred to the fetus by this process and includes human serum albumin, immunoglobulin G, immunoglobulin A, chorionic gonadotropin, and growth hormone.[29]

Various studies have indicated increased small intestinal permeability for intact food proteins during the neonatal period,[25] but passage of proteins has also been demonstrated in older children and even in adults.[30] The serum of infants contains a higher percentage of antibodies to food antigens than does the serum of adults,[31] which suggests that food proteins are absorbed intact in sufficient quantities for an immunologic response. On the basis of determination of serum concentration, the absorption of β-lactoglobulin was considered to be higher in preterm infants than in term infants.[25]

Clinical Correlations

Acid secretion limitations in premature infants should be kept in mind when considering the use of histamine-2 (H_2) blockers, which are widely prescribed in many neonatal intensive care units. Studies suggest that critically ill premature infants who receive treatment with H_2 blockers have a higher incidence of nosocomial sepsis and necrotizing enterocolitis.[32,33] Although speculative, it is possible that, with the already limited hydrogen ion production in the stomach of the premature infant, additional blockage further diminishes the acid barrier to microorganisms and allows for a higher load of bacteria in the more distal regions of the intestine.

In terms of protein absorption, the mechanisms for brush border hydrolysis are present early, dipeptides and tripeptides are absorbed faster than amino acids, and protein digestion and absorption rarely appear to be an issue in premature infants.[34]

Despite the potential limitations of digesting and enzymatic capability in premature infants, data showing significant benefits with use of hydrolyzed protein fractions appear to offer only minimal advantage over whole protein formulas.[32,35] Studies have yet to demonstrate a benefit of hydrolyzed protein formulas over human milk.

Hydrolyzed formulas are also extensively prescribed to prevent allergic and atopic disease. However, a recent Cochrane metaanalysis showed no evidence to support feeding with a hydrolyzed formula for the prevention of allergy compared with exclusive breast-feeding. Furthermore, in high-risk infants who are unable to be completely breast-fed, evidence is limited that prolonged feeding with a hydrolyzed formula compared with a cow's milk formula reduces infant and childhood allergy and infant cow's milk allergy.[36]

CARBOHYDRATE DIGESTION AND ABSORPTION

General

The overall digestion and absorption of carbohydrates is illustrated in Figures 115-1 and 115-3 and Box 115-2. Starches and complex carbohydrates must first be hydrolyzed to oligosaccharides by digestive processes in the mouth, stomach, and intestinal lumen. This is accomplished primarily via salivary and pancreatic amylases. Oligosaccharides must then be hydrolyzed at the epithelial brush border to monosaccharides before absorption, and the key catalysts in these processes are the brush border hydrolases, which include maltase, lactase, and sucrase. Dietary lactose, sucrose, and maltose come in contact with the surface of absorptive epithelial cells covering the villi where they engage with brush border hydrolases:

- Maltase cleaves maltose into two molecules of glucose.
- Lactase cleaves lactose into a glucose and a galactose.
- Sucrase cleaves sucrose into a glucose and a fructose.

Glucose and galactose are taken into the enterocyte by cotransport with sodium with use of the same transporter. Fructose enters the cell from the intestinal lumen via facilitated diffusion through another transporter.

Developmental Aspects of Carbohydrate Digestion and Absorption

Carbohydrate Digestion

In addition to the classic enzymes capable of digesting starches and glucose polymers (i.e., salivary and pancreatic α-amylase, intestinal glucoamylase), an enzyme present in breast milk, α-amylase, may participate in digestion. A high amylase activity

BOX 115-2

Monosaccharide Transport

Glucose uptake is Na^+ dependent.
Fructose is absorbed via facilitated diffusion.
Galactose and glucose are actively transported.

1. SGLT1 is the transport protein responsible for Na^+-dependent glucose transport.
2. GLUT2 transports glucose out of the cell and into the portal circulation.

exists in preterm colostrum, which slowly decreases during the first 2 months.[37-39] The individual variation in amylase activity is considerable. No difference is seen in amylase activity in preterm and term human milk. The isoamylase of preterm milk is of the salivary type, just as in term milk. Variation in amylase activity is not great during a feeding or from one feeding to another.[37,40] This can survive the relatively mild acidity and the lower activity of pepsin in the stomach of the newborn infant. Amylase in saliva is present in lower concentrations in children than in adults.[41]

Pancreatic amylase activity has been demonstrated in amniotic fluid and pancreatic tissue from 14- to 16-week-old fetuses.[42,43] Although salivary amylase activity rapidly increases shortly after term birth, pancreatic amylase remains low until 3 months and does not reach adult levels until nearly 2 years of age.[44]

The digestion of starch in the newborn infant has been examined by both carbohydrate tolerance type and balance studies. Gastric administration of a starch solution results in a slow, small increase in blood glucose levels in neonates; an equicaloric amount of glucose solution administered to the same children evokes a greater, earlier increase, with an earlier return to starting values within 120 minutes.[45]

Carbohydrate Absorption

Fetus

Activity of sucrase and lactase is lower in young fetuses than in specimens from the small-intestinal mucosa of adults.[19] Sucrase activity is present in the fetal colon and disappears before birth.[46] The presence of lactase in the fetal colon (13 to 20 weeks of age) has also been described previously.[19]

Sucrase-isomaltase in the human fetal intestine is present in a different form from that in adults, and it differs in degree of glycosylation (different electrophoretic mobility) and in the size of the polypeptide.[47] The difference in polypeptide length in fetuses and adults can be related to low activity of pancreatic proteases in the fetal intestinal lumen. Villa and co-workers[48] confirmed that intestinal lactase is low between 14 and 20 weeks gestation and exhibits a relatively high level of activity at 37 weeks; amounts of lactase messenger RNA (mRNA) correlated with the enzymatic activity. It is interesting that lactase mRNA was not detectable in the colon of normal adult subjects, whereas it was detectable at low levels in fetal colon.[49]

Postnatal

Studies suggest that colonic fermentation activity is adequate for colonic salvage of lactose even during the second week of life. Using a stable isotope method for serial assessment of lactose carbon assimilation, Kien[50] demonstrated efficient absorption of lactose in premature infants (30 to 32 weeks gestation and 11 to 36 days of age). In spite of that finding, in a study in 130 preterm infants fed standard preterm formula with and without lactase, lactase-treated infants were shown to grow faster the first 10 days of life but similarly thereafter, suggesting that limitations in lactose absorption are short-lived in the preterm infant.[51] In fact, infants absorb 98% of lactose from lactose-containing formulas.

Glucoamylase, an intestinal brush border enzyme, removes glucose units from the nonreducing ends of molecules of starch and dextran. This enzyme, which is present in the developing small intestine,[52] exhibits activity in an infant at the age of 1 month[53] that is comparable to the activity in young adults.[54]

Absorption of Monosaccharides

Prenatal

Transport of hexose monosaccharides increases between weeks 10 and 18 of gestation.[55] Developmental and regional expression of mRNAs encoding sodium-dependent and facilitative glucose transporter proteins in human fetal and adult small intestine demonstrated an abundance of mRNAs encoding the sodium glucose cotransporter isoform SGLT1, and the facilitative glucose transporter isoforms GLUT2 and GLUT5 exhibited highest levels in adult small intestine, but the levels of GLUT1 mRNA were found to be higher in fetal than in adult small intestine.[56] Immunohistochemical analysis of the fetal small intestine showed that GLUT5 was localized along the intercellular junctions of the developing villus. Other studies[57] also demonstrated expression of sodium-dependent glucose transporter mRNA in the small intestine of fetuses between 9 and 18 weeks gestational age. The sodium-dependent glucose transporter mRNA is also expressed in the fetal colon but in lower levels than in the adult colon.

Postnatal

Glucose absorption in infants is less efficient than in adults. The kinetics of glucose absorption are related to gestational age and appear to be affected by diet and exposure to glucocorticoids.[58] Other studies demonstrated that carrier-mediated monosaccharide absorption increases the first 2 postnatal weeks in infants born at 28 to 30 weeks gestation.[59]

Clinical Correlations

Because pancreatic secretion is poorly developed in the first several months after birth, this mode of starch hydrolysis could serve as a limiting factor that leaves substantial undigested starch in the intestine. Many infant formulas, including those formulated for preterm infants, contain partially hydrolyzed starches. The more extensively the starch is hydrolyzed, the less reliance is placed on an immature digestive capability, but the greater the osmolality. Whether there is any advantage of these hydrolyzed starch formulas over those containing disaccharides or lactose has not been established. Some data suggest that glucose polymers (18 to 29 glucose units) can be hydrolyzed by salivary amylases, but this digestion still falls substantially short of that accomplished by the usual concentrations of pancreatic amylase.[60,61] Whether limitations in salivary or pancreatic amylase play a significant role in premature infants is questionable because the major carbohydrate in human milk or cow's milk–based formulas is lactose. Furthermore, many premature infants are fed by tube in the neonatal intensive care unit, a practice that, at least partially, bypasses the action of salivary amylase.

If a shortage of lactase activity in premature infants actually is present, the scenario illustrated in Figure 115-4 is likely to occur. Insufficient lactase activity results in unhydrolyzed lactose in the distal intestine, which in turn is then hydrolyzed by luminal microbes into short-chain fatty acids, which can be absorbed. One study in premature infants was designed to ascertain whether the timing of feeding initiation affected the development of intestinal lactase activity and whether clinical ramifications of lower lactase activity exist.[62] Early feeding increased intestinal lactase activity in preterm infants. Lactase activity is a marker of intestinal maturity and may influence clinical outcomes. Whether the effects of milk on lactase activity were due to the greater

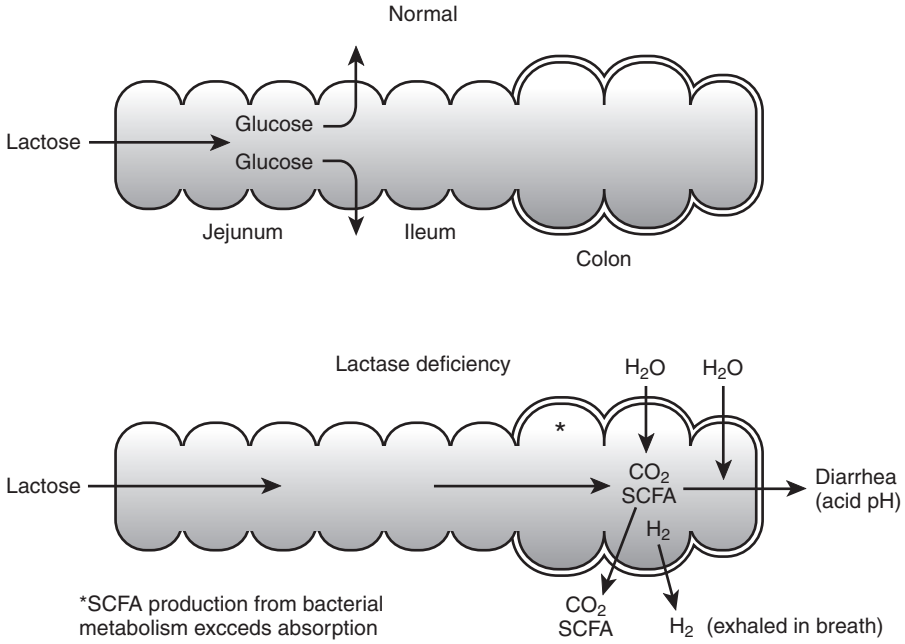


Figure 115-4 Lactose malabsorption. *SFCA,* Short-chain fatty acids.

concentration of lactose in human milk compared with that in formula has not yet been determined.[62]

The finding of low lactase activities in the intestines of fetuses has led to the notion that premature babies cannot tolerate lactose.[19] The presence of a high lactose concentration in human milk should not be a contraindication for its use in the very-low-birth-weight infant. Microbial salvage pathways that convert nonabsorbed lactose to short-chain fatty acids that can be absorbed and used for energy production are functional in these infants.[50] Furthermore, feedings for very-low-birth-weight infants rarely are initiated at levels intended to meet the infants' entire nutritional requirements and usually are advanced slowly. The rationale for using a lactose-free formula instead of human milk or even a commercial lactose-containing formula is weak and theoretically may be harmful. Slow initiation of enteral feedings is unlikely to exceed the lactose hydrolytic and salvage capability of the small and large intestines. This is especially unlikely when the quantity fed is less than 50% of the caloric requirement provided via the gastrointestinal tract (unless the infant has a bowel that has been radically shortened by surgery). Human milk also contains several lactose-derived oligosaccharides and other glycoconjugates that may play important roles in the infant's host defense. When formulas are provided for preterm infants, some evidence exists that, at full feedings, a combination of lactose and glucose polymers is better absorbed than lactose alone.[63] Furthermore, formulas that contain primarily glucose polymers appear to have a better calcium absorption in preterm infants.[64] However, a comparison with human milk, which contains primarily lactose, has not been reported.

Studies examining the crypt-to-villus gradient of intestinal carbohydrase activities demonstrate that most lactase activity is found at the mid to upper villus; sucrase, maltase, and glucoamylase are concentrated at the mid villus region.[65-68] This is likely to be pertinent to intestinal injury and villus damage. Lactase usually is the first enzyme to be lost and the last to be regenerated fully. Whether this necessitates a prolonged period of lactose-free feeding after intestinal injury (until the villus has undergone repair and restitution) remains debatable.

Infants in the neonatal intensive care unit undergo significant stresses. Chronic psychologic stress, such as maternal separation, impacts many functions of the gastrointestinal tract.[69] Studies using water immersion stress in rats have demonstrated that psychological stress induces major changes in glucose transport with a decrease in Na^+-dependent glucose absorption and an increase in GLUT2 expression at the brush border membrane level.[70] Whether similar changes occur in the sick neonate in the intensive care unit is not known, but the potential implications for nutrient absorption could be significant. Whether this contributes to feeding intolerance in these infants is unknown.

LIPID DIGESTION AND ABSORPTION

General

The bulk of dietary lipid is neutral fat or triglyceride, composed of a glycerol backbone with each carbon linked to a fatty acid via an ester moiety. Foodstuffs typically also contain phospholipids, sterols such as cholesterol, and many minor lipids, including fat-soluble vitamins. Finally, small intestinal contents contain lipids from sloughed epithelial cells and considerable cholesterol delivered in bile.

For the triglyceride to be absorbed, two processes must occur:

- Large aggregates of dietary triglyceride, which are virtually insoluble in an aqueous environment, must be broken down physically and held in suspension—a process called micellar emulsification (Figure 115-5).
- Triglyceride molecules must be enzymatically digested via triglyceride hydrolysis to yield monoglyceride and fatty acids, both of which can efficiently diffuse or be transported into the enterocyte (Figure 115-6).

The key mediators in these two transformations are *bile acids* and *lipases.* Bile acids are also necessary to solubilize other lipids, including cholesterol.

Emulsification, Hydrolysis, and Micelle Formation

Bile acids promote lipid emulsification. Bile acids have both hydrophilic and hydrophobic domains (i.e., they are amphipathic). On exposure to a large aggregate of triglyceride, the hydrophobic portions of bile acids intercalate into the lipid, with the hydrophilic domains remaining at the surface. Such coating with

bile acids aids in breakdown of large aggregates or droplets into smaller and smaller droplets. For a given volume of lipid, the smaller the droplet size, the greater the surface area, which provides greater surface area for interaction with lipase. Hydrolysis of triglyceride into monoglyceride and free fatty acids is accomplished predominantly by pancreatic lipase. The activity of this enzyme is to clip the fatty acids at positions 1 and 3 of the triglyceride, leaving two free fatty acids and a 2-monoglyceride. As monoglycerides and fatty acids are liberated through the action of lipase, they retain their association with bile acids and complex with other lipids to form micelles, which are small aggregates (4-8 nm in diameter) of mixed lipids and bile acids suspended within the ingesta. Micelles, providing much greater lipid surface area than the original fat globule, allow for amplified interaction with the brush border of small intestinal enterocytes, where the monoglyceride and fatty acids are taken up into the epithelial cells.

The major products of lipid digestion—fatty acids and 2-monoglycerides—enter the enterocyte by simple diffusion across the plasma membrane. A considerable fraction of the fatty acids also enter the enterocyte via a specific fatty acid transporter protein in the membrane.

Lipids are transported from the enterocyte into blood by a mechanism distinctly different from that of monosaccharides and amino acids. This process is dependent on the chain length of the fatty acids. Once inside the enterocyte, long-chain fatty acids and monoglyceride are transported into the endoplasmic reticulum, where they are used to resynthesize triglyceride. Beginning in the endoplasmic reticulum and continuing in the Golgi, triglyceride is packaged with cholesterol, lipoproteins, and other lipids into particles called chylomicrons. Chylomicrons are extruded from the Golgi into exocytotic vesicles, which are transported to the basolateral aspect of the enterocyte. The vesicles fuse with the plasma membrane and undergo exocytosis. Instead of being absorbed directly into capillary blood, chylomicrons are transported first into the lymphatic vessel that penetrates into each villus. Chylomicron-rich lymph then drains into the lymphatic system, which rapidly flows into blood. Blood-borne chylomicrons are rapidly disassembled and their constituent lipids used throughout the body. After entry into the cell, medium-chain triglycerides, which also require minimal emulsification by bile acids, undergo a relatively simple process of assimilation in which they do not undergo reesterification and chylomicron formation, as the long-chain lipids do. Medium-chain triglycerides are taken directly into the portal venous system; chylomicrons formed from long-chain fats enter the lymphatics. In conditions that involve obstruction of the lymphatics, feeding formulas containing primarily medium-chain triglycerides rather than long-chain triglycerides are recommended.

In the process of fat digestion, several lipases hydrolyze fatty acids from glycerol. Figure 115-7 illustrates some of these, which include the lipase found in human milk (bile salt–stimulated lipase [BSSL]) and lingual, gastric, pancreatic, and epithelial lipases. Bile acid–stimulated lipase is present in milk and becomes active in the small-intestine lumen in the presence of bile acids. Lingual lipase is secreted by glands at the base of the tongue and is involved in gastric lipid hydrolysis. Lipases produced in the stomach, secreted from the pancreas, and present in the mucosa also are involved in lipid hydrolysis.

Developmental Aspects of Lipid Digestion and Absorption

Prenatal Period

The presence of lipase in the stomachs of fetuses in early gestation, along with increased activities proceeding with gestation, has been reported.[71,72] Its activity increased with subsequent development. Ménard and colleagues[71] reported the appearance of lipase at 10 weeks gestation. In the pancreas, lipase immunoreactivity appears at week 21 of pregnancy; 5 weeks later, immunoreactivity for trypsinogens and chymotrypsinogen can be demonstrated.[21] Intensities of lipase increase greatly at birth. The ontogenesis of carboxyl ester hydrolase does not follow that of lipase.[21]

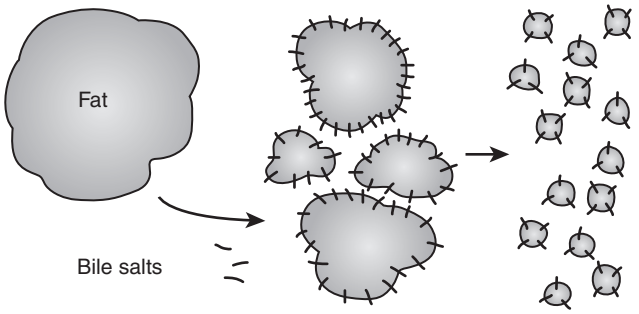


Figure 115-5 Digestion of lipids: micellar emulsification.

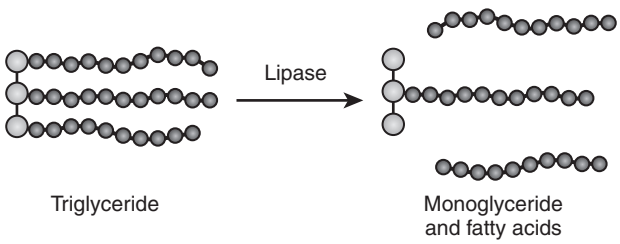


Figure 115-6 Triglyceride hydrolysis. *(From http://www.vivo.colostate.edu/hbooks/pathphys/digestion/smallgut/lipased.gif.)*

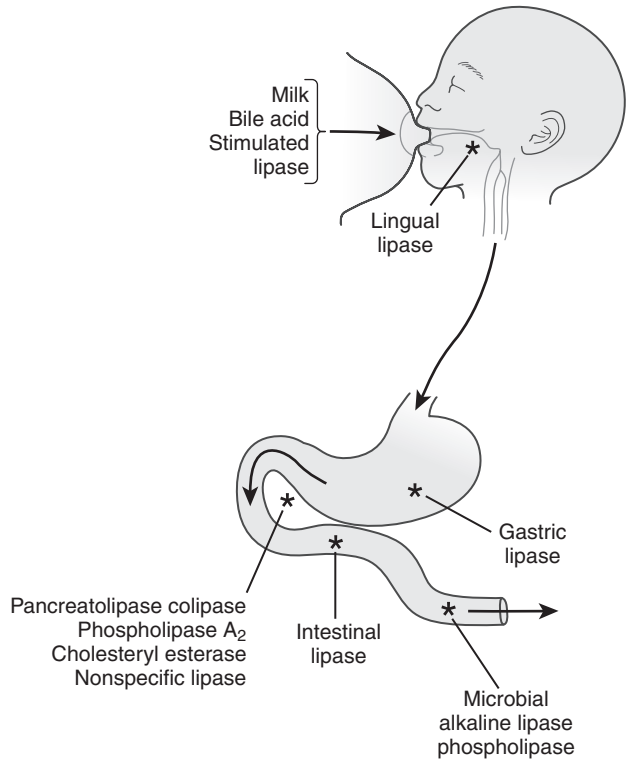


Figure 115-7 Lipases from different sources.

Postnatal Period

Bile Acids

Bile acids are critical to efficient fat digestion and absorption. These processes are limited in both term and preterm infants because the duodenal concentration of bile acids is low as a result of lower synthesis and ileal reabsorption.[73-75] Lower micellar solubilization leads to inefficient cell-mucosal interaction and subsequently lower absorption of the molecules of the mucosal-cell surface interface. Long-chain fatty acids (but not medium-chain fatty acids) depend on bile acids for solubilization and thus are the most susceptible to inefficient absorption.

Bile Salt–stimulated Lipase

Human milk contains esterolytic activity that is not detectable in bovine milk.[76] It has been shown that the digestion of long-chain triglycerides proceeded only in the presence of bile salts by an enzyme, later classified as BSSL, which is present in human colostrum and in preterm and term milk.[77-79] It has been estimated that in milk produced during the first 2 weeks of lactation 40% of triglycerides are hydrolyzed within 2 hours, and during later lactation only 20% of triglycerides are hydrolyzed.[79,80] This apparent decrease is caused by an increase in milk fat content during lactation, rather than a real change in absolute BSSL activity.

Properties of BSSL make it suitable to survive in the stomach with little or no loss of activity and to act in the duodenum when activated by bile acids (cholate, chenodeoxycholate) in concentrations close to those found in the infant duodenum.[78,79] Thus, at a low bile salt concentration, the action of milk BSSL may facilitate fat absorption. BSSL hydrolyzes long-chain triglycerides with no positional specificity and thus hydrolyzes 2-monoacylglycerol.[78] This property, to hydrolyze 2-monoacylglycerol, makes this enzyme complementary to the pancreatic lipase that delivers as its main products fatty acids and 2-monoacylglycerol. The significance of the presence of BSSL for the digestion of milk lipids is supported further by studies in low-birth-weight preterm infants (3 to 6 weeks old) fed raw or heat-treated (pasteurized or boiled) human milk. Fat from the former was absorbed more (74%) than from the latter two (54% and 46%).[81]

Lipoprotein Lipase

Milk also contains a serum-activated lipoprotein lipase (LPL).[78,82,83] Its activity is inhibited by bile salts. Although the concentration of BSSL activity in milk does not change during the duration of lactation, LPL activity increases during lactation, exhibiting a peak around the third week of life.[82] LPL has been implicated in the prolonged neonatal jaundice of breast-fed infants. Fresh milk samples from mothers of infants with jaundice exhibit an increased level of LPL.[84] The relationship between the high activity of LPL in milk and jaundice is not simple, because high levels of LPL in mother's milk are not always associated with jaundice.[85]

Preduodenal Lipases

At the end of the 1800s, von Ebner[86] described the presence of serous glands located on the proximal dorsal site of the tongue. These glands appear to be the source of lingual lipase. The release of these lipases is stimulated by feeding.[87] Preduodenal lipases exhibit partial stereospecificity for *sn*-3 esters over *sn*-1 esters.[88] Lipase activity is present in gastric aspirates as early as 6 months gestation and increases substantially around 8 to 9 fetal months.[89] With use of tributyrin as a substrate, it was shown that lipase activity in gastric aspirates collected from premature infants was lower at birth in infants at 26 weeks and less, peaked at 30 to 32 weeks, and declined to lower levels at term. Continuous feeding, as opposed to intermittent parenteral alimentation, appeared to promote the development of this lipase activity.[90]

Pancreatic Lipases

In adults, pancreatic juice contains two enzymes that are active against neutral lipids. The so-called pancreatic lipase is more active against insoluble, emulsified substrates than against soluble ones. The second lipase, also called pancreatic carboxylase esterase, is more active against micellar or soluble substrates than against insoluble, emulsified substrates. In contrast to the first lipase, it is strongly stimulated by bile salts. Colipase removes the inhibiting effect of bile salts on lipase. Studies usually do not differentiate between these lipases. Generally, lipases show the lowest values after birth.[91] The increase toward adult values occurs within the first 6 months of life, which is earlier than in the case of amylase. Premature and very-low-birth-weight infants have lower values than do term neonates.[92] During the first week of life, lipase activity increases about four-fold in premature infants.[91]

In healthy preterm infants between days 3 and 40 after birth, this activity increased linearly (in infants both at gestational age 29 to 32 weeks and at 33 to 36 weeks).[73] At 1 month of age, values reached 35% in 2- to 6-week-old infants. Premature infants fed soy-based formula at the age of 1 month exhibited higher lipase activity in the duodenal fluid after cholecystokinin-pancreozymin-secretin stimulation than did premature infants fed milk-based formula.[24]

Clinical Correlations

Although we have mentioned that, because of the lack of necessity for bile acid emulsification for medium-chain fats compared with long-chain fats, these should have a greater likelihood of absorptions, this does not appear to be the case. Studies have shown medium-chain triglycerides to be just as readily absorbed as long-chain triglycerides,[93] and the mechanisms of this are speculated to reside in greater gastric lipolytic activity of the longer-chain lipids. This observation is supported by a Cochrane review that showed no differences in growth, necrotizing enterocolitis, or other morbidities in infants fed primarily medium- versus long-chain triglycerides.[94]

Interest is considerable in the capability of neonates to convert the essential fatty acids into longer-chain fatty acids with 20 or more carbons. These long-chain polyunsaturated fatty acids are critical in the formation of eicosanoids and structural components of the central nervous system. They are found in relatively high concentrations in human milk but are not found in total parenteral nutrition or most neonatal formulas. Whether the addition of long-chain polyunsaturated fatty acids to formulas results in improved neurodevelopment is the subject of current intense investigation.

Most essential fatty acids provided to neonates are derived from the omega-6 family (linoleic acid). This is because much of the lipid derived from formulas or intravenous lipid solutions is from vegetable oil, which is rich in the omega-6 but not the omega-3 fraction. The likelihood of health benefits to infants provided greater quantities of the omega-3 lipids than they are currently receiving deserves additional study.

Absorption of fat from breast milk increases slightly during the first month in term infants, achieving values close to 90%. In premature infants, absorption values are close to those found in term infants.[95] Although absorption of long-chain fatty acid triglycerides is lower during the first 6 weeks of life, medium-chain fatty acid triglycerides and short-chain fatty acid triglycerides are already absorbed quite efficiently in premature infants soon after birth.[96]

By comparing the plasma triacylglycerol total and *sn*-2 position fatty acid levels in breast-fed and formula-fed term infants, Innis and associates[97] provided evidence that palmitic acid from

human milk is absorbed as *sn*-2 monoacylglycerol in infants receiving breast milk. Breast-fed infants have a significantly higher proportion of acetic acid in the fecal short-chain fatty acid spectra than the bottle-fed infants. This finding may be related to a different composition of the intestinal microflora and may be associated with protection against diarrhea and respiratory infection in the infant.[98]

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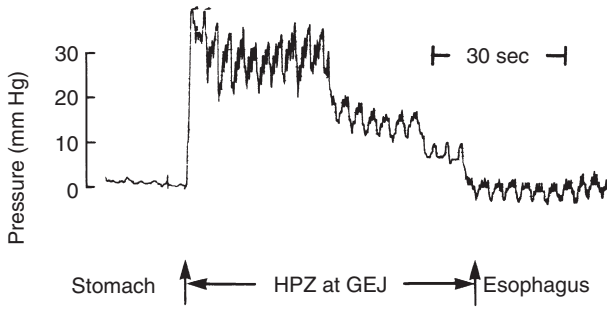


Figure 116-1 High-pressure zone *(HPZ)* at the gastroesophageal junction *(GEJ)* as measured by the station pull-through technique.[2,3] This zone is characterized by an elevated basal pressure and an oscillating pressure wave corresponding to respiratory rate.

diaphragm) allow the high-pressure zone to modify the gastroesophageal pressure gradient during a variety of physiologic processes, such as deglutition, esophageal distention, and abdominal straining.[5-9]

Lower Esophageal Sphincter

The LES is a muscular structure with distinctive mechanical and electrical characteristics, unique responses to drugs and hormones, and specialized neural innervation.[10-24] The muscles of the LES appear thicker than those of the more proximal esophagus when they are examined by ultrasound scan.[25] In contrast to the adjacent esophagus, in which the plexus lies only between the circular and longitudinal muscle layers,[26] the innervation of the LES is unique in that the myenteric plexus occupies multiple muscle planes.

The activity of the LES has been studied in a feline model in which esophageal distention results in changes in sphincter tone. During esophageal distention, the baseline high-pressure zone decreases to gastric baseline pressure; simultaneously, the magnitude of the respiratory-induced oscillations in the high-pressure zone is markedly reduced (Figure 116-2). This observation suggests that the response of the sphincter mechanism to esophageal distention involves a relaxation of the intrinsic LES. These events provide an ideal situation for the transit of a swallowed bolus through the gastroesophageal junction.

The neural pathways controlling such rapid changes in LES tone have been characterized. During swallowing, afferent signals from the pharynx travel to the nucleus solitarius in the medulla. Efferent signals then travel from the adjacent dorsal vagal nucleus and the nucleus ambiguus through the vagus nerve to the esophagus, thereby mediating both esophageal peristalsis and LES relaxation to propel swallowed material into the stomach.[27]

Measurements of lower esophageal pressure have also revealed high-amplitude (≤80 mm Hg) fluctuations in sphincter tone. These fluctuations occur at a frequency of approximately three per minute, and they correspond to the activity of the migrating motor complex of the stomach. Thus the LES allows the high-pressure zone to compensate for wide variations in gastric pressure caused by the motor activity of the stomach.[28]

The region of the stomach just below the LES also contributes to the intrinsic antireflux barrier. The smooth muscle fibers of this region form a sling around the greater curvature of the stomach.[29] Although they are not strictly part of the LES, the sling fibers help to prevent reflux by acting as a flap valve in which fundic distention presses the closed end of the sling against the distal esophagus.[30]

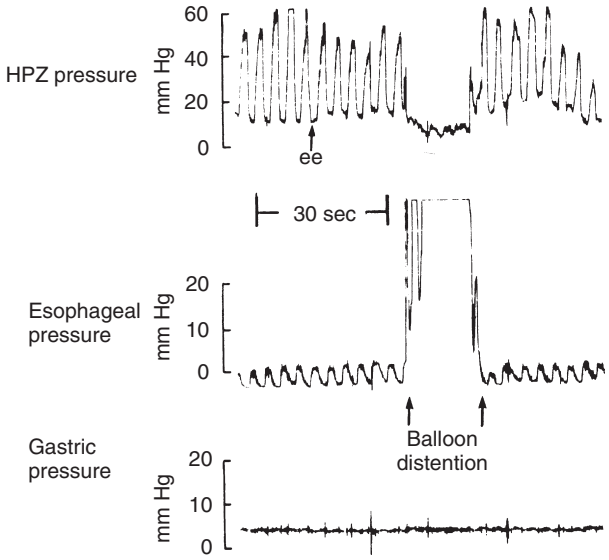


Figure 116-2 Effect of esophageal balloon distention on high-pressure zone *(HPZ)* pressure in a cat. During the period of balloon distention, baseline end-expiratory *(ee)* high-pressure zone pressure decreases to the level of gastric pressure, and a marked reduction is seen in the magnitude of the respiratory-induced oscillations in high-pressure zone pressure.

Crural Diaphragm

The crural diaphragm is distinguished from the costal diaphragm by its attachment to the vertebral column rather than the rib cage. Because the esophagus passes through the crural diaphragm, diaphragmatic contractions create an external sphincter mechanism at the gastroesophageal junction. The activity of the crural diaphragm varies with the respiratory cycle, thereby compensating for variations in thoracic and abdominal pressure that would otherwise favor gastroesophageal reflux during inspiration (see Figure 116-1). The activity of the crural diaphragm is increased or decreased in various other physiologic settings to allow forward passage of swallowed material but to prevent gastroesophageal reflux despite changes in abdominal pressure (see later). Indeed, in the absence of the LES (after resection), the crural diaphragm alone is able to maintain the high-pressure zone.[31]

In mammals, swallowing, distention of the esophagus, eructation, vomiting, regurgitation, and rumination are associated with an inhibition of diaphragmatic electrical activity, thereby facilitating passage of material through the gastroesophageal junction.[5,32-38] A consistent finding associated with transit through the gastroesophageal junction is that inhibition of the crural diaphragm occurs to a much greater degree than that of the costal diaphragm (Figure 116-3). In a feline model, swallowing and esophageal balloon distention resulted in concurrent relaxation of the LES and inhibition of crural inspiratory activity. The respiratory-induced oscillations in high-pressure zone pressure were abolished as a result of inhibition of crural inspiratory activity, whereas relaxation of the LES resulted in a decrease in baseline end-expiratory pressure. Costal diaphragmatic activity remained unchanged and allowed continued ventilation with little change in the respiratory rate or intrapleural pressure (Figure 116-4). During periods of increased intraabdominal pressure, such as the Valsalva maneuver, coughing, straight-leg raising, or abdominal compression, an increase occurs in crural diaphragmatic tone, thereby compensating for the increased pressure gradient favoring gastroesophageal reflux.[39]

In summary, the manometrically defined high-pressure zone at the gastroesophageal junction depends on the tone generated by

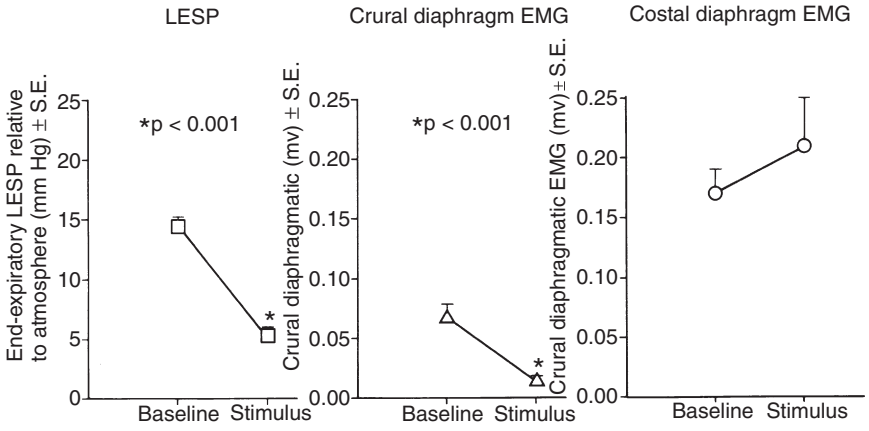


Figure 116-3 Summary of the effect of distention of the lower esophagus on intrinsic LES tone and diaphragmatic electromyographic *(EMG)* activity. It can be seen that distention (stimulus) caused a decrease in the activity of the crural diaphragm, together with a decrease in LES pressure *(LESP)*. In contrast, the activity of the costal diaphragm does not change significantly.

the intrinsic LES and on the magnitude of crural diaphragmatic contraction. Transit of material through the gastroesophageal junction is most likely to occur during simultaneous relaxation of the LES and inhibition of the crural diaphragm.

MECHANISMS OF GASTROESOPHAGEAL REFLUX

In theory, gastroesophageal reflux occurs in either of the following two situations: relaxation of the high-pressure zone (either transiently or for longer periods) or elevation of the gastroesophageal pressure gradient above that of the high-pressure zone (Figure 116-5, *A*). Using techniques that allow for simultaneous and continuous recording of LES pressure (manometry) and both acid and nonacid esophageal reflux (using pH and multichannel intraluminal impedance monitoring), investigators have been able to distinguish these two possibilities.[6-9,38-46] It has become clear that most gastroesophageal reflux is associated with a transient relaxation of the high-pressure zone involving decreased activity in both the LES and the crural diaphragm. However, the converse is not true; most transient relaxations are not associated with reflux events. Transient relaxations of the high-pressure zone occur in normal subjects, but it appears that those with gastroesophageal reflux disease have both a higher frequency of relaxations and an increased rate of reflux per episode of relaxation. Other factors must therefore also play a role in the pathogenesis of reflux disease, such as transient increases in intraabdominal pressure, impaired clearance of refluxed material, or the presence of hiatal hernia.

Transient Relaxation of the High-Pressure Zone

As illustrated in Figure 116-5, most episodes of gastroesophageal reflux occur during transient relaxation of the high-pressure zone (see Figure 116-5, *A*).[8,9,47,48] Transient relaxation is caused by an abrupt collapse of pressure in the high-pressure zone in the absence of a swallow or primary esophageal peristalsis. Pressure in the high-pressure zone before and immediately after these episodes is always greater than gastric pressure. The pressure profile of the high-pressure zone during transient relaxation is similar to that observed during a swallow and is characterized by decreases in both LES activity and crural diaphragm activity (Figure 116-6). The physiologic role of transient relaxations is to allow eructation (belching) to occur, consistent with the finding that gastric distention can trigger transient relaxations.[49-53]

Other triggers for transient relaxation remain unclear. Swallow-induced and transient relaxations of the high-pressure zone are identical manometrically, and, in humans, transient relaxations frequently occur within a few seconds of a previous swallow-induced relaxation or in association with an incomplete peristaltic sequence (Figure 116-7).[1,8,53-55] In an experimental

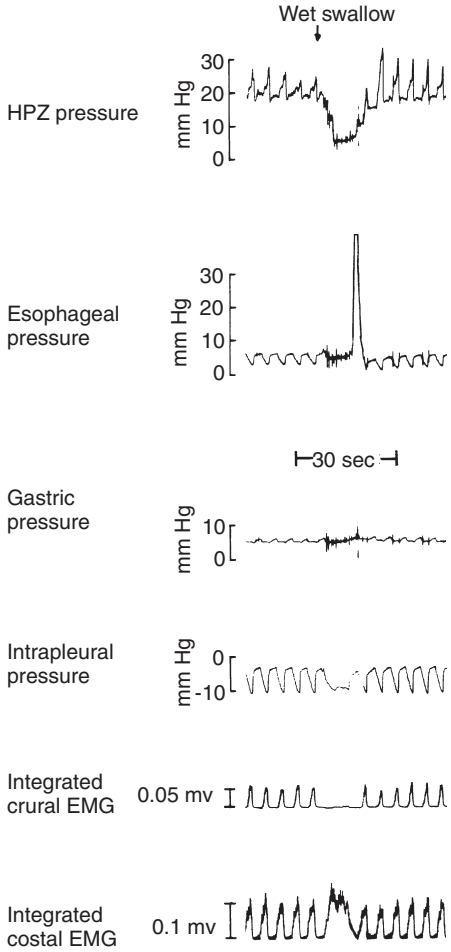


Figure 116-4 Effect of a wet swallow on high-pressure zone *(HPZ)* pressure and diaphragmatic activity. The wet swallow was induced by a bolus injection of water into the posterior pharynx. The swallow results in an esophageal contraction, with a simultaneous relaxation of the intrinsic LES and inhibition of integrated crural electromyographic *(EMG)* activity. The combination of these events results in end-expiratory high-pressure zone pressure decreasing to gastric baseline and an absence of respiratory-induced oscillations in high-pressure zone pressure. The costal diaphragm remains active during the swallow, thus allowing ventilation to continue. *(From Altschuler SM, Boyle JT, Nixon TE, et al: Simultaneous reflex inhibition of lower esophageal sphincter and crural diaphragm in cats,* Am J Physiol *249:G586, 1985.)*

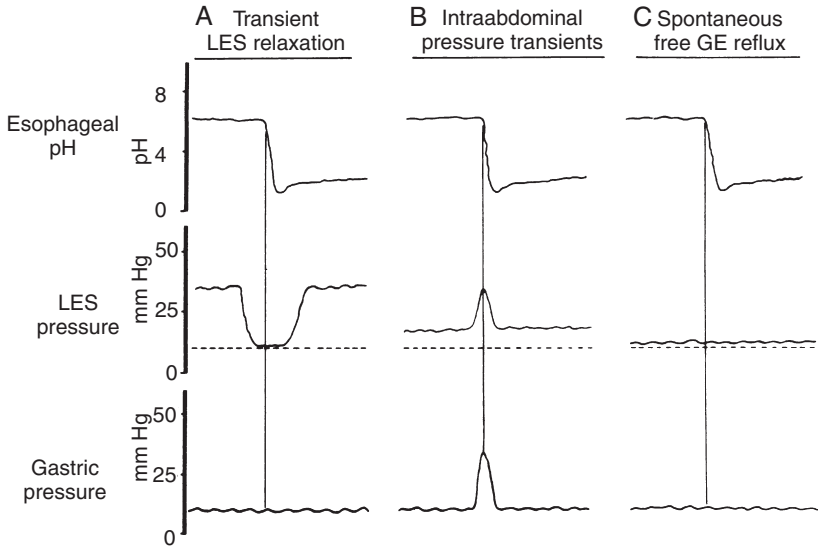


Figure 116-5 Schematic representation of different mechanisms of gastroesophageal *(GE)* reflux with use of the sleeve sensor to monitor high-pressure zone *(HPZ)* LES pressure. Episodes of gastroesophageal reflux occur during transient relaxation of the high-pressure zone **(A),** during increases in intraabdominal pressure that overcomes high-pressure zone pressure **(B),** or as spontaneous reflux across an atonic high-pressure zone **(C).** *(From Dodds WJ, Dent J, Hogan WJ, et al: Mechanism of gastroesophageal reflux in patients with reflux esophagitis,* N Engl J Med *307:1547, 1982.)*

model using the opossum, transient relaxations were produced by stimuli that tended to be less vigorous than the threshold required for inducing a full deglutition sequence or for the induction of secondary esophageal peristalsis.[56] Aggressive pharyngeal stroking tended to cause a full deglutition reflex, whereas a single pharyngeal stroke was most commonly associated with isolated LES relaxation. Electrical stimulation of the central end of the superior laryngeal nerve (vagal afferent stimulation) or the peripheral end of the cervical vagus (vagal efferent stimulation) at high frequencies resulted in a normal deglutition sequence, whereas low-frequency stimulation resulted in isolated LES relaxation. Studies in humans have shown that transient relaxation is frequently associated with mylohyoid and pharyngeal activity of a much lower magnitude than that observed during a swallow or with a nonperistaltic esophageal contraction.[54] As a result of these studies, it has been suggested that transient relaxations occur by the same neural pathways as swallowing-associated relaxation (see earlier) and may be induced by a long train of subthreshold stimuli originating in the pharynx.[56]

Studies in both normal subjects and in patients with gastroesophageal reflux disease have shown that most reflux episodes occur during transient relaxations.[50-52] Although transient relaxations of the high-pressure zone may last from 10 to 45 seconds, the actual episodes of reflux occur only during the period when the baseline high-pressure zone pressure is zero and respiratory-related pressure oscillations are absent (see Figure 116-6). Why are transient relaxations associated with gastroesophageal reflux disease in some subjects and not in others? Studies of transient relaxations in patients with and without reflux symptoms have found only a slight increase in the frequency of relaxations in those with symptoms.[52] Some of these patients, and children in particular, may have delayed gastric emptying resulting in gastric distention, which, in turn, may produce transient relaxations of the LES.[47,49,57,58] A more important difference appears to be an increase in the percentage of transient relaxations that result in reflux from 40% to 50% in physiologically normal persons to 60% to 70% in patients with gastroesophageal reflux disease.[52]

Intraabdominal Pressure Transients

Although transient relaxation of the high-pressure zone is the major criterion for episodes of gastroesophageal reflux in both physiologically normal persons and patients with symptoms, other mechanisms must contribute to reflux in patients with symptoms.[1,9,47,48] Some of these mechanisms are illustrated in Figure 116-5. Active increases in abdominal pressure occur during coughing, straining, and changes in position. In physiologically normal persons, an increase in crural diaphragm activity during active increases in abdominal pressure is thought to prevent episodes of gastroesophageal reflux.[39] In contrast, during passive abdominal compression (which may occur frequently in neonates and infants), crural diaphragmatic activity is inhibited and the activity of the LES is increased, thus leaving the LES as the sole barrier to gastroesophageal reflux (Figure 116-8). This adaptive response involves a vagally mediated increase in LES pressure in excess of the transmitted pressure increase in the stomach.[59] The degree to which failure of these responses to active or passive increases in intraabdominal pressure is responsible for symptomatic gastroesophageal reflux remains to be determined.

Spontaneous Free Gastroesophageal Reflux

Before the development of the sleeve sensor device to measure high-pressure zone transient relaxations and the simultaneous recording of esophageal pH, a depressed basal high-pressure zone pressure was considered the major factor leading to episodes of gastroesophageal reflux. A low resting high-pressure zone pressure was primarily attributed to a hypotonic intrinsic LES,[17] which allows free reflux of gastric contents into the esophagus. Strawczynski and colleagues[60] demonstrated that LES pressure is normally decreased in infants compared with that in adults. In addition, studies in the opossum have illustrated that lower esophageal pressure is depressed at birth and gradually achieves normal levels later in life.[61]

The presence of a hypotonic LES has also been observed in patients with neurologic impairment, connective tissue disorders such as scleroderma, previous surgery of the gastroesophageal

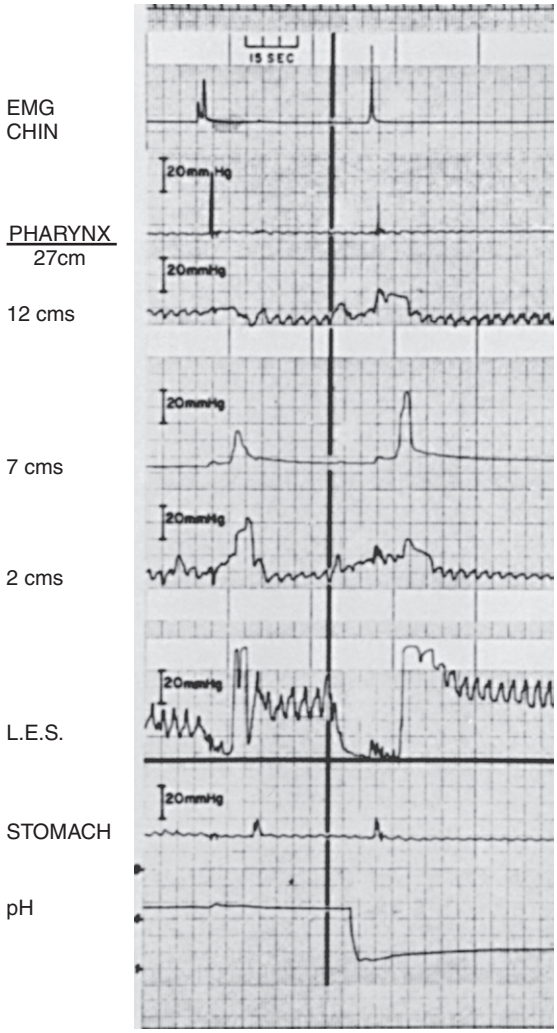


Figure 116-6 Comparison of swallow-induced high-pressure zone LES relaxation with transient relaxation of the high-pressure zone. The swallow-related high-pressure zone relaxation *(left* of the *dark vertical line)* is associated with a submental electromyographic *(EMG)* spike, a pharyngeal contraction, and an esophageal peristaltic contraction. The presence of the esophageal peristaltic contraction prevents the occurrence of gastroesophageal reflux. The *dark vertical line* indicates the start of a transient high-pressure zone relaxation. The relaxation is of longer duration than the swallow-induced high-pressure zone relaxation. An episode of gastroesophageal reflux occurs (esophageal pH falls from 5 to 1) when baseline high-pressure zone pressure approaches 0 and no respiratory-related oscillation occurs in high-pressure zone pressure. *L.E.S.,* Lower esophageal sphincter. *(From Mittal RK, McCallum RW: Characteristics and frequency of transient relaxations of the lower esophageal sphincter in patients with reflux esophagitis,* Gastroenterology *95:543, 1988. Copyright 1988 by the American Gastroenterological Association.)*

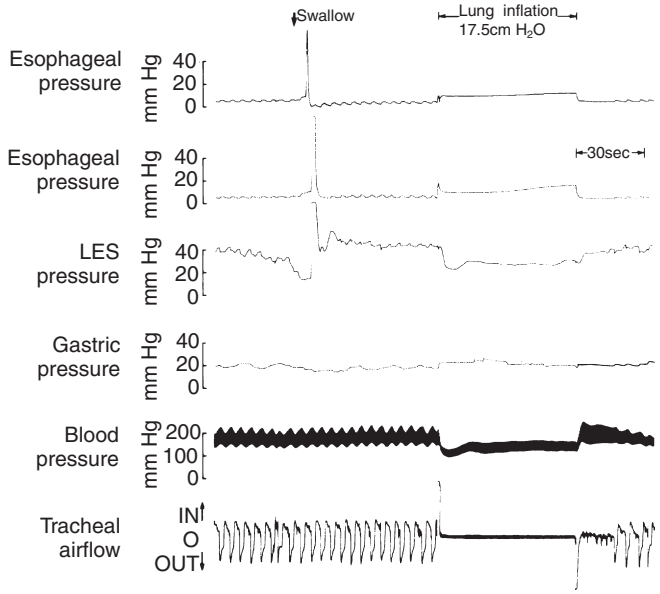


Figure 116-7 Effect of static lung inflation on high-pressure zone LES pressure. Lung inflation induces a reflex relaxation of high-pressure zone pressure that persists for the duration of the stimulus. During the lung inflation, no spontaneous respiration occurs, and thus respiratory oscillation in high-pressure zone pressure is absent. The high-pressure zone relaxation induced by lung inflation is not associated with any esophageal motor events. The swallow-induced high-pressure zone relaxation is terminated by a contraction of the LES. *(Courtesy J.T. Boyle, MD.)*

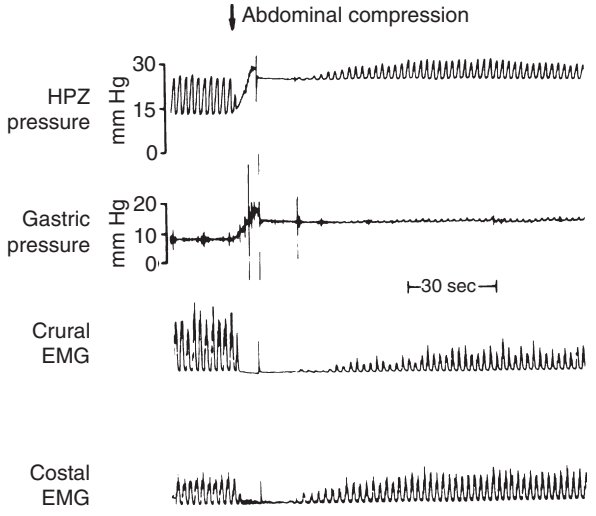


Figure 116-8 Effect of abdominal compression on the high-pressure zone *(HPZ)* pressure profile. With the onset of abdominal compression, end-expiratory high-pressure zone pressure increases and crural and costal diaphragmatic electromyographic *(EMG)* activity decreases. The crural diaphragmatic EMG activity remains inhibited for the duration of the stimulus, whereas costal activity returns to baseline levels. As a result of the inhibition of crural EMG activity, a decrease occurs in the magnitude of the respiratory-induced oscillations in high-pressure zone pressure. The increase in end-expiratory high-pressure zone pressure is greater than the increase in gastric pressure induced by the abdominal compression, a finding indicating that the LES has undergone an adaptive response during the period of abdominal compression. *(From Boyle JT, Altschuler SM, Nixon TE, et al: Responses of feline gastroesophageal junction to changes in abdominal pressure,* Am J Physiol *253:G315, 1987.)*

junction, and esophagitis.[1,48] An alteration in central neural input to the LES or an impairment of the contractile function of the LES muscle is likely present in these patients.[1,49] In many cases, it is difficult to establish the sequence of cause and effect, because reflux esophagitis itself causes esophageal dysmotility. Likewise, although hiatal hernia can exacerbate gastroesophageal reflux, it may be a secondary result of esophageal shortening caused by chronic esophagitis.[1] Although episodes of gastroesophageal reflux are now known to occur infrequently by the mechanism of a hypotonic LES, this mechanism may be important with regard to the previously mentioned disorders.

GASTROESOPHAGEAL REFLUX IN THE NEONATAL PERIOD

The mechanisms of gastroesophageal reflux in the neonate, and in particular in premature infants, have been less well described than in adults. Regurgitation is very common in this age group; up to 40% to 60% of normal 0- to 4-month-old infants regurgitate some amount of their feedings.[62] Basic mechanical considerations provide some explanation for the high frequency of regurgitation in infants. Newborns spend most of their time in the supine position, which has been shown to promote gastroesophageal reflux. The newborn term infant's esophageal length is only 8 to 10 cm, and the intraabdominal esophagus is less than 1 cm, thereby facilitating passage of refluxed material to a relatively higher anatomic level than in adults.

Esophageal pH monitoring, the current standard for the diagnosis of gastroesophageal reflux in adults, is of limited value in infants because gastric acid is buffered by frequent feedings. Nonetheless, it is possible to measure esophageal pressures in neonates and even premature infants and to identify ways in which their antireflux mechanisms differ from those of adults.[63-66] As suggested by prenatal ultrasound images of fetuses swallowing amniotic fluid, the primary esophageal mechanisms are present at an early stage. Manometric studies of both premature and term neonates have confirmed normal primary esophageal peristalsis. Moreover, even premature infants as young as 33 weeks postmenstrual age have been found to have a lower esophageal high-pressure zone although the pressure increases with age.[63-65] However, premature infants at 30- to 34-week gestational ages have lower esophageal peristaltic velocity and amplitude than term infants.[67,68] In response to mid-esophageal liquid infusion, premature infants have a longer delay to LES relaxation, but once the relaxation occurs it is of a longer duration than in term infants.[69] Premature infants have an elevated frequency of synchronous (i.e., nonperistaltic) esophageal contractions in the absence of a swallow, and this lack of coordination may lead to inadequate clearance of refluxed material.[63,64] As in adults, it appears that transient relaxations of the high-pressure zone are the primary mechanism of gastroesophageal reflux in neonates.[63-66] Thus it appears that the high frequency of gastroesophageal reflux in neonates reflects both anatomic factors and immaturity of the esophageal clearance mechanisms.

Manifestations of Gastroesophageal Reflux in the Neonate

Neonatal gastroesophageal reflux is considered pathologic if it is associated with growth failure resulting from poor feeding, excessive regurgitation, pulmonary complications, or neurobehavioral manifestations, such as severe irritability or Sandifer syndrome. In these cases, the diagnosis is made on the basis of a clear temporal association of the symptoms with feeding times or episodes of regurgitation. The relationship between gastroesophageal reflux and apnea of prematurity is less clear. Although it had been assumed that apnea of prematurity is caused at least in part by gastroesophageal reflux, several studies using combined pH monitoring, multichannel intraluminal impedance, and polysomnography have demonstrated that apnea is no more likely to occur after a reflux event (either acid or nonacid) than during a reflux-free period.[46,70-78] Thus it is not clear that measures directed toward reducing gastroesophageal reflux will be effective in reducing the frequency or severity of apnea of prematurity.

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117 Pathophysiology and Prevention of Neonatal Necrotizing Enterocolitis

Michael Caplan

Neonatal necrotizing enterocolitis (NEC) is an ischemic and inflammatory necrosis of bowel that primarily afflicts premature neonates after the initiation of enteral feeding.[1,2] Despite the significant morbidity and mortality associated with NEC, the pathophysiology has remained poorly understood. The disease is seen clinically in premature neonates with variable symptoms of intestinal bleeding, emesis, abdominal distention and tenderness, lethargy, apnea and bradycardia, thrombocytopenia, metabolic acidosis, respiratory failure, and, if severe, shock.[3] Clues to the origin are suggested by the pathologic changes observed in surgical specimens and autopsy material, including coagulation necrosis (suggesting some component of ischemic injury), inflammation (acute or chronic), and less commonly ulceration, hemorrhage, reparative change, bacterial overgrowth, edema, and pneumatosis intestinalis.[4]

Although most cases of NEC are diagnosed in premature neonates, term infants with specific underlying risk factors are at risk for this disease.[5-8] Previous studies have identified birth asphyxia, polycythemia, exchange transfusion, intrauterine growth restriction, cyanotic congenital heart disease, myelomeningocele, gastroschisis, and intrauterine cocaine exposure as potential events leading to the development of intestinal injury.[9] Nonetheless, the presentation of NEC in these cases is typically different from that in premature infants, with the onset in the first days after delivery and the course often less dramatic. For these reasons, the pathophysiology in term infants may be quite different from that in the premature neonate and is therefore not considered further in this chapter.

PREMATURITY: THE MAJOR RISK FACTOR FOR NECROTIZING ENTEROCOLITIS

The multifactorial theory has been suggested to explain the pathophysiology of neonatal NEC and purports that several risk factors (prematurity, formula feeding, ischemia or asphyxia, and bacterial colonization) result in the final common pathway of bowel necrosis. More than 90% of cases of NEC occur in premature infants, whereas stratification studies have shown that gestational age and birth weight inversely correlate with a higher incidence of disease.[10-12] Epidemiologic observations suggest an association between intestinal ischemia, infection, and feeding practice on the development of NEC; however, the presence of these factors is less consistent than the presence of prematurity. Although there are many differences between preterm and term neonates, the specific underlying mechanisms responsible for the predilection of NEC in the premature condition remain incompletely elucidated. Studies in humans and animals have identified alterations in multiple components of intestinal host defense,[13-15] motility,[16-19] bacterial colonization,[20-24] blood flow regulation,[25-27] and inflammatory response,[28-30] and these may contribute to the development of intestinal injury in this unique population (Box 117-1).

Host Defense

Intestinal host defense involves a complex combination of factors that function to prevent intraluminal pathogens and toxins from resulting in disease while allowing for normal absorption of nutrients. This intricate system includes the following: (1) physical barriers such as skin, mucous membranes, intestinal epithelia and microvilli, epithelial cell tight junctions, and mucin; (2) immune cells such as polymorphonuclear leukocytes, macrophages, eosinophils, and lymphocytes and secretory immunoglobulin A (IgA); and (3) various biochemical factors.[15,31-39] Although not exhaustively studied, many of these important functions appear to be abnormal in the premature infant and may

BOX 117-1

Premature Infants: Factors That May Increase Their Susceptibility to Necrotizing Enterocolitis

Compromised intestinal host defense
Physical barriers
- Skin
- Mucous membranes
- Epithelia and microvilli
- Tight junctions
- Mucin

Immune factors
- Neutrophils
- Macrophages
- Eosinophils
- Lymphocytes (including intraepithelial lymphocytes)
- Secretory IgA

Biochemical factors
- Antimicrobial proteins (trefoil factor, defensins and cryptidins)
- Oligosaccharides
- Glutamine
- Lactoferrin
- Polyunsaturated fatty acids
- Nucleotides
- Growth factors (EGF, TGF, IGF, erythropoietin)
- Gastric acid
- Cytokines

Intestinal dysmotility
Migratory motor complexes

Bacteria
Patterns of colonization or overgrowth
Pathogenicity of organisms

Altered autoregulation of intestinal circulation
Basal vascular resistance
Response to stress

Disordered inflammatory response
Increased proinflammatory response to stimuli
Suppressed antiinflammatory components

EGF, Epidermal growth factor; *Ig,* immunoglobulin; *IGF,* insulin-like growth factor; *TGF,* transforming growth factor.

therefore put this population at risk for NEC. Intestinal permeability to macromolecules including immunoglobulins, proteins, and carbohydrates is known to be greater in neonates compared with older children and adults, and in premature infants this permeability may be more pronounced.[37,40] Although mucosal permeability is beneficial for developing animals to augment passive immunity and nutrient absorption, the precise mechanisms accounting for these differences are poorly understood. The microvilli and tight junctional barrier may be deficient in the premature infant, but the data are inconclusive.[41] It is known that intestinal mucus (a complex gel consisting of water, electrolytes, mucins, glycoprotein, immunoglobulins, and glycolipids) protects against bacterial and toxin invasion and is abnormal in developing animals.[33,42] In addition, key bacteriostatic proteins are secreted from epithelium that bind to or inactivate the function of invading organisms. Intestinal trefoil factor is one such molecule that appears to be developmentally regulated and therefore deficient in the premature neonate.[43-45] Human defensins (or cryptidins) are bacteriostatic proteins synthesized and secreted from Paneth cells that protect against bacterial translocation and are altered in premature infants and those with NEC.[46,47]

Immunologic host defense is abnormal in developing animals.[48-50] It is known that intestinal lymphocytes are decreased in neonates (B and T cells) and do not approach adult levels until 3 to 4 weeks of life. Newborns have markedly reduced secretory IgA in salivary samples, reflecting the decreased activity presumed in the intestine.[51,52] Breast milk feeding provides multiple immune factors; formula-fed neonates have impaired intestinal humoral immunity, and this deficiency may predispose to the increased incidence of infectious diseases noted in this population.[53,54] A complete review on the importance of breast milk in immunologic host defense can be found in Chapters 149 to 158 of this textbook.

Several biochemical factors that are present in the intestinal milieu play an important role in the maintenance of gut health and integrity. Substances such as lactoferrin[55] and glutamine[56,57]; growth factors such as epidermal growth factor,[28] transforming growth factor-β,[58] insulin-like growth factor,[59] and erythropoeitin[60,61]; gastric acid; oligosaccharides[62]; polyunsaturated fatty acids[63,64]; nucleotides[65]; and many others affect mucosal barrier function, intestinal inflammation, and the viability of intraluminal bacteria. Many of these factors are deficient or absent in the preterm neonate, especially in those patients not receiving breast milk feedings. Intensive research is ongoing to define the specific role of each on gut integrity and the development of intestinal inflammation and necrosis.

Motility

The premature infant has altered gastrointestinal motility; term newborn motility patterns or "migratory motor complexes" do not appear until 34 to 35 weeks gestation.[16,66] Abnormal peristaltic activity may allow for bacterial overgrowth that could increase endotoxin exposure and predispose the infant to NEC. Furthermore, dysmotility causes intestinal dilatation and compromised intestinal blood flow, with further ischemic stress to compromised intestinal circulation.

Bacterial Colonization

Premature infants hospitalized in the neonatal intensive care unit have different patterns of gut bacterial colonization than healthy breast-fed term infants have.[67] Although epidemics of NEC have been described associated with specific bacteria (e.g., *Clostridia* spp., *Escherichia coli*, *Klebsiella* spp., *Staphylococcus epidermidis*), most cases occur endemically and demonstrate a variety of bacterial isolates from stool cultures.[24,68] Blood cultures are positive in only 20% to 30% of affected cases, and this likely represents the degree of mucosal damage at presentation. At birth, the intestine is a sterile environment, and no cases of NEC have been described in utero, a finding supporting the importance of bacterial colonization in the pathophysiology. Colonization develops from several species in breast-fed infants by 1 week of age that include probiotic (facultative anaerobes defined to confer benefit to the host) organisms such as *Bifidobacteria* spp. and *Lactobacillus* spp., whereas the intestine in the hospitalized, extremely premature infant has less species diversity and fewer facultative anaerobes.[54,69-71] This imbalance may allow for pathologic proliferation, binding, and invasiveness of otherwise nonpathogenic intestinal bacteria. Alternatively, probiotic organisms can modulate the inflammatory response and improve mucosal barrier function by multiple mechanisms that can contribute to overall intestinal health. It remains unclear whether bacterial translocation into submucosa is a prerequisite for NEC or, rather, the activation of the toll-like receptors from endotoxin and other pathogen-associated molecular patterns is adequate to initiate the final common pathway of intestinal injury.[22,72-74] Nonetheless, certain bacteria such as adherent *E. coli* produce disease in a rabbit model of NEC, whereas nonpathogenic strains of gram-positive organisms prevent disease.[75] Furthermore, reports have suggested that early enteral supplementation of probiotics (e.g., bifidobacterium and lactobacillis) reduces the risk of NEC in animals and humans.[20,76] Two randomized, placebo-controlled, single-center trials performed in Taiwan and Israel demonstrated a marked reduction of NEC incidence in subjects supplemented with separate probiotic preparations.[77,78] Large multicenter trials will be necessary to confirm the efficacy and safety of this approach, but this strategy might offer the first breakthrough in the prevention of NEC. In summary, bacterial colonization is an important factor in the initiation of intestinal injury, but the specific events in the pathophysiology are not well delineated.

Intestinal Blood Flow Regulation

Early observations on the pathophysiology of NEC suggested that profound intestinal ischemia was a critical predisposing factor.[79,80] Similar to the "diving reflex" observed in aquatic mammals, it was hypothesized that, in periods of stress, blood flow was diverted away from the splanchnic circulation, with resulting intestinal necrosis. Although early epidemiologic observations identified asphyxia as an important risk factor, subsequent studies have shown that most cases of NEC are not associated with profound impairment in intestinal perfusion.[3] In animal models, studies have shown that the reperfusion after intestinal ischemia is required for the initiation of bowel necrosis,[81,82] and therefore investigations of the role of intestinal ischemia in the pathophysiology of NEC are focused on this construct.

Intestinal and somatic growth is dramatic in developing animals, and therefore providing sufficient flow is mandatory. Neonatal animals have been shown to have differences in the intestinal circulation that may predispose them to NEC. The basal intestinal vascular resistance is elevated in the fetus and decreases significantly after birth, thus allowing for rapid increase in intestinal blood flow.[25] It has been shown that this change in the resting vascular resistance is dependent on the balance between the dilator (nitric oxide) and constrictor (endothelin) molecules and the myogenic response.[83,84] In findings perhaps more relevant than basal vascular tone, studies have shown that the newborn has alterations in response to circulatory stress, resulting in compromised intestinal flow and/or vascular resistance. In response to hypotension, newborn animals (3-day-old but not 30-day-old swine) appear to have defective pressure-flow autoregulation, resulting in compromised intestinal oxygen delivery and tissue oxygenation.[26,27,85] In addition, in the presence of arterial hypoxemia, the newborn intestinal circulatory response differs from that in older animals. Although intestinal vasodilation and

increased intestinal perfusion occur after modest hypoxemia, severe hypoxemia causes vasoconstriction, intestinal ischemia, and hypoxia, mediated in part by loss of nitric oxide production. Multiple chemical mediators (nitric oxide, endothelin, substance P, norepinephrine, and angiotensin) affect intestinal vasomotor tone, and, in the stressed newborn, abnormal production of these mediators may result in compromised circulatory autoregulation, leading to perpetuation of intestinal ischemia and tissue necrosis.[86-88]

Enteral Alimentation

Enteral alimentation has long been considered an important risk factor in the initiation of NEC; more than 90% of cases occurred in premature infants after feedings were introduced. Although the onset of disease used to occur several days after the first feeding, in reports of extremely low-birth-weight infants from the 1990s, NEC was often diagnosed several weeks later.[10,89] Whereas the precise relationship between enteral feedings and NEC remains poorly understood, studies have identified the importance of breast milk (versus formula), volume and rate of feeding advancement, osmolality, and substrate fermentation as important factors.[90-93]

Breast milk feeding appears to reduce the incidence of NEC in human studies and in carefully controlled animal models.[12,21] Breast milk contains multiple bioactive factors that influence host immunity, inflammation, and mucosal protection including secretory IgA, leukocytes, lactoferrin, lysozyme, mucin, cytokines, growth factors, enzymes, oligosaccharides, and polyunsaturated fatty acids, which are absent in or recently added to (e.g., polyunsaturated fatty acids) neonatal formula preparations. Specific intestinal host defense factors acquired from breast milk such as epidermal growth factor, polyunsaturated fatty acids, platelet-activating factor (PAF) acetylhydrolase, IgA, and macrophages are effective in reducing the incidence of disease in animals,[28,63,94,95] and some have been effective in human trials.[52,64] Nonetheless, breast milk is not completely protective against NEC in premature infants; the largest prospective trial identified a reduction by 50% in most birth-weight–specific groups.[12,96] Because most premature infants receive breast milk by the nasogastric route after artificial collection by mothers and subsequent freezing, it has been suggested that the methods of collection and feeding interfere with specific milk immunity and thereby reduce the protection against the neonate's microbial flora.

Specific components of milk feedings have been implicated in causing mucosal injury in the high-risk neonate. Studies have shown that hyperosmolar formulas resulted in NEC, and the addition of medication to feedings can markedly increase osmolality.[97,98] Animal studies have shown that short-chain fatty acids such as propionic or butyric acid can damage the developing intestine and that in situations of carbohydrate malabsorption colonic fermentation may lead to production of these acids by the host microflora.[99-101] This pathway may be especially problematic in the premature infant, deficient in lactase activity and other brush border enzymes.

Different approaches to feeding have been associated with the initiation of NEC. Early studies suggested that rapid volume increases with full-strength formula made the incidence of disease more prevalent, and protocols were designed to limit feeding advancement. Several studies have shown that early, hypocaloric, or trophic feedings are safe and improve gastrointestinal function in very-low-birth-weight infants.[102-105] Feeding advancement has been evaluated, and the results suggest that judicious volume increase may be safer. It has been postulated that overdistention of the stomach with aggressive volumes may compromise splanchnic circulation leading to intestinal ischemia.

In summary, the premature neonate has several unique features that may increase susceptibility to NEC, but the precise interrelationship of these factors in the final common pathway of intestinal necrosis remains unclear.

FINAL COMMON PATHWAY: THE INFLAMMATORY CASCADE

On the basis of a growing body of evidence obtained from humans and animal and tissue experimentation, the final common pathway of intestinal injury appears to result from the activation of the inflammatory cascade.[39,106,107] This cascade involves a complex balance of proinflammatory and antiinflammatory endogenous mediators, receptors, signaling pathways, second messengers, and a variety of downstream effects that ultimately results in end-organ damage in certain circumstances (Figure 117-1). Inflammation can be initiated by a variety of factors, most notably the exposure to the bacterial cell wall product, endotoxin. After endotoxin stimulation of the toll receptor family in animals, tissue, or cells, several mediators are rapidly produced including PAF, tumor necrosis factor, and interleukin (IL)-1.[108-112] With use of a neonatal mouse model of NEC that resembles the human condition after exposure to formula feeding and asphyxial stress, it was shown that the interaction of endotoxin with toll receptor TLR4 is a critical determinant in this disease model.[113] In intestine, subsequent events lead to chemotaxis, transmigration, and activation of leukocytes and to synthesis and release of many products from epithelial and inflammatory cells such as IL-6, IL-8, IL-10, IL-18, thromboxanes, leukotrienes, prostaglandins, nitric oxide, endothelin-1, and oxygen free radicals.[114-121] If counterregulatory responses are insufficient, pathologic changes to gut mucosa occur and may include accentuated apoptosis of epithelial cells, perturbation of tight junctional proteins and complexes, increased mucosal permeability, bacterial translocation, alterations of vascular tone and microcirculation, and additional neutrophil infiltration and accumulation. The process may then be perpetuated by the activation of the secondary inflammatory response, and the final common pathway will result in intestinal necrosis. Although these events remain localized in some cases, in others this activation results in the systemic inflammatory response syndrome, in which patients have capillary leak, hypotension, metabolic acidosis, thrombocytopenia, renal failure, respiratory failure, and, often, death.[122]

Although endotoxin is a well-characterized activator of inflammation, additional factors may play a role in stimulating the NEC cascade in premature infants. Asphyxia and ischemia-reperfusion activate the early mediators of inflammation in many tissues including intestine. Neonatal animal studies have shown that the stress of formula feeding accompanied by hypoxia stimulates phospholipase A_2 gene expression, intestinal PAF production, apoptosis, and the inflammatory response with resulting NEC.[107,123] Therefore many of the purported risk factors for NEC may activate the inflammatory response that results in the final common pathway described earlier.

The evidence suggests that the premature neonate may have an abnormal balance between proinflammatory and antiinflammatory mediator production, thereby increasing the neonate's predisposition for diseases such as NEC. PAF is a potent phospholipid inflammatory mediator that is associated with NEC in several experimental models and human analyses.[124-128] PAF infusion causes intestinal necrosis in animals, and PAF receptor antagonists prevent injury after hypoxia, endotoxin challenge, tumor necrosis factor infusion, and ischemia-reperfusion.[129-131] It has been shown that neonates are markedly deficient in their ability to degrade PAF because of decreased activity of the PAF-specific enzyme PAF acetylhydrolase.[29] PAF acetylhydrolase is present in breast milk but absent in commercial formula, and this may in part explain the beneficial effects of breast milk feeding. IL-10 is an antiinflammatory cytokine thought to be important in reducing intestinal inflammation and possibly NEC in animals and

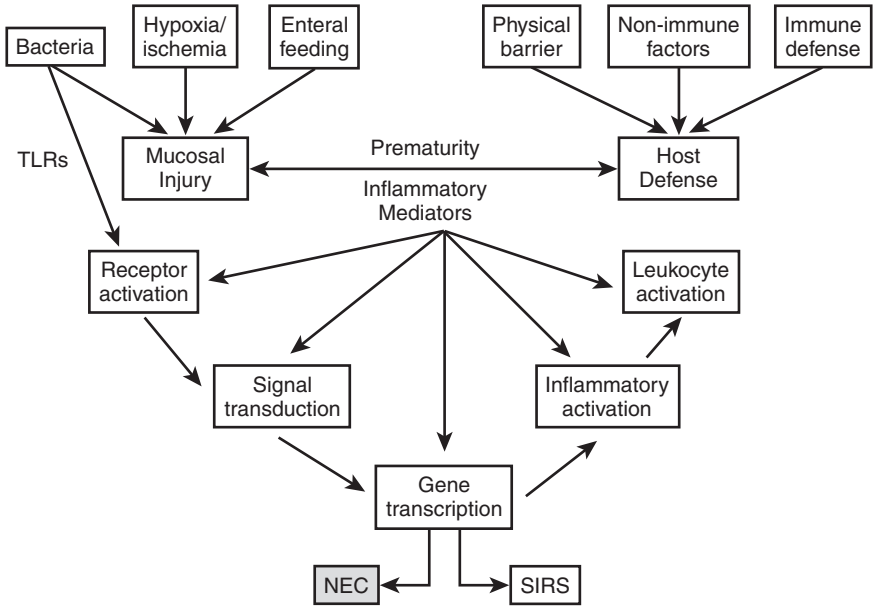


Figure 117-1 Hypothetical events leading to the final common pathway of inflammatory mediator activation and NEC in premature infants. Note the important balance between host defense and mucosal injury in the pathway. *SIRS,* Systemic inflammatory response syndrome.

TABLE 117-1

Published Reports of Successful Strategies to Prevent Intestinal Injury and/or Necrotizing Enterocolitis

Animals	Humans
Breast milk	Breast milk
IgA	IgA-IgG
Steroids	Steroids
Probiotics	Probiotics
PUFA	PUFA
Antibiotics	Antibiotics
PAF-AH	
Trefoil factor	
EGF	
PAF receptor antagonists	
Leukocyte depletion	
Oxygen radical scavengers	

EGF, Epidermal growth factor; *Ig,* immunoglobulin; *PAF,* platelet-activating factor; *PAF-AH,* platelet-activating factor acetylhydrolase; *PUFA,* polyunsaturated fatty acid.

humans.[132,133] In neonatal rats, maternal milk feedings increased IL-10 and reduced the incidence of NEC, whereas in human milk specimens, significant percentages of NEC patient-pairs were deficient in this important cytokine. Studies have compared proinflammatory response to endotoxin and/or IL-1 in different cell lines and have found that IL-8 response is significantly higher in fetal intestinal epithelium compared with mature, adult intestine.[30] Additional studies have shown persistent activation of a key proinflammatory transcriptional regulator, nuclear factor kappa beta (NF-κB), in premature or neonatal intestine compared with more mature samples.[134] These findings suggest that the balance of the inflammatory response in the neonate may be weighted toward the proinflammatory side and more likely to result in the pathologic outcome of NEC.

In conclusion, NEC is a multifactorial disease without one clear and precise cause. The well-described epidemiologic risk factors including bacterial colonization, intestinal ischemia or hypoxia, and formula feeding stimulate a final common pathway that results in intestinal injury in a subset of premature infants. Premature infants differ from term infants and older patients in multiple ways, including the complex system of intestinal host defense, intestinal motility, bacterial colonization patterns, autoregulation of splanchnic blood flow, and the regulation of the inflammatory cascade. Because each case of NEC is different, and the importance of each of the complex factors may vary between cases, no single approach has been completely successful in preventing this disease, although the prospect for probiotic supplementation in this context is compelling. Although preventing prematurity would be the most successful approach to prevent NEC, several strategies have been tested in humans and animals with intriguing results (Table 117-1). Carefully controlled trials in high-risk premature infants will be needed to significantly reduce this morbidity and mortality that currently plagues neonatal intensive care units worldwide.

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SECTION XV

Liver and Bilirubin Metabolism

118 Organogenesis and Histologic Development of the Liver

Steven Lobritto

The liver is the largest internal organ of the body, comprising approximately 6% to 7% of the total weight of an adult. The organ is unique in that it receives both a venous blood supply, predominantly from the intestines, pancreas, and spleen, and an arterial blood supply from the aorta. Together, this blood supply delivers nutrients, hormones, toxins, and oxygen to the liver tissues.[1] The hepatocytes, organized into linear plates, are exposed to these blood elements on their basal surface, which abuts a plexus of sinusoidal capillaries.[2-4] The hepatocytes are the predominant cellular element of the liver but are supported by interactions with other cellular components, including cholangiocytes, stellate cells, Kupffer cells, Ito cells, endothelial cells, and hematopoietic elements. An intercellular matrix that provides both structural integrity and pathways for intracellular communications supports these cellular elements.[5-7]

The hepatocytes perform various secretory and metabolic functions, including but not limited to detoxification of drugs and toxins, synthesis of key serum proteins (e.g., albumin, clotting factors, complement, apolipoproteins), synthesis and metabolism of dietary lipids, glucose homeostasis, and bile production.[8-12] Distortion of the normal architecture of the liver by either chronic disease states or congenital malformations can have a significant impact on the ability of the liver to perform these complex functions. As expected, the developmental steps necessary to ensure proper hepatic organization and function involve multiple and complex communications between the cellular and matrix components of the organ during organogenesis of the liver.[13-16] Investigators continue to discover new intracellular signals (specific growth factors and transcription factors) that appear to combine to initiate and propagate the transformation of fetal endodermal tissue into differentiated hepatic cellular elements and a functioning organ. This chapter summarizes some of the experiments by means of molecular genetics, molecular biology techniques, and tissue explant culture techniques that have shed light on our understanding of early steps in hepatic development.

EARLY EMBRYOGENESIS: AN OVERVIEW

Recent important discoveries have helped clarify some of the steps involved in the early development of the liver and the specific messengers and tissue signals that orchestrate this process, as well as the maturation of the hepatocytes.[13,17] Specific signaling molecules, hormones, growth factors, transcriptional factors, and intracellular matrix interactions have been shown to contribute to the induction of pluripotential primitive tissues into cells committed to a hepatic fate. In addition, these molecules are critical to the maturation of the cells into specialized epithelium, and they contribute to the proper organization of these cells into the correct configuration comprising the fully functioning organ. Much of this work has been derived from observations in genetically altered mice and tissue culture systems.

During the third to fourth week of gestation, a bud of proliferating endodermal tissue is observed originating from the ventral foregut constituting the hepatic diverticulum. The primitive cells of this bud, referred to as hepatoblasts, appear to be bipotential, with the ability to differentiate into either mature hepatocytes or cholangiocytes.[18] These cells are in contact with embryonic cardiac mesoderm, and they abut the septum transversum mesenchyme.[13,17] The hepatoblasts migrate as cords into the septum transversum, closely associated with primitive sinusoidal endothelial cells. As the process progresses, the sinusoidal structure is established, initially lacking the fenestrations observed at later stages of maturity. The undifferentiated hepatoblasts have few organelles at this stage, with a high nuclear-to-cytoplasmic ratio; scant, rough endoplasmic reticulum; and few lysosomes.[11] Intercellular communication appears to be mediated via cell surface adhesions with other hepatoblasts and surrounding mesenchymal cells. The anterior vitelline vessels of the yolk sac are the initial source of sinusoidal blood.[19] Early synthesis and secretion of alpha fetoprotein, transferrin, and α_1-antitrypsin can be observed from these immature prehepatic cells.[20]

LATE EMBRYOGENESIS: AN OVERVIEW

Early in the second month of gestation, the hepatoblasts gradually begin to differentiate into mature hepatocytes, with the necessary intercellular components (rough endoplasmic reticulum and Golgi apparatus) to conduct their multiple synthetic and metabolic functions.[11] These cells also acquire polarity with the increased production of specific membrane-associated proteins and transmembrane transporters.[21] This process creates both a basolateral hepatocyte domain with clusters of these membrane-associated receptors and transporters for protein secretion, in association with sinusoidal vessels, and an apical hepatocyte domain constituting the bile canaliculi, with transporters related to bile secretion.[22] Both these surfaces develop a microvillous architecture, presumably to optimize surface area for extracellular contact. Other hepatoblasts are thought to develop into cholangiocytes that organize into the hepatic biliary system.[23,24] The intrahepatic bile ducts are formed from periportal hepatoblasts constituting the ductal plate. Differentiation and maturation of the intrahepatic ducts occur via interactions with periportal connective tissue, glucocorticoid hormones, and basal laminar components.[18] Intrahepatic hematopoiesis increases at this stage and appears to progress with interactions between maturing hepatocytes and undifferentiated mesenchymal elements.[25-27]

SPECIFIC INTERACTIONS PROMOTING HEPATOGENESIS

The initial induction of the ventral foregut to commit to a hepatic fate has been shown in embryo tissue transplant studies in the chick to be a function of interactions of this endodermal

TABLE 118-1

Liver Cell Derivations

Cell type	Tissue of origin
Hepatocytes	Foregut endoderm, bipotential hepatoblast
Endothelial cells	Septum transversum mesenchyme, angioblast
Biliary epithelial cells	Foregut endoderm, bipotential hepatoblast
Hematopoietic cells	Septum transversum mesenchyme
Kupffer cells	Yolk sac and bone marrow
Ito cells	Septum transversum mesenchyme

region with cardiac mesoderm[28] (Table 118-1). The growth factors found to mediate this process produced by cardiac mesoderm are fibroblast growth factors (FGF)–1 and –2. Further studies with purified FGFs and FGF inhibitors confirmed this interaction.[29] The specificity of FGF for this region appears to be related to the fact that FGF migration is limited by high-affinity interactions with extracellular matrix.[30,31] In fact, without this induction stimulus, the fate of the ventral foregut that normally gives rise to the hepatic diverticulum will default to a pancreatic fate.[32] Specifically, FGF stimulation of the ventral foregut endoderm inhibits pancreatic genes and induces liver genes in this bipotential precursor cell population.

The induction stimulus provided by FGF is not sufficient to stimulate hepatocyte differentiation. Hepatocyte differentiation appears to be a function of a second induction stimulus from another mesoderm-derived tissue, the septum transversum mesenchyme. The signal proteins for this interaction appear to be bone morphogenetic proteins (BMP).[33] Specifically, BMP-2, BMP-4, and BMP-7 are produced by the septum transversum mesenchyme cells. Further studies with a BMP signal inhibitor, Noggin, demonstrated that BMP, as well as FGF, was needed to achieve hepatoblast induction from ventral gut endoderm.[33] The stimulatory effects of FGFs are focused on the prehepatic endodermal tissue by a network of transcription factors expressed in embryonic gut tissue. One such molecule is hepatocyte nuclear factor (HNF) 3B.[34] These transcription factors appear to be important mediators of hepatocyte differentiation; they bind to specific hepatic gene enhancer regions, promoting gene expression and cellular differentiation.[35]

The importance of the interaction between the septum transversum mesenchyme and the developing prehepatic endoderm cannot be overemphasized. The septum transversum mesenchyme is the source of BMP signaling, leading to induction of hepatoblast differentiation and propagation of the hepatocyte maturation process.[36] Extracellular matrix components of the septum transversum mesenchyme aid in the regulation of differentiation by binding and concentrating signaling molecules.[37,38] In addition, extracellular matrix components can mediate intracellular communication directly through interactions with integrins, focal adhesion kinase, and other signaling molecules.[7,39] Hepatocyte growth factor (HGF) is a potent hepatocyte proliferation stimulant that affects cell migration, as well as differentiation.[40,41] These are but a few of the recognized components of the septum transversum mesenchyme and its extracellular matrix that are part of an integrated and diverse signaling process ensuring integrity to the hepatocyte maturation process and the overall hepatic structural organization.[42]

HEPATIC VASCULAR ANATOMY

During fetal development, highly oxygenated blood is delivered to the liver through the falciform ligament from the placenta via the umbilical vein.[1,43,44] The blood supply to the right lobe is derived from the right branch of the portal vein. The left branch of the portal vein connects via the portal sinus to the umbilical vein. The blood supply to the left lobe of the liver is derived from direct branches off the umbilical vein. This difference in blood oxygen saturation gives preferential dominance to the left lobe in utero. The umbilical vein continues past these branch points as the ductus venosus, delivering well-oxygenated blood to the inferior vena cava, which is directed across the patent foramen ovale to the left side of the heart.[19,43,45] At birth, the ductus venosus and umbilical veins obliterate, and the normal adult hepatic vascular supply is established.

INTRAHEPATIC VASCULAR DEVELOPMENT

In the adult, the portal vein enters the liver and branches into smaller and smaller vessels that travel within the portal tracts, along with branches of the hepatic artery and the interlobular bile ducts. The portal veins terminate in the sinusoids of the liver that are characterized by fenestrated endothelial epithelium. Sinusoids are separated by single cell–thick sheets of hepatocytes, referred to as the hepatic plates. Therefore the hepatocytes are directly exposed to portal blood, permitting transfer of macromolecules. The sinusoidal blood empties into the central branches of the hepatic veins, which terminate into the left, right, and middle hepatic veins and return blood to the inferior vena cava and back to the heart. This important anatomic arrangement is critical for proper gland function and is under the control of a number of factors during hepatic development.[1,46]

Angioblasts, or primitive precursors to functional endothelial cells, have been found in an intermediate position between the prehepatic endoderm and the surrounding septum transversum mesenchyme[13] (see Table 118-1). These endothelial cell precursors intermingle with hepatoblasts as the endoderm organizes into the hepatic bud. The development of this cell population is under the influence of vascular endothelial growth factor 2.[47,48] Observations in transgenic mice and explant systems of liver bud tissue suggest that angioblasts stimulate development of the hepatic bud in endodermal tissues before the local blood vessels are formed.[49] The origin of the angioblast cells remains unknown. The current thinking is that vessel anatomy does not follow a rigid predetermined pattern, but rather vasculogenesis appears to be guided by local needs and flow dynamics. The vascular endothelium may well be equally important in providing signaling to the surrounding tissue, affecting the differentiation of organ cell precursors and the ultimate structural integrity of the organ.[50,51]

BILIARY DUCT DEVELOPMENT

In the adult liver, mature hepatocytes secrete bile into the canaliculi, which are small channels along the apical surface created by clusters of adjacent hepatocytes. These channels coalesce into a network of intrahepatic bile ducts and eventually into the main hepatic ducts lined by biliary epithelial cells or cholangiocytes. Bile is exported from the liver and stored and concentrated in the gallbladder for excretion during a meal.

The origin of biliary cells appears to be bipotential hepatoblasts originating at the hepatic bud[18] (see Table 118-1). Under the influence of the transcription factor HNF-6 (and its effect on HNF-1B) these cells undergo differentiation and proliferation.[52] These subsets of hepatoblasts strongly express biliary-specific cytokeratins.[24] The biliary precursor cells form the ductal plate that is characterized as a continuous, single-layered ring around the portal mesenchyme, which proliferates into a bilayer. Focal dilatations of the ductal plate along the bilayer give rise to the bile ducts. The newly formed ducts are incorporated into the hepatic mesenchyme. The whole process is moderated by

biliary precursor cell interactions with neighboring cells and with matrix proteins, including laminin, fibronectin, and collagen types I and IV.[37,38,53,54] The process of bile duct formation also appears to be influenced by interactions with the nearby developing blood vessels, as evidenced by abnormalities in bile duct morphology observed with genetic deficiencies in the vascular Notch pathways.[55]

HEPATIC HEMATOPOIESIS

The yolk sac contributes significantly to early fetal hematopoiesis.[25] The yolk sac delivers blood from the anterior vitelline vessels to the developing sinusoids of the liver. Around the 6th week of gestation, the earliest hematopoietic elements appear to differentiate from the undifferentiated mesenchymal cells derived from the septum transversum[56] (see Table 118-1). These cells appear to interact with the developing hepatocytes and may derive trophic signals stimulating growth and development.[26] This stimulatory effect appears to change with time, diminishing as the hepatocyte matures.[57] Hematopoietic elements produced in the liver migrate through regulated temporary migration pores (not between the intact endothelial cell lining of the sinusoids), a process termed diapedesis.[2,3] Interesting new evidence suggests that hepatocytes themselves may be derived from multipotential bone marrow cells, as well as from prehepatic endoderm.[58,59] As the hepatocyte matures and differentiates into the cells responsible for serum protein production, there appears to be a diminished role for intrahepatic hematopoiesis, which is then assumed by the marrow.

SINUSOIDAL CELLS: KUPFFER AND ITO CELLS

Kupffer cells are manifested during the second month of gestation. These specialized cells residing on sinusoidal surfaces of the endothelium have macrophage-like activity.[3] These cells appear to originate from both the embryonic yolk sac and from the bone marrow at later stages of development[60] (see Table 118-1). The position of these cells permits regulation of migration across the endothelium of the sinusoid.

Ito cells are specialized cells that reside in the perisinusoidal space of Disse between the endothelial cells and the developing hepatocytes of the hepatic plate.[61] These cells appear to originate from the undifferentiated mesenchyme of the septum transversum[4] (see Table 118-1). These cells store fat and vitamin A. Along with endothelial cells, these cells are involved with the production and secretion of basement membrane collagen and may therefore contribute to fibrosis in chronic liver injury.[61,62]

ACINAR ORGANIZATION

The functional unit of the liver is known as the acinus. This functional unit is defined by the hepatic blood flow from the portal vein branches within adjacent portal tracts, through the hepatic sinusoids separated by hepatocyte plates, terminating in the central branches of the hepatic veins. These basic units of function are recognized in the second and third months of gestation as the cell elements of the liver differentiate and as the matrix elements proliferate. In the mature liver, the hepatocytes within this functional unit perform different operations depending on their position within the acinus. The hepatocytes within the acinus have been divided into three zones. Zone 1 cells are closest to the portal tracts and therefore receive blood with the highest nutrient and oxygen content. These cells appear to be best suited to extract bile acids, perform gluconeogenesis, produce glycogen, synthesize and secrete protein, and metabolize lipids.[63-67] Zone 3 cells are located adjacent to the branches of the hepatic vein. Zone 3 hepatocytes appear to have a higher capacity to perform glycolysis; have higher cytochrome P-450 expression; and perform various metabolic reactions, including drug detoxification, glycosylation events, and ureagenesis.[10,68-70] Zone 2 cells are intermediate in position, and the hepatocytes in this zone appear to express activities similar to the cells of the other two zones depending on their relative proximity.

Investigations of the differences in the functions of the cells within these defined zones have led to two likely overlapping theories.[71] The first assumption is that differences in the oxygen and macromolecule content of the blood supply to these zones may influence the development and differentiation of hepatocytes. The second theory is that regional differences in cell signaling and matrix element interactions influence hepatocyte differentiation in local populations. Modulators of gene function and expression within the hepatocytes of a particular zone ultimately will define and refine their respective functions.[72] In addition, intercellular communications between adjacent hepatocyte populations will further affect hepatocyte differentiation and regional coordination of function. The likely conclusion is that both of these influences are responsible for the observed differences and similarities in hepatocyte function within the acinus.

REGULATION OF FETAL LIVER GROWTH AND MATURATION

As cell differentiation proceeds, the fetal liver also undergoes growth and structural organization. The molecules stimulating these processes are poorly understood. The process seems to be under the influence of circulating hormones and various growth factors (Box 118-1). Specifically, growth hormone and its gestational homologue placental lactogen are thought to be important stimuli for hepatocellular hyperplasia.[73,74] Furthermore, insulin, cortisol, and thyroid hormone have been shown to alter protein production and growth factor receptor expression in the developing liver and may be important mediators of growth and maturation of the organ both in utero and post partum.[75-77] In part, the effect of these hormones is a function of their effect on the production and binding of secondary messenger molecules, the insulin-like growth factors.[73,75-80] Differential production and binding of these messengers may contribute to cell growth and replication, cell migration, and tissue organization and maturation.

Other molecules contributing to cell hyperplasia, migration, and tissue organization are HGF and epidermal growth factor (see Box 118-1). HGF has emerged as an important tissue-specific signaling molecule between epithelial and mesenchyme tissues. This molecule is a ligand for the c-*met* proto-oncogene product of receptor tyrosine kinase and originates from mesenchymal tissues.[81] As such, this paracrine molecule supports

BOX 118-1

Stimuli of Hepatocellular Development

Hormonal

Growth hormone, placental lactogen
Thyroid hormone
Cortisol
Insulin

Growth factors

Insulin-like growth factor I
Insulin-like growth factor II
Hepatocyte growth factor
Epidermal growth factor

organogenesis, mature organ regeneration, and neoplastic processes.[81,82] HGF and the expression of its receptor in liver and other tissues have been demonstrated in association with tissue injury, suggesting a focused tissue-specific regeneration response.[82] The diverse biologic functions of this multipotent polypeptide now lead investigators to consider it a key molecule for tissue organization, organogenesis, and organ repair.

Epidermal growth factor has been identified as a key molecule involved with liver regeneration and may play a role in embryogenesis and fetal growth, because receptors have been found in fetal tissues.[83,84] The liver appears to modulate serum levels of this molecule, providing efficient clearance and degradation of the internalized protein.[85] Also, extensive data indicate interactions between epidermal growth factor and other circulating hormones.[84] Growth hormone increases epidermal growth factor binding in the liver.[86] Thyroid and steroid hormones modulate both epidermal growth factor and its receptors in multiple tissues.[87] Epidermal growth factor stimulates hormone release from the pituitary, the placenta, and the adrenal gland but inhibits hormone production by the gonads and thyroid. The effect on fetal growth and development may be mediated by gene activation, or it may be a function of other protein interactions.[88] The significance of this molecule in hepatic growth and maturation has yet to be proved.

CONCLUSION

In summary, the development of the liver from primitive endoderm and mesoderm is a complicated yet orchestrated process that mandates continued intercellular and matrix signaling. Our current knowledge of the multitude of contributing messengers to this process is likely just the tip of the "organogenesis iceberg." The expanding knowledge base of the steps ensuring proper hepatocyte differentiation, growth, and organization will permit the application of learned techniques to promote regeneration and restoration of the damaged mature organ. In addition, by increasing the knowledge of the signaling pathways governing normal growth and development of the liver, we may expand our understanding and ability to manage the uncontrolled growth of hepatic malignancies. The key to the evolution of this process in the short term is our expanding technical expertise with tissue culture systems and transgenic animal models.

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Bile Acid Metabolism During Development

Human milk contains a lot of fat (range, 20 to 90 g/L of milk), and infants derive almost 60% of their energy from fats. This energy-dense food fuels dramatic increases in body weight, but the hydrophobic nature of fats conflicts with the hydrophilic properties of digestive enzymes and transporter proteins. To solve this problem, the liver secretes bile acids, which act like detergents to facilitate fat digestion and absorption. Hence, bile acids are critically important for infant nutrition.

In this short chapter, we focus on bile acid metabolism during early development. In general, we will abide by the outline followed by earlier versions of this chapter, and initially and briefly describe bile acid structure and synthesis as well as enterohepatic circulation, which are generally similar between adults and neonates. We then describe bile acid metabolism unique to infants and the regulation of bile acid transport by the placenta, and we end with a brief description of disorders of bile acid metabolism that are initially detected in the young. We refer readers interested in the details of bile acid metabolism to numerous reviews (about 150 between 2002 and 2006) on this topic.

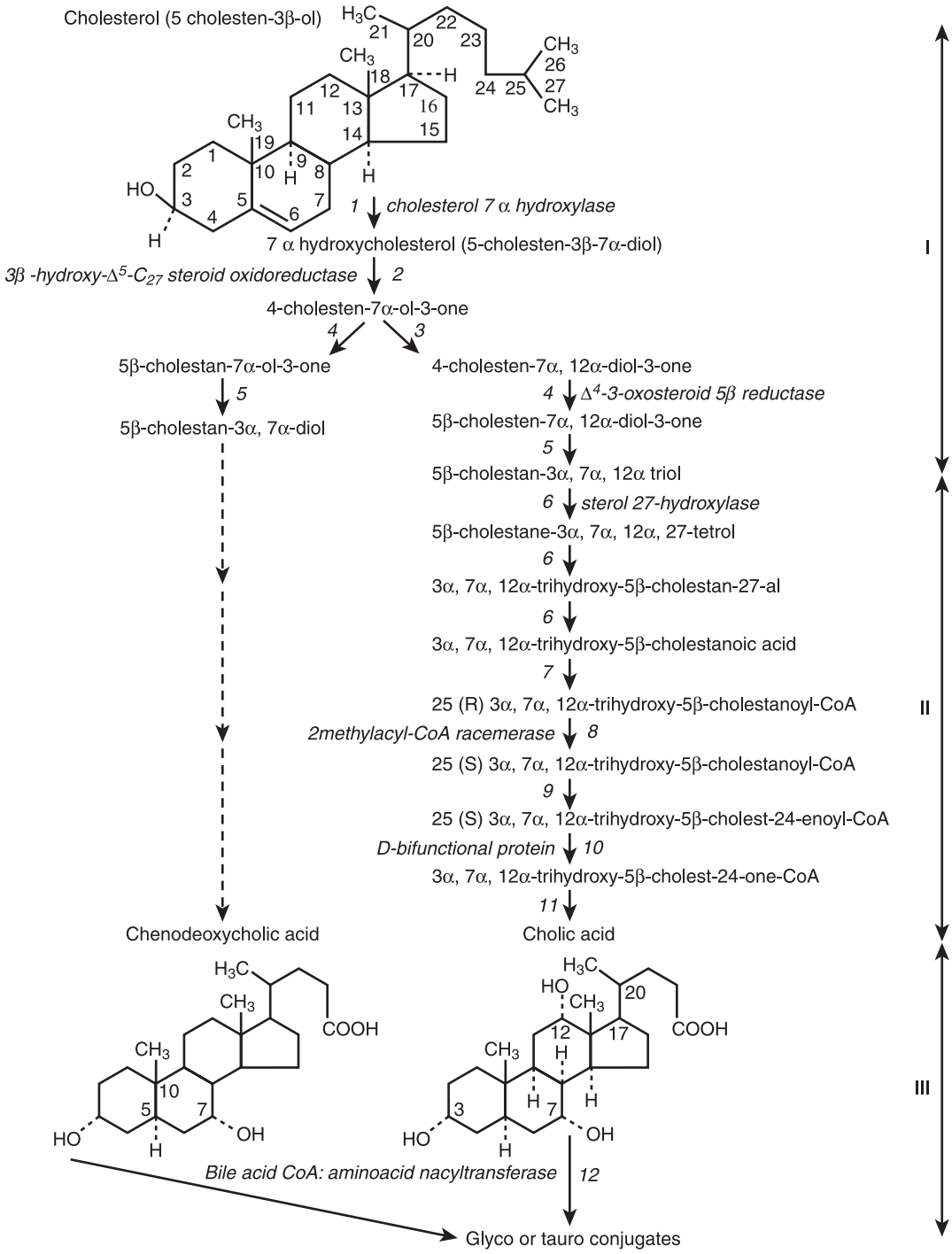


Figure 119-1 The classic pathway of bile acid synthesis. Numbers in italics by the *arrows* indicate different enzymes mediating the reaction. Enzymes for which defects have been identified to cause clinical problems are named and shown in italics. Roman numerals: *series I,* nuclear modification; *series II,* side-chain oxidation and shortening; *series III,* conjugation. In the absence of 12α-hydroxylation, chenodeoxycholic acid is formed by an alternative pathway (see text). *(Adapted from Vogh BP, Maren TH: Sodium, chloride, and bicarbonate movement from plasma to cerebrospinal fluid in cats,* Am J Physiol *228:673, 1975.)*

BILE ACID CHEMICAL STRUCTURE AND PROPERTIES

Bile acids are made up of a steroid nucleus with an aliphatic side chain consisting of 5 carbons and are very similar to the structure of cholesterol (Figure 119-1). However, the properties of bile acids and cholesterol differ markedly. Cholesterol is a flat, water-insoluble molecule, whereas the predominant human bile acids have a β configuration of the hydrogen atom at C5, giving them a kinked appearance and making them relatively more water-soluble. The number and orientation of the hydroxyl groups determine the capability of bile acids to form micelles and solubilize lipids. The α side of the bile acids is hydrophobic, and the β side is hydrophilic. When a micelle is formed, the β side forms a water-soluble exterior, and the α side forms a lipid-soluble interior.

Bile acids with a side chain containing 5 carbons are called cholanoic acids and comprise the primary human bile acids: cholic and chenodeoxycholic acids (see Figure 119-1, section III). Precursors of the primary bile acids are called cholestanoic acids and have 8 carbon side chains.

Cholic and chenodeoxycholic acids form conjugates by the formation of amides between the carboxyl group of the bile acid side chain with the amino group of glycine and taurine, to make glycocholic acid, taurocholic acid, glycochenodeoxycholic acid, and taurochenodeoxycholic acid in the liver. In proportionally smaller amounts, bile acids are also conjugated with glucoronate, sulfate, glucose, or *N*-acetylglucosamine. In humans, bile acids are primarily conjugated with glycine, but in early life, taurine conjugates predominate.[1] Conjugation increases the solubility of bile acids in aqueous solutions at the pH prevailing in the intestinal lumen. This decrease in hydrophobicity also minimizes potentially harmful bile acid interactions with plasma membranes and reduces the rate of passive absorption of bile acids in the more proximal intestinal regions, which have insignificant, if any, numbers of bile acid carriers. Reduced absorption in the proximal regions, in turn, leads to increased concentrations of bile acids in the lumen and contributes to luminal bile acids exceeding the critical micellar concentration, thus facilitating dietary fat digestion.

In the small intestinal lumen, small amounts of primary bile acids are converted into secondary bile acids by the action of the bacterial enzymes which remove hydroxyl groups. Most primary and secondary bile acids are reabsorbed through passive and active transport (see Enterohepatic Circulation) in the small intestine. A modest amount of bile acids enters the large intestine. Here, the remaining primary bile acids are mostly converted to secondary bile acids deoxycholic acid (from cholic acid), ursodeoxycholic acid, and lithocholic acid (from chenodeoxycholic acid) by colonic bacteria and are then reabsorbed passively.[2] Sulfation leads to formation of tertiary bile acids such as sulfolithocholic acid in the colon.

BILE ACID SYNTHESIS

In the liver, bile acids are synthesized from cholesterol through multiple enzymatic reactions (see Figure 119-1).[3,4] There are two basic changes that occur: the initial steps (see Figure 119-1, steps 1 to 5, series I) involve modification of the cyclopentanoperhydrophenanthrene ring or the nucleus, which is made up of the four closed rings; the second set of steps modify the 8 carbon side chain of the sterol linked to C17 (see Figure 119-1, steps 6 to 11, series II). Cholesterol, which is practically insoluble in water (solubility: 0.2 mg/100 mL water), is converted to cholic acid, which is more soluble in water (solubility: 28 mg/100 mL water), which by the process of conjugation (see Figure 119-1, step 12, series III) is converted to highly water-soluble bile salts (i.e., sodium taurocholate) (solubility: 100 mg/100 mL water). The enzymes involved in the conversion of cholesterol to bile acids are localized to different sites in the hepatocytes (i.e., endoplasmic reticulum, cytoplasm, mitochondria, and peroxisomes). The exact mechanism and sequence of the interorganelle movement of bile metabolites during bile acid synthesis have not yet been elucidated.

In the classical pathway, the first step in the modification of the sterol nucleus is the addition of an OH group on the α configuration at position C7 of cholesterol. This step is mediated by a microsomal P-450 enzyme expressed only in the liver, cholesterol 7α–hydroxylase (CYP7A1) (see Figure 119-1, step 1). In the alternate or oxysterol pathway, cholesterol is first hydroxylated either at carbon 24, 25, or 27 by the 24-, 25-, or 27-hydroxylase enzymes, respectively. These hydroxylated products are subsequently hydroxylated at C7 by oxysterol 7α-hydroxylase (CYP7B1). They then undergo side-chain oxidation and shortening similar to the classical pathway. The 24-hydroxylase enzyme is abundant in the brain and contributes little to overall bile acid synthesis but is important in turnover of cholesterol in the brain. 25-Hydroxylase messenger RNA (mRNA) is present in most tissues and is abundant in the retina and may have some role in bile acid synthesis. 27-Hydroxycholesterol is the most abundant oxysterol and is synthesized from cholesterol by sterol 27-hydroxylase. In mice, 25% of the bile acid pool comes from oxysterols, and 27-hydroxycholesterol is the major contributor.[5] In humans, the oxysterol pathway contributes to 5% to 10% of the bile acid pool.[6]

REGULATION OF BILE ACID SYNTHESIS

Classical Pathway

CYP7A1 is the rate-limiting enzyme in the synthesis of bile acids in the hepatocytes. Interruption of the enterohepatic circulation either by administration of bile acid binding resins,[7,8] fasting,[9] or surgical intervention[10] results in induction of this enzyme. Dietary cholesterol also induces CYP7A1 in the liver. Taurine feeding increases the CYP7A1 activity in rats fed cholesterol or bile acids.[8] Hence, reductions in luminal bile acid concentrations, increases in supply of its precursors, and increases in demand for bile acids apparently stimulate their synthesis. This adaptive mechanism is important in compensating for some pathologic conditions described below.

CYP7A1 is suppressed by bile acids and inflammatory cytokines. Inhibition appears to be mediated through hepatocyte nuclear factor (HNF)-3, -4, and -6 transcription factors essential for morphologic or functional expression of liver-specific genes. Interleukin-1β (IL-1β), chenodeoxycholic acid (through the JNK/c-jun pathway), and HNF-4 inhibit bile acid synthesis by inhibiting *CYP7A1* gene transcription in human primary hepatocytes and HepG2 cells.[11] A reduction in HNF-6 mRNA and protein expression in mouse liver after bile duct ligation is correlated with a reduction in hepatic CYP7A1 mRNA expression. Because bile duct ligation prevents bile acids from entering the intestinal lumen, this finding appears to contradict the previous finding that bile acid resins stimulate CYP7A1 synthesis. Bile duct ligation, however, markedly increases hepatic and plasma bile acid concentrations; therefore the signal regulating CYP7A1 may be the extracellular fluids bathing the hepatocytes. In fact, increases in HNF-6 levels in mice with bile duct ligation have been shown to increase CYP7A1 mRNA levels.[12]

Small heterodimer partner (SHP) is a nuclear receptor that modulates the activities of other nuclear receptors. The human SHP promoter is activated by sterol regulatory element binding protein-1 (SREBP-1), which regulates the expression of various genes involved in cholesterol and fatty acid synthesis. Overexpression of SREBP-1 activates the human SHP but not mouse SHP.[13] SHP physically interacts with HNF-3 and decreases CYP7A1 expression.[14]

Some of the nuclear receptors originally termed *orphan receptors* (i.e., farnesoid X receptor [FXR], pregnane X receptor [PXR], and liver X receptor [LXR]) play a major role in cholesterol and bile acid metabolism. These receptors are activated by a large number of physiologic ligands. Ligands for FXR that are relevant to this chapter are primary bile acids,[15-17] for PXR are secondary bile acids,[18,19] and for LXR are oxysterols.[20,21] Through coordinated regulation of transcriptional programs, these nuclear receptors regulate key aspects of cellular and whole-body sterol homeostasis, including cholesterol absorption, lipoprotein synthesis and remodeling, lipoprotein uptake by peripheral tissues, reverse cholesterol transport, and bile acid synthesis and absorption.[22]

FXR, when bound by primary bile acids in hepatocytes, suppresses bile acid synthesis by inhibiting CYP7A1, limits bile salt

uptake by repressing the sodium-taurocholate cotransporting polypeptide (*NTCP*) gene, and stimulates bile acid export from the cells, thus protecting the hepatocytes from bile acid toxicity.[16,23] PXR and vitamin D receptor (VDR) induce catabolism of secondary bile acids by stimulating CYP3A, a cytochrome P-450 enzyme that detoxifies lithocholic acid in the liver and intestine.[19,24,25] Hence, excess bile acids reduce bile acid synthesis and increase bile acid catabolism through FXR-, PXR-, and VDR-mediated pathways.

Conversion of cholesterol to bile acids in the liver is positively regulated by LXR.[26] Excessive dietary cholesterol leads to increased oxysterol formation. Oxysterols bind to LXR and induce transcription and synthesis of CYP7A1, thus increasing the removal of cholesterol by conversion to bile acids.[27] Hence, LXR and FXR have opposite effects on CYP7A1 transcription in the liver.

Hormones have been shown to modulate acute changes in bile acid metabolism. Addition of triidothyronine to human primary hepatocytes resulted in a decrease in bile acid production in parallel with decreases in mRNA expression of CYP7A1 and sterol 12α-hydroxylase (CYP8B1) (see Figure 119-1, step 3).[28] In mice, however, thyroid hormone induces bile acid synthesis in vivo, and in fact two thyroid hormone receptor binding sites on the *CYP7A1* gene have recently been reported.[29] Both sites are required for full induction of the CYP7A1 promoter by thyroid hormone. Addition of physiologic concentrations of insulin to human hepatocytes rapidly leads to stimulation of the *CYP7A1* gene; however, prolonged insulin treatment induces SREBP-1c, which inhibits human *CYP7A1* gene transcription.[11] Glucagon and cyclic adenosine monophosphate (cAMP) inhibit human *CYP7A1* expression by phosphorylation of HNF-4α.[9] Hence, glucagons, cAMP, chenodeoxycholic acid, and interleukins inhibit *CYP7A1* and may share the same HNF-4α–mediated inhibitory mechanism.

Alternative Pathway

The alternative, like the classical, pathway is also regulated by bile acids and bile salt binding resins. Oral administration of cholic acid, chenodeoxycholic acid, and deoxycholic acid in mice significantly down-regulated CYP7B1 (oxysterol 7α-hydroxylase), whereas administration of cholestyramine, which decreases luminal bile acid concentrations, up-regulated CYP7B1.[30] In rat primary hepatocytes, taurocholic acid decreased CYP7B1 activity.[31]

Bile acids repress the transcription of sterol 27-hydroxylase (CYP27 in the alternate pathway) in rat primary hepatocytes by decreasing HNF-1α binding to the CYP27 promoter.[32] Likewise, cytokines also modulate the alternative pathway because IL-1 and tumor necrosis factor decrease sterol 27-hydroxylase mRNA levels in human hepatocyte cell lines (HepG2).[33] Therefore, like in the classical pathway, reductions in luminal bile acid concentrations up-regulate enzymes in the alternative pathway, whereas increases in luminal bile acids down-regulate the alternative pathway.

These two pathways can interact. Humans with mutations of *CYP7A1*, who therefore have their classical pathway compromised, show evidence of up-regulation of the alternative bile acid pathway.[6] However, CYP27 was not regulated by cholesterol or cholestyramine feeding in CYP7A1 knockout mice.[5]

ENTEROHEPATIC CIRCULATION

After synthesis and conjugation with glycine and taurine, bile acids are secreted into bile and then stored in the gallbladder, where they become increasingly concentrated by the active transport of sodium and chloride followed by the passive resorption of water out of the gallbladder lumen. The gallbladder evacuates its contents in response to a meal by means of cholecystokinin, and more than 70% of the stored bile is expelled into the proximal small intestine.[34] In adults, the amount of bile acids entering the intestine is 4 to 6 g per meal (2 g/hour) or 12 to 18 g per day.[35] The average amount of bile acids synthesized by the liver is only 0.2 to 0.5 g per day, and the remainder is obtained from the enterohepatic recycling of bile acids (95% of bile acid pool). In the small intestinal lumen, bile acids reach critical concentrations to form mixed micelles and act as detergents in emulsifying dietary fats, facilitating absorption of dietary fats and of lipid-soluble vitamins.

About 90% of bile acids entering the intestinal lumen are absorbed in the distal third of the ileum. Intestinal absorption is a major step in the enterohepatic circulation of bile acids (Figure 119-2). To accomplish their enterohepatic circulation, bile acids cross four enterohepatic membrane barriers: the sinusoidal (basolateral) and canalicular (apical) membranes in the hepatocytes and the apical (luminal) and basolateral membranes in the enterocytes (Figure 119-3). The large number of membrane transporters involved in this process has been reviewed.[36,37] Bile acids absorbed from the intestines circulate in blood bound to albumin and to high-density lipoproteins. Upon return to the liver through the portal circulation, bile acids are reabsorbed, resecreted into the gallbladder, and then reenter the intestinal lumen. This completes the enterohepatic circulation of bile acids, which cycle 6 to 10 times per day depending mainly on the fat content of the meals.[38] A small fraction of the total bile acids enters the systemic circulation, 5 μmol/L, compared with the adult human portal venous plasma concentration of 20 to 50 μmol/L.[35]

The efficiency of enterohepatic circulation is very high, such that about 95% of circulating bile acids are conserved or recycled, and only 5% of the bile acid pool is lost. This amount is lost through fecal excretion but is replaced by the de novo synthesis of equivalent amounts of bile acids in the liver, keeping the size of the bile acid pool relatively constant.[39]

Hepatic Bile Acid Transport:

Basolateral

Along the sinusoidal (basolateral) membrane of the periportal hepatocytes, bile acids are extracted from the portal venous blood either by a sodium-dependent or a sodium-independent mechanism (see Figure 119-3). Sodium-dependent uptake of taurine- and glycine-conjugated bile acids is mediated by the sodium taurocholate cotransporting polypeptide NTCP (*SLC10A1*).[40] Up to 90% of conjugated bile acids are extracted at first pass by NTCP, irrespective of systemic bile acid concentrations.[41,42] NTCPs mediate the electrogenic, unidirectional, sodium-coupled uptake of taurocholate and other bile acids with the same stoichiometry (i.e., sodium–bile acid ratio of 2:1) and similar K_m values (taurocholate K_m ~ 6 μM, taurochenodeoxycholic acid K_m ~ 2 μM).[40,43] NTCP belongs to the same family as the apical sodium-dependent bile acid transporter in the ileum, ASBT (*SLC10A2*). However, NTCP transcription regulation or control, which is different from that of ASBT, is through a farnesoid X receptor and bile acid receptor (FXR/BAR)-dependent manner.[44] The *NTCP* gene is located on chromosome 14 in humans.[40]

Several members of the organic anion transporting polypeptide (OATP) family of anion exchangers, mainly OATP-A (*SLC21A3*), OATP-B (*SLC21A9*), and OATP-C (*SLC21A6*), and, to a lesser extent, OATP8 (*SLC21A8*), mediate the sodium-independent hepatic uptake of bile acids by facilitated exchange with intracellular anions.[38,45-47] The large OATP family has been reclassified, and the new names and symbols of these transporters are, respectively, OATP1A2 (*SLCO1A2*), OATP2B1

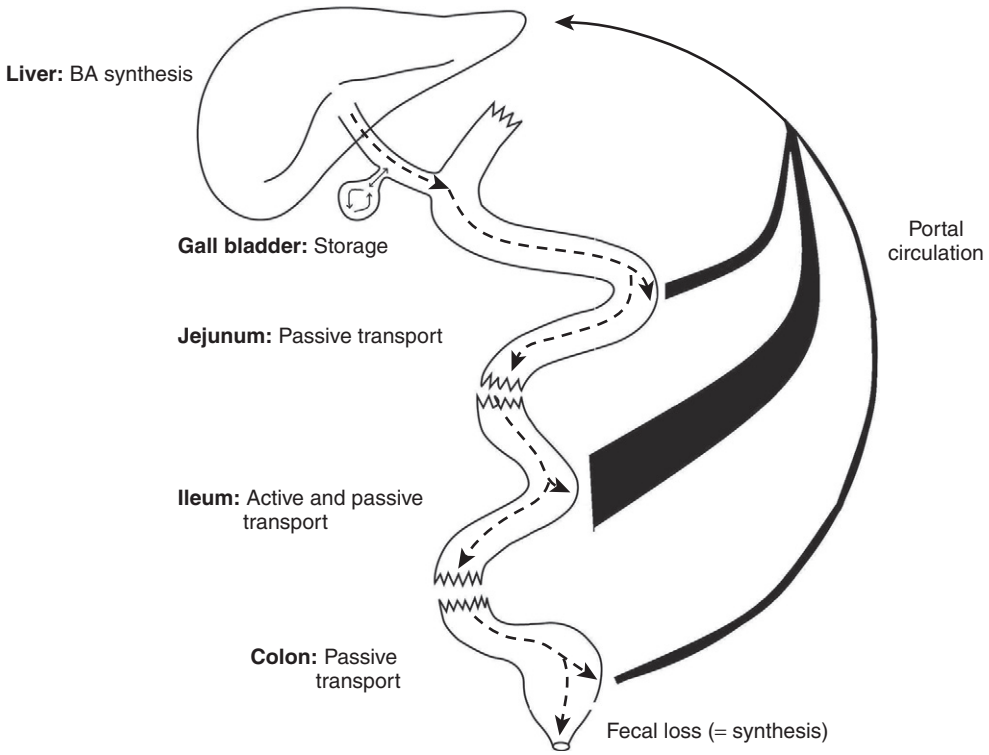


Figure 119-2 Enterohepatic circulation of bile acids. *Arrows* indicate the direction of bile acid movement, whereas *thickness of solid arrows* indicates relative amounts emanating from each intestinal region. Bile acids synthesized in the liver are stored in the gallbladder and then expelled into the gastrointestinal tract *(dashed arrows),* where they emulsify dietary fats; they are then reabsorbed into the portal circulation.

(*SLCO2B1*), OATP1B1 (*SLCO1B1*), and OATP1B3 (*SLCO1B3*).[48] Rat liver OATPs transport conjugated and unconjugated bile acids with K_m between 7 and 54 μM.[38] In general, the OATP transporters mediate anion exchange, coupling the cellular uptake of bile acids with the efflux mainly of bicarbonate[49] and of glutathione metabolites.[48] Cholestasis[50] and cholate feeding[51] down-regulate OATPs in mice.

Although the amount is modest, some bile acids also efflux out of the hepatocytes along the sinusoidal membrane into the portal blood through MRP3 (multidrug resistance protein 3) (*ABCC3*) and MRP4 (*ABCC4*),[37] especially during periods of cholestasis when these transporters are up-regulated[52,53] (see Figure 119-3). Patients with deficient canalicular MRP2 (*ABCC2*) expression have high levels of sinusoidal MRP3.[54] This efflux machinery therefore helps keep intracellular bile acid concentrations low when canalicular transport is compromised.

Cytosol

After entering the hepatocytes, unconjugated bile acids get reconjugated. The cytosolic hepatic bile acid binding proteins (HBABPs; see Figure 119-3) that have been identified in rats include 3α-hydroxysteroid dehydrogenase (3α-HSD), glutathione S-transferases, and liver–fatty acid binding protein (L-FABP).[55] These proteins act as cytosolic carriers for bile acids, moving them from the sinusoidal compartment to the canalicular side, to facilitate export by transporters.

Apical

Under physiologic conditions, conjugated bile acids are excreted into the bile across the canalicular (apical) membrane by the bile salt export pump, BSEP (*ABCB11*), also known as sister-to-P-glycoprotein, SPGP, a member of the *MDR* (multidrug resistance) gene family of adenosine triphosphate (ATP) binding cassette transporters (ABC).[56] BSEP plays a key role; the canalicular secretion of bile acids represents the rate-limiting step in the overall transport of bile acids from the portal blood into bile. While BSEP transports monovalent bile acids, which represent 98% of total bile acids, another transporter, the canalicular MRP2 (*ABCC2*) handles divalent, glucuronidated, and sulfated bile acids.[57] Meanwhile, the canalicular bile acid efflux pump breast cancer resistance protein BCRP (*ABCG2*), which functions as a xenobiotic transporter, transports sulfated bile acid conjugates into bile.[58] MRP2 is down-regulated in cholestasis.[59,60] MDR3 (*ABCB4*) mediates biliary excretion of phospholipids, which are second only to bile acids as the predominant solutes in bile. Bile then drains through bile ductules from the biliary canaliculi to the terminal branches of the biliary tree and bile ducts through the canals of Herring.

Role of Cholangiocytes

Cholangiocytes are epithelial cells lining the bile ducts, and they can modulate bile composition. Within the bile ducts, conjugated bile acids are reabsorbed at the cholangiocyte apical or luminal membrane by the apical sodium-dependent bile acid transporter ASBT (*SLC10A2*) and possibly by OATP-A (*SLC21A3,* now OATP1AC [*SLC01A2*]).[61-63] Unconjugated, relatively hydrophobic bile acids may be passively transported. The cholangiocytes secrete bile acids across the basolateral plasma membrane through MRP3 (*ABCC3*) along with a truncated form of ASBT, t-ASBT, that may mediate taurocholate efflux. This completes

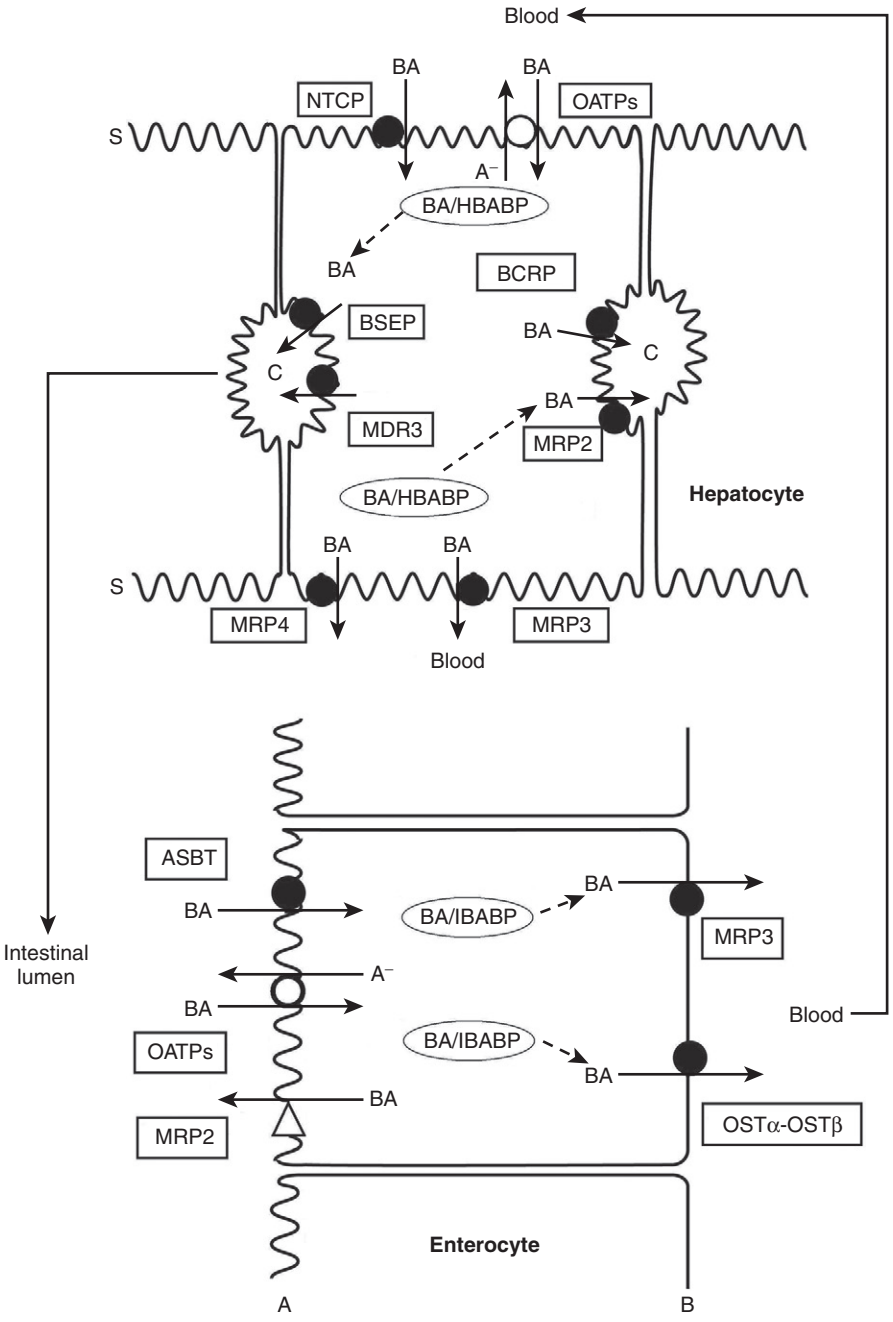


Figure 119-3 Bile acid (BA) transport across hepatic and intestinal membranes. *Arrows* denote direction of bile acid movement. *Hepatocyte:* At the basolateral or sinusoidal (*S*) membrane, the sodium-dependent bile acid transporter NTCP is the major uptake system for conjugated bile acids. OATPs (*SLC21*) mediate the sodium-independent bile acid uptake by facilitated exchange with intracellular anions A^-. A hepatic bile acid binding protein HBABP shuttles bile acids (BA/HBABP) across the cytosol of hepatocytes. At the canalicular membrane (*C*), the bile salt export pump BSEP (*ABCB11*) mediates excretion of monovalent bile acids, while the multidrug resistance–associated protein MRP2 (*ABCC2*) mediates divalent sulfated or glucuronidated bile acids. Other mediators of bile acid transport are the MDR3 (*ABCB4*) and BCRP (*ABCG2*). MRP3 (*ABCC3*) and MRP4 (*ABCC4*) mediate basolateral efflux for anionic conjugates and monovalent bile acids. *Enterocyte:* At the luminal or apical (*A*) membrane, the apical sodium-dependent bile salt transporter ASBT (*SLC10A2)* mediates bile acid uptake, while the anion exchanger OATPs mediate the sodium-independent bile acid transport. An ileal bile acid binding protein IBABP shuttles bile acids (BA/IBABP) from the apical to the basolateral membranes. Bile acid efflux across the apical membrane is carried out by MRP2. Other potential efflux transporters along the apical membrane include MDR1 and ABCG5/ABCG8 cholesterol transporter. At the basolateral (*B*) membrane, MRP3 and the heteromeric organic solute transporter α-β, OSTα-OSTβ, mediate bile acid transport into the portal circulation. (See text for references.)

the so-called cholehepatic shunt pathway[64,65] that allows the cycling of bile acids through the periductular capillary plexus back to the liver.

Intestinal Bile Acid Transport

Under physiologic conditions, more than 90% of bile acids secreted into bile are conjugated. While passing through the intestinal lumen, bile acids are absorbed along the entire gut, passively in the jejunum and colon, and both actively and passively in the distal ileum (see Figure 119-2).

Jejunal bile acid absorption is carried out through a sodium-independent, nonsaturable process, indicating uptake by passive diffusion or by low-affinity, passive transport systems.[66] Oatp3 (*Slc21a7* [now Oatp1as, *Slc01a5*]), the sodium-independent bile acid transporter cloned from rat ileum, is expressed in the brush border membrane of jejunocytes[67,68] and may mediate passive facilitated transport in this region. Ileal bile acid absorption is a combination of an active sodium-dependent, carrier-mediated process that predominates, and a sodium-independent transport. Active transport in the ileum results in a total transport rate that may be 5× faster in this region compared with that in the jejunum.[69]

Apical Absorption

The apical, sodium-dependent bile acid transporter, ASBT (*SLC10A2*), also known as ileal bile acid transporter, IBAT, is expressed in the distal ileum[70] and transports both conjugated and unconjugated bile acids with a higher affinity for chenodeoxycholic and deoxycholic acids than for taurocholate[71] (see Figure 119-3). This sodium-coupled transporter is critical for enterohepatic circulation and handles up to 90% of bile acids crossing the apical membrane of ileal enterocytes.[70-72] Like its close relative NTCP in the liver, it is electrogenic with a sodium–bile acid stoichiometry of 2:1.[73] In contrast to NTCP, the transcriptional regulation of *ASBT* gene expression is under control of HNF-1α and not regulated in an FXR/BAR-dependent manner.[44] ASBT expression has been shown to be up-regulated by glucocorticoids.[74-77] Thyroid hormone, although essential in regulating ASBT appearance during early rat development,[69] did not up-regulate its expression in weaning rats.[78] Bile acid feeding down-regulates total ileal bile acid transport.[79]

The sodium-independent transporters OATPs (particularly Oatp3, *Slc21a7* [now Oatp1as, *Slc01a5*]) have been found to be expressed also in the ileocyte brush border membrane[47,67] and may mediate passive transport of bile acids. Unconjugated bile acids, which are relatively hydrophobic, can also readily diffuse through the intestinal mucosal membrane after their passage through the intestinal mucosal "unstirred" water layer overlying the enterocytes.[80]

MRP2, which in the liver mediates the canalicular excretion of divalent, sulfated, or glucuronidated bile acids as well as non–bile acid organic anions such as bilirubin glucuronides, is also localized in the apical membrane of ileocytes. Here, MRP2 may mediate the efflux of bile acids back into the intestinal lumen. MDR1 (*ABCB1*), a PXR-inducible xenobiotic efflux pump, and the cholesterol transporter ABCG5/ABCG8 are also expressed along the enterocyte apical membrane.[37]

Cytosolic Transport

Bile acids absorbed at the apical membrane are moved intracellularly across the enterocyte cytosol by an ileal bile acid binding protein, IBABP, also known as ileal lipid binding protein (see Figure 119-3). Specific bile acids increase their own IBABP through the transcription factor FXR/BAR, up-regulating their expression.[16,81-83] FXR binds to a bile acid response element in the IBABP promoter. Reducing bile acid concentrations by cholestyramine decreases IBABP expression. The magnitude of changes in expression of IBABP and ASBT during development, including their biphasic regulation, parallel one another and like ASBT, IBABP is also up-regulated by dexamethasone, suggesting that both IBABP and ASBT might be under similar transcriptional control.[74,84-87]

Basolateral Transport

The anion exchange transport protein MRP3 (*ABCC3*) is localized in the basolateral membrane and may mediate bile acid efflux out of the enterocyte into the portal circulation (see Figure 119-3). However, human MRP3 has a lower affinity for conjugated bile acids than the rat MRP3, making it less likely to be the principal basolateral bile acid transporter in the ileocytes.[88-92] There is also an alternatively spliced form of ASBT, called t-ASBT, which is targeted to the basolateral membrane. t-ASBT expressed in the *Xenopus* oocytes mediates sodium-independent taurocholate efflux[65]; hence, in ileocytes, it may also mediate the exit of bile acids into the portal circulation. The recently described heteromeric organic solute transporter α-β, OSTα-OSTβ has been localized to the mouse ileal basolateral membrane and mediates sodium-independent basolateral bile acid transport.[93] α-β, OSTα-OSTβ may be responsible for bile acid efflux in ileum and other ASBT-expressing tissues. It remains to be resolved whether t-ASBT, MRP3, or α-β, OSTα-OSTβ is the principal basolateral bile acid efflux transporter.

Bile Acid Transport in the Colon

Absorption of bile acids (predominantly the secondary bile acids deoxycholic acid and lithocholic acids) in the colon is passive. The sodium-independent transporter OATP8 or OATP1B3 (*Slc21a7 or SLC01B3*) is expressed not only in the brush border membranes of jejunal and ileal enterocytes but also in the colonocytes.[94] Hence, they may mediate transport of bile acids in the colon. Here, the direction of net transport is likely dependent on concentration gradients across the membrane.

Alteration of Bile Acid Enterohepatic Circulation

Disruptions of the enterohepatic circulation lead to derangements in bile acid metabolism. Among the conditions that affect bile acid metabolism include conditions or disorders that involve cholestasis, intestinal bacterial overgrowth, and increased fecal loss of bile acids. Cholestasis and cholestatic disorders are discussed in another chapter. Disorders of bile acid synthesis, conjugation, and transport are discussed in a later section of this chapter.

Intestinal Bacterial Overgrowth Leads to Increased Bile Acid Deconjugation

Colonic bacteria entering through the ileocecal valve back into the small intestine deconjugate bile acids in the distal small intestine. Within the small intestine, bacterial overgrowth leads to bacterial modification of primary bile acids to form secondary bile acids (deoxycholic and lithocholic acid). Anaerobic bacteria deconjugate bile acids in the ileum, thereby reducing water solubility and compromising the ability of the bile acid to aid fat absorption because of decreased micelle formation. Secondary bile acids tend to be passively absorbed, and the efficiency of enterohepatic circulation may be reduced because of decreased ileal active transport. Increased levels of secondary bile acids in the small intestine thus lead to more bile acids in the colon and greater fecal excretion.

Luminal deconjugated bile acids like lithocholic acid may directly inhibit small intestinal absorption of carbohydrates and proteins because of their toxic effects on the intestinal

epithelium.[95] These unabsorbed sugars in turn ferment into organic acids, which reduce intraluminal pH in the colon, subsequently inducing osmotic diarrhea. Collectively, this bacterial backwash causing a combination of malabsorption and diarrhea symptoms is referred to as *bacterial overgrowth syndrome* (BOS). This syndrome also commonly occurs in patients with short bowel syndrome. High levels of deconjugated bile acids also can damage enterocytes and induce water secretion by the colonic mucosa, hence worsening the diarrhea. Lithocholate, which is hepatotoxic, has been detected in recirculated bile.[96]

Increased Fecal Bile Acid Loss in Short Gut Syndrome

Increased fecal loss of bile acids is secondary to decreased distal intestinal reabsorption of bile acids. Because the ileum absorbs more than 80% of intestinal bile acids, any pathology in this region leads to significant losses of bile acids in the feces and to decreases in total circulating bile acid pool. In an experimental rat model of necrotizing enterocolitis, unabsorbed bile acids induce ileal damage, causing further alteration in bile acid transport and increased intestinal-to-fecal loss.[97] Surgical removal of the terminal ileum secondary to neonatal necrotizing enterocolitis, among other neonatal pathologies that lead to short bowel syndrome, causes bile acid malabsorption and disrupts the enterohepatic circulation. The degree or severity and the location of ileal resection determine the adaptive behavior of the remaining segment. Minimal resection causes minimal perturbations in bile acid metabolism because rates of bile acid biosynthesis in the liver increase to compensate for potential losses (see earlier section on regulation of CYP7A1). Proximal ileal resection leads to diminished total bile acid pool and supraphysiologic ASBT expression in the remaining ileal segment with paradoxical reduction of cholesterol 7α-hydroxylase (see Figure 119-1, step 1), sterol 27-hydroxylase (see Figure 119-1, step 6), and hydroxymethylglutaryl coenzyme A reductase enzyme activities.[98] This finding seems contradictory to concepts of feedback regulation of bile acids on their synthetic enzymes. One possible explanation is the frequent cycling of bile acids secondary to the diminished bile acid pool, exposing the hepatocytes to a greater flux of bile acids.

Resection of the entire terminal ileum does not induce ASBT expression in the more proximal segments of the small intestine.[99] With no active and passive bile acid transport in the distal intestines, less bile acids will return to the liver for re-secretion, a situation that eventually leads to a diminished bile acid pool. The liver overcompensates by increasing rates of bile acid synthesis, up to 5 to 20 times more than normal, in an effort to maintain the bile acid pool size.[100] Hepatic compensation in ileal resection is usually insufficient to maintain adequate bile acid output.[35] This creates a vicious cycle of diminished bile acid concentration in the jejunum, which further leads to fat malabsorption and steatorrhea. The additional loss of water and electrolytes and other nutrients leads to a "short bowel syndrome" condition. Oral treatment with a conjugated bile acid preparation leads to improved fat absorption.[101] Resection may also induce bacterial overgrowth in the small intestine. Small intestinal bacterial overgrowth from the colonic flora (especially if the ileocecal valve is also removed) augments the loss of bile acids in the feces, as is observed in inflammatory bowel disorders,[102] the manifestations of which include diarrhea and steatorrhea.

BILE ACID METABOLISM DURING DEVELOPMENT

Composition

As early as in the 10th week of pregnancy, bile acids are synthesized from cholesterol and then conjugated with amino acids by the fetal liver (Figure 119-4).[103-105] The fetal gallbladder is functional and capable of storing bile. Bile secretion from hepatocytes into the gallbladder begins by the 12th week of gestation.[3] The bile acid composition in the fetus has unusual characteristics and differs from that in adults, as follows.

First, there is a high proportion of atypical C1-, C2-, C4-, and C6-hydroxylated bile acids (see cholesterol structure in Figure 119-1) in human fetal gallbladder bile, reflecting either synthesis of new bile from fetal liver[106] or an alternative pathway for converting maternally derived toxic bile acids, such as lithocholic acid, by hydroxylations at C1, C2, C4, and C6 positions, which render bile acids less toxic.[107] The presence of high proportions of hypocholic acid and several 1β-hydroxycholanoic acid isomers provides evidence for C1 and C6 hydroxylations by the fetal liver. Moreover, gas chromatographic and mass spectrometric analyses of bile by the 16th week of gestation show C4-hydroxylated bile acids (e.g. 3α,4β,7α-trihydroxy-5β-cholanoic and 3α,4β-dihydroxy-5β-cholanoic acids) accounting for 5% to 15% of total bile acids, suggesting that C4-hydroxylation is also an important pathway in bile acid metabolism during early fetal life.[1] These C1- and C6-hydroxylated bile acids have been detected in infants' meconium and in infants and adults with cholestasis. Bile acid patterns in human meconium show that in addition to cholic, chenodeoxycholic, deoxycholic, and lithocholic acids, several other atypical bile acids are also found, providing additional evidence for an alternative fetal bile acid synthesis pathway.[1,108]

Second, unlike human adult bile (which has a chenodeoxycholic acid–cholic acid ratio of 1:1.6), chenodeoxycholic acid concentration predominates over that of cholic acid in the fetal bile (chenodeoxycholic acid–cholic acid ratio of 1:0.85).[1] This is due in part to the preferential clearance by placental passive diffusion of bile acids formed from cholic acid by the rapid hydroxylation at the C1, C2, C4, and C6 positions[109] (see also later discussion of passive diffusion in the section Placental Bile Acid Transport). Amniotic fluid studies during preterm and full-term gestations not only confirm the predominance of chenodeoxycholic acid in the fetal bile but also show a reversal of the ratio in full-term newborns (chenodeoxycholic acid–cholic acid ratio of 1:2.5).[1,109] In fact, the adult ratio of 1:1.6 is achieved during the first year of life.[1]

Third, more than 80% of bile acids secreted in human fetal liver are conjugated to taurine, whereas glycine-conjugated and unconjugated bile acids account for only 5% to 10% of the total bile acid. In adults, glycine conjugation predominates (glycine-taurine ratio of 3.1:1),[110] whereas the level of unconjugated bile acids increases by approximately 50%. Based on duodenal bile acids measurements in infants, this adult pattern of predominantly glycine-conjugated bile acid is achieved by 2 to 7 months of life.[111]

The taurine conjugation of toxic bile acids (such as lithocholic acid conjugated to taurine, or taurolithocholic acid) in the fetus offers added fetal protection. Only trace amounts of bile acid sulfates are present in the fetal circulation, indicating that quantitatively, sulfation is not an important pathway in bile acid metabolism during development. The presence in the fetal circulation of bacteria-derived secondary bile acids, lithocholic acid in particular, implies maternal source and not synthesis by the fetus through the 3β-hydroxy-5-cholenoic acid pathway[1] because the fetal intestine is sterile and devoid of bacteria.

Pool Size

Figure 119-4 illustrates the timeline in the development of the bile acid pool. Serum bile acid levels progressively increase postnatally, peak by the second month, and then gradually decline to adult levels by 1 year of life.[112] The total bile acid pool increases progressively from the fetal to neonatal to older infants. It reaches

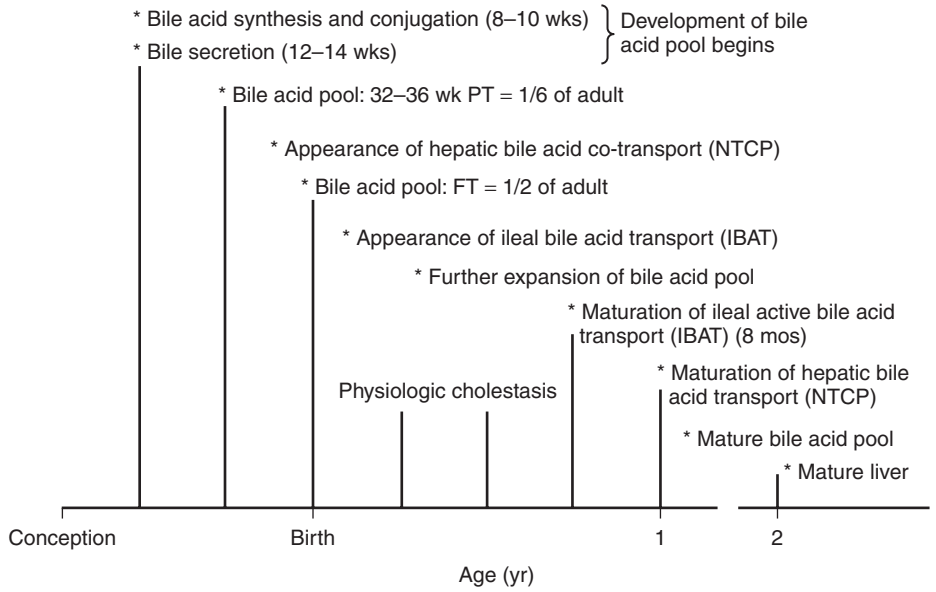


Figure 119-4 Timeline (not to scale) of developmental changes in the size of the human bile acid pool, compared with the adult (adult total bile acid pool size = 3 to 4 g). *FT,* Full-term; *PT,* preterm. See text for details.

adult levels of 3 to 4 g by 2 years of life, but when corrected for body surface area, pool size in grams per centimeter squared (g/cm^2) is similar between adults and infants 2 months of age.[113] The small size of the fetal bile acid pool is due to low synthesis rates and to placental clearance.[114] Between 32 and 36 weeks gestation, cholic acid levels in preterm infants and cholic acid synthesis rates are one third those of full-term infants.[115] In turn, the cholic acid levels in full-term infants are half those of adult levels[116] when normalized to body surface area. The clinical relevance of these phenomena is linked to the diminished intestinal luminal bile acid amount in the fetus, which is less than the critical micellar concentration (2 to 20 mM) that is effective in fat digestion.[1]

Development and Regulation

There are few studies on the regulation of bile acid synthesis and absorption in development. ^{14}C-labeled taurocholate tracer studies show during early development significant passive reabsorption of bile acids in the human jejunum and a poorly developed active taurocholate reabsorption in the ileum,[117] thereby affecting enterohepatic circulation. This immature nature of bile acid metabolism during early development affects the nutritional management of the preterm infant as it relates to malabsorption of fat and other nutrients. In rats, ASBT undergoes biphasic regulation; that is, it is expressed during the late fetal stage, down-regulated during the neonatal period, and up-regulated at weaning.[87] Lowered ASBT expression during certain developmental stages may be the mechanism underlying poor bile acid reabsorption.

Hormones also modulate bile acid metabolism in the neonate. Administration of glucocorticoids and phenobarbital allowed preterm cholic acid levels to approximate those of full-term infants.[115,118] Triiodothyronine (T_3) accelerated the maturation of bile acid metabolism in baboons,[119] primarily by altering the cholic acid–chenodeoxycholic acid ratio at the end of the preweaning period to near adult values. The combination of immaturity of ileal and hepatic bile acid transport systems, reduction in bile acid pool size, and increases in bile acid synthesis rates have been referred to as *physiologic cholestasis* or *hypercholanemia*[120] (see Figure 119-4).

FETAL LIVER-PLACENTA-MATERNAL LIVER PATHWAY

Although the fetal liver is capable of bile acid synthesis, it is incapable of bile acid elimination through the fetal hepatobiliary excretory pathway,[121] owing in part to underdeveloped intestinal function and the lack of necessity for digestion and absorption. Fetal bile acids have been detected in fetal gallbladder, but how they are secreted by the fetal hepatocytes is not fully known. If there is no significant excretion or loss, bile acids in the plasma should increase, and in fact, fetal bile acid concentrations in the plasma of rats and humans are higher than those of the mother.[122-124] If bile acids accumulate to high concentrations, these are potentially toxic to the fetus. Therefore the placenta and the maternal liver play key roles in bile acid elimination from the fetus.[125]

To be excreted, fetal bile acids have to cross five membranes through the fetal liver-placenta-maternal liver pathway (Figure 119-5): the basolateral membrane of the fetal hepatocytes, the fetal-facing (basolateral or basal) and maternal-facing (apical) trophoblastic membranes of the placenta, and the sinusoidal (basolateral) and canalicular (apical) membranes of the maternal hepatocytes. Efflux of bile acids across the basolateral membrane of fetal hepatocytes is handled by several members of the OATP family of transporters.[126]

Placental Bile Acid Transport

Simple diffusion across both trophoblastic membranes of the placenta has previously been widely accepted as the major route of transfer of bile acids based on their lipid nature and the concentration gradient. Transplacental bile acid diffusive transport is bidirectional, between the maternal and the fetal circulations, with a net balance favoring a fetal-to-maternal bile acid transfer.[127] However, the presence of secondary bile acids in the fetal bile acid pool (fetus should not have secondary bile acids because the fetal intestine is devoid of bacterial flora) is evidence of a maternal-to-fetal transplacental transfer of relatively hydrophobic secondary bile acids.[124]

Because of the conjugated, protein-bound, and ionized nature of fetal bile acids, passive diffusion is less likely to

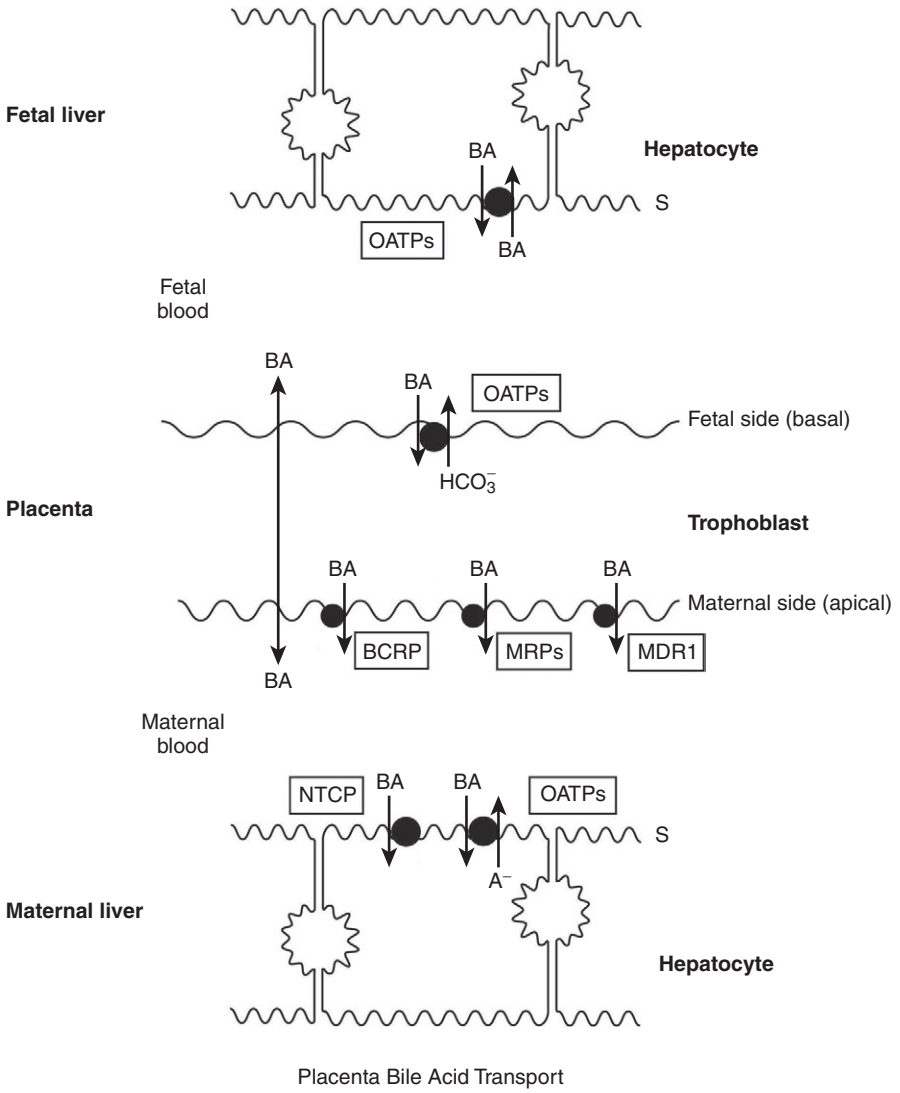


Figure 119-5 Bile acid exchange between fetal and maternal livers. Bile acids *(BA)* synthesized in the fetal liver are transported out of the fetal hepatocytes into the blood along the sinusoidal membrane *(S)* through members of the anion exchanger OATP family of transporters. Transport is bidirectional, and net transport is dependent on the gradient of bile acids across the basolateral membrane. There is no significant transport into the fetal canaliculus. Fetal blood through the umbilical arteries returns to the placenta. Along the basal trophoblastic (fetal-facing) membranes, the OATP anion exchangers transport BA into the cytosol mainly through HCO^-_3 exchange. Transport across the apical trophoblastic (maternal-facing) membranes is mediated by multidrug resistance–associated proteins (MRP1, MRP2, and MRP3), BCRP, and MDR1. Bile acids in the maternal blood from the placenta return to the maternal liver and are processed as in Figure 119-3.

be the major route of placental bile acid transfer.[128] Bile acid transport proteins in the basal and apical membranes of the trophoblasts account for unidirectional transport of bile acids from fetal to maternal circulation. The OATP family of transporters mediates bile acid transport across the placenta, where they are highly expressed.[129-131] Fetal bile acids cross the basal (fetal-facing) plasma membrane of the trophoblast by a sodium-independent bicarbonate anion exchange mediated by OATPs.[132] Several OATP transporters that are expressed in human placenta include OATP1B1 (formerly OATP3), OATP1B3 (OATP8), and OATP4A1.[129,133] In rats, the expression levels of these placental basolateral bile acid carriers OATP1a1, OATP1a4, and OATP1b2 are up-regulated during maternal cholestasis.[134] OATPs have also been shown to mediate the pregnenolone-sensitive placental uptake of fetal-derived sulfated steroids.[135]

Present in the apical (maternal-facing) plasma membrane of the trophoblasts are several ABC protein bile acid transporters, MRP1 (*ABCC1*), MRP2 (*ABCC2*), and MRP3 (*ABCC3*), whose expression have been confirmed in human placenta.[136] These transporters mediate the transfer of bile acids from the trophoblast cytosol to maternal circulation (see Figure 119-5). All MRPs are markedly up-regulated in pregnant rats with cholestasis.[134] BCRP (*ABCG2*) and MDR1 (*ABCB1*) also participate in the bile acid efflux across the apical trophoblastic membrane.[137] Carrier proteins in both the fetal and maternal blood (i.e., albumin and lipoproteins) ferry bile acids from the fetal liver to the basolateral membrane of trophoblasts and from the apical membrane of trophoblasts to the maternal liver.[137] Within the trophoblast, the identity of the cytosolic bile acid carrier or transporter that shuttles bile acids from the fetal (basal) to maternal (apical) trophoblastic membranes is not known. In the maternal liver, bile

acids are excreted following the pathways described in Figure 119-3.

DISORDERS OF BILE ACID METABOLISM

These can be subdivided into disorders of bile acid synthesis, of the tight junctions in the liver, and of membrane transport. We will only briefly touch on the transport defects because these are described further in the following chapter.

Disorders of Bile Acid Biosynthesis

CYP7A1 Deficiency

CYP7A1 converts cholesterol to 7α-hydroxycholesterol (see Figure 119-1, step 1). The gene is located on chromosome 8q11-12.[138] Patients with a homozygous deletion mutation of the *CYP7A1* gene have hyperlipidemia, increased hepatic cholesterol leading to formation of gallstones, and premature cardiovascular disease.[6] Oral cholic acid proved to be an effective treatment.

CYP7B1 Deficiency

Oxysterol 7α-hydroxylase is the major enzyme involved in the acidic or alternate pathway for bile acid synthesis. It is located on chromosome 8q21.3. One case has been described of an infant born of consanguineous parents, with severe neonatal cholestasis and cirrhosis. This infant had elevated 27α -hydroxycholesterol,[139] and cholic acid therapy proved ineffective. Identification of oxysterol 7α-hydroxylase deficiency in this infant with liver disease indicates the importance of the acidic pathway in early human life.

3β-Hydroxy-Δ⁵-C27 Steroid Oxidoreductase Deficiency

The gene for this enzyme is mapped to chromosome 16p11.2-12 (see Figure 119-1, step 2).[140] Accumulation of C24 and C27 steroids leads to hepatotoxicity. Patients present with neonatal jaundice, hepatomegaly, fat malabsorption, and fat-soluble vitamin deficiencies. Bile acid therapy with chenodeoxycholic acid, alone or in combination with cholic acid, leads to a decrease in pruritus and normalization of urinary steroids.[141]

Δ⁴-3-Oxosteroid 5β-Reductase Deficiency (AKR1D1 or SRD5B1)

Located on chromosome 7q32-33,[142] AKR1D1 deficiency leads to an increase in Δ4-3 oxo and allo bile acids (see Figure 119-1, step 3) and to severe cholestasis.[143] Mutations in the SRD5B1 gene have been recently demonstrated.[144,145] Oral bile acid therapy with a combination of cholic and chenodeoxycholic acid is effective; however, ursodeoxycholic acid therapy is not effective.[146]

Sterol 27-Hydroxylase (CYP27A1) Deficiency

This deficiency was one of the initially described defects in bile acid synthesis (see Figure 119-1, step 6). The gene has been localized to chromosome 2q33-qter.[147] This is an autosomal recessive condition that causes cerebrotendinous xanthomatosis. Decreased bile acid synthesis results in elevated plasma concentrations of cholesterol and cholestanol, which eventually cause neurologic symptoms, including dementia, psychiatric disturbances, pyramidal or cerebellar signs, and seizures. Other clinical manifestations are diarrhea, tendon xanthomas, and cataracts.[148] Treatment with chenodeoxycholic acid decreases plasma cholesterol and cholestanol concentration by about three-fold and results in normalization of electroencephalogram and biochemical abnormalities but not of the clinical symptoms. Cholic acid therapy is equally effective and has much less side effects.[149] Fatal neonatal cholestasis has been reported secondary to this deficiency.[150]

2-Methyl Coenzyme A Racemerase Deficiency

This enzyme is located in the peroxisomes and not only acts on bile acid synthesis but also participates in the degradation of methyl branched chain fatty acids (see Figure 119-1, step 8). The *AMACR* gene has been mapped to chromosome 5p11-13.[151] 2-Methyl coenzyme A racemerase (AMACR) deficiency leads to accumulation of pristanic acid and di- and tri-hydroxycholestanoic acids. Pristanic acid causes neurotoxicity. Adults present with neuropathy,[152] but an infant is described with coagulopathy, vitamin D and E deficiency, and mild liver impairment with no neurologic disease. AMACR-deficient mouse models consuming a diet supplemented with phytol, a source of branched-chain fatty acids, had impaired livers.[153]

D-Bifunctional Protein Deficiency (HSD-17B4)

The defect is in the 17β–hydroxysteroid dehydrogenase type IV enzyme (see Fig. 119-1, step 10). The gene is located on chromosome 5q2.[154,155] Loss of this enzyme is associated with accumulation of C27 bile acid intermediates and pristanic acid. Suzuki and colleagues[156] reinvestigated two Japanese patients thought to have L-bifunctional protein deficiency. Before D-bifunctional protein was described as an entity, these patients were thought to have L-bifunctional protein deficiency, but when the patients were retested, they actually had D-bifunctional protein deficiency. Many other patients previously diagnosed with L-bifunctional protein were subsequently established to have D-bifunctional protein deficiency. The infants present with neonatal seizures, hypotonia, hepatomegaly, and other defects. D-bifunctional protein deficiency is usually fatal before 2 years of age, although a few children have survived beyond this age.[157,158]

Disorders of the Tight Junction

Familial Hypercholanemia

This disorder has been described in 12 families of Amish descent.[159] The candidate gene is *TJP2* (tight junction protein 2) and is located on chromosome 9q12-9q13. Tight junctions separate bile in the lumen from plasma and the canalicular membranes from sinusoidal membrane domains. A proposed mechanism for this disorder is that the bile salts are transported into the lumen but then leak through the paracellular pathway represented by the tight junction back into the plasma. A second locus is on 9q22-9q23, which is a candidate gene for bile acid coenzyme A amino acid N-acyltransferase (BAAT) (see Figure 119-1, step 12). This enzyme conjugates bile acids with glycine and taurine. Unconjugated bile acids cannot be excreted by the BSEP into the canalicular lumen (see Figure 119-3), thereby leading to the accumulation of bile salts and their precursors in the hepatocyte cytosol and eventually in the plasma. Decreased levels of bile salts in the bile lead to fat malabsorption, vitamin K deficiency, and rickets. The increased bile salts in the plasma occasionally lead to pruritus, cholestasis, and chronic hepatitis. Ursodeoxycholic acid and rifampicin have been shown to improve the pruritus and normalize bile acid levels.[160]

Disorders of Transport of Bile Acids

These disorders are discussed in the next chapter and are mentioned here only briefly.

Progressive Familial Intrahepatic Cholestasis Type 1 (PFIC1)

This disease was first described in an Amish family, descendants of Jacob Byler, and was formerly called *Byler disease*. The underlying cause is a genetic defect in *ATP8B1* located on

18q21-q22.[161] The gene encodes a member (class V, type 10A) of the family of aminophospholipid-transporting ATPases or translocases. These aminophospholipid translocases transfer phosphatidylserine and phosphatidylethanolamine phospholipids from one side of a bilayer of the plasma to another. The PFIC1 protein is found on the apical membrane of the gastric pits, small intestine, pancreatic acinar cells, cholangiocytes, and hepatocytes in mice.[162] This is a well-characterized clinical syndrome in which patients have low bile salt concentrations in the intestinal lumen and increased bile acid concentrations in the plasma. These patients also have extrahepatic manifestations like steatorrhea, diarrhea, and failure to thrive. The patient response to ursodeoxycholic acid treatment varies. Partial external biliary diversion, a procedure that diverts some bile away from the intestine, may ameliorate cholestasis and pruritus.[163] Eventually, liver transplantation is required for liver failure.

Progressive Familial Intrahepatic Cholestasis Type 2 (PFIC2)

This defect is in the *ABCB11* gene, formerly called SPGP and BSEP, and is located on chromosome 2q24.[164] This gene encodes for BSEP, which is an ABC transporter found on the canalicular membrane of the hepatocytes (see Figure 119-3). About 13 different mutations have been found in this gene.[165] Infants with this disorder present with cholestasis. An increased risk for hepatocellular carcinoma has been reported.[165] Similar to those with PFIC1, some patients respond to ursodeoxycholic acid therapy or to partial external biliary diversion. Liver transplantation is required for end-stage liver disease.

Progressive Familial Intrahepatic Cholestasis Type 3 (PFIC3)

The defect is in the *MDR3* (*ABCB4*) gene (see Figure 119-3), a member of the multidrug resistance gene family, located on chromosome 7q21.[166,167] The MDR3 p-glycoprotein is a transmembrane protein that translocates phosphatidylcholine and may be involved in the transport of bile acids from the hepatocyte to the canaliculus as well as in transport of drugs. Bile salt secretion into the canaliculus is normal, but the bile is devoid of phosphatidylcholine. Phospholipids in the bile lumen normally form micelles with the bile acids and protect the canalicular membrane from damage by bile acids. Patients present with hepatosplenomegaly, portal hypertension, and jaundice and later in life develop liver failure. Some patients respond to ursodeoxycholic acid. Liver transplantation is required for end-stage liver disease.

Benign Recurrent Intrahepatic Cholestasis

The defect in this disorder is in the aminophospholipid translocase (*ATP8B1*) and *BSEP* (*ABCB11)* genes (see above for description). This appears to have the same genetic defect as those described previously, but phenotypically, it is a benign disorder with recurrent bouts unlike the ones discussed previously that typically progress to liver failure. Patients present with recurrent episodes of cholestasis. No effective medical therapy is available. Complete and long-lasting disappearance of pruritus and normalization of serum bile salt concentrations in cholestatic patients within 24 hours after endoscopic nasobiliary drainage have recently been reported.[168]

Intrahepatic Cholestasis of Pregnancy

This disease, which occurs in the third trimester of pregnancy, is prevalent in certain ethnic groups[169] and is due to a heterozygous non-sense mutation of the *MDR3* gene that has been demonstrated in familial intrahepatic cholestasis of pregnancy.[170] Recurrent prolonged intrahepatic cholestasis of pregnancy followed by periportal fibrosis or cirrhosis has been reported in four sisters.[171] The disease is reversible in the mother after delivery with excellent prognosis. However, the effects on the fetus include prematurity, stillbirth, and fetal distress. Estrogen and progesterone may play a role in the etiopathogenesis.[172] The severity of fetal complications is directly proportional to the magnitude of maternal hypercholanemia.[173] Ursodeoxycholic acid has been found to be effective in improving pruritus in the mother and also in improving fetal outcome.[174]

Lymphedema Cholestasis Syndrome (Aagenaes Syndrome)

Patients have severe neonatal intrahepatic cholestasis, which improves during childhood and becomes episodic as in benign recurrent intrahepatic cholestasis. Later, these children develop lymphedema. Bull and associates[175] mapped a locus for lymphedema cholestasis syndrome to chromosome 15q in Norwegian patients; however, no linkage was detected at chromosome 15q in a Serbian family.[176] Fifty percent of the patients can expect a normal life span.[177]

North American Indian Childhood Cirrhosis

This familial cholestasis with autosomal recessive inheritance in Ojibway-Cree children from Northwest Quebec presents with transient neonatal cholestasis progressing to biliary cirrhosis.[178] A missense mutation has been detected in the Cirhin (*CIRH1A*, cirrhosis autosomal recessive 1A) gene at chromosome 16q22.[179] No specific therapy is available, and patients with advanced cirrhosis require liver transplantation.

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Heidi Eigenrauch Karpen and Saul J. Karpen

Bile Formation and Cholestasis

The functions of the liver can be generally divided into three main areas: (1) synthesis and secretion of serum proteins including clotting factors; (2) energy metabolism, detoxification of endobiotics and xenobiotics; and (3) bile secretion. Perturbations in any of these functions, whether by genetically inherited or acquired conditions (e.g., sepsis and the acute phase response), can lead to liver disease, act as a cofactor in other conditions, and ultimately may lead to death. To date, no bioartificial device can satisfactorily replace a liver.

ANATOMY OF THE LIVER

The liver is the largest abdominal organ, receiving 20% to 25% of the cardiac output from two separate sources—the portal vein and the hepatic artery.[1] The portal vein delivers deoxygenated blood efflux from the intestines, spleen, and pancreas, contributing approximately 70% of total blood flow to the liver. Oxygenated blood is provided by the hepatic artery, which usually derives from the celiac axis. The blood from these two

circulations merges at the tissue level in the sinusoidal spaces that separate one- to two-cell-thick hepatocyte cords (Figure 120-1). The sinusoidal membranes are lined with receptors and transporters that efficiently accomplish numerous tasks. These include the uptake of nutrients, toxins, endobiotics, and xenobiotics; a robust response to circulating signals (e.g., cytokines); and delivery of secreted substances to the circulation. After filtering through the sinusoids, blood flows into central veins that coalesce into one of the three hepatic veins; these empty into the systemic circulation via the inferior vena cava, immediately inferior to the right atrium.

Blood flow through the liver changes around the time of birth. Before birth, the ductus venosus shunts blood from the left umbilical vein to the inferior vena cava. After birth, it collapses and begins to atrophy, eventually forming the ligamentum venosum, which is continuous with the ligamentum teres, the remnant of the left umbical vein (Figure 120-2).

On a cellular level, the liver is organized as functional units called hepatic lobules (see Figure 120-1). Each hexagonally shaped lobule comprises a central vein and contains a portal triad, usually at three or more of the corners. Blood flows in from the portal triad via the emptying of hepatic arterial and portal venous blood at the start of the sinusoids, then coursing toward the central vein. This arrangement of blood flow into the portal triad allows for the mixture of well-saturated arterial blood with the relatively desaturated portal blood. It also creates a zonal gradient within the lobular architecture whereby the periportal region is exposed the highest concentration of oxygen, nutrients, and toxins, which confers regional advantages and disadvantages for certain hepatic insults. Lobular heterogeneity in the expression of genes in the periportal and perivenous regions relate to the exposure and needs of these hepatocytes.

The polarized architecture of the hepatocyte allows for the majority (>90%) of the cell surface to contact blood (the sinusoidal surface), separated from the apical (canalicular) surface that is in contact with bile. Bile flows countercurrent to blood from the intercellular canaliculi at the apical regions, toward the bile ducts in the portal triad[2,3] (see Figure 120-1). The flow of bile is dependent on the active secretion of biliary solutes into the canalicular space, and impairment of this process leads to intrahepatic cholestasis[4,5] (see later discussion).

MICROARCHITECTURE

The hepatic parenchyma is comprised mainly of hepatocytes (>70%), but several other cell types are critical for coordinated function of this organ, including cholangiocytes, Kupffer cells, stellate cells, sinusoidal endothelial cells, and Pit cells.[6] Cholangiocytes (bile duct cells) are also polarized, with their apical surfaces in continuity of the flow derived from hepatocyte canaliculi.[7] These cholangiocytes line the bile ducts and gallbladder and originate in the ducts of Hering adjacent to portal triads. Kupffer cells are the resident macrophages within the liver and are the largest collection of macrophages in the body.[8] They can migrate from the sinusoids

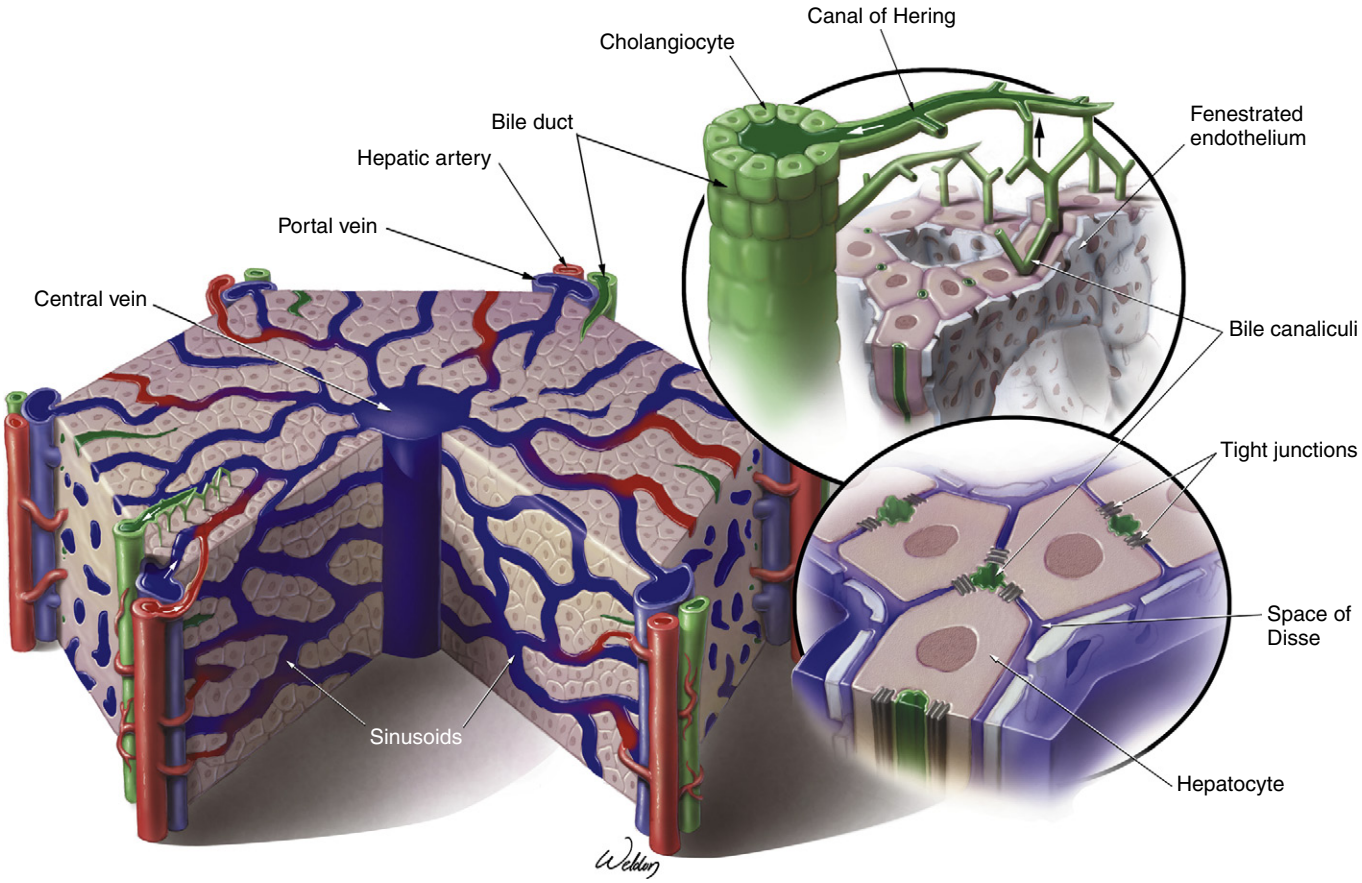


Figure 120-1 Schematic view of the liver lobule. The central vein is shown in the center of the lobule, separated by cords of hepatocytes forming sinusoids from six portal areas at the periphery. The portal areas contain a portal vein, hepatic artery, and bile duct. Blood flow is toward the center of the lobule, while bile flows toward the portal triads at the margins. Note the hepatic artery providing oxygenated blood to the hepatic sinusoids as well as the peribiliary plexus. The *top inset* provides a more detailed view of the fenestrated endothelium and the connections between intercellular canaliculi and the canals of Hering. The *lower inset* emphasizes the separate basolateral and canalicular membranes, as well as the junctional complexes.

to regions of insult or infections and are major components in the genesis of the inflammatory response within the liver. Pit cells may contribute to the immune defenses in the liver because they appear to have natural killer cell activity. Stellate cells are arguably the most complex and least well-characterized cell type within the liver. They express neuroendocrine markers, consistent with their neural crest origin, but express storage and fibroblastic-like activities.[9] Stellate cells occupy the perisinusoidal space with cytoplasmic extensions that encompass the sinusoidal endothelial lining, making them particularly well situated for their role in maintenance of the microvascular tone. In addition, they are the primary producers of collagen and extracellular matrix in response to inflammation and injury.[10,11] The combined effects of activated stellate cells, along with myofibroblasts that migrate to an injured liver, lead to fibrosis and, if prolonged, to cirrhosis.[12] Even sinusoidal endothelial cells have adapted to their unique environment and role in the liver, with fenestrated membranes to allow passage of relatively large molecules.[13]

Hepatocyte Polarity and Membrane Features

The distinct membrane domains found on the hepatocyte (basolateral, intercellular, and apical) not only contribute to their architecture as polarized cells but also are critical for their functions. The basolateral (sinusoidal) membrane is contiguous with the intercellular domain, both of which are in contact with the sinusoidal blood via the space of Disse (see Figure 120-1). The sinusoidal membrane contains numerous transporters, which import substances into the hepatocyte for metabolism and detoxification, as well as receptors for microbial products (e.g., Toll-like receptors) and cytokines.[14,15]

The intercellular (lateral) domain comprises almost 50% of the hepatocyte membrane and extends the sinusoidal surface up to the canalicular space, separated by tight junctions (zonula occludens). These tight junctions serve to define the polarity of the hepatocyte and create a semipermeable membrane between the blood and bile. They also serve as means of cell-cell communication and their permeability can be altered in response to certain conditions, mainly due to injury, inflammation, and toxins.[16]

Biliary Tree

The biliary tree begins as small (0.75 μM) tubules formed at the apical surface of two adjoining hepatocytes that define the canaliculus. Bile flows from the canalicular spaces into a network of canaliculi (see Figure 120-1), then to the canal of Hering, ultimately to drain into terminal bile ducts.[17] These terminal branches first become grossly visible as area ducts, which coalesce into segmental ducts, then into right and left hepatic ducts. In the biliary tree of the rat, at least seven orders of bifurcation define this arrangement that serves the entire liver.[7] Bile is then carried by the common hepatic duct to the gallbladder, where it is stored until hormonal action (primarily cholecystokinin) initiates a muscular contraction that delivers this stored gallbladder bile into the common bile duct via the cystic duct and then to the intestine to participate in digestion.

The cholangiocytes that line the biliary tree comprise only approximately 5% of the cells of the liver. These polarized epithelial cells modify the contents of bile via active transport of fluid and electrolytes in response to hormonal signals from the intestine.[18] Stimulation of the duodenum by fatty acids, acidic pH, and bile acids leads to the release of several hormones including secretin, which is carried by the portal circulation to the liver, altering the content and volume of bile secretion in a feedback mechanism. These alterations in bile composition are accomplished by the action of several transporters found on the apical membrane of the cholangiocyte, including the cystic fibrosis transmembrane conductance regulator and a chloride and bicarbonate exchanger.[19] The chloride and bicarbonate exchanger serves to alkalinize bile and increase fluid secretion and bile flow. The cystic fibrosis transmembrane conductance regulator transports chloride and mutation of the gene for the transporter results can lead to neonatal jaundice and liver disease in approximately 10% of patients with cystic fibrosis.[20]

Bile Composition

Bile is formed mainly of solutes and water secreted by both hepatocytes and cholangiocytes. The role of bile is mainly twofold: to aid in the digestion and absorption of long-chain fats and fat-soluble vitamins from the intestine and to provide a means of excretion for drugs, toxic substances, endobiotics, and xenobiotics that have been modified and/or detoxified by the hepatocyte.[5,21,22] To that end, the composition of bile reflects both of these roles. Although exact measurements of bile production in infants are not available, the average adult liver secretes approximately 600 to 800 mL of bile per day.[23]

Bile acids are the main organic solute contained in bile and are present in concentrations of 20 to 30 mmol/L. Secretion and recirculation of bile acids are the main forces driving bile flow[5,14] (see Figures 120-3 and 120-4). Four main bile acids comprise

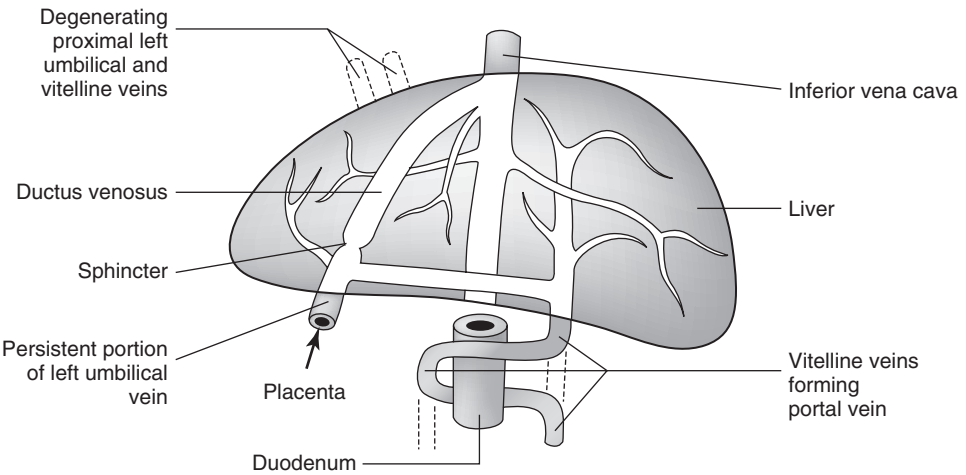


Figure 120-2 Dorsal view of the liver demonstrating the venous circulation at 7 weeks gestation. Note the path from the umbilical vein through the ductus venosus to the inferior vena cava and the vitelline veins forming the portal vein. *(From Moore KL:* The Developing Human: Clinically Oriented Embryology, *3rd ed, Philadelphia, 1982, WB Saunders, p 301.)*

more than 95% of the bile acid pool and are categorized as primary or secondary bile acids.[24,25] The two primary bile acids, cholic acid (CA) and chenodeoxycholic acid, are synthesized via a multienzyme series of reactions from cholesterol precursors solely within hepatocytes.[26-28] The more hydrophobic secondary bile acids, deoxycholic acid and lithocholic acid (LCA), are formed by the action of intestinal bacteria that 7α-dehydroxylate the primary bile acids. The vast majority of the bile acid pool is conjugated to either taurine or glycine in a 3:1 ratio, depending on the availability of dietary taurine. At high concentrations, bile acids function as detergents that can solubilize phospholipid membranes, if retained intracellularly. The more hydrophobic monohydroxy bile acids, such as LCA, are poorly soluble even when conjugated and are more damaging to membranes and hepatocytes when they accumulate.

In addition to bile acids, several other key components of bile include cholesterol, phospholipids, electrolytes, proteins, and conjugated bilirubin (Figure 120-3). Phosphatidylcholine is the most prevalent phospholipid in bile (approximately 95%) and is present in concentrations of 7 mmol/L. Cholesterol, which makes up approximately 3% of biliary solute, is secreted almost exclusively in the free or unesterified form. Phytosterols, plant-based sterols with similar structure to cholesterol, are secreted in small amounts.[29] Phospholipids, cholesterol, and bile acids form mixed micelles in bile and generally exist in a phospholipid:bile acid ratio of 2:1. Although normative ratios of cholesterol, bile acids, and phospholipids in neonatal bile are not known, it is thought that the formation of sludge and gallstones is a function of imbalances in the ratio of bile acid and cholesterol:phospholipids.[30] When bile is supersaturated by cholesterol, it can exist in several forms (micelles, lamellar phases, and crystals).

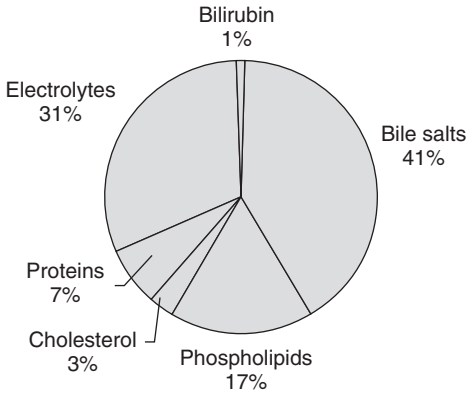


Figure 120-3 Solute composition of human bile. Note that bile acids are the most prevalent solute, followed by phospholipids and cholesterol. Conjugated bilirubin makes up a small fraction of biliary solute loads. *(From Vlahcevic et al: [chapter title. In Zakim D, Boyer TD, editors:* Hepatology: A Textbook of Liver Disease, *Philadelphia, 1996, Saunders, 381.)*

Bile flow is also driven by bile acid independent mechanisms, mainly via the secretion of glutathione, and conjugates of endobiotics or xenobiotics (glucuronides, glutathione, and sulfated forms primarily[31]; see Figure 120-4). Glutathione is used within the hepatocyte for many reducing reactions and is present in the cytosol at concentrations of 5 to 10 mmol/L. The characteristic yellow color of bile is due to the presence of bilirubin, which is secreted mainly in its diglucuronide form. Bilirubin concentrations in bile are typically approximately 0.2 mmol/L but can vary with certain disease states. Because bile is a primary mechanism for excretion of many toxic substances, small concentrations of drugs, vitamins, steroids, xenobiotics, and products of lipid metabolism are also present. In addition, bile is the main route for excretion of metals such as iron, copper, manganese, and zinc. It is readily apparent that retention of these substances during periods of cholestasis, from any mechanism, serves to promulgate ongoing liver damage. Moreover, impairments in the handling of potentially toxic substances due to inherited mutations in genes involved in these pathways can contribute to liver disease. Examples of clinical situations in which mutations or polymorphisms in select genes lead to retention of metals and liver disease include adult-onset hemochromatosis (iron) and Wilson disease (copper).[32,33] Recent research indicates that polymorphisms are likely in essential genes whose proteins encode products involved in the metabolism and transport that lead to "idiosyncratic" or idiopathic liver disease or drug toxicities.[34-37]

Enterohepatic Circulation of Bile Acids and Bile Flow

Bile flow is driven primarily by two factors: bile acid dependent mechanisms and bile acid independent mechanisms (Figures 120-3 and 120-4). The main determinants of bile acid dependent

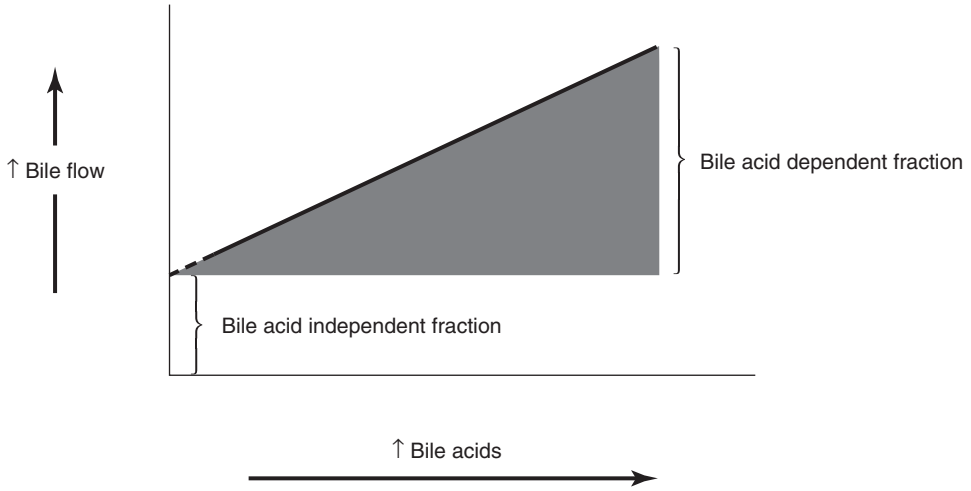


Figure 120-4 Bile acid dependent bile flow (BADF) and bile acid independent flow (BAIF). Note the linear relationship between bile acid secretion and BADF. The majority of bile flow is BADF that is reduced with smaller bile acid pool sizes, such as those of infants. The contribution made by BAIF to total bile flow is approximately 25% and estimated by the extrapolation of the relation between biliary flow and bile acid excretion to the *y*-axis, the imaginary point at which no bile acid secretes.

flow are the enterohepatic circulation of bile acids and the secretion of bile acids from the hepatocyte into the canalicular lumen, the rate-limiting step.[4,5,14] Bile acids are secreted by an active transport mechanism on the canalicular membrane, where the gradient from within the hepatocyte across the apical surface to the canalicular lumen can reach up to 1000-fold. After secretion into the duodenum from the extrahepatic biliary tree, bile acids are modestly absorbed in the jejunum but primarily reabsorbed in the terminal ileum, then returned to the liver via the portal circulation. Distinct but related transporters on the apical membrane of the ileal enterocytes and the sinusoidal membrane of the hepatocyte efficiently accomplish this process, leading to retention of nearly 95% of excreted bile acids. Despite being recirculated six to ten times per day, only a small fraction of the total pool (approximately 0.5 g of the 4- to 5-g pool in adults) is lost daily in the stool.

This process is well balanced, with the amount of de novo bile acid synthesis being relatively equal to fecal loss.[26,38] This feedback loop is effected by two mechanisms: bile acid sensing nuclear receptors in hepatocytes and factors released from the terminal ileum that travel through the portal vein to the hepatocyte.[14] Overall, this process allows for upregulation of bile acid synthesis in times of intestinal loss (e.g., diarrhea or short gut) or repression of synthesis in cholestasis.[39-41]

The relationship between bile acid secretion and bile flow is linear[42-45] (see Figure 120-4). Bile acids increase flow mainly by creating an osmotic gradient that draws in water, electrolytes, and other solutes. In addition to bile acid pool size and efficiency of enterohepatic circulation, the choleretic potency of different bile acids modulates this process. The bile acid pool size can be manipulated pharmacologically to produce increased bile flow via administration of certain bile acids (e.g., ursodeoxycholic acid) in patients with cholestasis.[46]

The bile acid independent pathway and cholangiocyte secretions drive the remainder of bile flow. Secretion of glutathione, glutathione conjugates, and organic anions by specific transporters on the canalicular membrane is the main determinant of bile acid independent flow and is influenced by hormones, certain drugs, and signaling properties of bile acids.[47,48] Cholangiocyte (ductular) cell secretion is mainly controlled by the hormone secretin, which promotes the alkalinization and dilution of bile via transport of ions into the canalicular space.[49,50] This process is species specific; secretin is a potent choleretic in humans and dogs, but has little influence in rodents.[24,51]

Water flows from the sinusoids to the bile canaliculi via several pathways: transcellular, paracellular, and transcytotic. The majority of water movement occurs through the paracellular pathway via tight junctions, with limited contribution from the other pathways.

BILE FORMATION IN THE NEONATE

Before birth, the placenta is responsible for bile acid metabolism and the detoxification and elimination of lipophilic organic anions in the fetus.[52] Abrupt disconnection from the placenta at birth unmasks the functional immaturity of the fetal/neonatal liver. During fetal life, the liver functions both as a metabolic and hematopoietic organ. The transition to an organ primarily responsible for metabolism and detoxification begins at birth and slowly reaches adult levels by 1 to 2 years of age.[53] Thus hepatic immaturity negatively affects the expected functionality of a premature newborn in multiple ways. In particular the immaturity of neonatal bile acid metabolism is clinically significant because adequate bile acid synthesis and secretion are essential for hepatic bile formation and for intestinal fat and fat-soluble vitamin absorption.

Bile acids have been detected in the fetal liver and gallbladder as early as 14 to 16 weeks gestation.[54] By the beginning of the third trimester (22 to 26 weeks), the primary bile acids found in the fetal gallbladder are taurine-conjugated dihydroxy bile acids, mainly tauro-chenodeoxycholic acid. As the third trimester progresses, tauro-CA, a trihydroxy bile acid, and glycocholate appear at approximately 28 weeks gestation.[55] These taurine conjugates are the predominant ones during fetal life, with a switch to tauro-CA as the principal bile acid at birth. The progression to the adult pattern of glycine conjugates is not accomplished until 2 to 7 months of life.[56-58]

The fetus does not generate the secondary bile acids because this process requires enzymatic cleavage by colonic bacteria, which are not present in the fetal intestine. A small amount of secondary bile acids is present in the fetal circulation, however, from the maternal pool. Specific transporters found on the brush-border and basolateral membranes of the placental syncytiotrophoblast provide bidirectional transport of bile acids between the fetal and maternal circulations.

Some degree of enterohepatic recirculation seems to be present; animal studies using radiolabeled taurocholate in utero showed passive reabsorption of conjugated bile acids in the proximal jejunum, but poorly developed active reabsorption of taurine-conjugated bile acids in the terminal ileum.[59,60] Poor reabsorptive capacity of the fetus coupled with efficient clearance through the maternal placenta lead to a small bile acid pool size in the fetus and neonate, with the term neonate bile acid pool approximately half that of the adult. The preterm neonate is at an even greater disadvantage because the CA synthesis rate and pool size is approximately one third that of a term infant. It is assumed that extremely preterm infants, less than 32 weeks gestation, are likely to have an even smaller bile acid pool size, although exact figures are not currently available.[61,62]

As with many other developmental processes, the adrenal axis appears to have a role in maturation of the synthesis and enterohepatic recirculation of bile acids in the fetus, likely through developmental increase in transporter expression and function.[62] Prenatal dexamethasone treatment has been shown to increase bile acid pool size in significantly preterm neonates to levels comparable with term infants. This is corroborated with animal studies that showed acceleration of CA conjugation levels after a 1-day treatment with cortisone.

The immaturity of the fetal liver and the small bile acid pool size are clinically relevant because bile acid synthesis and secretion are essential for bile flow and absorption of intestinal fat and fat-soluble vitamins.[63-65] Effects of this are manifest as fat malabsorption, poor growth, and deficiencies in fat-soluble vitamins, such as vitamin D.

MOLECULAR DETERMINANTS OF BILE TRANSPORTERS AND THEIR REGULATION

At physiologic pH, taurine and glycine conjugated bile acids exist in the anionic form and are unable to cross membranes by diffusion and thus are completely dependent on membrane transport proteins to enter or exit the hepatocyte. Thus the creation, maintenance, and regulation of bile formation rests mainly on the functional competency of membrane transporters, with central reliance on the transport of bile acids.[14] Recent research from a variety of laboratories have led to the discovery of transporters responsible for the import and export of specific substrates into and out of the hepatocyte[14,22] (Figure 120-5).

Basolateral Transporters

NTCP

The Na^+-taurocholate cotransporting polypeptide (NTCP, SLC10A1) is a high-affinity Na^+-dependent transporter expressed exclusively on the basolateral membrane of hepatocytes.[66,67] Approximately 75% of the conjugated bile acids and

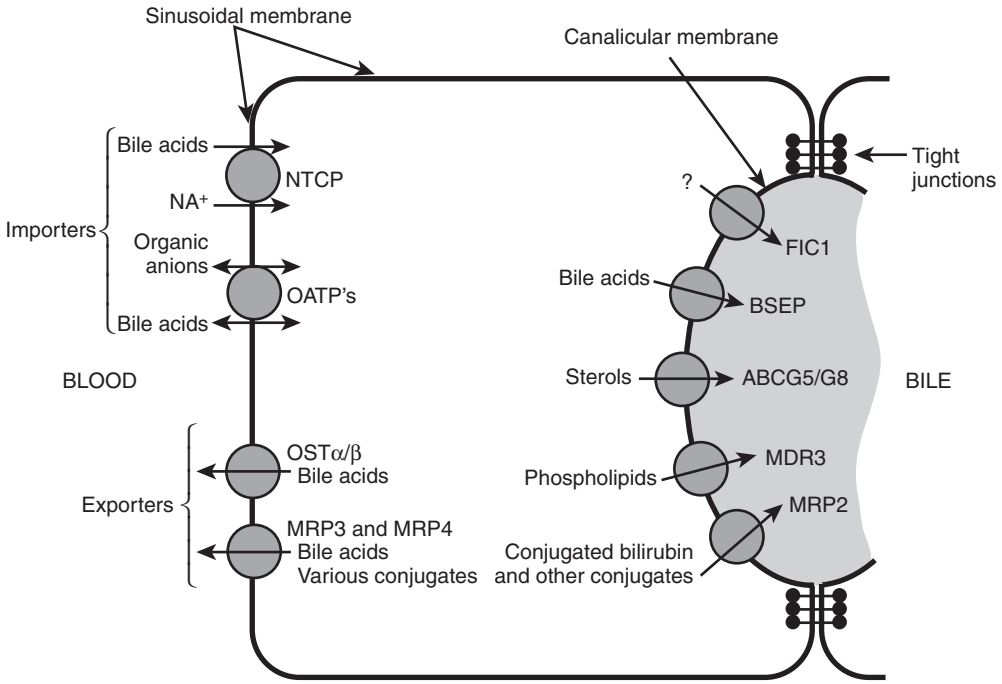


Figure 120-5 Composite schematic of select hepatobiliary transporters involved in bile formation. The hepatocyte is oriented with sinusoidal *(BLOOD)* transporters on the left, canalicular *(BILE)* on the right. *Arrows* connote general directions that the listed solutes may be transported. *Arrows* denote distinct membranes, and the tight junctions that help define these membrane domains. Note that the resident canalicular transporters are responsible for active secretion of nearly all components of bile. *BA,* Bile acids; *BSEP,* bile salt export pump; *MDR,* multidrug resistance protein; *MRP,* multidrug resistance-associated protein; *NTCP,* Na^+/taurocholate co-transporting polypeptide; *OA,* organic anion; *OC,* organic cation; *OATP,* organic anion transporting polypeptide; *PL,* phospholipid; *XOL,* cholesterol.

less than 50% of unconjugated bile acids are imported via this transporter in both rodents and humans. The inward Na^+ gradient necessary for this process is maintained by a Na^+/K^+ adenosinetriphosphatase and facilitates the uptake of conjugated bile acids such as taurocholate. This process is extremely efficient, with 75% to 90% first-pass clearance of bile acids from the sinusoidal blood.[68,69]

Because of its integral role in removal of bile acids from the portal circulation, the NTCP gene product is under substantial transcriptional regulation, which is particularly evident in times of cholestasis and inflammation.[14] Nearly all animal models of inflammation (cecal ligature or puncture, injection of endotoxin or proinflammatory cytokines: tumor necrosis factor-α (TNF-α), interleukin-1β and cholestasis (bile acid feeding, bile duct ligation) produce a rapid and profound decrease in NTCP RNA levels.[14,70-78] By suppressing NTCP expression, the hepatocyte attempts to insulate itself against the toxic effects of accumulating bile acid levels by decreasing import.[40,73,79] This transcriptional negative feedback loop helps protect the hepatocyte against increasing intracellular bile acid levels, which will eventually lead to cell damage and apoptosis.

NTCP was the first hepatobiliary transporter cloned, initially from the rat genome, and with its identification began the field of transporter biology.[80] In addition to the role in metabolism, bile acids also act as transcriptional regulators via certain nuclear receptors.[81] Bile acids are strong activators (ligands), for several NR family members including the farnesoid X receptor (FXR, NR1H4), the vitamin D receptor (NR1I1), the pregnane X receptor (NR1I2) and the liver X receptor (NR1H3).[82,83] Experiments in FXR+/+ mice fed a CA-enriched diet showed a marked reduction in NTCP RNA levels, whereas FXR−/− mice showed no change in NTCP levels in response to increasing bile acid concentrations.[78] The failure to initiate these hepatoprotective mechanisms in the FXR−/− mice led to continued import of bile acids with increased intracellular accumulation, ultimately resulting in bile acid–induced hepatocellular damage and apoptosis. These studies highlight the coordinated response designed to help shield the hepatocyte from bile acid damage. There are contributions from both the intestine and the hepatocyte that lead to the reduction in NTCP expression in cholestasis.[40]

Decreasing bile acid import also leads to decreased bile flux across the hepatocyte and an obligate decrease in bile flow, which contributes to the nutritional consequences of cholestasis. Given the high bile acid levels in infants and the low NTCP expression (inferred from studies in developing rats), it is likely that NTCP plays a key role in cholestasis seen in the sick or premature infant.[58,84]

Organic Anion Transporting Peptide Family

Although the majority of bile acid uptake is accomplished via the Na^+-dependent processes outlined above, approximately 25% of bile acids enter the hepatocyte through Na^+-independent mechanisms.[85,86]

The *organic anion transporting polypeptides* (humans OATP; other species Oatp) are 12 transmembrane domain glycoproteins that belong to the *SLC* gene superfamily of transporters.[31] Members of this group mediate sodium independent transport of a variety of structurally independent, mainly amphipathic organic compounds across the basolateral membrane of the hepatocyte, including bile salts, hormones, both conjugated and unconjugated bilirubin, toxins, and various drugs. OATP/Oatp are expressed in various epithelial cells, some in a single organ and others expressed ubiquitously. They participate in the bidirectional transport of solutes across membranes as diverse as the liver, kidney, intestine, choroid plexus, and retina.

The expression of the OATP transporters is critical for the coordinated import and metabolism of these compounds, so it not surprising that crossover in the transcriptional regulators between NTCP and the OATP genes is substantial, although regulation during development, inflammation, and cholestasis are not well characterized at this time. In animal models of sepsis and cholestasis, rapid, profound suppression of OATP-C (SLCO1B1) occurs, which primarily imports taurocholate, unconjugated bilirubin, and a variety of other organ anions into the hepatocyte.[87-89] The exact molecular mechanisms driving this suppression are still under investigation but, like NTCP, may involve intestinal products or altered function of nuclear receptors that regulate these genes. Developmental studies in rats show that adult levels of OATP expression were not achieved until postnatal day 29. Together, these studies suggest coordinated suppression of both NTCP and OATP transporters likely occurs in the sick, premature infant leading to reduced bile flow and hyperbilirubinemia.

Multidrug Resistance-Associated Protein 3

Multidrug resistance-associated protein 3 (MRP3, ABCC3) is a member of the adenosine triphosphate (ATP)-binding cassette protein superfamily of transporters.[40] It serves a unique role in the hepatic response to cholestasis by exporting retained bile acids and conjugated organic anions, including conjugated bilirubin, out of the hepatocyte. MRP3 is usually expressed in low levels on the basolateral membranes of cholangiocytes and perivenous hepatocytes, but its expression is induced under cholestatic conditions in both animal models and humans. Studies in rats have shown a coordinated increase in MRP3 expression when the canalicular transporter, MRP2 (ABCC2, see later discussion), is mutated.[31]

Organic Solute Transporters α and β

When partnered together, half-transporters organic solute transporters α and β (Octα and Ostβ) are strong exporters of intracellular bile acids.[14,41] They are transcriptionally inducible transporters that act as a sinusoidal "pop-off valves" when intracellular bile acid concentrations increase and exporting across the canalicular surface is not possible (e.g., bile duct obstruction). Both Ostα and Ostβ genes are activated by FXR.[90]

Canalicular Transporters

Bile Salt Export Pump

Bile acid export across the canalicular membrane is the rate-limiting step in the enterohepatic recirculation of bile acids and the primary determinant of bile flow.[86,91] Both newly synthesized and recirculated conjugated bile acids are transported into the bile canalicular space against a steep concentration gradient. This process requires ATP-dependent transport and is accomplished exclusively by the bile salt export pump (BSEP, ABCB11), which is expressed on the apical membrane of hepatocytes. Although BSEP has a high affinity for conjugated bile acids, it has a poor affinity for unconjugated bile acids and accomplishes little transport of these molecules.

In contrast to basolateral transporters, such as NTCP, expression of BSEP is maintained during periods of cholestasis as a coordinated mechanism designed to protect the hepatocyte against the toxic effects of accumulating intracellular bile acid levels. The importance of this process is underscored in patients with the disease *progressive familial intrahepatic cholestasis type 2* (PFIC2; see later), which presents in infancy with severe cholestasis, hepatomegaly, elevated serum bile acids, and aminotransferases, but low serum γ-glutamyltranspeptidase (GGT) levels.[37,91] In patients with PFIC2, mutations in the *BSEP* gene nearly eliminate canalicular *BSEP* expression, dropping biliary bile acid concentrations to nearly 1% of normal but allowing for marked accumulation of bile acids within hepatocytes. Because efflux of bile acids is the driving force for bile flow, this quickly leads to the consequences of cholestasis and liver injury.

As the sole bile acid transporter on the canalicular membrane, it is not surprising that *BSEP* expression is under extensive transcriptional regulation. Bile acids act in a feed-forward manner to increase the expression of BSEP via interactions with the nuclear receptor FXR, leading to enhanced bile acid secretion.[91] This mechanism explains the observation that bile acid feeding in mice results in induction of BSEP RNA and protein. In contrast, FXR−/− mice have low basal BSEP expression levels that fail to respond to a bile acid enriched diet.[78]

Multidrug Resistance-Associated Protein 2

Multidrug resistance-associated protein 2 (MRP2, ABCC2) is another ATP-binding cassette transporter that is found on the canalicular membrane of hepatocytes, as well as on the apical membrane of enterocytes of the duodenum and jejunum and renal proximal tubule epithelial cells. MRP2 mediates the export of glutathione, sulfated and glucuronidated conjugates of drugs, toxins, and bilirubin and is the main mechanism for bile acid–independent bile flow across the canaliculus.[92-96] MRP2 can also transport some unconjugated drugs such as ampicillin, ceftriaxone, methotrexate, irinotecan, and some statins.

As with other transporters, MRP2 RNA expression is severely reduced in animal models of sepsis and inflammation.[79,87] Mice fed diets of CA or ursodeoxycholic acid show enhanced expression of MRP2 RNA, but this is markedly reduced in response to inflammatory cytokines. Inflammation-mediated suppression of MRP2 gene expression is the likely cause of sepsis-associated increases in serum levels of conjugated bilirubin. Mutations in MRP2 lead to reduced bile acid independent flow and cholestasis seen in Dubin-Johnson syndrome (see later).

Multidrug Resistance Protein 3

Multidrug resistance protein 3 (MDR3, ABCB4) is an ATP-cassette transporter that belongs to a group of "flippases" responsible for translocating phospholipids from the outer leaflet of the canalicular membrane into the bile.[97] These phospholipids are critical for the formation of micelles, which protect the biliary tract cells from the detergent actions of bile. Mice deficient for the *MDR3* gene lack phospholipids in their bile, leading to ductular damage and cholestasis.[98] Mutations in the *MDR3* gene in humans result in *PFIC type 3* (see later), which is characterized by high serum bile acid and GGT levels, indicative of biliary tract damage. *MDR3* polymorphisms are associated with some forms of drug-induced liver injury and may be relevant in determining the length of cholestasis in some infants.[37]

ABCG5/G8

The ABCG5/ABCG8 transporters are a functional heterodimers that are coexpressed in the canalicular membrane of the hepatocyte and the apical membrane of the enterocytes. They serve to export cholesterol and phytosterols across both these membranes, thereby excluding them from the circulation. Mutations in either of these half-transporters results in defective biliary sterol transport, leading to the human disease sitosterolemia.[99,100]

CHOLESTATIC DISORDERS

Cholestasis is the clinical manifestation of impaired bile flow, yet practically, it is unlikely that one will ever directly measure this parameter in patients. Levels of serum conjugated bilirubin and bile acids serve as surrogate markers and are thus the most practical measures of cholestasis currently available. Clinically, patients with cholestasis appear jaundiced and often have dark urine and pale stools. With severe liver disease or long-standing

cholestasis, the results of hepatocyte damage are seen as impairments in hepatocellular synthetic dysfunction reflected in hypoalbuminemia, hypoglycemia, coagulopathy, hyperammonemia, impaired drug metabolism, and elevated levels of conjugated bilirubin and serum bile acids. Elevations in alanine aminotransferase and aspartate aminotransferase suggest hepatocellular damage, whereas elevated GGT and alkaline phosphatase levels suggest damage to cholangiocytes.[84]

Patients with cholestasis have defects in bile acid metabolism and the enterohepatic circulation of bile acids. Conversion of primary bile acids to secondary bile acids in the gut is enhanced, as are concentrations of alternatively modified bile acids (glucuronidation and sulfation). The intracellular accumulation of retained biliary constituents, especially hydrophobic bile acids, can be toxic to the liver and other tissues even at submicellar concentrations. Experimental studies have shown that infusions of LCA or feeding of LCA diets to rodents directly causes cholestasis. Exposure to micromolar concentrations of bile acids causes disruption of the lipid bilayer of cell membranes in a detergent-like manner, leading to dissociation of many membrane proteins. These accumulated bile acids can also increase calcium fluxes across membranes, leading to altered mitochondrial function, cell toxicity, and apoptosis. In addition, bile acids are potent modulators of cell signaling processes in the liver, including those linked to growth and the development of hepatocellular carcinoma. In fact subjects with PFIC2 who retain bile acids early in life are at high risk of developing hepatocellular carcinoma before 2 years of age.[101]

Separating cholestatic disorders anatomically into extrahepatic causes (e.g., biliary atresia, choledochal cyst) versus intrahepatic (e.g., Alagille syndrome, genetic or metabolic causes) is a useful first step in identifying the cause of cholestasis.[102,103] Imaging is helpful (ultrasonography) but usually is not definitive, except in choledochal cyst. Determining whether the process is acquired or congenital is the next issue, and here is where much of the difficulty lies in evaluating the infant with persistent elevation of conjugated bilirubin levels. In general, all forms of cholestasis lead to elevated conjugated (direct) bilirubin levels greater than 2 mg/dL and thus warrant consideration for evaluation for underlying liver disease. However, it is well known that sepsis, parenteral nutrition, and certain drugs and infections can lead to liver injury and elevated conjugated bilirubin levels, yet can improve with time. A high level of suspicion (e.g., elevated conjugated bilirubin levels within the first week of life or persistence after the administration of parenteral nutrition ceases) should lead to consideration of a prompt and detailed evaluation for a cholestatic disorder in an infant.[102]

GENETIC DISORDERS

A wide variety of genetic disorders involving intermediary metabolism, storage, bile acid synthesis, and lipid metabolism can lead to liver disease in infancy that can manifest as cholestasis. These include tyrosinemia, glycogen storage diseases, hereditary fructose intolerance, Niemann-Pick disease, amino acidopathies, and mitochondrial hepatopathies. It should come as no surprise that, given the central and essential role that liver metabolic processes play in the health of infants, any mutations in principal genes involved in metabolic pathways can lead to liver impairments and cholestasis.

Progressive Familial Intrahepatic Cholestasis Syndromes

FIC1 Deficiency and PFIC1

Homozygous loss of the *FIC1* gene (ATP8B1), which maps to chromosome 18, results in the disorder termed progressive familial intrahepatic cholestasis type 1 (PFIC1).[104,105] This gene is homologous to P-type ATP-ases, but the ultimate role, or transported substrate, of it remains unknown. The FIC1 protein is expressed mainly in the pancreas and intestine, with small amounts present on the canalicular membrane of the hepatocyte. It is interesting to note that *FIC1* deficiency (Byler disease) leads to reduced bile acid efflux into the canaliculus, yet it is not a bile acid transporter. The FIC1 protein seems to enhance the function of BSEP. One link could be the main transcriptional regulator of the BSEP gene, *FXR*, which is phosphorylated, and activated by mechanisms that include *FIC1* activity.[106]

BSEP Deficiency and PFIC2

Mutations in the primary canalicular bile acid transporter, BSEP (ABCB11), result in progressive familial intrahepatic cholestasis type 2 (PFIC2).[107] Patients typically present in infancy with progressive cholestasis, hepatomegaly, pruritus, elevated serum bile acids and aminotransferases, with normal serum GGT levels.[104] This disease progresses to end-stage liver disease in early childhood (2 to 10 years of age), and currently liver transplantation is the only available curative therapy.[108]

MDR3 Deficiency and PFIC3

The *MDR3* gene product (ABCB4) is responsible for maintaining an appropriate phospholipid concentration in bile, enhancing the formation of micelles that protect the biliary tract from the damaging effects of bile. Mutations in this canalicular transporter gene result in high bile acid:phospholipid ratios, leading to portal inflammation and bile duct proliferation (PFIC3). The high GGT levels seen in this disorder are a marker for the damaged caused by the highly detergent actions of hydrophobic bile acids on the biliary lining.[109,110]

Dubin Johnson Syndrome

Dubin-Johnson syndrome is a rare condition characterized by a benign elevation in serum and conjugated bilirubin levels. In addition to conjugated bilirubin, mutations in the *MRP2* (ABCC2) gene result in decreased transport of other organic anions and conjugated or glutathione-reduced products of drug metabolism.[111,112]

Disorders of Bile Acid Synthesis

The conversion of cholesterol to bile acids involves a 14-step enzymatic cascade.[28] At least seven of these genes have been noted to be mutated in cholestatic disorders that lead to the accumulation of bile acid synthetic intermediate species, liver damage, and low primary bile acid concentrations.[113,114] Many of these diseases are responsive to primary bile acid therapy, thus should be considered in liver disease with low GGT levels.

Acquired Cholestatic Disorders

Sepsis-Associated Cholestasis

As early as 1901, the functional impairment in bile flow associated with sepsis was described. Sir William Osler was among the first to make the connection between nonhepatitic infections with cholestasis and coined the phrase "toxaemic jaundice."[115] With greater recognition of this association, Dunham reported the concept of jaundice as the first presenting sign of sepsis in the neonate in 1933.[116] It has become apparent that sepsis-associated cholestasis does not require direct infection of the liver but is likely mediated by circulating bacterial products (i.e., endotoxin) and cytokines, such as TNF-α and interleukin-1β.[70,87]

In nearly all animal models, administration of endotoxin (bacterial lipopolysaccharides from gram-negative bacteria) results in a profound decrease in bile flow. The mechanism appears to involve the release of inflammatory cytokines from Kupffer cells in response to endotoxin, which in turn act on

receptors on the sinusoidal membranes of hepatocyte. In this manner, endotoxin causes direct effects on hepatocyte transporter trafficking and expression via nuclear receptor-mediated gene regulation.

During sepsis, the liver also plays an important role in the acute phase response, which functions to prioritize the functions of the liver and restore homeostasis to the body.[117,118] This coordinated transcriptional reprogramming in response to cytokines changes gene expression to increase the secretion of many factors that benefit the body as a whole (e.g., protease inhibitors), complement C-reactive peptide (to fight infection), and redirect nutrients to the periphery where they are needed. These cytokines, however, also serve to suppress the function and expression of critical hepatobiliary transporters.

Exposure to endotoxin results in a rapid (15 to 60 minutes) and significant decrease in membrane localization of both MRP2 and BSEP.[79,87] This rapid response appears to be a combination of enhanced degradation and trafficking of receptors to submembranous vesicles. Longer-term exposure to endotoxin and inflammatory cytokines results in activation of the mitogen-activated protein kinase (MAPK) family, which leads to marked alteration in the expression of a wide array of hepatic transporters, prolonging cholestasis.

It is likely that the enhanced susceptibility to inflammation-associated cholestasis in infants is related to hepatic immaturity, which is even more apparent in more premature infants. A common scenario involves the premature infant who has necrotizing enterocolitis, which therefore directly delivers bacteria and bacterial contents from a damaged gut wall to the immature liver. Treatment for this form of cholestasis involves proper attention to sepsis and gut integrity.

Drug-Induced Cholestasis

Because of the innate immaturity of the neonatal liver, infants display a reduced ability to handle drugs and toxic substances compared with older children and adults. They have reduced detoxification capacity and deficient antioxidant mechanisms with which to handle toxic substances. The enhanced susceptibility of infants to the cholestatic effects of certain drugs is not well understood but may be related to immature protective measures against apoptosis and necrosis, an enhanced inflammatory response to damaged tissues and decreased developmental expression of detoxification and transport genes.[37]

Some drugs lead to direct hepatocyte damage (e.g., acetaminophen); others interfere with the formation and flow of bile. Cholestasis is the result of many toxin-related injuries but may have involved a variety of mechanisms, including direct toxicity and necrosis of hepatocytes and cholangiocytes, impaired bile acid transport, and alterations in the viscosity of bile. Cephalosporins are an example of the latter; their high concentrations within bile can lead to the formation of sludge or stones and result in obstructive cholestasis. Interestingly, polymorphisms in ATP8B1 have recently been linked to prolonged cholestasis of the newborn.[119]

Parenteral Nutrition–Associated Liver Disease

The advent of intravenous nutrition in the early 1970s revolutionized the care of critically ill neonates, allowing survival of the most fragile infants in whom enteral feeding was not possible. Research demonstrated that administration of an intravenous solution composed of concentrated glucose solutions containing amino acids, micronutrients, vitamins, and minerals could support both growth and weight gain in children and adults.[120,121] The high glucose load in these early parenteral nutrition preparations caused hyperglycemia and poor immune function, leading researchers to experiment with ways to alter the composition of these solutions to supply a source of non–glucose-based energy and essential fatty acids. Since that time, the addition of a lipid emulsion, comprised of mainly soybean-derived oils rich in omega-6 polyunsaturated fatty acids (PUFA), has become standard practice.

Long-term parenteral nutrition has been associated, however, with the development of acquired cholestasis and progressive parenteral nutrition–associated liver disease (PNALD) in those infants unable to achieve significant enteral nutrition.[120,122-124] The infants most at risk for this disease process are extremely-low-birth-weight infants (<1000 g), infants with congenital abdominal wall and intestinal defects, necrotizing enterocolitis and hypomotility syndromes. The exact etiology of PNALD is not known, but it likely represents a multifactorial process with contributions from immaturity, repeated bouts of sepsis, inadequate bowel function, hydrophobic bile acids, and soy-derived lipid emulsions.[125] Clinically PNALD initially presents with jaundice, conjugated hyperbilirubinemia, and hepatomegaly. As this intrahepatic cholestasis progresses, babies develop hepatocellular dysfunction with elevated transaminases, portal hypertension eventually leading to hepatic failure as evidenced by hypoalbuminemia, elevated clotting times, hypoglycemia, and hyperammonemia. PNALD is a reversible condition in infants capable of achieving enteral nutrition, but the rapid progression from cholestasis to end-stage liver disease in nearly 65% of the most severely affected infants in approximately 4 to 6 months underscores the unique risk to the neonate.

Until the last few years, the only options for parenteral nutrition-dependent infants with end-stage liver disease were liver and/or small bowel transplantation. Recent studies have shown that parenteral fish oil (Omegaven, Fresenius, Bad Homburg, Germany) has been shown to reverse most of the biochemical derangements seen in PNALD.[126,127] This effect is likely due in part to the removal of the offending soy lipid component, likely stigmasterol, and partly as a result of the antiinflammatory effects of omega-3 fatty acids contained in fish oil emulsions.[128] Concern remains, however, that although the conjugated bilirubin is corrected, progressive fibrosis and cirrhosis continues to occur, which is unchanged by parenteral fish oil administration.[129] Further prospective studies, both clinical and laboratory, need to be undertaken to better elucidate the effects of omega-3 fatty acids on hepatic transporter regulation, bile flow, and fibrogenesis.

Work is ongoing into the development of newer lipid emulsions, which may contain a mixture of various fat sources to better balance the risks and benefits of these lipid cocktails. Soy-based oil products are high in omega-6 PUFAs but contain essential fatty acids.[130,131] In vitro work has shown that omega-6 PUFAs can initiate or worsen the inflammatory cascade by increasing TNF-α and interleukin-6 via activation of NF-κB and may also have immunosuppressive features. Fish oil emulsions are high in omega-3 PUFAs, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (reviewed in references 132 and 133). Studies in animal models and humans characterized the antiinflammatory properties of omega-3 PUFAs, although others have shown an increase in collagen deposition and fibrosis. Medium chain triglyceride oil originates mainly from palm kernel or coconut oils and contains less omega-6 PUFAs than soybean derived product. The metabolism of MCT is partially independent of the carnitine pathway, allowing for more rapid plasma clearance, metabolism, and energy utilization without accumulating in the liver. Olive oil is high in omega-9 PUFAs and α-tocopherol, a major antioxidant, and may be beneficial in patients at risk for oxidant damage or stress.

The future of intravenous lipid emulsion therapy may rest on a basic composition, which can then be customized with the addition of different oil components to benefit a particular patient population.

SUMMARY

The anatomy of the liver and the microanatomy of the hepatic lobule direct the abilities of the liver to act as a bioactive filter and modifier as well as a bile secretory organ. The formation of bile is mainly determined by active transport of bile acids and other biliary solutes across the canalicular membrane of the hepatocyte. Alterations in the expression of both sinusoidal and canalicular transporters directly affect bile flow and composition, with clinical consequences manifesting as cholestasis and hyperbilirubinemia in the infant. Neonates are particularly prone to develop cholestasis, likely because of an inherent developmental immaturity of the multiple components necessary to form bile (mainly the enterohepatic circulation of bile acids, hepatobiliary transport, and intermediary metabolism), combined with relatively poor hepatoprotective responses. Recently recognized patient-specific polymorphisms in genes integral to bile formation—transporters, synthetic enzymes, and nuclear receptors—may also contribute to neonatal liver disease. Recognition that infants with seemingly "innocent" cholestasis may rapidly develop cirrhosis and even end-stage liver disease can only help clinicians in determining the timing of potentially life-saving therapies.

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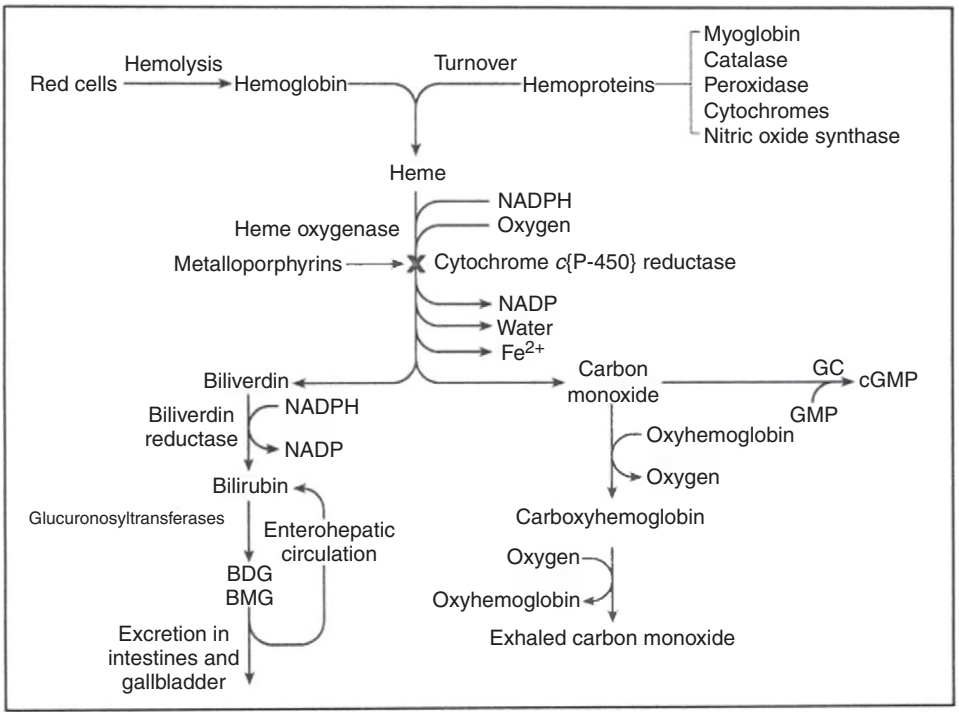


Figure 121-1 Metabolic pathway for the degradation of heme and the formation of bilirubin. *(From Dennery PA, Seidman DS, Stevenson DK: Neonatal hyperbilirubinemia,* N Engl J Med *344:581-590, 2001.)*

to albumin in the extracellular fluid and the plasma.[18] The albumin-bilirubin complex is in equilibrium with a very low concentration of unbound or free bilirubin, generally found in nanomolar concentrations at or below the limits of water solubility for unconjugated bilirubin.[18,19]

Albumin delivers bilirubin to fenestrated sites in the hepatic sinusoids within the space of Disse,[19] probably by passage of the albumin-bilirubin complex through the fenestrae and dissociation of bilirubin from albumin at the hepatic cell surface. Intracellular transport of bilirubin is facilitated by a cytosolic form of glutathione *S*-transferase, probably GSTA-2 (*g*lutathione *S*-*t*ransferase *a*lpha-2),[20,21] which was assigned the generic designation *ligandin* when first identified.[22] The ligandin-mediated function of intracellular transport appears to be shared between bilirubin and other hepatically metabolized molecules of low water solubility or similar structure and molecular weight.[21-23] It is not clear whether the initial passage of bilirubin across the basolateral portion of the hepatocyte membrane is carrier-mediated, contact-mediated, or caused by diffusion of free bilirubin.[19] Recent investigations have implicated still other hepatocellular membrane–associated polypeptides, notably OATP1B1 or SLC21A6, as specific or collaborative organic anion transporters in the sinusoidal or basolateral membrane uptake of bilirubin.[24,25] Although several investigators have identified a potentially important role for these transport polypeptides in bilirubin uptake and for their mutations in the risk for unconjugated hyperbilirubinemia,[24-27] a transfection study of SLC21A6 in HeLa and HEK293 cells did not support a role for it as an exclusive transporter of bilirubin.[28] Instead, transporters of the OATP (*o*rganic *a*nion–*t*ransporting *p*olypeptide) or SLCO (*sol*ute *c*arrier, *o*rganic) family may be collaborators with other transporters in a relay of extracellular to intracellular transport functions. Lin and ssociates[27] have recently estimated the frequency of coexpression for glucose 6-phosphate dehydrogenase (G6PD), OATP, and glucuronyl transferase polymorphisms in a reference population sample. To date, specific syndromic deficiencies of hepatic bilirubin uptake transporters have not been clearly delineated or genetically categorized.

CONJUGATION OF BILIRUBIN

Bilirubin is transported within the hepatocyte to the smooth endoplasmic reticulum, where its carboxyl groups are conjugated with glucuronic acid. Conjugation of bilirubin is catalyzed by bilirubin UDP-glucuronyl transferase (UDPGT-1),[29] one of a family of enzymes that catalyze the hepatic glucuronidation of a number of endogenously produced or exogenously introduced small molecules, such as bilirubin, barbiturates, and phenols.[30] Conjugation of bilirubin and similar small molecules increases their aqueous solubility in preparation for their canalicular transport to the bile.

The first step in the conjugation of bilirubin is formation of bilirubin monoglucuronide, which is sufficiently soluble for canalicular transport and in some instances may be the predominant form of conjugated bilirubin in fetal and early neonatal life.Addition of another molecule of glucuronic acid to bilirubin's second carboxyl group forms bilirubin diglucuronide, the fully conjugated form of bilirubin and the main component of "direct-reacting" bilirubin.

GENETIC VARIATIONS IN BILIRUBIN CONJUGATION

The gene that governs bilirubin conjugation, *UDPGT1,* is located on the long arm of chromosome 2 (at 2.q37)[31] and is part of a gene complex at that locus[29] with a common promoter region and a number of shared and individualized exons that catalyze glucuronidation of a variety of types of small molecules.[32] *UDPGT1* is the member of this gene family responsible for the conjugation of bilirubin.

More than 50 mutations and polymorphisms in the gene for UDPGT-1 have been identified, many of them in association with clinical manifestations of severe, prolonged, or recurrent unconjugated hyperbilirubinemia.[33] In general, the identified mutations have an autosomal recessive inheritance pattern, but the combined effects of heterozygosity for different mutations may produce clinical disease. Several genetic lesions of UDPGT-1 have distinctive clinical phenotypes, whereas others show variable patterns of unconjugated hyperbilirubinemia. The age at onset, severity, and duration of clinical jaundice may be related to (1) the rate of bilirubin production, (2) nutritional status, (3) developmental regulation of liver functions, and (4) intercurrent illness, as well as to specific mutations in the conjugating enzyme. All mutations and polymorphisms of clinical importance can cause severe or prolonged neonatal jaundice, especially if the rate of bilirubin production is increased by hemolysis.

Several *clinical syndromes* (or phenotypes) are associated with specific types of UDPGT-1 mutations. Other phenotypes of prolonged or severe hyperbilirubinemia associated with compound heterozygosity for various polymorphisms, or with the mixed effects of subtle hemolysis and inefficient conjugation, are not so easily classifiable according to the traditional terminology of recognized hyperbilirubinemia syndromes.

Examples of recognized syndromes are Crigler Najjar syndrome, types I and II, and Gilbert disease. Type I Crigler-Najjar syndrome is an autosomal recessive disorder in which no functioning UDPGT-1 is produced. Several nonsense mutations that terminate synthesis or delete key amino acid sequences of the enzyme have been described.[33] The most common variant (also found in the congenitally jaundiced Gunn rat) appears to be a single guanosine deletion at nucleotide 1206, resulting in a frame shift and deletion of 115 to 150 amino acids from the carboxyl-terminal (C-terminal) end of the protein. This defect prevents the binding of glucuronide for conjugation with bilirubin and related compounds. Severe, prolonged unconjugated hyperbilirubinemia begins in infancy and persists throughout life, usually with bilirubin levels of 20 to 45 mg/dL or higher, with eventual signs of central nervous system injury in nearly all cases. The nonsense or "stop" codons causing type I Crigler-Najjar syndrome preclude pharmacologic induction of the enzyme, so gene replacement and liver transplantation are the only effective long-term therapies.

Gilbert syndrome is a milder form of prolonged or recurrent unconjugated hyperbilirubinemia with occasional severe neonatal hyperbilirubinemia, usually followed by peak bilirubin levels of approximately 1 to 6 mg/dL, but with persistence or recurrences of more severe hyperbilirubinemia in older children and adults with the syndrome.[33] The most common genotype associated with the classic form of Gilbert syndrome is that involving an insertion of two extra bases (TA) in the 5′ promoter region of the gene for UDPGT-1, resulting in a sequence of A(TA)7TAA instead of the normal A(TA)6TAA.[29,30] A high carrier rate for this polymorphism has been documented in some families and ethnic groups, and polymorphisms incorporating other "abnormal" sequences of between 5 and 8 TA repeats in the TATAA portion of the promoter have been reported in affected patients with Gilbert syndrome and their family members.[34]

A variant form of the syndrome is caused by a substitution of adenine for guanine at nucleotide 211 in exon 1, with a glycine-to-arginine substitution in the amino terminus (N-terminus—the bilirubin-binding region—of the enzyme molecule. This and several less common substitution mutations are associated with prolonged breast-feeding jaundice and are responsible for a variant of Gilbert syndrome in Japanese kindreds, most members of which show no mutations in the TATAA sequence.[36] Newborns with Gilbert syndrome mutations may have severe hyperbilirubinemia and even kernicterus, especially with a concurrent disorder such as ABO incompatibility or G6PD deficiency.[35] Adults with this syndrome have mild recurrent jaundice, often exacerbated by intercurrent illness such as viral infections. Enzyme synthesis and activity can sometimes be increased and bilirubin levels decreased by administration of phenobarbital, for which a drug-responsive site is located in the promoter region of the gene.

A diagnosis of Crigler-Najjar syndrome, type II, is sometimes assigned to patients with a heterogeneous group of conjugating defects resulting from a number of single-site missense or insertion mutations that may retard synthesis of the enzyme or impair the glucuronide binding of its carboxyl terminus (C terminus).[33,37,38] Although small amounts of bilirubin conjugates may be found in the hepatocytes and bile of these patients, unconjugated hyperbilirubinemia may be severe in the newborn period and persist or recur in adulthood. Peak or intermittent bilirubin levels usually are 6 to 20 mg/dL but may be higher in newborn infants. Insertion or substitution mutations in exons 1, 2, 4, and 5 have been associated with the clinical diagnosis of type II Crigler-Najjar syndrome. Some persons with this phenotype have compound heterozygosity for two or more recessive mutations at different exons. In some patients, enzyme synthesis or activity may be induced or enhanced by phenobarbital, with an increase in bilirubin conjugates and a decrease in plasma levels of total and unconjugated bilirubin.

Persons heterozygous for two or more of the mutations associated with Crigler-Najjar or Gilbert syndrome may have neonatal or subsequent jaundice of intermediate severity, sometimes responsive to enzyme induction with phenobarbital. Exact classification of these mixed, heterozygous cases of unconjugated hyperbilirubinemia as manifestations of Gilbert or Crigler-Najjar syndrome may be difficult using clinical criteria, or even by determination of genotype in some patients. One possible approach would be to classify mutations that impair the promoter region, N-terminal binding of bilirubin, or initiation of enzyme synthesis as "Gilbert type" defects, and those that impair glucuronide binding or completion of C-terminal synthesis as Crigler-Najjar type defects. As an alternative to the classification of hereditary hyperbilirubinemia into two or three eponymous "syndromes," more detailed description and categorization of the specific types of gene defects and their physiologic consequences may ultimately be more useful.

HEPATIC AND ENTERIC EXCRETION OF BILIRUBIN

Conjugated bilirubin is excreted into canalicular bile by way of the multispecific organic anion transport (C-MOAT) system located in the canalicular membrane within the apical region of the hepatocyte.[39] The C-MOAT system shows genetic and structural homology with the multidrug resistance (MDR) family of proteins,[40] and with glutathione *S*-transferase,[41] but incorporates a different glutathione *S*-transferase isozyme from that involved in basolateral intracellular ligandin transport of unconjugated bilirubin.

In the small bowel, bilirubin may be transiently deconjugated by bacterial and brush border enzymes before further metabolism to other heme-derived compounds. Such metabolism occurs mainly in the large bowel and requires bulk transport of bile products by peristalsis and additional changes in the molecule by bacterial enzymes. Because bilirubin β-glucuronidase is indigenous to the small bowel brush border in the newborn, some bilirubin in the small bowel is deconjugated (by β-glucuronidase), reabsorbed at the brush border, and recirculated to the blood in a loop of excretion and reabsorption known as the enterohepatic recirculation of bilirubin.[42,43] Premature infants, surgical patients not being fed, and some infants poorly responsive to

phototherapy may have protracted unconjugated hyperbilirubinemia associated at least in part with a large enterohepatic recirculation of bilirubin before they are fully fed or fully colonized by normal intestinal flora.

Maturation of the hepatic conjugating system, development of adequate canalicular transport, elimination of bile products in the stool, and an eventual developmental close matching of the rates of bilirubin production and excretion allow plasma unconjugated bilirubin to decrease to normal adult levels in most newborns within 2 to 4 weeks or less. Persistent hyperbilirubinemia results if the rate of bilirubin excretion fails to match its rate of formation and production.

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122 Mechanisms of Bilirubin-Induced Brain Injury

Thor Willy Ruud-Hansen

Jaundice is a common transitional phenomenon in newborn infants and was purportedly first described in the Chinese textbook *On the Origins and Symptoms of Diseases* written in 610 AD.[1] In Western medical literature one of the earliest references to this phenomenon may have been in Bartholomaeus Metlinger's book from 1473 on the raising of children.[2] It is not clear when the association between neonatal jaundice and yellow staining of the brain was first noticed, but in work he did for his dissertation in 1847 the French medical student Jacques François Édouard Hervieux described jaundice of the brain during autopsy in 31 of 44 infants who had been noted to be jaundiced at the time of death.[3] Hervieux described some brains as being uniformly yellow, whereas in others some regions were more heavily stained. However, the staining phenomenon later known as *kernicterus* was first described in 1875 by Johannes Orth, who had performed an autopsy on an infant who died at 2 days of age with very intense jaundice.[4] The actual term *kernicterus*, the German word for jaundice of the nuclei (or basal ganglia), was coined by another German pathologist, Christian Georg Schmorl, in a landmark paper in 1904.[5] It is noteworthy that among the 120 jaundiced infants he had autopsied he found 114 jaundiced brains, but of these only six exhibited a pattern of more intense yellow color of the basal ganglia and medulla oblongata, which gave rise to the term. As will be discussed later, during the more than 100 years that have passed since then, the explanation for this localization phenomenon has eluded medical researchers.

The clinical picture in the infants who died included, in addition to severe jaundice, opisthotonos, seizures, spasticity, severe hypotonia, fever, high-pitched cry, and poor sucking. Arkwright[6] may have been the first to describe the acute picture of kernicterus. During the first half of the 20th century it became clear that the pathoanatomic finding of jaundiced brain nuclei had a clinical correlate in infants who survived extreme jaundice. These manifestations, as summarized by Byers and colleagues in 1955,[7] appeared to evolve over time. Thus during the first weeks to 2 to 3 months of life, hypertonicity, opisthotonos, absent Moro reflex, high-pitched cry, and poor feeding dominated the clinical picture. From then on until approximately 2 years of age marked delay of motor development became obvious, with decreased muscle tone, hyperreflexia, and persistence of immature postural patterns. Athetosis could begin toward the end of the second year of life but might not become apparent until 8 to 9 years of age. The degree of athetosis was quite variable, from hardly detectable except to the trained observer, to completely disabling athetosis. Some degree of hearing loss was present in most patients. Paresis of upward gaze appears to be fairly obligate in kernicterus but rare in other types of cerebral palsy. Intellectual deficits were not an obligate feature, and only a minority of the patients had developmental delay. This description is surprisingly compatible with the most recent discussions of kernicterus.[8] Recently, with the recognition that the long-term sequelae of bilirubin encephalopathy may present a many-faceted picture, a set of diagnostic criteria was proposed, which takes into consideration both the clinical findings (classified as isolated, mixed, and classic), the evolution over time (classified as acute, subacute, and chronic), and the severity (mild, moderate, and severe).[9] Time will show whether this tool will be helpful as far as increasing understanding of the processes and mechanisms involved in kernicterus.

Although it has been discussed whether the yellow staining seen in the brain of the infants described in the preceding paragraphs might be a phenomenon caused by other events, and where bilirubin just happens to be the coloring substance, there appears to be general agreement among present-day bilirubin researchers that bilirubin and its toxic influence on brain cells are the primary and causative factor. However, as yet no agreement exists on which mechanism (or mechanisms) might mediate brain bilirubin toxicity. The purpose of this chapter, therefore, is to give an overview of the research that has been carried out on bilirubin effects in and interactions with the brain and to discuss the relative merits and weaknesses of the various theories on the "basic mechanism of bilirubin neurotoxicity."

KERNICTERUS: WHAT IT IS AND WHAT IT IS NOT

Kernicterus strictly speaking refers to the pathoanatomic finding of intense yellow coloring of the basal ganglia superimposed on a paler yellow background as first described by Orth and Schmorl.[4,5] Areas that are found to have this staining include, in descending order of frequency, the hippocampus, thalamus, hypothalamus, corpus striatum, medulla, olives, pons, and dentate nucleus.[10] In these areas ultrastructural findings include membrane alterations, dense cytoplasmatic bodies thought to represent degenerated mitochondria, and calcium granules.[11] Changes of this nature are likely to be irreversible and to represent the pathoanatomic correlate of chronic bilirubin encephalopathy, also known as kernicterus. Thus a discussion of the mechanisms of bilirubin-induced brain injury must necessarily include events that cause or are associated with cell death.

However, evidence is ample that bilirubin has effects on the brain that are likely to be transitory. It is well described in the literature that neonates with significant jaundice exhibit lethargy or drowsiness, hypotonia, and feeding problems.[12] Evoked response studies, both in human infants and in experimental animals, have given more objective evidence of such apparently reversible toxicity,[13-19] although permanent changes have been found in some.[20] An increased incidence of apneas in jaundiced premature infants as compared with less jaundiced controls appears to abate after the first week of life and to be associated with the progression of changes in the auditory brain stem response.[21] This type of reversible toxicity cannot be called kernicterus, yet there is little doubt that it reflects bilirubin effects on the brain. At present no agreement exists on the terminology that should be used to describe this phenomenon.

It is not clear whether acute, and subsequently chronic, kernicterus represents the extreme end of a continuum, where the other end is represented by the slightly somnolent, moderately jaundiced term newborn whose somnolence improves in a couple of days, or whether separate and distinct mechanisms are involved in cell death versus apparently milder perturbation of neuronal signaling.[22] Recent data suggest that even

moderate degrees of hyperbilirubinemia may have discernible long-term effects on behavior,[23] and questions relating to the sensitivity and specificity of the tools used to study the long-term effects of neonatal jaundice will have to be resolved before we can be certain as to what is reversible. This chapter therefore discusses bilirubin-induced brain injury in a wide sense, including bilirubin effects on processes that may not result in cell death and residual damage. Whether such effects may permanently alter cell functions without causing cell death is at present not known.

BILIRUBIN CHEMISTRY AND SOLUBILITY

Certain phenomena relative to bilirubin chemistry and solubility appear inextricably linked to the questions of bilirubin neurotoxicity and therefore are briefly discussed here. The main bilirubin isomer in humans is bilirubin-IXα (Z,Z), which exists either as a charged dianion or as bilirubin acid. In dianion the presence of eight hydrophilic groups imparts some water solubility at neutral pH, whereas in the acid form the presence of intramolecular hydrogen bonds results in near insolubility in water.[24,25] The hydrophobic bilirubin-IXα isomer appears to be responsible for the toxic effects, whereas the water-soluble isomers are thought to be nontoxic.[26-29] Bilirubin becomes water soluble when it is conjugated in the hepatocytes and when bound to albumin for transport in serum. The isomerization of the bilirubin molecule that occurs during phototherapy also results in molecules that are more polar.[30]

Bilirubin is transported in plasma bound to albumin[31] and as such probably does not cross the blood-brain barrier, assuming the latter is intact.[32] However, a minute amount of bilirubin (in the nanomolar range) is always present in plasma as "free" or unbound bilirubin. According to the "free bilirubin theory," it is the unbound bilirubin molecule that enters the brain to produce neuronal injury.[33] Increased concentrations of unbound bilirubin in the blood will, by the laws of equilibrium, shift more bilirubin into the tissues, including the brain. Factors that are associated with increased concentrations of unbound bilirubin, and thus with risk for increased bilirubin entry into brain, include altered albumin characteristics[34,35] or the presence of exogenous or endogenous binding competitors.[36-38] Of these, bilirubin-displacing drugs (e.g., sulfisoxazole) previously used to treat sick infants have had a significant impact. Bilirubin IXα (Z,Z) is an amphipathic molecule but, with respect to membranes, behaves as lipophilic; thus it binds to and crosses phospholipid membranes.[39-41] It is presumably this characteristic that enables bilirubin to cross the intact blood-brain barrier and enter the brain.[42] However, the blood-brain gradient is high; in control animals with an unperturbed blood-brain barrier, the brain bilirubin concentration is only 1% to 2% of the serum bilirubin concentrations.[43-45] Bilirubin experts disagree on the issue of bilirubin solubility, leading to claims that many experiments on bilirubin toxicity have been carried out at concentrations that are unphysiologic and that the results are thus irrelevant.[46,47] This argument appears to rely exclusively on solubility figures from a single publication.[48] At this point the discussion about relevant concentrations of bilirubin for in vitro studies should probably still take into account the actual concentrations of bilirubin found in the brains of infants with kernicterus,[49] as well as those found in experimental animals with clinically relevant levels of serum bilirubin.[50-53]

THE BLOOD-BRAIN BARRIER AND BILIRUBIN-BRAIN KINETICS

Entry of bilirubin into brain is a sine qua non for neurotoxicity. Although the relative lipophilicity of bilirubin allows some passage through an intact blood-brain barrier, this occurs to a lesser degree than would be expected of a truly lipophilic molecule.[54] A recent review lists the characteristics of molecules that are able to cross the blood-brain barrier in significant amounts.[55] They must have a molecular weight (MW) <400 daltons, be lipid soluble, and not be a substrate for an active efflux transporter at the blood-brain barrier such as phosphoglycoprotein (P-gp). Bilirubin has an MW of 585, its lipophilicity depends on the isomeric form, and, as will be discussed later, it is a substrate for P-gp. These characteristics may help to explain the limited entry of bilirubin into brain in most circumstances. The blood-brain barrier may be more permeable to bilirubin in neonates than in more mature subjects, whereas albumin permeability is equally restricted in both young and old subjects.[56-58] Recent work with P-gp seems to support the concept of an "immature" blood-brain barrier as far as its role in keeping bilirubin out of the brain.[59]

An unstable state of equilibrium appears to exist between bilirubin in the blood and bilirubin in the brain, and this equilibrium is influenced by a number of factors (**Figure 122-1**). Bilirubin is bound to albumin, apparently at a primary and a secondary binding site with a high affinity, such that the concentrations of unbound bilirubin in serum are very low.[24,31,38] An epidemic of kernicterus occurred in the 1950s and was shown to be related to the use of sulfisoxazole.[60] In subsequent years it was shown that many drugs compete with bilirubin for binding to serum albumin.[61] Bilirubin displacers are now well recognized to increase the risk for bilirubin neurotoxicity in jaundiced infants, and this has significantly influenced the use and choice of drugs in neonatal medicine. Numerous animal studies show that the blood-brain equilibrium for bilirubin is shifted toward the brain when bilirubin-displacing substances are administered.[32,37,43-45,53]

Bilirubin may interact with biologic membranes in a way that affects their function[62-64]; thus it seems reasonable to ask whether bilirubin could also affect the blood-brain barrier. Bilirubin toxicity to glial cells might also conceivably translate into effects on blood-brain barrier function.[65,66] Some studies have addressed the question of bilirubin effects on the blood-brain barrier directly and found that preexposure to bilirubin increased the permeability of the blood-brain barrier both to a dye[67] and to bilirubin itself.[68]

P-gp is a member of the adenosine triphosphate (ATP)–binding cassette superfamily of membrane transporters. These transporters are expressed both in normal and in diseased tissues and appear to limit the entry of xenobiotics into cells.[69] Bilirubin is a substrate for P-gp and may also inhibit P-gp function.[70,71] This has several interesting implications for the interaction between bilirubin and brain. Thus brain bilirubin content in P-gp knockout mice after an IV bilirubin bolus was almost two-fold higher than in wild-type mice.[72] Drugs known to inhibit P-gp significantly enhanced bilirubin entry into rat brain after an IV bilirubin bolus.[73] P-gp function appears to be phosphorylation regulated, and phosphorylation has been linked to enhanced activity of P-gp.[74,75] This provides a possible link to understanding how bilirubin may increase blood-brain barrier permeability,[67,68] as bilirubin has been shown to inhibit phosphorylation of a wide range of protein-peptide substrates.[76,77]

Other organic anion transporters, such as multidrug resistance–associated proteins (MRPs), have also been hypothesized to play a role in limiting bilirubin accumulation in the cerebrospinal fluid.[78] Preliminary data appear compatible with a role for MRP1 (ABCC1) in keeping bilirubin out of brain cells.[79] In astrocytes bilirubin appears to mediate up-regulation of MRP1, resulting in decreased sensitivity to bilirubin toxicity.[80] In mouse embryo fibroblasts, intracellular accumulation of [^{3}H]bilirubin, as well as cytotoxicity, were significantly greater in cells from MRP1 knockout mice than from wild-type

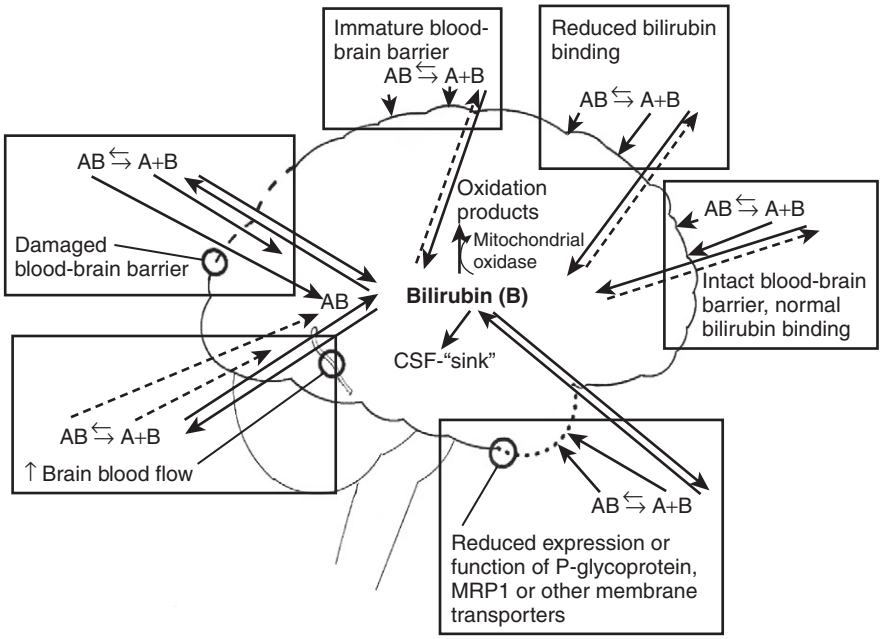


Figure 122-1 Factors that modulate the bilirubin-brain equilibrium. A number of factors can modulate bilirubin uptake into and clearance from brain. These include the avidity of bilirubin binding to serum albumin, the presence of competitors for the albumin binding site, immaturity or other factors that may affect blood-brain permeability, the presence and activity or expression of membrane pumps in the blood-brain barrier, and brain blood flow. *A,* Albumin; *AB,* albumin-bound bilirubin; *B,* (unbound) bilirubin.

controls.[81] The implications of these findings for blood-brain bilirubin transfer remain to be elucidated.

Opening of the blood-brain barrier produced by radiation, asphyxia, hyperosmolality, and hypercarbia has been shown to increase bilirubin entry into brain in a number of studies.[32,44,45,82,83] The latter three conditions may occur quite frequently in sick neonates and thus appear to be relevant for the clinical management of jaundice in infants in the neonatal intensive care unit. In hypercarbia most, but not all, of the bilirubin enters brain as the unbound molecule; in hyperosmolality significant entry of albumin into brain also occurs; and in the jaundiced individual a large proportion of this albumin will carry bilirubin.[32] In hypercarbia the acute entry of bilirubin into brain is increased relative to control conditions, but clearance is also rapid.[45] In hyperosmolality it appears that bilirubin remains in the brain significantly longer than under control conditions.[45] Whether such prolonged exposure is associated with increased risk for toxicity is speculation, but some clinical data suggest that not only the level of serum bilirubin but also the duration of hyperbilirubinemia are associated with risk for neurologic sequelae.[84]

Opening of the blood-brain barrier may contribute to or exacerbate bilirubin neurotoxicity.[82,83,85] In vitro studies suggest that albumin blocks the toxic effects of bilirubin when present in equimolar concentrations.[63,86,87] Opening of the blood-brain barrier permits the entry of albumin-bound, as well as unbound, bilirubin, and, because the equilibrium balance in serum is heavily tilted toward albumin-bound bilirubin, the observation of increased toxicity in vivo is perhaps somewhat surprising. Signs of toxicity may, however, best be predicted by total brain bilirubin content, and enhanced binding to albumin appears to be protective.[83]

The role of brain blood flow may best be illustrated by the studies of hypercarbia, which results in increased brain blood flow.[32,45,52,58,88] These studies show that increased brain blood flow in experimental animals is associated with increased entry of bilirubin into brain. One may speculate that with increased brain blood flow each circulating bilirubin molecule passes the blood-brain barrier more often and thus has more opportunity to equilibrate with bilirubin in the brain in a situation where a blood-to-brain concentration gradient exists.

The question of how bilirubin localizes to the basal ganglia will be addressed here, although whether the localization phenomenon is related to bilirubin entry into or clearance from brain is not at all clear. The predilection of bilirubin for certain brain regions has been described earlier and was primarily based on visual inspection of brain slices. However, more objective, albeit limited, data are available from human subjects. Thus Claireaux and colleagues[49] extracted bilirubin from the brains of four infants who died with severe jaundice. They found approximately 35 nmol/g in the nuclear regions and 8 nmol/g in the remainder of the brains, speculating that bilirubin concentrations may have been several-fold higher in the most intensely stained areas of the nuclei.

Attempts to recreate this staining pattern in animal models have met with variable success. Burgess and colleagues found regional differences in bilirubin concentration in piglets that had received a [^{3}H]bilirubin infusion plus either hypercarbia or hyperosmolality.[52,89] The question remains, however, whether these differences were due to variations in bilirubin entry into or disappearance from brain, or whether they might be related to redistribution or binding to specific tissue or cell elements. Cannon and colleagues[90] examined regional bilirubin concentrations in 15- to 19-day-old Gunn rat pups pretreated with sulfadimethoxine and found significant differences. Thus the cerebellar bilirubin concentration was 32 nmol/L, whereas the brain stem and cortex contained 18 nmol/L and 8 nmol/L, respectively. These numbers are surprisingly similar to those previously found in human brains.[49] The brain bilirubin concentrations were higher in the male pups, suggesting that sex may modulate brain bilirubin uptake or clearance. However, no clear explanation was given for how these regional differences arose, nor could they be said to mimic a typical kernicteric pattern. Attempts to recreate a kernicteric staining pattern in

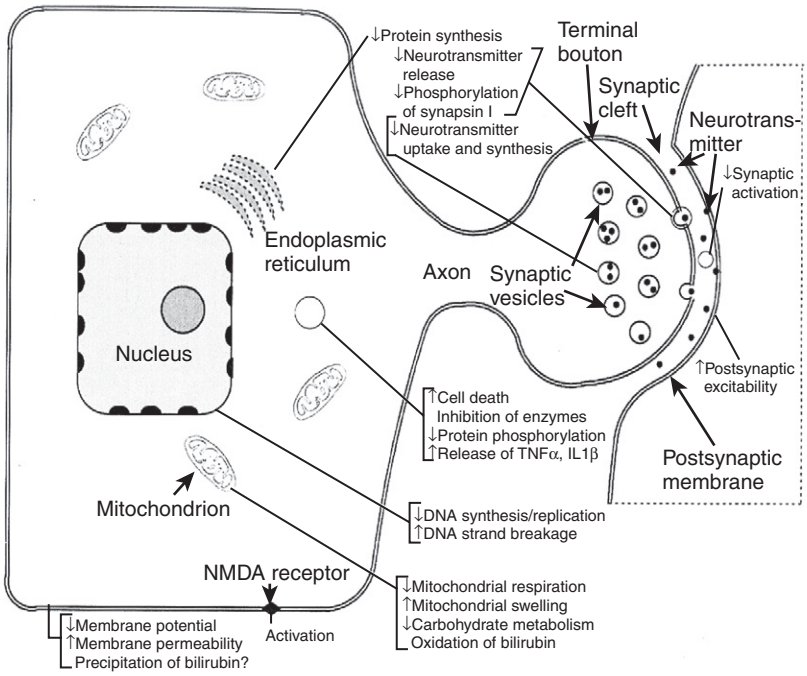


Figure 122-2 Interactions of bilirubin with cells and cellular processes. Bilirubin has been shown to interact with many different cellular processes and reactions. Although several of the studies have been performed in nonneuronal cells, in this figure these processes and reactions are depicted in a schematic and simplified neuron.

Sprague-Dawley rats through infusion of bilirubin and manipulations of bilirubin binding, blood-brain barrier opening, and brain blood flow have not been succesful.[32,43-45] These studies also failed to show interregional differences between bilirubin uptake into or clearance from brain. Other studies have examined bilirubin metabolism in brain, and, although differences were found, they did not conform to or explain the staining pattern of kernicterus.[91]

Hemolysis is believed to be implicated in the mechanisms underlying bilirubin neurotoxicity, and this is reflected in most therapeutic guidelines, which call for more aggressive management in the presence of hemolysis.[92,93] However, the mechanisms underlying this putative increased toxicity are not known. In rats with a chemically induced hemolytic anemia, neither entry into nor clearance of bilirubin from the brain was different from that in control animals after an IV bolus of bilirubin.[94] This obviously does not model the immunologic aspects of anemia in infants with blood group incompatibility and thus cannot discount the possibility that blood-brain barrier permeability may be affected by such mechanisms. However, immunology is not involved in the hemolysis of glucose-6–phosphate dehydrogenase deficiency, where increased risk for kernicterus is well described.[95] The question of whether the products of hemolysis, aside from bilirubin, could exacerbate the neurotoxic effects of bilirubin, appears not to have been the subject of study.

INHIBITION-UNCOUPLING OF OXIDATIVE PHOSPHORYLATION: "THE CLASSIC STORY"

The first experimental studies of bilirubin toxicity were performed in 1954[96] and showed that bilirubin inhibited respiration in a rat brain homogenate. In rat liver mitochondria in vitro, 300 μmol/L bilirubin partially inhibited respiration, and phosphorylation was nearly completely inhibited.[97] The interpretation was that bilirubin uncoupled oxidative phosphorylation. This, for many years, was the prevailing theory as far as the basic mechanism of bilirubin toxicity. A number of in vitro studies from recent years also lend some credence to the role of mitochondria in bilirubin toxicity. Thus bilirubin can affect the mitochondrial membrane, resulting in increased membrane permeability, decreased membrane potential, release of cytochrome *c*, and triggering of apoptosis.[98-102] A cartoon depicting some of the many different effects and interactions of bilirubin with cells and cellular processes is shown in Figure 122-2.

However, these data do not unequivocally confirm a role for the mitochondria as primary targets of bilirubin toxicity in vivo. Thus early electron microscopic observations in Gunn rats suggested that the mitochondrial changes might result from earlier effects in the cytoplasm.[103] A comparison of the effects of bilirubin with known uncouplers of oxidative phosphorylation and inhibitors of reduced nicotinamide adenine dinucleotide oxidase on L-929 cells in culture was more indicative of membrane perturbation than of toxic effects on the respiratory chain.[63] Studies in newborn pigs failed to show changes in cerebral oxygen, glucose, and lactate metabolism that would be compatible with perturbation of mitochondrial function.[104] Furthermore, bilirubin has been found to perturb neurotransmitter metabolism in permeabilized synaptosomes in vitro in the presence of high ATP, so that depletion of endogenous ATP could not have contributed to the observed effects.[105] However, some in vivo support exists for the role of mitochondria. Thus ultrastructural changes are found in the mitochondria of Gunn rats with bilirubin encephalopathy.[103,106] Rats with infusion-induced hyperbilirubinemia exhibited significantly decreased phosphocreatine and ATP in the brain, but only after opening of the blood-brain barrier with hyperosmolality.[107] In a recent study of magnetic resonance spectroscopy in infants with severe neonatal jaundice, one of five infants had an abnormally high lactate–*N*-acetyl aspartate ratio.[108] This was the only infant who also exhibited abnormalities in the basal ganglia on magnetic resonance imaging and one of two who demonstrated neurologic abnormalities during follow-up. The elevated lactate–*N*-acetyl aspartate ratio may have been the result of changes in mitochondrial function.

However, in another study in six infants with extreme jaundice, of whom four were neurologically abnormal at 1 year of age, none demonstrated elevated brain lactate levels.[109] In fact, the findings in this study were more compatible with changes in *N*-methyl-D-aspartate (NMDA) receptor sensitivity. For bilirubin to perturb mitochondrial function, it needs to get there in concentrations sufficient to cause toxicity. Whether this be the case has only been addressed by one study, in which [^{3}H]bilirubin was given as an IV bolus to rats. The animals were killed after 10 and 30 minutes, respectively, and their brains subjected to subcellular fractionation.[110] Although absolute values for bilirubin could not be computed, the bilirubin content relative to protein in mitochondria was much less than in the cytoplasm and membrane fractions. In conclusion, whether the mitochondria are the primary targets for bilirubin neurotoxicity remains a theory for which some experimental support exists, but a number of studies point in other directions.

INTERACTION WITH MEMBRANES

Evidence is ample that bilirubin may interact with biologic membranes.[62-64] Bilirubin content in rat brains subjected to subcellular fractionation was higher in the myelin (membrane) compartment than in any of the others.[110] In brain slices exposed to [^{3}H]bilirubin, bilirubin appeared to bind more avidly to neurons, suggesting a particular affinity for neuronal cell membranes.[111] An important question regarding the interaction of bilirubin with membranes is whether such interaction is primarily a phenomenon of physicochemical binding with perturbation of membrane integrity and potentials or is associated with effects on membrane-located enzymes, pumps, or transporters. Furthermore, it is unknown whether the putative effects of bilirubin on membranes are reversible or nonreversible.

It has been suggested that interaction of bilirubin with polar lipids may play an important role in the toxic mechanism.[112] The addition of bilirubin to cell cultures was associated with leakage of cytosolic enzymes into the medium.[63] Erythrocytes from jaundiced neonates examined by scanning electron microscopy demonstrated a crenated surface structure, compatible with an interaction of bilirubin with the outer half of the erythrocyte plasma membrane bilayer complex.[26] Phototherapy reversed the membrane changes. Others have also found evidence for crenation of the red cell outer surface in nonaggregating conditions in the presence of clinically relevant bilirubin concentrations.[113] Depression of the membrane potential of synaptosomes by bilirubin suggested a mechanism involving alterations in ion permeability.[114] However, it is not clear that perturbation of the membrane potential is an essential element in bilirubin toxicity. Thus in a model involving synaptosomes permeabilized with streptolysin O (excluding the importance of plasma membrane polarity) bilirubin was shown to inhibit Ca^{2+}-dependent neurotransmitter exocytosis and, at higher concentrations, to disrupt vesicular norepinephrine storage.[105] Bilirubin appears to have a high affinity for the phospholipids in cell membranes, and some evidence exists that it may form complexes with these phospholipids.[24,115,116] Partitioning into membranes was increased if they contained proteins, but the effect of proteins could not be attributed to specific binding sites.[116]

Bilirubin in its acid conformation may possibly precipitate, particularly in the presence of acidosis, resulting in irreversible aggregation.[40,117] Precipitation of bilirubin aggregates remains a theory that is supported by some investigators.[78] Others have proposed that bilirubin in the form of the monovalent anion of the acid might bind reversibly to membranes.[118] This is consistent with the finding that bilirubin binding to liposomes and red blood cells is reversible,[118] as well as with clinical and experimental observations regarding the reversibility of the milder signs of bilirubin influence on the brain.[119] However, reversibility of membrane changes caused by bilirubin appears to be dependent on model and experimental conditions. Thus in an erythrocyte model, washing with albumin could not fully reverse the membrane changes associated with bilirubin toxicity.[120] Of note, inherent in the proposed model of reversible bilirubin binding to membranes is also the possibility that decreasing pH could result in irreversible aggregation of bilirubin in tissues.[118]

Some effects of bilirubin on cell membranes may also be understood in terms of bilirubin interaction with membrane-localized enzymes, pumps, or transporters. Thus the break temperature for sodium, potassium–adenosine triphosphatase (Na^+,K^+-ATPase) activity was shifted to lower levels if tested with enzyme from young rats in the presence of bilirubin, whereas the enzyme from adult rats was unaffected.[121,122] This suggests an interaction between the enzyme, bilirubin, and the surrounding membrane lipid environment. Similar changes in the transition temperature under the influence of bilirubin have been found for NO synthase (NOS).[123] Bilirubin-induced changes in NOS activity may be attenuated by 7-nitroindazole, a specific inhibitor of neuronal NOS.[124] Na^+,K^+-ATPase and acetylcholinesterase activities in rat brain exhibited an age-dependent decrease (enzymes from young rat brains were more strongly inhibited)[125] after administration of bolus doses of bilirubin.

Bilirubin has been shown to inhibit the enzymes involved in the transfer of reducing equivalents across the inner mitochondrial membrane,[126] and also to inhibit vasopressin-stimulated water and Na^+ transport across the toad bladder membrane,[127] showing that bilirubin may exert a tissue-specific effect on membrane transport mechanisms. Inhibition of membrane ion transport, accompanied by increased water retention, is another bilirubin-associated perturbation of membrane function.[128]

NEUROTRANSMITTER METABOLISM

Both the inhibitory effects of bilirubin on evoked potentials and the reversible inhibition of synaptic activation in rat transverse hippocampal slices[129] suggested that neurotransmitter metabolism might be affected by bilirubin. Phosphorylation of synapsin I is an important step in neurotransmitter release at the synapse, and the ability of bilirubin to inhibit synapsin I phosphorylation[76] might therefore also predict bilirubin effects on neurotransmitter cycling.

Studies in synaptosomes (synaptic nerve endings that retain their function in vitro) showed that bilirubin was both able to inhibit the uptake of tyrosine, a precursor to dopamine, and to inhibit the formation of dopamine in these nerve endings.[130] Other studies showed that bilirubin inhibited direct uptake of dopamine into synaptosomes, but not its release.[131]

However, the release of endogenous acetylcholine was inhibited, and depolarization of the synaptosomal membrane was also observed. Of note, in preliminary studies other investigators observed inhibition of dopamine release by bilirubin using a different stimulus for release.[132]

Another contribution to decreased synaptic function in the jaundiced brain might be the inhibitory interaction between bilirubin and transport proteins in synaptic vesicle membranes, leading to inhibition of neurotransmitter uptake into these vesicles.[133] It is noteworthy in the studies cited above that uptake of dopamine and glutamate into synaptic vesicles was equally inhibited, though these are presumed to be driven by two different mechanisms (proton gradient and membrane potential, respectively). In a further attempt to delineate the relative importance of membrane potential versus energy metabolism, the same group used permeabilized synaptosomes to study the release of norepinephrine and found that bilirubin inhibits both exocytotic release and synaptic vesicular storage of brain catecholamines independently of ATP and in the absence of a membrane potential.[105]

The NMDA receptor ion–channel complex is localized at the surface of synaptic membranes, is activated by glutamate, and appears to be important during development.[134,135] In vivo administration of bilirubin in piglets resulted in increased affinity of the NMDA receptor for the blocking agent MK-801, suggesting that bilirubin modifies the function of the NMDA receptor–ion channel complex.[136] Acute bilirubin encephalopathy induced in homozygous Gunn rats by injection of a "displacer" was reduced by concurrent treatment with MK-801,[137] suggesting that NMDA-mediated excitotoxicity may be involved in bilirubin encephalopathy (see Figure 122-2). However, such interaction between bilirubin and the NMDA receptor was not observed in a different experimental model.[138] The effect of bilirubin on in vitro cell cultures has been studied by several groups. Glutamate uptake in rat cortical astrocytes was inhibited by bilirubin.[139] Apoptosis in developing rat brain neurons was reduced by the NMDA blocker MK-801,[140-142] and these observations have been confirmed by others working with human neurons.[143,144]

Exposure of astrocytes, microglia, and neurons in culture to bilirubin was followed by release of glutamate and cell death.[145-148] Immature cells were more vulnerable to loss of glutamate. L-carnitine, which protects neurons in culture from glutamate toxicity, has been shown to significantly reduce bilirubin toxicity in cerebellar granule cells in culture,[149] further pointing to the role of glutamate and excitotoxicity in bilirubin cell toxicity.

BILIRUBIN METABOLISM IN BRAIN

Bilirubin that has entered the brain is also cleared from the brain. In early studies the half-life of bilirubin in brain was estimated at 1.7 hours, and the clearance rate from blood was 1.6 hours.[58] However, this study was carried out in hyperosmolar conditions. Subsequent work has resulted in the much lower estimated half-life of 16 to 18 minutes during baseline conditions[43] and approximately 38 minutes during hyperosmolality.[45] A meta-analysis of these data indicated that clearance from brain could be more rapid than from blood, suggesting that additional mechanisms might be operating to metabolize bilirubin in brain.[91] Data from a study by Brodersen and Bartels could throw light on this phenomenon.[150] They described an enzyme capable of oxidizing bilirubin that was found on the inner mitochondrial membrane of brain cells, as well as in other tissues. Subsequent studies by others have verified the existence of a bilirubin-oxidizing activity in brain.[91,151-156] In brief, this enzyme appears to have some of the characteristics of the cytochrome oxidases, although it has not been unequivocally identified. Recent data suggest that cytochrome P-450 2A5 may play an important role in hepatic oxidation of bilirubin,[157] but it is not known whether hepatic and brain metabolic pathways are the same. As far as the brain enzyme, the activity is lower in the immature organism and in neurons versus glia.[152] These data appear compatible with clinical impressions suggesting greater vulnerability to bilirubin toxicity in infants compared with older children and adults, and in brain neurons compared with glia. The activity is also subject to genetic variability,[153] and one may speculate as to whether apparent interindividual differences in vulnerability to bilirubin neurotoxicity may, perhaps in part, have a genetic basis. However, the clinical implications of bilirubin metabolism in brain are as yet unknown, and further studies are needed to understand this phenomenon.

APOPTOSIS AND NECROSIS

Cell death is clearly involved in the devastating effect of bilirubin neurotoxicity seen in victims of kernicterus[158] and is observed in the brains of Gunn rats where Purkinje cells appear particularly vulnerable.[159] After the development of methods to study cell death processes in detail, a large number of studies have addressed the question of how cells die when exposed to bilirubin toxicity.

In vitro cell cultures have been especially useful, and several different cell lines have been used. In rat cerebellar granule cells bilirubin causes apoptosis, which can be blocked by inhibitors of RNA and protein synthesis inhibitors. This suggests that execution of the death process required de novo synthesis of RNA and protein.[160] In embryonic rat forebrain neurons in culture, apoptosis caused by toxicity was bilirubin dose dependent and was blocked by an NMDA receptor antagonist, suggesting a role for NMDA receptors.[140,141] In a comparison of bilirubin effects on rat brain astrocytes and neurons, toxicity was evident in both cell types but required higher bilirubin concentrations for the astrocytes.[161] In addition, glutamate uptake and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction (a measure of cell function) were inhibited, whereas neurons exhibited a greater tendency to necrosis and apoptosis. Apoptosis was preceded by signs of impaired mitochondrial metabolism and membrane perturbation in the form of altered lipid polarity and fluidity, protein order, and redox status.[98] However, when neurons and astrocytes were compared from young versus old rats, immature cells were more susceptible to the toxic effects of bilirubin, although mitochondria from the younger animals were more resistant.[162] This suggests that, although mitochondrial injury may play a role in mediating bilirubin toxicity, it is not likely to be the sole, nor probably the primary, mechanism.

In murine hepatoma cells, bilirubin also appeared to have an effect on cell membranes, possibly involving the aryl hydrocarbon receptor.[163] In rat oligodendrocytes bilirubin-induced apoptotic cell death was associated with induction of NOS.[164] Additional studies in rat neurons suggested that apoptosis triggered by mitochondrial depolarization and Bax translocation could be prevented by ursodeoxycholate.[99] It is interesting that hyperphosphorylation of enzyme activity (p38MAP kinase) (as a trigger for bilirubin-induced neuronal death) was observed in rat cerebellar granule cells.[165] This finding is intriguing in light of other evidence that bilirubin appears to have inhibitory effects on the protein–peptide phosphorylation interactions.[76,77,166-167] Minocycline, which acts partly by inhibiting glial caspase 1 and inducible NOS (iNOS) activity, appears to block bilirubin toxicity in rat cerebellar granule cells in vitro and to largely prevent loss of Purkinje cells and cerebellar hypoplasia in homozygous Gunn rat pups.[168] Somewhat surprisingly, phototherapy, which might also have been posited to be neuroprotective because of the formation of presumably nontoxic water-soluble photoisomers, appears to accentuate bilirubin toxicity in cultured cells.[169]

Although the studies just mentioned have been carried out in cells of animal origin, a human neuron-like cell line (NT2-N neurons) has been investigated with respect to bilirubin's ability to cause apoptosis or necrosis. These studies have shown that the induction of apoptosis as opposed to necrosis may depend on bilirubin concentration, such that high bilirubin concentrations induce early necrosis, whereas low-to-moderate concentrations predominantly induce delayed apoptosis.[170] Further studies with a specific caspase-3 inhibitor, a general caspase inhibitor, and an NMDA receptor antagonist indicated that both NMDA receptor–mediated and caspase–mediated pathways may be involved in bilirubin-induced cell death.[143] Concomitant inhibition of both pathways resulted in synergistic protection. However, in follow-up studies looking at recovery after short-term bilirubin exposure in NT2-N cells, caspase inhibition did not have a positive impact on cell survival, whereas NMDA blockade significantly increased the number of undamaged nuclei, without impacting MTT reduction, another measure of cell viability.[144]

INFLAMMATION AND INFECTION

Neonatal jaundice in the presence of infection has been believed to increase the risk for kernicterus, and this is supported by some clinical studies,[171] whereas others do not find this association.[172] In animal studies endotoxemia was found to increase the net accumulation of bilirubin in brain, which appeared to be caused by an increase in total and unbound bilirubin in the endotoxemic rats.[173] This is compatible with data from infected human infants, who had a lower reserve albumin binding capacity for bilirubin.[174] However, bilirubin metabolism in rat brain was not affected by endotoxemia or sepsis.[151]

In mouse fibroblasts in vitro the cytotoxicity of bilirubin was increased by endotoxin and tumor necrosis factor α (TNF-α).[175] In astrocytes exposed to bilirubin in conditions that induced <10% cell death, the release of TNF-α and interleukin (IL)-1β was significantly enhanced, whereas the production of IL-6 was inhibited.[148] Young astrocytes in culture were more prone to bilirubin-induced cell death and exhibited a greater inflammatory response than older cells, and bilirubin cytotoxicity was increased by endotoxin,[147] a finding also confirmed by other studies.[146] Microglia exposed to bilirubin in vitro were activated and released high levels of TNF-α, IL-1β, and IL-6,[145] suggesting that bilirubin-induced cytokine production can exacerbate neurotoxicity. The interaction of bilirubin with the immunologic cascade may be very complex. Thus in astroglia bilirubin induced a rapid rise in the levels of TNF-α receptor 1, which was followed by activation of mitogen-activated protein kinase p38 and nuclear factor κB (NF-κB), among others.[176] This suggests that NF-κB may play a role in astroglial response to bilirubin.

In apparent contradiction to these findings, others working with in vivo animal models have failed to find an effect of bilirubin on NF-κB or p38 mitogen-activated protein kinase. In fact, bilirubin may exert a cytoprotective effect through inhibition of iNOS expression, stimulation of local prostaglandin E_2 production.[177] In another study jaundiced rats were more resistant to endotoxin-induced hypotension or death compared with nonjaundiced controls, and iNOS expression was reduced.[178] Thus the questions concerning infection or inflammation and bilirubin neurotoxicity are unresolved at the present time.

EFFECTS OF BILIRUBIN IN OTHER SYSTEMS

Some investigators have studied the effect of bilirubin on enzyme activity. A 1979 review listed 25 separate enzymes and four pathways that bilirubin appeared to inhibit.[179] A number of other enzymes and pathways have subsequently been studied, and with few exceptions the effects of bilirubin are inhibitory.[121,126,166,180-182] The inhibitory effects of bilirubin on protein kinases are discussed elsewhere in this chapter.[76,77,183]

Several studies have found that bilirubin inhibits protein synthesis,[62,184-186] although at least one recent study came to the opposite conclusion.[142] Inhibition of DNA synthesis[62,187,188] and increased strand breakage in DNA in combination with phototherapy[189,190] have also been described. Finally, several older studies documented bilirubin inhibitory effects on carbohydrate metabolism.[180,191,192]

PROTEIN PHOSPHORYLATION

As will be evident from the topics discussed in this chapter, bilirubin toxicity has been found across a wide range of biologic reactions and systems. It is, of course, not clear that all of these reactions or systems are actually implicated in the effects of bilirubin observed in jaundiced infants. Similarly, no agreement exists as to which of these systems might be the principal or basic mechanism for bilirubin neurotoxicity.

Energy failure caused by inhibition of oxidative phosphorylation was first proposed as the basic mechanism,[96] and this theory continues to have support.[78] Perturbation of membrane function has been proposed as the triggering event in a cascade leading to excitotoxicity and mitochondrial energy failure.[193] However, studies in permeabilized synaptosomes (a model system in which the impact of both membrane polarity and endogenous energy metabolism is excluded) continue to show toxic effects of bilirubin on neurotransmitter metabolism.[105] Furthermore, studies with purified kinases and peptides in experimental systems devoid of membranes and with excess ATP present show widespread inhibitory effects of bilirubin on peptide-kinase interactions.[77]

Thus it seems reasonable to consider whether the many apparently divergent toxic and inhibitory effects of bilirubin may have something in common and whether bilirubin inhibition of such a putative basic reaction might explain the wide range of bilirubin toxicity. Protein-peptide phosphorylation has been shown to be an important regulatory mechanism for many cell processes.[194] A review of current knowledge regarding the many bilirubin-affected processes suggests that regulation by protein-peptide phosphorylation might indeed constitute a common theme. A detailed discussion would vastly exceed the limits for this chapter, but some aspects will be mentioned.

Early studies showed that bilirubin inhibited the binding of cyclic adenosine monophosphate (cAMP) to protein kinase, as well as the phosphorylation of histone,[166] and that protein phosphorylation in cell-free preparations from newborn rabbit brains was inhibited by bilirubin administered IV before the animals were killed.[167] Bilirubin inhibits phosphorylation of synapsin I, a protein that is preferentially localized to presynaptic vesicles.[76] Dephosphorylated synapsin I inhibits neurotransmitter release; thus this observation might explain why bilirubin inhibits synaptic activation in transverse rat hippocampal slices.[129] These observations are compatible with the clinical observation of drowsiness in jaundiced infants, as well as the documented changes in brain stem auditory evoked responses in such infants. Further, bilirubin has been shown to inhibit phosphorylation of endogenous proteins in fibroblasts.[195] Indeed, the inhibitory effect of bilirubin on synapsin I phosphorylation is not unique: a number of protein-kinase interactions are similarly inhibited by bilirubin, suggesting that inhibition of protein-peptide phosphorylation might explain the effects of bilirubin in many biologic systems.[77]

An intriguing new perspective may link the role of P-gp as a membrane-localized pump that may limit the intracellular and intracerebral accumulation of bilirubin (see earlier),[70,72,73] regulating by phosphorylation.[74,196-199] Although no studies that directly address the impact of bilirubin on P-gp phosphorylation have been published, the fact that both protein kinases A and C are involved in P-gp phosphorylation, and that bilirubin has been shown to inhibit the interaction between these two kinases and other peptide-protein substrates,[77] suggests that P-gp phosphorylation may have an integral role in bilirubin toxicity. The apparent ability of bilirubin to increase the permeability of the blood-brain barrier for bilirubin itself[68] could potentially be understood in terms of modulation of P-gp function. However, this has yet to be studied specifically.

Bilirubin binds to albumin in serum, as well as to ligandin in hepatocytes. Lysine seems to be common to both binding sites.[200,201] A review of the literature showed that lysine appears to be a constituent of the active sites of many of the reactions perturbed by bilirubin, including the ATP-binding subdomain II of the protein kinase family.[202] Lysine-containing peptides were shown to modulate the toxic effects of bilirubin in a model peptide-kinase system.[203] Thus binding of bilirubin to lysine may play a role in the mediation and/or modulation of bilirubin neurotoxicity.

Bilirubin has been shown to inhibit a wide range of phosphorylation reactions. Indirect evidence suggests that binding to lysine may play a role in these reactions. Given the key role of protein-peptide phosphorylation in the regulation of cell processes, widespread inhibition of such processes by bilirubin deserves consideration as a possible basic mechanism of bilirubin toxicity.

BILIRUBIN CELL TOXICITY: SELECTIVITY VERSUS GLOBAL EFFECTS

Bilirubin appears to be toxic in all in vitro cell systems that have been studied. However, evidence exists to suggest that some cell types are more vulnerable than others and that the age of the cells may also modulate toxicity. This is in accordance with clinical data. Thus, although clinical signs of cerebellar affection are quite rare when kernicterus is the result of severe neonatal jaundice,[8,204,205] cerebellar symptoms have been reported in persons with Crigler-Najjar syndrome type 1 who were neurologically normal in infancy and early childhood but who had signs of bilirubin encephalopathy later in life.[206]

Bilirubin effects in in vitro cell culture systems were studied in cells derived from a mouse neuroblastoma line, as well as in rat astrocytoma cells.[185] Bilirubin toxicity was evident in the neuroblastoma cells, but not in the glial cells. This is interesting in light of data suggesting that mitochondria from glial cells oxidize bilirubin at a greater rate than mitochondria from a pure neuronal source and thus may have a greater ability to protect themselves against bilirubin toxicity.[152] Bilirubin may also have a greater affinity for neurons, as suggested by binding of [^{3}H] bilirubin to hippocampal pyramidal and granular cells, as well as to Purkinje cells in rat brain slices,[111] though the mechanism for this phenomenon has not been explained.

The difference in reactions to bilirubin exposure between neurons and glia may not be simply one of tolerance versus sensitivity. Thus, in rat brain astrocytes and neurons exposed to 85.5 μmol/L bilirubin, apoptosis, cytoskeleton disassembly, and membrane leakage were greater in the neurons than the glial cells, whereas inhibition of MTT metabolism and glutamate uptake were more pronounced in astrocytes.[161] Differences may also exist between classes of glia. For example, microglia appear to be more sensitive to bilirubin toxicity than astrocytes.[145] Though regulation of MRP1 was curtailed, and the cells exhibited a significant increase in apoptosis. Cells exposed to the lower bilirubin concentration, however, became increasingly apoptotic if treated with a MRP1-blocking agent (MK571). Thus one potential explanation for differential sensitivity to bilirubin toxicity might be variable expression of membrane "flippases" such as MRP1.

Neuroblastoma cells that had differentiated under exposure to prostaglandin E_1 and cAMP lost their sensitivity to the toxic effects of bilirubin.[185] A similar observation was made in rat astrocytes exposed to dibutyryl cAMP.[207] It is noteworthy that both cAMP and prostaglandin E_1 increase phosphorylation of a membrane phosphoglycoprotein in human platelets.[208,209] Both MRP1 and P-gp are expressed in cultured rat astrocytes,[210,211] and data from human studies show that drug treatment may lead to overexpression of P-gp[212] and may even be associated with therapy resistance in epilepsy.[213] Evidence exists that MRP1 and P-gp are expressed in several different brain cells and with a different pattern of distribution.[214] Thus it is conceivable that a closer study of the importance of regulation of membrane transporter expression may shed light on aspects of differences in vulnerability to bilirubin toxicity.

Rat glial cells in culture became more resistant to bilirubin effects from day 2 to day 12 in culture, suggesting that immature cells are more vulnerable,[66] a finding recently confirmed with use of different cells and techniques.[146] It is noteworthy in this context that the expression of P-gp in both rat and mouse brain increases with maturation,[59,215] and, given the evidence that increased expression of MRPs may protect cells against bilirubin cytotoxicity,[81] it is possible that decreasing sensitivity to bilirubin with increasing age may be, at least in part, tied to increasing membrane transporter expression. In addition, increased bilirubin oxidation by more mature brain cells might also confer protection.[152]

Ngai and colleagues[216] compared cells with some relation to the central nervous system (neuroblastoma and glioblastoma cells) with fibroblasts and liver cells. With increasing bilirubin-albumin ratio, cells of central nervous system origin were clearly more vulnerable. When two neuronal cell lines, NBR10A and N115, were compared, the latter were more resistant to bilirubin toxicity.[188] Pathoanatomic data from kernicterus cases, as well as from Gunn rats, clearly show that not all neurons are damaged by bilirubin. The reasons for such differences in sensitivity are not clear. Although both P-gp and MRP1 may be expressed in neurons in experimental or refractory epilepsy,[217,218] they do not appear to be generally expressed in neurons.[219] Other MRPs appear to be found in neurons[220,221]; however, the implications for bilirubin neurotoxicity are unknown.

INTERINDIVIDUAL DIFFERENCES IN SENSITIVITY TO BILIRUBIN TOXICITY

Clinical data suggest a wide interindividual disparity regarding sensitivity to bilirubin neurotoxicity.[95,222-226] Thus, whereas some infants may tolerate serum bilirubin levels in excess of 500 to 600 μmol/L, the literature has reported cases of kernicterus in term infants with serum bilirubin levels barely in excess of 350 μmol/L,[227,228] and at even lower levels in preterm infants.[229]

Considerable discussion has occurred among bilirubin experts regarding this phenomenon. Although immaturity and diseases may explain vulnerability in some cases, apparently healthy, term infants remain in whom kernicterus develops at serum bilirubin levels that many others tolerate without residual damage. The explanation for this remains speculative. Future research should probably investigate genetic differences in the expression of membrane transporters, as well as how their expression may be modulated by such factors as disease, drugs, and even bilirubin itself. Bilirubin oxidation in brain may be hypothesized to be protective, but this remains to be proved. Whether genetic differences in such oxidation might play a role is also awaiting further studies.

IMPLICATIONS OF BILIRUBIN NEUROSCIENCE FOR CLINICAL MANAGEMENT

It will be obvious from this chapter that our understanding of the mechanism(s) of kernicterus and bilirubin encephalopathy is far from complete. It is not surprising, then, that the challenge of translating these data into clinical guidelines has been daunting. The resulting guidelines indicate great variability between practitioners: infants may receive an exchange transfusion in one neonatal intensive care unit for serum bilirubin levels that would not even trigger phototherapy elsewhere.[92] Nevertheless, our current state of knowledge regarding the mechanism(s) of bilirubin neurotoxicity does provide some input into the practical management of a newborn with jaundice.

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SECTION XVI

The Kidney

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William E. Sweeney, Jr., and Ellis D. Avner

Embryogenesis and Anatomic Development of the Kidney

EMBRYOLOGIC FEATURES OF THE MAMMALIAN RENAL EXCRETORY SYSTEM

Embryonic development is regulated by sequential activation and inactivation of developmental gene programs. Such genes encode proteins that determine cell interaction and developmental function. As a result of sequential and reciprocal cell and tissue interactions progenitor cells develop into complex organs. The possibility of creating gene-targeted mouse models has led to new insights into the complex hierarchy of genes that control organogenesis from the earliest developmental stages. Gene targeting approaches have revealed that relatively small gene families encoding transcription factors, proto-oncogenes, and secreted signaling molecules regulate organogenesis in a variety of systems. However, how signaling by such relatively similar signals leads to establishment of a panoply of unique organ structures with highly specialized cells is a challenge for future research.

The mammalian kidney is an excellent model system for investigating developmental mechanisms. The process can be modeled in vitro in organ culture, which provides a means to carry out gain- and loss-of-function and knock-down experiments. The combination of classic and modern experimental embryologic methods has the power to reveal developmental control mechanisms relevant to multiple organ systems. The genes that regulate development are often reactivated in various pathologic conditions, and changes in such genes may predispose organisms to diseases and to become targets of epigenetic changes. Hence, research in the field of developmental biology can be expected not only to influence knowledge and understanding of normal mechanisms of ontogeny, evolution, and development of diseases but also to lead to new therapeutics.

A detailed compilation of the regulatory pathways of renal development is beyond the scope of this chapter, and the following will highlight only those studies that are supported by multiple models, provide novel insights, or raise interesting new questions regarding renal organogenesis. Exhaustive, regularly updated reviews of (1) the developing mammalian urogenital tract, at http://www.gudmap.org,[1] and (2) the Kidney Developmental Database,[2] the molecular basis of kidney development, at http://golgi.ana.ed.ac.uk/kidhome.html, are excellent sources of current findings.

EXCRETORY SYSTEM

During embryonic development, three sets of kidneys form within the mammalian intermediate mesoderm.[3,4] The three kidneys form in a significant temporal and anterior-to-posterior sequence such that the differentiation of the definitive mammalian kidney, or metanephros, will not take place unless it has been preceded by the successive formation and involution of two embryonic kidneys, the pronephros and the mesonephros.[5,6]

The mesoderm forms during gastrulation, and rudimentary or embryonic kidneys subsequently develop from intermediate mesoderm (nephrogenic cords), between the dorsal somites and the lateral plate mesoderm. The pronephros and mesonephros develop segmentally as tubules are induced to differentiate from the intermediate mesoderm by the adjacent pronephric and mesonephric ducts.

Early in development (day 22 in humans), the pronephric duct arises just ventral to the anterior somites.[6] The cells of this duct migrate caudally, and the anterior region of the duct induces the adjacent mesenchyme to form the tubules of the initial kidney, the pronephros. In humans, the pronephros is a rudimentary, nonfunctional kidney that develops during the third embryonic week (eight- to nine-somite stage); it contains rudimentary tubules opening into the pronephric duct and regresses by the fifth week.[6] The human pronephric duct persists as a remnant after pronephric tubular regression. It becomes the mesonephric or wolffian duct, which, in turn, evolves into the ureteric bud (UB), an epithelial structure required for the formation of the metanephros. The mesonephros appears in the third to fourth week of gestation immediately caudal to the last pronephric tubules.[5,6] The mesonephros is the second transient kidney of higher vertebrates but is the definitive functioning kidney of fish and amphibians. Mesonephric nephrons develop a vascularized, glomerulus-like filtering component connected to proximal and distal tubules draining into the mesonephric duct that constitutes the first definitive functional renal unit in the human embryo.[4,5] As with the pronephros, the mesonephros degenerates in a cephalocaudal direction from the fifth to the 12th week of gestation and ceases to function as an excretory organ.[4,5] The distal end of the mesonephric duct gives rise to the UB, and other mesonephric structures participate in genitourinary development. In the male, mesonephric tubules in the area of the gonad form the efferent ductules, whereas the mesonephric duct gives rise to the epididymis and the ductus deferens. In the female, the mesonephric duct completely disappears during the third month, whereas some mesonephric tubules persist as the epoöphoron and paroöphoron, which have no known function in humans.

FORMATION OF THE METANEPHROS

The formation of the metanephros gives rise to the permanent kidney in humans, as well as in other mammals, reptiles, and birds. Unlike other organs that develop through simple epithelial branching morphogenesis, the metanephros develops from reciprocal, sequential inductive interaction of the epithelial UB with the mesenchymal nephrogenic blastema or metanephric mesenchyme (MM).[7,8] Whereas the mesenchyme induces the UB to extend and branch (branching morphogenesis), the UB

induces the formation of epithelial nephrons from MM cells (mesenchyme-to-epithelial [MET] transformation).[9]

The UB in the human embryo arises as an outgrowth from the dorsal aspect of the caudal end of the mesonephric or wolffian duct in the fourth to fifth week of gestation. It extends dorsally into the caudal-most portion of the nephrogenic cord and then draws the MM in a cranial direction. The upward movement of the MM from a pelvic position to its final lumbar position is complete by the eighth embryonic week. On emerging from the pelvis, the metanephros undergoes a 90-degree rotation so that the original ventral hilum takes its final medial position.

The UB undergoes a complex development program of growth, branching, and remodeling in response to inductive signals from the MM, to eventually give rise to the entire urinary collecting system during kidney development. UB branches form the epithelium of the collecting ducts, calyces, pelvis, ureter, and bladder trigone.[4,5] The MM differentiates into glomeruli, proximal tubules, loop of Henle, distal tubular segments, and interstitial stroma.[10]

Normal development and embryogenesis of the UB are fundamental to renal organogenesis. The patterned migration and division of the UB (branching morphogenesis) determine the elaborate three-dimensional branching pattern of the mammalian kidney and are responsible for formation of the urinary collecting system. Further, the specific induction of MM by the ampullary portion of the UB is responsible for individual nephron formation (nephrogenesis). The two basic organogenetic processes of pattern formation (architectonics) and nephrogenesis occur simultaneously during the period of UB ampullary activity, which in the human embryo extends from week 6 through week 36 of gestation. The kidney has thus become the prototype organ for studying the developmental processes of branching morphogenesis, inductive signaling, MET transformation, and epithelial cell polarization.

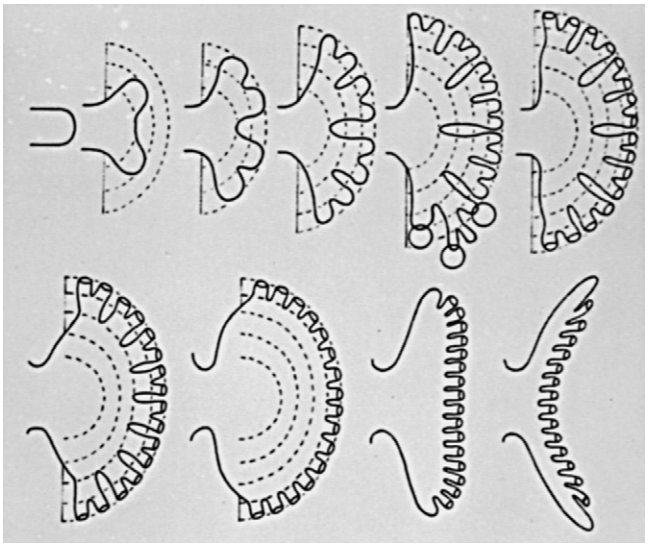


Figure 123-1 Development of a minor calyx and papilla. After the dilation of the first three to five generations of the UB that forms the renal pelvis and major calyces, a second series of three to five generations of UB divisions occurs in rapid succession. This second series of rapidly formed short tubules expands to produce the cavity of the minor calyx, whereas the tubules of the next generations expand to become the cribriform plate that covers the papillary surface. It is thought that the definitive cup shape of the calyx and the conical form of the papilla are formed by the combination of intratubular pressure generated by urine formation and the extratubular pressure generated by enlargement and differentiation of nephrons distally. *(From Potter EL:* Normal and abnormal development of the kidney, *Chicago, 1972, Year Book Medical Publishers.)*

RENAL ARCHITECTONICS

Mammalian kidney function requires the coordinated development of specialized cell types within a precise architectural framework so that body fluid composition can be monitored and optimally regulated. The morphogenesis of the collecting system and the distinct zonal arborization of the developing kidney are controlled by the patterned divisions of the nephron-inducing UB. The renal pelvis, calyces, and intrarenal collecting ducts arise from phases of rapid dichotomic branching of the UB, which alternate with phases of enlargement and structural reorganization (Figure 123-1). Dilation of the early generations of UB branches and final structural modification of the pelvis, calyces, and papillae occur concomitantly with the formation of functional nephron units and probably result from accumulation of urine.

During renal embryogenesis, the pattern of UB branching determines the topographic distribution of nephrons and the elaborate patterns of arborization by which they attach to successive branches of the cortical collecting ducts. The branching of the UB is highly structured, and dissection of early embryonic kidneys revealed several types of repeating patterns of divisions.[11-13] Lateral branches originate from a main stem at equally spaced or at random intervals. The budding of the UB from the wolffian duct is the first lateral branch that then divides into two, producing the first bifid branch point. In bifid branches, the main stem divides into two daughter branches similar in size. Each daughter branch can divide again in a bifid manner until a terminal set of branches is reached. The angle of bifurcation is variable, but in the first bifurcation of the UB it is as large as 180 degrees. Formation of the characteristic collecting duct system of the kidney requires both lateral and bifid branches, which then form three different patterns of branching (lateral, bifid, and trifid) that unfold sequentially. The formation of lateral branches is followed by terminal bifid branching (bifurcation of one stem into two daughter branches). As each ureteric tip connects to a nephron, it is effectively removed from further branching events. This type of branching appears to induce the majority of initially formed nephrons.[14] The remaining fraction develops through the poorly understood process of late-phase branching.[13,15]

The early patterns of renal branching morphogenesis had been previously inferred from static observations derived from microdissection, and serial reconstruction of sectioned kidneys.[6,11,12] Dynamic observations of murine renal organ cultures[13,16] and in vivo time-lapse analysis of UB branching in a transgenic mouse strain expressing enhanced green fluorescent protein specifically in the UB epithelium[17] confirmed many of the hypothesized branching events and also provided new insights into this complex, highly regulated process. Readers interested in more detailed analysis of branching morphogenesis are referred to recent reviews.[18-20]

Differences in embryonic behavior of the ampullae of UB branches make it possible to artificially divide the structural development of the human kidney into four periods[5]: period 1 (embryonic weeks 5 through 14), in which ampullae actively divide and induce formation of nephrons only when not carrying an attached nephron (Figure 123-2); period 2 (embryonic weeks 14 to 15 through 20 to 22), when ampullae rarely divide but induce new nephrons while carrying an attached nephron, resulting in arcade formation (Figure 123-3); period 3 (embryonic weeks 20 to 22 through 32 to 36), in which ampullae do not divide but induce new nephron formation when not carrying an attached nephron (Figure 123-4); and period 4 (embryonic weeks 32 to 36 through adult life), when ampullae are inactive and do not divide or induce formation of new nephrons. All renal growth from period 4 through adulthood is a result of enlargement of existing structures and expansion of interstitial tissue.

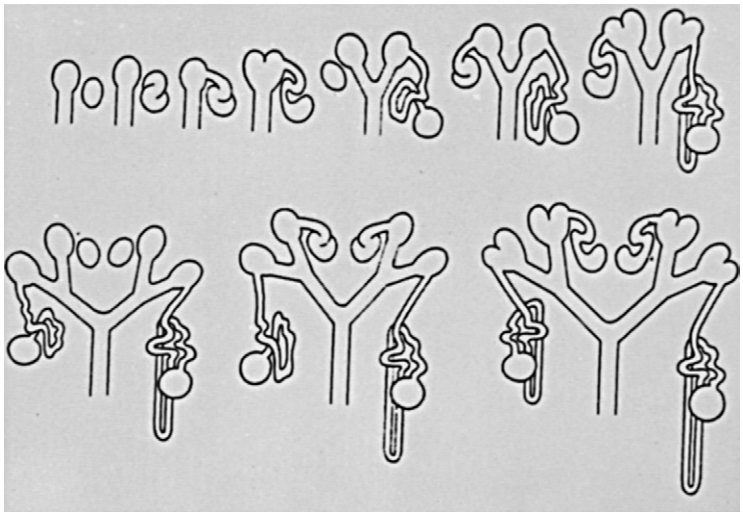


Figure 123-2 Period 1 of nephron formation (embryonic weeks 5 to 14). Ampullae actively divide and induce formation of nephrons only when not carrying an attached nephron. *(From Potter EL:* Normal and abnormal development of the kidney, *Chicago, 1972, Year Book Medical Publishers.)*

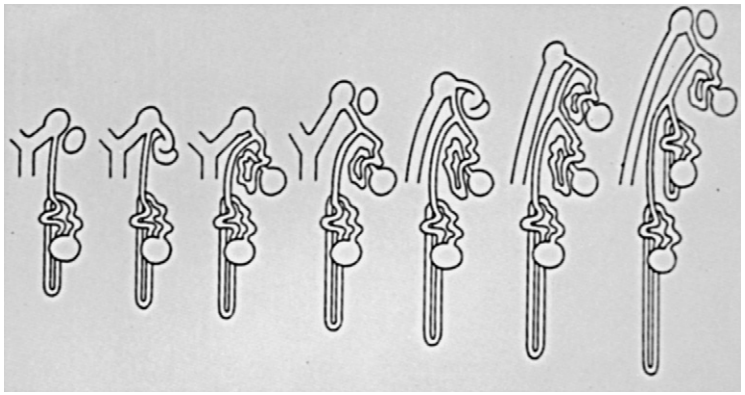


Figure 123-3 Period 2 of nephron formation (embryonic weeks 14 to 15 through 20 to 22). Ampullae no longer actively divide and become capable of inducing new nephrons, even though they already carry an attached nephron. Although two nephrons may be attached to each terminal ampulla temporarily, the connecting piece of the more mature nephron shifts to communicate only with the connecting piece of the younger nephron. In this fashion, arcades of four to six nephrons become attached to each tubule. *(From Potter EL:* Normal and abnormal development of the kidney, *Chicago, 1972, Year Book Medical Publishers.)*

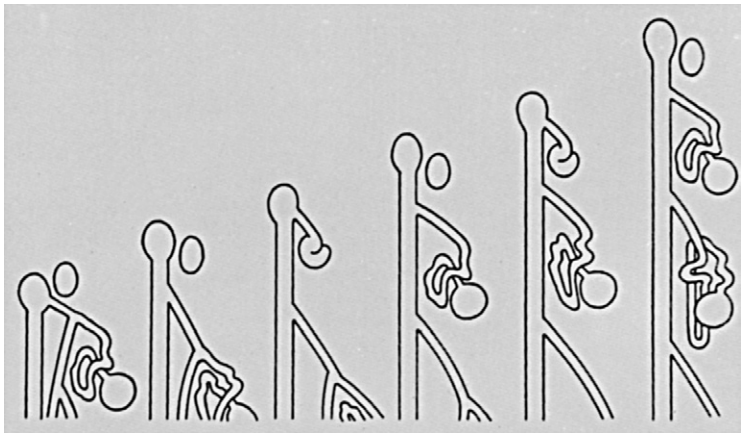


Figure 123-4 Period 3 of nephron formation (embryonic weeks 20 to 22 through 32 to 36). During this period, ampullae branch rarely, induce formation of new nephrons when not already carrying nephrons, and permit attachment of new nephrons only behind the active growth zone at the junction of ampulla and collecting tubule. This results in direct attachment of nephrons to the terminal portion of each collecting tubule. *(From Potter EL:* Normal and abnormal development of the kidney, *Chicago, 1972, Year Book Medical Publishers.)*

The actual differentiation of individual nephrons occurs continuously and without variation in pattern during the different periods of ampullary activity.

The programmed stages of UB division and induction result in a predictable pattern of nephron arborization in the newborn kidney (Figure 123-5). The connecting tubules of all nephrons in the mature kidney are connected to the last two to three generations of collecting ducts produced by UB divisions (13th to 15th generation of UB branches). Because nephrons are successively attached to the UB within a zone of accelerated interstitial growth, they advance with the ampullae. As the ampullae divide, nephrons of different ages and at different stages of development are carried along one ramification of an extending UB branch. The nephron attachments are thus carried along in a centrifugal fashion from generation to generation of UB branches.

The development of the renal vascular system parallels the branching pattern of UB division and nephron induction.[5,6,21,22] Branches of the aorta and vena cava invade the MM soon after initial UB division. Such branches proliferate, arborize, and extend to the periphery of the developing kidney, at which point they merge into a subcapsular network. At approximately 14 embryonic weeks, arterial branches at the future corticomedullary junction elongate at right angles to the basic centrifugal branching to form the arcuate arteries. Branches of the interlobar arteries directly supply the medulla as the vasa recta and return to the corticomedullary area as venules, which drain into the interlobar

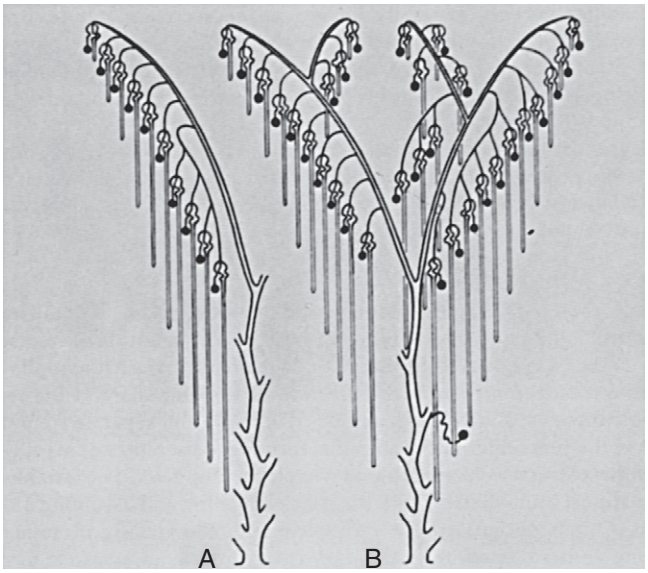


Figure 123-5 Nephron arborization in the newborn kidney. The programmed periods of UB division and induction result in a predictable pattern of nephron arborization in the newborn kidney. **A**, The most common arrangement, whereas **B** shows possible variations. *(From Potter EL:* Normal and abnormal development of the kidney, *Chicago, 1972, Year Book Medical Publishers.)*

veins. The vascular supply of the human fetal kidney is similar to that of the newborn kidney by 15 embryonic weeks.

NEPHROGENESIS

Nephrogenesis is a complex process involving the coordinated growth and differentiation of endothelial, mesangial, neuronal, and epithelial cells that occur throughout the previously noted periods of renal architectonic development. Cytodifferentiation and spatial assembly of different cells during nephron formation are governed by complex interactive events and changing gene expression patterns among transcription factors and protooncogenes, polypeptide growth factors and their receptors, cell adhesion molecules and extracellular matrix (ECM) glycoproteins, ECM receptor molecules, and ECM degrading proteases. These reciprocal signals coordinate developmental processes such as cell proliferation, apoptosis, migration, adhesion, differentiation, vascularization, and neurogenesis that occur concurrently during nephrogenesis. Experimental studies have provided considerable insight into the molecular basis of mammalian nephron formation and thereby provide a basis for exploration of the pathoembryologic characteristics of renal developmental abnormalities.

NORMAL DIFFERENTIATION OF THE INTERMEDIATE MESODERM

The intermediate mesoderm, from which the pronephric, mesonephric, and metanephric kidneys originate, is specified along the mediolateral axis by the temporal expression of specific proteins, and the anterior-posterior axis can be represented by the pronephric, mesonephric, and metanephric kidneys themselves. Therefore a unique combination of mediolateral and anterior-posterior markers can be used to specify any position within the developing mesoderm. The proper differentiation and specification of the intermediate mesoderm along both axes are critical for the eventual development of the metanephros or adult kidney. Disruption in any of the sequential steps of pronephros development and involution, mesonephros development, and metanephric induction leads to abnormal or even complete absence of kidney development.

Developmental profiling of murine embryos reveals a number of proteins initially expressed in the lateral plate mesoderm that become restricted to the intermediate mesoderm before any morphologic indication of urogenital development. The expression pattern of these proteins is the first indication that intermediate mesoderm is differentiating from surrounding cells. Normal differentiation of the intermediate mesoderm has been shown in targeted gene deletion experiments to be dependent on expression of several transcription factors including *Lim1*[23] and *Odd1* (odd-skipped related 1).[24] *Odd1* (*Osr1,* Mouse Genome Informatics) expression is confined to undifferentiated renal progenitor tissue, and its expression precedes all known renal regulatory genes. Murine loss-of-function studies revealed an absolute requirement for *Odd1* in the establishment of the MM and in the activation of the earliest known genes expressed during metanephric kidney formation, *Lim1* and *Pax2.*[25] The paired-domain genes *Pax2* and *Pax8* are expressed subsequent to *Odd1* and *Lim1* activation and further restrict the area fated to become MM and eventually renal epithelia.[25] Targeted deletion experiments demonstrate that *Odd1, Lim1,* and *Pax2/8* are all required to delineate the mediolateral margin of the intermediate mesoderm destined to become MM.[25-31] A complex temporal and spatial relationship exists among these marker proteins suggesting that other positional signaling cues remain to be identified. Recent evidence suggests that bone morphogenetic proteins (BMPs) and other members of the transforming growth factor β (TGF-β) superfamily of growth factors are involved.[24,32]

At the posterior end of the nephric duct, the evagination of the UB is a critical event that is tightly regulated along the anterior-posterior axis. Failure to correctly position the UB along the axis can have severe consequences, resulting in clinical phenotypes ranging from complete renal agenesis to congenital abnormalities of the urinary tract including reflux and hydroureter, to hypertension and progressive renal failure as a result of reduced nephron number.[14]

During embryonic development, anterior-posterior body axis is specified in part by activities of *Hox* genes. The *Hox* genes are conserved among all metazoans and specify positional information along the body axes. Targeted gene deletion experiments revealed that the *Hox11* paralogous genes are essential for early patterning of the MM. The loss of *Hoxa11* and *Hoxd11* resulted in misrouted ureters and hypoplastic kidneys, and the deletion of all three *Hox11* paralogs (*Hoxa11, Hoxc11,* and *Hoxd11*) resulted in complete failure of the MM to induce UB evagination.[33,34]

Before UB induction, the condensing MM expresses a unique combination of markers, including the *Hox11* paralogs (*Hoxa11, Hoxc11,* and *Hoxd11*), *Odd1, Pax2, Eya1, Wt1, Six1, Six2,* and *Gdnf.*[35]

In *Hox11* triple mutants, the expression of both *Six2* and *Gdnf* is absent whereas the remaining early kidney patterning markers in the uninduced MM are unperturbed.[34] Gong and coinvestigators[36] recently demonstrated that the Hox11 paralogous proteins formed a complex with Pax2 and Eya1 that directly activated the expression of both *Six2* and *GDNF* in the MM. These data reveal a novel Hox11-Eya1-Pax2 cassette that activates critical target genes (*Six2* and *Gdnf*) during early renal development, thereby translating anterior-posterior positional information within the developing mammalian kidney.

URETERIC BUD OUTGROWTH

Directed by inductive signals emanating from the MM, and as previously described earlier, the wolffian duct forms the UB as an epithelial outpouching, which invades the adjacent MM. In vitro studies have shown that glial cell–derived neurotrophic factor

(GDNF), produced in the MM, is a primary inducer of ureteric budding.[37] GDNF, a member of the TGF-β family, signals through a receptor tyrosine kinase, RET (*RE*arranged during *T*ransfection proto-oncogene), and the glycosylphosphatidylinositol (GPI)-linked cell surface coreceptor Gfrα1.[38,39]

The initial outgrowth of the UB is a critical step in the development of the urogenital system. The formation of a single, properly positioned UB is critically dependent on appropriately regulated expression of GDNF. Therefore mechanisms to ensure the restricted expression of GDNF involve a complex network of positive and negative regulatory elements that are required for normal development. Common birth defects such as failure to form a ureter and/or the kidney, as well as incorrect positioning of the ureter, result from faulty UB development, and more subtle defects in UB branching can trigger reduced nephron numbers, which may lead to hypertension and renal disease later in life.[6,13,14]

The regulation of GDNF-RET signaling occurs at the level of both gene expression and signal transduction regulation.[35] Positive regulators include the *Hox11* paralogs,[34] *Odd1*,[25] *Pax2/8*,[27,28,40] *Eya1*,[41] *Wt1*,[42] *Six1*,[43] *Six2*,[44] and *Sall1*.[45] In addition to positive regulatory genes that specify the metanephric mesenchyme, negative regulatory mechanisms exist that restrain GDNF expression and therefore limit the area along the wolffian duct from which the UB can evaginate.[35]

GDNF functions as a chemoattractant for RET-expressing cells providing guidance that ensures proper alignment of the UB with the MM. The transcription factors FOXC1 and FOXC2 are normally expressed in the MM and help to define the anterior edge of GDNF expression. Deletion of either gene results in anterior expansion of GDNF expression resulting in evagination of multiple UBs anterior to the normal site of UB outgrowth.[35,46] Similarly, deletion of the secreted protein Slit2 or its receptor Robo2 also results in expanded GDNF expression resulting in multiple, ectopic UBs anterior to the normal position.[47]

Deletion of Sprouty1, an inhibitor of the Ras/Erk/MAPK pathway, results in multiple, ectopic ureters, without altering GDNF expression.[48] Instead, Sprouty1 appears to regulate the response to GDNF by an unknown mechanism. Bmp4, which is expressed in the MM along the wolffian duct before the onset of kidney development, in the mesenchyme surrounding the nascent UB before invasion, and in mesenchyme cells adjacent to the ureter trunk once induction is complete, appears to play a critical role in limiting the site and number of UBs elaborated from the wolffian duct.[32]

The transcription factor Gata-3 is required for proper expression of RET in the wolffian duct.[40] *Gata-3* mutations exhibit increased duct cell proliferation and misdirected UBs.[40]

REGULATION OF GROWTH AND BRANCHING OF THE URETERIC BUD BY THE METANEPHRIC MESENCHYME

The initiating step in renal development is the sprouting of the UB off of the wolffian duct into the genetically preprogrammed MM. Reciprocal inductive signaling between the UB and MM leads to multiple iterations of branching and elongation of the UB while the mesenchyme is induced to condense and epithelialize around the branched ampullary tips of the UB (MET transformation or MET).[9] It has long been hypothesized that the MM controls many aspects of UB growth and branching through inductive interactions, and more recently it has been learned that the renal stroma, which is derived from a distinct cell population in the metanephric blastema, is also important for continued UB development.[6,14,49,50] A number of growth factors expressed by the MM and/or stromal cells or the UB itself, including members of the fibroblast growth factor (FGF), TGF-β, and Wnt families, have been implicated in the initiation of the MET process. Cell surface and ECM proteins such as integrins, heparan sulfate proteoglycans (HSPs), and matrix metalloproteinases (MMPs) are also likely to play roles in MET.

It is likely that inductive signaling from the UB constitutes a complex set of secreted factors, and several UB-derived signals may be required in a temporal and spatial sequence for the proper execution of the induction program.

The balance of data to date suggests that members of the Wnt family of proteins, specifically *wnt9b,* are critical renal MET-inducing factors. These data include the observations that (1) LiCl, a specific inhibitor of glycogen synthase kinase 3 (GSK3) in the canonical Wnt signaling pathway, mimics the early response to induction[51]; (2) cells expressing the Wnt proteins can effectively replace the potent inductive effects of spinal cord[52,53]; (3) Wnt9b is expressed in the UB, and *wnt9b* mouse mutants fail to induce the mesenchyme, despite undergoing early branching morphogenesis[54]; (4) cells that form a "pretubular aggregate," the precursor to the renal vesicle, express Wnt-4[55]; (5) HSP synthesis is required for early kidney development,[56] and Wnt proteins require HSP for activity; (6) a cocktail of factors used to promote epithelial differentiation in isolated rat mesenchyme depended on Wnt ligand activity[57]; (7) Wnt1 can substitute for the UB as an inducer of tubulogenesis (although it is not expressed in the developing kidney)[52]; and (8) six Wnts are expressed in developing kidneys (Wnt2b, Wnt4, Wnt6, Wnt7b, Wnt9b, and Wnt11[54,58,59]). These data strongly suggest that a Wnt signal is essential and permissive for epithelialization and induction, which is a multistep process requiring an initiation signal, factors that suppress apoptosis, and factors that promote proliferation of induced mesenchyme.[35]

EXPANSION AND SURVIVAL OF RENAL STEM CELLS

Only the MM has the competence to respond to the UB to form kidney tubules, and, if induced by other tissues (such as embryonic salivary gland or neural tube tissue), the MM will respond by forming kidney tubules and no other structures.[60] Thus the MM is preprogrammed to produce the diverse epithelial cell types of the nephron.[61,62] However, not all cells of the MM become epithelia. A portion of the MM cells must maintain their mesenchymal character and remain as interstitial stromal cells between the developing tubules. Signaling between induced mesenchyme fated to become epithelia and stroma is essential for proliferation of both cell types as the kidney grows. Failure to expand and renew the epithelial precursor cells leads to developmental arrest and rudimentary kidneys, without inhibiting the differentiation of already induced cells.[35] Reciprocal, inductive interactions are essential for survival and differentiation of both the mesenchymal and epithelial components within the early kidney.[7,63]

The competence of the MM to respond to UB inducers is regulated by the transcription factor called WT1. MM that lacks expression of WT-1 undergoes apoptosis resulting in renal agenesis.[42] GDNF is synthesized in the MM and, as previously described, acts as a chemoattractant to induce the outgrowth of the UB, which expresses c-RET, the receptor for GDNF.[64] The UB also expresses the receptor c-Met, which responds to a MM-produced growth factor HGF.[65] The absence of either GDNF or HGF expression in the MM leads to apoptosis of the MM and renal agenesis. The synthesis of both GDNF and HGF is thought to be regulated in part by WT-1.

Proteoglycan production by the MM has also been shown to provide a critical survival cue to the UB once it has entered the MM. Mesenchymal proteoglycans are necessary to stimulate the continued growth of the UB by maintaining the expression and secretion of Wnt11 from the tips of the UB.[51]

The UB also secretes antiapoptotic factors such as BMP7 and FGF2, which are necessary for the survival of the MM.[66,67,68] In vitro, BMP7 inhibits apoptosis of the uninduced MM and,

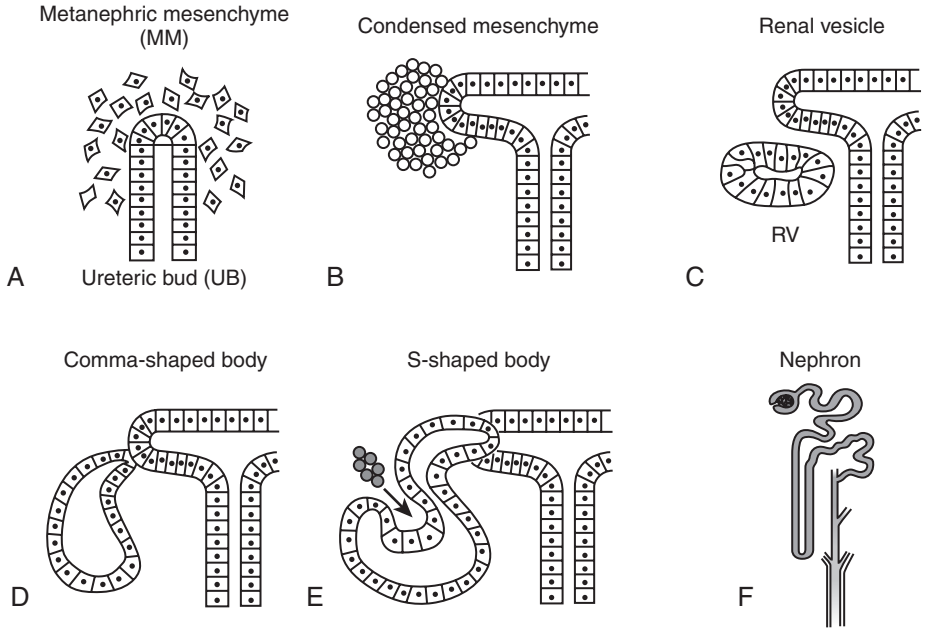


Figure 123-6 The stages of normal nephron formation. Development of the nephron begins when the UB invades a population of prespecified MM **(A).** Shortly thereafter, a group of mesenchymal cells within the MM condense and form a cap around an ampullary tip of a UB branch **(B).** A subset of cells within the condensate will aggregate and undergo a MET transition, forming a renal vesicle *(RV)* **(C).** The majority of the nephron, from the glomerulus through the distal tubule, will differentiate from the RV. The RV will undergo morphogenesis, forming a comma **(D)** followed by an S-shaped body **(E).** Vascular endothelial progenitor cells invade the cleft of the proximal part of the S-shaped body and begin to form the endothelial network of the glomerulus. The distal portion of the S-shaped body will fuse to the UB, forming one continuous lumen. Elongation and subsequent maturation of distinct segments lead to one of approximately 1 million nephrons of the human kidney **(F).**

together with FGF2, maintains the competence of the MM to respond to inductive signals and increase the population of stromal cells.[69] BMP7 and FGF2 work concurrently to inhibit tubulogenesis, suggesting that expansion of the available stem cell pool requires a finely tuned balance between differentiation, survival, and renewal. If cells differentiate too quickly, they can outpace their ability to self-renew, leading to a small, rudimentary kidney.[35]

A recent study by Marose and colleagues[70] demonstrated the necessity of maintaining a similar balance between differentiated and undifferentiated UB-derived epithelia. β-Catenin activity was shown to be necessary for maintaining wolffian duct cells and the duct-derived UB–collecting duct system in an undifferentiated or a precursor state. Decreased expression of β-catenin leads to the premature expression of differentiation markers leading to an imbalance between differentiated and undifferentiated epithelia.[70] Such an imbalance could lead to aberrant branching and a decrease in the number of nephrons.

DEVELOPMENT OF THE NEPHRON

Normal nephron development encompasses four progressive stages that follow embryonic inductive interaction of the metanephric blastema and UB (Figure 123-6, *A*). Ultrastructural glomerular, proximal tubular, and distal nephron development can be seen in the larger context of this framework of overall nephrogenesis. Concomitantly with branching of the UB, the tips of the UB branches induce epithelialization of the MM and subsequent development of the nephron from the glomerulus through the distal tubule.

Nephron development proceeds through a series of intermediate forms, beginning with the condensation or aggregation of induced mesenchyme around the UB tips (see Figure 123-6, *B*). The perpendicular cells form a corona adjacent to the tip of the UB and express Pax2, Wnt4, cadherin-11, and α8 integrin.[58] These cells comprise a subpopulation of what is traditionally called "condensing mesenchyme." These aggregates begin to proliferate and express epithelial properties as a "pretubular aggregate" or the renal vesicle begins to takes shape just beneath the tips of the UB at the juncture of the ampulla and stalk (see Figure 123-6, *C*).

Nephron development continues to proceed through morphologically defined stages known as the comma (see Figure 123-6, *D*) and S-shaped bodies (see Figure 123-6, *E*) before elongation and eventual fusion with the duct. The S-shaped body is generally regarded as the precursor of two distinct processes, that is, glomerulogenesis and tubulogenesis, which are related to the spatial segmentation of the S-shaped body. Glomerular development proceeds from the differentiation of two distinct visceral and parietal epithelial layers during the S-shaped vesicle stage when mesenchymal invagination and development of capillary loops occur (through vasculogenesis and angiogenesis).[71] This is followed by the progressive development of specialized epithelial intercellular connections and basal lamina formation with consequent endothelial fenestration and basilar modification of visceral epithelium into foot processes and slit pores.[72-74]

Nephronegenesis begins as condensations of induced MM cells surrounding the ampullary tip of the UB that are phenotypically and structurally converted to an epithelial vesicle. The proximal tubule and loop of Henle arise from the lower limb and midlimb of the S-shaped body and the distal tubule arises from the upper limb, which ultimately fuses with the collecting duct (see Figure 123-6, *F*). Continued branching of the UB and inductions of new nephrons continue in a radial fashion, such that the first nephrons that develop lie near the corticomedullary junction and the final nephrons form in the outer cortex.

Basic questions about the critical molecular and cellular events that control normal and abnormal nephrogenesis have largely been addressed with in vitro model systems and manipulation of

targeted "developmental" genes in vivo.[1] Model systems that have proved particularly valuable in this regard include both transfilter and whole organ murine metanephric organ culture, isolated mature renal cell lines, isolated embryonic UB and MM cell lines, and in vivo models including zebrafish, *Xenopus,* and the generation of transgenic and genetically engineered mice.[1,10,60,75-81] These approaches have provided important insight into the regulation and modulation of renal organogenesis by characterizing (1) the normal differentiation of the intermediate mesoderm that gives rise to the wolffian duct, UB, MM, and gonadal tissue; (2) induction of the UB outgrowth from the wolffian duct; (3) regulation of growth and branching of the UB by the MM; (4) early nephron morphogenesis and epithelialization; (5) ECM remodeling and cell adhesion changes that pattern early nephron morphogenesis and epithelialization; and (6) the process of nephron vascularization. Studies, to date, have identified more than 400 genes (e.g., secreted growth factors, tyrosine kinase receptors, transcription factors, extracellular components, proteases) involved in renal developmental pathways.

EARLY NEPHRON MORPHOGENESIS AND EPITHELIALIZATION

The differentiation of each nephron starts with mesenchymal cell aggregation around a UB branch tip (ampullae). Each condensate subsequently forms a lumen (the vesicle shape) and elongates to form a tubule (the comma-shape body), which then undergoes regional specialization into primitive glomerular and proximal tubular epithelia (the S-shaped body). The proximal end of each S-shaped body becomes the glomerular epithelia, and the distal end fuses with the adjacent branch of the UB. Soon afterward, the cells of this epithelial structure begin to differentiate into regionally specific cell types, including the capsule cells, the podocytes, and the distal and proximal tubule cells.

To date, the use of mice with targeted mutations has conclusively identified a few important molecules involved in the early events of nephron formation and epithelialization, but these data provide little information regarding the orchestration of nephrogenesis beyond the S-stage. These results demonstrate that nephrogenesis becomes "frozen" at the condensation stage in mice with homozygous mutations in *Wnt-4*[58] or *Foxd1* (originally BF-2)[82] and *Emx2.*[83] The epithelialization of MM is delayed or reduced in mice lacking expression of cadherin-6, and a significant number of tubules that form do not fuse to the UB.[84] Genetic deletion of *Wnt-9b* leads to an arrest of epithelialization in the MM before occurrence of Wnt-4–positive pretubular aggregates. In addition, epithelialization can be rescued in *Wnt-9b−/−* animals by overexpressing Wnt-1 from the UB, indicating that a Wnt signal is essential and permissive for epithelialization.[54]

Few data are available regarding the specification or polarization along the proximodistal or glomerulo-collecting duct axis during nephrogenesis. Asymmetric expression of Brn-1,[85] E-cadherin, and cadherin-6[86] provides the first known evidence of polarization of the proximodistal axis. However it is not readily distinguishable until the S-shaped body stage when Pax2 is highly expressed within the distal portion of the S-shaped body, including the region that fuses to the UB.[87] Podocyte precursors reside in the proximal limb of the S-shaped body and express high levels of Wilms tumor-1 (Wt1).[42] Adjacent to the podocyte in the cleft of the proximal limb, the vascular endothelial network of the future glomerular filtration apparatus starts to assemble.

Previous studies had indicated the likelihood of the importance of Notch activity in establishing the proximodistal axis, as podocytes and proximal tubules were lost when γ-secretase activity was abolished.[88,89] Cheng and colleagues[87] recently demonstrated that Notch2 activity is essential for patterning of the proximal regions of the nephron and a central determinant of segmented pattern in the mammalian nephron.

This induction process of MM must be initiated by secreted signals at the tip or ampullae of the UB because contact is not required. In vivo and in vitro UB cells have been shown to secrete FGF2 and 9, TGF-α, tissue inhibitor of metalloproteinases (TIMP) 1, TIMP2, and MMP2.[90] Conditioned medium from an isolated UB cell line was found to trigger MET conversion of isolated MM grown in three-dimensional gel cultures.[91] The MM generated several differentiated epithelial cell types, cysts, tubules, and segmented nephrons. Isolation and purification of the UB conditioned medium demonstrated that it contained FGF2, which was required for MM survival, and leukemia inhibitory factor (LIF), a member of the interleukin (IL)-6 cytokine family, which initiated an epithelialization program through activation of STAT3.[92] In vitro, at least three IL-6 family members can substitute for LIF, and all can bind to their own receptor or heterodimerize with gp130 receptors and activate STAT3, demonstrating a remarkable degree of redundancy.[92]

The development of chemically defined serum-free medium to promote nephrogenesis in organ culture models provided the opportunity to explore the role of soluble growth factors in nephron growth and differentiation.[75,93-96] During experimental nephrogenesis, transferrin and prostaglandin E_1 have major roles in organotypic metanephric growth and differentiation.[94,96] Additional organ culture studies have suggested important roles for certain polypeptide growth factors in segmental nephron differentiation.[93,97-99] Thus epidermal growth factor (EGF) and TGF-α increase overall renal growth and specific differentiation of distal nephron elements (thick ascending limb to early distal tubule, collecting tubule) but retard epithelial differentiation of glomeruli and proximal tubules. These effects are mediated by the tyrosine kinase activity of the EGF receptor.[99] Insulin-like growth factors I and II stimulate overall renal growth and promote segmental differentiation of tubular, but not glomerular, epithelia. TGF-β globally retards growth and differentiation of all nephron segments.[97,98] These findings indicate that inductive signaling from the UB probably constitutes a complex set of secreted factors, and several UB-derived signals may be required in a temporal and spatial sequence for the proper execution of the induction program.[55] Postinductive nephrogenesis thus appears to be regulated by the overall balance of a number of local autocrine and/or paracrine growth factor systems. Alterations in the critical balance of positive or negative regulatory factors might produce a variety of hypoplastic or dysplastic nephropathies.[100]

THE STROMA IN KIDNEY DEVELOPMENT

As previously mentioned, the MM also gives rise to cell types other than those that contribute to the nephron. One such cell type is the stromal cell, which does not differentiate into nephrons or into the collecting-duct system. Gene-targeting studies in mice have demonstrated that, in addition to the UB and the MM, the stroma is an important source of signaling in renal organogenesis. Ablation of the forkhead box D1 *(Foxd1)* gene (formerly called the *Bf2* gene), expressed in the stromal-cell compartment, led to defects in the collecting-duct system and the nephrons[82] despite its lack of expression in these cell types. This suggests that *Foxd1* might contribute to kidney development by regulating the expression of a stromal cell–derived signal that promotes tubulogenesis and UB development.

Evidence also suggests that stromal cell–derived signaling through retinoic acid maintains *RET* expression in the UB, which then reciprocally regulates the differentiation of mesenchymal cells.[49,101] Stromal cells have been found to secrete FGF7, a growth factor the receptor of which is found on the UB. FGF7 is seen to be critical for maintaining UB epithelia growth and ultimately ensuring an appropriate number of nephrons in the kidney.[102] These data indicate that a signaling loop might exist

between the stroma, UB, and kidney mesenchyme that coordinates kidney development.

The recent progress in identifying crucial regulatory molecules involved in renal organogenesis has been remarkable. The use of genetically engineered animals and the development of new models have provided new insights into the complexities of renal development. Future progress will require the continued development of cell lines and transgenic models with inducible gene expression[103] so that the temporal expression, relative prioritization, and interaction of specific signaling pathways can be elucidated. These ongoing studies of the molecular and cellular biology of renal development form the basis for future investigations into the molecular mechanisms of gene activation and signal transduction in renal embryogenesis and will provide insight into the complex processes that underlie normal and abnormal renal organogenesis.

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Robert L. Chevalier and Victoria Fay Norwood

Functional Development of the Kidney in Utero

In early embryogenesis, a primitive kidney, the pronephros, appears at 3 weeks gestation, to disappear after another 2 weeks.[1] An intermediate kidney, the mesonephros, then appears from 5 to 12 weeks gestation and also undergoes involution through apoptosis (programmed cell death). Development of the permanent kidney or metanephros overlaps that of the mesonephros, appearing at 5 weeks gestation and first producing urine at 10 weeks (Figure 124-1).[1] Nephrogenesis progresses most rapidly in midtrimester and is complete by 34 weeks gestation as described in the preceding chapter. During this time, renal mass increases exponentially, reflecting the rapid development of renal tubules in the third trimester (see Figure 124-1).

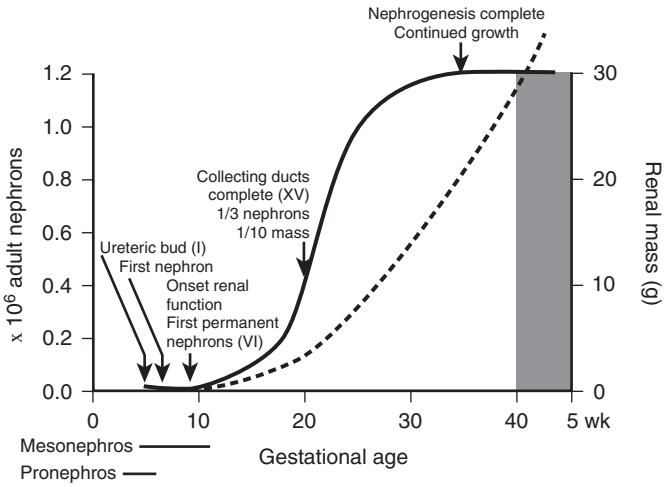


Figure 124-1 Human fetal renal development. The most rapid period of nephrogenesis occurs between 20 and 30 weeks gestation *(solid line).* This is also the period of exponential increase in renal mass *(dashed line). (Modified from Harrison MR, Golbus MS, Filly RA, et al: Management of the fetus with congenital hydronephrosis,* J Pediatr Surg *17:728, 1982.)*

DEVELOPMENT OF RENAL FUNCTION IN THE FETUS

Function of the developing fetal kidney is closely linked to the rapid changes resulting from ongoing morphogenesis. Although fetal homeostasis is primarily dependent on placental function, the contribution of fetal renal function in the third trimester has a significant impact on fetal health. Bilateral nephrectomy of fetal sheep does not cause fetal death after 2 weeks, but animals become hypoxic without increase in fetal plasma erythropoietin. In addition, fetal plasma renin becomes undetectable, chloride falls 20%, and phosphate doubles.[2] Management of the condition of the preterm infant, particularly the very-low-birth-weight infant, requires knowledge of the adaptive potential of the immature kidney and the process of transition from the intrauterine to extrauterine environment.

Urine Flow Rate

Fetal urine flow rates increase rapidly in the second half of gestation, averaging 40 mL/hour at 40 weeks (Figure 124-2).[3] These data were generated from serial ultrasound measurements of fetal bladder volume at 2- to 5-minute intervals. The increasing fetal urine production is paralleled by amniotic fluid volumes that also increase dramatically after 20 weeks (Figure 124-3).[4] Fetal oliguria or anuria resulting from renal or urinary tract anomalies impairs pulmonary development, which can result in neonatal morbidity and mortality from respiratory failure.[5] The determinants of urine flow are complex and involve the dynamics of renal blood flow, glomerular filtration rate, tubular function, and hormonal regulation. These are individually addressed in the following sections.

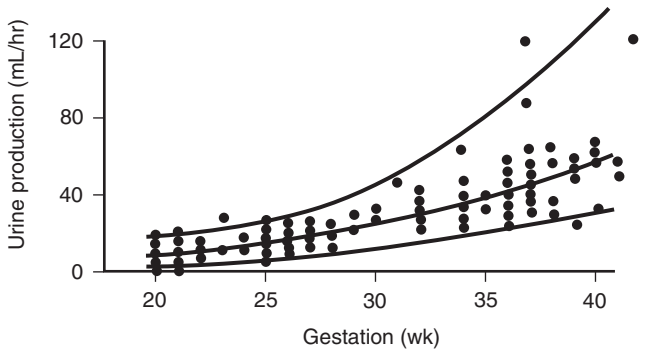


Figure 124-2 Hourly fetal urine production rate measured by maternal real-time ultrasonography, showing individual measurements *(circles),* mean, and 95% confidence intervals. *(Modified from Rabinowitz R, Peters MT, Vyas S, et al: Measurement of fetal urine production in normal pregnancy by real-time ultrasonography,* Am J Obstet Gynecol *161:1264, 1989.)*

Renal Blood Flow

Color-pulsed Doppler measurements of fetal renal blood flow in human fetuses reveal a nearly 10-fold increase from 20 to 40 weeks gestation (Figure 124-4).[6] In contrast to the fetal kidneys, which receive less than 5% of the cardiac output, neonatal kidneys receive 15% of cardiac output.[7] After birth, renal blood flow increases linearly in concert with increasing systemic blood pressure and decreasing renal vascular resistance.[8] Renal blood flow is tightly regulated by a process termed autoregulation, by which blood flow to the kidneys is maintained within a narrow range independent of blood pressure fluctuations above a critical minimum pressure. Autoregulation of renal blood flow has been demonstrated in fetal sheep in response to a modest increase in blood pressure,[9] as well as in immature rats subjected to decrease in blood pressure.[10] Of importance, the range of blood pressure within which renal blood pressure is maintained in the immature rat is the normal range for this age: the "autoregulatory range" increases as blood pressure increases into adulthood.[10]

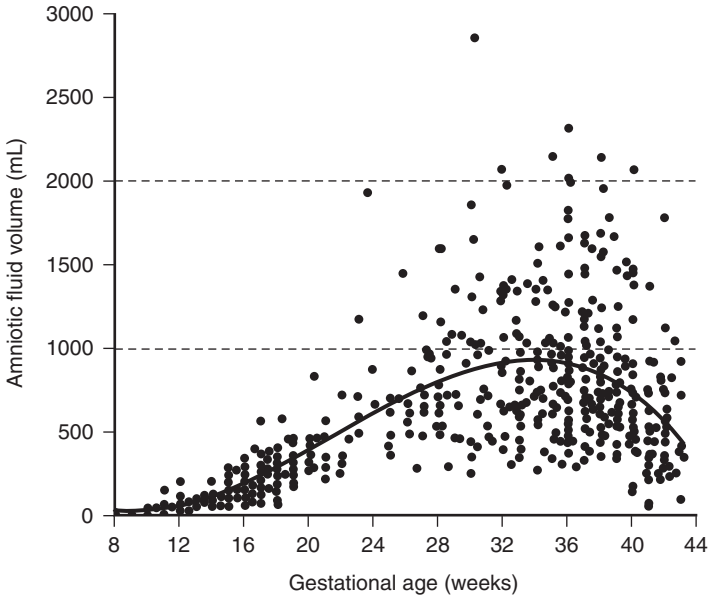


Figure 124-3 Amniotic fluid volume in human pregnancies in the second and third trimesters (*individual data points* [n = 705 pregnancies]). *Solid line* is polynomial regression. *(Modified from Brace RA, Wolf EJ: Normal amniotic fluid volume changes throughout pregnancy,* Am J Obstet Gynecol *161:382, 1989.)*

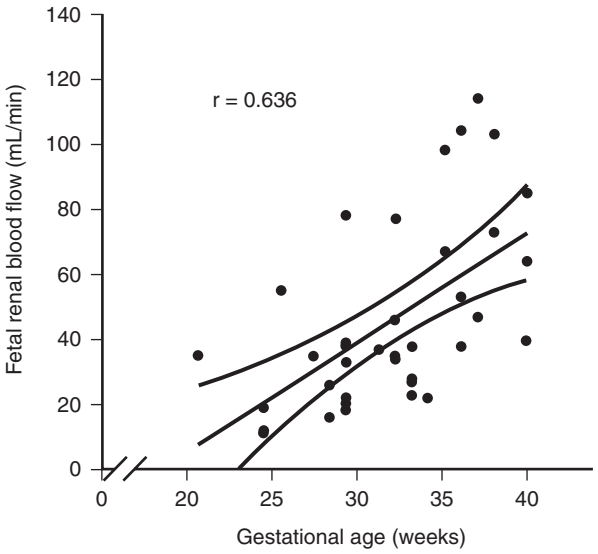


Figure 124-4 Renal blood flow in human fetuses in the second half of gestation measured by color-pulsed Doppler evaluation of the renal artery (*individual data points* [n = 22 fetuses each studied three times], with lines showing mean and 95% confidence intervals). *(Modified from Veille JC, Hanson RA, Tatum K, et al: Quantitative assessment of human fetal renal blood flow,* Am J Obstet Gynecol *169:1399, 1993.)*

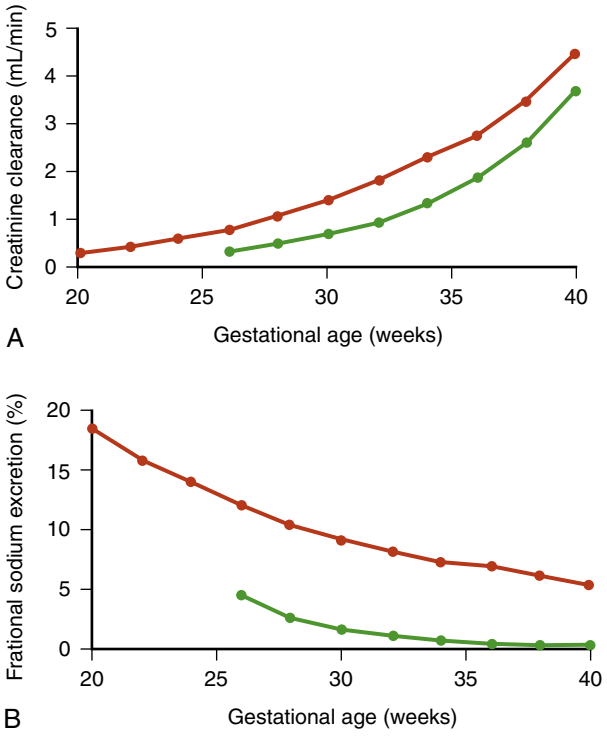


Figure 124-5 Renal function in the human fetus *(red circles)* and neonate *(green circles)* in the second half of gestation. **A,** Estimated creatinine clearance. **B,** Calculated fractional sodium excretion. Fetal values were calculated from several groups of data from a single human fetal research center,[3,12,13] and postnatal values were previously reported.[20] *(Modified from Haycock GB: Development of glomerular filtration and tubular sodium reabsorption in the human fetus and newborn,* Br J Urol *81[suppl 2]:33, 1998.)*

Glomerular Filtration Rate

Concurrently with renal blood flow, glomerular filtration rate increases progressively during the second half of gestation: values for neonates at corresponding gestational ages are similar (Figure 124-5, *A*).[11-13] Compared with that in older infants and children, fetal glomerular filtration rate is very low, ranging from <1 mL/minute at 20 weeks to 5 mL/minute at term (see Figure 124-5, *A*). After birth, glomerular filtration rate increases rapidly. Although glomerular filtration rate at birth is proportional to gestational age, the rate doubles within 2 weeks regardless of gestational age (Figure 124-6).[14] Catch-up in glomerular filtration rate for very-low-birth-weight infants may not be achieved for many months.[15] Determinants of glomerular filtration rate in the fetus and neonate include the number of functioning nephrons, arterial blood pressure, blood volume, glomerular filtration surface area, and glomerular permeability. Glomerular filtration surface area is the most important factor, which is determined by the number of glomeruli, as well as the state of glomerular

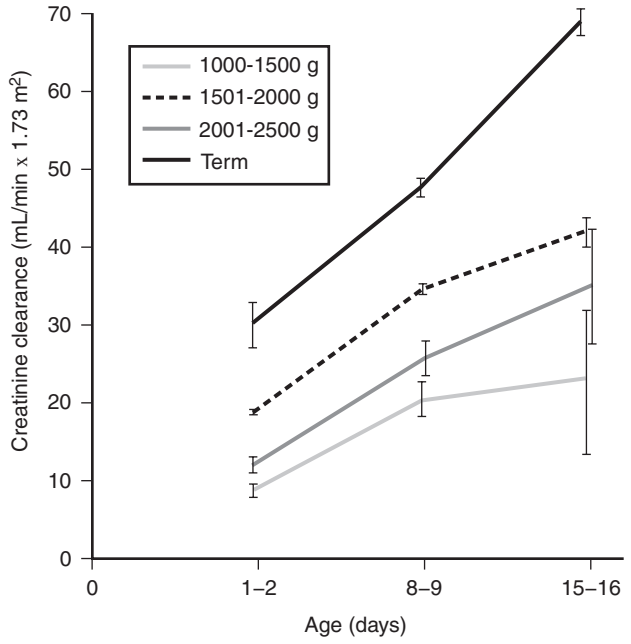


Figure 124-6 Creatinine clearance (corrected for 1.73 m^2 surface area) during the first 2 weeks of life for preterm and term neonates. *(Modified from Bueva A, Guignard JP: Renal function in preterm neonates,* Pediatr Res *36:572, 1994.)*

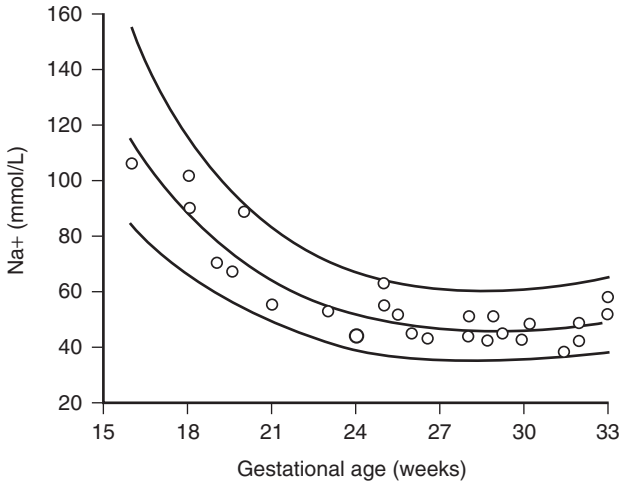


Figure 124-7 Fetal urinary sodium concentration based on data from 26 human fetuses 16 to 36 weeks gestation with normal urinary tracts (mean and 95% confidence intervals). *(Modified from Nicolini U, Fisk NM, Rodeck CH, et al: Fetal urine biochemistry: an index of renal maturation and dysfunction,* Br J Obstet Gynaecol *99:46, 1992.)*

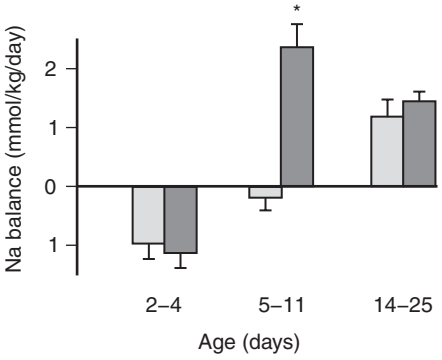


Figure 124-8 Net external sodium balance for preterm infants in the first 3 weeks of life (*gray bars,* control infants; *black bars,* infants receiving sodium supplementation 4-5 mEq/kg/day, $p < .0005$ vs. controls). In the first 2 to 4 days after birth, infants undergo natriuresis regardless of sodium intake, whereas, by 1 week, infants receiving supplementation achieve positive sodium balance sooner than controls. *(Modified from Al-Dahhan J, Haycock GB, Nichol B, et al: Sodium homeostasis in term and preterm neonates: III. Effect of salt supplementation,* Arch Dis Child *59:945, 1984.)*

maturation.[16] In addition to genetic factors, a number of factors in the intrauterine environment can modulate the number of nephrons, which can vary by almost 10-fold among normal individuals.[17] Of particular importance in preterm infants, a number of drugs and therapies (including angiotensin and prostaglandin inhibitors) administered to critically ill infants can reduce glomerular filtration rate (see later).[18]

Tubular Function

As with renal hemodynamics and glomerular filtration rate, renal tubular function is dependent on the stage of renal maturation, as well as the intrauterine or extrauterine environment.

Sodium

Fetal urinary fractional sodium excretion decreases throughout the second half of gestation (see Figure 124-5, *B*), and urinary sodium concentration progressively decreases from 16 to 32 weeks gestation (Figure 124-7).[19] Contributing factors to this state of relative natriuresis in the fetus include a greater proportional extracellular space and immaturity of tubular transporters. Most important, dramatic changes in sodium homeostasis take place at the time of birth, such that fractional excretion of sodium decreases markedly in the first 2 weeks of life.[14,20] Thus transition from the intrauterine to extrauterine environment significantly decreases urinary fractional sodium excretion for corresponding gestational ages in the third trimester (see Figure 124-5, *B*).[11] For a given intratubular pressure, proximal tubular reabsorptive rate is 10-fold greater in the immature compared with the adult kidney.[21,22] Activity of the sodium-hydrogen exchanger increases immediately after birth,[23] and the basolateral sodium-potassium adenosine triphosphatase (ATPase) activity increases steadily after birth.[24] The increase in sodium-potassium ATPase appears to be regulated by increasing sodium-hydrogen exchanger activity.[25] In the neonatal rabbit, intrarenal distribution of the sodium-potassium ATPase α-subunit parallels nephron maturation, with greatest concentration in the medullary region.[26] Sodium enters tubular epithelial cells through the amiloride-sensitive sodium channel: subunits mature in concert with maturation of the transporters to enhance sodium conservation. Infants with mutations of this channel are unable to conserve sodium, and pseudohypoaldosteronism type 1 and life-threatening volume depletion develop.[27]

These changes are reflected by an initial negative sodium balance in the first days after birth, reflecting renal excretion of expanded extracellular fluid and loss of up to 15% body weight, followed by a transition to positive sodium balance by the second week of life (Figure 124-8).[28] The transition can be accelerated by sodium supplementation, but this does not prevent the initial postnatal natriuresis (see Figure 124-8). Transition to positive sodium balance is soonest in infants of gestational age greater than 32 weeks and is delayed in very-low-birth-weight babies.[29] In these infants, fractional distal sodium reabsorption approaches that of term infants by 2 weeks of age.[30] Throughout the animal kingdom, positive sodium balance is essential for

normal somatic growth during early development, a response mediated by numerous overlapping regulatory mechanisms.[31]

Potassium

Urinary potassium excretion is regulated by tubular secretion rather than by reabsorption, and fetal tubular potassium transport mechanisms are mature in the third trimester.[32] Bilateral nephrectomy in the lamb fetus does not alter fetal plasma potassium concentration after 2 weeks, indicating that nonrenal mechanisms are responsible for regulating potassium homeostasis before birth.[2] However, transition from fetal to extrauterine life depends on intact renal function: neonatal plasma potassium concentration ranges from 4.5 to 6.5 mmol/L, and hyperkalemia is a common electrolyte abnormality in the preterm neonate.[33]

Calcium and Phosphorus

Compared with that in the older child and adult, neonatal urinary calcium concentration is increased, with even higher levels in the preterm infant.[34] This becomes important in the critically ill neonate receiving calciuric drugs such as furosemide or glucocorticoids, which can lead to nephrocalcinosis or nephrolithiasis.[35]

Phosphorus is actively transported across the placenta from mother to fetus,[36] and fetal renal phosphorus reabsorption is similar to that in the adult.[37] Tubular reabsorption of phosphorus normally exceeds 95% in term infants and 75% in preterm infants after the first week of life.[34] Lower values point to a proximal renal tubular abnormality or hyperparathyroidism.

Glucose and Amino Acids

Tubular reabsorption of glucose is very efficient in the fetus, exceeding that of the adult.[38] Both the maximal tubular excretory capacity and plasma threshold for glucose reabsorption are increased in the fetus, the latter increasing with gestational age.[38] In very-low-birth-weight infants, fractional excretion of glucose is increased,[39] but neonatal glucosuria is generally a consequence of excessive IV dextrose administration. Tubular amino acid reabsorption is lower in the neonate than in the older child, such that the normal neonate has relative generalized amino aciduria.[40]

Organic Acids and Bases

Renal excretion of organic acids and bases is of importance with respect to elimination of toxins and drugs from the circulation. Compared with that in the adult, fetal excretion of organic acids (such as penicillin) is very low, whereas excretion of organic bases (meperidine) is more similar.[41] Fortunately, efficient maternal renal excretion of organic acids generally protects the fetus from toxic exposure.

Acid-Base Balance

Fetal urine pH is consistently less than plasma pH, a consequence of efficient proximal tubular bicarbonate reabsorption and titratable acid excretion.[42] Increasing bicarbonate reabsorption with increasing gestational age may depend on maturation of carbonic anhydrase activity.[43] Although reduced in magnitude compared with that in the adult, the fetal renal response to metabolic acidosis is similar to that of the adult.[44] The tubular threshold for bicarbonate reabsorption is lower in the preterm neonate compared with the adult, ranging from 14.5 to 24.5 mmol/L.[45] Excretion of an acid load is also lower in the neonate, reaching adult levels within 6 weeks of birth.[46]

Renal Concentrating Capacity

Fetal urine concentration normally ranges from 100 to 250 mOsm/kg H_2O but can be regulated beyond this range in response to changes in maternal hydration or fetal infusion of arginine vasopressin.[47,48] A number of factors contribute to the reduced renal concentrating capacity of the fetus compared with the adult. The loops of Henle are markedly shorter in the fetus compared with the adult,[49] and redistribution of renal blood flow to the inner cortex increases relative blood flow to the vasa recta, washing out the medullary concentration gradient. Because urea contributes significantly to generation of the renal concentrating gradient, the relatively low protein intake of the fetus also limits maximal urinary concentration. The fetal collecting duct is relatively insensitive to the action of arginine vasopressin, which is produced by the fetus.[9,50,51] This may result from decreased coupling between receptor binding and cyclic adenosine monophosphate generation or a decreased number of receptors.[52,53] Increased water movement across the collecting duct in response to arginine vasopressin is dependent on the expression and activation of water channels (aquaporins). Aquaporin-1 is expressed in the kidney of the first-trimester fetal sheep, increasing throughout gestation.[54] In very-low-birth-weight neonates, maximal urinary concentration is below 500 mOsm/kg H_2O.[55] In term infants, maximal concentrating capacity can exceed 1000 mOsm/kg by 2 months of age.[56] The clinical importance of maximal urinary concentration relates to calculation of fluid requirements for the preterm neonate, who has greater obligatory urinary losses compared with the term infant.

Regulation of Renal Function in the Fetus

Renal function is controlled by a variety of endocrine, paracrine, and autocrine factors with coordinated actions on renal hemodynamics, glomerular filtration, and tubular function. Maintenance of homeostasis in the fetus and neonate therefore depends on the relative maturation of these factors, as well as on renal morphogenesis itself.

Renin-Angiotensin Aldosterone System

The renin-angiotensin system is a highly conserved vital regulatory system necessary for normal growth, renal organogenesis, and maintenance of homeostasis. All components of the renin-angiotensin system are present in fetal kidney and are even expressed in the mesonephros.[57,58] Plasma renin concentration in the fetus exceeds that of the mother.[59] Fetal angiotensin II regulates blood pressure and renal blood flow, but vasoconstrictor responses are attenuated compared with those in the adult.[60] However, fetal renin and angiotensin are released in response to hemorrhage, preserving perfusion to vital organs.[61,62] Because angiotensin II preferentially constricts the efferent compared with the afferent glomerular arteriole, administration of an angiotensin receptor blocker or angiotensin-converting enzyme inhibitor to the fetal sheep decreases glomerular filtration rate and urine flow while increasing renal blood flow.[63]

As in the adult, increased activity of the renin-angiotensin system enhances fetal tubular sodium reabsorption.[64] Avid renal sodium reabsorption in the neonate is mediated by endogenous angiotensin II acting through the angiotensin II type 1 receptors,[65] an effect that can be suppressed by chronic sodium loading.[66] Although aldosterone moves from the maternal to fetal circulation across the placenta, the majority of fetal aldosterone is fetal in origin, is regulated by fetal angiotensin production, and contributes to tubular sodium reabsorption.[67-69] However, the kaliuretic effect of aldosterone is decreased in the fetus and neonate compared with the adult,[70] which may contribute to relative hyperkalemia in the preterm infant.

In addition to the physiologic role of the renin-angiotensin system in fetal hemodynamics and renal function, this system plays a major role in renal morphogenesis, with angiotensin behaving as a growth factor.[71] In contrast to the systemic renin-angiotensin system, a number of tissue-specific systems have been identified, including the brain, vasculature, gonads, and kidneys. The study of mutant mice lacking functional components of the renin-angiotensin system has revealed a variety of abnormalities in renal morphogenesis, including abnormal glomeruli, thickened truncated renal vessels, and primitive renal tubules.[72] Experimental

placental insufficiency in the sheep results in suppression of renal renin and angiotensinogen gene expression, which may impair renal development.[73] Of clinical importance, exposure of the fetus to maternal administration of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers during the second and third trimesters results in fetal hypotension, growth retardation, hypocalvaria, and neonatal renal insufficiency with increased mortality.[74-76] Even fetal exposure to angiotensin-converting enzyme inhibitors in the first trimester increases cardiovascular and central nervous system malformations.[77]

Prostaglandins

Fetal prostaglandins counterbalance endogenous vasoconstrictors by promoting systemic and renal vasodilation and diuresis. Thus inhibition of fetal prostaglandins by maternal administration of prostaglandin synthase inhibitors to stop preterm labor is associated with oligohydramnios and neonatal oliguria,[78] and renal insufficiency develops in preterm neonates receiving indomethacin to close patent ductus arteriosus.[79]

Maternal exposure to prostaglandin synthesis inhibitors can also result in abnormal fetal renal morphogenesis, including cystic dilatation of developing nephrons.[80] Mice lacking functional cyclooxygenase 2 have abnormal nephron maturation, developing hypoplastic glomeruli and glomerulosclerosis.[81] As for angiotensin, prostaglandins therefore not only are necessary for maintenance of hemodynamics but also play a critical role in renal development.

Endothelin

Endothelin is a 21–amino acid peptide that is a potent vasoconstrictor acting on receptors on vascular smooth muscle cells and is active in the fetal circulation.[82] Fetal blood concentration of endothelin is elevated compared with that in adults, and it is present in amniotic fluid.[83,84] In direct contrast to its effects on the adult kidney, endothelin promotes diuresis in the ovine fetus, which suggests a relatively lower vasoconstrictor effect on the fetal afferent glomerular arteriole.[85]

Kallikrein-Kinin System

Kinins are peptides produced by the kidney in late gestation and in the adult are expressed by the connecting tubule.[86] Bradykinin has been localized to the distal tubules of the embryonic kidney and presumably plays a role in renal morphogenesis.[87,88] The physiologic role of kinins in the fetus has not been clarified, and their synthesis increases rapidly after birth.[89,90] In contrast to the case in the adult, in which bradykinin is associated with vasodilation and diuresis, the reverse is the case in the fetal and neonatal sheep.[89] The underlying mechanisms for these differences remain unclear.

Nitric Oxide

Nitric oxide is produced in the kidney by nitric oxide synthase (NOS) and has multiple actions as a regulator of cell survival, inflammation, vasodilation, and renal function. Inhibition of nitric oxide synthesis in the fetal sheep increases renal vasoconstriction and reduces glomerular filtration and urinary sodium excretion.[91] Just as angiotensin plays an exaggerated role in maintenance of renal vascular tone in the developing kidney, nitric oxide has a greater role in modulating these effects in the developing kidney than in the adult.[92] Three isoforms of NOS exist: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). In the adult kidney, nNOS is expressed by cells of the macula densa and inner medullary collecting duct, iNOS is found in proximal tubule and collecting duct, and eNOS is distributed throughout renal endothelial cells.[93,94] In the fetal rat, nNOS appears first in the inner cortex, intensifying in the macula densa during the immediate postnatal period.[95] In contrast, eNOS is produced by endothelial cells of capillaries surrounding fetal medullary collecting ducts and is also expressed by proximal tubular cells at birth.[96] Mice lacking functional eNOS develop vascular dysfunction and progressive tubular injury in adulthood.[97,98] Thus, as for angiotensin and prostaglandins, impaired fetal renal production of NOS can lead to alterations in renal structure and function with long-term consequences.

Atrial Natriuretic Peptide

Atrial natriuretic peptide (ANP) is produced by cardiac myocytes and binds biologically active receptors increasing intracellular cyclic 3′,5′-guanosine monophosphate.[99] Generated cyclic 3′,5′-guanosine monophosphate inhibits sodium reabsorption by blocking sodium channels in tubular epithelial cells. In addition to binding to its biologically active receptors coupled to guanylate cyclase, ANP binds to an even greater population of so-called clearance receptors that regulate plasma levels by removing ANP from the circulation.[100] ANP has potent vasodilatory effects, increases glomerular filtration rate, and promotes natriuresis. Fetal plasma ANP levels exceed maternal concentrations and are further increased by acute volume expansion.[101] In both the fetus and adult, ANP decreases blood pressure and blood volume, with an increase in urine flow and natriuresis.[102-104] Of interest, the midtrimester fetus responds to ANP infusion with a greater increase in glomerular filtration rate and natriuresis than does the late-gestation fetus,[105] an effect that may be mediated by greater numbers of clearance receptors in early gestation.[106]

After birth, plasma ANP concentration decreases with postnatal diuresis in preterm infants, which is paralleled by decreasing right atrial size.[107] The production of cyclic 3′,5′-guanosine monophosphate per ANP glomerular binding site decreases in the postnatal period, and extrusion of cyclic 3′,5′-guanosine monophosphate from glomeruli exposed to ANP is reduced in the neonatal period.[108,109] These adaptations contribute to the transition from fetal natriuresis to postnatal sodium conservation necessary for rapid growth.[31]

Renal Sympathetic Nerves

Renal nerves develop in the fetal kidney,[110] and fetal renal vasoconstriction is more sensitive to α_1-adrenoceptor low-level renal nerve stimulation than in neonatal or adult sheep.[111,112] Counterbalancing these effects, norepinephrine activates β_2-adrenoceptors to induce fetal renal vasodilation.[113] Renal sympathetic nerves play an important role in stimulation of renal renin production during the transition to extrauterine life and in attenuating diuresis and natriuresis during this transition: another mechanism to promote sodium conservation necessary for postnatal growth.[114]

Cortisol

Glucocorticoid receptors are expressed in the rat embryonic liver, lung, heart, and kidney.[115] Administration of cortisol to third-trimester fetal sheep increases glomerular filtration rate and urinary phosphate excretion but does not affect fractional sodium excretion.[116] Although exogenous cortisol reduces proximal sodium reabsorption, distal reabsorption is increased by accelerated maturation of Na-H exchanger and Na^+, K^+-ATPase.[117,118] Maternal administration of glucocorticoids also has an impact on fetal nephrogenesis, however, and dexamethasone results in a decrease in the number of nephrons when the fetus is exposed during a critical period (15-18 days gestation in the rat).[119] Dexamethasone-induced reduction in nephron number appears to be due to inhibition of ureteric branching morphogenesis in the fetus.[120] Administration of dexamethasone to pregnant ewes also reduces the number of nephrons in the fetus and leads to adult hypertension.[121] Of greater physiologic relevance, administration of corticosterone (the natural glucocorticoid) to pregnant rats also reduces fetal nephron number and induces postnatal hypertension.[122]

LONG-TERM CONSEQUENCES OF FETAL RENAL FUNCTION

Fetal renal function is closely linked to both the developmental stage of the kidney and the intrauterine or extrauterine environment of the fetus. Adaptation of the kidney after birth of the very-low-birth-weight infant is remarkable, but clinical management of the condition of these patients requires knowledge of normal fetal renal function. Whereas function of the immature kidney had previously been regarded as "limited," advances in developmental renal physiology have revealed that renal function is finely adapted to the needs of the infant.[31,123] As discussed in this chapter, maternal exposure to a variety of drugs not only may alter function of the fetal kidney but also may cause permanent impairment of renal development and function. Recent studies now suggest that although very-low-birth-weight infants have favorable short-term outcomes, nephrogenesis may not progress in the extrauterine environment.[124,125] Reduced nephron number, in turn, may predispose these patients to renal and cardiovascular disease in adulthood.[126] These considerations underscore the lifetime importance of optimal prenatal and neonatal care.

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Michael J. Solhaug and Pedro A. Jose

Postnatal Maturation of Renal Blood Flow

Newborn mammals, including humans, exhibit lower renal blood flow compared with their adult counterparts.[1,2] The low neonatal renal blood flow is maintained by a high renal vascular resistance and establishes the newborn's unique renal functional state characterized by a low glomerular filtration rate. The unique renal hemodynamic state at birth affects the clinical management of the newborn. The low renal blood flow and resultant low glomerular filtration rate contribute to the newborn's altered pharmacokinetics of renally excreted medications,[3] as well as avid tubular sodium reabsorption in the distal nephron. This blunts the ability to excrete an acute saline load in the term infant.[4] The newborn's renal hemodynamic state modifies the development and severity of pathophysiologic conditions, such as acute renal failure resulting from adverse perinatal events and the complications of respiratory distress syndrome.[5] Having an understanding of the regulation of renal blood flow in the newborn may provide insights into creating therapies directed at the prevention and treatment of pathologic conditions in the neonate.

The postnatal maturation of renal hemodynamics involves a progressive increase in renal blood flow to reach adult capability.[1,6-8] The major factor influencing the maturational increase in renal blood flow is the synchronous drop in renal vascular resistance, which occurs most notably in the immediate postnatal period.[7,9] Renal blood flow in the postnatal developing kidney is influenced by structural factors, such as the number of existing vascular channels, as well as functional factors, offered by the glomerular resistance vessels.[10] Several studies confirmed that in the developing kidney the functional maintenance of vascular tone (principally through a balance of vasoactive factors) is the paramount mechanism affecting renal hemodynamics.[11-13] The maturational changes in renal blood flow and renal vascular resistance must proceed normally to achieve adult capability for fully integrated renal-cardiovascular homeostasis. Disruption of the maturation of renal hemodynamics may lead to inadequate renal-cardiovascular function in the adult and may produce pathologic conditions such as hypertension.

CHARACTERISTICS OF RENAL BLOOD FLOW IN THE IMMATURE KIDNEY

Total Renal Blood Flow

In most mammalian species, renal blood flow in the neonate is lower than in the adult compared on the basis of body weight, kidney weight, or surface area.[1] In human newborns and infants, total renal blood flow has been determined by the clearance of *p*-aminohippurate (PAH), which measures effective renal plasma flow (ERPF)[6] and by Doppler ultrasonography.[14,15] Renal blood flow measured by Doppler and ERPF by clearance of PAH is lowest in newborns, and it correlates with gestational age. The increase in renal blood flow after birth in preterm infants is influenced by postconceptional rather than postnatal age. ERPF increases from 20 mL/min/1.73 m^2 at 30 weeks gestation to 45 mL/min/1.73 m^2 by 35 weeks gestation and 83 mL/min/1.73 m^2 at term gestation.[16] During the first 3 months of postnatal life, ERPF increases rapidly to 300 mL/min/1.73 m^2. Thereafter ERPF increases gradually, reaching values of 650 mL/min/1.73 m^2 by 12 to 24 months of age[6,17] (Figure 125-1). However, the clearance of PAH underestimates ERPF in the neonatal period because the renal extraction of PAH is only 60% during the first 3 months of age compared with 95% by 5 months of age.[18] The low renal extraction of PAH in the neonate[9,19,20] has been attributed to shunting of blood to non-PAH-extracting tissues (e.g., relatively greater medullary blood flow, intracortical efferent arteriovenous shunting).

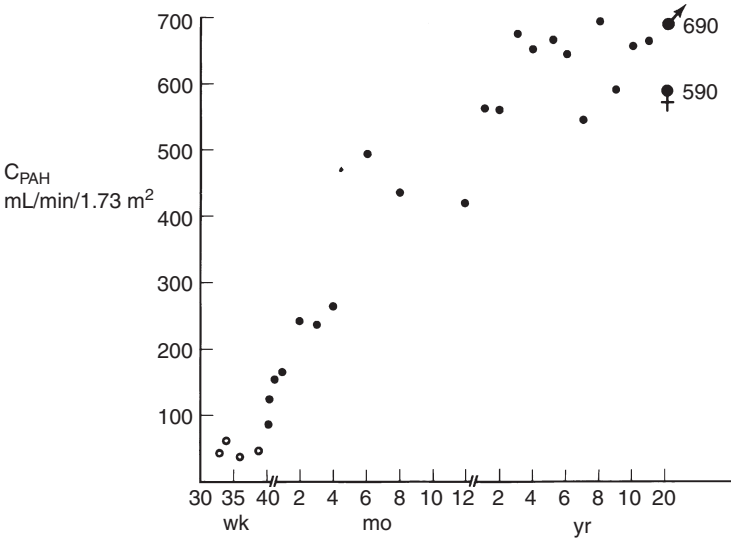


Figure 125-1 The clearance of *p*-aminohippurate (C_{PAH}) with age. *(From Rubin MI, Bruck E, Rapoport M, et al: Maturation of renal function in childhood: Clearance studies,* J Clin Invest *28:1144, 1949, by copyright permission of the American Society for Clinical Investigation.)*

Intrarenal Blood Flow

The renal vasculature is characterized by two capillary networks—the glomerular and peritubular capillary systems—linked in series with each other. The major sites of renal vascular resistance are the glomerular arterioles. Blood enters the glomerulus via the afferent arteriole that arises from the interlobular artery, and it exits via the efferent arteriole. Vasoconstriction or vasodilation at these sites regulates blood flow to the glomerulus (hence, glomerular filtration rate) as well as the intrarenal distribution between the cortex, which contains all the glomeruli, and the medulla, which contains vasa recta and tubules but not glomeruli. In the mature kidney, the afferent arteriole of inner cortical nephrons accounts for the entire preglomerular resistance to blood flow, whereas in superficial cortical nephrons, the interlobular arteries offer the largest resistance to blood flow.[21,22] Blood flow to each region of the kidney (cortical, medullary, and papillary) increases with maturation.[9,20,23-27] The distribution of intrarenal blood flow in the young, however, is different from that reported in adults. The neonatal kidney has a greater percentage of blood flow to the inner cortical and medullary areas than does the adult kidney. The low extraction ratio of PAH in infants younger than 3 months of age may be related to a relatively greater perfusion of juxtamedullary nephrons. As total renal blood flow reaches adult levels with maturation, a greater fraction of renal blood flow is received by the outer cortical nephrons. The duration of this maturational period varies from species to species.

Autoregulation of Renal Blood Flow in the Young

The mature kidney exhibits autoregulation; that is, renal blood flow remains constant even though renal perfusion pressure (determined by mean arterial pressure) varies throughout a range from low to high. Autoregulation depends on intrarenal mechanisms and is modulated by intrarenal factors. In the newborn, the range of autoregulation is set at lower perfusion pressures than seen in the adult, and the renal pressure-flow relationship changes with renal growth.[28-31] Autoregulation of renal blood flow is less efficient in the young than in the adult. Furthermore, uninephrectomy impairs the autoregulatory response in young rats but does not affect this response in adult rats. This reduced autoregulatory efficiency in the neonate is apparently the result of prostaglandin-dependent renin release, which causes vasoconstriction at lower levels of perfusion pressure.[29]

Maturational relationships between tubular flow and glomerular filtration rate (tubuloglomerular feedback) also occur with growth. The tubuloglomerular feedback mechanism is maximally sensitive at a tubular flow range that corresponds to the normal operating range. As the glomerular filtration rate increases with maturation, the maximal response and flow range also increase so the relative sensitivity of the tubuloglomerular feedback mechanism is unaltered during growth.[32] The relative roles of endothelial cell, smooth muscle, and tubuloglomerular feedback have not been defined in the developing animal.

REGULATION OF POSTNATAL RENAL HEMODYNAMICS

The low renal blood flow of the neonate and the increase that occurs with maturation are the result of a combination of effects, including alterations in cardiac output, perfusion pressure, and renal vascular resistance. Lower cardiac output and perfusion pressure may partially account for the decreased renal blood flow noted in the newborn infant. In the dog, however, cardiac output corrected for body weight is highest in the youngest puppies, which also have the lowest renal blood flow per body weight.[20,33] The proportion of cardiac output distributed to the kidneys is 4% to 6% in the first 12 hours of life and increases to 8% to 10% in the first week of life. In comparison, 25% of cardiac output is distributed to the kidneys in the normal adult. Systemic vascular resistance decreases markedly after birth. This may cause a redistribution of blood flow to organs other than the kidney and may immediately contribute to the low neonatal renal blood flow. Systemic vascular resistance gradually increases with maturation and therefore is not a factor in the increase in renal blood flow with age. Renal vascular resistance, however, is the most important regulating component contributing to postnatal renal hemodynamics. Gruskin and colleagues[7] demonstrated that, in the developing piglet, the major factor influencing the maturational increase in renal blood flow was an 86% decrease in renal vascular resistance. Renal vascular resistance in the developing kidney is influenced by *structural* factors, the number and size of vascular channels, as well as by *functional* vasoactive factors, the modulators of the resistance offered by the glomerular arterioles[10,34,35] (Figure 125-2).

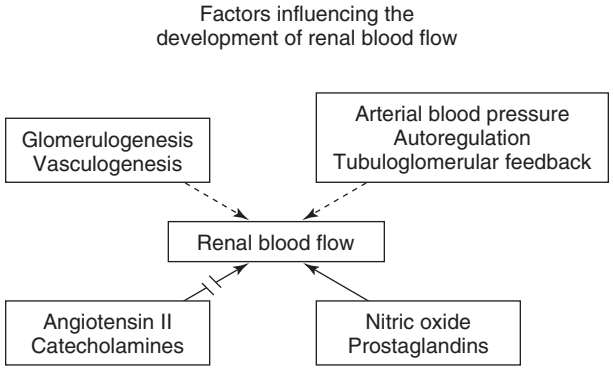


Figure 125-2 Factors that influence the development of renal blood flow include anatomic factors (glomerulogenesis and vasculogenesis), physical factors (arterial blood pressure, myogenic autoregulatory response), and vasoactive factors (autoregulation, tubuloglomerular feedback, angiotensin II, catecholamines, renal nerves, nitric oxide, and prostaglandins). Other vasoactive agents can regulate renal blood flow; however, renal vascular resistance in the newborn regulated by vasoactive agents is probably the result of a balance between the vasoconstrictor influences of angiotensin II and catecholamines or renal nerves and the vasodilatory influences of nitric oxide and prostaglandins.

Role of Anatomic Development

The contribution of structural changes to the maturational changes of renal vascular resistance in the developing kidney is an important consideration. Renal vascular resistance in the developing kidney is a function of the number of existing vascular channels, as well as the arteriolar resistance offered by each channel.[10,34,35] The main structural development that could influence renal hemodynamics postnatally is the addition of vascular channels by nephrogenesis, resulting in a decrease in renal vascular resistance. The increase in renal blood flow after birth is caused by development and formation of new glomeruli and vascular remodeling. This increase may play a role in the postnatal renal hemodynamic development of several of the mammalian species in which nephrogenesis continues after birth, such as canines, swine, and rodents. Renal blood flow, however, continues to increase in these species long after glomerulogenesis has completed.[6,19,20,36] In addition, the increase in the diameter of resistance vessels during maturation is greater in the kidneys than in other organs.[37] In studies in canine puppies, Evan and colleagues[34] found substantial differences, both quantitative and qualitative, between neonatal and adult renal vasculature.[10] However, morphologic changes in renal resistance vessels cannot account for the rapid decreases in renal vascular resistance that occur in the period of renal hemodynamic maturation.

Specifically, in humans, nephrogenesis is completed at 34 weeks gestation, yet renal blood flow continues to rise and renal vascular resistance continues to decrease with postnatal age, a finding indicating that the functional vasoactive characteristics of the immature renal vessels determine this alteration. Several studies confirmed the major contribution of the functional vasoactive attributes of the developing renal vasculature in altering renal blood flow.[9,12,13,38,39] Thus, although structural development may contribute to the changes in developing kidney by decreasing renal vascular resistance during nephrogenesis, the functional vasoactive changes of the resistance vessels are the main factors producing the maturational changes in renal hemodynamics.

Role of Vasoactive Factors

High renal vascular resistance accounts for the low renal blood flow at birth, and the increase in renal blood flow with maturation mainly results from the progressive reduction in renal vascular resistance.[7,9,11,13,19,20] Vasoactive factors modulate renal blood flow through the alteration of renal vascular resistance by (1) the extent of participation of resistance vessels (interlobular artery and afferent and efferent arterioles), (2) the intrinsic properties of immature resistance renal vasculature, and (3) neurohormonal factors unique to the immature kidney. The site of high renal vascular resistance in the newborn animal has been localized mainly to the preglomerular resistance vasculature, the interlobular artery, and the afferent arteriole.[40] In older animals, increases in renal blood flow are associated with a decrease in resistance in all the glomerular vessels: the interlobular, afferent, and efferent arterioles.[8] The decrease in renal vascular resistance with age may be modulated by developmental changes in the intrinsic properties of the renal resistance vasculature. However, little is known about the myogenic internal vasoactive capabilities of these vessels or about any developmental differences in resistance vessel responsiveness to vasoactive factors. Ultimately, the characteristics of postnatal renal hemodynamics are considered to be a balance of neurohormonal vasoactive factors. Both the vasoconstrictors and the vasodilators producing this immature renal condition have differing effects, intrarenal levels, and sites of action compared with the mature adult. Several vasoactive agents participate in the regulation of renal blood flow in the postnatal maturing kidney, including adenosine, arginine vasopressin (AVP), angiotensin II, atrial and other natriuretic peptides, bradykinin, endothelin, nitric oxide (NO), prostaglandins, renal nerves, and the adrenergic nervous system.

Adenosine

The intrarenal vasodilator action of adenosine has been implicated as an important mediator of tubuloglomerular feedback in the adult, and it may play a role in protection of the outer medulla from hypoxia. Tubuloglomerular feedback is important in the renal autoregulation of glomerular filtration rate and blood flow. Adenosine, formed by the breakdown of adenosine triphosphate, can be a renal vasodilator. Several adenosine receptor subtypes have been cloned: A_1, A_{2A}, A_{2B}, and A_3.[41] In the adult, adenosine decreases the glomerular filtration rate and increases renal blood flow apparently by reducing efferent vascular resistance.[42-44] In one study, intrarenal adenosine reduced cortical blood flow via adenosine A_1 receptors and increased medullary blood flow via adenosine A_2 receptors.[45] The stimulation of renal adenosine A_1 receptors is associated with inhibition of renin release and constriction of afferent arterioles, whereas adenosine A_2 receptors promote renin release and dilate afferent and efferent arterioles. Xanthines block all the adenosine receptor subtypes. In the adult, methylxanthines produce no consistent change in renal blood flow. Theophylline, however, via its adenosine receptor blocking property (independent of phosphodiesterase inhibition), increases renal vascular resistance in newborn rabbits.[46] The role of adenosine receptors in the control of renal blood flow during development remains to be determined. It is possible that adenosine A_2 receptors may act to modulate the high neonatal renal vascular resistance.[47]

Arginine Vasopressin

AVP functions as an intrarenal vasoconstrictor. There are three major AVP receptor subtypes: V_{1a}, V_{1b}, and V_2.[41] The V_2 receptors found in collecting ducts of the kidney increase hydraulic conductivity. V_{1a} receptors in arterioles mediate vasoconstriction, whereas V_{1b} receptors in pituitary corticotrophs and the limbic system may mediate corticotroph responsiveness and control of emotional processes. In conscious dogs, AVP has been shown to vasodilate the kidney via V_2 receptors.[48] AVP has the potential to contribute to the increased renal vascular resistance present in the neonate through its action on vascular and glomerular V_1 receptors. The role it plays in regulating basal renal hemodynamics remains to be defined. Infusion of synthetic AVP does not alter renal blood flow and renal vascular resistance in fetal sheep. AVP may still play a role, however, in certain stress-induced reductions in renal hemodynamics. For example, the marked decrease in renal blood flow and renal vascular resistance has been closely correlated with the rise in plasma AVP during hemorrhage but not with hypoxemia.[49]

Angiotensin II

The highly activated renin-angiotensin system, through the vasoconstriction properties of angiotensin II, contributes to the high renal vascular resistance in the immature kidney.[26,50-55] Circulating renin and angiotensin II levels are high in most neonatal mammals, including humans, and they decrease with age.[51,53,54] Angiotensin II is the predominant vasoconstrictor in the developing renal vasculature. There are four angiotensin II receptors: AT1, AT2, AT3, and AT4.[41] In rodents, there are two AT1 receptors: AT1a and AT1b. AT1a and AT2 receptors are differentially expressed during development. AT2 but not AT1 receptors are expressed in the very immature fetal structures, a finding implicating a role in nephrogenesis.[54,56-58] Angiotensin II, AII, perhaps via AT1 but more probably via the AT2 receptor, may be involved in fetal renal development including angiogenesis. The AT2 receptor gene is expressed to the greatest extent in fetal kidneys; its expression decreases rapidly after birth in the rat. In contrast, AT1 receptor expression increases progressively after birth.[59-61] AT1 receptors, which mediate vasoconstriction, are expressed at later stages in the glomerulus, resistance arterioles, and medulla,[56,62] including the period of hemodynamic development. Studies show that AII via the AT receptors has a significant role in renal formation and postnatal function. Abnormalities in renal development are noted in human infants and neonatal rats treated with angiotensin-converting enzyme inhibitors. In neonatal rats, AT1, but not AT2, receptor antagonists produce renal defects similar to those noted with angiotensin-converting enzyme inhibitors, a finding further attesting to the importance of this receptor subtype in kidney development.[58,59,63,64] However, disruption of both AT1 and AT2 receptors can lead to malformations of the kidney and urinary tract.[58] Specific blockade of the AT1 receptor in neonatal rats results in significant functional changes later in life that may show sex differences. AT1 inhibition for 2 weeks after birth in rats produces increased blood pressure and decreased nephron number in both males and females. However, only males exhibit decreased papillary volume, significant proteinuria and reduced GFR at 3 months of age.[64] Although nonspecific inhibition of AII has not revealed developmental differences in renal hemodynamics, the regulatory role of the renin-angiotensin system has been clarified with the use of nonpeptide antagonists of AII receptors. Systemic infusion of an angiotensin-converting enzyme inhibitor in conscious newborn sheep—although decreasing renal vascular

resistance—did not change renal blood flow.[65] Administration of the nonselective angiotensin receptor antagonist saralasin by Osborn and colleagues[66] in piglets and Jose and colleagues[30] in puppies, did not significantly alter renal hemodynamics. However, the intrarenal infusion of a nonpeptide-specific AT1 receptor antagonist produces greater increases in renal blood flow in newborn piglets compared to the adult, suggesting that AII, via the AT1 receptor, is a more important renal vasoconstrictor in the immature kidney than the adult.[55] Chappallez and Smith[67] determined the individual roles of the AT1 and AT2 receptors in renal hemodynamics of conscious lambs. AT1 receptor inhibition significantly decreased RVR and increased RBF, while altering mean arterial pressure only at high doses. Further, the pressor responses to aII infusion were abolished by AT1 inhibition. In contrast, AT2 blockade did not change any of these parameters.[67] AT2 receptors, in fact, may be vasodilatory.[68] This may explain the inability of angiotensin-converting enzyme inhibition to affect renal blood flow in newborn piglets.[69] Thus angiotensin II, via the AT1 receptor, plays an important role in the regulation of renal blood flow in the newborn and during the postnatal period. In contrast to the apparently increased vasoconstrictor effect of angiotensin I via AT1 receptors affecting RBF in the immediate neonatal period, angiotensin II may actually be needed to maintain glomerular filtration rate in the neonatal rabbit. Interruption of the renin-angiotensin system in neonatal rabbits decreases glomerular filtration rate without affecting renal blood flow.[70]

Atrial and Other Natriuretic Peptides

There are several natriuretic peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP).[71] ANP and BNP (originally described in brain) are produced in the atria and ventricles, whereas CNP is found mainly in the brain, pituitary gland, vascular endothelium, kidney, and female genitourinary tract. ANP is present in both atria and ventricles of the fetus in several species, and circulating levels of this peptide in plasma are significantly elevated compared with those of the adult.[72-74] Within the first few weeks of life, however, the plasma concentrations of ANP fall to adult levels. During the early developmental period, the release of ANP appears to respond to various stimuli that are associated with volume overload. The most important vascular and renal effects of ANP in mature animals are as follows: (1) vasodilation and decrease in mean blood pressure; (2) increase in renal blood flow, glomerular filtration rate, and filtration fraction; (3) inhibition of sodium and water reabsorption in both proximal and distal tubules; and (4) decreased concentrating ability. Although ANP may play a role in sodium and volume homeostasis during the perinatal period, its role in the regulation of renal hemodynamics is uncertain. In rabbits, clear age differences in the renal response to intravenous infusions of α-human ANP have been noted. In response to ANP, newborn rabbits demonstrate a decrease in diuresis and kaliuresis and no change in natriuresis, whereas an immediate increase in diuresis, natriuresis, and kaliuresis occurs in adult rabbits.[75] The infusion of recombinant ANP into fetal sheep results in minimal declines in mean arterial blood pressure. In contrast, significant decreases in blood pressure and increments in heart rate have been observed in newborn and adult sheep during continuous infusion of pharmacologic doses of ANP. ANP reduces renal blood flow in fetal and newborn sheep. This effect, however, decreases with maturation,[76] and its significance is not clear. Several natriuretic peptide receptors (NPR) have been cloned: NPR-A, NPR-B, NPR-C, and NPR-D.[77] ANP and BNP bind to NPR-A, whereas CNP binds to NPR-B receptors. Both receptors generate cyclic guanosine monophosphate; NPR-C, which has an equal affinity to all the natriuretic peptides, is not linked to cyclic guanosine monophosphate. NPR-C acts as a clearance receptor to regulate circulating levels of natriuretic peptides but may also generate NO. NPR-D has been identified only in eels. Systemic clearance of ANP is increased in the newborn and decreases with development.[78] The decreased effects of ANP in the newborn period cannot be explained by increased clearance of ANP or by decreased production of cyclic guanosine monophosphate.[79]

Bradykinin

Bradykinin is the major functional vasodilator produced by the kallikrein-kinin system. Bradykinin exerts its effects via B_1 and B_2 receptors.[41] Under physiologic conditions, most of the effects of bradykinin are mediated by the B_2 receptor. NO may, in part, mediate the vasodilation of bradykinin.[80,81] Kallikreins are proteinases that liberate vasoactive kinins from the protein precursor kininogen. The bradykinin-synthesizing enzyme, kininase II, is identical to angiotensin-converting enzyme, which produces angiotensin II. Although bradykinin may play a role in renal morphogenesis,[82-84] the role of this vasodilator in the renal hemodynamics is less certain. Several studies indirectly suggest that bradykinin may participate in the maturational increase in renal blood flow. Urinary kallikrein excretion corrected for either renal mass or glomerular filtration rate increases with maturation.[85,86] Kininase II mRNA and enzymatic activity are low in newborn rat kidney and peak at 2 to 3 weeks of age.[87] However, studies have failed to demonstrate a significant role for bradykinin in immature renal hemodynamics. Newborn and 6-week-old lambs failed to show any renal hemodynamic response to acute intrarenal injection of a selective kinin B_2 antagonist.[88] Long-term administration of the same B_2 antagonist to neonatal rats from birth to 3 weeks did not alter the maturational increase in renal blood flow.[89] The possibility remains, however, that bradykinin opposes the basal vasoconstriction of angiotensin II in the immature rat kidney.[89]

Endothelin

Endothelin, one of the vasoconstricting factors produced by the endothelium, is one of the most potent endogenous vasoconstrictor, second only to urotensin II. There are three different endothelin peptides encoded by three distinct genes: endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3). ET-1 produces renal vasoconstriction in both the afferent and efferent arterioles. Two ET receptors, ETA and ETB, mediate endothelin action in the kidney. However, it is most likely that ETA receptors facilitate the ET-1 vasoconstrictor action; endothelins can also mediate vasodilation.[90,91] On the basis of pharmacologic evidence, two types of ETB receptors have been postulated: ETB1, which is a relaxant; and ETB2, which is a constrictor. In ETB knockout mice, both vasodilatory and vasoconstrictor effects of ETB receptors were eliminated, a finding suggesting that the ETB1 and ETB2 receptors are the same receptor.[90] The differences in ETB1 and ETB2 actions are related to the sites where these receptors are expressed. Thus ETB (ETB1) receptors expressed in endothelial cells (tunica intima) cause vasodilation because of their linkage to NO and prostaglandins, whereas ETB receptors expressed in vascular smooth muscle cells (tunica media) cause vasoconstriction.[92,93] In full-term infants, the urinary excretion of ET-1 remains constant in the first month of life; in premature infants, ET-1 excretion increases with maturation such that, after the first week, postnatal values exceed those found in older children.[94] In newborn rabbits, ET-1 failed to change renal hemodynamics at doses that decreased renal blood flow in adults; however, at higher doses, exogenous endothelin induced a marked reduction in renal blood flow and glomerular filtration rate in the newborn as well.[95] Blockade of endogenous ET-1 activity in newborn rabbits by ET-1 antiserum increased renal vascular resistance and decreased renal blood flow.[96] These studies suggest that ET-1 may not be responsible for the increased renal vascular resistance in the newborn period. Rather, ET-1, via endothelial ETB, may function as a renal vasodilator to counteract the vasoconstrictor effects of other systems (e.g., angiotensin II and catecholamines).

The finding that ETA and ETB blockade in the newborn increases renal blood flow but does not affect glomerular filtration indicates that endothelin is probably not an important factor in the increase of renal blood flow with age.[97]

Nitric Oxide

Nitric oxide (NO) synthesized by nitric oxid synthase (NOS) is an important endogenous regulator of renal hemodynamics in the immature kidney, functioning as a critical vasodilator to counterbalance highly activated vasoconstrictors, such as angiotensin II.[55] Three NOS isoforms exist: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS). However, only eNOS and nNOS participate in regulating basal renal hemodynamics. The immature renal vasculature is highly responsive to alterations of the NO synthesizing enzyme, NOS. Figure 125-3 shows the renal blood flow responses of the 3-week-old piglet to sequential intrarenal infusions of acetylcholine, a NOS stimulator, and L-NAME, an arginine analogue that nonselectively competitively inhibits all NOS isoforms.[12] Several reports identify NO as a vital participant in postnatal developing renal hemodynamics under physiologic and pathophysiologic conditions.[12,36,98,99] The intrarenal infusion of the NOS inhibitor, L-NAME, in both whole animals (piglets and newborn lambs)[36,100] and isolated perfused kidneys (newborn rabbits),[101] produces greater renal hemodynamic responses than the adult. These data suggest that renal NO production by NOS is greater in the immature kidney compared with the adult. This increased NO production is supported by the distinct developmental patterns in the postnatal maturing porcine kidney of two NOS isoforms: nNOS and eNOS.[102,103] Whole kidney and corticomedullary nNOS protein abundance is greatest in the newborn (and at all immature ages) but decreases with age to reach the lowest values in the adult.[102] This distinct pattern suggests that nNOS may be the more important NOS isoform participating in immature renal hemodynamics. The ability of NO to counteract the effects of vasoconstrictor agents under physiologic conditions suggests that NO may be even more important in pathologic conditions (e.g., perinatal hypoxemia).[99] In newborn rabbits, the renal blood flow responses to L-NAME are significantly greater during hypoxemia. These conditions induce an intense renal vasoconstriction, mediated by angiotensin II, often resulting in vasomotor acute renal failure.[5] Thus NO may serve to protect the immature kidney from the deleterious effects of adverse perinatal events that lead to vasomotor acute renal failure.

Prostaglandins

Arachidonic acid is an essential fatty acid that is metabolized through different pathways: 5-lipoxygenase leads to formation of leukotrienes, 15-lipoxygenase leads to formation of lipoxins, cytochrome P450 leads to formation of epoxy-20:3, and cyclooxygenase (COX) leads to formation of prostaglandins and thromboxanes. In rats, arachidonic acid metabolism in the kidney shifts from a lipoxygenase-dependent to a cytochrome P450-dependent pathway during development.[102] Two COX isoforms are present in the kidney: COX-1 and COX-2. COX-1 is found in the renal vasculature, glomeruli, and collecting ducts across species lines.[103] However, COX-2 intrarenal localization is species dependent. In rats and dogs, COX-2 localizes to tubules, macula densa, and thick ascending limb of the loop of Henle, whereas in humans, COX-2 co-localizes with COX-1.[104,105] Several therapeutic agents such as nonsteroidal antiinflammatory drugs, including aspirin and indomethacin, nonselectively inhibit both COX-1 and COX-2. Celecoxib and rofecoxib are selective COX-2 inhibitors. Although COX-1 may not undergo postnatal regulation, COX-2 is highest after birth (between 1 and 2 weeks in rats) and declines with age.[103,106-108] COX-2 involvement in nephrogenesis may contribute to renal dysgenesis syndromes associated with COX inhibition. In fetal kidneys, COX-2 strongly localizes to tubular

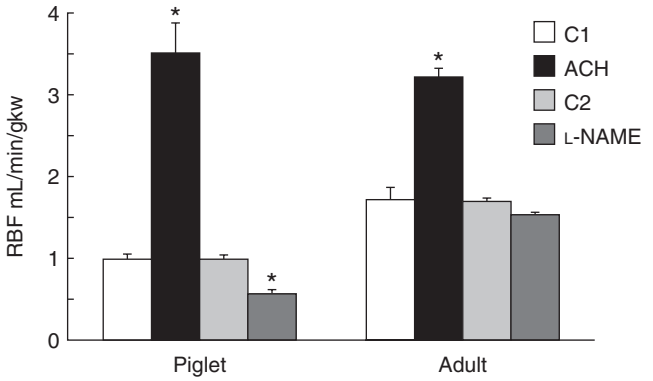


Figure 125-3 Comparison of the renal blood flow (RBF) responses in immature and adult renal vasculature to vasoactive agents that act on the nitric oxide (NO) synthesizing enzyme, nitric oxide synthase (NOS). RBF responses were directly measured by electromagnetic flow probe in anesthetized 3-week-old piglets and adults (n = 4) during the sequential intrarenal infusions of NOS stimulation with acetylcholine, 0.05 μg/kg/min (ACH), and NOS inhibition with L-NAME, 3 μg/kg/min (L-NAME) with interval control periods (C1 and C2). The immature kidney demonstrates lower baseline RBF in both control periods. RBF responses were greater in the immature kidney to NOS stimulation, ACH, and inhibition with L-NAME. *gkw,* Gram kidney weight. *$p < .05$ versus others within each group. *(Modified from Solhaug MJ, Ballèvre LD, Guignard JP, et al: Nitric oxide in the developing kidney,* Pediatr Nephrol *10:529, 1996.)*

structures, which differ from the adult.[98,99] Selective COX-2 inhibition leads to disruption of nephrogenesis in both mice and rats.[109] Genetic ablation of COX-2 results in severe morphologic abnormalities, including impaired glomerulogenesis, cortical dysplasia, and diffuse tubular cyst formation.[110] These studies confirm the teratogenic potential of prenatal use of nonsteroidal antiinflammatory drugs and selective COX-2 inhibitors leading to acute renal failure and renal dysgenesis.[111-113]

Despite the increasing information about the role of COX isoforms in nephrogenesis, the functional vasoactive role of the COX products (prostaglandins) in the immature kidney is uncertain. In fact, the relation of specific COX isoforms to renal function is not well understood. In general, thromboxanes and leukotrienes vasoconstrict the kidney, whereas prostaglandins and lipoxins vasodilate it. Cytochrome P450 metabolites of arachidonic acid can act as vasoconstrictors or vasodilators. Renal prostaglandin production is increased during the perinatal period. In preterm infants, the urinary excretion of prostaglandin E and a prostacyclin metabolite is five times that noted at term and is 20 times greater than that observed in physiologically normal children.[113] Glomerular prostaglandin synthesis and adenylate cyclase response to prostaglandin E_2 also decrease with maturation.[114,115] Moreover, when prostaglandin synthesis is inhibited by indomethacin in the unstressed adult or neonate, blood flow is either unchanged or redistributed to the outer cortex. Therefore, if prostaglandin synthesis was deficient in the newborn infant, one could expect a pattern of blood flow converse to that normally found in the neonatal kidney.[57] Likewise, the increased renal blood flow found in the uninephrectomized young rat is not maintained by increased prostaglandin or decreased thromboxane effects.[29] Although neonatal rabbits may be more sensitive to the vasoconstrictor effects of acetylsalicylic acid,[116] prostaglandin inhibition does not alter basal renal blood flow in the immature piglet kidney.[12] Moreover, the COX inhibitor ibuprofen does not affect renal blood flow in preterm neonates.[117] Thus, in the newborn, as in adults, prostaglandins play little or no role in the

control of renal blood flow in the normal animal at rest. Prostaglandins may, however, attenuate renal vasoconstriction in pathologic conditions. In contrast, prostaglandins may be important in the fetus in the regulation of renal blood flow under both basal and stress conditions.[118] In young and adult rats, the major pathway of arachidonic acid metabolism in glomeruli occurs via the lipoxygenase pathway[114]; however, the effects of this pathway on basal renal blood flow during development remain to be determined. Moreover, the renal development of the eight prostanoid receptors has not been determined.[41,119]

Renal Nerves and the Adrenergic System

The influence of renal innervation and the adrenergic nervous system is both maturation and species dependent. The renal circulation of the pig is under tonic neural vasoconstrictor influence, whereas that of the sheep is not. As such, renal denervation does not alter basal renal blood flow in fetal lambs but does result in an increase in renal blood flow in piglets. Overall, the renal vascular bed of the newborn seems to be more sensitive, but less reactive, than that of the adult in response to renal nerve stimulation.[120,121] Low-level stimulation of the renal nerve of fetal sheep and 1- to 2-week-old piglets results in a greater increase in renal vascular resistance than in their adult counterparts. During higher levels of renal nerve stimulation, however, renal vascular resistance increases to a greater extent in older animals than in younger animals. In the presence of α-adrenergic blockade, renal nerve stimulation induces an increase in renal blood flow in fetal and newborn lambs but not in adults. This effect is apparently the result of a greater density of α- (versus β-) adrenergic receptors in neonatal sheep. After the immediate newborn period, the neonatal renal circulation is more sensitive to α-adrenergic stimulation in several species (dogs, pigs, guinea pigs, sheep) compared with adults.[14,122,123] An age-dependent increase in renal blood flow and a concomitant decrease in renal vascular resistance with α-adrenergic blockade have been shown in piglets[122] and in canine puppies.[13,123] Isolated renal vessels of fetal lambs, studied in vitro, are also more sensitive and reactive to α-adrenergic agonists than are their newborn or adult counterparts. The rabbit seems to be an exception in that the renal vasculature in the adult is more sensitive to catecholamines compared with newborns. β-Adrenergic agonists increase renal blood flow to a greater extent in neonatal than in adult sheep. An opposite effect has been noted in piglets. The renal vasodilator effect of dopamine increases with maturation in several species (pigs, dogs, sheep).[123,124] The earlier reports of apparent absence of ontogenic differences in the renal catecholamine response in sheep can now be attributed to bolus versus constant intrarenal infusions. Fetal and newborn sheep are more responsive to the vasoconstrictor effects of α-adrenergic ligands and less responsive to the vasodilator effects of dopaminergic ligands.[26,122,125-128]

CONCLUSION

The low renal blood flow in the young is the result of several factors, including smaller size, decreased number of glomeruli, lower systemic pressure, and higher renal vascular resistance. The increased renal vascular resistance in the newborn is probably caused by increased activity of the renin-angiotensin system as well as increased sensitivity to vasoconstrictor catecholamines. The latter is the result of receptor and postadrenergic receptor mechanisms. Critical vasodilators, such as NO, may act to counterbalance these vasoconstrictor forces. The increase in renal blood flow with age presumably occurs as the vasoconstrictor influences decline. New ligands and receptors continue to be discovered, but their roles, if any, in the development of renal blood flow remain to be determined.

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Development of the Renin-Angiotensin System

The *renin-angiotensin system* (RAS) regulates arterial blood pressure, renal hemodynamics, and fluid and electrolyte balance.[1,2] The RAS cascade comprises a series of enzymatic reactions that culminate in the generation of angiotensin peptides, the main effectors of the system (Figure 126-1). Renin, the key regulatory enzyme of the system, is produced mainly by the juxtaglomerular (JG) cells of the kidney and released into the circulation, where it catalyzes the hydrolysis of angiotensinogen to generate angiotensin I. Angiotensin I is subsequently hydrolyzed by angiotensin-converting enzyme (ACE) to form the octapeptide angiotensin II. Most of the known actions of the RAS are exerted through the binding of angiotensin II to cell membrane–bound receptors, AT_1 and AT_2. Additional receptors and alternative pathways for the generation of other angiotensin peptides with potential biologic actions have been identified (see Figure 126-1).[3]

In addition to the endocrine effects of the circulating RAS, a locally acting intrarenal RAS regulates renal function by paracrine and/or autocrine effects.[4,5] The extrarenal tissue RASs remain controversial, but components of the system and local generation of angiotensins have been described in tissues outside the kidney including brain, heart, blood vessels, adrenal gland, testis, and eye.[6-8] In addition to the well-known hemodynamic effects of the RAS, angiotensin II is also involved in normal and abnormal growth processes.[9,10] These actions seem to be well conserved across the phylogenetic scale and are important in the development and maintenance of normal nephrovascular architecture.[11]

MATURATION OF THE RENIN-ANGIOTENSIN SYSTEM

The major components of the RAS are present from early gestation, but their anatomic distributions and activities are in many cases different from those seen in the adult mammal.These differences are likely to result in distinct functions of the RAS within different tissues in the growing, differentiating, and maturing fetus. Although little information exists regarding the components of the RAS in human fetal or infant tissues, several animal models have been thoroughly studied and are mentioned here.

Angiotensinogen

Angiotensinogen is a 55- to 60-kDa glycoprotein precursor of the angiotensin peptides and is the only known substrate for renin.[12] Angiotensinogen is encoded by a single gene on human chromosome 1, and its messenger RNA (mRNA) product encodes a prohormone. Removal of the leader peptide results in a mature protein of which the angiotensin I decapeptide is the amino terminus.[12]

In adult animals, the primary source of circulating angiotensinogen is the liver, but angiotensinogen mRNA levels have also been detected in other organs and tissues including kidney, brain, heart, vasculature, adrenal gland, ovary, placenta, and adipose tissue.[13] However, in the fetal rat liver, angiotensinogen mRNA levels are almost undetectable.[14] In contrast, angiotensinogen is expressed at relatively high levels in the yolk sac and fetal placenta, as well as fetal brain, fat, and kidney,[14] and circulating

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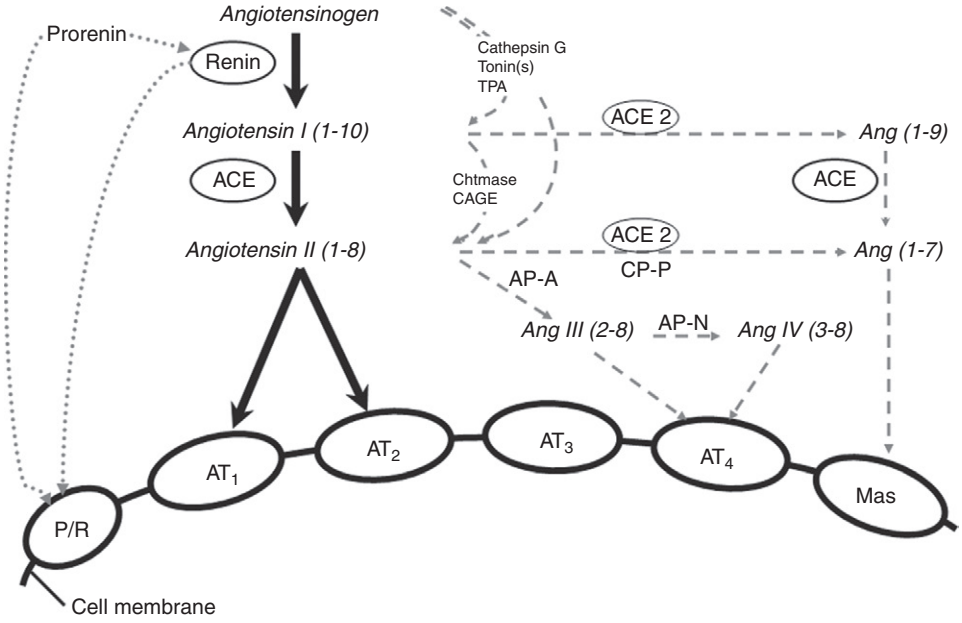


Figure 126-1 The renin-angiotensin cascade. The *thick arrows* depict the traditional enzymatic cascade. The *dashed arrows* depict alternative pathways for angiotensin *(Ang)* generation. The *dotted arrows* show renin and prorenin binding to a prorenin-renin *(P/R)* receptor. *ACE,* Angiotensin-converting enzyme; *AP-A/AP-N,* aminopeptidase A–aminopeptidase N; *AT,* angiotensin receptor; *CAGE,* chymostatin-sensitive angiotensin II–generating enzyme; *CP-P,* carboxypeptidase P. *(Modified from Gomez RA, Norwood VF: Developmental consequences of the renin-angiotensin system,* Am J Kidney Dis *26:409, 1995.)*

levels of angiotensinogen are similar to or higher than those seen in adult animals.[15] In sheep, angiotensinogen mRNA levels increased steadily throughout the last third of gestation.[16] In the human fetus, however, angiotensinogen mRNA is expressed in the fetal liver from early stages of development.[17]

In the umbilical cord of the human fetus angiotensinogen levels are low.[18] Levels rise in the neonatal period and are higher in the infant than the adult.[18] In newborn rats, a 20-fold increase in the expression of angiotensinogen in the liver occurs within 24 hours of birth, and this expression remains elevated until the pups are weaned.[14] Concomitantly, circulating angiotensinogen levels rise quickly after birth.[19]

The production of angiotensinogen by the adult liver is regulated by various physiologic stimuli. Salt depletion, sex steroids, thyroxine, and angiotensin II stimulate angiotensinogen expression, whereas it is suppressed by insulin.[20-23] The fetal liver is also clearly responsive to hormonal stimuli. In the rat, dexamethasone or thyroxine administration stimulates fetal liver angiotensinogen expression.[24]

The functional importance of angiotensinogen in the fetus remains unclear, and the secretion of the protein from its various sources has not been determined. However, its presence in the yolk sac and placenta implies potential actions in the control of fetal perfusion and fluid and electrolyte transport. Its presence in numerous developing organs may also suggest a role in tissue morphogenesis and blood flow regulation.[25]

Overall, these studies indicate that, as the animal matures, angiotensinogen synthesis and regulation mature in preparation for the transition from fetal to extrauterine life. These changes in angiotensinogen availability are accompanied by adjustments in the control of blood flow patterns, blood pressure, and salt and water homeostasis.

Renin

Renin is an aspartyl protease with a unique substrate, angiotensinogen. Renin substrate specificity is so stringent that only homologous angiotensinogen is cleaved efficiently.[26] Human renin is encoded by a single gene in chromosome 1 and is synthesized as pre-prorenin, an inactive single polypeptide precursor molecule. Removal of the signal peptide results in prorenin, which is subsequently glycosylated and is stored in immature granules before further processing into mature active renin. Renin is thought to be secreted by two pathways: a regulated pathway for mature renin and a constitutive pathway for prorenin, which accounts for approximately 70% to 90% of the renin in the human circulation.[27] Therefore prorenin may be a reservoir for local generation of active renin and angiotensin II, but the biologic significance of this finding is still poorly understood. Renin is mainly synthesized in the kidney vasculature. However, extrarenal production of renin has been demonstrated in adrenal, liver, gonads, and brain, although in much lower abundance.[28] The characterization, regulation, and roles of extrarenal renin are areas of active investigation.

In the normal adult, renin is synthesized and released to the circulation by JG cells, a small group of myoepithelioid granulated cells located in the wall of the afferent arteriole at the entrance to the glomerulus. However, during early development, renin-expressing cells are broadly distributed along large intrarenal arteries, inside the glomeruli, and in the renal interstitium.[29-33] As maturation continues, the number of renin cells is reduced as they become progressively restricted to a few cells in the JG area found in the adult kidney. Because adult JG cells contain myofilaments, it has been postulated that they derive from smooth muscle cells.[34] However, it is now clear that renin cells are in fact progenitors for arteriolar smooth muscle cells, mesangial cells, and a subset of proximal and distal tubular cells.[35] Renin cells are also progenitors for specific cell types in extrarenal sites.[35]

The developmental pattern of renin cell distribution and the importance of renin cells in generating other cell types are relevant to understand the phenomenon of recruitment. Recruitment is a survival mechanism whereby a threat to homeostasis (such as dehydration or hypotension) results in an increase in the number of renin-expressing cells along the preglomerular arteries, inside the glomeruli, and in the kidney interstitium resembling the embryonic and fetal pattern (Figure 126-2). The increase in the number of renin-expressing cells, with the attendant increase in circulating renin, is due to dedifferentiation of preexisting adult cells and does not involve migration or replication of cells. It was recently demonstrated that the cells that retain the plasticity to develop phenotypic characteristics of renin cells are the same ones that expressed *renin* earlier in development.[35] Recruitment of renin cells extends to other organs beyond the kidney including the adrenal gland[36,37] and extrarenal arteries,[38] which also express *renin* early in development, again, pointing to the relationship between cell lineage and recruitment.

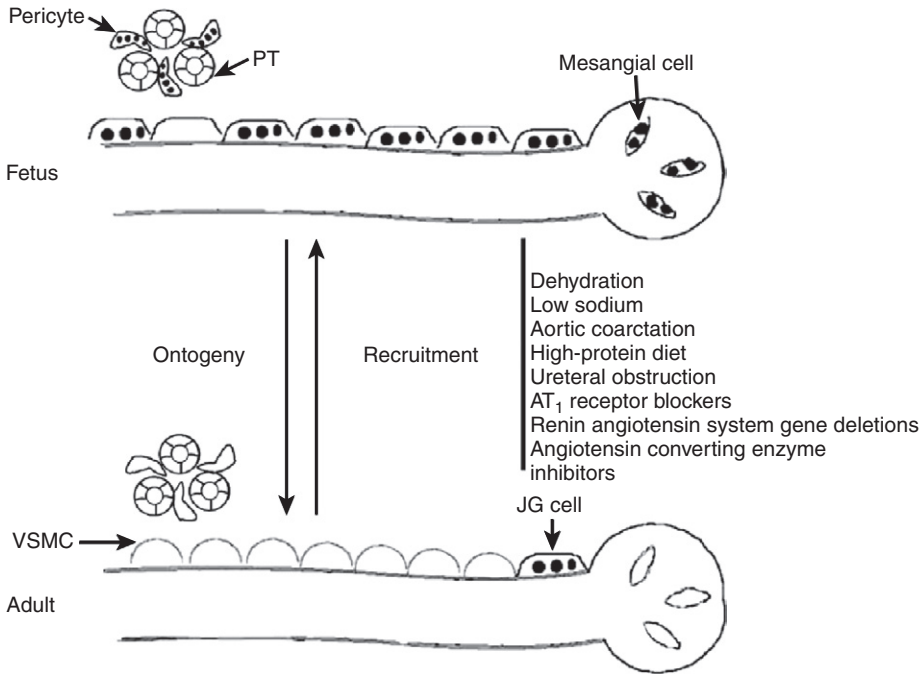


Figure 126-2 Ontogeny and recruitment of renin-expressing cells. Renin-expressing cells are represented with *black dots. JG,* Juxtaglomerular; *PT,* proximal tubule; *VSMC,* vascular smooth muscle cell.

In humans, *renin* expression and activity are markedly different during fetal and neonatal life compared with the mature animal. Renin is observed early in human development, first localized to the mesonephros at 5 to 6 weeks gestation.[29] By 8 weeks gestation, renin is found within the metanephros, several weeks before the onset of fetal urine production.[29] Although renin production is strictly localized to the juxtaglomerular apparatus in the normal adult, its localization (in other mammals) is more widely distributed within the developing renal microvasculature. After birth, renin expression decreases in the larger vessels until it remains only in the classic juxtaglomerular location.[32,33] This developmental pattern of renin expression has been found in every species examined.[29]

The major source of circulating renin during fetal life is the kidney,[39] and fetal plasma renin concentration and activity are elevated compared with levels in newborns who likewise have higher levels than adults.[40-42] In preterm infants, plasma renin activity is high and inversely correlates with gestational age.[43] At birth, plasma renin activity increases further for several weeks and then begins a slow decline through infancy and childhood until adult levels are reached by 6 to 9 years of age.[40]

Renin release is modulated by many different positive and negative stimuli, and control of this process is well established in the late-gestation fetus and in the newborn.[44,45] The principal mechanisms controlling renin release are the macula densa mechanism, the baroreceptor, the sympathetic nervous system, and various hormonal mechanisms. The macula densa mechanism stimulates renin release when macula densa cells (that are in close proximity to JG cells) sense a decrease in sodium chloride concentration in the distal tubule.[46,47] The renal baroreceptor responds to decreases in renal perfusion pressure in the afferent arteriole with increases in renin secretion.[48] The sympathetic nervous system, acting through the renal nerves and cell-surface β-adrenergic receptors, also stimulates renin production and release.[49,50] All of the above mechanisms are active during development, and both humoral and intracellular signals including cyclic adenosine monophosphate, Ca^{2+}, and prostaglandins mediate the release of renin from its storage pools and are active during development.[51,52] Angiotensin II generated by the RAS cascade in turn exerts a negative feedback on renin synthesis and release, thereby preventing uncontrolled hypertension or fluid overload once the homeostatic challenge that triggered renin release has been controlled. These stimuli result in changes in renin release that are qualitatively similar to those seen in adults. The physiologic, pharmacologic, and pathophysiologic factors that affect the RAS during development are shown in Box 126-1.[44] The end result of these integrated regulatory mechanisms is a precise and physiologically responsive control system that accurately governs renin secretion in the immature animal.

Angiotensin-converting Enzyme

Angiotensin-converting enzyme (ACE) is a zinc-containing protease that cleaves carboxy-terminus dipeptides from small peptides, most notably angiotensin I and bradykinin. Human ACE is the product of a single gene that ultimately produces two extensively glycosylated polypeptides: a larger form found in endothelial cells and most somatic tissues and a smaller form found in testis.[53] ACE has hydrophobic amino acids at the N terminus that provide an anchor to the cell membrane. The soluble form of ACE present in plasma and seminal fluid is derived by posttranslational cleavage of the C-terminal region and may reflect turnover of membrane-bound ACE.[54]

A novel enzyme similar to ACE, named ACE 2, has recently been identified, which hydrolyzes only one amino acid from angiotensin I and generates angiotensin (1-9) and from angiotensin II to generate angiotensin (1-7).[55] In turn, the "classic" ACE can convert angiotensin (1-9) to angiotensin (1-7) (see Figure 126-1). ACE 2 is expressed mainly in the airway epithelium and vascular endothelial cells of the lung[56] and in epithelial cells of the kidney.[57] ACE 2 may balance the activity of the RAS counterregulating the actions of ACE on angiotensin II generation and at the same time enhancing angiotensin (1-7) formation.[58]

In contrast to renin expression, ACE activity is relatively low during fetal life and increases slowly as term approaches.[59] After delivery, renal ACE activity in the rat increases to a maximum within the first 2 weeks, followed by a decline to adult values by 1 to 2 months.[60,61] In human infants, plasma ACE levels are similar to those seen in adults.[18] At every age studied, ACE has the same substrate affinity.[59] However, the maximum velocity (V_{max}) of the enzyme is four-fold lower in fetal than in adult tissues, a finding suggesting that increasing ACE activity during development is the

BOX 126-1

Factors that Increase and Decrease the Activity of the Renin-Angiotensin System in the Fetus and Newborn Mammal

Increase
- Prematurity
- Vaginal delivery
- Furosemide
- Hemorrhage
- Hypotension
- Hypoxemia
- Aortic constriction
- Exchange transfusion (withdrawal)
- Upright position

Decrease
- Volume expansion
- Indomethacin
- Phenylephrine
- Cortisol
- Arginine vasopressin
- Recumbent position
- Exchange transfusion (replacement)

Modified from Gomez RA, Pupilli C, Everett AD: Molecular and cellular aspects of renin during kidney ontogeny, *Pediatr Nephrol* 5:80, 1991.

result of the production of more enzyme rather than further activation of preexisting enzyme.[59] During renal organogenesis, ACE is first detected in proximal tubules, endothelial cells invading the S-shaped glomerulus, and the afferent and efferent arteriolar endothelium of more mature glomeruli.[62] With maturation, ACE is predominantly expressed by proximal tubules and peritubular capillaries.[62] The presence of vascular and intraglomerular ACE during nephrogenesis suggests that local angiotensin production may be necessary for normal development of the glomerulus and renal microvasculature.

Angiotensin II

Angiotensin II regulates blood pressure and fluid and electrolyte homeostasis through various actions. Angiotensin II is an extremely potent vasoconstrictor; intravenous infusion results in a pressor response within 15 seconds that lasts for 3 to 5 minutes.[63]

Angiotensin II concentration in the fetus is twice that of maternal plasma, accordingly with the high levels of active renin during development.[18] Circulating angiotensin II levels remain high during the newborn period and decrease with age, paralleling the changes in active renin. In addition, the enzymes responsible for degradation of angiotensin II, the angiotensinases, are relatively inactive in the developing animal, and therefore high circulating levels of angiotensin II are maintained during early life.[64]

Although angiotensin II is the main active product of the RAS, other metabolites of angiotensin I and II (see Figure 126-1) may have significant biologic activity, mostly in tissues. For example, angiotensin (1-7) has a vasodilatory effect, inhibits cell growth, and causes natriuresis, in contrast to the vasoconstrictor and growth-promoting actions of angiotensin II.[65,66] Angiotensin III (2-8) and IV (3-8) are generated by the removal of amino acids from the N terminus of angiotensin II by the action of aminopeptidases (see Figure 126-1). Angiotensin III may play an important role in regulating blood pressure at the central nervous system, and angiotensin IV seems to cooperate with angiotensin II in the brain.[67]

Angiotensin II Receptors

Angiotensin II receptors have been located by radioligand binding in every angiotensin II–responsive tissue or cell type and mediate the physiologic and pathologic effects of angiotensin II. At least four receptor subtypes have been described.[68] The major receptor subtypes, AT_1 and AT_2, have widely divergent patterns of tissue distribution and second-messenger signaling mechanisms.

Both AT_1 and AT_2 receptors contain seven transmembrane domains, but the glycosylated proteins share only 32% homology.[69] AT_1 receptors are coupled to G proteins.[70] Stimulation of AT_1 by angiotensin II induces hydrolysis of inositol phosphates, increases intracellular calcium (Ca^{2+}) and diacylglycerol, inhibits adenylate cyclase, and releases arachidonic acid metabolites.[71] Most of the established actions of angiotensin II are mediated by AT_1 receptors that are widely distributed on many cell types. These physiologic and pathologic effects include (1) cardiovascular effects: vasoconstriction, increased blood pressure, increased cardiac contractility, cardiac and vascular hypertrophy; (2) renal effects: tubular sodium reabsorption, inhibition of renin release; (3) nervous system effects: sympathetic system and adrenal cortex (aldosterone release); and (4) miscellaneous effects: proinflammatory and profibrogenic effects, growth stimulation, and oxidative stress.[70,72-74]

AT_2 receptors are not coupled to G proteins; they activate an intracellular phosphotyrosine phosphatase, thereby inactivating mitogen-activated protein (MAP) kinase and decreasing cyclic guanosine monophosphate levels.[75-78] In addition, a growth-inhibitory function has been described for AT_2.[75,79] The role for AT_2 and other putative receptor subtypes during development remains elusive. AT_2 mRNA is abundant in brain, kidney, and other tissues during fetal life but decreases rapidly after birth.[69] Stimulation of AT_2 receptors decreases blood pressure by the release of nitric oxide, induces differentiation, inhibits growth, and mediates apoptosis.[78]

AT_4 receptors have been identified as insulin-regulated aminopeptidases[80] and may mediate the release of plasminogen activator inhibitor I by angiotensin II, III, and IV.[68] The function of the AT_3 receptors is still unknown.

Some evidence exists that angiotensin (1-7) acts through a Mas receptor (product of the Mas proto-oncogene).[68]

Both AT_1 and AT_2 subtypes are developmentally regulated and are found in fetal membranes as early as 10 days of gestation in the rat.[81] During embryonic life, the AT_2 subtype predominates and is expressed in most mesenchymal tissues.[82] AT_1 receptors are also present, although in fewer numbers.[82] In the fetal rat kidney at 14 days of gestation (the beginning of metanephric development), 80% of angiotensin receptors are the AT_2 subtype and are localized to the undifferentiated renal mesenchymal cells.[83] By 17 days of gestation, AT_1 expression has increased and is found primarily in deep, more mature glomeruli but also diffusely throughout the nephrogenic cortex.[83,84] At birth, AT_1 and AT_2 are equally prevalent. AT_2 continues to be expressed in undifferentiated cells or differentiating structures, whereas AT_1 predominates in glomeruli, arteries, and vasa recta.[83-85] After birth, AT_2 is rapidly down-regulated, but it remains detectable at low levels in the adult adrenal gland, renal glomeruli, and nephrons.[86] AT_1 persists in the renal microvasculature, mesangial cells, and proximal tubules, where it is maintained throughout adulthood.[83-85]

In addition to maintaining fluid and blood pressure homeostasis, during development angiotensin II receptors regulate morphogenetic events in various tissues including the kidney. The AT_1 receptor is linked to certain G protein–associated second-messenger systems (phospholipase C in vascular smooth muscle, phospholipase A_2 in proximal tubules, and adenylcyclase in adrenal cortex) through which smooth muscle contraction, Na^+ reabsorption, and aldosterone secretion, respectively, are mediated.[70] Few of these effects have been studied in the developing human.

The classic representation of the RAS is that of circulating renin acting on constitutively secreted angiotensinogen to produce circulating angiotensin II. This endocrine system is highly functional and is up-regulated during development. As shown in Figure 126-3, circulating levels of angiotensinogen, renin, angiotensin II, and aldosterone are all higher in developing animals than in adults.[87] Evidence supports the additional existence of local, or tissue, RASs that may generate angiotensin II for paracrine or autocrine functions.[4,88] Because all components of the RAS are present early in the developing embryo and are found in the kidney before the onset of fetal urine production, it is possible that the local system may regulate growth and differentiation of developing nephrons and other tissues. Once kidney structures are formed, the RAS then begins to regulate renal hemodynamics and Na^+ transport.

Renin Receptors

Renin and prorenin receptors have recently been described in several tissues, including heart, kidney, brain, and placenta.[89] Two renin-prorenin receptors have been cloned and sequenced, and the existence of a third receptor has been suggested.[89-92] The putative (pro)renin receptor consists of a 350–amino acid protein with a single transmembrane domain, and it is highly homologous in human, mouse, rat, chicken, fish, *Xenopus*, and *Caenorhabditis elegans*.[89,93] Cell culture experiments on cells expressing the (pro)renin receptor in the presence of angiotensin receptor blockers, ACE inhibitors, and/or renin inhibitors showed that both renin and prorenin signal in an angiotensin-independent manner by activation of mitogen-activated protein kinases (MAPK p42/44 and p38) and subsequent heat shock protein (HSP) 27 phosphorylation.[89,94-96] Prorenin stimulation of the MAPK p38/HSP27 pathway in cardiomyocytes showed an alteration in actin filaments and may be the cause of cardiac hypertrophy in transgenic animals overexpressing *prorenin*.[96,97] In the kidney, by up-regulation of transforming growth factor-β1, renin has been linked to the development of renal fibrotic disease.[94,98,99]

FUNCTIONAL EFFECTS OF ANGIOTENSIN II DURING DEVELOPMENT

The production of angiotensin II by the active renin-angiotensin cascade in the fetus and newborn results in systemic and local effects that control systemic blood pressure, renal hemodynamics, and fluid and electrolyte homeostasis by interaction of angiotensin II with its receptors. In addition, angiotensin II regulates various growth and developmental processes that are important to the care of the maturing infant. Although the role of angiotensin II in the maintenance of physiologic processes has been more thoroughly studied in adults,[70] appropriate functional responses to stimuli of the RAS have been well established during development. Hypotension, aortic constriction, hemorrhage, furosemide, ACE inhibition, sympathetic nervous system activity, prostaglandins, vasopressin, and atrial natriuretic peptide can all influence renin secretion and thereby the production of angiotensin II in the fetus (see Box 126-1).

Blood Pressure Control

The role of angiotensin II in the control of systemic blood pressure is exerted through effects on several systems (Figure 126-4). Blood pressure is increased by elevations in both peripheral vascular resistance and cardiac output, which, in turn, are increased by both heart rate and stroke volume. The direct vasoconstrictor effects of angiotensin II, mediated by AT_1 receptor–initiated increases in vascular intracellular Ca^{2+}, provide an exceedingly powerful mechanism for raising systemic pressure rapidly by increasing small-vessel resistance. Additional mechanisms by which angiotensin II can increase blood pressure include alterations in cardiac output through AT_1-mediated increases in myocardial contractility and centrally mediated increases in sympathetic nervous system activity to increase heart rate. Angiotensin II directly stimulates proximal tubular Na^+ reabsorption and also leads to intrarenal vasoconstriction that ultimately results in further Na^+ and water reabsorption and extracellular volume expansion. An additional long feedback loop allows angiotensin II to stimulate aldosterone secretion and thereby increase distal renal Na^+ reabsorption. Meanwhile, angiotensin II in the central nervous system stimulates thirst and salt appetite and further increases extracellular volume. These aggregate effects of angiotensin II provide a closely balanced and integrated endocrine and paracrine system capable of tight regulation of arterial blood pressure and body fluid homeostasis. Studies in genetically engineered animals have confirmed the primary role of the RAS in blood pressure regulation. Disruptions of the genes for *angiotensinogen*,[100] *renin*,[101,102] *ACE*,[103,104] or the AT_{1A} receptor[105] in mice result in marked decreases in blood pressure. These findings confirm the importance of the RAS in the normal regulation of blood pressure and illustrate that no other mechanism completely compensates for the lack of angiotensin II activity.

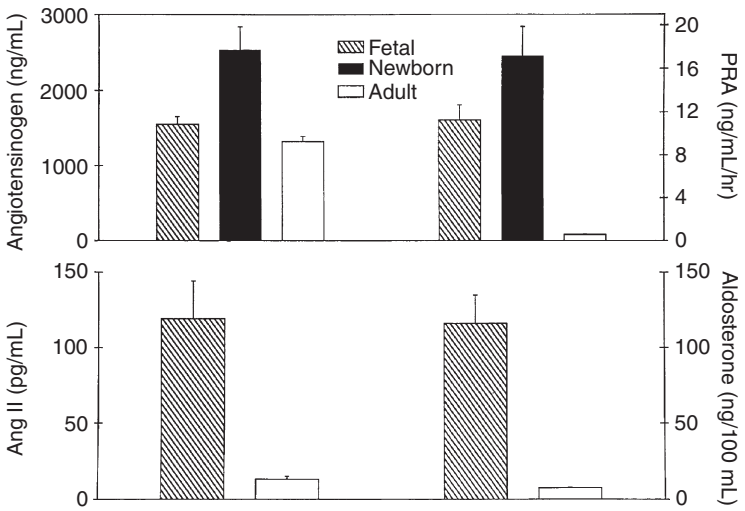


Figure 126-3 Circulating levels of angiotensinogen, plasma renin activity *(PRA)*, angiotensin II *(Ang II)*, and plasma aldosterone in fetal cord blood, newborn infants, and physiologically normal adults. *(From Tufro-McReddie A, Gomez RA: Ontogeny of the renin-angiotensin system,* Semin Nephrol *13:519, 1993; data from Godard C, Gaillard R, Vallotton MB: The renin-angiotensin-aldosterone system in mother and fetus at term,* Nephron *17:353, 1976.)*

The potency of angiotensin II changes with development. The pressor response to exogenous angiotensin II is lower in fetal and newborn lambs than in adults,[106] and it may be caused by occupancy of the receptors by high concentrations of endogenous angiotensin II, as well as maturational differences in receptor density or function. The hypotensive response to fetal hemorrhage is mediated in part by angiotensin II, but it also increases as maturation proceeds. A 20% reduction in fetal placental blood volume results in hypotension in young fetuses but has no effect near term, a finding suggesting that the control of fetal blood pressure by the RAS increases with maturation.[107]

As in the adult, alterations in the activity of the RAS during early life may lead to pathologic situations. In the human infant, hypertension usually results from activation of the RAS. Renal artery thromboembolism caused by umbilical artery catheter use, coarctation of the aorta, renal artery stenosis, obstructive uropathy (especially ureteropelvic junction obstruction), glucocorticoid administration, and bronchopulmonary dysplasia all stimulate angiotensin II production. These infants are usually given treatment with ACE inhibitors such as captopril or enalapril. However, the potency of these drugs is enhanced in the first 6 months of life, and care must be taken to avoid hypotension and oliguria.[108] Effective reductions in blood pressure have been obtained with doses much lower than those required by older infants. Current recommendations suggest a starting captopril dose of 0.05 mg/kg with careful increases until the appropriate effect is obtained. Adverse effects of these drugs include neutropenia, rash, hyperkalemia, and decreased glomerular filtration rate (GFR) necessitating careful follow-up for infants requiring these medications. Although of benefit in the treatment of hypertension, the angiotensin-receptor blockers have not yet been as thoroughly evaluated in infants or young children.

Renal Hemodynamics

The intrarenal actions of angiotensin II include control of renal blood flow, GFR, and tubular Na^+ excretion. The effects on renal blood flow and GFR are primarily mediated through AT_1 receptors in the afferent and efferent arterioles, with the dominant vasoconstriction occurring in the efferent arteriole.[4] As in adult animals, administration of angiotensin II to fetal lambs decreases renal blood flow and maintains GFR by increasing efferent arteriolar tone.[106] Within the adult glomerulus, angiotensin II binding to mesangial cell AT_1 receptors results in contraction and decreased filtration by decreasing the surface area available for filtration.[109] This response may serve as a counterbalance to the increase in GFR caused by efferent arteriolar constriction. AT_1 receptors are also present in fetal mesangial cells,[110] but their role in intraglomerular hemodynamic control is unknown. Treatment of the fetus with ACE inhibitors decreases renal vascular resistance and GFR by decreasing efferent arteriolar tone.[111] When given on a long-term basis to fetuses, ACE inhibitors can dramatically decrease fetal GFR and urine flow,[111] and this may explain the neonatal anuria and renal compromise seen in human

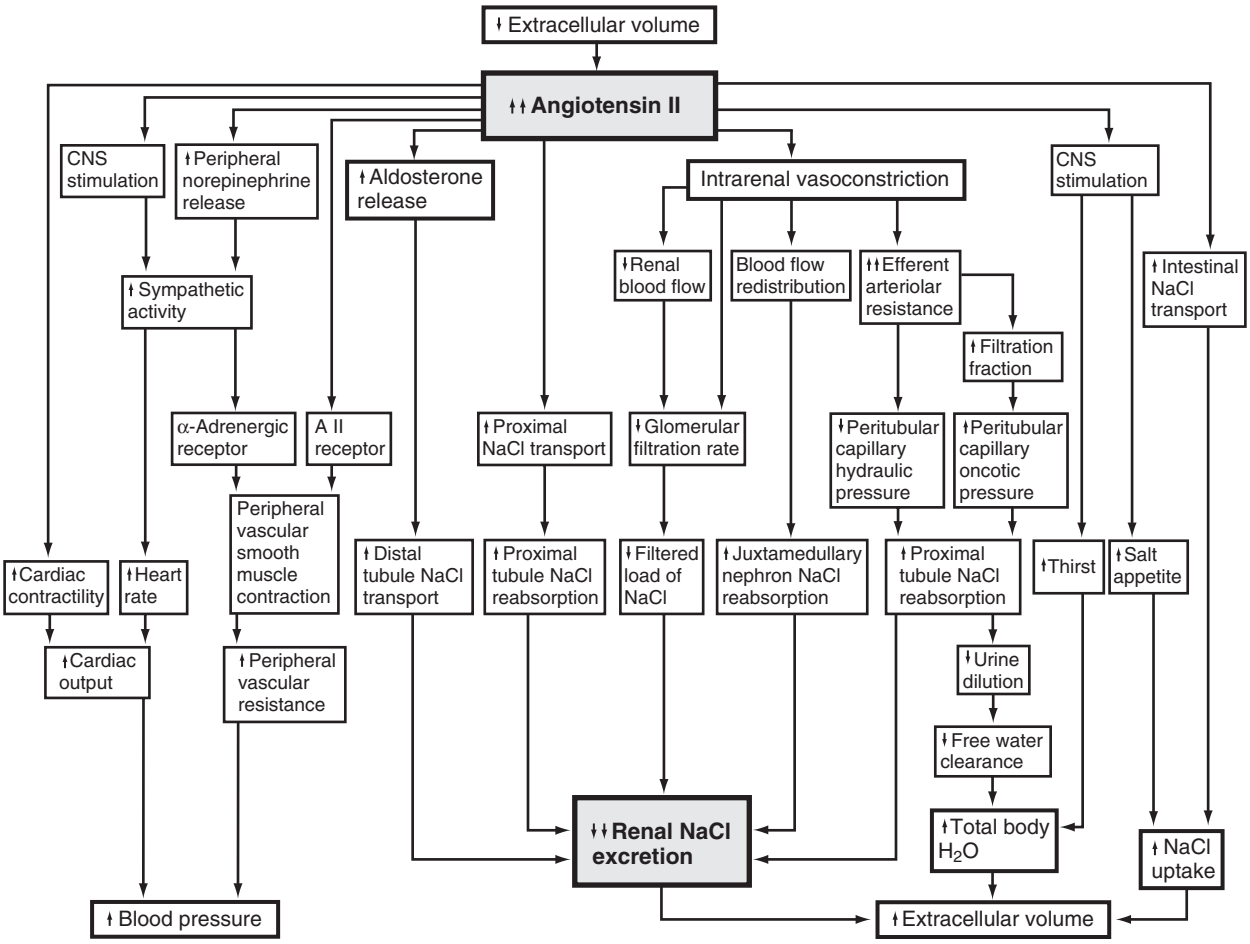


Figure 126-4 The integration of angiotensin II *(A II)* effects to maintain arterial pressure and extracellular fluid volume. *CNS,* Central nervous system. *(From Ballermann BJ, Zeidel ML, Gunning ME, et al: Vasoctive peptides and the kidney. In Brenner BM, Rector FC, editors:* The Kidney*, ed 4, Philadelphia, 1991, Saunders, pp 510-583.)*

infants exposed to these drugs in utero. The use of these drugs during the second and third trimesters of pregnancy is associated with renal dysplasia, oligohydramnios, pulmonary hypoplasia, and renal failure in the fetus, features that make these drugs contraindicated during pregnancy.[112] These effects are most likely the result of loss of angiotensin II–dependent blood flow to the fetal kidney, as well as inhibition of the angiotensin II–dependent growth effects.

Fluid and Electrolyte Balance

Angiotensin II mediates the control of systemic fluid and electrolyte balance through various mechanisms, some directly affecting renal hemodynamics or tubular transport and others indirectly altering intake or output of water and solutes. In the brain, angiotensin II, through AT_1 receptors, stimulates thirst, salt appetite, and vasopressin release, all of which increase extracellular fluid volume.[113] AT_1 receptors in the adrenal cortex respond to angiotensin II stimulation by production and secretion of aldosterone that subsequently enhances basolateral Na^+, potassium–adenosine triphosphatase (Na^+,K^+-ATPase) and Na^+ channel activity in the distal nephron, resulting in increased Na^+ reabsorption. Although the fetal adrenal gland responds to angiotensin II stimulation by secretion of aldosterone, the rise in plasma aldosterone is less than that seen in adult animals, a finding suggesting relative insensitivity of the fetal adrenal gland to angiotensin II stimulation.[106] In the proximal tubule, AT_1 receptors activate both luminal and basolateral Na^+ transport mechanisms including the Na^+/H^+ antiporter,[114] the Na^+/bicarbonate cotransporter,[115] and Na^+,K^+-ATPase,[116] all of which result in net Na^+ reabsorption. In the cortical collecting duct, angiotensin II, acting through AT_1 receptors, stimulates H^+-ATPase activity, a finding suggesting additional roles for angiotensin II in the control of acid-base balance.[117] An additional mechanism by which angiotensin II controls fluid and electrolyte balance is via control of peritubular blood flow. The capillaries that compose the peritubular network are downstream from the glomerulus, having branched from the efferent arteriole. As a result, factors that affect afferent and efferent blood flow and GFR directly affect peritubular flow. Efferent vasoconstriction decreases postglomerular flow and hydrostatic pressure and increases peritubular oncotic pressure, resulting in enhanced Na^+ and water reabsorption. Therefore the net effect of angiotensin II stimulation in the renal tubule is to encourage Na^+ and water reabsorption.

Although direct evidence of the function of the various components of the tubular RAS in the fetus and newborn is not available, it is likely that the relative immaturity of all or some of these control mechanisms may account for the negative Na^+ and water balance seen in premature infants. The relatively poor capacity for Na^+ reabsorption seen in the immature newborn explains the tendency for development of hyponatremia in these infants when they are given diets with Na^+ contents appropriate for older infants. As maturation proceeds, renal reabsorptive capacity for Na^+ increases, and the term infant maintains a positive Na^+ balance on various diets. In fact, acquisition of adequate Na^+ stores is required for cellular growth and bone mineralization.[118] It has been hypothesized that the ongoing deposition of Na^+ into growing bone may be the mechanism driving the higher set point for the RAS in the developing animal.[118]

Angiotensin II as a Growth Factor

The role of angiotensin II as a modulator of growth is now well established in various cardiovascular, renal, and other cell types and tissues. These effects have important implications for organogenesis, tissue maturation, glomerulosclerosis, and aging. In vascular smooth muscle cells, angiotensin II stimulation of AT_1 receptors elicits hypertrophic,[119,120] hyperplastic,[121] or dual effects.[122] In contrast, the AT_2 receptor inhibits proliferation in coronary endothelial cells.[123] Angiotensin II has also been shown to induce angiogenesis in the chick embryo chorioallantoic membrane.[124] Angiotensin II may also play a role in the architectural development and growth of the renal vasculature.

Within the various cell types of the nephron, angiotensin II has both hypertrophic and proliferative effects. The type of growth response to angiotensin II appears to be both species specific and cell specific, as well as developmentally regulated. Human and murine fetal mesangial cells respond to angiotensin II with proliferation,[110] whereas adult mesangial cells respond primarily with hypertrophy.[125,126] Proximal tubular cells from rabbits, mice, and pigs all respond to angiotensin II by increasing cell size and protein content,[119,127] whereas medullary thick ascending limb cells proliferate in response to the peptide.[128] The mechanisms by which angiotensin II exerts these effects remain incompletely understood, but to date all have been ascribed to the AT_1 receptor.[125,126] These responses may be mediated directly by angiotensin II, or they may involve various growth factors, protooncogenes, kinases, and phosphatases.[10]

The growth effects of angiotensin II have also been explored in whole animal models. Blockade of the neonatal rat AT_1 receptor with losartan results in decreased ratios of kidney to body weight and suppressed renal cell proliferation.[52] Similar results have been shown with use of ACE inhibitors.[129] The kidneys from treated animals exhibited fewer, smaller glomeruli and tubular dilation, as well as vascular malformations.[130,131] Kidneys from animals receiving an AT_2-specific inhibitor showed no abnormalities,[130] a finding indicating that the nephrogenic effects of angiotensin II are mediated through AT_1. The lack of AT_2 effect in this model is most likely the result of the rapid down-regulation of this receptor after birth.

Transgenic animals carrying targeted disruptions of components of the RAS raise new questions regarding the importance of angiotensin II in renal developmental processes. Animals lacking renin,[101,102] angiotensinogen,[100] ACE,[104] or both AT_{1A} and AT_{1B} receptors[132,133] demonstrate an increased neonatal mortality and progressive development of renal arterial and cortical disease after birth. Abnormalities include delayed glomerular maturation, tubular atrophy, decreased renal vascular branching, arterial and arteriolar wall thickening, and interstitial fibrosis. *AT_2*[134,135] homozygous null mice have no renal abnormalities on some genetic backgrounds but various genitourinary abnormalities on other backgrounds, suggesting that AT_2 control of renal development may be modified by other factors.[136] These results imply a central role for angiotensin II and its receptors in the appropriate development of the kidney and in the maintenance of structural integrity of the adult kidney.

Unfortunately, the growth effects of angiotensin II have also been demonstrated in human infants, in whom ACE inhibition during pregnancy has been associated with intrauterine growth restriction, renal dysplasia and anuria, pulmonary hypoplasia, bone malformations, and neonatal death.[112] These drugs are consequently contraindicated during pregnancy. Although nephrogenesis is complete in the human fetus by 32 to 36 weeks gestation, further growth and maturation of glomeruli and tubules occur until approximately 2 years of age. This brings into question the safety of ACE or other angiotensin II inhibitors during infancy. Premature and term newborns are very responsive to ACE inhibition, and they require lower doses and have more significant problems with decreased GFR, hyponatremia, and hyperkalemia than do older children.[108] These effects are explained by the highly activated state of the RAS during development and the known effects of RAS blockade. The potential effects of ACE inhibitors on renal growth and differentiation in premature and newborn infants have not been defined. Therefore the risks of these agents must be carefully weighed against the benefits of managing renin-mediated hypertension.

BOX 126-2

Primary Renal Tubular Dysgenesis

- Oligohydramnios
- Potter facies
- Anuria
- Hypotension
- Skull ossification defects
- Respiratory distress
- Perinatal death

BOX 126-3

Secondary Causes of Renal Tubular Dysgenesis

- Renal ischemia
- Twin-twin transfusion
- Major cardiac malformations
- Severe liver disease
- Infantile renal artery stenosis
- In utero exposure to renin-angiotensin system inhibitors

Recently, mutations in genes of the RAS (*renin, angiotensinogen, ACE*, or AT_1 receptor) in humans have been associated with autosomal recessive renal tubular dysgenesis, a severe disorder of tubular development characterized by persistent fetal anuria with oligohydramnios, Potter facies, hypotension, respiratory distress, and perinatal death, probably because of pulmonary hypoplasia (Box 126-2).[137,138] In the kidney this disease is characterized by an absence of proximal tubules. It has been proposed that renal lesions and early anuria result from chronic low perfusion pressure of the fetal kidney.[137] This syndrome should be differentiated from secondary causes of renal tubular dysgenesis resulting from renal ischemia as encountered in twin-twin transfusion, major cardiac malformations, severe liver disease, infantile renal artery stenosis, and in utero exposure to RAS inhibitors (Box 126-3). Altogether, those findings further support the crucial role of the RAS in human kidney development.

SUMMARY

The RAS is present and active at the earliest phases of development and appears to acquire different functions as maturation proceeds. Initially, the complex interplay between the mitogenic and trophic effects of angiotensin II and other growth mediators suggests an important role for angiotensin II in the tightly regulated processes of organogenesis and remodeling in the fetus. Along with other organ systems, these coordinated effects direct the kidney to become a properly differentiated and architecturally organized organ. At a slightly later time during development, coincident with differentiation and maturation of the kidney, the RAS gains the capability to control water and solute balance and renal hemodynamics. After birth, these mechanisms become more tightly regulated and efficient and allow the maintenance of homeostasis in the extrauterine environment.

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Jean-Pierre Guignard

Postnatal Development of Glomerular Filtration Rate in Neonates

The production of urine begins with the formation of an ultrafiltrate of plasma by the glomerulus. The function of the tubule is to modify this ultrafiltrate to allow an efficient excretion of waste products and the retention of those substances required to maintain body fluid volume and homeostasis. Glomerular filtration is also essential for the elimination of drugs. Alterations in glomerular filtration rate (GFR) have severe consequences on the body fluid homeostasis. An estimate of GFR may be required to prescribe fluids, electrolytes, or drugs excreted by the kidney. This chapter reviews the factors that regulate GFR and the methods available to assess GFR in the newborn infant.

PHYSIOLOGY OF GLOMERULAR FILTRATION

Each kidney contains approximately 1 million nephrons (227,327 to 1,825,380) consisting of a glomerulus and a tubule. The number of nephrons correlates with birth weight[1,2] (Figure 127-1), the count increasing by 25,743 for each 100 g of birth weight.[1] The glomerulus is a unique structure made out of a capillary network, the glomerular capillaries, supplied and drained by two resistance vessels, the afferent and the efferent arterioles. Urine formation starts by the production of an ultrafiltrate of plasma across the permselective glomerular capillary wall.[3,4] This wall consists of three layers: (1) the endothelial cell lining of the

glomerular capillaries; (2) the glomerular basement membrane, composed of connective, noncellular tissues; and (3) the visceral epithelial cells of the Bowman capsule. The endothelial cells have numerous fenestrae with a diameter of 70 to 100 nm. The capillary endothelium acts as a screen to prevent blood cells and platelets from entering into contact with the basement membrane. The endothelial cells contain on their surface negatively charged sialoproteins and proteoglycans. The endothelial glycocalyx could contribute to a primary glomerular anionic barrier that diminishes the load of macromolecules being passed into the glomerular basement membrane (GBM) and up to the slit diaphragms.[5] Under the endothelium, the GBM forms a continuous layer that probably behaves as the filtration barrier of large molecules. It is an amorphous, 300- to 350-nm–thick extracellular structure formed of negatively charged glycoproteins, mainly triple-helical type IV collagen, proteoglycans, laminin, and nidogen. The GBM-specific proteoglycans perlecan and agrin probably contribute to its charged selectivity because they contain negatively charged heparan sulfate side chains. The basement membrane is the main filtration barrier. The epithelium is formed by highly specialized cells called podocytes, which are attached to the basement membrane by foot processes known as *pedicels*.[6] Membrane proteins such as nephrin and prodocin are found in the foot processes and slit membranes. Mutations in these proteins result in massive proteinuria.[7] Adjacent pedicels are separated by filtration slits measuring approximately 25 by 60 nm in width, and each gap is bridged by a thin diaphragm. The diaphragms, in turn, contain rectangular "pores" with a dimension of 4 by 14 nm. Thus the filtration slits with their diaphragms could also constitute a size-selective filtration barrier. The podocytes may help in the phagocytosis of macromolecules. The size of the apertures in the glomerular filtration "barrier" is not the only factor that limits the passage of compounds through the glomerular capillary wall. The shape of the molecule, its flexibility and deformability, and its electrical charge also play important roles. The molecular weight (MW) cutoff for the glomerular filter is approximately 70,000 Da. Thus 69,000 Da MW albumin passes through the filter in minute quantities. Smaller molecules pass the filter more easily. Molecules with MW less than 7000 Da pass freely. The glomerular ultrafiltration thus initially contains small solutes and ions in the same concentration as present in the plasma.

The central part of the glomerular tuft is composed of irregularly shaped cells, the mesangial cells, that hold the delicate glomerular structures. By contracting, the mesangial cells can modify the filtering surface area of the glomerular capillaries. The mesangial cells also act as phagocytes to prevent the accumulation in the basement membrane of macromolecules that have escaped from the capillaries.

The rate of ultrafiltration is governed by several factors: the balance of Starling forces across the capillary wall, the rate at which plasma flows into the glomerular capillaries, the permeability of the glomerular capillary wall to water and small solutes, and the total surface area of the capillaries.[3] The ultrafiltration coefficient (K_f) is defined as the product of the glomerular capillary permeability and the area of the capillary available for filtration. The permeability of the glomerular capillaries is approximately 100 times greater than the permeability of other capillaries elsewhere in the body. The mean hydrostatic pressure within the glomerulus favors filtration. It is opposed by the oncotic pressure in the glomerular capillary. ΔP and $\Delta\pi$ represent the glomerular transcapillary hydrostatic and oncotic pressure, respectively. The net ultrafiltration pressure (P_{UF}) is defined as $\Delta P - \Delta\pi$. GFR is proportional to the sum of the Starling forces across the glomerular capillaries ($\Delta P - \Delta\pi$) times the K_f:

$$GFR = K_f\,(\Delta P - \Delta\,\pi)$$

In normal conditions, P_{UF} and GFR are highly dependent on the arterial pressure within the glomerular capillaries, on renal blood flow (RBF), and on the glomerular plasma flow rate. The transcapillary hydrostatic pressure is also regulated by the balance between the afferent and efferent arteriolar resistance (Figure 127-2). Pathologic conditions and drugs can affect GFR by

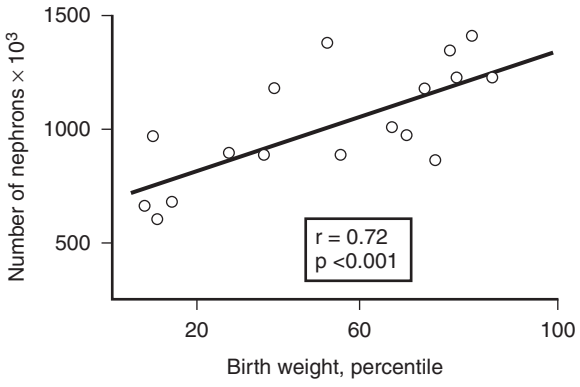


Figure 127-1 In humans the number of nephrons correlates positively with birth weight, here expressed in percentiles. *(Modified from Merlet-Bénichou C, Gilbert T, Vilar J, et al: Nephron number: variability is the rule—causes and consequences,* Lab Invest *79:515, 1999.)*

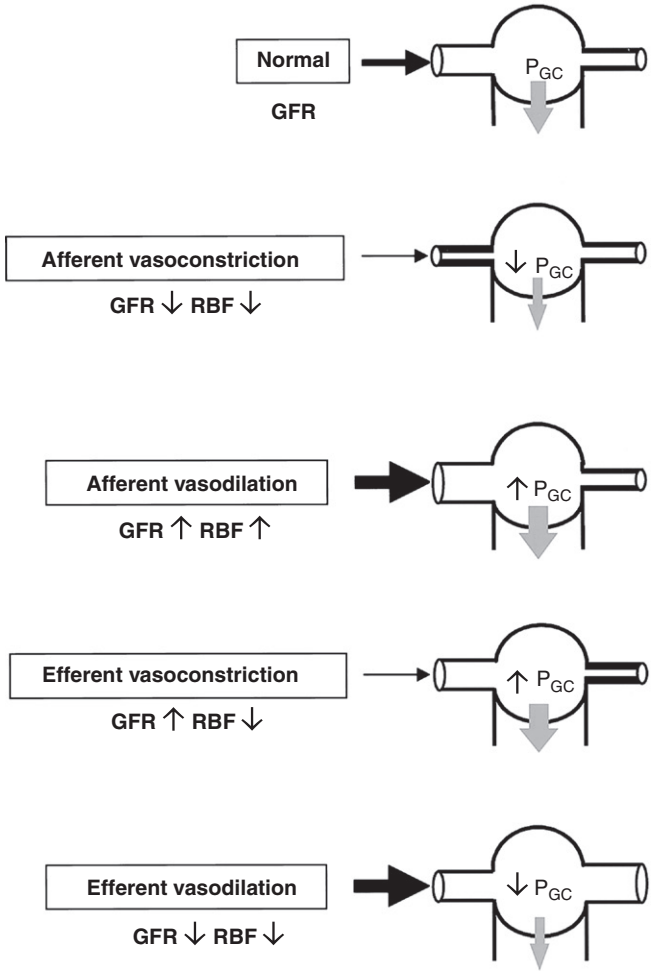


Figure 127-2 Effects of changes in the afferent or efferent arteriolar tone on renal blood flow *(RBF)* and glomerular filtration rate *(GFR)*. P_{GC}, Glomerular capillary hydraulic pressure.

modifying the pressure within the glomerular capillary (severe hypotension), the K_f (drugs, diseases), or the oncotic pressure within the glomerular capillary (changes in plasma proteins).

Vasoactive Agents

Several vasoactive agents modulate GFR and RBF during fetal and postnatal life.[8] Such agents include angiotensin II, the prostaglandins, atrial natriuretic peptide, endothelin, nitric oxide (NO), bradykinin, and adenosine. Sympathetic nerves can also affect vascular tone. All these factors modulate GFR by affecting afferent or efferent vascular tone (see Figure 127-2), as well as mesangial contractility. The main actions of the vasoactive agents are as follows.

Angiotensin II: This peptide is a very potent constrictor of the efferent arterioles and, to a lesser extent, of the afferent arterioles, acting on two types of receptors, the AT_1 and the AT_2 receptor subtypes. The AT_1 receptors are widely distributed and appear to mediate most of the biologic effects of angiotensin II. The exact role of the AT_2 receptors remains uncertain. Angiotensin II predominantly vasoconstricts the efferent arteriole, thereby increasing the intraglomerular pressure.[9] This mechanism serves to maintain GFR when the renal perfusion pressure decreases to low levels.

Prostaglandins: The prostaglandins are potent vasoactive metabolites of arachidonic acid.[10] They are of major importance for maintaining the GFR of the newborn kidney perfused at low arterial pressures. They further protect the kidney when vasoconstrictor forces are activated, as for instance during hypotensive, hypovolemic, and sodium depletion state or during congestive heart failure.[10]

Atrial natriuretic peptide: This 28–amino acid polypeptide increases GFR by producing afferent vasodilation and relative efferent vasoconstriction, thus increasing GFR without significantly affecting renal blood flow.[11] The kidney also produces a 32–amino acid natriuretic peptide, urodilatin, with the same local actions as atrial natriuretic peptide.

Nitric oxide: NO is a very potent vasodilator synthesized from L-arginine in endothelial cells throughout the body. In superficial nephrons, NO appears to decrease the preglomerular resistance with much less effect on the postglomerular resistance.[12] In juxtamedullary nephrons, NO decreases both the afferent and the efferent resistance. NO appears essential to fine-tune the vasoconstrictor action of angiotensin II.[13]

Bradykinin: This vasodilator and diuretic peptide is produced in the kidney by the effect of the enzyme kallikrein on kininogen. Bradykinin exerts its renal effects via β_2-receptors. The expression of this receptor is higher in neonatal than in adult kidneys, a finding suggesting a role for this peptide during renal development. Bradykinin vasodilates the newborn kidney, as evidenced by the renal vasoconstriction that results from β_2-receptor blockade.[14] It decreases the ultrafiltration coefficient.

Adenosine: Adenosine is an end product of adenosine triphosphate metabolism. Adenosine decreases GFR by vasodilating the efferent arteriole via A_2 receptor stimulation and by vasoconstricting the afferent arteriole via A_1 receptor stimulation.[15] By constricting the mesangial cells, adenosine further decreases the ultrafiltration coefficient.

Endothelin: This peptide released from endothelial cells constricts the afferent and the efferent arterioles, thereby decreasing both GFR and RBF. However, circumstantial evidence exists that, at low endogenous concentrations, endothelin may actually vasodilate the glomerular vessels in fetuses and neonates.[16,17] Endothelin stimulates whole kidney production of vasodilating prostaglandins that can partially balance its vasoconstrictive effects.

Sympathetic nervous system: The sympathetic nerve endings are primarily of the α_1- and β-adrenergic subtypes and secrete norepinephrine. Modest increases in renal nerve activity produce equivalent constrictions of both the afferent and efferent arterioles, reducing RBF without significantly affecting GFR.[18] At elevated concentrations, as occurring in severe acute hemorrhage fetal distress and asphyxia, norepinephrine vasoconstricts the mesangial cells, thus decreasing GFR.

Autoregulation of Glomerular Filtration Rate and Renal Blood Flow

Autoregulation is necessary to prevent abrupt changes in GFR and RBF when blood pressure varies acutely. Two systems are responsible for renal autoregulation: (1) a myogenic mechanism and (2) a tubuloglomerular feedback mechanism.

The myogenic mechanism refers to the intrinsic ability of arteries to constrict when blood pressure rises and to vasodilate when it decreases. This phenomenon prevents excessive changes in RBF and GFR when blood pressure varies.

The tubuloglomerular feedback mechanism involves the macula densa and the juxtaglomerular cells. The macula densa cells sense the changes in sodium chloride delivery to the distal tubule that follow changes in blood pressure. A drop in blood pressure and the consequent decrease in sodium chloride delivery stimulate angiotensin II formation by the juxtaglomerular cells. By constricting the efferent arteriole, angiotensin II increases the intraglomerular hydrostatic pressure and thus returns GFR toward normal levels.

CONCEPT OF CLEARANCE

The most common measurement of GFR is based on the concept of *clearance,* which relates the quantitative urinary excretion of a substance per unit time to the volume of plasma that, if "cleared" completely of the same contained substance, would yield a quantity equivalent to that excreted in the urine. The clearance *(C)* of a substance *(x)* is expressed by the following formula:

$$C = U_x \cdot V / P_x$$

where U_x represents the urinary concentration of the substance, V the urine flow rate, and P_x the plasma concentration. For its clearance to be equal to the rate of glomerular filtration, a substance must have the following properties: (1) it must be freely filterable through the glomerular capillary membranes, that is, not be bound to plasma proteins or sieved in the process of ultrafiltration; (2) it must be biologically inert and neither reabsorbed nor secreted by the renal tubules; and (3) it must be nontoxic and not alter renal function when infused in quantities that permit adequate quantification in plasma and urine.

Several substances, endogenous or exogenous, have been claimed to have the foregoing properties: inulin, creatinine, iohexol, ethylenediaminetetraacetic acid, diethylenetriaminepentaacetic acid, and sodium iothalamate. The experimental evidence that this is true has been produced only for inulin. The most commonly used markers in neonates are creatinine and inulin.

GLOMERULAR MARKERS

Inulin

Inulin, a fructose polysaccharide derived from dahlia roots and Jerusalem artichokes, has an Einstein-Stokes radius of 1.5 nm and a MW of approximately 5200. It diffuses as would a spherical body of such radius. Inulin is inert, is not metabolized, and can be recovered quantitatively in the urine after parenteral administration.

The rate of excretion of inulin is directly proportional to, and a linear function of, the plasma concentration of inulin over a wide

range. The clearance of inulin *(U • V/P)* is consequently independent of its plasma concentration. Evidence that inulin is neither reabsorbed nor secreted by the renal tubules has been obtained in experimental micropuncture studies showing that (1) the concentration of inulin was identical in the Bowman space fluid and plasma, (2) 99.3% of inulin injected in the proximal tubule could be collected in the distal tubule, and (3) the rate of recovery was the same when the peritubular plasma was loaded with inulin.[19]

Because the renal excretion of inulin thus occurs exclusively by glomerular filtration, its clearance is the most accurate index of GFR. Estimates of inulin clearance provide the basis for a standard reference against which the route or mechanisms of excretion of other substances can be ascertained.

Inulin as a Marker of Glomerular Filtration Rate in Neonates

The hypothesis that inulin may not be freely filtered across the immature glomerulus has not been confirmed by studies of inulin handling in several species. The same conclusion was reached from studies in preterm infants that showed that higher-MW inulin did not accumulate in the plasma of very immature babies infused with inulin for several days, thus excluding any retention of the larger molecules.[20,21]

In clinical practice, sinistrin, a readily soluble preparation of polyfructosan with side branching (extracted from bulbs of *Urginea maritima*), is more widely used. The clearance of sinistrin is identical to that of inulin. Because complicated analytic methods are required for its measurement, inulin and sinistrin cannot be used for routine clinical purposes. In neonates, only creatinine has been used broadly to assess GFR.

Creatinine

Creatinine is the anhydride of creatine, a compound that exists in skeletal muscle as creatine phosphate. It has a MW of 113 Da. Conversion of creatine to creatinine is nonenzymatic and irreversible. The serum creatinine level reflects total body supplies of creatine and correlates with muscle mass. Creatinine is excreted through the kidneys in quantities proportional to the serum content. The renal excretion of endogenous creatinine is very similar to that of inulin in humans and several animal species. However, in addition to being filtered through the glomerulus, creatinine is secreted in part by the renal tubular cells. In spite of this, creatinine clearance correlates well with inulin clearance when the GFR is normal. This agreement results from the balance of two factors: (1) excretion rate of creatinine is higher than the filtered rate because of the occurrence of tubular secretion of creatinine, and (2) the measured plasma creatinine is higher than the true creatinine because of the presence of noncreatinine chromogens that interfere with the colorimetric analysis of creatinine *(Jaffe reaction).*

Overestimation of GFR by creatinine clearance is usually more evident at low GFR. As GFR falls progressively during the course of renal disease, the renal tubular secretion of creatinine contributes an increasing fraction to urinary excretion, so that creatinine clearance may substantially exceed the actual GFR.

The use of creatinine clearance to estimate GFR may be unreliable when one studies uremia in patients. Creatinine is uniformly distributed in the body water, and it diffuses into the gut. At a normal plasma concentration, the amount of creatinine entering the gut is negligible; it may become significant during renal failure when the plasma creatinine increases.[22] This phenomenon may also explain why creatinine clearance overestimates true GFR in patients with renal failure.

Although in use for decades, the methods available for the chemical determination of creatinine still present important drawbacks. The traditional assay for measuring creatinine is based on the alkaline picrate Jaffe reaction. Because of the presence of interfering pseudochromogenic constituents in the blood, this method substantially overestimates true serum creatinine levels. The major drawback for routine use in neonates is interference by bilirubin. Adaptations of the alkaline picrate assay have reduced the overestimation without totally eliminating the interference.[23] Although more specific than the Jaffe method, the enzymatic techniques are still biased by various interfering substances.[23] A new method coupling high-performance liquid chromatography and isotope dilution mass spectrometry appears to have an excellent specificity and low relative standard deviation.[24]

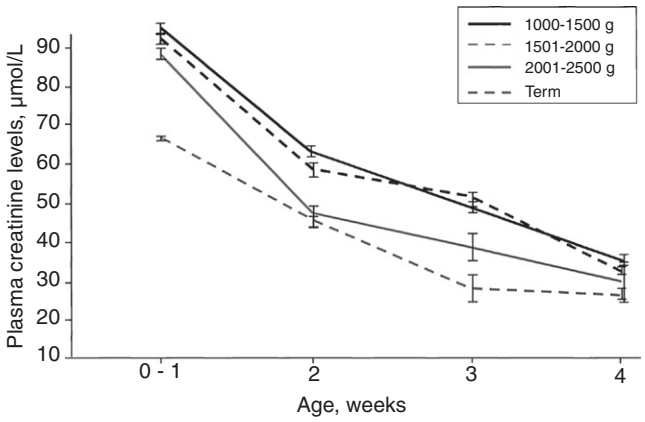


Figure 127-3 Plasma creatinine concentrations (micromoles per liter) during the first weeks of life. *(From Bueva A, Guignard JP: Renal function in preterm neonates,* Pediatr Res *36:572, 1994.)*

Creatinine as a Marker of Glomerular Filtration Rate in Neonates

The plasma creatinine concentration varies during the first postnatal weeks.[25,26] It is elevated at birth and decreases rapidly during the first week of life (Figure 127-3); values stabilize at approximately 0.40 mg/dL (35 µmol/L; range, 0.14 to 0.70 mg/dL [12 to 61 µmol/L]) on the fifth postnatal day in term infants and somewhat later in very-low-birth-weight infants.[26,27] The elevated plasma creatinine concentration at the time of birth reflects maternal creatinine levels. Indeed, a perfect equilibrium between fetal and maternal plasma creatinine concentrations has been observed throughout gestation.[28,29] In very premature neonates, the elevated plasma creatinine at birth increases transiently; the highest levels are reached by the third day of life (Figure 127-4 and Table 127-1).[30,31] The plasma urea level also rises significantly over time, but it does so in a more variable manner. This postnatal transient increase in plasma creatinine values is probably the consequence of creatinine reabsorption (back diffusion) across leaky tubules,[32] as suggested by studies in piglets and newborn rabbits.[32,33] Plasma creatinine levels may take a month to reach *neonatal* levels in very-low-birth-weight infants.[27,28]

Iohexol

Iohexol is a nonionic contrast agent with a MW of 821 Da that appears to be eliminated exclusively by glomerular filtration. In spite of reports showing a significant correlation between the plasma disappearance curve of iohexol and the standard clearance of inulin, its usefulness in clinical pediatric practice remains to be demonstrated. It should not be used in the neonatal period.

Iothalamate Sodium

Iothalamate sodium has a MW of 637 Da. It is only minimally bound to plasma proteins,[34] and its clearance is independent of variations in plasma activity. The clearance of iothalamate was

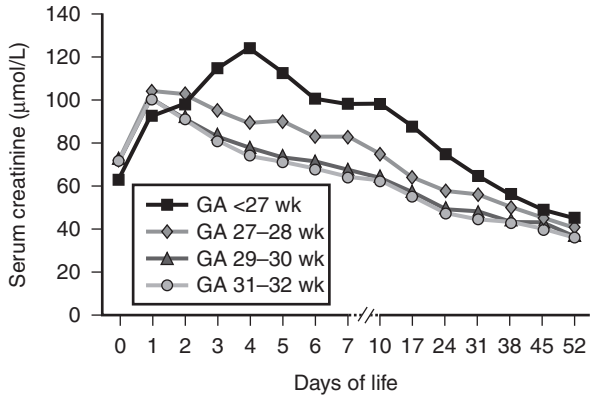


Figure 127-4 Changes in serum creatinine in preterm neonates during the first 52 hours of postnatal life. Peak increases were noted on day 4 in the most premature infants. *(Modified from Gallini F, Maggio L, Romagnoli C, et al: Progression of renal function in preterm neonates with gestational age ≤32 weeks,* Pediatr Nephrol *15:119, 2000.)*

TABLE 127-1

Changes in Plasma Creatinine Over Time for Different Gestation Groups

Group gestation age (wk)	Birth plasma creatinine (μmol/L)*	Peak plasma creatinine (μmol/L)*	Time to peak plasma creatinine (hr)*
23-26	67-92	195-247	40-78
27-29	65-89	158-200	28-51
30-32	60-69	120-158	25-40
33-45	67-79	99-140	8-23

Modified from Miall LS, Henderson MJ, Turner AJ, et al: Plasma creatinine rises dramatically in the first 48 hours of life in preterm infants, *Pediatrics* 104:e76, 1999.
*95% confidence intervals.

initially shown to correlate well with that of inulin. However, later studies unequivocally demonstrated that iothalamate is actively secreted by the renal tubules and perhaps also undergoes tubular reabsorption in humans and animal species.[35] The agreement of iothalamate clearance with inulin clearance appears to be a fortuitous cancellation of errors between tubular excretion and protein binding. Iothalamate sodium has rarely been used in human neonates and in neonatal animal studies. Its use during the first month of life should be avoided.

Radioisotopic Clearance Studies

Radioisotopic substances should not be used in the neonatal period and will not be discussed here.

CLINICAL ASSESSMENT OF GLOMERULAR FILTRATION RATE IN NEONATES

Standard Clearances

Inulin Clearance

Classic inulin clearance studies have been performed in premature and term neonates to provide data on (1) the reliability of inulin as a marker of GFR in human neonates, (2) the development of GFR in early postnatal life, and (3) the effect of disease states on GFR.

In the classic method, inulin is administered as a priming dose to achieve plasma concentrations close to 300 to 400 mg/L and is constantly infused to maintain such levels. Accurate urine collection is performed by using bladder catheterization, spontaneous voiding into plastic bags, or a collection tray. The clearance study is performed over a period of 3 to 4 hours.

Several studies clearly demonstrated that inulin is freely filtered in even the most immature of human patients.[20,21] The standard inulin clearance must thus be considered the reference test in human neonates with which all other methods of estimating GFR should be compared.

Creatinine Clearance

Creatinine is the most commonly used marker of GFR. Its clearance has been claimed to approximate true GFR in both term and preterm neonates. The validity of creatinine clearance has been assessed in low-birth-weight infants (mean birth weight, 1600 g; range, 1040 to 2275 g; postnatal age, 10 hours to 10 days). A significant correlation was found ($r = 0.738$) between inulin and creatinine clearance, but the scatter of values was substantial.[36] In infants with the lowest GFR (C_{in} <12.5 mL/minute per 1.73 m^2), creatinine clearance overestimated true GFR, whereas it underestimated it in infants with higher filtration rates. A substantial underestimation of GFR by creatinine clearance has usually been reported in preterm and mature neonates.[37] Measurement of creatinine by a manual resin adsorption method, rather than by the automated method, improved the correlation.[37] Several factors can account for the variability of creatinine clearance determinations in very premature infants. These factors fall into two major categories: (1) those related to the transport of creatinine by the premature kidney and (2) those affecting the accuracy of plasma creatinine assays. Because of the low-normal levels of creatinine in the blood of neonates, small variations in laboratory measurement may spuriously alter the estimated concentration. Most clinical laboratories determine creatinine with the Jaffe reaction, which may be affected by many interfering substances, such as bilirubin. Values obtained by this method greatly overestimate the true creatinine concentration at values lower than 1.0 mg/dL.[38]

Inaccuracies in determining the true creatinine concentration in neonates, as well as uncertainties in renal tubular handling, point to a possible drawback in the use of creatinine as a glomerular marker, at least in very-low-birth-weight infants. With these limitations in mind, the true creatinine clearance probably remains the best index of GFR for routine use.[39,40]

Estimation of Glomerular Filtration Rate Without Urine Collection

Constant Infusion of Inulin Without Urine Collection

The constant infusion technique[41] assumes that the rate of intravenous infusion needed to maintain the plasma concentration of inulin at a constant level is equal to the rate of its excretion. At constant plasma levels, after inulin has equilibrated in its diffusion space, the $U \cdot V/P$ clearance must be equal to the rate of infusion (I) divided by the plasma concentration: $C = U \cdot V/P = I/P$. To accelerate the achievement of a steady plasma concentration of inulin, a loading dose of inulin precedes the constant intravenous infusion. An estimate of the extracellular fluid volume (where inulin distributes) and of GFR is required to select the correct loading dose and infusion rate.

In studies in which inulin was constantly infused for only a few hours, significant overestimation of true GFR has been observed.[42] As demonstrated by Coulthard and Ruddock,[20] reliable estimates of GFR can be obtained, provided inulin is constantly infused for 24 hours, with or without bolus injection at the start of the test. This method has the obvious advantage of eliminating the need for urine collection. Its main disadvantage is that it requires a constant infusion of long duration, as well

as careful supervision of the test. Should the infusion stop for a moment, a long extra period of infusion would be necessary because the plasma inulin falls exponentially but only rises again asymptomatically. The constant infusion method also has the disadvantage of not reflecting acute changes in GFR.[41,43]

Single-Injection (Plasma Disappearance Curve) Technique

The mathematical model for this technique is an open two-compartment system. The glomerular marker is injected in the first compartment, equilibrates with the second compartment, and is excreted from the first compartment by glomerular filtration. To obtain a well-defined plasma disappearance curve, and therefore an accurate calculation of the plasma clearance, numerous blood samples are required. Extension of the sampling period to 4 to 5 hours improves the precision of the results.

The single-injection method has been used in neonates, most often by using inulin as a glomerular marker. Inulin is injected intravenously at a dose of 100 mg/kg, and the plasma concentration is measured at regular intervals over a few hours. Simplified techniques have been proposed that are based on a single-compartment model. They obviate the need for frequent blood sampling, but they are less accurate.

Results comparing data obtained by the single-injection technique with those obtained by the standard inulin clearance method are conflicting. Early studies using the plasma disappearance curve of inulin or polyfructosan claimed it to be a reliable index of GFR.[44] Later studies, however, questioned the validity of the single-injection technique in neonates. The overestimation in the younger neonates was ascribed to incomplete equilibration of inulin in its diffusion space during the 130 minutes of the test.[45]

Simple Creatinine Clearance Method in Neonates Without Urine Collection

A formula often used to estimate GFR in children is

$$\text{GFR (mL/min)} \times 1.73\ \text{m}^2 = k \cdot L / P_{Cr}$$

where k is a constant, L (centimeters) represents body length, and P_{Cr} (milligrams per deciliter) is the plasma creatinine concentration. This formula is based on the assumption that creatinine excretion is proportional to body height and is inversely proportional to plasma creatinine.[46] The value of factor k can be obtained from the formula k = GFR • L/P_{Cr}. Under steady-state conditions, k should be directly proportional to the muscle component of body weight, which correlates reasonably well with the daily urinary creatinine excretion rate. The value of k has been determined in term and low-birth-weight neonates, with use of the 8- to 12-hour creatinine clearance as an estimate of GFR in the formula $k = C_{Cr} \cdot L/P_{Cr}$.[47] The mean value of k, calculated in 118 low-birth-weight infants with a corrected age of 25 to 105 weeks, was 0.33 ± 0.01. It rose to 0.45 in term infants up to 18 months.[46] When the plasma creatinine was expressed in micromoles per liter, the corresponding values were 29 and 40, respectively. In both groups, a large scatter of values for k was observed, which the authors ascribed to the variability in body composition, differences in diet and creatinine excretion, errors in collection of urine, and inaccuracies in the measurement of creatinine. In spite of these limitations, the formula was claimed to be useful, because it correlated well with the inulin single-injection technique.[47] It is unfortunate that the $k \cdot L/P_{Cr}$ formula has not been validated in neonates by comparing its results with those given by the standard $U \cdot V/P$ inulin clearance.

The accuracy of the $k = L/P_{Cr}$ formula as an estimate of GFR has been questioned.[42,48] In a study in infants younger than 1 year of age, the value of k varied from 0.17 to 0.82 (15 to 72 when P_{Cr} was given in micromoles per liter), even though it was derived from the standard inulin clearance. Factor k was found to vary markedly with the state of hydration. Moreover, the regression line relating the clearance estimated from the formula with the results obtained from the standard inulin clearance differed significantly from the identity line.[41]

It is true that the $k \cdot L/P_{Cr}$ formula may be more informative clinically than the plasma creatinine alone because the creatinine value, in addition to renal function, is critically dependent on the percentage of muscle mass. Caution should be exercised, however, when using the formula as an estimate of GFR in studies aimed at defining pathophysiologic mechanisms in neonates.

Detection of an Abnormal Glomerular Filtration Rate by Cystatin C

Cystatin C, a nonglycosated 13-kDa basic protein, is a proteinase inhibitor involved in the intracellular catabolism of proteins.[49] It is produced by all nucleated cells, freely filtered across the glomerular capillaries, almost completely reabsorbed, and catabolized in the renal proximal tubular cells. Its production rate is apparently constant and was initially claimed to be independent of inflammatory conditions, muscle mass, and sex. This claim has been questioned by a large recent study in 8058 adult inhabitants of Groeningen.[50] In this study, male sex, older age, greater weight, higher serum C-reactive levels, and cigarette smoking were independently associated with higher cystatin C levels after adjusting for creatinine clearance. It thus appears that, in adults at least, cystatin C levels are influenced by factors other than renal function alone. Cystatin C does not appear to cross the placental barrier, and no correlation exists between maternal and neonatal serum cystatin C levels.[51] Cystatin C concentrations are highest at birth and then decrease, to stabilize after 12 months of age (Figure 127-5). It is uncertain whether cystatin C is significantly higher in premature infants as compared with term infants.[52,53] Mean values of 1.63 ± 0.26 mg/L (mean ± SD) were recorded during the first month of life, 0.95 ± 0.22 mg/L during months 1 to 12, and 0.72 ± 0.12 mg/L after the first year of life.[54] The concentration of cystatin C has been claimed to offer a greater sensitivity than creatinine in detecting an abnormal GFR in newborn infants, as well as in children or adults. Whether this advantage is clinically significant is doubtful, however. The use of cystatin C to predict GFR has important drawbacks. Because cystatin C is not excreted in the urine, the GFR predicted from its plasma concentration cannot be verified by its clearance. Cystatin C only predicts the presence of renal failure and not the precise level of GFR. In children, cystatin C has also been shown to be less reliable than the Schwartz formula in distinguishing impaired from normal GFR.[55] In addition, the assessment of GFR by the Schwartz formula has the obvious advantage of providing rational semiquantitative values of GFR related to the body surface area.

DEVELOPMENT OF GLOMERULAR FILTRATION RATE

Glomerular Filtration Rate at Birth

GFR is low at birth. Standard inulin clearance studies performed by Guignard and co-workers[56-58] on the first 2 days of life indicated that GFR related to the body surface area increases rapidly from the 28th to the 35th week of gestation (Figure 127-6). At the 35th week of gestation, GFR reaches a plateau that is maintained up to the time of birth, a finding reflecting the parallel increase in kidney size and function. In these studies, GFR approximated 20 mL/minute × 1.73 m^2 at birth in term infants and 12 to 13 mL/minute × 1.73 m^2 in preterm infants of 28 to 30 weeks of gestation. Low values of GFR of 0.85 mL/kg/minute, as assessed by the inulin constant infusion technique, were also recorded by van der Heijden and colleagues[59] on the second day of life of neonates. Using standard creatinine clearance techniques to assess GFR, Siegel and Oh[40] and Gallini and associates[31] reported somewhat lower values of GFR in 2-day-old premature infants with gestational age of less than 30 weeks. This underestimation

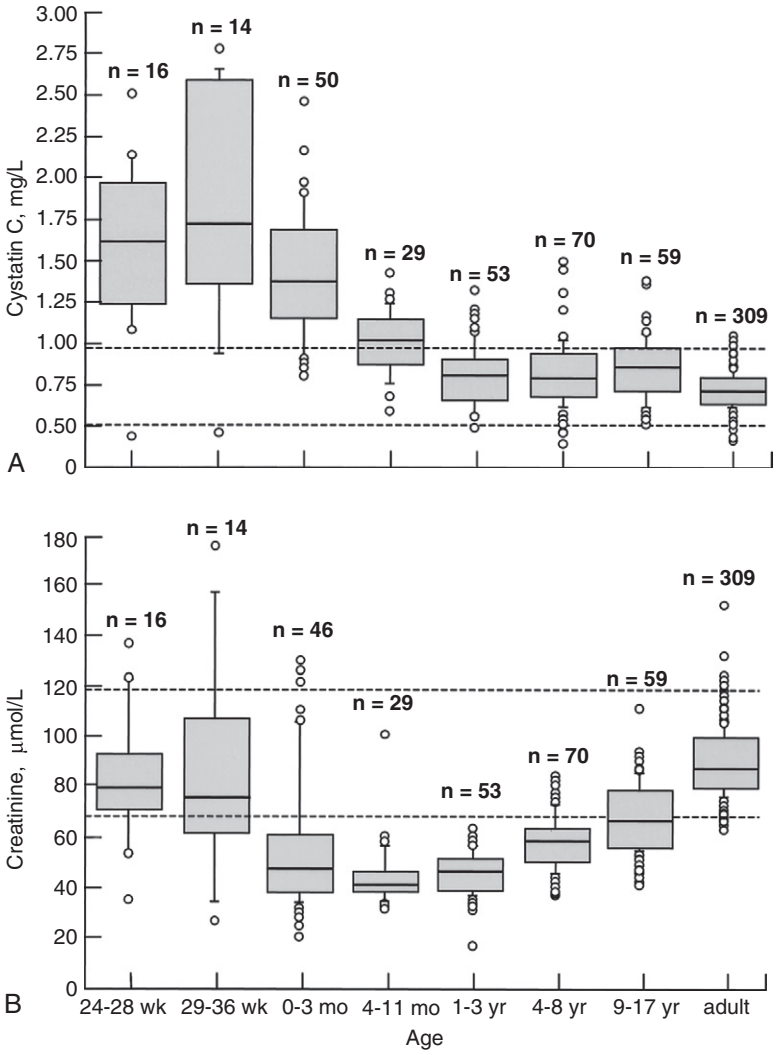


Figure 127-5 Box plot distributions showing **(A)** cystatin C and **(B)** creatinine values (10th, 25th, 50th, and 90th centiles) across the age-groups. The categories of 24 to 28 weeks and 29 to 36 weeks refer to gestational ages of preterm infants. *Dotted lines* indicate the 95% confidence interval of the adult range. Preterm infants born between 24 and 36 weeks gestation were 1 day old. *(Modified from Finney H, Newman DJ, Thakkar H, et al: Reference ranges for plasma cystatin C and creatinine measurements in premature infants, neonates, and older children,* Arch Dis Child *82:71, 2000.)*

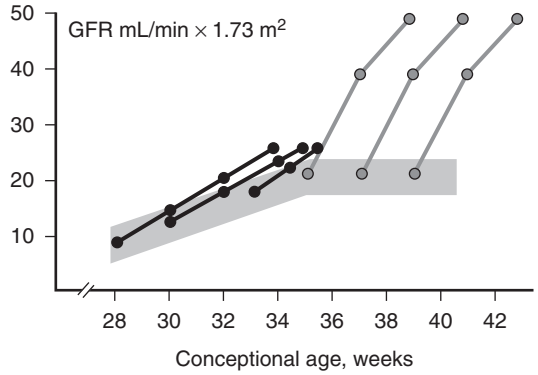


Figure 127-6 Development of GFR in relation to conceptional age during the last 3 months of gestation and the first month of life. The *shaded area* represents the range of normal values. The postnatal increase in GFR observed in neonates born before (•) or after (◦) 35 weeks gestation is schematically represented. *(Modified from Guignard JP: Renal function in the tiny, premature infant,* Clin Perinatol *13:377, 1986.)*

of GFR by the clearance of creatinine was also observed by Coulthard and co-workers.[60] Such an underestimation can be accounted for by the reabsorption of creatinine across leaky immature tubules.[28,32] In all these studies performed on the first days of life, GFR correlated with gestational age.

Maturation of Glomerular Filtration Rate in the First Month of Life

A rapid increase in GFR takes place in the first month of life. As shown in Figure 127-7, inulin clearance doubles in the first 15 days of life.[56-59] The rate of increase in GFR is somewhat lower in the most premature infants. In spite of this impressive increase in GFR, as expressed in milliliters per minute per 1.73 m^2, the values achieved at 1 month of life are still only 50% of the adult values that are reached during the second year of life (Figure 127-8). This can be considered a state of "physiologic" renal insufficiency. Using the 24-hour constant infusion method in preterm infants, van der Heijden and colleagues[59] confirmed that GFR increases rapidly after birth; the postnatal increase is independent of body weight.

A similar pattern of maturation was observed in 66 physiologically stable term and premature infants undergoing creatinine clearance studies.[27] The lowest values of creatinine clearance were observed in the infants with the lowest birth weight (mean birth weight, 1332 ± 40 g; mean gestational age, 31.3 ± 0.5 weeks). This was true both for absolute values of creatinine clearance and for values expressed in relation to the body surface area. The progressive increase in creatinine clearance observed in the first 15 days of life also correlated significantly with postnatal age.[27,59-62] The study by Gallini and associates[31] confirmed the occurrence of a steady increase in creatinine clearance over the first 52 days of life, at which time values close to 42 mL/minute × 1.73 m^2 and 27 mL/minute × 1.73 m^2 were recorded in term

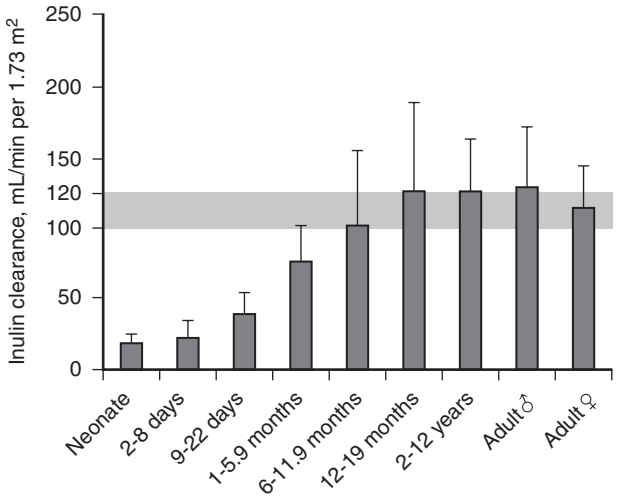


Figure 127-7 Changes in GFR (inulin clearance) as a function of age.

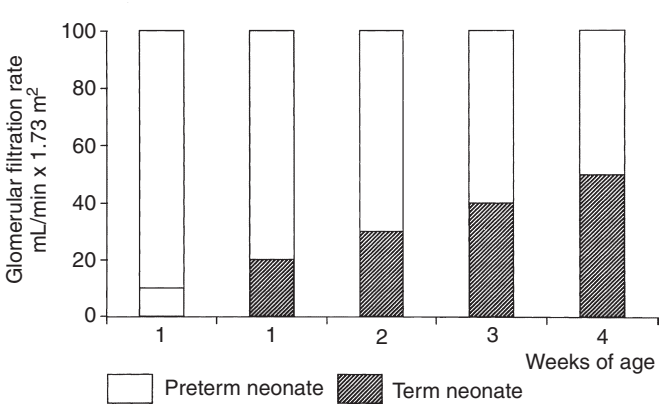


Figure 127-8 Maturation of GFR during the first month of life of newborn infants. The *open columns* represent normal adult values. *(From Guignard JP, Drukker A: Clinical nephrology. In Barratt TM, Avner ED, Harmon WE, editors:* Pediatric Nephrology, *Baltimore, 1999, Lippincott Williams & Wilkins.)*

neonates and neonates born at less than 27 weeks gestational age, respectively.

Determinants of the Postnatal Increase in Glomerular Filtration Rate

Several factors account for the striking postnatal maturation of GFR: (1) a decrease in renal vascular resistance and consequent increase in RBF, (2) an increase in systemic blood pressure and in glomerular plasma flow rate, (3) an increase in the effective filtration pressure, and (4) an increase in the K_f.

1. Renal vascular resistance is elevated at birth. It decreases rapidly in the first postnatal weeks. In rats, both afferent and efferent arteriolar resistances decrease by a factor of 3 during maturation.[63] The elevated renal vascular resistance is mainly the result of the high activity of vasoactive agents and hormones and the relative responsiveness of the newborn infant to these vasoconstrictor and vasodilator substances. The decrease is associated with a rise in RBF and an improvement in glomerular plasma flow rate. In human neonates, the maturational increase in GFR parallels the increase in RBF.[56] Improved RBF and glomerular plasma flow rate also probably explain the 30% immediate increase in inulin clearance associated with delayed clamping of the umbilical cord.[64]
2. The striking increase in systemic blood pressure occurring during the first weeks of life (Table 127-2) is associated with an increase in the glomerular capillary hydrostatic pressure. This favors filtration. Creatinine clearances measured on the first 2 days of life in neonates of 28 to 43 gestational weeks have been shown to correlate significantly with blood pressure.[27,56]
3. The low oncotic pressure present in newborn infants resulting from low plasma protein concentrations favors the ultrafiltration pressure. Furthermore, because the glomerular hydrostatic pressure increases more rapidly than the oncotic pressure during development, the effective filtration pressure rises. Because of the low systemic blood pressure, the glomerular capillary hydraulic pressure is extremely low in early life and increases in parallel with blood pressure. The glomerular transcapillary hydraulic pressure difference increases by 10 mm Hg from the third week of life to adulthood in rats.[65] In guinea pigs, the effective filtration pressure increases 2.5 times in the first 50 days of life.[66] Increases in glomerular capillary hydraulic pressure and glomerular transcapillary hydraulic pressure difference could contribute to changes in GFR during early maturation.

TABLE 127-2

Normal Mean Arterial Blood Pressure in the Newborn Infant

Age	<1.0 kg	1.0-1.5 kg	>2.5 kg
Birth	33 ± 15	39 ± 18	49 ± 19
1 week	41 ± 15	47 ± 18	60 ± 19
2 weeks	45 ± 15	50 ± 18	64 ± 19
4 weeks	48 ± 15	53 ± 18	68 ± 19

Values expressed in mm Hg as mean ± 95% confidence limits for single measurements; measurement via umbilical artery catheterization or by Dynamap, GE Health Care, Missouri City, TX.

From Ong WH, Guignard JP, Sharma A, Arandaet JV: Pharmacological approach to the management of neonatal hypertension, *Semin Neonatol* 3:149, 1998.

4. The K_f reflects both the surface and the permeability of the filtration barrier. Maturational changes in K_f also account for the postnatal rise in GFR. The K_f is low in the newborn. It is significantly lower in 40- to 64-day-old rats than in adult animals.[67,68] The glomerular basement membrane surface area has been shown to increase 3.5-fold from birth to adulthood in rats,[69] whereas the glomerular capillary cross-sectional area increased 10-fold from 3 weeks to adulthood in dogs.[70] A 40% increase in glomerular corpuscular diameter has been observed in humans from birth to adulthood.[71] If they correspond to increases in the glomerular filtering area, these changes should improve GFR. The increases in the area of the endothelial fenestrae observed in growing rats can also explain the postnatal rise in GFR. From studies in 1- and 6-week-old dogs, Goldsmith and colleagues[72] concluded that an increase in both the glomerular filtering area and pore density accounted for the postnatal increase in GFR.

The increase in the number of negatively charged sites in the GBM[73] and the 2.5-fold increase in GBM thickness[68] observed in growing rats could account for the decreased permeability to large molecules (proteins) in the first postnatal weeks of human neonates. Clearance studies in human infants infused with inulin or polyfructosides with variable MWs have clearly shown that the high-MW inulin did not accumulate in preterm infants (birth weight of 850 to 1250 g) infused with inulin for 2 to 10 days.[20] The filterability of inulin (MW 5200) appears similar in neonates and older children. This does not, however, exclude subtle changes in glomerular capillary permeability during growth. Experimental studies on isolated rat glomeruli also failed to demonstrate an increase in glomerular hydraulic permeability during growth.[74]

FACTORS THAT CAN IMPAIR GLOMERULAR FILTRATION RATE IN THE PERINATAL PERIOD

The low GFR of the very-low-birth-weight infant is maintained by a delicate balance of intrarenal vasoconstrictor and vasodilator forces. In immature animals, various conditions such as hypoxemia, hypercapnia, metabolic acidosis and alkalosis, hypothermia, and hyperthermia can dramatically increase renal vascular resistance. Similarly human neonates are prone to the development of vasomotor nephropathy and acute renal failure, the main causes including hypotension, hypovolemia, hypoxemia, and perinatal asphyxia.[75] Other causes include the administration of angiotensin-converting enzyme inhibitors, nonsteroidal anti-inflammatory drugs, and tolazoline. Positive-pressure artificial ventilation also impairs GFR. In all these situations, the interaction between the vasoconstrictive and vasodilation forces that are stimulated are rather complex. Several of the vasoconstrictor or vasodilatory forces indeed differ in their effects on the systemic and the intrarenal circulations.

Perinatal hypoxemia and asphyxia are common causes of impaired GFR in neonates. The renal vasoconstriction is the consequence of the overactivation of the renin-angiotensin system, intrarenal adenosine, vasopressin, the catecholamines, and other vasoconstrictor substances.[75] Contraction of blood volume (hypovolemia) with or without failure of the cardiac pump is often presented in the neonates.[76] Experimental neonatal studies on the hypoxemia-induced decrease in GFR suggest a key role for intrarenal adenosine[77] in mediating the vasomotor nephropathy. By decreasing the postglomerular resistance, this agent induces a fall in the intraglomerular pressure and consequently GFR (Figure 127-9). The hypothesis that adenosine plays a key role in the pathogenesis of the hypoxemic vasomotor nephropathy is supported by the fact that theophylline, an antagonist of adenosine cell surface receptors, protects the immature kidney from the deleterious hypoxemic stress. Recent clinical studies using theophylline in high-risk asphyxiated neonates with respiratory distress syndrome[78-80] or in neonates with severe respiratory distress syndrome[81] suggest a beneficial effect of theophylline in protecting GFR. Interestingly enough, in the neonatal rabbit model, the specific adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine does not offer the same protection as theophylline.[82] Other conditions that impair GFR by stimulating vasoconstrictive forces are hypothermia, hyperthermia, and severe respiratory or metabolic acidosis.[75]

Ventilation of human neonates and newborn animals with high airway pressures has deleterious effects on renal function resulting from decreased venous return and low cardiac output, increased renal sympathetic nervous activity, and high serum vasopressin levels.[83]

ACE inhibitors can lead to a dramatic fall in mean arterial pressure accompanied by persistent oliguria.[75] The oliguric renal insufficiency is similar to that observed in fetuses and neonates of women who are administered angiotensin-converting enzyme inhibitors shortly before birth. It is explained by the interference with the renin-angiotensin system, which is physiologically overactive during fetal and neonatal life.[75]

High prostaglandin activity is physiologically necessary to maintain sufficient perfusion of the newborn kidney. Serum prostaglandin levels are also high in infants with chronic cardiac failure, patent ductus arteriosus, and all instances of hypotension and hypovolemia. Prostaglandin synthesis inhibitors, used before birth to prevent the premature onset of labor or after birth to promote the closure of a hemodynamically active patent ductus arteriosus, reduce GFR and RBF.[84,85] They do so by blunting the effect of vasodilator prostaglandins on the afferent arteriole. Decreases in GFR have been described in neonates exposed to indomethacin before birth, as well as in neonates administered indomethacin for the closure of patent ductus arteriosus. The deleterious effect is usually transient and results in water retention and hyponatemia.[84,85] Other nonselective (ibuprofen) or selective (nimesulide) cyclooxygenase inhibitors likely share the same adverse effects.[87,88] In experimental immature animals, cyclooxygenase–nonselective and cyclooxygenase-2–selective inhibitors similarly impair GFR and RBF.[89]

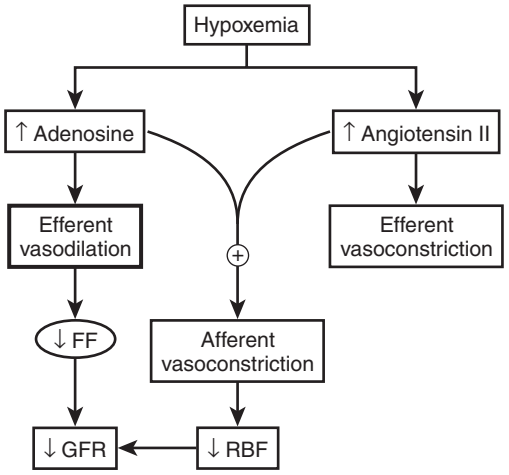


Figure 127-9 Hypoxemia activates the release of intrarenal adenosine and angiotensin II. Whereas angiotensin II preferentially vasoconstricts the efferent arteriole, adenosine effectively vasodilates it. The decrease in the filtration fraction induced by hypoxemia reflects net efferent vasodilation. The combined net effect of angiotensin II and adenosine is thus afferent vasoconstriction and efferent vasodilatation. This results in a decrease in the intraglomerular pressure and consequently in GFR.

CONCLUSION

Glomerular filtration is the first essential step in urine formation. Its maintenance within narrow limits is mandatory and requires subtle autoregulatory mechanisms that involve the action of autacoids, vasoactive substances, and intrinsic myogenic mechanisms. Because not only pathologic changes but also physiologic changes in GFR during maturation may affect body fluid homeostasis and the excretion of drugs by the neonate, an estimation of GFR will help the neonatologist to rationally prescribe fluids, electrolytes, and drugs.

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Leonard G. Feld and Howard E. Corey

Renal Transport of Sodium During Early Development

The regulation of intracellular and extracellular sodium is a prerequisite to life in a nonaqueous environment. Whereas mature animals maintain sodium homeostasis, immature animals must maintain a positive balance of sodium. This positive sodium balance is achieved by at least two complementary mechanisms.

First, newborn animals universally prefer to ingest a salt solution rather than sodium-free water.[1] In the term rat, this "sodium preference" can be demonstrated by day 5 of life.[2] Second, developing nephrons tend to retain sodium. In the term infant, approximately 65% of ingested sodium is retained rather than excreted by the kidneys.[3,4] Together, "salt preference" and "renal immaturity" promote a positive sodium balance.

Several investigations have associated sodium transport with somatic growth.[5-8] Bickel and associates[9] evaluated the relative abundances of renal sodium transporters in young lean and obese Zucker rats by semiquantitative immunoblotting. The abundances of the α_1-subunit of sodium, potassium–adenosine triphosphatase (Na^+,K^+-ATPase), the thiazide-sensitive Na-Cl cotransporter (NCC or TSC), and the β-subunit of the epithelial sodium channel (ENaC) were all significantly higher in the kidneys of obese rats. No differences were found for the sodium-hydrogen exchanger (NHE3), the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2 or BSC1), the type II sodium-phosphate cotransporter (NaP_i-2), or the α-subunit of ENaC.

Leptin, an adipose-induced hormone, up-regulates the expression of the erythrocyte sodium-hydrogen exchanger (NHE1).[10] In normal individuals, insulin also induces the expression of erythrocyte NHE1. In contrast, insulin has no effect on NHE1 activity of erythrocytes from obese individuals.[11] These findings suggest a link between obesity, insulin resistance, and hypertension on the one hand and the active renal transport of sodium on the other.

To better understand sodium handling by the kidney during development, investigators have traditionally used clearance methods to measure the fractional excretion of sodium (FE_{Na}) and the free water clearance (C_{H2O}). These studies point to the distal tubule as the site of the most avid sodium conservation. Using modern techniques of molecular biology and targeted proteomics, more recent investigations have now established the cellular basis of these earlier observations.

FRACTIONAL EXCRETION OF SODIUM DURING DEVELOPMENT

Sodium handling by the kidney may be expressed as the clearance of sodium divided by the clearance of creatinine (FE_{Na}).

[Urinary concentration of sodium (U_{Na}, mEq/L) × Urine volume (mL) / Unit time / [Creatinine or inulin clearance × Plasma sodium concentration (P_{Na}, mEq/L)] becomes

$$(U_{Na} \times P_{cr}) / (U_{cr} \times P_{Na}) \times 100\,\%$$

FE_{Na} measures renal tubular sodium transport "normalized" for the glomerular filtration rate (GFR). As GFR rises dramatically after birth, FE_{Na} is a useful measurement, particularly in the newborn infant.

Several studies have indicated that FE_{Na} varies inversely with the gestational and postconceptional age.[12,13] The entire spectrum of FE_{Na} related to gestational age is shown in Figure 128-1 and summarized in Table 128-1.

In the human, the fetus produces large amounts of sodium-rich urine with a mean FE_{Na} of 12.8%.[3-7] Healthy premature infants, particularly those less than 30 weeks gestational age, continue to excrete large amounts of sodium (average FE_{Na} 5%) (Figure 128-2)[14] and are at risk for negative sodium balance.[15] All preterm infants of less than 30 weeks have a negative sodium balance (Figure 128-3).[14] The incidence decreases to 45% to 70% by 32 weeks gestation and to 0% to 29% after 36 weeks.[15,16]

Vanpeé and colleagues[17] compared the FE_{Na} in infants with a gestational age of 25 to 30 weeks with values found in a cohort of older infants with a gestational age of 31 to 34 weeks. During the first 2 weeks, FE_{Na} fell rapidly in both groups, eventually reaching the low levels found in term infants 4 to 6 weeks after birth (Figure 128-4).[17] In very sick preterm infants of less than 30 weeks gestation, negative sodium balance persisted through the first week of life. In infants of greater than 33 weeks gestation, a positive sodium balance was achieved by days 2 to 4 of life.

In the term infant, the FE_{Na} decreases from 3.4% to 1.5% in the first few hours of life and to even lower values in the subsequent

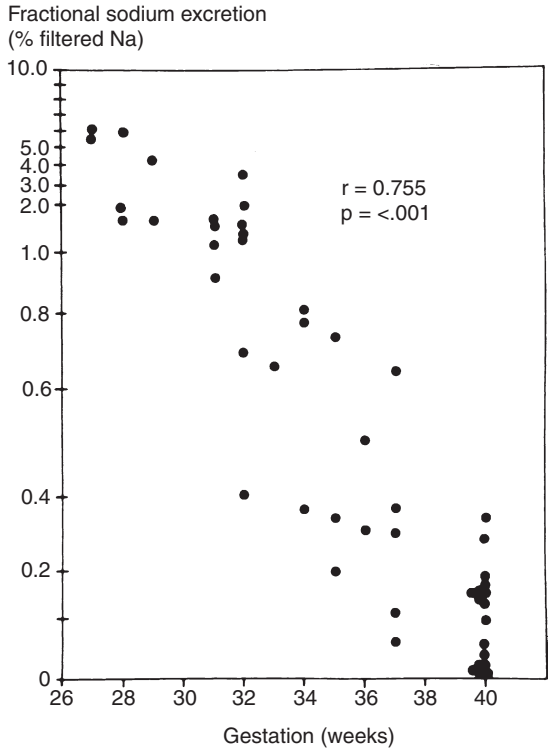


Figure 128-1 Scattergram showing the indirect correlation between the fractional excretion of sodium and gestational age. *(From Siegel SR, Oh W: Renal function as a marker of human fetal maturation,* Acta Paediatr Scand *65:481, 1976.)*

TABLE 128-1

Fractional Excretion of Sodium at Different Postconceptual Ages for Infants of Different Birth Weights

Weight (g)	1-2 d (%)	8-9 d (%)	15-16 d (%)
1000-1500	2	1.4	0.7
1501-2000	2.2	0.8	0.4
2001-2500	0.4	0.6	0.2
>2500	1.0	0.6	0.3

From Bueva A, Guignard JP: Renal function in preterm neonates, *Pediatr Res* 36:572, 1994.

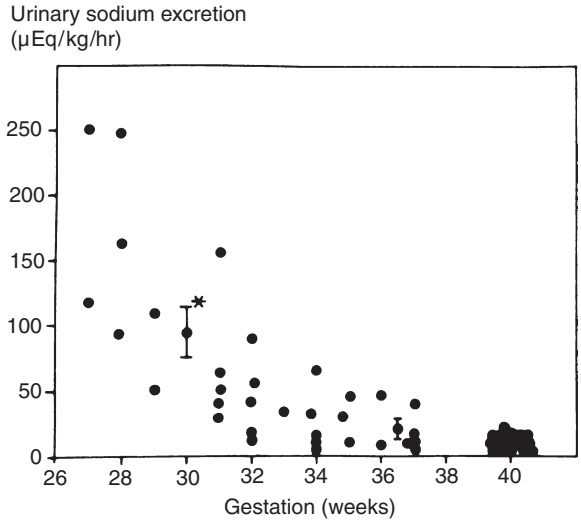


Figure 128-2 Urinary sodium excretion in newborns from 27 to 40 weeks gestational age. Circles with bars at 30 and 37 weeks are mean values for 27- to 32- and 33- to 40-week-gestation infants. *Significant difference between groups. *(From Siegel SR, Oh W: Renal function as a marker of human fetal maturation,* Acta Paediatr Scand *65:481, 1976.)*

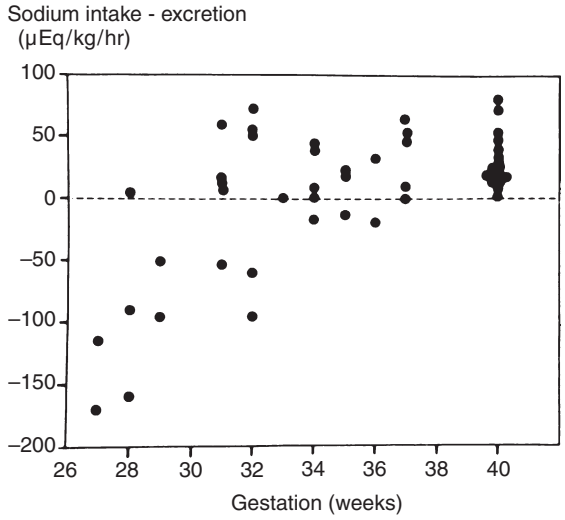


Figure 128-3 Sodium balance is represented by the difference in sodium intake and excretion of infants at 27 to 40 weeks gestational age. *(From Siegel SR, Oh W: Renal function as a marker of human fetal maturation,* Acta Paediatr Scand *65:481, 1976.)*

24 to 48 hours. In most cases, urine sodium excretion over the first few days of life still exceeds the dietary sodium intake (breast milk or low-salt formula) by the infant.[18]

These observations suggest that maturation of the renal tubules is crucial for the newborn infant to conserve sodium. Additional studies (see later) indicate that the major site for sodium conservation by the kidney is the distal portion of the nephron.

Sodium supplementation may prevent a negative sodium balance in the neonate (Figure 128-5).[19] In very-low-birth-weight infants (<1.3 kg at birth) between 2 and 6 weeks of age with a low sodium intake (≤2 mEq/kg/day), the incidence of hyponatremia (≤130 mEq/L) is nearly 40%.[20] A sodium supplementation of approximately 3 mEq/kg/day (provided until a postnatal age of 6 to 7 weeks) will reduce the occurrence of hyponatremia to less than 2%.

However, it is unclear that sodium supplementation is beneficial for the neonate. First, sodium supplementation may interfere with the capacity of the kidney to conserve sodium. In the neonatal rat, GFR does not increase after 1% volume expansion. In nonsupplemented animals, urine sodium excretion increases minimally. In rats that chronically receive sodium supplementation, urine sodium excretion increases markedly, similar to that of the adult.[21] The investigators suggested that the limited sodium in maternal milk may condition the tubules toward antinatriuresis.

Second, excessive administration of sodium to term newborn infants can result in extracellular volume expansion, edema, and in some cases life-threatening hypernatremia.[22] Fortunately, hypernatremia in preterm infants is rare and usually is related to excessive insensible water loss, not to sodium administration.[13] Hypernatremia typically occurs in the first postnatal week when transcutaneous water losses are high.

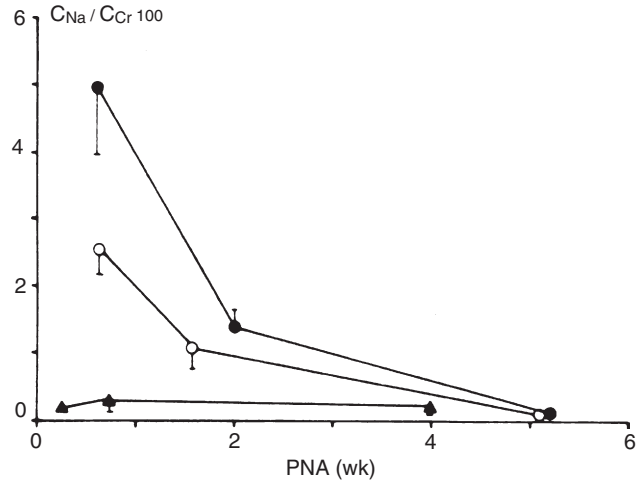


Figure 128-4 Fractional sodium excretion ($FE_{Na} = C_{Na}/_{cr} \times 100\%$) in infants with a gestational age of 28 weeks *(closed circles)* and 32-week *(open circles)* and term *(triangles)* infants. A significant difference was found between the 28- and 32-week groups at 1 week of age. *(From Vanpeé M, Herin P, Zetterström R, Aperia A: Postnatal development of renal function in very low birthweight infants,* Acta Paediatr Scand *77:191, 1988.)*

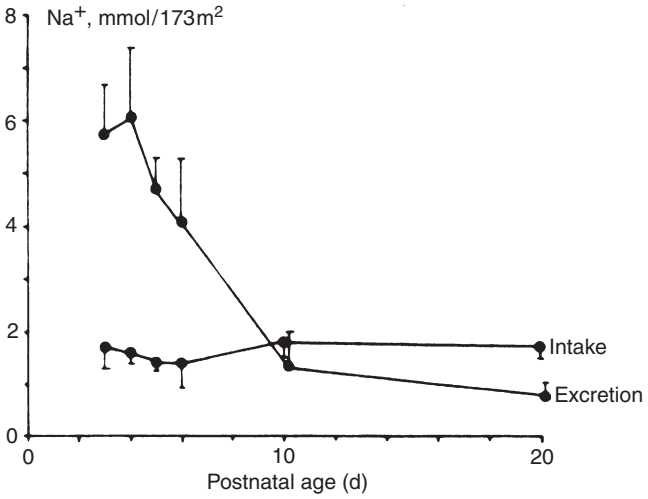


Figure 128-5 Average hourly sodium intake and excretion in preterm infants 3 to 21 days postnatal age. Each circle represents the average of four to nine observations. *(From Aperia AS, Broberger O, Herin P, Zetterström R: Sodium excretion in relation to sodium intake and aldosterone excretion in newborn pre-term and term infants,* Acta Paediatr Scand *68:813, 1979.)*

CLEARANCE STUDIES IN ANIMALS

Newborn animals have a blunted response to saline loading.[23] Banks[24] compared the effects of saline volume expansion on renal tubular reabsorption between newborn and adult animals. Proximal and distal tubule function was estimated by the distal nephron blockade technique with use of ethacrynic acid and amiloride. During saline infusion, which increased extracellular volume by approximately 30% for both age groups, total nephron fractional Na reabsorption was 0.91 for the adult and 0.98 for the puppy ($p < .01$). However, proximal tubule fractional Na reabsorption was greater in the adult (0.64) than in the puppy (0.48, $p < .01$), whereas distal nephron fractional Na reabsorption was much greater in the newborn (0.51) than in the adult (0.26, $p < .01$).

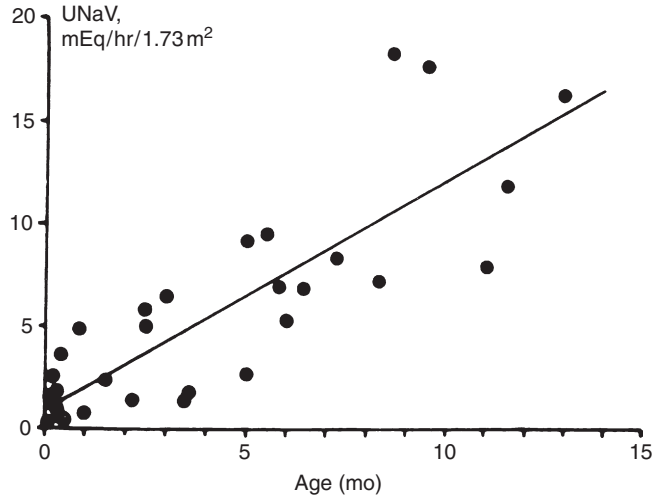


Figure 128-6 Average hourly urinary sodium excretion during the first 13 months of life. Correlation coefficient = 0.764. *(From Aperia A, Broberger O, Thodenius K, Zetterström R: Development of renal control of salt and fluid homeostasis during the first year of life,* Acta Paediatr Scand *64:393, 1975.)*

Using renal micropuncture re-collection techniques in proximal tubules of guinea pigs, Schoeneman and Spitzer[25] found a low proximal fractional reabsorption at all ages; however, a higher whole kidney fractional reabsorption was observed in newborn when compared with adult animals. On the basis of these studies, the tendency to retain an IV sodium load was primarily due to enhanced sodium reabsorption in segments beyond the proximal convoluted tubule.

These observations suggest that factors affecting the function of the distal tubule are mainly responsible for developmental changes in sodium conservation by the nephron. These factors may include the distal delivery of sodium, the activity of natriuretic factors, the secretion of or renal response to aldosterone, and the expression of sodium transport proteins.

CLEARANCE STUDIES IN THE NEONATE

Aperia and collaborators[26] studied preterm (<35 weeks gestation) and term infants under maximal water diuresis after an oral load of sodium chloride. Both groups were unable to excrete the sodium load as efficiently as older children (8 to 14 years).[27] In term infants, the renal response to an oral sodium load of 2.2 mEq/kg body weight is poor in the immediate postnatal period. The urinary sodium excretion rate per hour corrected for body surface area (0.1 to 3 mEq/hour/1.73 m^2) is only approximately 10% of that found in older children (16 mEq/hour/1.73 m^2). However, the renal response to an oral sodium load reaches the latter value by 10 to 13 months of age and remains constant thereafter (Figure 128-6).[28]

Sulyok and co-workers[29] also studied preterm and term neonates, although the conditions of maximal water diuresis were not met. Sodium delivery to the distal tubule was significantly greater and distal sodium reabsorption was significantly less in premature than in term infants, suggesting the inability of the distal tubule to handle the increased sodium load caused by defective proximal tubule sodium reabsorption. However, the fractional distal tubule reabsorption in preterm infants rose to term values of approximately 85% by 2 weeks after birth.[30]

Rodriguez-Soriano and associates[31] studied premature (<34 weeks gestation) and term infants during maximal water diuresis. Distal tubule sodium reabsorption was significantly higher, and sodium delivery was significantly greater, in the more immature infants (Figure 128-7).[31] Fractional distal sodium reabsorption, however, was significantly lower (82%) than in term infants (92%) (Figure 128-8).[31] Therefore a lower percentage of the sodium load presented to the distal tubule was reabsorbed in preterm than in term infants.

These findings suggest that both proximal and distal tubule reabsorption of sodium are inversely related to gestational age and that the major site of adaptation to extrauterine life is the distal tubule. In the premature newborn infant, sodium reabsorptive capacity lags behind the increase of GFR ("glomerular-tubular imbalance"). The high excretion of β_2-microglobulin (a small peptide reabsorbed entirely in the proximal tubule) found in the preterm infant supports this proposition. In infants with gestational ages greater than 33 weeks, sodium reabsorption is more efficient, suggesting glomerular-tubular balance. In healthy term neonates, an enhanced ability of the distal tubule to reabsorb sodium exists, which explains the blunted sodium excretory response to a sodium load.[32]

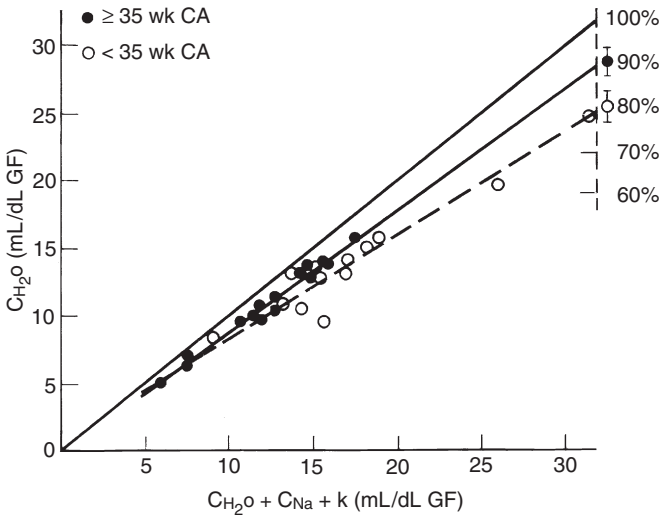


Figure 128-7 Free water clearance (C_{H2O}) and fractional distal sodium delivery ($C_{H2O} + C_{Na} + k$). *(From Rodríguez-Soriano J, Vallo A, Oliveros R, Castillo G: Renal handling of sodium in premature and term neonates: a study using clearance methods during water diuresis,* Pediatr Res *17:1013, 1983.)*

TRANSPORT STUDIES

Plasma sodium is freely filtered by the glomerulus ("filtered load") and is then reabsorbed along the renal tubule, by means of transport systems that are unique to the individual nephron segments (proximal tubule, loop of Henle, distal tubule). The bulk of the filtered load is reabsorbed by the proximal tubule. The proximal tubule is a relatively permeable epithelium with both low-resistance paracellular and transcellular pathways. In the rat proximal tubule, approximately one third of sodium is reabsorbed by active transport, one third is reabsorbed by electrical transference, and one third is reabsorbed by solvent drag.[33] Both active and passive transport forces change with maturation.

Passive Transport During Development

Studies in newborn puppies, guinea pigs, rabbits, and rats have revealed developmental changes in proximal sodium reabsorption.[34-37] In the guinea pig, the single-nephron GFR and the rate of proximal fluid reabsorption increase 20-fold between 1 and 40 days of age.[38] During the same period of time, the length of the proximal tubule increases six-fold. Therefore the rate of sodium and fluid reabsorption per millimeter of tubule length increases approximately fourfold during development. However, the fractional sodium reabsorption along the proximal convoluted tubule remains constant at approximately 60%.[38,39] Without a change in fractional sodium reabsorption in this segment, fractional reabsorption in segments distal to this site must also remain unchanged.[38]

A significant portion of the reabsorption of sodium in young animals is due to passive transport. The leakage of the proximal tubule epithelium to various molecular species is referred to as the reflection coefficient (lower values equal increased permeability). During development, the proximal tubule has a lower reflection coefficient for microperoxidase and mannitol[40] than that in adult animals. This finding may be due in part to changes in membrane permeability with development.

In renal proximal tubule brush-border membrane vesicles (BBMVs), early uptake of ^{22}Na is more rapid in BBMVs from kidneys of 7-day-old than of adult rats. Furthermore, fluorescence

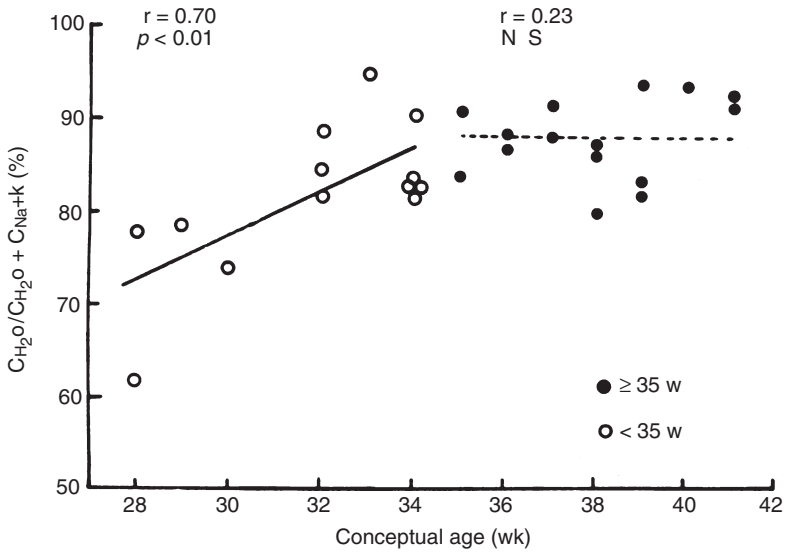


Figure 128-8 Percentage of distal sodium reabsorption versus conceptual age (gestation + postnatal age). *(From Rodríguez-Soriano J, Vallo A, Oliveros R, Castillo G: Renal handling of sodium in premature and full-term neonates: a study using clearance methods during water diuresis,* Pediatr Res *17:1013, 1983.)*

polarization studies reveal that lipids from BBMVs of 7-day-old rats are more fluid than lipids from BBMVs of adult rats.[41] These differences are likely due to age-related changes in the lipid composition of the membranes. These differences in turn could influence the transport and permeability characteristics of the developing tubular membrane. These findings would explain, in part, the alterations of renal BBMV solute transport with growth.

Spitzer and colleagues[37,38] have estimated Starling's hydraulic fluxes ("passive forces") across the proximal convoluted tubule of young and adult rabbits (Table 128-2). By subtracting the passive flux from the total reabsorptive volume flux, one may estimate the relative contribution of active and passive forces to the reabsorption of solute during development.

The reabsorptive flow rate per unit of hydrostatic pressure ($J_{\Delta P}$) is greater in young than in mature animals, possibly because of a higher tubular membrane permeability in the young animal.[38,40,41] Spitzer and associates[37,38] estimated that the hydrostatic pressure–driven flow in the young animal accounts for 7% of the total proximal convoluted tubule reabsorptive flow (J_v) (0.33 nL/min × mm), compared with only 0.6% in the mature animal (J_v = 1.2 nL/min • mm) (Figure 128-9 and see Table 128-2).

The peritubular capillary oncotic pressure is low in the immature animal because of a low plasma oncotic pressure. This results in a two- to three-fold lower reabsorptive flow per unit of applied oncotic pressure ($J_{\Delta\Pi}$) in immature animals compared with adults (0.14 vs. 0.38 nL/min • mm) (see Figure 128-9 and Table 128-2).[38] Therefore, in newborn animals, oncotic pressure–driven flow accounts for 42% of total absorptive flow compared with 32% in adult animals.[38,42] The total reabsorptive flow (J_v) of the young animal is nearly four times lower, and the sum of the hydrostatic ($J_{\Delta P}$)- and oncotic ($J_{\Delta\pi}$)-driven flow is approximately 2.5 times lower than that in the adult.

In summary, active and passive forces may contribute equally to the reabsorption of solute across the immature proximal tubule. With development, protein transport carriers mature and play an increasingly important role in solute reabsorption (see Figure 128-9).[38,42] For example, if total flow is 50 pEq/min • mm in the young animal, 25 pEq/min • mm would occur by active transport. Conversely, if total flow is 180 pEq/min • mm in the mature animal, 60 pEq/min • mm would be accounted for by active processes.[38]

TABLE 128-2

Estimates of Hydraulic Fluxes Across the Proximal Convoluted Tubules of Young and Adult Rabbits

Rabbit	$J_{\Delta P}$	$J_{\Delta\Pi}$	$J_{\Delta P} + J_{\Delta\Pi}$	J_v	Non-Starling
Young (%)	0.023 (7)	0.14 (42)	0.163 (49)	0.33	0.167 (51)
Adult (%)	0.007 (0.6)	0.38 (32)	0.387 (32)	1.2	0.813 (68)

$J_{\Delta P}$, Hydrostatic pressure–driven flow, nL/min • mm; $J_{\Delta\Pi}$, oncotic pressure–driven flow, nL/min • mm; $J_{\Delta P} + J_{\Delta\Pi}$, total pressure-driven flow, nL/min • mm; J_v, reabsorptive volume flux, mL/min • mm; *Non-Starling,* active transport + passive transport caused by transepithelial effective osmotic and ionic gradient. Because the latter term is negligible,[24] "non-Starling" forces are mainly due to active transport.

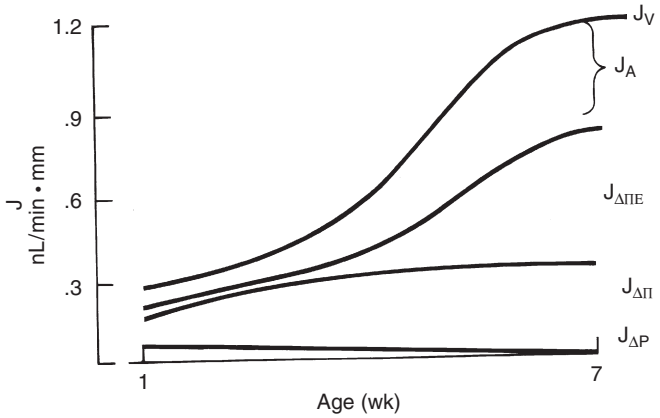


Figure 128-9 Developmental time course of net reabsorptive flow (J_v), oncotic ($J_{\Delta\pi}$), and hydrostatic ($J_{\Delta P}$) pressure-driven flows, and possible course of development of active transcellular flux (J_A) and of fluxes driven by cell-generated effective osmotic and ionic gradients ($J_{\Delta\pi E}$). *(From Barac-Nieto M, Spitzer A: The relationship between renal metabolism and proximal transport during ontogeny,* Pediatr Nephrol *2:356, 1988.)*

Active Transport During Development

Different sets of transport proteins are distributed to the apical and basolateral surfaces of the plasma membranes of renal epithelium.[42] The asymmetric distribution of membrane proteins is generated by an intracellular machinery that addresses the moieties to the appropriate domain. The extracellular environment regulates the protein sorting by conveying the positional information that creates the plasma membrane domains. These interactions are mediated by macromolecular assemblies of cell adhesion molecules, cytoskeleton, and signaling molecules.

To determine the ontogeny of sodium transport proteins in embryonic rat tissue, Schmitt and associates[43] used in situ hybridization and immunohistochemistry to localize the Na-P_i cotransporter type 2 (NaP_i-2), the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2), the thiazide-sensitive Na-Cl cotransporter (NCC), the Na-Ca exchanger (NaCa), the epithelial sodium channel (rENaC), and 11β-hydroxysteroid dehydrogenase. These proteins were first observed in the post–S-shape stages. NKCC2 was initially expressed at the macula densa region and was observed later extending along the nascent ascending limb of the loop of Henle. In comparison, NaP_i-2 expression along the proximal tubular part of the loop of Henle was observed later in development. The NCC was initially found at the distal end of the nascent distal convoluted tubule and later extended toward the junction with the ascending limb of the loop of Henle. Strong coexpression of rENaC and 11β-hydroxysteroid dehydrogenase was observed in early nascent connecting tubule and collecting ducts and later also in the distal portion of the distal convoluted tubule.

The sodium transporters do not contribute equally to the maintenance of sodium balance. In knockout mice, severe salt wasting results from loss of function of the NKCC2 in the thick ascending limb, or of the ENaC in the collecting duct. However, knockout mice with loss of function of the proximal absorptive NHE do not exhibit severe salt wasting.[44] Apparently, the severity of Na dysregulation is unrelated to the basal rate of absorption of NaCl in a given nephron segment. At least for some nephron segments, a tubuloglomerular feedback mechanism may lower the rate of sodium delivery to compensate for a lower rate of sodium reabsorption.[44]

SODIUM, POTASSIUM–ADENOSINE TRIPHOSPHATASE

Bistritzer and co-workers[45] measured Na^+,K^+-ATPase activity in cord red blood cells of newborn infants at different gestational ages. Red blood cell Na^+,K^+-ATPase activity was significantly lower ($p < .01$) in preterm babies with a gestational age below 35 weeks, compared with those aged 35 weeks and above: 2.3 ± 0.8 and 6.7 ± 1.3 nmol reduced nicotinamide adenine dinucleotide/minute/mg protein, respectively. Two weeks after birth, regardless of gestational age, the enzyme activity of the

preterm babies increased to values similar to those observed in the term neonates at birth. These findings suggest that the Na^+,K^+-ATPase enzyme system undergoes important developmental changes.

Basolateral Na^+,K^+-ATPase provides the electrochemical driving force for solute reabsorption along the proximal tubule and the remainder of the nephron. In animals, the increase in active transport with maturation of the kidney parallels the threefold increase in Na^+,K^+-ATPase activity per millimeter tubule length during maturation.[46-49] The activity of Na^+,K^+-ATPase has been shown to increase in rats during development and to increase after aldosterone treatment.[50] Glucocorticoids can increase the rate of Na^+,K^+-ATPase production and may account for some of the increase in postnatal sodium handling in premature infants when compared with unborn fetuses at the same developmental age.[51] However, the activity of this enzyme system is not rate limiting for sodium transport, because the increase in the tubular membrane area precedes the increase in Na^+,K^+-ATPase activity.[52,53]

In addition to enzyme activity, the distribution of the Na^+,K^+-ATPase to the basolateral tubular membrane also appears to be developmentally regulated. In the distal nephron, messenger RNA (mRNA) expression for the β_2-subunit is expressed apically and is down-regulated after birth, whereas mRNA expression for the β_1-subunit is expressed basolaterally and is up-regulated after birth.[54,55]

Symporters

In primordial prokaryotic cells, families of transport proteins arose to serve a variety of cellular functions, including the regulation of cell pH and the provision of carbohydrate for cellular energy. The protein sequences have been conserved and through evolution modified proteins that now perform additional tasks, including the bulk transport of solutes.

In the proximal tubule, the filtered solutes glucose, phosphate, and amino acids are transported from the lumen into the tubular cells by proteins (symporters or antiporters) located in the brush-border luminal membrane.[56-59] Sodium travels down an electrochemical gradient, and the solutes are coupled to the movement of sodium. Bicarbonate is reabsorbed via the apical NHE3 and the basolateral Na-bicarbonate cotransporter (NBC).

PROXIMAL TUBULE

Sodium-Hydrogen Exchanger (NHE)

Neonates have a lower serum bicarbonate level than do adults because of a lower renal threshold for bicarbonate. Eighty percent of bicarbonate reabsorption occurs in the proximal tubule, in which proton secretion is predominantly mediated by an electroneutral luminal NHE.

The NHEs are plasma membrane proteins that are either sensitive (NHE1, NHE2, and NHE4) or insensitive (NHE3) to inhibition by amiloride. Their pattern of expression differs. NHE1 is transcribed in all tissues tested and functions in the regulation of intracellular pH and cell volume. NHE2 is highly expressed in the kidney and gastrointestinal tract and may play a role in sodium reabsorption. NHE3 is expressed in the kidney and contributes to sodium and bicarbonate reabsorption, and NHE4 is expressed mainly in the stomach. The renal NHEs are sorted either to the apical (NHE3) or to the basolateral (NHE1, NHE 4) membranes.[60,61]

Housekeeping Role

NHE3 plays a "housekeeping role" in maintaining the cell actin cytoskeleton. Members of the ezrin (VIL2)-radixin (RDX)-moesin (MSN) (ERM) protein family are highly concentrated in the apical aspect of polarized epithelial cells. These cells are studded with microvilli containing bundles of actin filaments, which must attach to the membrane to assemble and maintain the microvilli. The ERM proteins, together with merlin, the NF2 gene product, join integral membrane and cytoskeletal proteins and bind directly to actin. Actin cytoskeleton reorganization requires the activation of NHE3 (SLC9A3).[62]

Sodium Reabsorption

Schultheis and co-workers[63] created knockout mice lacking NHE3 function. Homozygous mutant mice survived but had slight diarrhea and mild acidosis. As expected, bicarbonate and fluid absorption were low in proximal convoluted tubules, the blood pressure was low, and a severe absorptive defect was observed in the intestine. These findings indicate that NHE3 is the major absorptive NHE in kidney and intestine and that lack of the exchanger impairs acid-base balance and Na^+-fluid volume homeostasis.

It is important to note that the mutant mice were not severely hyponatremic. Two mechanisms may account for their ability to maintain sodium balance. First, sodium transporters other than NHE were induced. Plasma aldosterone was increased in NHE3-deficient mice, and expression of both renin and the AE1 (Slc4a1) chloride-bicarbonate exchanger mRNAs were induced in kidney. In the colon, epithelial Na^+ channel activity was increased, and colonic H^+,K^+-ATPase mRNA was induced. Presumably, the high activity of these induced transporters compensated for the loss of function of NHE.

Second, the reduction in proximal fluid absorption was accompanied by a proportional decrease in the GFR. Compensation of the transport defect by a reduction in filtered load was so efficient that clinically symptomatic Na losses were not observed.[44,63]

Developmental Changes

The V_{max} of embryonic apical membrane NHE3 is three-fold lower than that in the adult, suggesting a lower abundance or turnover rate.[64] Na-H activity increases during the transition from fetus to newborn sheep.[65] The administration of glucocorticoid to the mother increases V_{max} to the adult level.

To examine the maturation of apical NHE3 antiporter activity in rat proximal convoluted tubules, Shah and associates[66] perfused rat proximal convoluted tubules in vitro. Na^+-H^+ antiporter activity was assayed as the proton secretory rate on luminal sodium removal. Na^+-H^+ antiporter activity was 121.2 ± 18.4 pmol/mm × minute in neonatal and 451.8 ± 40.6 pmol/mm × minute in adult proximal convoluted tubules ($p < .001$). Adult renal cortical NHE3 mRNA abundance was 10-fold greater than that in 1-day-old neonates ($p < .001$). A comparable developmental increase occurred in renal BBMV NHE3 protein abundance ($p < .001$). These observations indicate an age-related increase in rat apical membrane Na^+-H^+ antiporter activity, renal cortical NHE3 mRNA, and BBMV NHE3 protein abundance.

The abundance of mRNA for the basolateral NHE1 and NHE4 and the pH-dependent renal sodium uptake is higher in 2- to 4-week-old rats than in the adult.[67] The functional significance of this observation is unclear, because the major mechanism for the transport of HCO_3^- across the basolateral membrane is via the electrogenic Na^+-HCO_3^- cotransporter (NBC).

Sodium Bicarbonate Cotransporter (NBC)

Molecular cloning experiments have so far identified three NBC isoforms (NBC-1, NBC-2, and NBC-3) in the kidney.[68] Under normal conditions, all appear to mediate the cotransport of Na^+ and HCO_3^-. In addition, they may be functionally altered in certain pathophysiologic states. For example, NBC-1 may be up-regulated in metabolic acidosis and potassium depletion, and also in response to glucocorticoid excess, and may be down-regulated in response

to HCO_3^- loading, or alkalosis.[69] Mutations in the *SLC4A4* gene for NBC cause proximal renal tubular acidosis, with bilateral glaucoma, cataracts, and band keratopathy. Such mutations may increase the bicarbonate concentration in the corneal stroma, which would facilitate calcium deposition, leading to band keratopathy.[70]

Sodium-Phosphate Type IIa

In the intestine and the kidney, the apical sodium Na-P_i cotransport is the rate-limiting step for the reabsorption of phosphate.[71] Three different Na-P_i cotransporters have been identified:

1. Type I cotransporters, such as NPT1 (SLC17A1), are found mainly in proximal tubule and do not appear to be regulated by dietary phosphate levels. These proteins also show anion channel function and may play a role in secretion of organic anions.
2. Type II cotransporters, such as NaP_i-3 (SLC34A1), determine Na^+-dependent transcellular P_i movements in the renal proximal tubule (type IIa) and in the small intestine (type IIb).
3. Type III cotransporters are expressed in many different tissues, where they serve housekeeping functions. Type III transporters also can function as membrane receptors for primate retroviruses.

The Na-P_i symporters derive from ancient lineage. A NaP_i-2 homologue cloned from *Vibrio cholerae* is a functional P_i transporter when expressed in *Xenopus* oocytes.[72]

In the small intestine, expression of IIb protein varies in response to altered P_i intake and levels of 1,25-dihydroxyvitamin D_3. In the proximal tubule, P_i intake, vitamin D levels, and parathyroid hormone all regulate the expression of the IIa cotransporter.

Contribution of Sodium-Phosphate Type 2 to Sodium Reabsorption

The NHE is the major apical sodium transporter in the proximal convoluted tubule. The relative contribution of other transport systems to the reabsorption of sodium can be estimated by using "targeted proteomics." In this method, the kidneys of genetically deficient mice with loss of NHE3 function are probed for renal sodium transporters. Brooks and associates[73] used semiquantitative immunoblotting to estimate the percentage change in abundance of each transporter in knockout compared with wild-type mice. In NHE3 knockout mice, three changes were identified that could compensate for the loss of NHE3-mediated sodium absorption: (1) in the proximal tubule, the sodium-phosphate cotransporter NaP_i-2 was markedly up-regulated; (2) in the collecting duct, the 70-kDa form (aldosterone stimulated) of the γ-subunit of the epithelial sodium channel ENaC was up-regulated; and (3) glomerular filtration was significantly reduced. These findings suggest that the NaP_i-2 transport system plays an important role in sodium, as well as phosphate, handling by the kidney.

Sodium-Phosphate During Development

The renal tubules of the neonate have a high rate of phosphate reabsorption. To determine the mechanism, Woda and colleagues[74] performed renal micropuncture experiments in acutely thyroparathyroidectomized adult (>14-week-old) and juvenile (4-week-old) male Wistar rats in the presence and absence of parathyroid hormone. P_i reabsorption was greater in proximal convoluted and straight tubules of the juvenile compared with adult rats, whether or not parathyroid hormone was present. These findings were consistent with a greater P_i uptake in BBMVs from both superficial and outer juxtamedullary cortices of juvenile animals. This high rate of P_i uptake is due to a high V_{max} of the NaP_i-2 symporter.

In kidneys of newborn rats, the appearance of NaP_i-2 protein and mRNA coincides with the development of the brush border (assessed by actin staining) on proximal tubular cells. Traebert and colleagues[75] found that NaP_i-2 was not detectable in the nephrogenic zone or in the outgrowing straight sections of proximal tubules, which lack a brush border. In 13-day-old suckling rats, strong NaP_i-2 staining was seen in the brush border membrane (BBM) of convoluted proximal tubules of all nephron generations. In contrast, in 22-day-old weaned rats, NaP_i-2 staining in the BBM of superficial nephrons was weaker than that in the BBM of juxtamedullary nephrons. Western blotting demonstrated that the overall abundance of NaP_i-2 protein in the BBM of 22-day-old rats was decreased to approximately 70% of that in 13-day-old rats. These observations suggest a greater abundance of NaP_i-2 in newborn compared with mature animals.

Woda and colleagues[74] found that dietary phosphate restriction in juvenile rats resulted in a significant increase in phosphate reabsorption in the PCT and PST segments. NaP_i-2 expression in the proximal tubule BBM was also increased, as was the expression of intracellular NaP_i-2 protein. In addition, dietary phosphate restriction in the juvenile rat was associated with up-regulation of BBM NaP_i-2 expression, which was associated with a further increase in proximal tubular phosphate reabsorption.

The ancient molecular lineage and the high plasticity of NaP_i-2 suggest an important role in maintaining sodium, as well as phosphate, homeostasis.

DISTAL TUBULE

The thick ascending limb of the loop of Henle in neonates has only 20% of the capacity to transport sodium compared with adults.[76,77] As in the proximal tubule, Na^+,K^+-ATPase activity is significantly lower in the differentiating basolateral membrane of the thick ascending limb. This accounts for an isotonic fluid delivered to the early distal tubule in the immature kidney, in contrast to a hypoosmotic fluid delivered in the adult kidney.

Aperia and Elinder[78] studied distal tubular sodium reabsorptive capacity in immature (24-day-old) and mature (40-day-old) rats during hydropenia and after volume expansion. Under hydropenic conditions, the fraction of filtered sodium entering the early distal tubule was higher, although it was similar in the late distal tubule in the young compared with mature rats (Figure 128-10).[78] This indicates that immature nephrons deliver a greater fraction of sodium from the ascending loop of Henle but

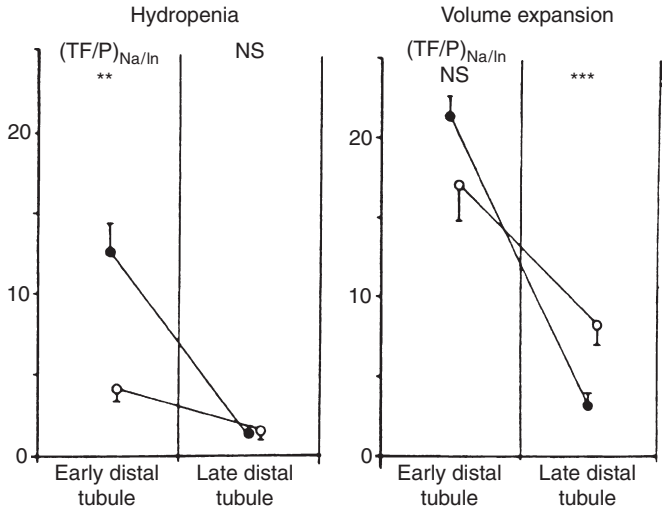


Figure 128-10 Changes in $(TF/P)_{Na/inulin}$ from early distal to late distal tubule in hydropenic and volume-expanded 24-day-old *(closed circles)* and 40-day-old *(open circles)* rats. *NS,* No significant difference. $^{**}p < .01$. $^{***}p < .001$. *(From Aperia A, Elinder G: Distal tubular sodium reabsorption in the developing rat kidney,* Am J Physiol *240:F487, 1981.)*

also have a higher fractional reabsorption of sodium along the distal tubule.

Epithelial Sodium Channel

Canessa and colleagues[79,80] cloned and characterized subunits of a rat ENaC that had the functional properties of the distal renal sodium channel—high sodium selectivity, low conductance, and amiloride sensitivity. The functional channel is composed of at least three subunits, alpha (SCNN1A), beta (SCNN1B), and gamma (SCNN1G). The three subunits show sequence similarities to one another, indicating descent from a common ancestral gene. Each encodes a protein containing two transmembrane domains, with intracellular amino and carboxyl termini.

In the distal nephron, ENaC mediates electrogenic Na transport and is the rate-limiting step of sodium reabsorption. In the adult, ENaC is expressed all along the connecting tubule (70% of the distal convolution) and the cortical collecting duct.[81]

Regulation of the Epithelial Sodium Channel by Aldosterone and Angiotensin II

The level of sodium reabsorption is primarily determined by the action of aldosterone on ENaC in the distal nephron. To conserve sodium, aldosterone must first bind to the mineralocorticoid receptor (MR). MR-deficient knockout mice develop symptoms of pseudohypoaldosteronism, such as weight loss, dehydration, and renal sodium wasting. By day 8, MR-deficient knockout mice develop hyperkalemia, hyponatremia, and high circulating levels of renin, angiotensin II, and aldosterone. GFR is similar to that of controls, but the FE_{Na} is more than eight-fold higher.[82]

In an aldosterone-sensitive renal cell line (A6), aldosterone regulates sodium reabsorption by short- and long-term processes. In the short term, aldosterone regulates sodium transport by inducing expression of the small G-protein K-Ras2A, by stimulating the activity of methyltransferase and *S*-adenosylhomocysteine hydrolase to activate Ras by methylation, and, possibly, by subsequent activation by K-Ras2A of phosphatidylinositol phosphate-5-kinase (PIP-5-K) and phosphatidylinositol-3-kinase (PI-3-K), which ultimately activate ENaC. In the long term, aldosterone regulates sodium transport by altering trafficking, assembly, and degradation of EnaC.[83,84]

Brooks and co-workers[85] examined renal homogenates from mice in which the gene for the angiotensin II type 1a (AT[1a]) receptor had been deleted to determine which sodium transporters and channels are regulated by the AT(1a) receptor. In mutant compared with wild-type mice maintained on a low-NaCl diet, the abundance of the thiazide-sensitive cotransporter and the three subunits of the amiloride-sensitive ENaC was markedly increased. No significant changes were found in the abundances of the proximal tubule NHE or the Na^+-K^+-$2Cl^-$ cotransporter of the thick ascending limb. These observations suggest that angiotensin II directly alters renal sodium transport independently of aldosterone secretion.

Ontogeny of Epithelial Sodium Channel

The total expression of ENaC subunits is very low in the embryonic rat kidney but rises rapidly to adult levels by the first postnatal day.[86] ENaC is expressed in a variety of tissues, including lung and kidney. In knockout mice that do not express ENaC subunits, severe hyponatremia and/or failure to clear pulmonary fluid may occur (Table 128-3).[86-91] These findings indicate that EnaC is critically important to sodium balance.

TABLE 128-3

Epithelial Sodium Channel Subunit Loss of Function Mutations

Type	Lung	Kidney
a-ENaC	Failure of pulmonary fluid clearance	Salt wasting, metabolic acidosis, and high aldosterone
b-ENaC	Cleared fluid	Compensated on normal sodium intake but PHA-1 on low sodium
g-ENaC	Fluid problems	Severe hyperkalemia

ENaC, Epithelial sodium channel.

Cation-Chloride Cotransporters

The cation-Cl cotransporters (CCC) mediate the coupled movement of Na and/or K to that of Cl across membranes of epithelial cells. Eight CCC have been identified to date: two Na-K-Cl cotransporters (NKCC), four K-Cl cotransporters (KCC), one Na-Cl cotransporter (NCC), and one CCC interacting protein (CIP). All the NKCC and KCC are inhibited by loop diuretics.[92]

Sodium-Potassium-Chloride Cotransporter

The Na-K-Cl cotransporters are a family of integral membrane proteins that mediate the coupled transport of Na^+,K^+, and Cl^- across the plasma membrane. Quaggin and colleagues[93] observed that the kidney-specific Na-K-Cl cotransporter (previously called NKCC2) mediates active reabsorption of sodium chloride in the thick ascending limb of the loop of Henle and is the site of action of the clinically important diuretics furosemide and bumetanide. The protein of the transporter gene, symbolized as SLC12A1 in human and mouse, is structurally related (64% amino acid identity in the mouse) to another Na-K-Cl cotransporter (SLC12A2, previously called NKCC1), which is expressed in many tissues, including the basolateral membrane of secretory epithelia, where it mediates active chloride secretion.

Sodium-Chloride Cotransporter

The thiazide-sensitive NCC of the distal convoluted tubule is the principal mediator of sodium and chloride reabsorption in this nephron segment, accounting for a significant fraction of net renal sodium reabsorption. Simon and colleagues[94] and others[95] have associated Gitelman's syndrome of inherited hypokalemic alkalosis with mutations in the electroneutral Na-Cl transporter. Gamba and associates[96] have cloned this transporter from rat kidney. Simon and co-workers[94] have cloned the human homologue, which they designated TSC for thiazide-sensitive Na-Cl cotransporter, and showed that the protein is encoded by 26 exons. The human protein showed 89% identity with the rat homologue and 63% identity with the flounder homologue.

Mastroianni and co-workers[97] also cloned the *SLC12A3* gene. The predicted protein sequence of 1021 amino acids (112 kDa) showed a structure common to other members of the Na-K-Cl cotransporter family: a central region harboring 12 transmembrane domains and the two intracellular hydrophilic amino and carboxyl termini. The expression pattern of the gene confirmed the kidney specificity. By fluorescence in situ hybridization, these investigators mapped the gene to 16q13.

In the NCC knockout mice, the abundance of the 70-kDa form of the γ-subunit of ENaC was higher than in controls. However, the abundance of other sodium transport proteins was similar to that of wild-type mice. Compared with the renal Na transporters of the NCC knockout mouse, the renal Na transporters of NH3 knockout mice undergo extensive adaptation. This observation suggests that NHE3 is more important for bulk sodium transport than is NCC.[73]

REGULATORY SYSTEMS

Development changes in a variety of endocrine and paracrine systems (e.g., the renin-angiotensin-aldosterone system, prostaglandins, catecholamines, atrial natriuretic peptide, glucocorticoids, the kallikrein-kinin system, nitric oxide, and endothelin)

may influence the renal handling of sodium. Detailed reviews have been published.[98,99]

Renin-Angiotensin-Aldosterone System

Studies in the Fetus

In the fetus, the expression of renin in the kidney is different from that in the adult. Reddi and associates[100] observed a shift in renin distribution from interlobar and arcuate arteries in the fetus to the afferent arterioles in the adult. In addition, these investigators identified seven types of renin distribution along the afferent arterioles. In type I, renin was distributed continuously along the whole length of the afferent vessel. This pattern was most frequently observed in the fetus. In type II, renin extended upstream from the glomerulus but did not occupy the whole length of the arteriole. This type was relatively constant throughout postnatal life. In type III, renin was present as bands along the afferent vessel; it was most frequently observed in the fetal and early perinatal periods. In type IV, renin was restricted to the "classic" juxtaglomerular localization. It was the most frequent type observed in the adult rat. In type V, no renin was found in the arteriole. It was the second most frequent type observed in the adult rat. The distribution of renin-expressing cells was spatially and temporally associated with the development of blood vessels. During this process, renin-expressing cells were distributed along the whole of the newly formed vessel. As the vessel matured, renin-expressing cells became restricted to the juxtaglomerular portion of the afferent arteriole.

In chronically catheterized fetal sheep, the renin-angiotensin system is active during intrauterine life.[101] Levels of angiotensin II in fetal sheep are similar to those in maternal sheep. The fetal kidney, like other vascular beds, has high levels of the AT2 angiotensin receptor subtype. With maturation, the proportion of the AT1 receptor subtype increases.

Blockade of the fetal renin-angiotensin system with angiotensin-converting enzyme inhibitors or with the nonpeptide angiotensin II antagonist losartan causes a fall in fetal GFR and a rise in renal blood flow. Angiotensin II reverses the fall in GFR even though renal blood flow decreases. Lumbers[101] observed that the fraction of the filtered sodium load reabsorbed by the proximal tubule was not affected when the fetal renin-angiotensin system was blocked by captopril or losartan. High doses of infused angiotensin II had no effect on renal reabsorption of sodium in the short term, but in the long term had an effect on depressed fractional proximal reabsorption. These studies suggest that the renin-angiotensin system is active in the fetus but that the proximal renal tubule is resistant to the sodium-retaining effects of angiotensin II.

Studies in the Neonate

After birth, the kidneys of newborn animals become sensitive to the salt-retaining effects of angiotensin II. FE_{Na} in rat pups given daily injections of the ATI receptor inhibitor losartan is higher than in control animals administered vehicle.[102]

Plasma renin activity (PRA)[103,104] and plasma aldosterone (PA) concentrations[105,106] are higher in newborn animals and humans. Sulyok and co-workers[107] studied the relationship of PRA, PA, and urinary aldosterone excretion (UAE) in infants given sodium supplements and no supplements with a mean gestational age of 31 weeks. Among infants not given supplements, PRA, PA, and UAE increased dramatically from week 1 to week 3 after birth and then gradually decreased over the subsequent 2 weeks of the study (Figure 128-11).[107] In contrast, no consistent change was seen in the supplemented group, with values similar to those of term neonates. In fact, PRA, PA, and UAE were significantly higher in infants who did not receive supplements versus those who did receive supplements at a postnatal age of 2 to 4 weeks. Godard and co-workers[108] also found PRA to be inversely correlated with urinary sodium excretion in term infants. Highest values were noted in the first 2 days of life and decreased over the subsequent week.[108]

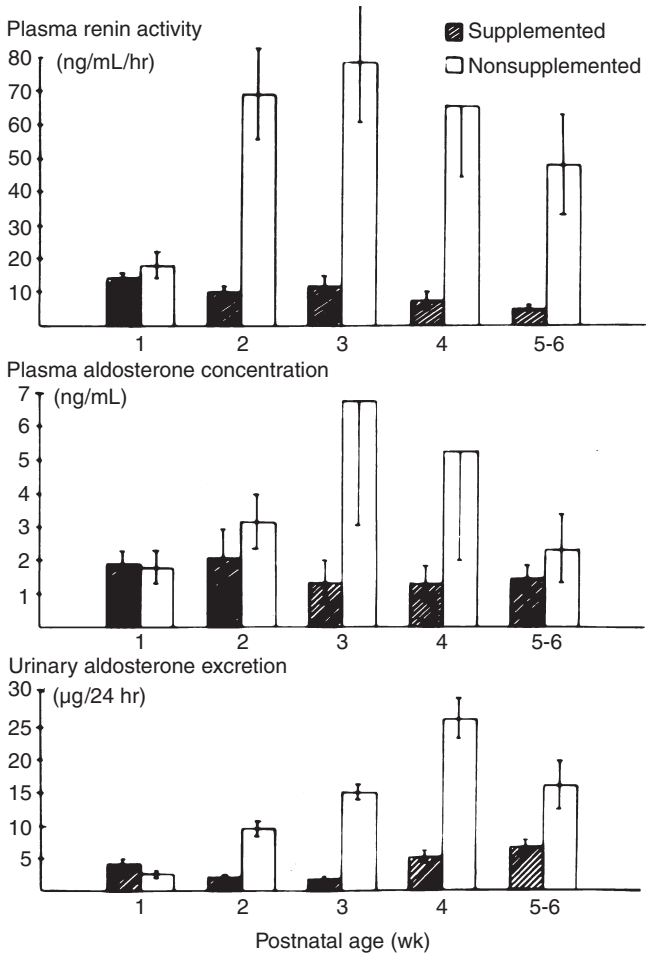


Figure 128-11 Plasma renin activity, plasma aldosterone concentration, and urinary aldosterone excretion in preterm infants with and without sodium supplementation in the first 5 to 6 weeks of life. *(From Sulyok E, Németh M, Tényi I, et al: Relationship between the postnatal development of the renin-angiotensin-aldosterone system and electrolyte and acid-base status of the NaCl-supplemented premature infants. In Spitzer A, editor:* The Kidney During Development, *New York, 1982, Masson, pp 273-281.)*

Aperia and associates[109] have shown that the pattern of UAE was similar in preterm (less than or equal to 34 weeks gestation) and term infants. During the first 3 postnatal weeks, UAE was higher in both groups compared with normal adult values (Figure 128-12).[109] Because aldosterone affects tubular sodium and potassium transport in opposite directions, the urinary sodium–urinary potassium ratio (U_{Na}/U_K or U_K/U_{Na}) has been used to assess the effect of this hormone on distal tubular transport. It is interesting that the urinary potassium–sodium ratio showed a linear relationship to UAE with time only in term infants, whereas the ratio in the preterm group showed a correlation to UAE only after 10 days of age. The hyponatremia found at 2 weeks of age in the premature infants corresponded to the highest UAE and a low urinary potassium–sodium ratio.

An impaired end-organ response to aldosterone seemed to exist, with improvement with increasing postnatal age. Because the absolute urinary sodium excretion was similar in both

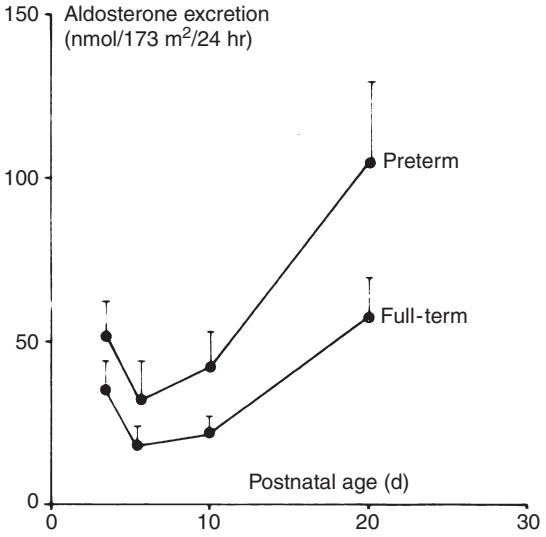


Figure 128-12 Changes in urinary aldosterone excretion in the first 30 days after birth in preterm and term infants. *(From Aperia A, Broberger O, Herin P, Zetterström R: Sodium excretion in relation to sodium intake and aldosterone excretion in newborn pre-term and full-term infants,* Acta Paediatr Scand *68:813, 1979.)*

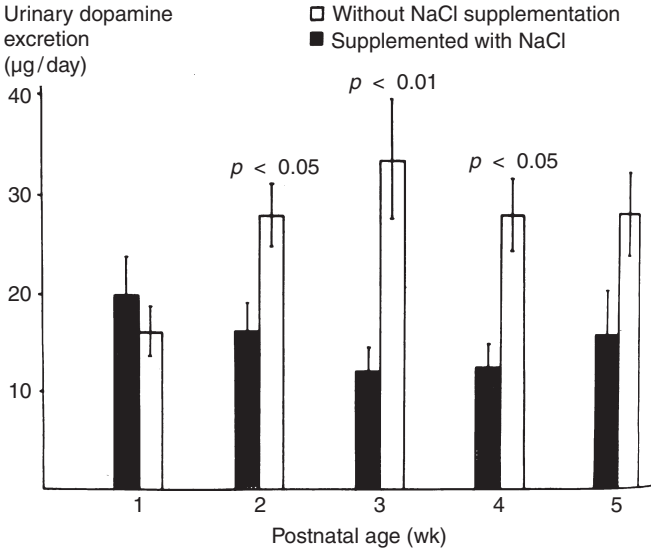


Figure 128-13 Urinary dopamine excretion in preterm infants with and without sodium supplementation in the first 5 weeks of life. *(From Sulyok E, Gyódi G, Ertl T, et al: The influence of NaCl supplementation on the postnatal development of urinary excretion of noradrenaline, dopamine, and serotonin in premature infants,* Pediatr Res *19:5, 1985.)*

groups, factors other than sodium balance contributed to the hyponatremia found in very premature infants. The lack of renal tubular sensitivity to aldosterone may be related to lack of receptors or lack of Na^+,K^+-ATPase activity in the premature infant. However, Stephenson and co-workers[110] found that immature rats (in contrast to older animals) were essentially insensitive to subcutaneously injected aldosterone. In addition, equivalent high-affinity aldosterone-binding sites existed in both groups of rats. They concluded that the resistance of immature rat kidneys to aldosterone was not a defect in signal presentation or receptors but rather was a postreceptor phenomenon.

Prostaglandins

Renal prostaglandin (PGE) production, as measured by urinary PGE_2 and prostacyclin, is highest in preterm neonates and decreases with increasing gestational and postnatal age.[111] PGE concentrations decrease progressively with postnatal age in premature infants receiving sodium supplementation but show no significant change with advancing age in a nonsupplemented group.[112] The steady decline in PGE concentrations in the sodium-supplemented group corresponds to decreased activity in the renin-angiotensin-aldosterone system under similar conditions. From the studies, Ertl[112] suggested that increased PGE synthesis would be needed to offset the vasoconstrictor effect resulting from activation of the renin-angiotensin system to maintain normal blood pressure and reduce renal vascular resistance. In premature neonates, inhibition of PGE synthesis with indomethacin can significantly reduce glomerular blood flow and glomerular filtration, but the role of PGE synthesis inhibition in renal tubular sodium handling is minimal.[113]

Catecholamines

Both neuronally derived and locally produced (renal tubular cells) catecholamines exert control over renal sodium handling. Tubule cells possess receptors for dopamine, norepinephrine, and epinephrine. An association exists between increased sodium excretion and increased dopamine excretion in preterm infants who do not receive supplementation (Figure 128-13).[114,115] Conversely, infants who receive supplementation show no increase in dopamine excretion. These studies demonstrate that the renal sympathetic nervous system enhances sodium reabsorption. However, its relationship to the renin-angiotensin-aldosterone system still requires additional investigations.

The IV administration of dopamine to sick premature infants increases sodium excretion and PRA without a significant change in PA.[116] This could be interpreted as an unresponsiveness of the adrenal gland to additional stimulation. Even though age-related differences exist in the effect of dopamine administration, the results are similar to those reported in adults.

To sort out the differences in the renal response to endogenous versus exogenous dopamine, metoclopramide was given to premature infants to block dopamine production.[117] Urinary sodium excretion was increased in the low–salt intake group, whereas no change was seen in the high–dietary sodium group. No effect also was seen on PRA in either group. Conversely, PA concentration and UAE decreased. These data suggested that increased urinary sodium excretion was due to metoclopramide suppression of aldosterone secretion in these young infants. Therefore endogenous dopamine stimulated renal tubular sodium reabsorption, and increased aldosterone production possibly played a role in restoration of positive sodium balance in premature infants with late hyponatremia.

Studies in fetal sheep have examined the role of renal innervation in the development of sodium homeostasis. The relative distribution of α_1-adrenoceptors (which modulate tubular sodium and water reabsorption) and α_2-adrenoceptors (which modulate sodium, water, and potassium excretion) changes during development.[118] Stimulation of α_1-adrenoceptors in fetal, newborn, and adult sheep showed a maximal ability to decrease urine flow and sodium excretion in newborn sheep, with significantly less conservation of sodium in fetal sheep.[119] The ability to reabsorb sodium and water in the immediate postnatal period is, in part, mediated by development of the renal response to catecholamines. Incomplete development of this system in premature neonates may play a role in the hyponatremia of prematurity.

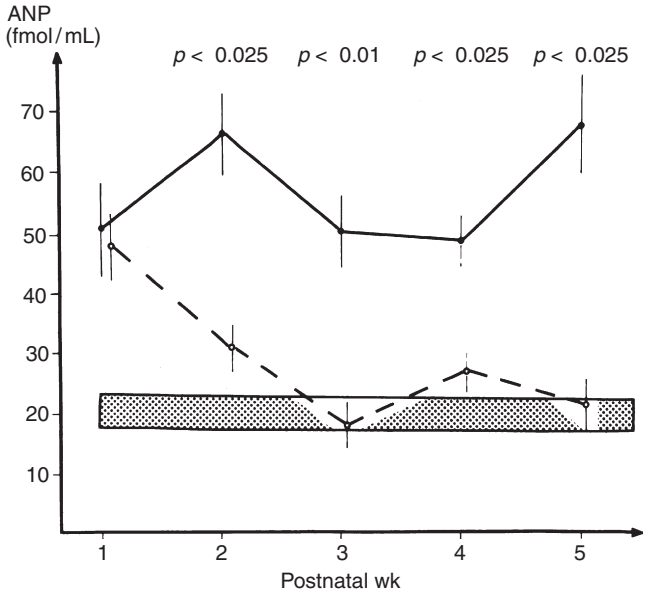


Figure 128-14 Plasma atrial natriuretic peptide (ANP) concentrations in preterm infants on a low-sodium *(dashed line)* and a high-sodium *(solid line)* intake. The stippled area is ANP values for 7- to 10-day-old term infants. *(From Tulassay T, Rascher W, Seyberth HW, et al: Role of atrial natriuretic peptide in sodium homeostasis in premature infants,* J Pediatr *109:1023, 1986.)*

Atrial Natriuretic Peptides

Atrial natriuretic peptide (ANP) is detectable early in fetal development.[120] The distribution of ANP is relatively widespread in both atria and ventricles during early development and localizes to the right atrium near the time of birth.[121] Circulating levels of ANP increase throughout gestation, continue to increase after birth for 7 to 10 days, and are higher in premature infants than in term neonates.[122] The highest ANP levels are seen in the most premature neonates, particularly those with respiratory disease.[123] ANP concentrations are elevated in the plasma of infants who receive sodium supplementation (Figure 128-14).[124] Although the exact mechanism modulating this increase is unknown, it may be a result of an expanded extracellular fluid volume because of the augmented sodium diet. The lower value in infants given a low-sodium diet may be a response of the premature kidney to conserve sodium for tissue growth.

Although circulating levels of ANP are high during development and in the newborn period, the renal natriuretic and diuretic response to ANP is blunted. Possible reasons for this blunted response include the following: (1) lower renal perfusion pressure seen in fetal and newborn kidneys,[125] (2) incompletely developed system of ANP receptors in the kidney,[126] and/or (3) inhibition of ANP effect by high circulating levels of angiotensin II and aldosterone.

The importance of ANP in sodium handling in the young animal is unclear. Deloof and colleagues[127] placed pregnant rats on either a high-sodium or a normal-sodium diet. The high-sodium diet was not associated with a change in the plasma ANP concentrations but significantly decreased the PA concentrations in both the maternal and fetal rats. In response to the high-salt diet, the density and affinity of total ANP, ANPb, and ANPc receptors were not altered in the maternal isolated renal glomeruli or the adrenal zona glomerulosa membranes, or the fetal adrenal gland and kidney membrane preparations. These results suggest that ANP is not involved in the regulation of water and electrolyte balance in maternal and fetal rats during salt-loaded intake.

Digoxin-like immunoreactive substances in both the urine and plasma have been suggested to be natriuretic by virtue of their known inhibition of Na^+,K^+-ATPase activity in vitro.[128] Ebara and colleagues found a correlation between these substances and FE_{Na} in preterm infants.[128] The urine and plasma concentrations of digoxin-like immunoreactive substances remained elevated for 4 weeks in infants of 24 to 29 weeks gestation and decreased in those infants with a gestational age greater than 30 weeks. Additional studies will be required to assess the significance of this study.[129]

SUMMARY

Primordial cells have developed specialized proteins to aid in the maintenance of cellular integrity, pH, and metabolism. More evolved cell types have co-opted and modified these ancient proteins to serve a variety of functions useful to the organism as a whole, including the bulk transport of solutes across epithelial cell membranes. In immature animals, the transport proteins not only maintain homeostasis but also promote growth.

Newborn animals maintain a positive balance for sodium. Clearance studies have indicated that the distal tubule is mostly responsible for the adaptation to extrauterine life. Because of advances in molecular biology, a variety of specialized sodium transporters are now recognized. Studies performed with use of targeted proteomics have revealed the special importance of ENaC and the NCC proteins in maintaining sodium balance.

Some investigators have postulated the existence of growth-specific transporters that are not expressed in the adult. Future studies will determine exactly how these proteins are sorted, distributed, and regulated. The contribution of regulatory paracrine systems such as the renin-angiotensin system can then be rigorously investigated.

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129 Potassium Homeostasis in the Fetus and Neonate

Corinne Benchimol and Lisa M. Satlin

Potassium is the most abundant intracellular cation. Maintenance of a high intracellular potassium concentration (100 to 140 mEq/L) is essential for many basic cellular processes, including cell growth and division, DNA and protein synthesis, conservation of cell volume and pH, and optimal enzyme function. The steep gradient between potassium concentration in the cell and that in the extracellular fluid, maintained by the ubiquitous sodium-potassium adenosine triphosphatase (Na^+,K^+-ATPase), is the major determinant of the resting membrane potential across the cell membrane, and thus it affects neuromuscular excitability and contractility. Approximately 98% of the total body potassium content in the adult resides within cells, primarily muscle (Figure 129-1), whereas the remaining 2% is located within the extracellular fluid. The extracellular potassium concentration (generally ranging from 3.5-5.0 mEq/L) is tightly regulated by mechanisms that govern the *internal* distribution between the intracellular and extracellular compartments and the *external* balance between intake and output.

POTASSIUM HOMEOSTASIS

The homeostatic goal of the adult is to remain in zero potassium balance. Thus, of the typical daily potassium intake of 1 mEq/kg body weight, approximately 90% to 95% is ultimately eliminated from the body in the urine; the residual 5% to 10% of the daily potassium load is lost through the stool (see Figure 129-1). Normally, the amount of potassium lost through sweat is negligible.

In contrast to the situation in the adult, infants greater than approximately 30 weeks gestational age (GA) must conserve potassium for growth.[1,2] Postnatal growth is associated with an increase in total body potassium from approximately 8 mEq/cm body height at birth to more than 14 mEq/cm body height by 18 years of age.[3,4] The rate of accretion of body potassium per kilogram body weight in the neonate is greater than in later childhood (Figure 129-2), a finding reflecting an increase in both cell number and potassium concentration (at least in skeletal muscle) with advancing age.[4-6] Given the requirement of the growing organism for potassium conservation, it is not unexpected

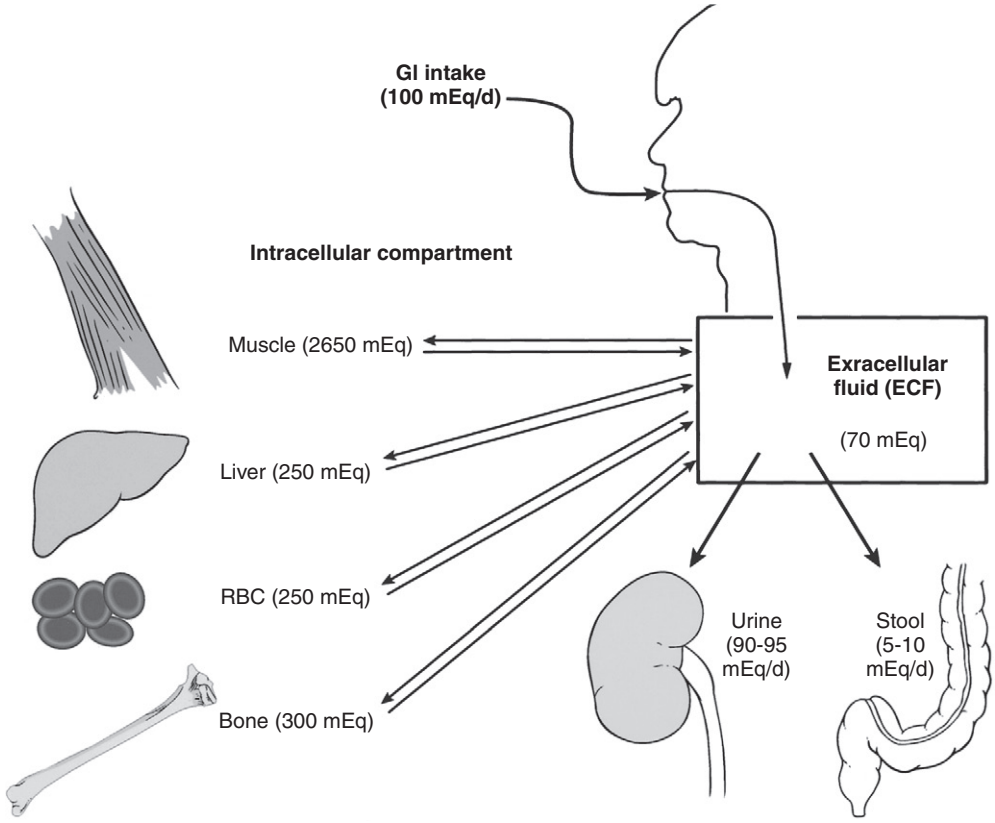


Figure 129-1 Potassium homeostasis in the adult: *internal* and *external* balance. *External* potassium balance is maintained by the urinary (90% to 95%) and fecal (5% to 10%) excretion of the daily potassium intake of approximately 1 mEq/kg/day in the typical adult. *Internal* potassium balance depends on the distribution of potassium between the extracellular fluid (ECF) compartment and the vast intracellular storage reservoirs provided by muscle, liver, erythrocytes (RBC), and bone. *GI,* Gastrointestinal.

that infants maintain a state of positive potassium balance.[2,7] This tendency to retain potassium early in postnatal life is reflected, in part, in the higher plasma potassium values in infants, and particularly in preterm neonates.[2,7]

In fetal life, potassium is transported across the placenta from mother to fetus. Indeed, the fetal serum potassium concentration is maintained at levels exceeding 5 mEq/L even in the presence of maternal potassium deficiency.[8,9]

Urinary potassium excretion varies considerably, depending in large part on dietary intake. Children and adults ingesting an average American diet that contains sodium in excess of potassium excrete urine with a sodium-to-potassium ratio greater than 1.[2,10] Although breast milk and commercially available infant formulas generally provide a sodium-to-potassium ratio of approximately 0.5 to 0.6, the urinary sodium-to-potassium ratio in the newborn up to 4 months of age generally exceeds 1. This high ratio may reflect the greater requirement of potassium over sodium for growth. In fact, some premature (<34 weeks GA) newborns may excrete urine with a sodium-to-potassium ratio greater than 2, a finding suggesting significant salt wasting and a relative hyporesponsiveness of the neonatal kidney to mineralocorticoid activity.[2] Because of the many vital processes dependent on potassium homeostasis, multiple complex and efficient mechanisms have developed to regulate total potassium balance and distribution.

REGULATION OF INTERNAL POTASSIUM BALANCE

The task of maintaining potassium homeostasis is complex, in large part because the daily dietary intake of potassium (approximately 100 mEq in the adult) typically approaches or exceeds the total potassium normally present within the extracellular fluid space (approximately 70 mEq in 17 L of extracellular fluid with a potassium concentration averaging approximately 4 mEq/L) (see Figure 129-1). To maintain zero balance in the adult, all the dietary intake of potassium must be ultimately eliminated, a task performed primarily by the kidney. However, renal excretion of potassium is rather sluggish, requiring several hours to be accomplished. Approximately 50% of an oral load of potassium is excreted during the first 4 to 6 hours after it is ingested, yet life-threatening hyperkalemia is not generally observed during this period because of the rapid (within minutes) hormonally mediated translocation of extracellular potassium into cells, particularly those of muscle and liver. The buffering capacity of the combined cellular storage reservoirs, capable of sequestering up to approximately 3500 mEq of potassium, is vast compared with the extracellular pool (see Figure 129-1).

Cells must expend a significant amount of energy to maintain the steep potassium and sodium concentration gradients across their cell membranes. This is accomplished by the Na^+,K^+-ATPase cation pump, which catalyzes the hydrolysis of cytosolic adenosine triphosphate, thereby providing energy for the active extrusion of sodium from cells in exchange for the uptake of potassium in a ratio of 3:2, respectively. A cell interior negative potential is created by the unequal cation exchange ratio and the subsequent leak of potassium out of the cells through potassium-selective channels in the plasma membrane. Na^+,K^+-ATPase consists of a catalytic (α) and a regulatory (β) subunit.

Thirty percent to 50% of very-low-birth-weight and premature infants <28 weeks GA exhibit nonoliguric hyperkalemia (defined as a serum potassium concentration of >6.5 mEq/L) during the

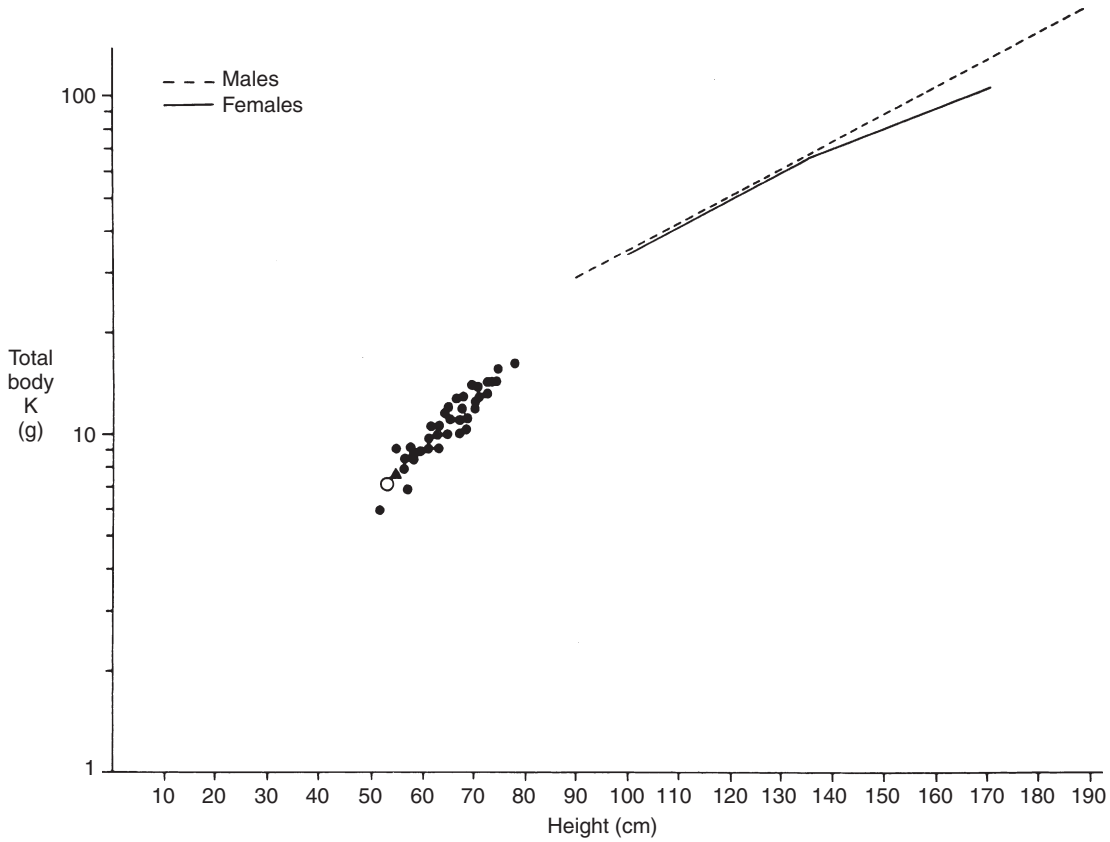


Figure 129-2 Relationship between total body potassium (in grams) and height (in centimeters) for infants and children. The rate of accretion of body potassium in the neonate is faster than in later childhood, likely reflecting an increase in both cell number and potassium concentration, at least in skeletal muscle, with advancing age. *(From Flynn MA, Woodruff C, Clark J, et al: Total body potassium in normal children,* Pediatr Res *6:239, 1972.)*

first 48 hours after birth, despite the intake of negligible amounts of potassium.[11-15] This phenomenon is not observed in mature infants or very-low-birth-weight infants after 72 hours.[13,14] This biochemical observation has been proposed to reflect a shift of potassium from the intracellular to the extracellular fluid space, because of low Na^+-K^+ pump activity, as well as a limited renal potassium secretory capacity.[13,14,16] Prenatal steroid treatment may prevent this nonoliguric hyperkalemia via induction of pump activity in the fetus.[17,18]

Na^+,K^+-ATPase activity is regulated by numerous circulating hormones that exert short-term and long-term control over it.[19] Whereas long-term stimulation of pump activity is generally mediated by changes in gene and protein expression, short-term regulation generally results from alterations in the phosphorylation status of the pump, changes in the subcellular or cell surface distribution of pumps (i.e., membrane trafficking), and/or interaction with regulatory proteins.[19] Regulation of internal potassium balance in the neonate may be influenced by developmental stage–specific expression of potassium transporters (such as the cation pump) and channels, receptors, and signal transduction pathways.

The chemical, physical, and hormonal factors that acutely influence the *internal* balance of potassium are listed in Table 129-1. Potassium uptake into cells is acutely stimulated by insulin, β_2-adrenergic agonists, and alkalosis and is impaired by α-adrenergic agonists, acidosis, and hyperosmolality. Generally, deviations in extracellular potassium concentration arising from fluctuations in internal distribution are self-limited as long as the endocrine regulation of *internal* balance and mechanisms responsible for regulation of *external* balance are intact.

Plasma Potassium Concentration

Active basolateral cellular potassium uptake in large part determines the intracellular pool of potassium. An increase in plasma potassium, either because of a dietary or parenteral potassium load or resulting from a chronic progressive loss of functional renal mass, decreases the concentration gradient (dependent on the ratio of intracellular to extracellular potassium concentration) against which the Na^+,K^+-ATPase pump must function and thus favors an increase in cellular potassium uptake. In those cells of the kidney and colon specifically responsible for potassium secretion, the resulting increase in intracellular potassium maximizes the concentration gradient between cell and lumen, thereby promoting potassium diffusion into the tubular lumen and thus potassium excretion.

TABLE 129-1

Factors, Relevant to the Infant, That Acutely Regulate the Internal Balance of Potassium

Factor	Effect on Cell Uptake of Potassium
Physiologic factors	
Plasma K concentration	
Increase	Increase
Decrease	Decrease
Insulin	Increase
Catecholamines	
α-Agonists	Decrease
β-Agonists	Increase
Pathologic factors	
Acid-base balance	
Acidosis	Decrease
Alkalosis	Increase
Hyperosmolality	Enhances cell efflux
Cell breakdown	Enhances cell efflux

Hormones

Insulin, the most important hormonal regulator of *internal* potassium balance, stimulates Na^+,K^+-ATPase-mediated potassium uptake and sodium efflux in the kidney, skeletal muscle, adipocytes, and brain, a response that is independent of the hormonal effects on glucose metabolism.[20] The mechanism of insulin action in these tissues differs, in part, because of differences in the isoform complement of the catalytic α-subunit of the pump. Insulin stimulates Na^+,K^+-ATPase activity by promoting the translocation of preformed pumps from intracellular stores to the cell surface (as in skeletal muscle)[21-24] and/or increasing cytoplasmic sodium content (as in adipocytes)[25-27] or by increasing the apparent affinity of the enzyme for sodium (as in kidney).[28] Basal insulin secretion is necessary to maintain fasting plasma potassium concentration within the normal range. An increase in plasma potassium in excess of 1.0 mEq/L in the adult induces a significant increase in peripheral insulin levels to aid in the rapid disposal of the potassium load, yet a more modest elevation of approximately 0.5 mEq/L is without effect.[29]

The effect of epinephrine on potassium balance in the adult is biphasic and is characterized by an initial increase, followed by a prolonged fall in plasma potassium concentration to a final value lower than baseline. The initial transient rise in plasma potassium results from α-adrenergic receptor stimulation causing release of potassium from hepatocytes.[30,31] β_2-Receptor stimulation, via stimulation of adenylate cyclase leading to generation of the second messenger cyclic adenosine monophosphate, activates Na^+,K^+-ATPase and thus promotes enhanced uptake of potassium by skeletal and cardiac muscle, effects that are inhibited by the β_2-blocker propranolol.[30-35] The observation that the potassium-lowering effects of insulin and epinephrine are additive suggests that their responses are mediated by different signaling pathways.

The effects of these hormones on the distribution of potassium between the intracellular and extracellular compartments have been exploited to effectively treat disorders of homeostasis. For example, the β_2-adrenoreceptor agonist albuterol has been used to treat life-threatening hyperkalemia in premature and term neonates, children, and adults.[36-38] Administration of this drug to treat hyperkalemia is associated with fewer side effects (mild, reversible tachycardia and tremors)[39] than are observed with many conventional protocols. Administration of glucose, insulin, and furosemide may be associated with significant fluid, electrolyte, and glucose derangements in premature infants.[40-42] Administration of sodium bicarbonate has been associated with an increased incidence of intraventricular hemorrhage.[43] Oral or rectal administration of ion exchange resins can increase total body sodium, can cause cecal perforation,[44] and is often ineffective in small infants.[40,45]

Aldosterone is best known for its effect on transporting tissue, increasing potassium secretion in distal segments of the nephron and colon (see later). Thyroid hormone may also promote the cellular uptake of potassium as a result of its long-term stimulation of Na^+-K^+ pump activity.[19]

Acid-Base Balance

It is well known that the transcellular distribution of potassium and acid-base balance are interrelated.[46] Whereas acidemia (increase in extracellular hydrogen ion concentration) is associated with an increase in plasma potassium resulting from potassium release from the intracellular compartment, alkalemia (decrease in extracellular hydrogen ion concentration) results in a shift of potassium into cells and a consequent decrease in plasma potassium. However, the reciprocal changes in plasma potassium that accompany acute changes in blood pH differ widely among the four major acid-base disorders; metabolic disorders cause greater disturbances in plasma potassium than do those of respiratory origin, and acute changes in pH result in larger changes in plasma potassium than do chronic conditions.[46]

Acute metabolic acidosis after administration of a mineral acid that includes an anion that does not readily penetrate the cell membrane, such as the chloride of hydrochloric acid or ammonium chloride, consistently results in an increase in plasma potassium. As excess extracellular protons, unaccompanied by their nonpermeant anions, enter the cell where neutralization by intracellular buffers occurs, potassium (or sodium) is displaced from the cells, thus maintaining electroneutrality. However, comparable acidemia induced by acute organic anion acidosis (lactic acid in lactic acidosis, acetoacetic, and β-hydroxybutyric acids in uncontrolled diabetes mellitus) may not elicit a detectable change in plasma potassium.[46-48] In organic acidemia, the associated anion diffuses more freely into the cell and thus does not require a shift of potassium from the intracellular to the extracellular fluid.

In respiratory acid-base disturbances, in which carbon dioxide and carbonic acid readily permeate cell membranes, little transcellular shift of potassium occurs because protons are not transported in or out in association with potassium moving in the opposite direction.[46]

Changes in plasma bicarbonate concentration, independent of the effect on extracellular pH, can reciprocally affect plasma potassium concentration. Movement of bicarbonate (outward at a low extracellular bicarbonate concentration and inward at a high extracellular bicarbonate concentration) between the intracellular and extracellular compartments may be causally related to a concomitant transfer of potassium. This may account for the less marked increase in plasma potassium observed during acute respiratory acidosis, a condition characterized by an acid plasma pH with an elevated serum bicarbonate (hence inward net bicarbonate and potassium movement), as compared with acute metabolic acidosis with a low serum bicarbonate concentration (hence outward net bicarbonate and potassium movement).

Other Factors

Many other pathologic perturbations alter the *internal* potassium balance. An increase in plasma osmolality resulting from severe dehydration causes water to shift out of cells. The consequent increase in intracellular potassium concentration exaggerates the transcellular concentration gradient and favors movement of this cation out of cells. The effect of hyperosmolality on potassium balance becomes especially troublesome in patients with hyperglycemia, as observed in patients with diabetes in whom the absence of insulin exacerbates the hyperkalemia.

REGULATION OF EXTERNAL POTASSIUM BALANCE

Renal Contribution

The kidney is the major excretory organ for potassium. In adults, urinary potassium excretion parallels dietary intake (see Figure 129-1), and the speed of renal adaptation depends on the baseline potassium intake and the magnitude of the change in dietary potassium intake. Extreme adjustments in the rate of renal potassium conservation cannot be achieved as rapidly as for sodium, nor are the adjustments as complete; whereas urinary sodium can be virtually eliminated within 3 to 4 days of sodium restriction, a minimum urinary potassium loss of approximately 5 mEq/day occurs in the adult, even after several weeks of severe potassium restriction. An increase in dietary potassium intake is matched by a parallel increase in renal potassium excretion within hours, yet maximal rates of potassium excretion are not attained for several days after increasing potassium intake. In adults, renal potassium excretion follows a circadian rhythm, presumably determined by hypothalamic oscillators, and it is characterized by maximum output during times of peak activity.[49] It is unknown whether a circadian cycle of urinary potassium excretion prevails in infancy.

The processes involved in renal potassium handling in the fully differentiated kidney include filtration, reabsorption, and secretion (Figure 129-3).[50] Filtered potassium is reabsorbed almost entirely in proximal segments of the nephron, and urinary potassium is derived predominantly from distal secretion. Therefore potassium balance, at least in the adult, is maintained by renal secretion rather than reabsorption.

The renal potassium clearance is low in newborns, even when it is corrected for their low glomerular filtration rate.[2,7] Infants, like adults, can excrete potassium at a rate that exceeds its glomerular filtration when given a potassium load, a finding indicating the capacity for net tubular secretion.[51] However, the rate of potassium excretion per unit body weight or kidney weight in response to exogenous loading is less in newborn than older animals.[52,53] Clearance studies in saline-expanded dogs also provide indirect evidence of a diminished secretory and enhanced reabsorptive

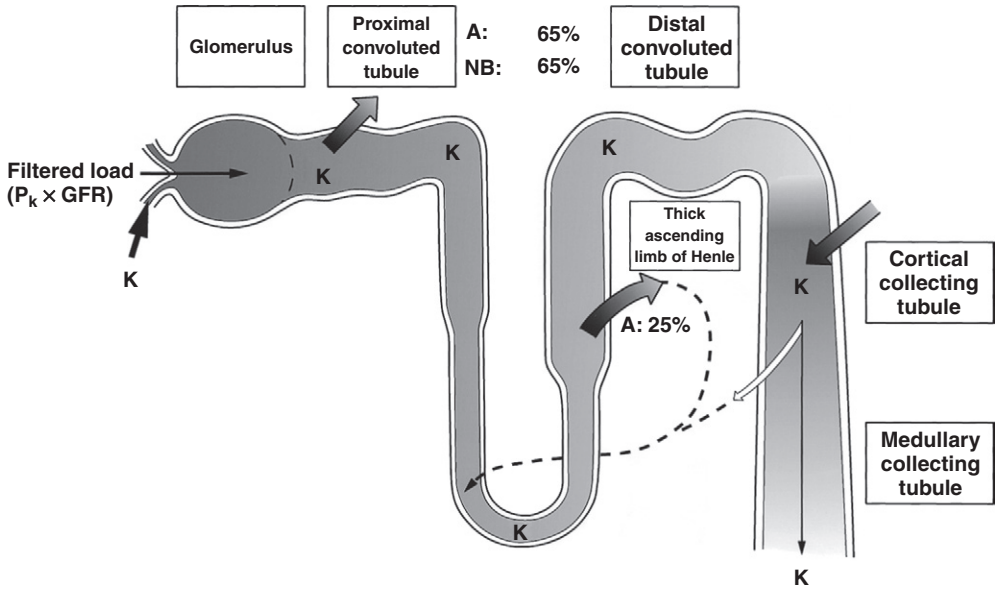


Figure 129-3 Tubular sites of potassium (K) transport along the nephron. The percentages of filtered potassium reabsorbed along the proximal tubule and TALH are indicated for the adult *(A)* and, when known, the newborn *(NB)*. *Arrows* identify the direction of net potassium transport as either out of (reabsorption) or into (secretion) the urinary fluid. *GFR,* Glomerular filtration rate.

capacity of the immature distal nephron to potassium.[54] A similar conclusion can be drawn from a longitudinal prospective study in premature neonates demonstrating a 50% reduction in the fractional excretion of potassium between 26 and 30 weeks GA, in the absence of significant change in absolute urinary potassium excretion.[1] To the extent that the filtered load of potassium increased almost three-fold during this same time interval, the constancy of renal potassium excretion could be best explained by a developmental increase in the capacity of the kidney for potassium reabsorption.[1] In general, the limited potassium secretory capacity of the immature kidney becomes clinically relevant only under conditions of potassium excess. As stated earlier, under normal circumstances, potassium retention by the newborn kidney is appropriate and is required for somatic growth.

Sites of Potassium Transport Along the Nephron

Potassium is freely filtered at the glomerulus. Approximately 65% of the filtered load of potassium is reabsorbed along the proximal tubule of the suckling rat, a fraction similar to that measured in the adult (see Figure 129-3).[55-58] Reabsorption is passive in this segment, closely following water reabsorption, and is driven, in part, by the positive transepithelial voltage that prevails along part of the proximal tubule.

Approximately 10% of the filtered load of potassium reaches the early distal tubule of the adult, a finding reflecting significant further net reabsorption of this cation in the thick ascending limb of the loop of Henle (TALH) (see Figure 129-3).[56] In contrast, up to 35% of the filtered load of potassium reaches the distal tubule of the very young (2-week-old) rat.[55] The observations in the maturing rodent that the fractional reabsorption of potassium along the TALH, expressed as a percentage of delivered load, increases by 20% between the second and sixth weeks of postnatal life, and that both the diluting capacity and TALH Na^+,K^+-ATPase activity increase after birth are consistent with a developmental maturation of potassium absorptive pathways in this segment.[55,59,60] However, direct functional analysis of the potassium transport capacity of the TALH in the developing nephron has not been performed.

The avid potassium reabsorption characteristic of the fully differentiated TALH is mediated by a Na^+,K^+-$2Cl^-$ cotransporter that translocates a single potassium ion into the cell accompanied by a sodium and two chloride (Cl^-) ions (Figure 129-4, *A*). This secondary active transport is ultimately driven by the basolateral Na^+,K^+-ATPase that generates an electrochemical gradient favoring sodium entry at the apical membrane. Diuretics such as furosemide and bumetanide that inhibit the Na^+,K^+-$2Cl^-$ cotransporter block potassium reabsorption at this site and uncover potassium secretion, leading to profound urinary potassium losses.

Activity of the Na^+,K^+-$2Cl^-$ transporter requires the presence of a parallel potassium conductance in the urinary membrane. This luminal secretory potassium channel is encoded by the *ROMK* (rat outer medullary K^+ channel) gene.[61,62] Loss-of-function mutations in *ROMK* lead to antenatal Bartter syndrome, also known as the hyperprostaglandin E syndrome, which is characterized by severe renal salt and water losses, consistent with a pattern of impaired TALH function[63] and similar to the clinical picture observed with the chronic administration of loop diuretics. The typical presentation of antenatal Bartter syndrome includes polyhydramnios, premature delivery, and life-threatening episodes of dehydration during the first week of life. This group of patients also has severe growth failure, hypercalciuria with early-onset nephrocalcinosis, hyperreninism, and hyperaldosteronism but normal blood pressure.[64]

In the healthy adult, regulated potassium secretion by the distal tubule and the collecting duct contributes prominently to urinary potassium excretion, which can approach 20% of the filtered load (see Figure 129-3).[50] Two major populations of cells comprise the distal nephron. Principal cells reabsorb sodium and secrete potassium, whereas intercalated cells primarily function in acid-base homeostasis but can reabsorb potassium in response to dietary potassium restriction or metabolic acidosis. Potassium secretion by the collecting duct requires potassium to be actively transported into principal cells in exchange for sodium at the basolateral membrane by the action of Na^+,K^+-ATPase (see Figure 129-4, *B*). Potassium accumulates within the cell and then passively diffuses across the apical membrane through secretory potassium channels. The magnitude of potassium secretion is determined by its electrochemical gradient and the apical permeability to this cation. The electrochemical gradient is established by the potassium concentration gradient between the cell and lumen, and lumen negative voltage, generated by apical sodium entry through epithelial sodium channels (ENaCs) and its basolateral electrogenic extrusion.

Two apical potassium-selective channels have been functionally identified in the distal nephron. The small conductance secretory potassium (SK) channel, encoded by the *ROMK* gene, is considered to mediate baseline potassium secretion, whereas the high-conductance, stretch- and calcium-activated maxi-K channel has been proposed to regulate flow-stimulated potassium secretion.[65] Any factor that enhances the electrochemical driving force or increases the apical membrane permeability to potassium will favor potassium secretion.

The direction and magnitude of net potassium transport in the distal nephron vary according to physiologic need. Thus, in response to dietary potassium restriction or potassium depletion, the distal nephron may reabsorb potassium. Potassium reabsorption is mediated by a H^+,K^+-ATPase, an enzyme that exchanges a single potassium ion for a proton (see Figure 129-4, *C*), localized to the apical membrane of acid-base–transporting intercalated cells.

Potassium secretion in the distal nephron, and specifically in the cortical collecting duct (CCD), is low early in life and cannot be stimulated by high urinary flow rates.[66] The limited capacity of the neonatal CCD for baseline potassium secretion appears not to be due to an unfavorable electrochemical gradient. Although Na^+,K^+-ATPase activity in neonatal collecting duct segments is only 50% of that measured in the mature nephron, cell potassium content of this segment is similar at both ages, presumably reflecting a relative paucity of membrane potassium channels early in life.[59,67,68] The rate of sodium absorption in the CCD at 2 weeks of age is approximately 60% of that measured in the adult and is not considered to be limiting for potassium secretion.[66] Electrophysiologic analysis has confirmed the absence of functional potassium secretory channels in the luminal membrane of the neonatal CCD.[69] Cumulative evidence now suggests that the postnatal increase in the potassium secretory capacity of the distal nephron is due to a developmental increase in number of SK and maxi-K channels, reflecting an increase in transcription and translation of functional channel proteins.[69-71] Molecular analyses demonstrate that *ROMK* messenger RNA and protein are first detectable in the second week of postnatal life in the rodent, immediately preceding the appearance of functional channels and potassium secretion in this segment.[66,69-71] Messenger RNA encoding the maxi-K channel first becomes detectable after weaning in the rodent, as does functional maxi-K channel activity.[78]

Indirect evidence suggests that the neonatal distal nephron absorbs potassium. As indicated earlier, saline-expanded newborn dogs absorb 25% more of the distal potassium load than adult animals.[52] Functional analysis of the rabbit collecting duct has shown that the activity of apical H^+,K^+-ATPase in neonatal intercalated cells is equivalent to that in mature cells.[72] The latter data alone do not predict transepithelial potassium absorption under physiologic conditions. However, high distal tubular fluid potassium concentrations, as measured in vivo in the young rat, may facilitate lumen-to-cell potassium absorption mediated by the H^+,K^+-ATPase.[55]

The major factors that influence the *external* balance of potassium are listed in Box 129-1 and are discussed in the following sections.

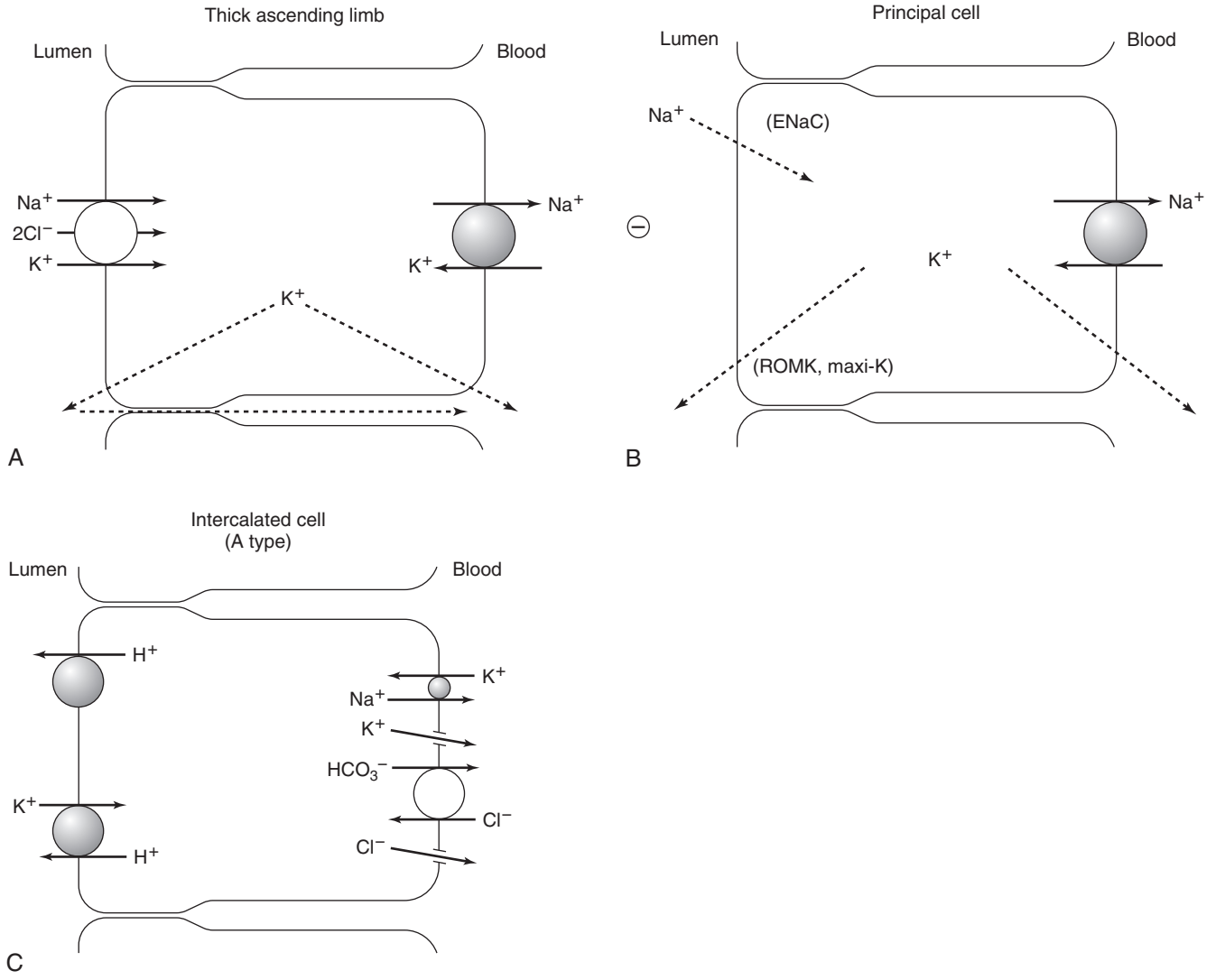


Figure 129-4 Potassium (K^+) transport pathways in specific renal tubular cells. A, In the TALH, potassium is avidly absorbed by specialized luminal Na^+-K^+-$2Cl^-$ cotransport. A luminal secretory potassium channel, encoded by the *ROMK* gene, in this cell, allows potassium to recycle back into the tubular fluid, thereby ensuring a continuous and abundant supply of potassium for the cotransporter. **B,** In the CCD, potassium is pumped into the cell in exchange for sodium by the basolateral Na^+,K^+-ATPase. After entry into and accumulation in the cell, potassium is secreted preferentially across the apical membrane through the *ROMK* or maxi-K channel, a process driven by a favorable electrochemical gradient. The electrochemical gradient is composed of two components: the cell-to-lumen concentration, or chemical gradient, and the cell-to-lumen electrical gradient. The latter is generated by apical sodium entry through amiloride-sensitive ENaCs and its electrogenic basolateral extrusion. **C,** Intercalated cells in the collecting duct mediate potassium absorption via apical H^+,K^+-ATPase, a pump that catalyzes the exchange of a single proton for potassium. *HCO_3^-,* Bicarbonate.

Distal Sodium Delivery and Transepithelial Voltage

As predicted from the principal cell model (see Figure 129-4, *B*), an increase in sodium absorption enhances the electrochemical driving force for potassium diffusion into the lumen. The magnitude of passive apical sodium entry and its electrogenic basolateral extrusion determine the apical membrane electrical potential and rate of basolateral Na^+-K^+ exchange. The dependence of potassium secretion on distal sodium delivery becomes evident at tubular fluid sodium concentrations less than 30 mEq/L, a value below which potassium secretion falls sharply.[73,74] In vivo measurements of the sodium concentration in distal tubular fluid generally exceed 35 mEq/L in both adult and suckling rats and thus should not restrict distal sodium secretion.[55,60,73,75]

Extracellular volume expansion or administration of many diuretics (osmotic diuretics, carbonic anhydrase inhibitors, loop and thiazide diuretics) is accompanied by an increase in excretion of both sodium and potassium. The kaliuresis is mediated not only by the increased delivery of sodium to the distal nephron but also by the increased tubular fluid flow rate, which maximizes the chemical driving forces, as described later, favoring potassium secretion. Other potassium-sparing diuretics, such as amiloride and triamterene, block distal sodium reabsorption, which reduces the electrical potential gradient favoring potassium secretion.

Sodium delivered to the distal nephron is generally accompanied by chloride. Chloride reabsorption, which occurs predominantly via the paracellular pathway, tends to reduce the lumen-negative potential that would otherwise drive potassium secretion. When sodium is accompanied by an anion less reabsorbable than chloride, such as bicarbonate (in proximal renal tubular acidosis), β-hydroxybutyrate (in diabetic ketoacidosis), or carbenicillin (during antibiotic therapy), luminal electronegativity is maintained, thereby eliciting more potassium secretion than occurs with a comparable sodium load delivered with chloride.

BOX 129-1

Factors Regulating the *External* Balance of Potassium

Renal factors
Distal sodium delivery and transepithelial voltage
Tubular (urinary) flow rate
Potassium intake–plasma potassium concentration
Hormones (mineralocorticoids, vasopressin)
Acid-base balance

Gastrointestinal tract factors
Stool volume
Hormones

Tubular Flow Rate

High rates of urinary flow in the mature, but not the neonatal or weanling, distal nephron stimulate potassium secretion.[66] Thus volume expansion and diuretics, both of which increase distal tubular fluid flow rate, enhance potassium secretion in the fully differentiated kidney. A number of factors are responsible for the flow stimulation of potassium secretion. First, flow stimulates sodium reabsorption, which enhances the electrochemical gradient favoring potassium secretion.[76,77] Second, the higher the urinary flow rate in the distal nephron the slower the rate of rise of tubular fluid potassium concentration because secreted potassium is rapidly diluted in urine of low potassium concentration. Maintenance of a low tubular fluid potassium concentration maximizes the potassium concentration gradient (and thus the chemical driving force) favoring net potassium secretion. Finally, increases in tubular fluid flow rate increase the intracellular calcium concentration, which activates the maxi-K channel in the collecting duct to secrete potassium, thereby enhancing urinary potassium excretion.[65,78]

The capacity for flow-stimulated potassium secretion appears to be developmentally regulated. Flow-stimulated potassium secretion, studied in single rabbit CCDs, does not appear until the 5th week of postnatal life, approximately 2 weeks after baseline potassium secretion is first detected.[66,78] Maxi-K channel messenger RNA and protein are not consistently detected in the CCD until the 4th and 5th weeks of life, respectively,[78] suggesting that the postnatal appearance of flow-dependent potassium secretion is determined by the transcriptional and/or translational regulation of expression of maxi-K channels in the distal nephron.

Potassium Intake and Cellular Potassium Content

Ingestion of a potassium-rich meal is rapidly followed by enhanced urinary excretion of potassium. The increase in potassium entry into principal cells from the basolateral (blood) side maximizes the concentration gradient favoring apical potassium secretion into the urinary fluid. Simultaneously, the increase in circulating levels of plasma aldosterone that accompanies potassium loading enhances the electrochemical driving force favoring potassium secretion in the distal nephron. It has also been suggested that a reflex increase in potassium excretion via vagal afferents follows activation of potassium-specific sensors in the gut or hepatic portal circulation, a control system that may be regulated in the absence of change in the plasma potassium concentration.[49]

Chronic potassium loading leads to *potassium adaptation,* an acquired tolerance to an otherwise lethal acute potassium load.[50] This adaptation occurs in the distal nephron and colon; the rate of potassium secretion in these segments varies directly with body stores of potassium. A similar adaptive response is seen in renal insufficiency such that potassium balance is maintained during the course of many forms of progressive renal disease. The mechanisms underlying this adaptation in the principal cell include not only an increase in the density of apical membrane potassium channels but also an increase in the number of conducting sodium channels and activity of the basolateral Na^+-K^+ pump. The latter two processes result in increases in transepithelial voltage and the intracellular potassium concentration, events that enhance the driving force favoring potassium diffusion from the cell into the urinary fluid.

When potassium intake is chronically reduced, potassium secretion by principal cells falls as reabsorption by intercalated cells increases. Stimulation of luminal H^+,K^+-ATPase activity in intercalated cells results not only in potassium retention but also in urinary acidification and metabolic alkalosis.

Hormones

Mineralocorticoids stimulate sodium reabsorption and potassium secretion in principal cells of the distal nephron.[79,80] In the adult, the mineralocorticoid-induced stimulation of renal potassium secretion is considered to be due primarily to an increase in the electrochemical driving force favoring potassium exit across the apical membrane, generated by stimulation of apical sodium entry and reabsorption. Aldosterone action requires its initial binding to the mineralocorticoid receptor, followed by translocation of the hormone-receptor complex to the nucleus in which specific genes are stimulated to code for physiologically active proteins (e.g., Na^+,K^+-ATPase). Cellular effects within the fully differentiated CCD include increases in the density of active ENaC channels, caused by recruitment of intracellular channels to the apical membrane, de novo synthesis of ENaC subunits, and activation of preexisting channels.[81,82] Aldosterone also stimulates Na^+,K^+-ATPase activity through both recruitment of preexisting pumps to the plasma membrane and increased total amounts of sodium pump subunits.[83] The sum effect of these actions is the stimulation of net sodium absorption, leading to an increase in lumen negative transepithelial voltage, thereby facilitating net potassium secretion. The effects of aldosterone on ENaC and, to some extent, the Na^+-K^+ pump appear to be indirect, mediated by aldosterone-induced proteins, including serum- and glucocorticoid-inducible kinase (sgk).[84]

Plasma aldosterone concentrations in the newborn are high compared with those in the adult.[2,85] Yet clearance studies in fetal and newborn animals demonstrate a relative insensitivity of the immature kidney to the hormone.[2,86-88] The density of aldosterone-binding sites, receptor affinity, and degree of nuclear binding of hormone receptor are thought to be similar in mature and immature rats.[88] Thus the early hyposensitivity to aldosterone is considered to represent a postreceptor phenomenon.[2,88]

The transtubular potassium gradient (TTKG) provides an indirect, semiquantitative measure of the renal response to mineralocorticoid activity in the aldosterone-sensitive cortical distal nephron and is calculated by using the equation: $TTKG = \{[K^+]_{urine} / (U/P)_{osmolality}\} / [K^+]_{plasma}$, where $[K^+]$ equals the potassium concentration in either urine (U) or plasma (P), as indicated.[89-91] Measurements of TTKG have been reported to be lower in 27- than 30-week–GA preterm infants followed over the first 5 weeks of postnatal life.[92] The low TTKG has been attributed to a state of relative hypoaldosteronism[92] but may also reflect the absence of potassium secretory transport pathways (i.e., channel proteins).

Acid-Base Balance

Disorders of acid-base homeostasis can induce changes in tubular potassium secretion. Acute metabolic acidosis causes the urine pH and potassium excretion to decrease, whereas both acute respiratory alkalosis and metabolic alkalosis result in increases in urine pH and potassium excretion. Chronic metabolic acidosis has variable effects on urinary potassium excretion.

The alkalosis-induced stimulation of potassium secretion reflects two direct effects on principal cells: (1) the stimulation of Na^+,K^+-ATPase activity and basolateral potassium uptake, and (2) an increase in the permeability of the apical membrane to potassium resulting from an increase in duration of time the potassium-selective channels remain open.[50] Alkalosis also decreases acid secretion in intercalated cells, thereby reducing H^+,K^+-ATPase–mediated countertransport.

Acute metabolic acidosis reduces cell potassium concentration and leads to a reduction in urine pH, which in turns inhibits apical potassium channel (SK/ROMK) activity.[50] The net effect is a fall in urinary potassium secretion. The effect of chronic metabolic acidosis on potassium secretion is more complex and may be influenced by modifications of the glomerular filtrate (e.g., chloride and bicarbonate concentrations), tubular fluid flow rate, and circulating aldosterone levels.[50] The latter two factors may lead to an increase rather than a decrease in potassium secretion and excretion.

Contribution of the Gastrointestinal Tract

Under normal conditions in the adult, 5% to 10% of daily potassium intake is excreted in the stool (see Figure 129-1).The colon is considered to be the main target for regulation of intestinal potassium excretion.[93] Potassium transport in the colon represents the balance of secretion and absorption.[94] Under baseline conditions, net potassium secretion predominates over absorption in the adult, whereas the neonatal gut is poised for net potassium absorption.[93]

Potassium secretion requires potassium uptake by the Na^+,K^+-ATPase and Na^+,K^+-$2Cl^-$ cotransporter located in the basolateral membrane of colonocytes; potassium is then secreted across the apical membrane through potassium channels, including a calcium-activated maxi-K channel similar to that found in the distal nephron.[95-98] Stool potassium content can be enhanced by any factor that increases colonic secretion, including aldosterone, epinephrine, and prostaglandins.[99-101] Indomethacin and dietary potassium restriction reduce potassium secretion by inhibiting the basolateral transporters and apical potassium channels. Diarrheal illnesses are typically associated with hypokalemia, presumably because of the presence of nonreabsorbed anions (which obligate K^+), an enhanced electrochemical gradient established by active chloride secretion, and secondary hyperaldosteronism resulting from volume contraction.

Potassium adaptation in the colon is demonstrated by increased fecal potassium secretion after potassium loading and in the face of renal insufficiency. Fecal potassium excretion may triple in patients with severe chronic renal insufficiency.[99,102-104] The enhanced colonic potassium secretion characteristic of chronic renal insufficiency requires induction and/or activation of apical maxi-K channels in surface colonic epithelial cells.[105]

Net colonic potassium absorption is significantly higher in young compared with adult rats.[93] The higher rate of potassium absorption during infancy is due to robust activity of potassium absorptive pumps located in apical membrane and limited activity of the basolateral transporters that mediate secretion.[106,107]

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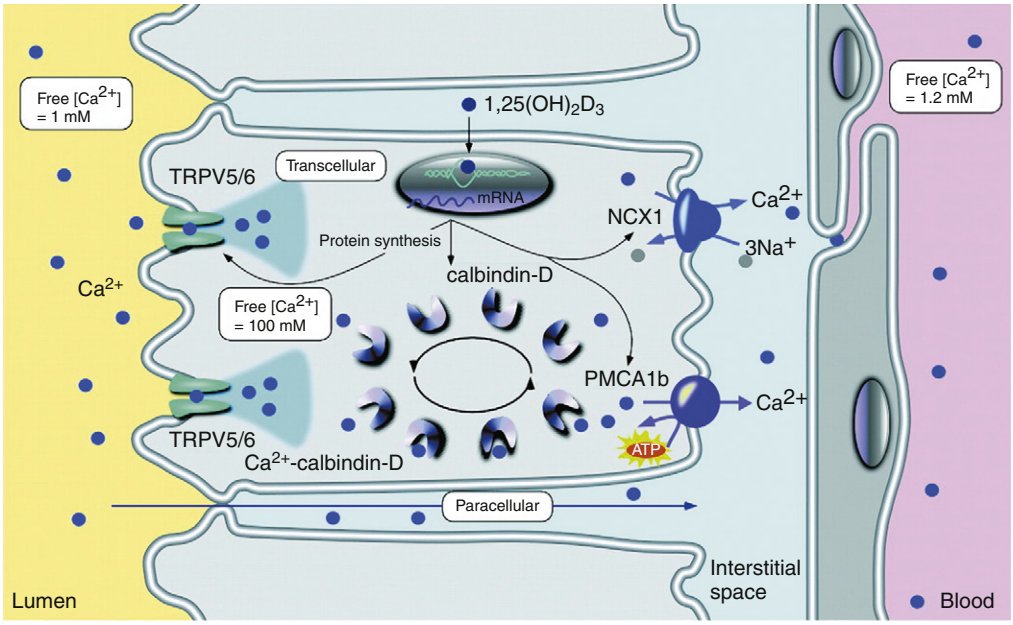


Figure 130-1 Mechanism of epithelial Ca^{2+} transport. Entry of Ca^{2+} is facilitated by the apical Ca^{2+} channel (TRPV5/6). In the cell, Ca^{2+} binds to calbindin-D and diffuses through the cytosol to the basolateral membrane. There, Ca^{2+} is extruded via a Na^{+}-Ca^{2+} exchanger (NCX1) and a Ca^{2+}-ATPase (PMCA1b). *(Modified from Hoenderop JG, Nilius B, Bindels RJ: Calcium absorption across epithelia,* Physiol Rev *85:373-422, 2005. Used with permission.)*

compartment via a sodium calcium exchanger and a calcium adenosine triphosphatase (ATPase).[13,16-18]

Epithelial Calcium Channels

Transient receptor potential (TRP) channel proteins constitute a large and diverse family of proteins that are expressed in many tissues and cell types.[19,20] Within the TRP superfamily is the TRPV subfamily consisting of six members. This group of channels includes TRPV1 through 4, which respond to heat, osmolality, odorants, and mechanical stimuli.[21,22] TRPV5, a highly Ca^{2+}-selective channel in the TRP family, was the first TRP channel identified in the kidney. TRPV5 is abundantly expressed in the distal convoluted tubule (DCT) and the connecting tubule (CNT) and has been recognized as the gatekeeper of active Ca^{2+} reabsorption in the kidney. Its function is further characterized by TRPV5 knockout (TRPV5−/−) mice, which exhibit profound hypercalciuria, intestinal Ca^{2+} hyperabsorption, and reduced bone thickness. TRPV6, a homologous highly Ca^{2+}-selective channel, was originally identified from rat duodenum but is also expressed in DCT, CNT, and cortical and medullary collecting ducts. TRPV6 is involved in intestinal Ca^{2+} absorption, but its role in the kidney remains largely unknown.[21,22]

Genomic cloning has demonstrated that these Ca^{2+} channels are transcribed from distinct genes rather than being splice variants. TRPV5 and TRPV6 are juxtaposed on the human chromosome 7q35 with a distance of only 22 kilobases, which suggests an evolutionary gene duplication event. TRPV5 and TRPV6 have been cloned from many species including rabbit, rat, mouse, and human. The identified sequences have an overall homology of 75%. Several domains in TRPV5 and 6 are completely conserved within these species including the core structure of the protein consisting of six transmembrane (TM) segments and the pore region, ankyrin repeats, PDZ motifs, and putative protein kinase C (PKC) phosphorylation sites, of which three are conserved among all identified TRPV5 and TRPV6 channels.[23,24]

The epithelial Ca^{2+} channels are responsible for the active transcellular entry of calcium at the apical membrane and are the initial, rate-limiting step in the process of transcellular Ca^{2+} transport. They are expressed in tissues such as intestine, kidney, pancreas, brain, prostate, and testis. Immunohistochemistry has shown TRPV5 to be present in the apical membrane of DCT, in CNT in the kidney, and in the brush border membrane of duodenal and jejunal villi. It is colocalized with other proteins of transcellular calcium transport such as sodium-calcium exchanger (NCX1), plasma membrane Ca^{2+}-ATPase (PMCA), and calbindin-D.[25-27]

Ca^{2+} movement across TRPV5 is controlled in multiple ways. TRPV5 gene expression is regulated by calciotropic hormones such as vitamin D_3 and PTH.[28,29] Channel activity is modulated by intracellular Ca^{2+} by feedback inhibition. TRPV5 is also controlled by mobilization of the channel toward the plasma membrane. The recently discovered antiaging hormone Klotho has been shown to enhance TRPV5 activity via a novel mechanism modifying its glycosylation status and entrapping the channel at the cell surface resulting in a prolonged expression of TRPV5 at the plasma membrane.[30] The immunosuppressant tacrolimus has also been shown to decrease activity of TRPV5 and calbindin-28K, causing hypercalciuria.[31] Ca^{2+} uptake in TRPV5 expressing *Xenopus laevis* oocytes is inhibited by acidification of the incubation medium. Extracellular pH determines the cell surface expression of TRPV5 via a unique mechanism. Extracellular alkalinization causes a pool of TRPV5-containing vesicles to be rapidly recruited to the cell surface without collapsing into the plasma membrane, resulting in increased TRPV5 activity. Extracellular acidification on the other hand causes the vesicles to be retrieved from the plasma membrane, resulting in decreased TRPV5 activity. This effect could explain the molecular basis of acidosis-induced calciuresis.[32]

In the distal tubules intracellular calcium concentration is increased by osmosensitive, nonselective ion channels such as TRPV4 (previously described as OTRPC4, VR-OAC, TRP 12, and VRL-2) especially under hypotonic stimulus.[33]

Calcium Buffering Within the Cell

The vitamin D–dependent Ca^{2+}-binding proteins, named calbindins (CaBPs), are expressed in cells that are challenged by a high Ca^{2+} influx such as in brain, bone, teeth, inner ear, placenta,

mammary gland, kidney, and intestine. In these tissues, CaBPs (i.e., $CaBP_{9K}$ and $CaBP_{28K}$) are a key component in cellular Ca^{2+} handling. In the kidney, calbindin-D ($CaBP_{28K}$) facilitates Ca^{2+} diffusion from the luminal Ca^{2+} entry side of the cell to the basolateral side, where Ca^{2+} is extruded into the extracellular compartment. CaBP (28K) provides protection against toxic high intracellular Ca^{2+} levels by buffering the cytosolic Ca^{2+} concentration during high Ca^{2+} influx.[34] Studies using protein-binding analysis, subcellular fractionation, and evanescent-field microscopy have shown that $CaBP_{28K}$ translocates toward the plasma membrane and directly associates with TRPV5 at a low Ca^{2+} concentration. Uptake measurements, electrophysiologic recordings, and transcellular Ca^{2+} transport assays of lentivirus-infected primary rabbit CNT-DCT cells have shown that associated $CaBP_{28K}$ tightly buffers the flux of Ca^{2+} entering the cell via TRPV5, facilitating high Ca^{2+} transport rates by preventing channel inactivation. Therefore $CaBP_{28K}$ acts in calcium-transporting epithelia as a dynamic Ca^{2+} buffer, regulating Ca^{2+} in close vicinity to the TRPV5 pore by direct association with the channel. Calbindins are regulated by calciotropic hormones including vitamin D, estrogens, PTH, and dietary Ca^{2+}.[35]

Previously $CaBP_{28K}$ was identified as a high-capacity Ca^{2+} buffer with Ca^{2+} affinities fitting the classical properties of a Ca^{2+} buffer. However, sequential Ca^{2+} binding and conformational changes suggested that $CaBP_{28K}$ can act as a Ca^{2+} sensor controlling downstream cellular processes.[36-39] It was shown in cerebellar Purkinje neurons that $CaBP_{28K}$ directly interacts with membrane-targeted inositol-1,4,5-trisphosphate and acts as a Ca^{2+} sensor to regulate the degradation of inositol messengers in an activity-dependent manner.[40]

Inside the cell, calbindin directly interacts with a calmodulin-binding isoleucine-glutamine motif present in the C-tail of TRPV5 and TRPV6 channels. Deletion of the first eight amino acids of the isoleucine-glutamine motif in the carboxyl terminus tail of the Ca^{2+}-channel subunit a1C eliminates Ca^{2+}-dependent inactivation of voltage-gated, L-type Ca^{2+} channels.[41] Ca^{2+}-dependent interaction also occurs between calbindin and a novel site in the C-terminal domain of the 1A subunit of P/Q-type Ca^{2+} channels (calbindin-binding domain). In the presence of low concentrations of intracellular Ca^{2+} chelators, Ca^{2+} influx through P/Q-type channels enhances channel inactivation, increases recovery from inactivation, and produces a long-lasting facilitation of the Ca^{2+} current.[26] Ca^{2+} "shuttling" across the intracellular organelles such as mitochondria or endoplasmic reticulum depends on a Ca^{2+} electrochemical gradient, driven by internally negative membrane potential across the inner mitochondrial or endoplasmic reticular membrane.

Calcium Transport Across the Basolateral Membrane

The extrusion of Ca^{2+} across the basolateral membrane into the interstitium occurs against an electrochemical gradient and, as such, is an energy-dependent process. The transport is primarily mediated by two calcium transporters located at the basolateral membrane: Na^+-Ca^{2+} exchanger (NCX)[42] and the Ca^{2+}-ATPase (plasma membrane Ca^{2+}-ATPase [PMCA]).[43]

Na^+-Ca^{2+} Exchanger

Three genes for NCX known as NCX1, NCX2, and NCX3 have been identified in mammals. In the kidney the expression of this transporter is restricted to the distal part of the nephron where it predominantly localizes along the basolateral membrane.[44,45]

NCX1 is widely distributed in many different mammalian tissues, whereas NCX2 and NCX3 are expressed only in brain and skeletal muscle.[46,47] It has been demonstrated that NCX1 is the primary extrusion mechanism, whereas only a minor amount of Ca^{2+} in the distal tubular cells is extruded by the plasma Ca^{2+} pump.[48] It has also been shown that targeted deletion of NCX1 results in NCX1-null embryos that do not have a spontaneously beating heart and die in utero.[49,50] Studies in oocytes and mammalian cell systems show that NCX is regulated by several factors including the membrane potential, PKC activation, protons, nucleotides, and calciotropic hormones.[51] PTH markedly stimulates Ca^{2+} reabsorption in the distal part of the nephron primarily by augmenting NCX1 activity via a cyclic adenosine monophosphate (cAMP)–mediated mechanism.

Plasma Membrane Ca^{2+}–Adenosine Triphosphatase

PMCA belongs to a class of P-type, ion-motive ATPase proteins with molecular weights ranging from 120,000 to 140,000 daltons. PMCAs are high-affinity Ca^{2+} efflux pumps present in almost all cells and are responsible for the maintenance and resetting of the resting intracellular Ca^{2+} levels.[51]

Four different isoforms—PMCA1 to A4—are encoded by separate genes. PMCAs are a universal system for the extrusion of Ca^{2+} in cells. In the kidney PMCAs are present in all nephron segments with highest expression in the basolateral membrane of cells lining the distal part of the nephron. The DCT possesses the highest Ca^{2+}-ATPase activity and exhibits the strongest immunocytochemical reactivity for PMCA protein expression.[52-54]

In humans PMCAs are found along the DCT and, in contrast to other species, along the cortical collecting duct.[44] Studies suggest that PMCA1 and PMCA4 are the major isoforms expressed in the kidney, whereas PMCA2 and PMCA3 are more tissue specific. PMCA1 and PMCA4 are involved in the maintenance of cellular Ca^{2+} homeostasis.[55] PMCA4b plays a significant role in basolateral Ca^{2+} extrusion. PMCA1b is the predominant isoform and abundantly expressed in the small intestine, where NCX1 is expressed at a low level. It is the major Ca^{2+} extrusion mechanism in intestinal Ca^{2+} absorption. PMCA also plays a role in cytosolic acidification, which, in turn, is a signal for activation of the sarcoplasmic reticulum Ca^{2+}-ATPase and other cellular functions in muscle.

Limited data are available regarding the regulation of PMCA by hormones or signaling mechanisms. Several studies indicate that PMCA are positively regulated by 1,25-dihydroxyvitamin D_3 ($1,25\text{-}[OH]_2D_3$) in the intestine to increase Ca^{2+} absorption. In addition, several potassium (K^+)-dependent Na^+-Ca^{2+} exchangers (NCKX) have been described.[51,56] Northern blot analysis demonstrated that some isoforms (i.e., NCKX4 and NCKX6) of this family are expressed in epithelia including small intestine and kidney.[57,58]

These exchangers are found in various tissues suggesting a key role in regulating intracellular Ca^{2+} homeostasis in mammalian cells. Ca^{2+} extrusion from the cell is a Na^+-Ca^{2+} countertransport driven by the Na^+ concentration gradient across the basolateral membrane maintained by Na^+,K^+-ATPase. Ca^{2+} removal by this exchanger is slowed when extracellular Na^+ concentration is diminished or when Na^+,K^+-ATPase is inhibited with ouabain.[59]

Regulation of Calcium Transport

Parathyroid Hormone

The parathyroid glands play an important role in maintaining the extracellular Ca^{2+} concentration. They have the capacity to sense changes in the level of blood Ca^{2+} from its normal level via the Ca^{2+}-sensing receptor (CaR). In response to low blood Ca^{2+} levels, PTH is secreted into the circulation and then acts primarily on kidney and bone, where it activates the PTH-PTHrP receptor. In the kidney, PTH stimulates transcellular Ca^{2+} reabsorption via a slow genomic pathway and a rapid nongenomic pathway.

The rapid pathway is via activation of adenylyl cyclase, accumulation of cAMP, and stimulation of protein kinase A (PKA), resulting in an increase in the cytosolic concentration and transcellular Ca^{2+} transport. PKA inhibitors reduce the PTH-mediated rise of cytosolic Ca^{2+}, which suggests that cAMP–dependent phosphorylation is an essential step in short-term PTH stimulation.

In addition to cAMP, PKC is also involved in the short-term PTH response. The slow pathway results in an elevated expression of the Ca^{2+} transport proteins TRPV5, calbindin-D28K, NCX1, and PMCA1b.

The PTH receptor directly enhances the tubular Ca^{2+} reabsorption, and it stimulates the activity of 1α-OHase and, thereby, increases the $1,25\text{-}(OH)_2D_3$–dependent absorption of Ca^{2+} from the intestine. PTH binds to two types of receptors: PTHR1, which also binds the PTH-related peptide (PTHrP),[60] and PTHR2, which binds only PTH.[61] The PTHR1 receptor is the predominant receptor found in the kidney, and it is expressed in glomeruli and all tubule segments, except the TAL and collecting duct. The PTHR2 receptor is distributed primarily in brain, lung, pancreas, and vasculature (including the vascular pole of the glomerulus). Signal transduction for both PTH and PTHrP is via adenylate cyclase and phospholipase C.[62,63] PTH stimulates active Ca^{2+} reabsorption in the distal part of the nephron. PTH increases transepithelial Ca^{2+} transport via a dual signaling mechanism involving PKA- and PKC-dependent processes.[64,65] PTH has various actions such as membrane insertion of apical Ca^{2+} channels, opening of basolateral chloride channels resulting in cellular hyperpolarization, and modulation of PMCA activity.[66-68] PTH activates dihydropyridine-sensitive channels responsible for Ca^{2+} entry. Once inserted or activated, these dihydropyridine-sensitive channels could mediate Ca^{2+} entry into Ca^{2+}-transporting epithelial cells. It is thought that dihydropyridine-sensitive Ca^{2+} channels play a role in signal transduction processes to maintain the cellular Ca^{2+} homeostasis. Studies of parathyroidectomy in rats showed a decrease in serum PTH levels and hypocalcemia, which was accompanied by decreased levels of TRPV5, calbindin-D_{28K}, and NCX1.[69] Supplementation with PTH restored serum Ca^{2+} concentrations and abundance of these Ca^{2+} transporters in kidney. This suggests that PTH affects renal Ca^{2+} handling through the regulation of the expression of the active renal Ca^{2+} transport proteins, including the epithelial Ca^{2+} channel TRPV5. PTH also stimulates the PMCA activity by increasing the affinity for Ca^{2+} in the distal tubule, unlike $1,25\text{-}(OH)_2D_3$ that does not directly affect the basolateral membrane PMCA activity.

In bone, PTH can induce a rapid release of Ca^{2+} from the bone matrix and can also mediate long-term changes in Ca^{2+} metabolism by acting directly on the bone-forming osteoblasts and indirectly on bone-resorbing osteoclasts by increasing their number and activity.

Calcium-Sensing Receptor

The CaR was discovered in 1993. It is a plasma membrane–bound G-protein–coupled receptor, found in many tissues including the parathyroid gland, thyroid, kidney, intestine, bone, bone marrow, brain, skin, pancreas, lung, and heart. CaR plays a critical role in Ca^{2+} homeostasis by inducing changes in PTH secretion and renal Ca^{2+} reabsorption in response to variations in the extracellular concentration of Ca^{2+}. CaR is not specific for calcium alone as it responds to other cations such as magnesium, aluminum, and gadolinium, but it has the highest affinity for calcium. The human CaR is encoded by six exons of the CaR gene located on chromosome 3q13.3–21.[70] The receptor is expressed abundantly in the parathyroid glands and, to a lesser extent, along the length of the kidney tubule.[45]

Activation of the CaR on the parathyroid gland cells by elevated serum Ca^{2+} leads to activation of many secondary messengers resulting in inhibition of PTH synthesis and secretion. Activation of the CaR in response to hypercalcemia produces several different effects in the renal tubules. In the TAL, decrease occurs in Na^+, K^+, and Cl^- reabsorption and decrease in K^+ transfer across a specific potassium channel at the apical membrane causing a decrease in the lumen positive voltage in the tubular lumen leading to decreased calcium and magnesium reabsorption and increased distal delivery. In the distal tubule, CaR stimulation on the basolateral membrane causes inhibition of calcium transport. In the medullary collecting duct epithelium, CaRs are found on the apical side. Here they reduce the insertion of aquaporin-2 water channels when the luminal calcium concentration is high, decreasing water reabsorption, and thereby decreasing calcium concentration and calcium crystal formation in the urine.[71-73]

Mutations in the CaR gene are associated with familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, and autosomal dominant hypocalcemia.[74-78]

Vitamin D

The physiologic actions of vitamin D are mediated by its metabolite $1,25(OH)_2D_3$, which is formed by 1-hydroxylation of 25(OH) D in proximal tubules. The classic action of vitamin D is to stimulate transcription by binding to nuclear receptors (VDR). It binds with VDR in a ligand-dependent manner. VDR is expressed in epithelia that play a role in Ca^{2+} reabsorption. Microarray analysis of $1,25(OH)_2D_3$-stimulated carcinoma cells has shown TRPV6 as one of the most highly vitamin D–responsive genes.[79]

Rats fed a vitamin D–deficient diet showed diminished Ca^{2+} reabsorption in the kidney, because of the down-regulation of the Ca^{2+} transport proteins TRPV5, calbindin-D28K, and NCX1. Supplementation of $1,25(OH)_2D_3$ restored the expression of the renal Ca^{2+} transport proteins and normalized the hypocalcemia. TRPV5 knockout mice showed renal Ca^{2+} wasting because of impaired reabsorption in the DCT and CNT. However, elevated $1,25(OH)_2D_3$ plasma levels, intestinal Ca^{2+} hyperabsorption, and reduced bone thickness prevented hypocalcemia in these knockout mice. The underlying mechanism of this compensatory hyperabsorption is hypovitaminosis D, which increases the expression of the intestinal Ca^{2+} transport proteins TRPV6, calbindin-D9K, and PMCA1b. The recently developed 1α-OHase and TRPV5 double-knockout (1α-OHase−/−/TRPV5−/−) mice have hypocalcemia and absence of hypervitaminosis D, again reinforcing the role of vitamin D in maintaining calcium levels in TRPV5 knockout mice.[80,81]

Calcitonin

Calcitonin is a 32–amino acid peptide and is secreted by the parafollicular cells of the thyroid gland. It is released in response to hypercalcemia and lowers serum calcium by various mechanisms. Its main action for lowering calcium is by inhibiting bone resorption. Calcitonin receptors are found on osteoclasts. The signal transduction pathways for calcitonin receptors are adenylate cyclase–PKA and phospholipase C–PKC via linking with G-proteins. In the kidney, calcitonin increases the urinary excretion of calcium and phosphorus, independent of PTH.[82-85] Calcitonin has not been shown to affect intestinal calcium absorption but may decrease phosphorus absorption. Calcitonin is a major regulator of renal 1,25-hydroxylase gene expression.[86]

Plasma Calcium Concentration

Hypercalcemia

Hypercalcemia results in an increase in Ca^{2+} excretion caused by a net increase in the filtered load and a decrease in tubular reabsorption. Hypercalcemia, in the presence of intact parathyroid glands, decreases the glomerular ultrafiltration coefficient (K_f) and thus causes a decline in glomerular filtration rate (GFR). Yet, the filtered load of Ca^{2+} may increase as a result of the elevation in serum Ca^{2+} concentration.[87,88] Hypercalcemia also causes a decline in the tubule reabsorption of Ca^{2+} by PTH-independent mechanisms.[89] This effect is mediated by stimulation of the CaR, which inhibits the apical K^+ channel and K^+ recycling, necessary for the activity of the Na^+-K^+-$2Cl^-$ transporter.[90] Decreased activity of the transporter decreases the lumen-positive potential difference and thus Na^+, Ca^{2+}, and Mg^{2+} reabsorption. In addition,

a decrease in intestinal Ca^{2+} absorption is brought about by diminished synthesis of $1,25(OH)_2D_3$.

Hypocalcemia

In hypocalcemic conditions, an increase occurs in PTH secretion that results in the mobilization of Ca^{2+} from the bone and soft tissues. This produces a fall in PTH that, in turn, promotes a decrease in fractional excretion of Ca^{2+} and a decline in net Ca^{2+} excretion. The CaR is thought to play a significant role in the enhancement of Ca^{2+} reabsorption in the TAL.[91] Decreased Ca^{2+} concentration in the vasa recta contributes to enhanced extrusion of Ca^{2+} at the basolateral membrane. Hypocalcemia also stimulates the production of $1,25(OH)_2D_3$, which increases the intestinal absorption of Ca^{2+}.

Phosphate and Magnesium

Hypophosphatemia produces a decrease in PTH resulting from an increase in plasma Ca^{2+} concentration. The ensuing hypercalciuria is, however, only partially corrected by the administration of PTH, a finding suggesting a direct effect of phosphate (PO_4^{3-}) deprivation on renal Ca^{2+} transport.[92] Phosphate infusion enhances Ca^{2+} reabsorption in the distal nephron and reduces Ca^{2+} excretion, even in the setting of volume expansion. Mg^{2+} infusion produces an increase in Ca^{2+} excretion that is not corrected by PTH infusion.[93]

Volume Status

Expansion of intravascular space produces natriuresis and increases Ca^{2+} excretion by inhibiting Na^+ (and therefore Ca^{2+}) reabsorption in the proximal tubule and Ca^{2+} reabsorption in the distal tubule. This effect, which is independent of circulating PTH levels,[94] is used in treating patients with hypercalcemia. Contraction of the extracellular volume increases proximal tubular reabsorption of Na^+ (and Ca^{2+}) and results in decreased Ca^{2+} excretion.

Acid-Base Status

Both acute and chronic metabolic acidosis have been shown to induce the release of Ca^{2+} from bone and to inhibit distal reabsorption of Ca^{2+}, resulting in hypercalciuria.[95] The effect of acidosis on tubular transport is mediated by a reduction in TRPV5 activity.[96] Metabolic alkalosis increases Ca^{2+} reabsorption in proximal tubule and decreases Ca^{2+} excretion.[97]

Insulin, Glucagon, and Glucose

Ca^{2+} excretion is enhanced by glucose infusion. Insulin infusion and hyperinsulinemia are associated with reduced proximal reabsorption of Na^+, water, and Ca^{2+}, but only Ca^{2+} excretion is increased. Insulin induces a rise in near-membrane Ca^{2+} but not in free intracellular Ca^{2+} in muscle cells. The rise in near-membrane Ca^{2+} is the result of an increase in influx through L-type Ca^{2+} channels.[98,99] Glucagon has a natriuretic and calciuretic effect caused by increases in renal blood flow and GFR.[100]

Mineralocorticoids

Acute mineralocorticoid excess is associated with the retention of Na^+ but not Ca^{2+}. Chronic mineralocorticoid excess, conversely, results in an escape from Na^+ retention (after 3 to 5 days) and a concomitant rise in Ca^{2+} excretion.[101] This is the case in the Bartter syndrome, which is characterized by high aldosterone levels, salt wasting, hypokalemic alkalosis, hypercalciuria, and normal plasma Mg^{2+} levels. These manifestations are consequent to genetic defects in the Na^+-K^+-$2Cl^-$ transporter or the adenosine triphosphate–sensitive K^+ channel (renal outer medullary potassium [ROMK]) in the TAL and are similar to those consequent to the administration of loop diuretics.[102] The Gitelman syndrome is also associated with salt wasting and hypokalemia, but, unlike in the Bartter syndrome, the excretion of Ca^{2+} is reduced and plasma levels of Mg^{2+}s are low. The genetic defect resides in the thiazide-sensitive Na^+-Cl^- cotransporter of the distal convoluted tubule encoded by the human thiazide-sensitive Na^+-Cl^- cotransporter (SLC12A3) gene. Reduced function of this cotransporter results in an increase in Ca^{2+} reabsorption because of stimulation of the basolateral extrusion of Ca^{2+} through the NCX transporter and a decrease in Mg^{2+} reabsorption because of inhibition of an apical Na^+-Mg^{2+} exchanger.[103]

Diuretics

Loop diuretics (furosemide, ethacrynic acid, and bumetanide) inhibit the Na^+-K^+-$2Cl^-$ (NKCC2) transporter present in the apical membrane of the TAL resulting in a reduction in NaCl reabsorption and K^+ recycling across the apical membrane. This action diminishes the lumen-positive potential, which is the driving force for paracellular Ca^{2+} reabsorption in this particular nephron segment and explains the hypercalciuric effect of furosemide. The calciuric effect of furosemide enhances the delivery of Ca^{2+} to DCT and CNT, which are the primary sites of active Ca^{2+} reabsorption. It is at present unknown whether these latter nephron segments partly compensate the hypercalciuric effect of the loop diuretics.[104]

Thiazide diuretics cause a fall in urinary Ca^{2+} excretion. They increase renal Na^+ excretion by inhibiting the Na^+-Cl^- cotransporter present in the apical membrane of DCT cells. This inhibition of Na^+ reabsorption results in increased renal salt and water loss and thereby decreases extracellular volume.[105] The underlying mechanism is an enhancement of proximal Na^+ reabsorption observed in volume contraction by diuretics and Na^+ restriction, which leads to an increase in the electrochemical driving force for passive Ca^{2+} reabsorption.[106-109] Thiazide diuretics can reduce Ca^{2+} excretion in the absence of PTH or volume contraction. They increase Ca^{2+} reabsorption and inhibit Na^+ reabsorption, thus revealing the dissociation in the transport of these ions in distal nephron.[110]

Autocrine and Paracrine Calcitropic Hormones

Arginine Vasopressin

Arginine vasopressin is the key regulator of water reabsorption in the distal nephron. In addition, it has a Ca^{2+}-sparing effect, mediated by an increase in the paracellular transport.[111]

Prostaglandin E_2

In the cortical collecting duct, prostaglandin E_2 has a dual effect on Ca^{2+} transport. By interacting with apical and basolateral prostaglandin EP2 or EP4 receptors, prostaglandin E_2 stimulates Ca^{2+} transport, whereas via interaction with basolateral EP3 receptors it inhibits the stimulatory action of other calciotropic hormones.[112] The G-protein–coupled EP3 receptor modulates cAMP: via G_i activation, it inhibits adenylyl cyclase, and, via G_s activation, it stimulates adenylyl cyclase activity.[113] The molecular mechanism by which EP2 or EP4 stimulates and EP3 inhibits the action of calcitropic hormones is unknown.

Adenosine

Acting via apical A1 receptors, adenosine increases transcellular Ca^{2+} transport to the same extent as PTH, arginine vasopressin, and prostaglandin E_2.[114]

Adenosine Triphosphate

Adenosine triphosphate inhibits the action of stimulatory calciotropic hormones. The effect is mediated via both apical and basolateral P2y receptors.[115]

Nitric Oxide

Nitric oxide activates Ca^{2+} reabsorption via cyclic guanosine monophosphate.[116]

Thyroid Hormone

Ca^{2+} uptake is increased in renal brush-border membrane vesicles from hyperthyroid rats and is decreased in those from hypothyroid rats.[117]

Fetal and Neonatal Aspects of Renal Transport of Calcium

Ca^{2+} is vital for the adequate mineralization, growth, and development of the fetal skeleton. Approximately 30 g of Ca^{2+} is transferred via the human placenta from the mother to the fetus, mainly during the third trimester. The syncytiotrophoblasts actively transport 80% of Ca^{2+} from maternal to fetal circulation where it is needed for fetal skeleton growth, especially during the third trimester of pregnancy.[118,119] Syncytiotrophoblasts of the placenta have mechanisms of calcium transport that are similar to those found in the distal renal tubules.[120] TRPV6 expression in human placenta was shown to be much higher compared with its level in the kidney and small intestine. This higher TRPV6 placental expression is in line with the fact that transplacental Ca^{2+} transport is critical for normal fetal growth and development. This is also an indication that the transcellular, rather the paracellular, pathway of the transepithelial Ca^{2+} transport, in which the TRP proteins do not participate, may be dominant in the placenta.[121]

Toward the end of gestation, the Ca^{2+} levels in fetal plasma (total and ionized) are higher than they are in the mother.[122] Hormones such as calcitonin, PTH, and PTHrP have been found in maternal and fetal circulations, and they originate from mother, fetus, and placenta.[123] PTHrP has been found in the parathyroid glands of fetal sheep, in a 7-week-old human fetus, and in human placenta.[124] This protein is reported to stimulate placental Ca^{2+} transfer from mother to fetus in animals and in humans.[125] However, fetal PTH is not required for transplacental Ca^{2+} transfer.[126] The placenta has most of the G-protein–coupled receptors that bind the calcitropic hormones. CaR is expressed in both villous and extravillous regions of the human placenta and contributes to the local control of transplacental Ca^{2+} transport and to the regulation of placental development.[127] Polycystin-2, a ubiquitous transmembrane glycoprotein, mutated in autosomal dominant polycystic kidney disease, is present in term human syncytiotrophoblast, in which it behaves as a nonselective cation channel. Fetal renal cortex adenylate cyclase activity increases in response to PTH, as evidenced by an increase in urinary cAMP excretion after PTH administration in fetal sheep preparations[128] and by adenylate cyclase responsiveness in fetal rabbit[129] and fetal rat kidney preparations.[130] PTH infusion into fetal lambs results in a rise in plasma Ca^{2+}, a decline in plasma phosphate, and increases in urinary flow rate and urinary Ca^{2+} excretion.[131] Several studies in young animals, as well as in premature and term human neonates, revealed an increase in PTH levels during hypocalcemia and a calcemic response to PTH.[124,132] Yet, premature infants given exogenous PTH had minimal increases in urinary cAMP until day 6 of life, a finding suggesting a maturational delay in response to PTH by the nephron. Perfusion of isolated newborn guinea pig kidneys with a PTH-containing solution increased Ca^{2+} reabsorption and cAMP excretion, but it did not affect the reabsorption of phosphate.[133] Similar effects have been observed in full-term human newborns.[134] Vitamin D–dependent Ca^{2+}-binding proteins, thought to be involved in transepithelial Ca^{2+} transfer, have been found in human kidneys as early as 14 weeks gestation.[135] Yet, the major function of the kidneys in fetal Ca^{2+} homeostasis appears to be the production of $1,25(OH)_2D_3$, rather than renal regulation of Ca^{2+} excretion.[136] The urinary Ca^{2+}-creatinine ratio (millimoles to millimoles) was reported to increase from 0.05 to 1.2 in term neonates and from 0.3 to 2.3 in preterm neonates during the first week of life. Children older than 1 year of age have a mean urinary Ca^{2+}-creatinine ratio (millimoles to millimoles) of 0.40, and school-aged children have a ratio of less than 0.21.[137] These findings suggest that the fractional excretion of Ca^{2+} is high in newborns, especially in those born prematurely.[138] However, the high excretion rates observed in these infants may have been caused, at least in part, by a low phosphate intake because these children were largely breast-fed.[139] Urinary Ca^{2+} excretion in preterm neonates has been also found to vary directly with urinary flow rate and with urinary Na^+ excretion. Yet, young animals given a saline load had an attenuated natriuretic response but a similar calciuretic response when compared with adult animals.[140] This finding suggests linked proximal tubular Ca^{2+} and Na^+ reabsorptive mechanism and an unlinked distal mechanism in newborn animals.[141]

PHOSPHORUS

The importance of maintaining proper phosphate balance is illustrated by the wide variety of biologic processes that use phosphate, including adenosine triphosphate production, coenzymes, and signaling proteins, in addition to its role as a major constituent of bone, muscle, and even the cellular bilipid membrane. Although an acute decrease in circulating plasma phosphate produces symptoms consisting of myopathy (weakness and myalgia), sinus bradycardia, and hematologic disturbances, chronic hypophosphatemia can contribute to development of congestive heart failure, as well as bone demineralization (leading to osteomalacia in adults and rickets in children).[142] Thus a positive phosphate balance in neonates and children is critical for proper growth and development.

During the perinatal period, phosphate homeostasis, like that of calcium, involves the integrated action of several hormonal systems and certain key factors. The high metabolic demand of the neonate for phosphate is met through a relatively high intake of dietary phosphate, efficient intestinal phosphate absorption, and reduced urinary phosphate loss. The kidney plays a critical role in this process by limiting the urinary excretion of phosphate through enhanced tubular phosphate reabsorption.[143-149] The rate of renal phosphate uptake largely determines the level of extracellular phosphate and, ultimately, regulates phosphate homeostasis.

Of the total body phosphorus, approximately 80% is present in the skeleton, with the remainder being in muscle, soft tissues, and finally extracellular fluids. Approximately two thirds of the circulating pool of phosphorus is in the form of organic phosphates (such as esters and phospholipids) and one third is as inorganic phosphate, essentially all in the form of orthophosphate. It is estimated that the latter fraction, which represents the plasma phosphate concentration, exists as free phosphate, complexed with calcium or magnesium, or protein bound. The plasma phosphate concentration is highest in infants (4.5-9.3 mg/dL) and higher in children (4.5-6.5 mg/dL) than in adults (3.0-4.5 mg/dL).[150] This section focuses on the adaptations in renal phosphate handling and response to regulators of phosphate transport that facilitate the increased renal retention of phosphate during development. Furthermore, this discussion concentrates on new advances in renal phosphate handling since the publication of excellent reviews.[151-153]

Phosphate Reabsorption and the Transport Maximum

Ninety percent of plasma phosphate is freely filterable across the glomerular capillary as 10% is protein bound. However, because of the Donnan effect (the inability of charged molecules to evenly distribute across a semipermeable membrane) and correction for plasma water, the filtered load of phosphate is still calculated as the product of the total plasma phosphate concentration (not 90%) and the GFR.

Under normal conditions, the kidney reabsorbs 75% to 85% of the filtered phosphate load with the remainder delivered to the collecting duct acting as a titratable acid and excreted in the urine. The tubular reabsorption of phosphate is a saturable process characterized by a transport maximum (TmPi), although the TmPi is not a fixed value but rather influenced by the actions of hormones, dietary phosphate, and growth.[151,153] To facilitate comparison of TmPi values from individuals or animals of different sizes, values are normalized by kidney, body mass, or more commonly the GFR. On this basis, the TmPi-GFR in infants[154,155] and neonatal animals[134,154,156] is greater than in corresponding adults. Furthermore, in contrast to the adult, the developing animal has a renal threshold that is far greater than the normal plasma phosphate concentration, suggesting that the newborn can maintain avid phosphate uptake in the face of high plasma phosphate levels.

For many years, the limited ability of the newborn to excrete phosphate was thought to be a consequence of the reduced GFR. Although this may be a limiting factor in the very early postnatal period, studies performed in weaned rats,[143-153,156] newborn guinea pigs,[157] and older infants[155] indicate that the reduced urinary excretion of phosphate occurs because of a high rate of tubular phosphate reabsorption and does not result from the low GFR.

The bulk of filtered phosphate is reabsorbed in the proximal convoluted tubule. In adult animals under normal conditions, approximately 70% of the phosphate present within the glomerular ultrafiltrate is reabsorbed within this segment, and the rate of renal reabsorption could increase to 85% to 90% in states of phosphate retention such as hypoparathyroidism,[151-153] dietary phosphate deprivation,[158-161] and respiratory alkalosis.[158,161] This increase in phosphate retention is facilitated by reabsorption in segments beyond the proximal convoluted tubule, such as the proximal straight tubule (pars recta),[161-163] DCT,[159,164] and cortical collecting tubule,[165] particularly in states of phosphate conservation.[158,160,163,166] The postnatal period of rapid growth and development may represent a state of phosphate conservation that involves avid renal phosphate retention. Results of micropuncture experiments indicate that a proportionally greater fraction of the filtered phosphate load is reabsorbed in the early part of the proximal convoluted tubule of newborn guinea pigs compared with adults.[157] Kinetic studies in newborn guinea pigs suggest that the elevated phosphate uptake in the proximal tubule is a result of a higher maximum velocity (V_{max}) of phosphate transport, equating to an increased number of Na-dependent P_i cotransporters,[167] whereas studies in newborn rats demonstrated that a rise in the affinity (K_m) of the sodium-phosphate transporter may also play a role.[168] Of interest, fractional phosphate reabsorption along the proximal convoluted tubule in neonatal rats is reduced until 3 weeks of age and then equals or even exceeds the rate seen in older rats.[168]

Studies using nuclear magnetic resonance techniques on isolated kidney cells have reported lower intracellular phosphate concentrations in kidneys of neonates compared with adults,[169,170] despite the known higher rate of phosphate entry into these tubular cells. This suggests that the rate of basolateral phosphate efflux must also be elevated in the newborns and may contribute to their high reabsorptive capacity for phosphate. The concentration of intracellular phosphate has been directly correlated with postnatal age and dietary phosphate supply and inversely correlated with the TmPi. However, it is not significantly altered when TmPi is increased or decreased because of changes in the demand for phosphate that result from reductions in the rate of body growth or bone mineralization.[170] This suggests that variations in intracellular phosphate concentration do not contribute to those renal adaptations.

Estimates based on the differences between proximal tubular phosphate reabsorption and urinary phosphate excretion indicate that phosphate uptake in nephron sites beyond the superficial proximal tubule is enhanced in the newborn compared with the adult.[157] Micropuncture studies in immature rats demonstrated a significant decrease in the delivery of phosphate from the end of the proximal convoluted tubule to the early DCT, compared with older animals, implying a significant amount of phosphate reabsorption within the pars recta of superficial nephron.[149] The descending and ascending limb of Henle probably do not contribute to the reabsorption of phosphate because the permeability of these segments to phosphate is extremely low.[151-153] Heterogeneity of phosphate reabsorption between superficial and juxtamedullary nephrons is another important element to consider in the tubular handling of phosphate by the newborn. The intrinsic capacity to transport phosphate in single nephron proximal tubules, in vivo, is greater in deep than superficial nephrons.[171] This observation could have significant implications for the developmental period, in view of the centrifugal pattern of nephron maturation. Because nephrogenesis begins in the juxtamedullary region and continues with the development of outer cortical nephrons,[172] the relative preponderance of deep nephrons (with a higher capacity for phosphate transport) in the immature kidney may contribute to the high capacity for phosphate reabsorption during development.[144]

Sodium-Phosphate Transporters

The rate-limiting step in the transcellular transport of phosphate (P_i) within the nephron is the movement of phosphate across the luminal brush border membrane and into the tubular cell. Once inside the cell, inorganic phosphate can be incorporated into many organic compounds through a multitude of biosynthetic pathways or simply can pass through the cell to enter the circulatory system. Phosphate entry into the renal proximal tubule cell is mediated by an electrogenic sodium (Na)-coupled, secondary active, transport process that is dependent on an electrochemical gradient for sodium generated by a basolateral sodium-potassium ATPase pump.[151-153]

Three Na-dependent P_i (NaPi) cotransporter families have been described to date. Type I NaPi cotransporters are expressed on the proximal tubular brush border[173] but appear to influence intrinsic cellular phosphate transport.[174] Type II NaPi cotransporters have three known isoforms (NaPi IIa, NaPi IIb, and NaPi IIc), with IIa and IIc almost entirely present within the proximal tubular apical membrane,[175-177] and IIb, primarily found in the small intestine, involved in intestinal phosphate absorption.[178] NaPi IIa is localized throughout the proximal tubule, whereas NaPi IIc is restricted to the first portion (S1) of the proximal tubular cells.[179] Finally, type III NaPi cotransporters are expressed on the basolateral surface of proximal tubular cells and serve a housekeeping function.[180] The movement of P_i across the basolateral membrane is thought to be a passive process.[151-153] Of interest, NaPi IIa transports three Na cations for each divalent P_i anion whereas NaPi IIc remains electroneutral by moving two Na ions across the apical membrane along with each P_i molecule.[181] The isolation and cloning of these specific renal NaPi symporters in a variety of species[182-185] including humans[182,184] has opened opportunities to further elucidate the regulation of these transporters. For instance, NaPi II transporter messenger RNA and protein expression are up-regulated in adult rat brush border membranes of proximal tubular cells exposed to a low-phosphorus diet,[186] a process now known to be microtubule dependent involving the up-regulation of both NaPi IIa and IIc.[187-189] However, although placing mice on a low P_i diet (for 5 days) caused an increase in the proximal tubular expression of apical NaPi IIa and IIc transporters, only NaPi IIc messenger RNA levels were up-regulated under these conditions,[177] suggesting that mechanism underlying the increase in NaPi IIa is via posttranscriptional means. As expected, administration of a high-phosphate diet

leads to a drop in the expression of renal NaPi IIa[188,190] and NaPi IIc[179,188] protein, without affecting the intestinal expression of NaPi IIb.[190] Although thyroparathyroidectomized rats display an up-regulation of NaPi II messenger RNA and protein,[191] infusion of PTH, a factor strongly linked to increasing urinary P_i excretion, has been shown to affect NaPi IIa and IIc expression via the endocytic removal of the proteins from the brush border membrane and subsequent lyosomal degradation.[177,187,189,192] Of note, addition of the microtubule inhibitor colchicine prevented the down-regulation of apical NaPi IIc, but not NaPi IIa transporter, after PTH administration in thyroparathyroidectomized rats.[179] Additional studies are needed to fully elucidate the complex mechanisms involved in the insertion and removal of the NaPi IIa and IIc cotransporters from the renal tubular brush border membrane.

Genetic manipulation over the past decade has confirmed the importance of the NaPi transporters in regulating renal phosphate homeostasis. NaPi IIa gene ablation in mice produces a phenotype that is hypophosphatemic and hypercalciuric and eventually develops skeletal abnormalities, as a result of decreased renal phosphate reabsorption.[193,194] This occurs despite the significantly enhanced expression of brush border membrane NaPi IIc protein in these animals. In addition, NaPi IIa knockout mice have a poor response to PTH or changes in dietary intake,[195] indicating that NaPi IIa is the major cotransporter involved in phosphate homeostasis in the mouse model. Ablation of NaPi IIc produces animals with mild phenotypes compared with the NaPi IIa knockout mice described earlier.[196] Of interest, in 2006, a single nucleotide deletion of the NaPi IIc gene in humans was associated with hereditary hypophosphatemic rickets with hypercalciuria,[197,198] an autosomal recessive disorder characterized by elevated urinary phosphate losses, hypercalciuria (resulting from significant intestinal calcium absorption), and skeletal abnormalities such as rickets and osteomalacia.[199] Thus NaPi IIc appears to play a greater role in renal phosphate handling in humans as compared with the rodent.

Recent findings have determined that renal NaPi II transporter expression is significantly greater in juvenile animals, and a developmental decrease in the transporter protein occurs into young adulthood. These changes in NaPi II expression directly coincide with the increased tubular phosphate uptake seen in these juvenile animals.[148] It is now known that the expression of NaPi IIc is also elevated in weaning animals, suggesting a possible role for this transporter as well during the growth process.[176,200] Thus, although these findings clearly indicate that up-regulation of NaPi II transporters is a key adaptation facilitating avid phosphate uptake in the developing animal, it is not clear the extent that NaPi IIa or NaPi IIc plays during growth, as well as whether other unique, yet undiscovered, transporters are also involved. Further studies are needed to help shed light on this issue.

Age-Dependent Effects of Parathyroid Hormone on Renal Phosphate Reabsorption

PTH, the principal hormonal regulator of renal phosphate transport, is released from the parathyroid glands in response to hypocalcemia (or hyperphosphatemia) and acts on the kidney to increase calcium reabsorption while decreasing phosphate uptake. Of interest, recent evidence shows that exposure of the gut to a high P_i diet rapidly (within 10 minutes) can affect PTH secretion, independent of changes in serum calcium or phosphate.[201,202] In the adult animal, the phosphaturic effect of PTH occurs through a direct action of the hormone on both proximal[162,166] and distal nephron segments.[164,166] In most cases, the action of PTH is mediated by an increase in intracellular cAMP,[151-153] although evidence indicates that both PKA and PKC second-messenger systems are also affected by PTH binding to its receptor.[203,204] Finally, it is interesting to note that juxtamedullary (deep) nephrons elicit a greater phosphaturic response to PTH than do superficial nephrons[205]; whether this effect is due to an increase in PTH receptors or increased maturation of the deep nephrons is not known.

The response of the juvenile animal to the phosphaturic effect of PTH is attenuated compared with that of the adult.[143,146,148,163,206] This reduced sensitivity to PTH occurs despite normal circulating levels of PTH during the postnatal period.[207] Of interest, the blunted phosphaturic response to PTH in the juvenile animal is restored to adult levels when growth hormone (GH) is suppressed, an effect that is mediated through NaPi II transporters.[146,149] Furthermore, immunofluorescence studies demonstrated that, although PTH causes the internalization of the NaPi II cotransporter from the brush border membrane of juvenile rats, it appears to remain within the subapical compartment[148] rather than degraded in the lysosomal system, as noted in adults.[187,188] Although the reason for this is unknown, it may be related to the fact that with the high demand of the growing animal to reabsorb filtered P_i, this allows for the transporters to be available for rapid reinsertion into the apical surface. This potential age-related difference appears to be GH dependent, because the endocytic removal and degradation of the PTH-induced NaPi II transporter can occur in the juvenile kidneys after GH suppression.[149] This indicates that the PTH mechanism is intact in the juvenile kidney, but the ability of PTH to decrease NaPi II transporters is overridden by the ability of circulating GH to prevent the lysosomal degradation on these transporters. Further studies are required to help elucidate this pathway.

Age-Dependent Effects of Dietary Phosphate on Renal Phosphate Reabsorption

Dietary phosphate restriction elevates phosphate reabsorption in the adult animal,[147,148,151,153] through increases in phosphate transport in the proximal tubule.[148,159,162,166] In the adult, phosphate deprivation is associated with increased membrane fluidity and reduced membrane cholesterol content, which may facilitate the observed increase in V_{max}.[167] Conversely, aging is associated with loss of membrane fluidity, increased cholesterol,[208] and a reduction in phosphate reabsorption.[209] The adaptation to enhance tubular phosphate reabsorption during dietary phosphate deprivation is also present in immature dogs[154] and rats.[145,148] Indeed, studies in the juvenile rat have shown that the adaptive increase in phosphate reabsorption is far greater than that observed in the phosphate-deprived adult and is PTH independent.[145,148] Moreover, this increase occurs despite the already high rate of phosphate transport in the immature animal. The elevated TmPi in the phosphate-deprived newborn serves to maintain avid reabsorption of phosphate and facilitate the accelerated rate of growth that occurs on the restoration of phosphate to the diet.[145,148] Micropuncture and NaPi II transporter studies in the juvenile rat demonstrate that sites beyond the proximal convoluted tubule significantly contribute to the high renal P_i uptake seen in P_i-deprived growing animals[148] (Figure 130-2).

Thyroid Hormone

Thyroid hormone may also be integral to proper phosphate homeostasis in the young animal. Thyroid hormone receptors are present in the fetus, with the hormone peaking at birth in humans and by 4 or 5 weeks after birth falling to levels maintained through adulthood. The kidney is a target organ for thyroid hormone and contains the iodothyronine deiodinase enzyme that converts the main circulating hormone T_4 to the more potent T_3. Increases in thyroid hormone stimulate sodium-dependent phosphate transport in adult rats[210] and mice.[211] This effect is probably related to the hormone's ability to up-regulate the V_{max} of the transporter.[212,213] Thyroid hormone may also be involved

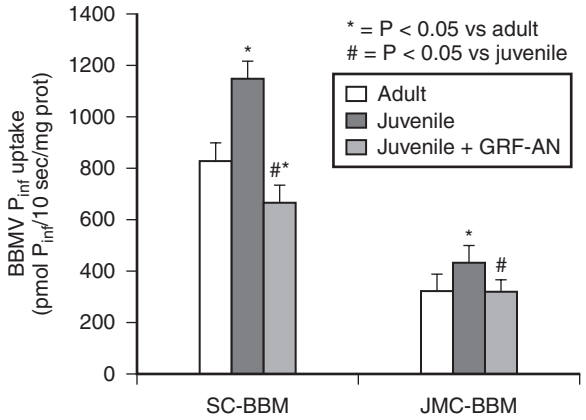


Figure 130-2 Brush border membrane vesicle (BBMV) NaPi uptake in superficial cortex (SC-BBM) and outer medullary cortex (JMC-BBM) in parathyroid gland intact adult, juvenile, and GH-suppressed juvenile rats. BBMVs from control juvenile rats *(dark gray bar)* have significantly greater P_i uptake in both SC-BBM and JMC-BBM compared with adult rats. Suppression of GH (with use of a GH–releasing hormone antagonist, GRF-AN) significantly reduced NaPi uptake to levels at or below that in adults. *(From Woda CB, Halaihel N, Wilson PV, et al: Regulation of renal NaPi-2 expression and tubular phosphate reabsorption by growth hormone in the juvenile rat,* Am J Physiol Renal Physiol *287:F117-F123, 2004, with permission.)*

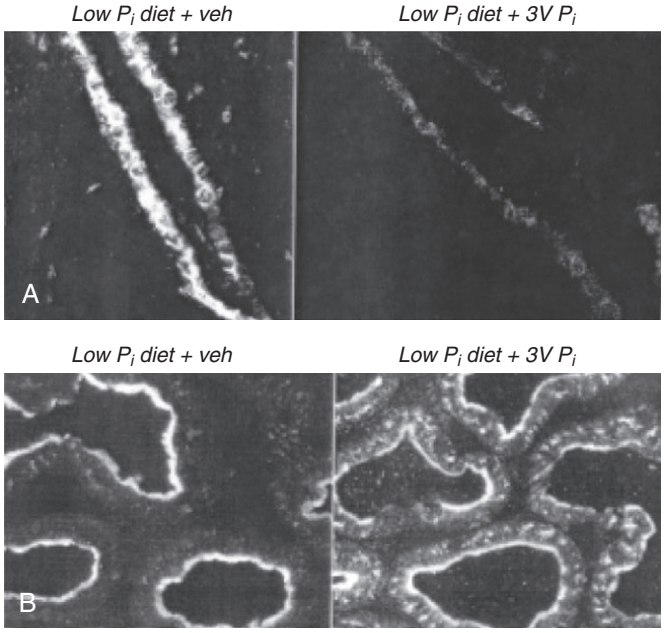


Figure 130-3 Effect of third-ventricle injection of vehicle or P_i in young rats fed a low-phosphate diet. Within 15 minutes, P_i injection into the third ventricle of the brain results in the rapid reduction of NaPi transporters from ependymal cells surrounding the third ventricle and translocation of transporters from the renal brush border to cytosol. *(From Mulroney SE, Woda CB, Halaihel N, et al: Central control of renal sodium-phosphate [NaPi-2] transporters,* Am J Physiol Renal Physiol *286:F647-F652, 2003, with permission.)*

in the maturational increase in the K_m of the proximal tubule brush border phosphate uptake that occurs with weaning, because prolonged weaning in rats is associated with a decrease in both thyroid hormone levels and the K_m for tubular phosphate transport.[214]

Growth Hormone

GH is a key factor regulating renal phosphate reabsorption, particularly in the postnatal period. GH hypersecretion is associated with hyperphosphatemia and a reduction in urinary phosphate excretion.[215,216] Conversely, GH deficiency (dwarfism) is associated with an attenuated growth rate and increased excretion of phosphate.[216] When GH-deficient individuals are given GH injections, growth rate acutely increases and phosphate excretion diminishes.[216] Studies in adult dogs[215-217] and rats[218] have reported that administration of GH increases the tubular reabsorption of phosphate, independent of PTH. Conversely, when adult animals are hypophysectomized to prevent pituitary GH release, an increase in urinary phosphate excretion occurs with a correlated decrease in the TmPi.[218] It is not known whether the increase in phosphate retention results directly from binding to renal GH receptors or indirectly from GH activation of renal insulin-like growth factor (IGF)-I, because IGF-I is also capable of directly increasing phosphate reabsorption in vitro and in vivo.[218,219]

Results from animal studies have established an important link between the growth-promoting effects of GH and renal phosphate reabsorption. Suppressing GH release significantly attenuates the growth rate and reduces the positive phosphate balance in juvenile rats.[144,220] These effects are associated with a decrease in the TmPi to levels observed in the adult animal. Reductions in NaPi II transporters account for the reduced proximal and distal tubular phosphate reabsorption.[148] GH or IGF-I replacement prevents the reduction in TmPi in GH-suppressed juvenile rats. Of importance, suppression of GH has no effect on phosphate handling in adult rats. In addition, suppression of GH release in weaning and juvenile rats produces a phosphaturic response to PTH comparable with that observed in adult rats and drives the increase in tubular phosphate reabsorption in response to dietary phosphate deprivation.[146,148,149] These findings indicate that many of the adaptations to conserve phosphate in the developing animal are facilitated directly, or indirectly, by GH.

Central Regulation of Renal Phosphate Reabsorption

One of the most intriguing findings is that central control of renal NaPi II transporters exists in juvenile animals. Addition of phosphate directly into the third ventricle of the brain in phosphate-deprived juvenile rats rapidly (within 15 minutes) down-regulates renal NaPi IIa transporters, despite the low plasma phosphate milieu (Figure 130-3).[148] The reduction in NaPi II transporters correlates with a significant reduction in the TmPi of these animals. These findings open new approaches for investigating the regulation of phosphate homeostasis in development and aging.

NEW PERSPECTIVES IN THE CLINICAL RELEVANCE OF RENAL P_I REGULATION

Phosphatonins

Phosphatonins, a term coined in 1994, are a collective set of circulating and/or autocrine-paracrine factors shown to inhibit renal tubular phosphate uptake and play a crucial role in a variety of phosphaturic disorders such as autosomal dominant hypophosphatemic rickets (ADHR), tumor-induced osteomalacia (TIO), and X-linked hypophosphatemic rickets (XLH).[221,222] The initial report on the existence of phosphatonins, by Cai and colleagues,[223] demonstrated that opossum kidney cells exposed to media housing cells from patients with TIO, a disease

characterized by renal phosphate wasting, had a reduction in Na-dependent P_i transport. Several recently studied phosphatonins include secreted frizzled-related protein-4 (sFRP-4), matrix extracellular phosphoglycoprotein (MEPE), and fibroblast growth factor 23 (FGF23); with FGF23 being the only factor currently linked to human phosphate wasting diseases.[224-229]

FGF23

FGF23, considered the most potent phosphatonin, was isolated from patients with ADHR, XLH, and TIO, three diseases characterized by hypophosphatemia (subsequent to renal phosphate wasting), as well as skeletal abnormalities.[230-234] These studies implicate FGF23 in the pathogenesis of phosphate wasting disorders and also contribute to our general understanding of renal phosphate homeostasis regulation, particularly during growth and development.

Opossum kidney cells exposed to FGF23 have decreased Na-dependent P_i transport[235] through down-regulation of NaPi IIa expression.[236] Furthermore, FGF23 infusion or overexpression of FGF23 in mice caused a decline in renal P_i uptake, resulting from the decreased NaPi IIa expression.[229,231,233] In vivo studies have demonstrated that NaPi IIc messenger RNA and protein also decrease in response to FGF23.[237,238] As expected, FGF23 ablation studies resulted in hyperphosphatemia and renal P_i retention, as well as elevated levels of circulating $1,25\text{-}(OH)_2D_3$.[232,234] Of note, FGF23 may also be involved in the pathogenesis of McCune-Albright syndrome because circulating levels appear to correlate with the severity of hypophosphatemia and bone dysplasia in these individuals.[239] Because binding of FGF23 to the FGF receptor elicits mitogen-activated protein kinase activation and tyrosine kinase signaling,[240] FGF23 inhibitors or blockers of the FGF receptor signaling cascade may have the potential of preventing the significant P_i losses, and bone abnormalities, seen in these patients.

Of clinical importance is the fact that patients with chronic kidney disease have exceptionally high levels of circulating FGF23,[241-243] and the levels inversely correlate with the maximum tubular capacity of phosphate reabsorption.[241] Furthermore, hemodialysis plays no role in lowering the elevated FGF23 levels in these patients.[244] Although the significance is not currently known of the high circulating FGF23 levels in patients with chronic kidney disease, it is thought that they may contribute to the suppression of $1,25\text{-}(OH)_2D_3$ levels, which is involved in maintaining efficient intestinal calcium absorption, as well as leading to secondary hyperparathyroidism, which has implications in producing bone mineralization abnormalities in these patients.Thus FGF23 suppression may be a novel therapeutic target in the near future for individuals with end-stage renal disease who are receiving dialysis.

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Aaron L. Friedman

Transport of Amino Acids During Early Development

GENERAL FEATURES

The reclamation of filtered amino acids by the proximal tubule of the kidney undergoes a process of maturation coincident with other maturational changes in renal function.[1] To understand the ontogeny of renal amino acid transport, it is useful to review general features of renal amino acid transport.

Glomerular filtrate contains amino acids in concentrations essentially identical to those in plasma. It is from this filtered load that amino acids are reabsorbed exclusively by the proximal tubule. Glycine and taurine are reabsorbed in later segments of the proximal tubule, but for most amino acids, more than 80% of reabsorption occurs in the early proximal tubule.[2,3] This reclamation of amino acids back into the extracellular space is not merely movement across a single plasma membrane but is passage through a cell layer–transepithelial transport.

Figure 131-1 depicts a proximal tubule cell with its major reabsorptive surface, the brush-border membrane, oriented into the lumen of the tubule and the remainder of the cell membrane that any reabsorbed solute must transverse: the basolateral membrane. The directional flux for amino acids is from the tubule lumen to the peritubular capillary. Thus the heavier arrows in the figure are pointed in that direction. The net flux is composed of movement into the cell from the lumen and the interstitial space surrounding the basolateral membrane, as well as movement back into the tubule lumen and out of the cell into the surrounding interstitium. This view is somewhat simplistic in that it does not take into account any contribution made by intracellular metabolic processes or the possibility of amino acid movement by paracellular processes across the tight junction. However, Figure 131-1 does define the general components of transepithelial amino acid transport.

The net movement across the renal proximal tubule cell is primarily an active transport process requiring energy.[4] Passive diffusion plays a small role. For every amino acid studied thus far, the movement across the brush-border membrane is inextricably tied to sodium (Na^+) transport. The present model states that energy is expended by the cell to pump Na^+ from the cell into the extracellular space. The pump at the basolateral membrane is sodium-potassium-adenosine triphosphatase (Na^+-K^+-ATPase). The extrusion of Na^+ from the cell creates an inward (into the cell) driving force for Na^+ (down a concentration gradient) at the brush-border membrane. Thus amino acids and Na^+ are cotransported. In studies with brush-border membrane vesicles, in

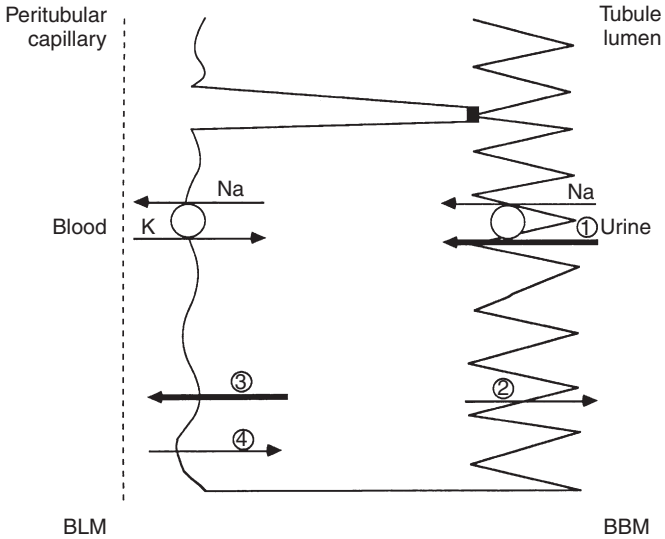


Figure 131-1 Representation of process involved in transepithelial transport of amino acids by the renal proximal tubule: (1) sodium amino acid co-transport; (2) efflux back into tubule lumen (backleak); (3) efflux from tubule to peritubular space (extracellular space) and return to plasma; (4) transport from peritubular space into tubule cell. *BBM,* Brush border; *BLM,* basolateral membrane; *circle with opposite-pointing arrows,* Na^+,K^+-ATPase.

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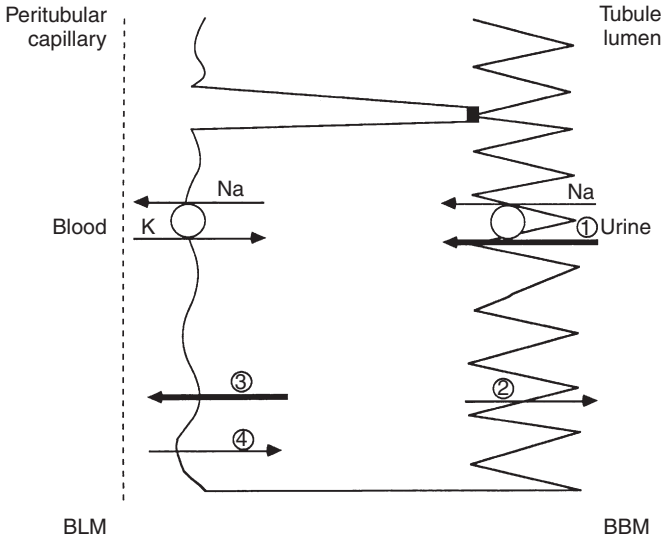


Figure 131-1 Representation of process involved in transepithelial transport of amino acids by the renal proximal tubule: (1) sodium amino acid co-transport; (2) efflux back into tubule lumen (backleak); (3) efflux from tubule to peritubular space (extracellular space) and return to plasma; (4) transport from peritubular space into tubule cell. *BBM,* Brush border; *BLM,* basolateral membrane; *circle with opposite-pointing arrows,* Na^+,K^+-ATPase.

which transport across the brush-border membrane can be studied, amino acid accumulation in the vesicles is nearly completely blunted when Na^+ is excluded from the external medium.[4-6]

Transport characteristics of the brush-border carrier (transporter) for amino acids and Na^+ suggest that the transporter is saturable. In other words, increasing the concentration of amino acids above a certain level does not lead to significant further accumulation. All amino transporters follow Michaelis-Menten kinetics, meaning that under proper experimental conditions, it should be possible to determine the maximal velocity (V_{max}) of transport and the concentration at which half-maximal velocity occurs (K_m). The V_{max} can be viewed as a measure of transport capacity, with the higher the V_{max}, the greater the capacity. The K_m is a measure of affinity. The lower the K_m, the more avidly the substrate is "attracted" to the transporter. As Scriver and Rosenberg[7] have pointed out, the transporters have a higher affinity for the L-isomers of amino acids, and they demonstrate specificity. In many instances, groups of amino acids are transported by the same carrier. Group-specific carriers are, with respect to any single amino acid, high-capacity but low-affinity systems. In a disease such as cystinuria, a group carrier is often involved, and most patients excrete excessive quantities of cystine, ornithine, lysine, and arginine. More selective transporters for single amino acids have lower capacities but higher affinities.

Transport properties at the basolateral membrane resemble those of the brush-border membrane, although Na^+-*independent* transport may play a greater role in transport at the basolateral membrane. Efflux properties are less well understood, although diffusion down a concentration gradient (intracellular → extracellular) may be important in the transepithelial movement of amino acids from the tubule lumen to the extracellular space. With respect to the reclamation of amino acids by the proximal tubule, and thereby the final amino acid concentration in the urine, the important features are the filtered load of amino acids and the transport capability, especially the proximal tubule brush-border membrane transporter properties.

DEVELOPMENTAL ASPECTS

It is generally recognized that very young, developing mammals (including humans) have "physiologic" aminoaciduria. As the term suggests, more amino acid is in the urine compared with in adults, and this aminoaciduria is not pathologic. Perhaps the term *developmental aminoaciduria* would be more appropriate because the failure of the young mammal to reabsorb amino acids to the degree seen in the adult appears to be a developmental process.

The excretion of amino acids by humans is measured by numerous methods. Using any method, the hyperexcretion of amino acids by the human infant can be documented. Table 131-1 shows the normal range of urinary amino acid excretion in infants, children, and adults as measured after a timed collection (μmol amino acid/g creatinine/24 hours). Two other techniques for quantitating the excretion of amino acids have been employed. Scriver and Rosenberg[7] proposed the following renal clearance method:

$$(C_{AA})\ \text{clearance of amino acids} = \frac{(U)\ \text{urine concentration (μmol/mL)} \times \text{volume (mL/minute)}}{(P)\ \text{plasma concentration (μmol/mL)}}$$

and Silbernagel[3] quantified excretion by determining the fractional excretion (FE) of amino acids:

$$FE = \frac{C_{AA}}{C_{cr}} = \frac{U_{AA} \times P_{cr}}{P_{AA} \times U_{cr}}$$

where C_{AA} = amino acid clearance, C_{cr} = creatinine clearance, U_{AA} = urine amino acid concentration, P_{cr} = plasma creatinine concentration, P_{AA} = plasma amino acid concentration, and U_{cr} = urine creatinine concentration.

Fractional excretion and clearance data clearly indicate that higher concentrations of amino acids are present in the urine of newborn infants. Importantly, however, these data also suggest that the excess amino acids are not secondary to higher filtered loads. What then could be the cause of this maturational delay in the reabsorptive capacity of the proximal tubule of the infant?

Scriver and colleagues,[8] in their discussion of the expression of mutations that could lead to hereditary disorders of tubular transport, postulated at least three mechanisms to explain the developmental tubular differences seen in the infant. The first of the possible developmental differences could be at the brush-border membrane transporter site (see *site 1* in Figure 131-1). Diminished transporter function could lead to a decrease in amino acid influx and an increase in amino acid excretion. An increase in amino acid exit at *site 2* in Figure 131-1, that is, increased backflux into the lumen, could represent a second developmental difference that could explain developmental aminoaciduria. A third possibility (see *site 3* in Figure 131-1) is a decrease in efflux. This would lead to increased intracellular concentrations of amino acids that would then result in increased backflux into the tubule lumen, or a direct inhibitory effect on transporters to reduce the amount of amino acid carried into the proximal tubule cell, or both. A further possibility discussed by Scriver and colleagues[8] deals with the metabolic fate of amino acids. In disease states, it is possible that intracellular accumulation may occur because the metabolic end point of an amino acid may be blocked, leading to diminished metabolic "runout" and abnormal intracellular accumulation. This last possibility is not likely to explain developmental aminoaciduria because amino acids that do not undergo metabolic change or incorporation into protein are excreted in high quantities in the young of many mammalian species (see Table 131-1). Certain amino acids seem to contribute more to the high urinary amino acid excretion than do others.[9] This finding suggests that a single defect such as altered energy use cannot explain the developmental changes seen. Rather, it is more likely that any developmental effect is related to the maturation of influx or efflux capabilities and that the influx-efflux transport systems "mature" at separate rates.

Numerous laboratories have studied the ontogeny of amino acid transport. Early studies by Baerlocher and associates[10,11] examined glycine and proline transport in rat kidney cortex slices (Long-Evans rat) from neonatal animals. Their studies were consistent with the presence of a low-affinity, high-capacity transporter at birth and the emergence of a high-affinity, low-capacity system at 1 week of age for proline and 2 weeks of age for glycine. Studies by Segal and colleagues[12] in Sprague-Dawley rats found similar glycine uptake systems in the newborn and the adult Sprague-Dawley rat. Roth and associates[13] reexamined glycine transport in isolated rat tubule segments, and Reynolds and colleagues[14] reexamined glycine transport in the cortex slice. Both groups of investigators found high- and low-affinity uptake systems in newborn and adult renal tissues. However, the efflux of glycine from rat cortex slides[12] or from isolated rat tubule segments[13] was slower in the newborn than in the adult. These data, along with work reported by Meadow and colleagues,[15] suggest that slowed efflux is an important determinant of intracellular amino acid pools and therefore of transepithelial transport in the newborn.

Cystine is another amino acid whose fractional excretion is higher in the immature animal and in the human neonate. Studies with cystine have shed light on additional aspects relevant to amino acid transport. Hwang and colleagues[16] and Foreman and colleagues[17] documented the presence of two transport systems for cystine (high-affinity, low-capacity, and low-affinity, high-capacity). In the report by Foreman and colleages,[17] the affinity

TABLE 131-1

Plasma and Urine Amino Acids*

	PLASMA (μmol/L) NORMAL RANGES BY AGE			
	<1 mo	**1-24 mo**	**2-18 yr**	**>18 yr**
Taurine	46-492	15-143	10-170	54-210
Aspartic acid	20-129	0-23	1-24	1-25
Threonine	90-329	24-174	35-226	60-225
Serine	99-395	71-186	69-187	58-181
Glutamic acid	62-620	10-133	5-150	10-131
Glutamine	376-709	246-1182	254-823	205-756
Sarcosine	0	0	0	0
Proline	110-417	52-298	59-369	97-329
Glycine	232-740	81-436	127-341	151-490
Alanine	131-710	143-439	152-547	177-583
Citrulline	10-45	3-35	1-46	12-55
Valine	86-190	64-294	74-321	119-336
Cystine	17-98	16-84	5-45	5-82
Methionine	10-60	9-42	7-47	10-42
Cystathionine	0-3	0-5	0-3	0-3
Isoleucine	26-91	31-86	22-107	30-108
Leucine	48-160	47-155	49-216	72-201
Tyrosine	55-147	22-108	24-115	34-112
Phenylalanine	38-137	31-75	26-91	35-85
β-Alanine	0-10	0-7	0-7	0-12
Homocysteine	0	0	0	0
Tryptophan	0-60	23-71	0-79	10-140
Ethanolamine	0-115	0-4	0-7	0-7
Ornithine	48-211	22-103	10-163	48-195
Lysine	92-325	52-196	48-284	48-284
1-Methylhistidine	0-43	0-44	0-42	72-124
Histidine	30-138	41-101	41-125	41-125
3-Methylhistidine	0-5	0-5	0-5	0
Anserine	0	0	0	0
Carnosine	0	0	0	0
Arginine	6-140	12-133	10-140	15-128
	0-91	0-63	3-45	0-53

	URINE (mmol/g CREATININE) NORMAL VALUES	
	<1 yr	**>1 yr**
Taurine	0.64 ± 1.16	0.83 ± 0.69
Aspartic acid		
Threonine	0.47 ± 0.38	0.17 ± 0.12
Serine	1.43 ± 0.80	0.48 ± 0.28
Glutamic acid	0.10 ± 0.21	0.05 ± 0.07
Glutamine	1.30 ± 0.70	0.61 ± 0.37
Sarcosine		
Proline	0.57 ± 0.78	0.07 ± 0.04
Glycine	5.07 ± 3.10	1.48 ± 0.98
Alanine	0.92 ± 0.49	0.46 ± 0.37
Citrulline	0.04 ± 0.03	0.02 ± 0.01
Valine	0.10 ± 0.08	0.06 ± 0.05
Cystine	0.16 ± 0.15	0.05 ± 0.03
Methionine	0.28 ± 0.23	0.07 ± 0.06
Cystathionine		
Isoleucine	0.14 ± 0.10	0.05 ± 0.04
Leucine	0.16 ± 0.11	0.08 ± 0.06
Tyrosine	0.33 ± 0.23	0.18 ± 0.12
Phenylalanine	0.16 ± 0.10	0.09 ± 0.06
β-Alanine		
Homocysteine		
Tryptophan	0.10 ± 0.12	0.07 ± 0.07
Ethanolamine		
Ornithine	0.08 ± 0.07	0.05 ± 0.06
Lysine	0.52 ± 0.69	0.21 ± 0.19
1-Methylhistidine	0.13 ± 0.22	0.25 ± 0.20
Histidine	1.34 ± 0.01	0.96 ± 0.54
3-Methylhistidine	0.23 ± 0.08	0.32 ± 0.13
Anserine		
Carnosine		
Arginine	0.07 ± 0.06	0.04 ± 0.06

*Urine values are given as mmol/g creatinine in a 24-hour urine collection. Plasma values are given as concentration in μmol/L. From University of Wisconsin Metabolic Program, with permission of Dr. Jon Wolff. See also references 3 and 7.

for cystine was actually *higher* in the newborn infant than in the adult, but the V_{max} (capacity) was much lower. This could clearly lead to a decrease in renal cystine reabsorption. These authors further commented on the metabolic potential of the neonatal proximal tubule. Cystine is reduced intracellularly to cysteine. In their studies, tubules from the immature kidney metabolized cystine just as readily as did those from adult kidneys. Therefore, at least for cystine (and perhaps other intracellularly metabolized amino acids), the explanation for developmental aminoaciduria does not rest with an altered intracellular metabolic process. In the same study, Foreman and colleagues[17] investigated the effect of lysine on cystine transport. Lysine inhibited the high-affinity system in the immature kidney tissue just as it did in tissue from adult animals. These data suggest that the properties of shared transport for cystine and dibasic amino acids (lysine, ornithine, citrulline) are intact even in the newborn. This group of studies points to the luminal brush-border membrane as another important site responsible for developmental aminoaciduria (see Figure 131-1).

A structure-function analysis of amino acid transporters has resulted in classification of transporters into four genetic families:

1. *GAT* family: Na^+- and chloride-dependent transporters
2. *CAT* family: Cationic amino acid transporters
3. Glutamate transporters
4. *rBAT* family: Cationic-neutral amino acid transporters

Further follow-up of these gene families has demonstrated two different membrane protein structures. *GAT, CAT,* and glutamate families have multiple transmembrane domains and are considered carriers, whereas *rBAT* family members are considered activators or part of oligomeric carriers. These findings raise an interesting question regarding maturation of amino acid transporters. No information is yet available on the maturation of these transport systems.[18,19]

Chesney and Jax[20] studied the ontogeny of the transport system for the β-amino acid taurine in the rat. Using dietary alterations to help uncover the adaptive response of the kidney to taurine, most of these investigations focused on the brush-border membrane vesicle. The results of these studies can be summarized as follows:

1. Thin renal cortex slices from young rats demonstrated greater accumulation of taurine than did cortical slices from adult rats. This observation is especially striking considering that urinary excretion and fractional excretion of taurine during the first 28 days of life in the rat are much greater than those of the mature rat.[20]
2. Studies with cortical slices and proximal tubule segments revealed two transport systems—a high-affinity, low-capacity system and a low-affinity, high-capacity system.[21-23] Thus maturation of a specific transport system could not explain the developmental taurinuria present in the young mammal.
3. Experiments using brush-border membrane vesicles (isolating the apical portion of the proximal renal tubule) revealed no difference in transport kinetics between the adult and the still-maturing 28-day-old rat.[24,25]
4. In dietary alteration studies in which the mother was fed a low-, normal-, or high-taurine diet, taurine excretion in the 14- and 21-day-old pup mirrored dietary intake in the mother. With a low-taurine diet in the mother, urinary excretion of taurine was reduced in the 14- or 21-day-old pup. A similar positive correlation was seen with a high-taurine diet.[26]
5. This adaptation to dietary intake was reflected in the brush-border membrane, with an increase in V_{max} seen at the brush-border membrane during low dietary taurine intake. This adaptation was observed in rats ranging in age from 14 days to maturity.[25-28]
6. A plausible explanation for the finding of developmental taurinuria (but similar transport kinetics between young [≥14 days] and mature animals) is that efflux from the proximal tubule cell is diminished in the maturing animal (see *site 3* in Figure 131-1), thereby resulting in an increased backleak of amino acid into the tubule lumen and into the urine.
7. Total taurine accumulation was lower in brush-border vesicles prepared from 7-day-old rats compared with any other age studied (14-, 21-, 28-day-old or mature 60-day-old rats). Further, no dietary influence on taurine uptake could be demonstrated in 7-day-old pups. These findings suggest that, in the most immature animal, the developmental aminoaciduria observed may in part result from diminished uptake across the brush-border membrane and not just diminished efflux across the basolateral membrane.[27]

Amino acids are transported across the renal epithelium primarily by NaCl cotransport systems.[29] Studies examining proline transport in experimental animals revealed a reduced uptake of proline in the immature animal and a less pronounced characteristic uptake pattern in the immature animal, attributed to differences in Na^+ permeability and Na^+-K^+ exchange activity in the immature versus the mature animal.[30,31] A faster or higher accumulation of Na^+ would more quickly dissipate the extracellular-to-intracellular Na^+ gradient, thereby reducing the driving force for Na^+-dependent transport, such as amino acid transport. In this way, amino acid accumulation by the renal epithelium of the very young rat could be diminished. Such a hypothesis could explain the aminoaciduria, diminished brush-border vesicle accumulation of amino acids, and failure of the brush border to demonstrate an adaptive response to a dietary deficiency.

Protein phosphorylation has been proposed as a controlling factor for amino acid transport. Intracellular protein kinases, including protein kinase A, protein kinase C, and calcium calmodulin–dependent protein kinase, have been shown to modulate amino acid transport. Studies by Zelikovic and associates[32-34] showed that exogenous and endogenous calcium-dependent protein kinases inhibit proline uptake in brush-border membrane vesicles and differential regulation of calcium-dependent and calcium-independent protein kinase C isoenzymes exists during development.[32-34] Together, these data point to a role for age-related changes in calcium-dependent protein kinases and their differential regulation during development in the maturation of renal amino acid transport.

Overall, studies in the developing animal point to maturational events at both the brush border and basolateral membrane that are necessary for the efficient reabsorption of amino acid seen in the adult mature animal. At present, the two most likely explanations are diminished efflux at the basolateral membrane leading to backleak into the tubule urine (see *site 3* in Figure 131-1) and diminished apical transport in the most immature animals (see *site 1* in Figure 131-1). Of course, questions remain: Is there a qualitative, generalized membrane difference that could explain developmental aminoaciduria? What is the maturational signal?

The interest in developmental amino acid transport is not merely the study of a fascinating biologic question. Geggel and associates[35] reported abnormal retinograms and low plasma taurine levels in infants receiving long-term taurine-free parenteral nutrition. More recently, Zelikovic and colleagues[36] reported that low-birth-weight infants receiving total parenteral nutrition can become taurine depleted, in part because of immaturity of the reabsorptive function of the proximal tubule. For some amino acids, such as taurine, the kidney may be the major regulator of that amino acid's body pools. With immaturity (developmental aminoaciduria), the kidney may not be able to adapt sufficiently during periods of deficient intake, and body stores of certain amino acids will be depleted. This could lead to important growth and maturational effects.

Much has been learned[37] and much remains to be learned, but the studies to date of developmental renal amino acid transport suggest that at least two aspects of transport across the proximal tubule undergo change in the postnatal period: efflux from the tubular epithelial cell increases with age and transport across the apical membrane into the tubular epithelial is lower in the infant compared with the adult. Future study should delineate the exact membrane changes that underlie this developmental change.

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Deborah P. Jones and F. Bruder Stapleton

Developmental Aspects of Organic Acid Transport

Renal tubular capacity for organic acid secretion undergoes prenatal and postnatal maturation, as do glomerular filtration and other tubular functions. Organic acids include endogenous compounds, such as uric acid, hippurate, and prostaglandins, as well as many exogenous xenobiotics (Box 132-1). Because many drugs are secreted by the organic acid transporter, the development of this system is important. Studies of the developmental aspects of renal organic anion transport primarily have concentrated on renal tubular transport of para-aminohippurate (PAH), the standard substrate for clearance studies. The original developmental studies of organic anion transport often use the tissue slice technique. In this method, the uptake of radiolabeled compounds into tissue slices can be quantified and expressed as a slice-to-media (S-M) ratio. Because the patterns of postnatal development of renal organic acid secretion may differ among species, care must be exercised in generalizing findings of animal models to humans.

ORGANIC ACID TRANSPORT

Organic acids (or anions), such as PAH, are secreted by the proximal tubule. The net direction of movement is from the basolateral (blood side) membrane of the proximal tubular cell and then out into the proximal tubular fluid. The relative magnitude of organic

BOX 132-1

Example of Common Organic Acids Secreted by the Proximal Tubule

Exogenous Acids
- Furosemide
- Thiazides
- Acetazolamide
- Antimicrobials–β-lactam
  - Penicillins
  - Cephalosporins
- Azidothymidine
- Salicylates
- Indomethacin
- Probenecid
- Iodinated contrast media
- Morphine
- Methotrexate
- Losartan

Endogenous Acids
- Uric acid
- Hippurates
- Oxalate
- Prostaglandins
- Bile salts
- Benzoates
- Cyclic adenosine monophosphate
- Folate

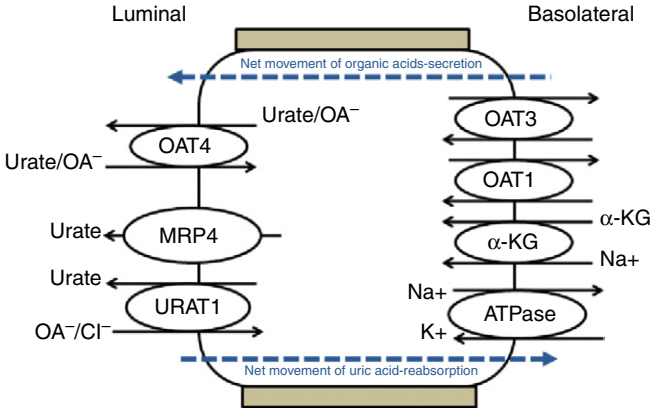


Figure 132-1 Schematic representation of organic acid and uric acid transport by the renal proximal tubular cell. Basolateral systems include the anion exchangers *OAT1* and *OAT3* that exchange organic acids *(OA)* or uric acid for alpha-ketoglutarate *(α-KG),* which is exchanged for sodium by the sodium dicarboxylate cotransporter (NaDC3). Although net movement may be bidirectional, secretion occurs as substrates are extracted from blood down the chemical concentration gradient of sodium. Apical systems include *OAT4,* which exchanges organic acids or uric acid for anions. Addition luminal transport systems for uric acid include the secretory pathway of *MRP4* and the bidirectional exchanger *URAT1.*

acid transport along the proximal tubule segments (S1, S2, and S3) is species specific.[1] PAH secretion is accomplished by two basic steps: at the basolateral membrane, exchange of extracellular organic anions with α-ketoglutarate (OAT1 and 3) allows the transport of substrate against a concentration gradient. Basolateral transport of PAH is indirectly dependent on basolateral sodium because of the coupling of PAH transport to a dicarboxylic acid–Na^+ cotransporter.[1] The latter cotransporter relies on the Na^+,K^+–adenosine triphosphatase (ATPase)–generated Na^+ gradient (Figure 132-1). Once inside the proximal tubular cell, PAH exits the cell in exchange for an anion at the luminal membrane by the organic anion–OH^- antiporter (URAT1), a transport system shared with urate.[1] Additional efflux mechanisms for PAH secretion include MRP4 and OAT4.

The SLC family of organic anion transporters (OAT) includes numerous isoforms.[2,3] There is structural homology of the OAT family of transporters with the organic cation transporters (OCT) and the uric acid transporter, URAT1. Substrates are shared between the two systems: verapamil, clonidine, cimetidine, cortisol, and aldosterone. OAT1 (SLC22A6) and OAT3 (SLC22A8) are localized to the basolateral membrane and OAT4 (SLC22A11) to the apical membrane of the proximal renal tubule. OAT1 is the classic PAH-dicarboxylate exchanger elucidated by physiologic studies.[3] Although the two homologues share substrates, they are distinct: OAT3 does not demonstrate significant affinity for PAH yet has a high affinity for estrone sulfate, whereas the opposite is true for OAT1. These transporters are exchangers that are part of a tertiary transport system, working in parallel with the sodium-dicarboxylate cotransporter (NaDC3) and the Na^+,K^+–ATPase.

Developmental expression of messenger RNA for each OAT isoform has been studied in the rat and sheep. Renal cortical expression OAT1 was primarily localized to the kidney and reached adult levels by day of life 30.[4] Recent studies in the mouse indicate that there is a tendency for expression of organic anion transporters to increase during fetal development with the largest fold increase occurring between the immediate postnatal to mature stages.[5,6] In the ovine model, which is more similar to humans than is the rat, renal cortical expression of OAT1 and OAT3 was present in early gestation but was relatively low until late gestation when parallel increases in both were observed before birth.[7] One theory proposes that a preparturient rise in fetal cortisol levels might stimulate transcription of OAT1 and OAT3.[8] The effect of premature birth on the activity of these transport systems is unknown.

The ability of the kidney to produce a concentration gradient of organic anions is present in early gestation. Both mesonephros and metanephros of the developing chick are able to accumulate PAH.[9] Organic acid transport of the chick mesonephros peaks as early as 9 days after fertilization and then begins to decline by 17 days, when the metanephros displays significant PAH accumulation. Uptake of ^{3}H-PAH in tissue slices from fetal mice increases from 24% of adult values in 16-day-old fetuses to 79% in 19-day-old fetuses.[7] Organic cation transport has a different pattern of maturation, occurring later. Maximal PAH-concentrating ability peaks before that of organic bases or tetraethylammonium (TEA, the standard substrate) in both the mesonephros and the metanephros of the chick embryo. Similarly, the uptake of radiolabeled TEA into tissue slices is only 24% of adult values in 19-day-old fetal mice, whereas PAH is 79% of adult values. The contrasting patterns of development between the organic cation and anion transport systems was also demonstrated in gene expression studies in which the cation transporter OCT1 and OAT2 were late events.[5]

Studies performed in newborn humans,[10] dogs,[11] and rats[12] demonstrate a gradual increase in renal extraction of PAH with age. When PAH transport is examined by using renal tissue slices from rabbits, S-M ratios peak at approximately 150% of adult values by 1 month of age and then decline to values similar to those in adults by 3 months of age. Both clearance and renal extraction of PAH are reduced in human infants and neonates. Calcagno and Rubin[10] found PAH extraction and clearances in infants 3 months of age and younger to be approximately 30% lower than values in older children. Postnatal maturation of PAH clearance

and extraction has been found in most animal species studied. In the neonatal puppy, clearance studies indicate that the maximal tubular transport of PAH gradually increases by ten-fold over the first 8 weeks of life, although values are still reduced compared with those in adult animals.[13] Parallel studies, using the tissue slice technique, demonstrated a rather dramatic peak in the S-M ratio of PAH at 4 weeks of age, after which the S-M ratio declined to adult values over the next several weeks. Kim and colleagues[14] found S-M ratios for PAH to be 3.23 ± 0.27 in tissue slices from 2-day-old rats compared with 6.93 ± 0.337 in 10-day-old rats and 4.94 ± 0.28 in 14-day-old rats. By 20 days of age, S-M ratios were still less than adult levels.

This apparent discrepancy between in vivo and in vitro findings may be explained by the somewhat artificial nature of the slice method, as well as the possible contributions made by maturational differences in PAH efflux and protein binding. Cole and co-workers[15] found different maturational patterns in PAH uptake, efflux, and ligandin binding. Consistent with previous reports, intracellular-media PAH ratios in rabbit cortical slices gradually increased up to 4 weeks of age, at which time the ratio exceeded levels in adults and then declined to adult values by 10 weeks of age. The rate of ^{14}C-labeled PAH uptake reached adult values by 4 weeks of age and was maintained at adult levels from 6 to 10 weeks to adulthood. Efflux of PAH in fetal, 2-week-old, and 4-week-old rabbits was lower than in adult animals and attained adult levels by 6 weeks of age. In addition, ligandin levels were lower in immature animals and gradually increased with age. Thus developmental patterns of PAH transport, observed in vitro, may be the result of the sum of changes in efflux and transport, as well as the fraction of protein-bound PAH at a given age.

Enhancement of PAH transport occurs when organic acids (substrates for the transport system) are administered to animals before the time of study. In rabbits, the S-M ratios of PAH increase from birth to 4 weeks of age, then gradually decline to adult levels.[16] When procaine penicillin was given to 2- and 4-week-old rabbits for 3 consecutive days, PAH transport increased in the 2-week-old animals but not in the 4-week-old animals, suggesting that administration of organic acids to immature animals may induce maturation of the tubular transport process.[16] In addition, when pregnant animals received daily procaine penicillin during the last half of their pregnancy, S-M ratios of PAH in the offspring were markedly increased when compared with those in age-matched controls during the first 2 postnatal weeks, after which time values were equal to those in controls.[16] Subsequent studies have corroborated these findings.[17,18] Species-related or compound-related differences may exist in the response to organic acid exposure. Stopp and Braunlich[18] failed to induce increases in PAH transport after treatment of neonatal (5 day old) rats with sulfamethoxypyridazine, cyclopenthiazide, and phenobarbital, although 2-week-old rabbits did exhibit increases in PAH transport after administration of these compounds.

In vivo administration of thyroxine (T_3) enhances PAH uptake into cortical tissue slices from weanling rats without affecting PAH uptake of adult slices.[15] Other investigators have found conflicting responses of PAH secretion to T_3. Braunlich[19] demonstrated that T_3 stimulates PAH transport in both 10- and 30-day-old rats (Figure 132-2). The stimulatory action of T_3 on PAH transport can be prevented by the simultaneous administration of inhibitors of protein synthesis. One may conclude that the effect of T_3 on PAH uptake is dependent on protein synthesis (possibly reflecting synthesis of new transporters) and that pharmacologic, rather than physiologic, doses are required to stimulate PAH transport.

Kinetic analysis of age-related changes in PAH transport in tissue slices has revealed that transporter affinity (K_m) for PAH remains constant at 0.50 mmol/L, whereas the maximal PAH uptake increases as a function of age.[18] The stimulatory effect of organic anions on renal tubular secretion of PAH during early development is related to their ability to increase the maximal transport capacity for PAH rather than to an effect on K_m.[20] No detectable effect on efflux appears to occur. It is speculated that de novo synthesis of transporters occurs both with maturation and after the exposure to organic acids.

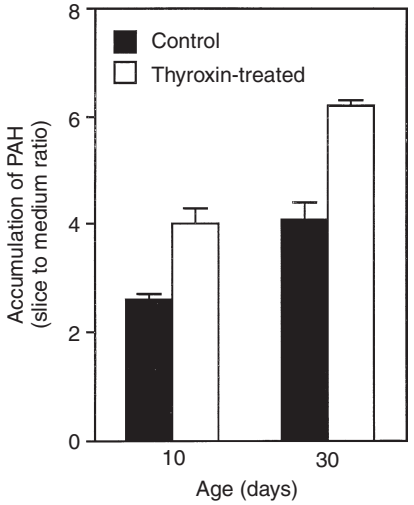


Figure 132-2 Effect of thyroid hormone treatment on PAH accumulation by renal cortical slices from 10- and 30-day-old rats. *(Modified from Braunlich H: Hormonal control of postnatal development of renal tubular transport of weak organic acids,* Pediatr Nephrol *2:151, 1988.)*

Rapid renal growth, as well as the possibility of postnatal nephrogenesis in some species, could explain some of the maturational changes observed in PAH secretion. Schwartz and colleagues[21] measured PAH uptake in isolated cortical tubules from 8- and 16-day-old rabbits. Transport capacity was then standardized for tubule length. An almost five-fold increase in transport capacity was observed in the older animals. Although tubular elongation might explain a fraction of the increasing transport of PAH observed with maturation, acquisition of transport sites was the major factor. Additional studies revealed that penicillin pretreatment increases intrinsic transport capacity by 89%.

Age-related changes in organic anion excretion result from increases in intrinsic renal tubular transport capacity and, to a lesser extent, increases in glomerular filtration and renal blood flow. Exposure to similar organic compounds stimulates the immature renal tubule to enhance transport, probably by increasing the density of transporters. Many drugs, such as antibiotics and diuretics, are secreted by the organic anion transport system. The newborn is less able to eliminate drugs when their elimination is dependent on renal tubular secretion.[22] Continued administration of some drugs, however, such as the penicillins, to the developing kidney may result in alterations of drug clearance via the mechanism of substrate stimulation. Such changes might require modification of drug dosage or dosing interval.[23] In addition, PAH clearance is not a reliable estimation of renal plasma flow in the newborn animal because of the reduced tubular secretion observed with immaturity.

URIC ACID TRANSPORT

Uric acid (or urate) is freely filtered at the glomerulus; however, a wide variation in urate handling has been observed in different species.[24,25] For example, in the monkey, human, rat, and mongrel dog, the fractional excretion of urate is less than 100%, indicating

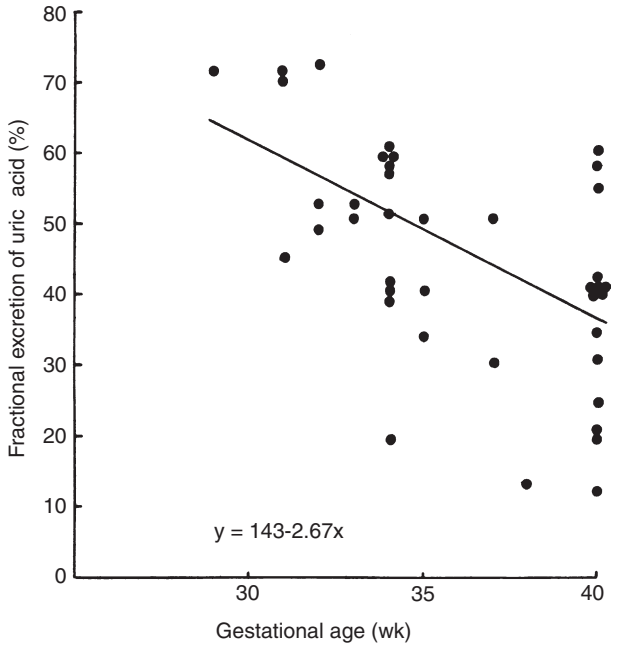


Figure 132-3 Fractional excretion of uric acid during the first day of life in infants according to gestational age. *(From Stapleton FB: Renal uric acid clearance in human neonates,* J Pediatr *103:290, 1983.)*

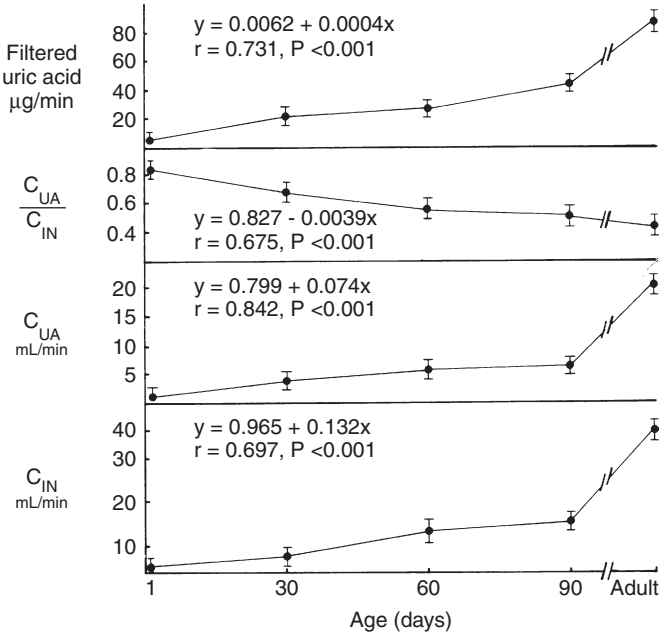


Figure 132-4 Patterns of renal clearance of uric acid and inulin in mongrel puppies during early development are compared with values in adult dogs.

net reabsorption. In other species, such as the rabbit and pig, urate excretion is greater than the filtered rate, which is indicative of net secretion. Species-dependent differences in net urate excretion and the parallel presence of both secretory and reabsorptive processes along the proximal tubule make characterization of the precise mechanisms of urate transport elusive.

The net reabsorption of urate across the proximal tubular epithelium involves an increasingly recognized number of transport systems. Only the major ones will be discussed here; for a more in-depth review, the reader is referred the work of Buist and colleagues.[4] There are three main transporters found on the luminal membrane: the anion exchange transporter, URAT1 (SLC22A12); the organic acid antiporter, OAT4 (SLC22A11); and the multidrug resistance protein, MRP4 (ATP-binding cassette family, ABCC4).[24] URAT1 transports urate in exchange for Cl or organic anions to achieve net reabsorption from the tubular lumen; this process is inhibited by uricosuric agents such as probenecid and losartan.[24] OAT4 reabsorbs urate in addition to organic acids via exchange with dicarboxylates, a process inhibited by hydrochlorothiazide. As a secretory pathway, MRP4 requires ATP to pump urate out of the cell and into the tubular lumen. Basolateral mechanisms for urate transport include OAT1 and OAT3, discussed previously in the section on organic acids. These transporters move urate from the basolateral or blood side into the proximal renal tubular cell.

Details regarding the maturational pattern of uric acid transport are limited. Clearance studies have been performed in humans and mongrel puppies. Newborns have higher serum uric acid levels, as well as elevated urinary urate excretion. As a rule, uric acid clearance increases and fractional excretion decreases during childhood.[26] Values for the fractional excretion of urate in 3- to 4-day-old term newborns were 20% to 59% compared with 13% to 26% in older infants up to 1 year of age and 11% to 17% in children aged 1 to 7 years. When fractional excretion of uric acid was examined during the first 24 hours of life in newborns of differing gestational ages, mean fractional excretion was 61.24% ± 12.21% in infants of 29 to 33 weeks gestation, 44.52% ± 15.23% in neonates of 34 to 37 weeks gestation, and 38.19% ± 13.61% in term infants (Figure 132-3).[27] Serum uric acid levels and uric acid clearances were not significantly different among infants of differing gestational age.

In the mongrel dog, plasma uric acid decreases with age in a manner similar to that in humans. Stapleton and Arant[28] demonstrated that the clearance of uric acid increased and fractional excretion of uric acid decreased as a function of postnatal age (Figure 132-4). Uric acid clearance increased from 0.39 mL/minute at 1 day of age to 3.72, 5.54, and 6.3 mL/minute at 30, 60, and 90 days of age, which is still lower than the adult value of 20.16 mL/minute. At 1, 30, 60, and 90 days of age, the fractional excretion of urate was 83%, 69%, 57%, and 51%, compared with the adult value of 44%. A decreased fractional excretion of urate in the face of an increased filtered load can be explained by either increased reabsorption or decreased secretion with increasing age. Studies done to date have not answered this question; however, the net reabsorption of urate in the mongrel dog increased from 0.2 µg/minute at 1 day of age to 8.8, 11.9, and 22.2 µg/minute at 30, 60, and 90 days. Adult animals exhibited a net reabsorption of urate of 39 µg/minute.[27]

Urate uptake has been measured by using tissue slices obtained from fetal and newborn mice.[29] Accumulation of ^{14}C-labeled uric acid by slices from 16-day fetuses was 49% of adult levels and increased to 79% of adult values at 19 days gestation. At the postnatal age of 1 day, a transient decline in urate accumulation to 58% of adult values was observed; however, urate accumulation increased to 70% of adult values by 7 to 8 postnatal days.[29] This developmental pattern was similar to that observed for PAH. In contrast to PAH, however, uric acid uptake into renal cortical slices is not enhanced by pretreatment of neonatal animals with acetate or procaine penicillin.[22]

The mechanisms responsible for the increase in net tubular urate reabsorption associated with maturation are not fully understood. One might anticipate that nonspecific cellular events, as well as increased transport capacity of either or both luminal and basolateral transport systems for urate, could account for the decrease in fractional excretion with increasing age; however, these events have yet to be characterized by kinetic or molecular studies.

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†*Michael A. Linshaw*

Concentration and Dilution of the Urine

PHYSIOLOGY OF THE URINARY CONCENTRATING MECHANISM

The ability to concentrate and dilute the urine greatly expands our range of habitable environments. To accomplish this, the mammalian kidney must deliver sufficient solute to the medulla, establish a solute gradient that increases from cortex to papilla, preserve the gradient despite its tendency to dissipate from continuous flow of blood, and stimulate or suppress reabsorption of water as needed. A solute gradient can develop because membranes of tubule segments as well as microvessels have varying solute permeability characteristics. When a solute gradient is established, water movement from tubule lumen toward the interstitial gradient depends largely on the tubular response to antidiuretic hormone (ADH, vasopressin) and the anatomic integrity of the tubulovascular structures (Figure 133-1). Because fluid is reabsorbed isotonically in proximal tubules and is not concentrated in distal convoluted tubules, major steps in the concentrating process involve events occurring in the loops of Henle and collecting tubules. A vital site of active salt transport occurs at the thick ascending loop of Henle; deeper nephron segments can move solute and water passively, thereby facilitating conservation of energy. The actual process of urinary concentration requires the presence of ADH and occurs in collecting ducts where water is avidly reabsorbed down an osmolar gradient generated initially by active salt reabsorption in the thick ascending limb of Henle. Good reviews with detailed discussion and extensive references on the urinary concentrating mechanism are available.[1]

Development of the Medullary Gradient

Several processes illustrated in Figure 133-2 occur simultaneously, but it is convenient to start a discussion of the concentrating mechanism at the thick ascending limb of Henle in the outer medulla.[2-4] This segment has a very low hydraulic conductivity, but it can reabsorb NaCl in the relative absence of water (reabsorbs hypertonic NaCl). The result is dilution of fluid remaining in the tubular lumen and an increase in the interstitial concentration of NaCl surrounding the thick limbs and collecting ducts. Isotonic fluid from the proximal tubule enters the descending loop of Henle at the level of the outer medulla and is exposed to the increased interstitial osmolality. Descending loop segments have a high hydraulic conductivity, but a low sodium permeability. As a consequence, water in the descending limb moves osmotically into the interstitium, and fluid in the lumen of this segment becomes more concentrated.

†Deceased

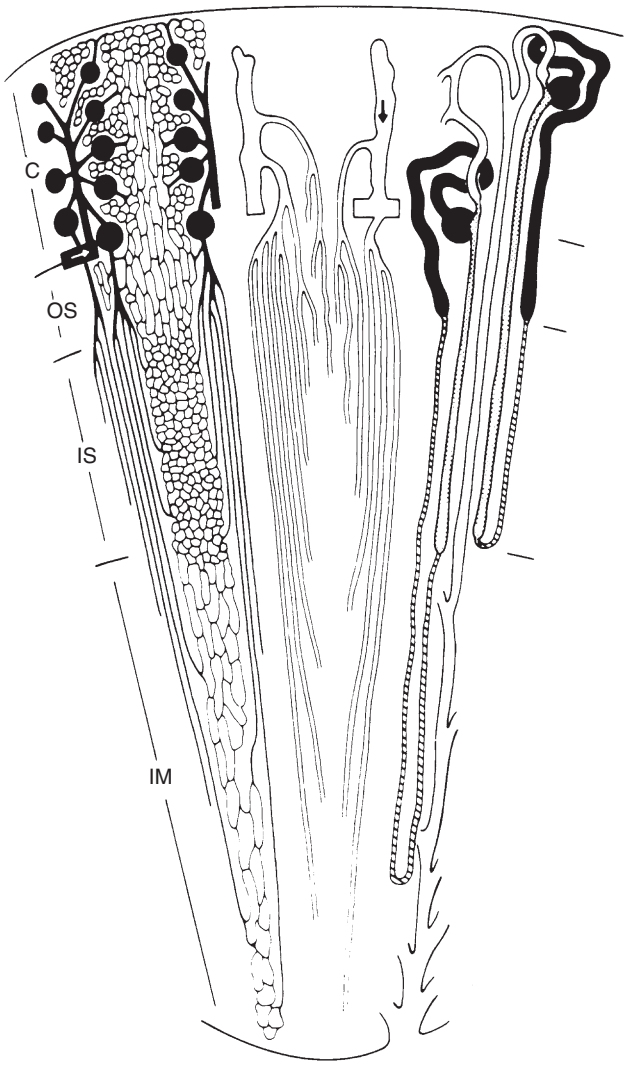


Figure 133-1 Anatomy of tubules and blood vessels and relationship of superficial and deep nephrons. *C,* Cortex; *IM,* inner medulla; *IS,* inner stripe; *OS,* outer stripe. *(From Zimmerhackl BL, Robertson CR, Jamison RL: The medullary microcirculation.* Kidney Int *31:641, 1987.)*

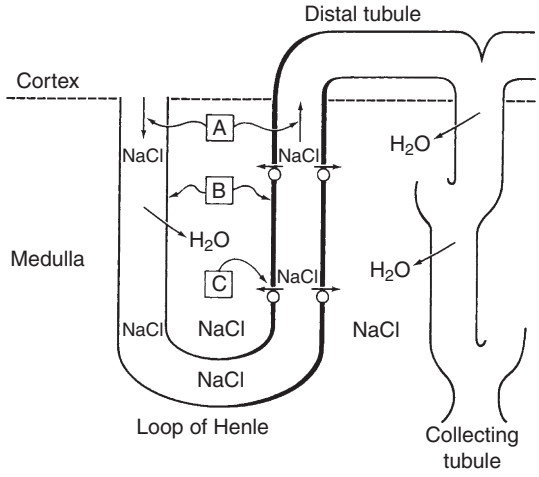


Figure 133-2 The countercurrent movement of water and solutes between the loops of Henle and the collecting tubule. Heavier lines of the ascending thin limb in the inner medulla and of the ascending thick limb in the outer medulla depict impermeability to water. NaCl is actively reabsorbed from the thick limb, diluting urine and increasing osmolality of the outer medulla (1). Water reabsorption continues in the distal tubule and the cortical and outer medullary collecting duct, leading to increased urea concentration (2). Water and urea are reabsorbed from the inner medullary collecting duct (3). Urea accumulates in the interstitium, osmotically abstracts water from the descending limb, and allows NaCl concentration *(A)* in descending limb fluid to increase and *(B)* in interstitium to decrease (4). The thin ascending limb is permeable to NaCl and receives fluid rich in NaCl. NaCl enters the interstitium down its concentration gradient, and urine becomes hypoosmotic to the surrounding interstitium (5). See text for details. *(Reprinted by permission of the* New England Journal of Medicine: *Jamison RL, Maffly RH: The urinary concentrating mechanism.* N Engl J Med *295:1059, 1976.)*

Urine, flowing through the thick ascending limb, becomes progressively dilute by virtue of the relative impermeability of this segment to water and then enters the distal convoluted tubule, a cortical segment in which water and salt reabsorption and equilibration with blood produce a largely isotonic urine entering the outer medullary collecting duct. In a hypertonic outer medulla, water is osmotically removed from the tubule lumen, and solutes in the collecting duct become increasingly concentrated. Urea movement in distal tubules is limited, but water reabsorption is substantial, so cortical collecting tubules receive urine with a high urea concentration. This segment has low urea permeability both in the presence and absence of ADH, so a high urea concentration is maintained in urine entering the outer medullary collecting duct. The outer medullary collecting duct has a low permeability to sodium and urea and is not thought to transport NaCl actively. This segment probably does not contribute to the high interstitial salt content, but its water permeability is high in the presence of ADH.

Vasopressin also stimulates NaCl reabsorption by the medullary thick ascending limb, thereby increasing NaCl content in the outer medullary interstitium.[5-7] This latter step may help counteract the dilution of interstitial NaCl that follows when ADH induces water reabsorption from the outer medullary collecting duct. The net effect of ADH is to increase the osmotic driving force and subsequent reabsorption of water as urine from the collecting tubule traverses the outer medulla. The high water and relatively low urea permeability cause urea to become a dominant solute in the outer medullary collecting duct. The outer medullary interstitium contains relatively little urea, but a significant amount of NaCl reabsorbed from the ascending thick limb. A high luminal urea concentration can drive water toward the tubule lumen. Conversely, a high interstitial NaCl concentration can drive luminal fluid to the interstitium.

Although osmolality is equal across the collecting duct epithelium, the net driving force favors water reabsorption from the lumen because NaCl has a higher reflection coefficient than does urea and exerts a more effective osmotic pressure.[8] This further increases urea concentration in urine entering the inner medullary collecting duct. In contrast to the more proximal collecting duct, the inner medullary collecting duct is permeable to urea in the presence of ADH.[6,7] Vasopressin also increases the permeability of this segment to water, although urea and water may traverse different channels.[9]

Urea diffuses down a chemical gradient to enter the medullary interstitium. Urea can also enter the descending limb, and its concentration actually rises in this segment. Accumulation of urea in the interstitium provides an osmotic driving force that further abstracts water from medullary portions of the descending loop. This increases the luminal concentration of NaCl and

urea. The water leaving the descending loop dilutes interstitial NaCl. When urine flows through the bend of the loop and enters the thin ascending limb of Henle, the high NaCl concentration in this segment is exposed to an interstitium of similar osmolality but now lower NaCl concentration; the remainder of the osmolar concentration is composed mainly of urea. The thin ascending limb has a low hydraulic conductivity, a high NaCl permeability, and a moderate permeability to urea[10]; NaCl can thereby diffuse down its concentration gradient into the interstitium whereas, water tends to remain in the lumen.

The urine begins to become progressively less concentrated, and NaCl accumulates in the interstitium. The presence of this added interstitial NaCl abstracts more water from urine in the inner medullary collecting duct as this segment courses through the interstitium, a step that further concentrates the final urine. In addition, urea can re-enter the lumen of thin ascending limbs to recycle back to the collecting tubule.

Although the hypertonic medulla provides the driving force for water reabsorption and urinary concentration, it is noteworthy that cells in the renal medulla are under considerable osmolar stress from the hyperosmolar state. The major solutes are urea and salt, the latter capable of damaging cellular DNA and causing cell death when salt concentration is sufficiently high. The medullary cells adapt by synthesizing and accumulating relatively nonpermeant organic solutes. A regulatory protein, tonicity-responsive enhancer binding protein, stimulates genes to produce proteins that transport or synthesize the protective organic osmolytes.[11,12]

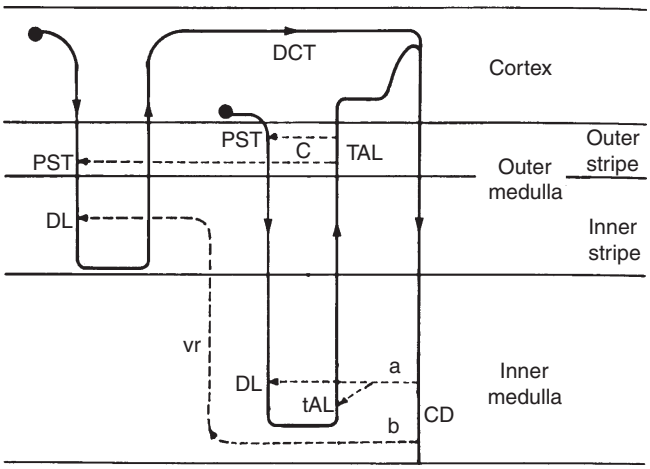


Figure 133-3 Urea recycling pathways in mammalian kidney. Short-looped nephron *(left)* and long-looped nephron *(right)* are depicted by *solid lines.* Urea transfer between segments at sites a, b, and c is indicated by *dashed arrows.* See text for details. *AL,* Thin ascending limb; *CD,* collecting duct; *DCT,* distal convoluted tubule; *DL,* descending limb; *PST,* proximal straight tubule; *TAL,* thick ascending limb; *vr,* vasa recta. *(Reprinted by permission of the* New England Journal of Medicine*, 31:629, 1987.)*

Role of Short-Loop Nephrons and Urea

Recycling of urea (to maintain a high medullary urea content) is critical for maximal efficiency of the concentrating process and is facilitated by short-looped nephrons that do not descend deep into the inner medulla.[13,14] These nephrons reabsorb a large portion of filtrate, reducing the volume of fluid and solute that needs to be concentrated. Short-looped nephrons also exhibit permeability and anatomic characteristics that place them in good position to receive urea and facilitate its recycling to the inner medulla.[5] For example, in the rat, urea permeability of thin limbs is greater in short-looped than in long-looped nephrons. Moreover, in contrast to long-looped nephrons, short-looped thin descending limbs run within vascular bundles and are closely apposed to vasa rectae that ascend from the inner medulla and contain blood with increased amounts of urea.

The intertubular recycling of urea is thought to be accomplished through three major routes (Figure 133-3). First, urea in the ascending thin limb can remain in the tubule lumen, travel through the distal nephron to the collecting ducts, and then recycle to the interstitium. Second, urea in thin ascending limbs can reach the thick ascending limb, a segment that, in the outer medulla, is permeable to urea and is in close relationship to proximal straight tubules (and therefore descending limbs) of both long- and short-loop nephrons. At this site, urea can return to the inner medulla directly via proximal straight tubules and descending limbs of long-loop nephrons, or it can cycle through loops and collecting ducts of short nephrons to re-enter the medulla. Third, urea can leave the inner medullary interstitium through ascending vasa rectae and re-enter descending limbs of short-looped nephrons. This urea can similarly be carried back through superficial distal nephrons and transported to collecting ducts in the inner medulla.

Urea transfer from vasa rectae to short nephron descending limbs is likely facilitated by the anatomic proximity of these structures in vascular bundles of the inner stripe of the outer medulla. The solute delivered to deep thin ascending limbs of the inner medulla can recycle to maintain a continued presence of deep medullary solute. The solute helps concentrate fluid in the descending limbs of long-looped nephrons and is available to enhance movement of water from the collecting duct before the urine reaches the renal pelvis and ureter. The importance of urea transport in this process is underscored by the presence of urea transporters located in endothelial cells of medullary descending vasa rectae[15] and the ability of ADH to induce insertion of urea transporters in the luminal membrane of the inner medullary collecting duct.[16,17] An additional important site of urea transfer is from ascending to descending vasa rectae (see later discussion).

The role of urea is complex. Urea had been thought to cross cell membranes solely by passive diffusion, but rapid urea transport rates in some tissues suggested a facilitative transport mechanism. As reviewed recently,[18] several urea transporter isoforms (UTs) derived from the UT-A gene have been identified in renal tissue that can facilitate transmembrane movement of urea. These transporters may serve different functions. UT-A genes express medullary proteins in inner medullary collecting ducts and thin descending limbs of Henle. UT-A1 and UT-A3 are restricted to the terminal portion of the inner medullary collecting duct. UT-A2 is localized in the lower part of thin descending limb of short-loops of Henle in the inner stripe of the outer medulla, but also in the thin descending limb of long-loop nephrons in the inner medulla under more prolonged antidiuretic conditions. These transporters can help generate the hypertonic medulla. Thirsting increases levels of UT-A2 protein, a response mediated by ADH. Rapid movement of urea actually may serve to dissipate a transmembrane osmotic urea gradient.[19-21] By contrast, the UT-B gene produces a transporter protein located in endothelial cells of medullary descending vasa recta that allows urea leaving the ascending vasa recta to re-enter the descending vasa recta, become "trapped" in the medulla, and maintain a high medullary urea concentration. It is interesting that the UT-B1 transporter protein is down-regulated by ADH. This may represent a metabolic adaptation to reduce the need for urea transport and allow for reduced urea synthesis in descending medullary vasa recta endothelial cells. It could serve either to favor nitric oxide over urea synthesis (for vasodilation and improved nutrient delivery) or to limit urea production in endothelial cells when a very high inner medullary content of urea is already present.[22,23]

Fenton and Knepper[18] have summarized several interesting experiments with knockout mice designed to clarify aspects of

the concentrating mechanism. According to this model, during antidiuresis, water is reabsorbed in the cortical collecting duct, a segment poorly permeant to urea. The increased urea concentration in urine reaching the urea permeable medullary collecting duct favors movement of urea to the medullary interstitium. The nearly equal concentration of urea across the medullary collecting duct membrane, as proposed by Berliner and colleagues[24] more than 50 years ago essentially eliminates any tendency for urea to cause an osmotic diuresis. The high interstitial urea content helps draw water from the descending loop of Henle into the interstitium, concentrating the NaCl in the loop and diluting the NaCl in the interstitium. As urine enters the medullary thin ascending loop of Henle, a segment not thought to transport NaCl actively, the surrounding interstitial milieu has drawn water from the descending limb by virtue of the high urea content. The now lower concentration of interstitial NaCl favors movement of NaCl from the ascending limb, and perhaps also from the medullary collecting duct. Accordingly, one would expect that if UT-A1 and UT-A3 in the inner medullary collecting duct were nonfunctional, a urea-induced osmotic diuresis would occur. This was tested in knockout mice with no functional UT-A1/A3 transporters on different protein intakes.[25] When these animals with free access to water were placed on a high protein intake, their urine volume and fluid intake was high and urine osmolality was low compared with controls. When they were water restricted, the mice could not reduce their urinary output or increase urine osmolality and became volume depleted. On a low protein intake, where urea production is low, no polyuria or change in osmolality was observed when given access to water, and when water was restricted no difference was observed in urine flow or osmolality between control and UT-A1/A3–deficient animals. Moreover, compared with control mice, knockout mice had essentially identical inner medullary concentrations of Na^+, Cl^-, and K^+, but the knockout mice showed a substantial decrease in urea concentration. These data were interpreted to indicate that a concentrating defect in animals devoid of inner medullary collecting duct urea transporters was mainly the result of a urea-dependent osmotic diuresis. In further studies,[26] an attempt was made to test the relationship between urea transport in the inner medulla as a factor helping generate an increase in interstitial NaCl (see previous discussion). It would be predicted that knockout mice devoid of functional UT-A1/A3 would have impaired ability to concentrate NaCl in the inner medulla because urea movement would not be increased into the medulla from the medullary collecting duct to induce water flow from the descending thin limb that in turn would facilitate reabsorption of NaCl from the ascending limb. Experiments were performed in mice on low, normal, and high protein diets. Compared with control mice, those devoid of UT-A1/A3 had a progressive increase in water intake and urine flow with increasing protein intake but had no increase in urine osmolality. In additional studies, mice were subjected to either a low or high protein diet and measurements were made of the osmolality, urea, and Na^+ concentrations in the cortex, outer medulla, and two levels within the inner medulla. In control mice, increasing protein intake from 4% to 40% led to a dramatic increase in corticomedullary osmotic gradient, related primarily to an increasing corticomedullary urea concentration. By contrast, in knockout mice, no appreciable change in corticomedullary osmolality or urea gradient on either diet was observed, and, on the high protein diet, the inner medullary osmotic and urea content were markedly reduced. However, no differences were seen between controls and knockout mice in the Na^+ concentrations from cortex, outer medulla, or inner medulla and Na^+ profiles, and corticomedullary Na^+ gradients were virtually identical in animals on both diets. Therefore depleting medullary urea either by reducing urea production on a low protein diet or deleting medullary collecting duct urea transporters had no impact on the ability of these mice to accumulate inner medullary Na^+ or develop a well defined corticomedullary Na^+ gradient. It follows that although the passive model of urinary concentration as described is still convenient conceptually, clearly the mechanism is more complex.

Knockout mice have also been used to clarify the relative importance of urea transporters because they help recycle urea to maintain high medullary urea content (see earlier discussion). The lower portion of thin descending limbs of short-looped nephrons contains UT-A2 and descending vasa recta contain UT-B. Part of urea recycling into ascending Henle's limb may involve transfer of urea from ascending vasa recta to thin descending limbs and ascending to descending vasa recta. If these transporters were deleted, less accumulation of inner medullary urea and consequently urea–induced osmotic diuresis from the high urea content in the urine of medullary collecting ducts as it coursed through deeper portions of the medulla would occur. In recent studies,[27] compared with control mice, when the UT-A2 transporter was deleted, knockout mice on normal protein intake had no decrease in urinary concentrating ability or accumulation of inner medullary urea in response to water deprivation. On a low protein intake, UT-A2–depleted mice had only a modest decrease in urinary concentrating ability and medullary urea content without a drop in medullary chloride. It appears that urea secretion into the thin descending limb is not a critical part of the process. By contrast, studies with UT-B–depleted mice are consistent with this transporter playing a larger role.[28,29] Mice were subjected to basal conditions, acute urea loading (intraperitoneal injection) and chronic urea loading (high protein diet). Compared with control animals, knockout mice (deletion of UT-B) on a normal protein intake had increased urine output and lower urine osmolality and had a significantly decreased maximal urine osmolality when water deprived. This was due primarily to a decrease in medullary urea content. After an acute urea load, control animals showed a well-defined increase in urine osmolality and urine urea concentration but only a modest increase in urine output. Knockout mice had a blunted increase in urine osmolality and urine urea concentration and a higher urine output. Even after the urea load had been excreted during 6 to 8 hours, control mice maintained a much higher urine osmolality and urine urea concentration and a lower urine output than the UT-B–depleted mice. These findings are consistent with the need to sequester inner medullary urea to allow for increased urine concentration and decreased urine output. Animals were also placed on 10% (low), 20%, or 40% (high) protein diets for a week. The diets did not induce changes in urine osmolality within groups. However, urine osmolality was consistently lower and urine output higher on each diet in the UT-2–depleted mice. Urea excretion increased to the same extent in both groups of mice with the increase in dietary protein, but this occurred with an increase in urine urea concentration in control animals, whereas the knockout animals required an increase in urine volume with little change in urine urea concentration. Even with a week of high protein intake, the UT-2–depleted mice could not translate the urea load to an improvement in urine concentrating ability. It would appear that UT-B urea transport between descending and ascending vasa rectae is critical to trapping urea in the inner medulla and maximizing conservation of water, and this exchange may contribute quantitatively more than recycling of urea between ascending vasa rectae and thin descending limbs to the urinary concentrating mechanism.

In a series of experiments, Halperin and colleagues[30,31] provided insight about urea as an effective osmole for water transfer in the inner medulla. Only solutes with a concentration difference can affect water movement across a semipermeable membrane. ADH increases water and urea permeability in inner medullary collecting ducts. If urea is highly permeant across this membrane, urea concentration equilibrates and adds to final urine osmolality but induces little increase in urine flow.

By contrast, salts remaining in higher concentration within the collecting duct lumen will act as effective osmoles and obligate water. In one set of experiments, subjects were either fasted or placed on a normal diet and then water-depleted. Those on a regular diet excreted largely urea and NaCl as their nonurea urinary osmoles. Fasted subjects excreted less urea, and their nonurea osmoles were largely NH_4^+ and β-hydroxybutyrate, not NaCl. Their urea excretion rate and total urine osmolality were lower, but both groups had similar nonurea osmolality and urine flow rates. Therefore urine flow was related mainly to nonurea osmolar load. Urea did not act as an effective osmole and did not induce more water excretion as long as the nonurea osmolar load was similar. When water-deprived subjects on a normal diet received a urea load, urine flow rate nearly doubled, and rates of excretion of both urea and nonurea osmoles increased. The total urine osmolality was unchanged because a decrease in nonurea osmolality (diluted by increased urine flow) accompanied the increase in urea osmolality generated by the urea load. However, both urea and nonurea solutes in this setting acted as effective osmoles. Urea could potentially decrease the nonurea osmolality of the inner medullary interstitial fluid by the following process. Interstitial osmolality increases as NaCl, in high concentration in the relatively water impermeant ascending limb, enters the interstitium without water. The interstitial NaCl concentration falls, however, as electrolyte-free water is reabsorbed from the inner medullary collecting duct, in part from added salt and in part from water moving osmotically with urea. As more urea enters the deeper medullary collecting duct, the intratubular nonurea solute will be necessarily lower because total osmolality will be equal across the tubular membrane. This should help drive even more electrolyte-free water toward the interstitium. As a result, interstitial nonurea osmolality should be lower, maintaining the effectiveness of residual intratubular salt as effective osmoles.[30]

In further studies,[31] human subjects on a regular diet were water deprived, given vasopressin, and then given hydrochlorothiazide to increase the proportion of urinary electrolytes comprising total urinary osmoles. After the diuretic, the proportion of urea osmoles in urine dropped from 50% to 20% of total urinary osmoles. When these subjects, now excreting a high proportion of nonurea osmoles, also received a urea load, urea concentration increased, nonurea osmolality or nonurea excretion rate did not change, and urine flow rate did not change. By not increasing urinary flow, urea acted as an ineffective, highly permeant osmole. Under a different protocol, subjects received furosemide to induce negative NaCl balance and were placed on a low-salt diet to generate an electrolyte-poor urine. Subjects were then water deprived and given a urea load. With a low nonurea (electrolyte) osmolality, the urea load now induced a marked increase in urine flow rate and a decrease in nonurea osmolality, but no change in total urine osmolality. In this setting, urea acted as an effective, less permeant osmole.

Rats behaved similarly. When rat papillary tip and urine solute content were compared, total as well as urea and nonurea osmolalities were similar when urine had a high proportion of urinary electrolyte osmoles, indicating equilibration of urea and nonurea solutes between papillary interstitium and urine. By contrast, when rats were electrolyte deprived on a low-salt diet and given a urea load, urea concentration was higher in urine than in interstitium, nonurea (electrolyte) concentration was higher in the interstitium, and the total omolalities were similar. Urea now acted as a relatively impermeant osmole and did not equilibrate across the interstitium.

The authors concluded that urea acts as an ineffective urine osmole, unable to obligate more water excretion, when there is a high rate of salt excretion. Conversely, when the rate of electrolyte excretion is low, urea acts as an effective osmole, thereby obligating water and increasing urine flow. When a urea load is sufficiently large, it becomes an effective osmotic agent, increasing urine flow to help remove excess urea. However, at low or modest excretion rates of urea, this solute equilibrates between collecting duct and interstitium so that urea exerts no significant osmotic effect and thus causes no increase in urine flow. In addition, relatively impermeant nonurea solutes such as NH_4^+ and β-hydroxybutyrate can ensure a minimal urine flow even during chronic fasting, reducing the risk for hyperkalemia and formation of calculi.[30]

The importance of these findings is apparent when considering that kidneys must reduce urine volume when water conservation is needed (dehydration) without reducing volume so much as to increase the risk for hyperkalemia or stone formation. In the setting of volume depletion and low renal electrolyte excretion, urea becomes less permeant and obligates water, maintaining urine flow to more than severe oliguria. In the setting of adequate urinary electrolytes, the kidney excretes urea (main protein metabolic product) without increasing the minimum urine volume needed to excrete other solutes. Were urea not highly permeable, it would remain in the collecting duct lumen (obligate water excretion) and blunt the ability of the kidney to lower water excretion in times of water depletion. This is accomplished because urea becomes more permeant, equilibrates across the medullary interstitium, and thereby is excreted without obligating more water. It is unclear how the kidney alters urea permeability in these situations, but the mechanism may involve a change in the function or number of urea transporters.

Halperin and colleagues[32] have more recently suggested a mechanism by which the renal concentrating mechanism is regulated using the observations that the majority of thin descending limbs of Henle (those from short-looped nephrons) lack aquaporin-1 and that inner medullary loops of Henle lack urea transporters. In their conception, NaCl is reabsorbed across the thick ascending limb via the Na-K-2Cl co-transporter along with some passive Na reabsorption driven by a lumen-positive voltage. Reabsorption depends on K movement to the lumen across a K channel. Water reabsorbed from the collecting duct in response to this interstitial NaCl may dilute an inhibitor of the K channel, allowing more NaCl to be reabsorbed. The authors suggest, for several reasons, that the calcium sensing receptor (Ca-SR) may provide such an inhibitor. When enough Ca is reabsorbed to occupy the receptor, the K channel will be inhibited, interfering with the cotransporter and preventing excessive NaCl reabsorption that in turn, if not curbed, might lead to excessive water reabsorption, increasing the potential to form kidney stones. The absence of aquaporin-1 in the majority of descending thin limbs serves to limit the amount of water that could potentially interfere with this inhibitory feedback system. In the absence of substantial water abstraction from this segment, it is likely that NaCl enters this segment so as to provide sufficient salt in the deeper portions of the medulla. As mentioned previously, the mechanism is unclear, but does not appear to be dependent on water and urea reabsorption from the medullary collecting duct resulting in dilution of interstitial NaCl. Therefore this permits passive reabsorption of NaCl from the ascending limb, as suggested by the passive model of urinary concentration, because interfering with the medullary collecting urea transporters does not interfere with medullary interstitial content of NaCl. Urea recycling is critical to inner medullary function. The large amount of reabsorbed urea, along with water from the medullary collecting duct, leaves via the ascending vasa rectae. Urea can enter the lumen of descending thin limbs to reach the distal convoluted tubule and later enter the collecting duct, but this occurs only in the lower portion of the descending thin limbs of short looped superficial nephrons that contain the UT-A2 transporter. The site where urea re-enters this segment occurs deeper in the outer medulla than the site where most of the water is reabsorbed to the interstitial compartment. This facilitates the medullary recycling of urea. As a final point, the authors again point to the need for an adequate

number of effective osmoles in the urine, namely NaCl. When NaCl in the urine is insufficient (e.g., on a low salt diet), urea concentration in the lumen is higher than in the papilla, and urea then acts as an effective osmole, obligating water to prevent a urinary concentration high enough to increase the risk for stone formation. The purpose of the inhibitory mechanism involving the thick ascending reabsorption of Na is to ensure that not only can adequate water be conserved, but also that the amount of water to eliminate urinary solutes will be adequate.

Role of Vasa Rectae

Vasa rectae are blood vessels coursing through the interstitium. Vasa rectae provide substrate for, and remove end products of, metabolic reactions and also, because they are highly permeable, remove solute and fluid reabsorbed by tubules. If the vessels traversed the medullary interstitium only once, they would remove reabsorbed urea and NaCl, depleting the interstitium of solutes and compromising the solute gradient. To avoid this, they descend into the medulla to varying degrees, break up into small capillaries that course through localized areas of interstitium, and rejoin to ascend toward the cortex (Figure 133-4 and described later). A recent review of the renal medullary microcirculation is available for the interested reader.[33] Juxtamedullary glomeruli send efferent arterioles into the outer medulla. In the outer stripe these vessels divide into descending vasa rectae that form vascular bundles in the inner stripe of the outer medulla. Peripherally located vessels can form an interbundle capillary plexus in close proximity to metabolically active thick ascending limbs and collecting ducts. More centrally located descending vasa rectae penetrate to the deep portion of the inner renal medulla. Vascular bundles form in the inner stripe of the outer medulla. As the descending vasa rectae move into the inner medulla, vascular bundles are not present, but the descending vasa rectae course close to nephron segments and at the bottom of the medulla form a capillary plexus that eventuates in ascending vasa rectae that course toward the vascular bundles of the inner stripe. In the inner stripe vascular bundles, descending and ascending vasa rectae as well as thin descending loops of Henle from short-looped nephrons are in close apposition. Ascending vasa rectae are fenestrated and highly permeant. Descending vasa rectae have continuous endothelial cells and pericytes (contractile smooth muscle remnants). They also contain the urea transporter UT-2 as well as aquaporin-1. Descending vasa rectae are well situated adjacently in vascular bundles and also in deeper portions of the medulla to receive NaCl and urea from ascending vasa rectae and to deliver water toward the ascending vessels down concentration gradients.[14,34] Accordingly, solutes such as NaCl and urea found in high concentration in ascending vasa rectae, can enter descending vasa rectae that contain fluid of lower solute concentration, whereas water can move in the opposite direction. As a result, fluid in the descending vessel penetrates deep in the medulla and carries a high solute concentration close to that in the interstitium. The driving force for passive loss of this solute from interstitium to vessels is thereby minimized. Part of the vascular bundle may include descending limbs of short-loop nephrons. This segment can take up solute from ascending vasa rectae and recycle it to collecting ducts traversing the deep medulla, where solute and water are further reabsorbed.

Role of the Renal Pelvis

The renal pelvis may contribute to urinary concentration.[35] Epithelium covering the inner medulla and papilla and facing the pelvic space is similar to that of papillary collecting ducts. During water diuresis, these cells are closely apposed and intercellular spaces are narrow. During antidiuresis, individual cells are more distinct, and intercellular spaces are widely dilated. This

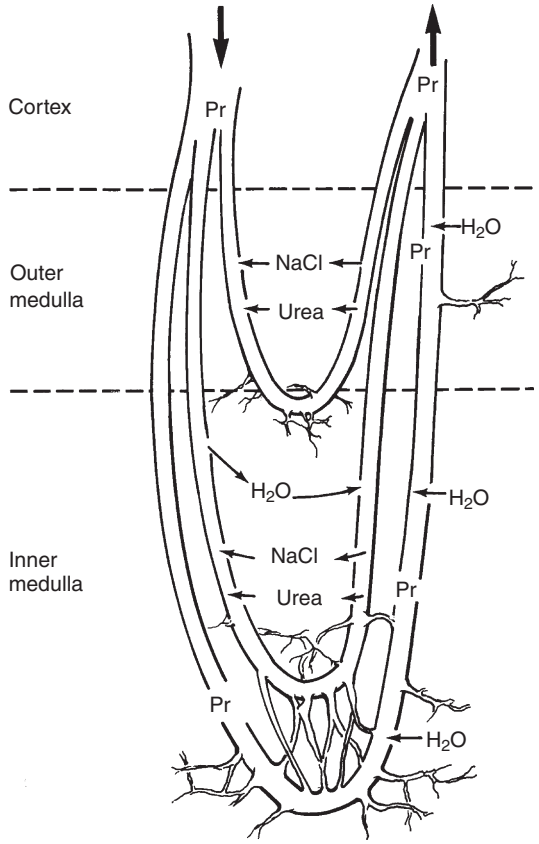


Figure 133-4 Countercurrent exchange in vasa recta. Medullary circulation includes network of interconnecting vessels and main thoroughfares. Vessels are freely permeable to NaCl, urea, and water. Type size denotes solute concentration relative to its location in medulla but not relative to concentration of another solute. Loops of Henle and collecting ducts are responsible for increasing concentration of interstitial NaCl and urea. Solutes can enter descending vasa recta and leave ascending vasa recta to remain trapped in medulla. As water leaves and descends the vasa recta, protein concentration increases. High osmotic and oncotic pressures in ascending vasa recta enhance capillary fluid uptake, returning water, reabsorbed from tubules, to general circulation. Therefore vasa rectae trap solute and remove water, preserving hyperosmolality of the renal medulla. *Pr,* Plasma protein. *(Reprinted by permission of the* New England Journal of Medicine: *Jamison RL, Maffly RH: The urinary concentrating mechanism.* N Engl J Med *295:1059, 1976.)*

may reflect enhanced transepithelial movement of fluid.[36] Pelvic extensions in some species allow the urinary space to surround vascular bundles and much of the papillae and may reach the cortex.[13] Increased urine concentration has been related to an increase in contact surface area between papilla and pelvic urine.[36,37] Moreover, an intact renal pelvis may be necessary to produce a maximally concentrated urine. If one excises the ureter to expose the renal papilla of a rat, urine osmolality at the papillary tip drops sharply by nearly 40%.[38] Urine in the renal pelvis circulating around the papillae and inner medulla may reach an area in the medullary interstitium where urea concentration is less than that in the urine. Urea could then recycle from pelvic urine to the medullary interstitium and help maintain a high medullary urea concentration gradient, enhancing abstraction of water from thin descending limbs of Henle. In this regard, the concentration of urea in fluid bathing the papilla, rather than the total osmolality, seems to be a critical factor in determining the

final urine concentration. If the papilla is bathed with solutions of varying osmolality and urea concentration, the osmolality of urine from the papillary tip increases when the urea bath concentration is increased, but not necessarily when the bath osmolality is altered.[39] This hypothesis remains uncertain. Other investigators have not found significant urea reabsorption across this epithelium. In addition, not all rodents show a relation between extensions of renal pelvis and fornices around their papillae and the ability to maximally concentrate urine.[35]

Confusion about the impact of urine flow and pelvic reflux on papillary function is evident from some of the following considerations. After urine leaves the papillary tip, it can descend the ureter, reflux slightly to surround a small portion of the papillary tip, or reflux fully over the entire fornices and pelvic extensions and cover more of the medulla in which solute and water exchange may occur. Full retrograde flow of urine over the entire papillary surface and up toward the outer medulla occurs with bladder and retrograde ureteric contractions. It can be stimulated by increasing hydrostatic pressure in the pelvis by as little as 1 cm of H_2O. When the renal pelvis is removed, urine flow is nonpulsatile and continuous, a state not associated with full pelvic reflux. In contrast, when the renal pelvis is intact and performs its contractile or pulsatile action, urine flow in terminal collecting ducts is intermittent rather than continuous. During antidiuresis, when urine flow is slow and urine becomes highly concentrated, full pelvic refluxes also do not occur, even with an intact pelvis. Therefore the importance of an intact renal pelvis to an efficient urinary concentrating ability may be related more to the contractile nature of the pelvis than to the actual degree of reflux. During a peristaltic contraction, a urine bolus through the terminal papillary collecting duct is seen to enter the renal pelvis quickly, after which urine flow ceases and collecting ducts become empty and remain collapsed for 95% of the time. When urine flow rates are slow, the velocity of a urine bolus is rapid, and the contact time between urine and epithelium at a site approximately 1 mm from the papillary tip is brief. At higher rates of urine flow, the urine bolus leaves the papillary tip more slowly, and contact time between urine and papillary epithelium is actually greater. Therefore, during antidiuresis, when urine flow is slow and urine becomes highly concentrated, the urine is in contact with the reabsorbing epithelium for a shorter time, and retrograde flow of pelvic urine, rich in urea, is an uncommon event.[35,40-42]

Schmidt-Nielsen and colleagues[35,40-42] proposed that during antidiuresis, urinary concentration is augmented by peristaltic action of the renal pelvis that causes intermittent flow of urine and helps urine equilibrate with the hypertonic papillary interstitium. The relatively small "tip" reflux in an antidiuretic state, coupled with the small area occupied by the papillary tip and the short contact time of urine with papillary epithelium, might lead one to conclude that solute and water exchange at this site are rather limited. More important, other papillary structures are also affected by the milking, contractile action of the renal pelvis. When the pelvis contracts and constricts the papilla, blood flow in the vasa rectae stops. This may reduce medullary solute washout by allowing more complete solute equilibration between ascending vasa rectae and interstitium. Flow in the loops of Henle is also slowed. This could help accumulate interstitial solute. Therefore, without full reflux, pelvic contraction may alter flow of solute in papillary structures to facilitate the increase in the interstitial solute gradient.[35,40-42]

Role of Aquaporins

Aquaporins are proteins that traverse the cell membrane lipid bilayer. They appear to represent molecular water-transporting channels and are expressed in a wide variety of tissues.[43-46] Several reviews are available.[47-55] The first water channel protein to be identified, originally named CHIP-28 for the 28-kDa channel-forming integral protein, was abundant in red blood cells and proximal renal tubules. These tissues have high water permeability and facilitate the constitutive movement of water down an osmotic gradient. To determine whether this protein behaved as a water channel, investigators employed *Xenopus* oocytes, which can survive in fresh water owing to their very low water permeability. When complementary RNA of CHIP-28 was expressed in these oocytes, a dramatic increase in membrane water permeability was observed. The membranes behaved as though they contained water channels. This observation was confirmed by reconstituting the purified protein into proteoliposomes and demonstrating a dramatic increase in osmotic water permeability.[55-57] Seven aquaporins now identified in kidney tissue appear to have specific cellular localization.[42] Some features of aquaporins 1 to 4 are summarized in Table 133-1. Little is known yet about aquaporins 6 to 8.

TABLE 133-1

Characteristics of Aquaporins Found in Kidney Tissue

Genetic Designation	Renal Localization	Proposed Physiologic Role
Aquaporin-1	Proximal tubule S1, S2, S3 segments of short- and long-looped nephrons	Major transmembrane pathway for H_2O flow, including entry and exit sites
	Descending thin limb of Henle in long-loop nephrons	Not under hormonal control
	Nonfenestrated descending vasa rectae	
	Limited in vesicles and vacuoles	
	Present in both apical and basolateral membranes (basal and lateral aspects)	
Aquaporin-2	Outer and inner medullary collecting duct	The vasopressin-regulated H_2O channel; ADH induces movement of an intracellular vesicle pool of H_2O channels to the apical membrane
	Primarily apical membrane and intracellular vesicles	
Aquaporin-3	Collecting duct principal cells in cortex and medulla; strongest medullary label is at base, not tip, of papilla	Major exit site for osmotically driven H_2O transport; not regulated through vesicular trafficking via ADH; may provide for small nonelectrolyte solute movement
	Mainly in lateral and basolateral infoldings, not in basal aspect, of basolateral plasma membrane	
	Virtually absent from apical membrane; minimally found in intracellular vesicles	
Aquaporin-4	Inner medullary collecting duct principal cells, especially proximal two thirds of segment; little in cortex and outer medulla	Basolateral exit site of cellular H_2O transport of inner medullary collecting ducts
	Distributed roughly equally in basal and lateral domains of basolateral plasma membrane, and not in intracellular vesicles	

ADH, Antidiuretic hormone.

Aquaporin-1 is found in renal proximal tubules, long-loop thin descending limbs of Henle, and nonfenestrated endothelium of descending vasa rectae. It is not found in other tubule segments, including thin descending limbs of short-looped nephrons, collecting ducts, or in ascending vasa rectae. Aquaporin-1 imparts high osmotic water permeability. In nephron segments, this protein is identified in both apical and basolateral membranes, including both basal and lateral infoldings, but not to any substantial degree in cytoplasmic vesicle and vacuole membranes. Accordingly, aquaporin-1 is poised to facilitate transcellular water movement in segments responsible for reabsorbing a major portion of the glomerular filtrate, but it does not appear to require hormonal regulation and therefore is not the ADH-sensitive water channel.[58-60] This protein is also abundant in red blood cells, where it is thought to increase membrane permeability. Although individuals without functional CHIP water channels do not appear to have clinical abnormalities, such as polyuria,[61] rare individuals lacking aquaporin-1 have a urinary concentrating defect in response to ADH or to water deprivation.[62] The role of aquaporin-1 in maintaining urinary concentrating ability is appreciated from studies of transgenic knockout mice lacking aquaporin-1. These mice are polyuric and do not tolerate water deprivation.[63] Moreover, perfused proximal tubules[64] as well as descending thin limbs[65] from such mice have reduced osmotic water permeability. Thus aquaporin-1 is necessary for maximal concentrating ability. This is likely related to the need for rapid equilibration of water across the thin descending limb of Henle in helping establish the countercurrent multiplication process.

As mentioned earlier, it has recently been shown that aquaporin-1 is not expressed in descending thin limbs of short-loop nephrons.[66] Water permeability across this segment appears to be lower than that of descending thin limbs of long-loop nephrons, probably because of the absence of aquaporin-1. Because osmotic equilibration across ascending thin limbs of short-loop nephrons occurs, likely some other feature facilitates fluid movement, perhaps an as yet unidentified aquaporin. Alternatively, there may be solute transfer across this segment associated with aquaporin independent water flow.

Aquaporin-2 appears to be the ADH-regulated water channel and mediates the short-term renal response to the hormone. This conclusion is supported by the following cumulative observations. Vasopressin binds to a basolateral membrane receptor and initiates a cyclic adenosine monophosphate (cAMP)-mediated chain of signaling events leading to insertion of water channels and increased osmotic water permeability of the collecting duct apical membrane.[67,68] Aquaporin-2 protein is present primarily in cytoplasmic vesicles and in apical plasma membranes of collecting duct principal cells. Also some staining occurs of basolateral plasma membranes of inner medullary collecting duct principal cells.[51] When expressed in *Xenopus* oocytes, aquaporin-2 induces a dramatic increase in osmotic water permeability.[69] In the water-loaded state, staining for this protein is more prominent in cytoplasmic vesicles. After stimulation by ADH, apical membrane staining for aquaporin-2 intensifies, but it decreases in the subapical vesicles (i.e., aquaporin-2 redistributes from vesicles to membrane).[70,71] That there is a reservoir of cellular aquaporin-2 protein capable of actually recycling between intracellular cytoplasmic vesicles and the plasma membrane is suggested from studies using LLC-PK1 epithelial cells.[72] Despite inhibition of protein synthesis, aquaporin-2 staining was primarily localized to intracellular vesicles in nonstimulated cells and quickly redistributed (within 10 minutes) to the plasma membrane after exposure to ADH. The pattern was reversible on removal of ADH.

There is also evidence that aquaporin-2 plays a major role in the long-term adaptation to pathophysiologic stimuli known to alter urinary concentrating ability. For example, lithium, a drug used in affective disorders, induces an ADH-resistant concentrating defect characterized by a down-regulation of aquaporin-2 expression in rat inner medullary membranes that coincides with the development of polyuria.[73,74] The ADH-resistant urinary concentrating defect occurring after release of bilaterally obstructed ureters is also associated with a marked down-regulation of inner medullary aquaporin-2 expression that correlates with polyuria. In addition, the slow recovery is marked by persistence of decreased aquaporin-2 expression.[75]

Chronic hypokalemia may lead to ADH-resistant nephrogenic diabetes insipidus. When hypokalemia was induced by potassium deprivation for 11 days, polyuria correlated with down-regulation of aquaporin-2 in both the cortex and inner medulla of rat kidneys. Polyuria expression of aquaporin-2 corrected within a week of potassium repletion.[75] Hypercalcemia is another electrolyte disorder in which a concentrating defect and polyuria have been associated with down-regulation of aquaporin-2.[76,77] Thirsting increases expression of aquaporin-2 in rat collecting ducts, and in Brattleboro rats, a model of central diabetes insipidus, expression of aquaporin-2 in collecting ducts is reduced in the basal state and is significantly increased upon exposure to ADH.[78,79] The decreased urinary concentrating ability associated with protein-depleted or malnourished subjects is thought to relate to a decrease in the deep medullary urea content. However, rats kept on a low-protein diet for 2 weeks, without malnutrition, demonstrate decreased maximal urine osmolality after water deprivation and reduction in ADH-stimulated osmotic water permeability, as well as expression of aquaporin-2 protein in terminal portions of the inner medullary collecting ducts.[80]

These examples of limitations in urinary concentrating ability associated with long-term alterations in aquaporin-2 are of particular interest considering the presence of aquaporins in fetal and neonatal renal tissues (see later discussion). Moreover, aquaporin-2 appears to be necessary for ADH-dependent concentration of urine in humans, as evidenced by a patient with autosomal recessive nephrogenic diabetes insipidus who had two mutations in the gene encoding aquaporin-2. Expression of the defective proteins in *Xenopus* oocytes showed nonfunctional water channel proteins that failed to increase osmotic water permeability.[81] Such abnormalities may become definable by analyzing urine from such patients.[82,83] Tsukahara and colleagues[84] reported urinary excretion of aquaporin-2 in premature and term infants. Levels were lower than in adults and correlated with urinary osmolality and ADH, suggesting that early in life (within the first 4 days) aquaporin channels contribute to urinary concentrating in the newborn infant.[84]

In some studies, microdissected arcade segments (branched tubule segments that connect distal convoluted tubules of deep and midcortical nephrons to cortical collecting ducts) were found to express both aquaporin-2 and the V2 ADH receptor protein. Staining of aquaporin-2 was not as intense as in cortical collecting ducts but nevertheless was present in large amounts and was up-regulated in segments dissected from animals that had been thirsted. This indicates that the tubule arcades represent another site of water reabsorption stimulated by water deprivation and probably regulated by ADH.[85] This finding is of interest because extensive water reabsorption in these segments, which are present in the renal cortex, will foster absorption of large amounts of water in the cortex. Water not reclaimed in the cortical vascular circulation but delivered to the deeper medulla might dilute the medullary interstitium and impair maximal concentrating ability.

Aquaporin-3 is expressed along the connecting tubule and entire length of the collecting duct (in principal cells) from the cortex and the outer and inner medulla, especially the base, rather than the tip, of the inner medulla.[86,87] The protein is found primarily in lateral and basal infoldings of the basolateral membrane, not the basal portion of the membrane. Labeling is virtually absent from the apical plasma membrane and is modest for intracellular cytoplasmic vesicles. Limited labeling of this protein

in cytoplasmic vesicles suggests aquaporin-3 does not regulate short-term H_2O movement by vesicular trafficking. However, both water restriction for 48 hours and chronic ADH infusion for 5 days in rats increase expression of aquaporin-3, suggesting that this protein is under long-term influence of ADH and therefore integrally involved in urinary concentration.[88] Moreover, experiments have shown that transgenic knockout mice lacking aquaporin-3 have a concentrating defect and polyuria.[89] Aquaporin-3 also appears to conduct small, nonelectrolyte solutes such as urea.[90]

Aquaporin-4 expression is found primarily in inner medullary collecting duct principal cells, not in the cortex or outer medulla. It is more prominent in the inner medullary base than in the papillary tip and is found in basolateral (both basal and lateral domains) rather than apical membranes. Little staining is found in intracellular vesicles. When rats were either water restricted for 48 hours or infused chronically during 5 days with ADH, no increase in expression of aquaporin-4 over a baseline water-loaded state was observed.[87,88] These findings are consistent with a role for aquaporin-4 as a basolateral exit pathway for transcellular movement of water in the inner medulla—perhaps in response to osmotic gradients, but not in response to secretion of ADH. The role of aquaporin-4 has been further assessed in transgenic knockout mice lacking aquaporin-4. Such mice had a mild urinary concentrating defect,[91] and their perfused inner medullary collecting ducts had reduced ADH-stimulated osmotic water permeability.[92]

Three other aquaporins have been identified in kidney tissue, but their physiologic roles are unclear.[51] Aquaporin-6 is expressed in collecting duct intercalated cells from cortex to inner medulla. The protein appears to be present in intracellular vesicles, not plasma membranes, and may represent an intracellular water and ion channel.[93] Aquaporin-7 is present in the proximal tubular brush border, particularly in the S3 segment,[51] and aquaporin-8 is found in proximal tubule and collecting duct cells as well as other tissues.[94]

Role of Chloride Channels

Rapid abstraction of NaCl down its concentration gradient occurs in medullary thin ascending limbs and is an integral part of the countercurrent multiplication process. This segment is particularly permeant to chloride. Chloride channels would be poised to facilitate transmembrane movement of solute and, in fact, such channels (e.g., ClC-K1) have been identified in the inner medullary thin ascending limbs of Henle in rat kidney. The channel was localized to both apical and basolateral membranes in one study[95] and primarily on the basolateral membrane in another.[96] The channel was not observed in the descending medullary thin limb, a segment that largely abstracts water, rather than NaCl. Although this channel is also found in other, more distal segments in both cortex and medulla, its location in the thin ascending limb underscores its role in concentrating and diluting the urine. In this regard, dehydration was shown to increase expression of the ClC-K1 channel in cortical and medullary segments. Moreover, knockout mice deleted of ClC-K1 showed clinical nephrogenic diabetes insipidus, a defect that was attributed to impaired generation of inner medullary hypertonicity rather than decreased collecting duct water permeability.[97,98] The role of chloride channels in urinary concentration as well as other ion channels along the nephron has recently been reviewed.[99,100]

To summarize, as a result of the countercurrent relationship of loops of Henle, vasa rectae, and proximal straight and collecting tubules, several cycles of solute transfer appear to take place more or less simultaneously. At all levels of the medulla (outer and inner stripes of the outer medulla as well as the inner medulla), but particularly in the inner stripe, in which the vessels located within the vascular bundle are separated only by a thin layer of interstitium, solute has the potential to leave ascending vasa rectae and enter descending vasa rectae. Some of this solute can be taken up by descending loops of Henle from short-loop nephrons that are also within the bundle. This solute can ascend to the cortex and then move to collecting tubules. When deep in the medulla, the solute can be transferred to ascending vasa rectae or to ascending loops of Henle and can then re-enter the descending loop of Henle to recycle in the medulla. In the outer stripe of the outer medulla, fluid in the ascending loop, as well as the ascending vasa recta, may also be able to enter the proximal straight tubules of short- and long-looped nephrons. These relationships provide an ongoing, if complex, transfer of solute between vessels and tubule segments to maintain sodium chloride and urea gradients in the medulla, allowing extraction of water from collecting ducts when there is need to concentrate the urine. Urine leaving the renal papillary collecting ducts enters the renal pelvis and may be able to circulate in the pelvic spaces to bathe portions of the outer medulla. Then is the potential for urea to recycle back to the medulla and contribute further to urinary concentration. Functional water channels appear to be amply present in both apical and basolateral membranes and are neatly poised to reclaim water by processes that are dependent on, as well as independent of, ADH.

URINARY CONCENTRATION IN THE FETUS

The placenta, not the kidneys, seems to maintain normal extracellular salt concentration in utero, and the practical need for the fetus to concentrate or dilute urine during pregnancy is little. However, kidneys may play a homeostatic role under conditions of fetal stress, and survival of premature infants 24 to 26 weeks of age must depend on kidneys deprived of 3 to 4 months of anatomic and functional in utero development. Such infants, fetal in some of their functions, require special attention to their fluid and electrolyte needs. Information on human fetal renal tubular reabsorption of water and response to ADH is limited. Most information comes from animals in which chronic catheters are inserted. Fetal metabolism, ontogeny, and ADH secretion are conveniently studied in the third trimester of sheep. This model is similar to humans because both placentas are impermeant to ADH, so that fetal blood ADH levels reflect fetal production.[101] To maximally concentrate urine, ADH must be synthesized, released to the circulation, and carried to collecting tubules, which must respond to the hormone. Loops of Henle, vasa rectae, and tubules must have established the necessary anatomic relationship. Fetal urine, in a variety of species, is usually hypotonic to plasma.[102-108] This observation led to the conclusion that fetal kidneys could not concentrate urine, perhaps because ADH is either unavailable or the fetus is unresponsive to it. That such is not the case is evident from the following considerations.

Maturation of Fetal Water Reabsorption

A slight increase in urinary concentration occurs during fetal development.[107,109] At a time when plasma solute concentrations are stable, fetal sheep late in gestation (130 days of a normal 145-day gestation) have a urine osmolality significantly higher than that in younger fetuses of less than 130 days. Both urea and nonurea urinary solutes are seen to increase, but whereas some investigators[110,111] find that intrarenal urea and salt gradients are present in fetal lamb medulla by midgestation (Figure 133-5), the actual contribution of urea to total urinary osmolality is relatively small (Figure 133-6). During late gestation, urine flow rates decrease, but osmolar clearance remains unchanged. Therefore free water clearance, defined as urine flow rate minus osmolar clearance, is reduced, indicating that free water is more effectively separated (reabsorbed) from solute in the older fetal kidney.[109] Although the degree of urinary concentration in late

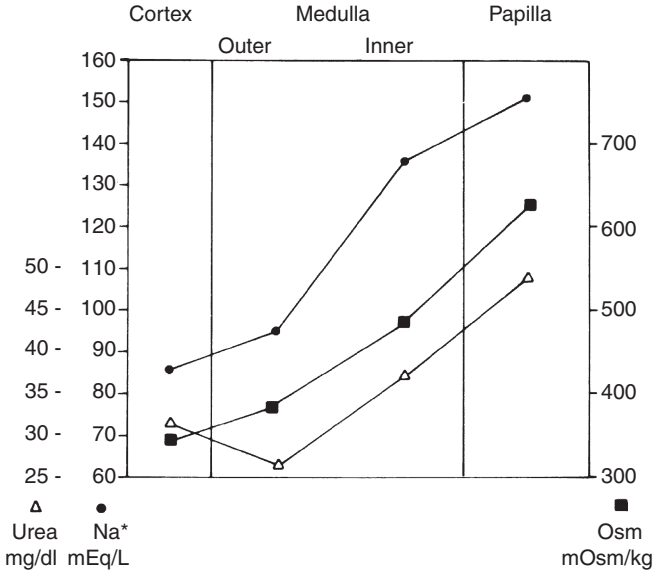


Figure 133-5 Intrarenal solute gradients in fetal lambs at midgestation. *(From Moore ES, Kaiser BA, Simpson EH, McMann BJ. Ontogeny of intrarenal solute gradients in fetal life, In Spitzer A [ed]:* The Kidney During Development: Morphology and Function. *New York, 1982, Masson, pp 223-231.)*

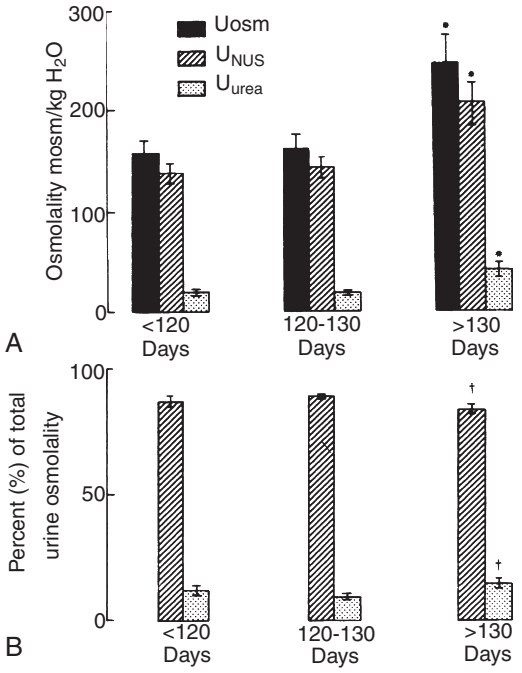


Figure 133-6 Comparison of fetal urine at different gestational ages in lambs. $^*p < .05$ and $^\dagger p < .02$ when fetuses 130 days old are compared with other groups. **A,** Urine composition and osmolality. **B,** Percentage of total urine osmolality contributed by urea and nonurea solute. U_{NUS}, Urine nonurea solute; *Uosm,* urine osmolality; *Uurea,* urine urea. *(From Robillard JE, Matson JR, Sessions C, Smith FG Jr: Developmental aspects of renal tubular reabsorption of water in the lamb fetus.* Pediatr Res *13:1172, 1979.)*

gestation is unimpressive, the increase in water reabsorption is consistent with a heightened response to endogenous levels of ADH in the older fetus.

Availability of and Responsiveness to Vasopressin

Although fetal pituitary content at term is much less than that of the adult, ADH has been identified in the human pituitary by the week 12 of gestation, with a demonstrable increase over ensuing weeks.[112-114] Neurosecretory granules and material are found in hypothalamic nuclei, the hypothalamohypophysial tract, and the infundibular process of the neurohypophysis by 16 weeks gestation in the human fetus.[115] Clearly, ADH is available at an early fetal age. Vasopressin mediates its tubular effect on water permeability by stimulating the generation of cAMP.[116-118] Compared with adults, fetal ADH causes a smaller increase in cAMP from renal medullary tissue obtained from the early fetus.[119-122] To achieve osmotic equilibrium (i.e., osmolality approximately 300 mOsm/kg H_2O), the fetal kidney needs higher plasma levels of ADH (approximately 5 μU/mL) than the adult, who requires only approximately 0.7 μU/mL. Moreover, maximal urine osmolality is achieved in both the fetus and adult at similar plasma levels of ADH, 4 to 6 μU/mL, but adult urine is much more concentrated.[109] Therefore the fetal nephron appears to be less sensitive than the adult to vasopressin.

Exogenous Vasopressin

The fetus responds to exogenous ADH by increasing urine osmolality,[123] a response linearly related to age over the third trimester of the fetal ewe[124,125] (Figure 133-7). The human fetal nephron responds to ADH as shown in an isolated, perfused human medullary collecting duct obtained from a 5.5-month male abortus with trisomy D.[126] This tubule increased transmembrane water flow from 2.1 to 12.0 μL/cm^2/Osm/minute on exposure to peritubular ADH. Cells swelled with conspicuous dilation of intercellular spaces, indicating outward net flow of water through intercellular spaces and through cell membranes. Thus collecting duct receptors to ADH are well developed functionally in prenatal life.

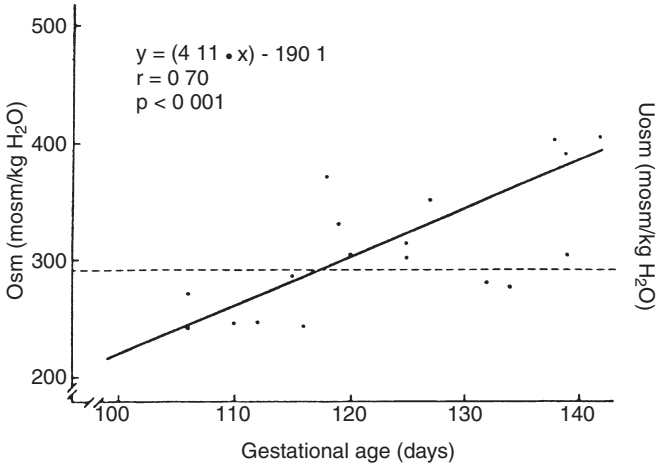


Figure 133-7 The relationship between gestational age and urine osmolality in fetal lambs during an infusion of vasopressin, 600 μU/min/kg. The *horizontal dotted line* represents mean plasma osmolality (291 mOsm/kg) during infusion of vasopressin. *(From Robillard JE, Weitzman RE: Developmental aspects of the fetal renal response to exogenous arginine vasopressin.* Am J Physiol *238:F407, 1980.)*

Endogenous Vasopressin

In addition to the availability of ADH and tubular reactivity to the hormone, the fetus responds to osmotic, nonosmotic, and volume stimuli (hypertonic saline, hypoxia, hemorrhage, furosemide, and dehydration) by a prompt increase in endogenous plasma levels of ADH.[127-132] Infusing hypertonic saline into

a fetus causes an increased level of plasma ADH that closely relates to plasma osmolality.[107,108,131] Fetal infusion of hypertonic saline increases fetal urine osmolality and decreases fetal urine flow rate. This response can be blocked by an ADH antagonist.[133] When water deprivation is imposed on a pregnant ewe, the older fetus, compared with the young fetus, has a higher plasma ADH level for a given plasma osmolality and, for a given plasma ADH level, the older fetus has a greater reduction in urinary water clearance.[127,129] When varying degrees of hemorrhage are induced in lamb fetuses during the third trimester of pregnancy, plasma ADH levels increase nearly 50-fold and correlate with the degree of blood removed.[134] In late gestation, more than 130 days, a hypoxic stimulus causes fetal plasma ADH levels to rise, which are accompanied by an increase in urine osmolality and a decrease in water clearance without change in osmolar clearance. This response occurs in nearly all (9 of 10) near-term fetuses of more than 130 days and approximately half (5 of 9) of young fetuses of less than 120 days.[109,129,135] Therefore fetal hypoxemia induces antidiuresis, particularly in a near-term fetus.

It appears then that in a fetus during the last trimester, volume, osmotic, and nonosmotic receptors for production and release of ADH are functional, ADH is available, and tissue response to the hormone is intact. Other factors may contribute more to limited concentrating ability. Loops of Henle are short,[136,137] and salt reabsorption in thick ascending limbs is limited in early life.[138] Total cortical flow exceeds that to the medulla, but blood flow/nephron is proportionately greater in the medulla than in the outer cortex in early development.[139-141] These factors tend to limit the efficiency with which a medullary osmotic gradient can be generated and maintained in fetal kidneys. Regardless of quantitative limitations on urinary concentration, a mature fetus or a premature infant is establishing means to tolerate stresses that may alter the osmolality or volume of its fluid environment.

Other Effects of Vasopressin

Vasopressin receptors are found in mesangial cells, aortic smooth muscle, liver, brain, and anterior pituitary. Thus ADH has other potential roles,[142] including (1) blood pressure control by effects on vasculature and baroreceptor reflexes, (2) platelet aggregation, (3) regulation of Factor VIII and von Willebrand factor, (4) release of adrenocorticotropic hormone, (5) glomerular mesangial cell contraction with some control of filtration, (6) synthesis of prostaglandins, and (7) control of/effects on hepatic metabolism by stimulating gluconeogenesis and exerting a glycogenolytic effect on hepatocytes. Some of the responses require high ADH concentration and may be more of pharmacologic interest.

Vasopressin may influence fetal water and electrolyte balance in ways separate from its effect on the kidney. Vasopressin appears to exert some control over fetal osmolar homeostasis through an effect on the placenta.[112] An osmolar gradient, generated by infusing hypertonic saline or mannitol into the mother,[112,129,143] favors transfer of fluid from fetus to mother, and water leaves the fetus to enter the maternal circulation. In the presence of hypertonic saline, neither sodium nor ADH appears to cross the placenta, but fetal serum sodium and plasma ADH increase and fetal ADH levels exceed those in the mother. Infusing hypertonic saline into the mother causes net water flux from the fetus to the maternal fluid compartment, inducing fetal water loss and increased fetal secretion of ADH. This effect is blunted if the fetus is given an ADH infusion. Similarly, mannitol infused into the mother does not cross the placenta but induces sufficient fetal water flow to raise fetal serum osmolality. Infusing ADH into the fetus blunts this response and may even drop fetal and slightly raise maternal osmolality. Therefore fetal ADH can induce transplacental water flow and cause a net gain of fetal water by inhibiting fetal to maternal water flow despite a hyperosmolar stimulus sufficient to move water from the fetus to the mother.

Vasopressin may help regulate the volume of amniotic fluid by its direct effect on urine flow and its regulatory effect on lung fluid production.[144] Vasopressin also affects fetal cardiovascular homeostasis.[145,146] Vasopressin infusion into a sheep fetus slows heart rate and raises blood pressure within 4 to 5 minutes. Vasopressin also causes redistribution of blood flow so that the proportion of cardiac output to gastrointestinal and peripheral circulation drops, and the percentage of cardiac output to umbilical-placental, cerebral, and myocardial circulations increases. These changes are similar to those following hypoxia and suggest that ADH release helps support the fetal cardiovascular response to stress. Moreover, fetal hemorrhage drops blood pressure, but ADH provides some protection; the fall in pressure is less, and the time for fetal blood pressure recovery toward normal after hemorrhage is shorter in the presence of the hormone.

Aquaporins

Aquaporin-1 expression was shown to increase dramatically (approximately seven-fold) from 60 to 140 days gestation in the ovine fetus, reaching adult levels by 6 weeks of postnatal age. This correlates with the maturational changes occurring in the kidney. Although nephrogenesis is complete by birth in the ovine fetus, there is a marked increase in postnatal glomerular filtration rate (GFR) and considerable tubular growth. This relates to the need for an increased capability for Na and water reabsorption and the need for functional and abundant water channels in portions of the nephron where a large amount of filtrate is absorbed.[147] Expression of aquaporin-2 is limited early in gestation in the ovine fetus. None was detected in 40-day-old or younger fetuses. It was present in low levels at 64 days, and expression increased from 80 through 140 days gestation and much more by adulthood. The increased expression of aquaporin-2 during gestation correlates with the heightened sensitivity of the older fetal kidney to ADH in obtaining a concentrated urine. It is emphasized that this process is physiologic. The purpose of fetal urine is mainly to provide an adequate volume of amniotic fluid. Maximal concentrating ability is not needed. ADH and aquaporin-2 expression are poised low enough to allow continued excretion of high volumes of dilute urine in the fetal kidney.[148]

In summary, fetal renal concentrating ability increases with gestation, but actual concentrating capacity is small, and the impact of ADH on urine concentration is relatively unimportant. Adult and immature subjects differ in tissue sensitivity to ADH, generation of cAMP, and tubule permeability to salt and urea. However, ADH is present early in gestation, nephrons respond to the hormone, and volume and osmotic responses for ADH release appear to function in the last trimester of pregnancy in the fetal lamb. Therefore the absence of or nonresponsiveness to ADH does not explain limited concentrating ability in the premature or term neonate. A more likely explanation is inadequate generation and maintenance of a hypertonic medulla. ADH under adverse circumstances helps alter electrolyte and water transfer across the placenta, maintain adequate blood pressure, and redistribute circulating blood volume, all of which would help stabilize fetal circulation. The response to exogenous ADH and to adverse stimuli for release of ADH both mature with advancing age. Therefore, although kidneys are not needed to maintain electrolyte balance in utero, fetal kidneys respond to hypoxemia, asphyxia, and volume depletion and probably contribute to electrolyte, volume, osmolar, and blood pressure homeostasis. In late gestation, amniotic fluid is regulated through fetal urine and lung fluid. Accordingly, fetal kidneys and ADH may be important for normal fetal and lung development.

TABLE 133-2

Average Maximal Urine Osmolality in 212 Children 3 Days to 18 Years of Age

Age	Maximal Urine Osmolality (mOsm/kg)
Third day	515
Sixth day	663
First month (10 - 30 days)	896
1 - 2 months	1054
End of first year	1118
Puberty (14 - 18 years)	1362

Data from Polacek E, Vocel J, Neugebauerova L, et al: The osmotic concentrating ability in healthy infants and children. *Arch Dis Child* 40:291, 1965.

URINARY CONCENTRATION IN THE NEONATE

In practical terms, low neonatal concentrating capacity is of little importance unless the infant is given insufficient water to compensate for dietary solute load and high rate of insensible water loss. A low concentrating ability makes the infant more vulnerable to extrarenal water depletion, as occurs in diarrhea, febrile states, and excessive insensible skin loss. Water needs of premature infants are even greater because of higher skin losses, but the higher water intake needed to offset these losses must be provided with care to prevent overhydration since the ability to excrete a water load is limited by a very low GFR until approximately 34 weeks gestation. In general, 100 to 160 mL/kg/day of water can be given to infants without major concern for dehydration or fluid overload.

Neonatal Urinary Concentrating Capacity

In the presence of ADH, human newborn infants produce a urine that is only modestly more concentrated than plasma and much less concentrated than that in the adult.[149-154] Svenningsen and Aronson[153] gave 10 μg of 1-deamino-(8-D-arginine)-vasopressin (DDAVP) to 20 healthy term and preterm infants (30 to 35 weeks gestational age) and found that by 4 to 6 weeks of age, their mean maximal urinary concentrating capacity was only 565 and 524 mOsm/kg, respectively, although some infants concentrated urine to greater than 600 mOsm/kg.[153] Pratt and Snyderman[152] found that premature infants, 11 to 23 days of age, ingesting evaporated milk and small amounts of water, concentrated their urines to a maximal osmolality of 637 to 985 mOsm/kg.[152] Calcagno and colleagues[149] found maximal urine osmolality to range from 588 to 648 mOsm/kg in five premature infants 5 to 25 days of age after 12 to 18 hours of water restriction.[149]

The dramatic maturation in concentrating ability is appreciated from data in a larger group of children reported nearly 40 years ago.[151] Urine osmolality exceeded 600 mOsm/kg within a week of life, was greater than 1000 mOsm/kg by 1 to 2 months, and was more than 1100 mOsm/kg by 1 year (Table 133-2). Children reached the adult level of maximal urine concentrating ability, 1300 to 1400 mOsm/kg by 2 years of age.[155] These values are comparable with the concentrating ability of a group of children 2 to 16 years of age reported by Edelmann and colleagues.[156] After nearly 20 hours of standardized water deprivation, these children concentrated urine to a mean osmolality of 1089 mOsm/kg (range, 873 to 1305 mOsm/kg), with little change occurring beyond 2 years of age. Clearly, with a urine osmolality of 1000 mOsm/kg, the 1- to 2-month-old infant is well protected from transient mild to moderate reductions in water intake. In fact, a nonhighly concentrated urine of 600 to 700 mOsm/kg is adequate for physiologic needs of the neonate and would even be adequate for an adult under most clinical circumstances. Physiologic and anatomic factors contributing to a low neonatal renal concentrating capacity are summarized in the next section.

Factors Limiting Concentrating Ability in the Neonate

Physiologic Considerations

Fluid Transport in Superficial Nephrons

Micropuncture studies reveal a maturation in water and solute transport in loops of Henle from superficial nephrons.[138,157-159] For example, in the rat,[159] the tubular-fluid-to-plasma inulin ratio of fluid distal to the loop (a measure of water reabsorption) increased from 13 through 39 days of age, a time when there was no change in the fraction of filtered water remaining at the end of the proximal tubule. Therefore the increase in tubular-fluid-to-plasma inulin ratio was due to increased water reabsorption along the loop. This increase in water reabsorption does not necessarily reflect greater water reabsorption in the thick ascending limb. It may relate to increased water reabsorption from the descending limb as this segment courses through the increasingly hypertonic outer medulla. In the final urine, inulin concentration increased by a factor of 5 during this time interval, indicating increased water reabsorption along the collecting ducts. In addition, the osmolality of the final urine increased from 700 to 1500 mOsm/kg. An increase in fractional reabsorption of sodium and chloride occurred along the loop. Furthermore, the potential diluting ability was also enhanced, as shown by a drop in sodium and chloride concentrations in early distal tubule fluid.

Ascending Thin Limb

Urinary concentration requires a countercurrent multiplication process allowing the medullary interstitium to become progressively hypertonic and the osmotic equilibration of medullary collecting duct fluid with the hypertonic medullary interstitium. To accomplish this, cells require a variety of channels and transporters. The ascending thin limb of Henle is highly permeable to chloride, likely related to its chloride channel ClC-K1. By facilitating efflux of NaCl to the interstitium, salt can osmotically induce movement of collecting duct water to the interstitium and increase the osmolality of collecting duct fluid. Failure to express this channel causes diabetes insipidus in the mouse.[97] Kobayashi and colleagues[160] related concentrating ability in the rat to expression of this channel. Maturation of the channel and the thin ascending limb correlated with an increase in concentrating ability. After dehydration, urine osmolality remained low until postnatal day 19. Concentrating ability then increased rapidly, with a marked increase by day 21.

The chloride channel was first noted near the papillary tip on day 5, increased by day 14, and was actually comparable with that of the adult by day 19. Its expression during development coincided with the increase in concentrating ability. Aquaporin-1 was present in inner medullary tubules by postnatal day 1, but its expression was weak. It increased to reach adult levels by day 14. Aquaporin-2 was present in the premature kidney throughout the inner medullary collecting duct, and its pattern did not change with maturation. Therefore water channels were present to allow for urinary concentration by water abstraction, and chloride channels matured to facilitate solute movement from the medullary ascending thin limb to interstitium to help establish and maintain a medullary gradient.

Fluid Transport in Collecting Tubules

The adult collecting tubule is a "tight" epithelium with high transepithelial electrical resistance and a low rate of reabsorbed sodium backfluxing to the lumen. Basal permeability to water is low, and the epithelial cells can maintain transepithelial osmotic and sodium gradients. By contrast, newborn (3 to 5 days of age) rabbit collecting tubules allow substantially more sodium backflux to the tubule lumen and have a lower transepithelial voltage and electrical resistance (Table 133-3). Therefore the newborn collecting tubule may be unable to maintain an adequate sodium (osmolar)

TABLE 133-3

Comparison of Baseline Characteristics of Newborn (3 - 5 Days) and Adult Rabbit Cortical Collecting Tubules

Characteristic	Neonate	Adult
Net sodium flux (pEq/mm/min)	−83.9 ± 21.3 (19)	−1.4 ± 25.3 (8)
Water absorption (nL/mm/min)	0.35 ± 0.13 (19)	0.12 ± 0.11 (8)
Transepithelial voltage (mV)	−0.1 ± 0.3 (32)	−2.8 ± 0.6 (10)
Transepithelial electrical resistance (Ω/cm^2)	83.2 ± 11.6 (10)	220.6 ± 55 (6)

Data from Reyes JL, Roch-Ramel F, Besseghir K: Net sodium and water movements in the newborn rabbit collecting tubule: lack of modifications by indomethacin. *Biol Neonate* 51:212, 1987.

Values expressed in mean ± SE; (*n* = number).

With bath to lumen sodium gradient (142.5 versus 100 mEq Na/L), adults maintained gradient, but neonates showed net luminal entry of Na; PD of newborn tubules is similar to zero; with perfusate hypotonic to bath, water reabsorption in adult tubules is similar to zero.

gradient and, consequently, be unable to build a driving force to abstract water efficiently from more distal parts of the nephron.[161]

Urea

It is curious that a urinary waste product performs a homeostatic function. The importance of urea to mammalian urinary concentration was recognized nearly 70 years ago when Gamble and colleagues[162] noted that adult rats fed urea excreted a more concentrated urine than rats fed a nonurea diet made equimolar by adding inorganic solutes. High-protein diets and urea loads increase urine concentration in the human adult.[163] Urea, a protein metabolic waste product, normally represents approximately 50% of the solute in maximally concentrated urine, but this is not the case in the neonate. Infants and adults have similar urinary content of nonurea (electrolyte) solute, but the urinary urea solute content of the infant is distinctly lower. Most of the increase in concentrating capacity occurs after birth and can be augmented by dietary alterations.[164,165]

Edelmann and colleagues[156] determined maximal urine concentrating ability in nine full-term and premature infants (7 to 39 days of age) who had been placed on diets varying in protein. Before a protein load, concentrating ability in infants was about half that of adults. When given a high-protein diet or a urea load, infants increased their maximal urine osmolality independent of fluid intake or urine output (Table 133-4). The low neonatal concentrating ability seemed to reflect the type of solute available to the renal medulla rather than an intrinsic problem with the newborn kidney. It was postulated that because the infant is in a highly anabolic state, concentrating ability was limited by the efficient use of protein, which would tend to minimize the amount of urea available to build up a medullary gradient. However, despite the high-protein diet, urine osmolality still did not reach adult levels, and the levels actually reached required an extremely high protein intake (i.e., 8 g/kg) and a very high blood urea nitrogen ranging to 45 mg/dL.

It is evident from other studies as well that urinary concentrating ability does not depend on diet alone.[166-169] Rats are weaned and accordingly change their diet during the third week of life when concentrating capacity is increasing sharply. Rane and colleagues[169] found that maximal concentrating ability increased during the third week both in rats who were normally weaned and in those who continued to nurse, indicating that the improvement in concentrating ability was independent of dietary composition or protein content (Figure 133-8). Also no concomitant change occurred in the serum level of ADH. Therefore the maturational effect is intrarenal. Rather than simply an insufficient urea content, the low level of neonatal urinary concentration is more likely related to some difference in the manner in which urea is handled by the kidney. Although the gradual increase in urea

TABLE 133-4

Effect of High Protein Intake on Maximal Urine Concentration

	Protein Intake (g/kg)	Fluid Intake (mL/kg)	BUN (mg/dL)	Urine Osmolality (mOsm/kg)
Group 1	8.75	115	29	931
	2.3	215	9	571
Group 2	8.4	172	26	844
	2.4	164	9	657

Data from Edelmann CM Jr, Barnett HL, Stark H, et al: A standardized test of renal concentrating capacity in children. *Am J Dis Child* 114:639, 1967.

Group 1 included four infants 13 to 38 days old, birth weight 1840 to 2800 g, first on high-protein-low-fluid intake, then on low-protein-high-fluid intake. Group 2 included five infants 7 to 32 days of age, birth weight 1980 to 3620 g, first on high-protein then low-protein diet without altering fluid intake.

BUN, Blood urea nitrogen.

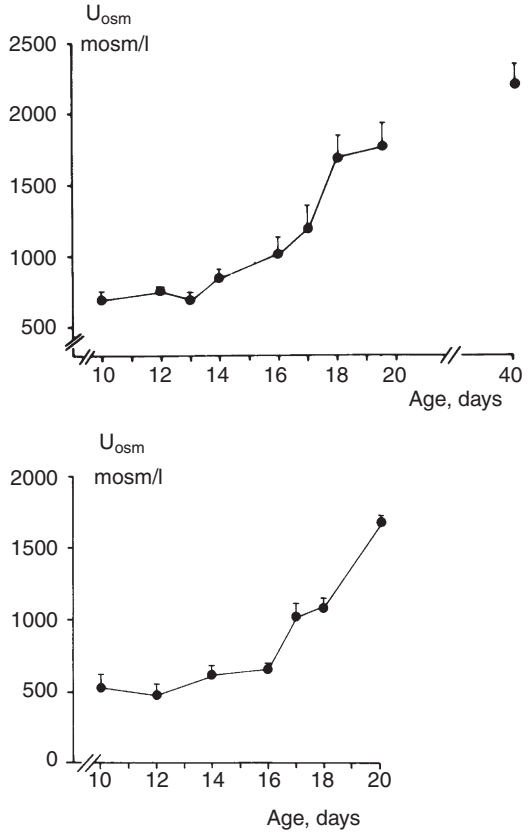


Figure 133-8 Effect of weaning on maturation of concentrating capacity (urine osmolality) after 24 hours of water and food deprivation in rats. Values are mean ± SE. *Top*, Normally weaned rats with free access to food. *Bottom*, Unweaned rats fed only breast milk. Urinary concentration was unaffected by weaning. *(From Rane S, Aperia A, Eneroth P, Lundin S: Development of urinary concentrating capacity in weaning rats.* Pediatr Res *19:472, 1985.)*

synthetic function that occurs during the neonatal period might contribute in part to the progressive increase in concentrating ability,[170] the search for other physiologic as well as anatomic limitations on neonatal concentrating ability has continued.

Response to Vasopressin

Vasopressin attaches to receptors on basolateral membranes of collecting tubule cells, but its permeability effect is at the apical membrane. In responsive cells, ADH-receptor interaction

TABLE 133-5

Concentrations of Vasopressin in Newborn Lambs

Condition	PLASMA VASOPRESSIN (μU/mL)	
	Baseline Values	Peak Values
Hypertonic saline (10 mEq/kg; n = 11)	2.9 ± 0.7	22.2 ± 9
Phlebotomy (10 mL/kg; n = 10)	1.9 ± 0.4	72.0 ± 40
Dehydration (18 hr; n = 9)	0.6 ± 0.1	4.8 ± 1.8
Water loading (100 mL/kg; n = 7)	3.4 ± 1.2	1.1 ± 0.3

Data from Jamison RL, Maffly RH: The urinary concentrating mechanism. *N Engl J Med* 295:1059, 1976.

Plasma vasopressin levels in newborn lambs (1 to 7 weeks) change appropriately with above stimuli. Values are mean ± SE; $p < .05$ for all peak versus baseline values.

TABLE 133-6

Postnatal Maturation of Vasopressin-Dependent Adenylate Cyclase in Rat Medullary Collecting Tubules

Age (days)	ΔcAMP Formed in Collecting Tubule (fmol/mm/30 min)
3 (n = 4)	203 ± 50 (28%)
28 (n = 3)	536 ± 56 (75%)
60 (adult; n = 6)	719 ± 68

From Imbert-Teboul M, Chabardès D, Clique A, et al: Ontogenesis of hormone-dependent adenylate cyclase in isolated rat nephron segments. *Am J Physiol* 247: F316, 1984.

Values (stimulated minus basal adenylate cyclase activity) are mean ± SE; response to 1 μM vasopressin of immature rat as percentage of the response of cyclic adenosine monophosphate (cAMP) formed in adult rat tubules given in parentheses. Note progressive sensitivity of tubule cAMP stimulation by vasopressin.

stimulates adenylate cyclase to form cAMP from adenosine triphosphate. The cAMP binds a protein kinase that catalyzes phosphorylation of apical membrane proteins. Cytoplasmic tubular structures are present in cells at rest. In the presence of ADH, these structures, containing small particles or aggregates, fuse with the apical membrane. The particles represent water channels (membrane proteins) that promote transmembrane osmotic water movement. This requires an intact cytoskeleton; disassembling microtubules interferes with the cellular response to ADH.[171,172] The newborn lamb responds to stimuli such as hypertonic saline, water loading, furosemide, dehydration, and blood loss[131,173] (Table 133-5), by appropriately elevating or suppressing ADH levels. The human newborn infant responds to hypertonic saline.[174] Human neonatal concentrating capacity remains limited even in the presence of elevated plasma ADH levels typical of the immediate newborn period in term and preterm infants.[112,175-178] Clearly lack of ADH does not explain a gross reduction in neonatal concentrating ability. Nonetheless, investigators still consider that the neonatal ADH-renal axis may be inadequate because, in the immediate newborn period of the rat, urinary osmolality is lower than papillary tip osmolality, and hormone levels in young animals are further increased (to levels comparable with that of older animals) in response to dehydration. Rather than an insufficient circulating level of ADH, the limited newborn concentrating ability may reflect decreased responsiveness to the hormone or a decreased number of ADH receptors.

Supporting this hypothesis, Schlondorff and colleagues,[121,122] studying isolated cortical collecting tubules of 10- to 12-day-old and adult rabbits, found that in the newborn rabbit, basal adenylate cyclase activity was considerably lower and stimulation of adenylate cyclase by ADH was much less than in the adult. The adult basal level of adenylate cyclase was 2.5 times that of the newborn rabbit. After stimulation with ADH, adult levels increased 10-fold in contrast to the 2.5-fold increase observed in newborn tubules.[121,122] In isolated rat collecting tubules, Imbert-Teboul and colleagues[119] reported a blunted ability of ADH to stimulate adenylate cyclase in the neonate[119] (Table 133-6). They found an initial increase in ADH-dependent adenylate cyclase during the first week of life, a plateau of activity from days 7 to 14, and a second period of increase in enzyme activity after day 14. Adult levels were reached by day 35.[119] However, the period of greatest increase in activity corresponded to a time when Edwards and colleagues[167] observed the sharpest increase in concentrating ability. In studies of the ontogenetic development of ADH receptors, Rajerison and colleagues[120] found a progressive increase in ADH stimulation of medullary adenylate cyclase in animals over a 2- to 46-day period with a concomitant, although slightly earlier, development of ADH binding capacity.[120,179,180] Vasopressin binding rapidly increased after birth so that the 3- to 4-week-old rat had a capacity to bind ADH to kidney medullopapillary membranes that was similar to that of the adult.[120,179,180] These findings suggest that the cAMP system or the binding properties or response to ADH are immature in the neonate.

In more recent studies, immature, isolated, perfused cortical collecting ducts were shown to have basal osmotic water permeability similar to mature tubules. However, the increase in water permeability after exposure to ADH was considerably less than that observed in adult tubules. The blunted response of immature tubules to ADH could not be abolished by exposing tubules to the stimulating effect of cAMP or to the inhibition of prostaglandin synthesis by indomethacin. The investigators concluded that in addition to any impairment in ADH-stimulated cAMP generation or antagonism of cAMP by prostaglandins, there is likely a more distal pathway disruption, perhaps at the level of the basolateral membrane.[181]

Sulyok[182] suggested that the response of a premature infant kidney to ADH may become blunted by the presence of hyponatremia, a state that may occur in the first few weeks of life. Premature infants with late hyponatremia show an *increase* in ADH excretion with age, even though their serum sodium levels and osmolality fall and their urine osmolality *diminishes* as urine flow rate increases. Renal salt wasting and hyponatremia may hinder establishment of an intrarenal salt gradient at a time when salt, rather than urea, would be the primary medullary solute. Although there might be a delay in maturation of concentrating ability, a blunted response to ADH could minimize the retention of excess water and may thereby serve to limit the severity of the hyponatremia. The late development of hyponatremia in premature infants can be prevented by administration of supplemental NaCl. Such infants have increased sodium excretion; nevertheless, they are in positive sodium balance and their ADH secretion is increased. The salt supplement appears to enhance ADH secretion with resultant retention of water, although in proportion to salt retention, because serum sodium remains relatively constant.[183]

Aquaporins

Aquaporin-1 is present in rat renal proximal tubules and thin descending limbs of Henle and is expressed in these tissues shortly before birth. However, the amount of renal aquaporin-1 messenger RNA is small in the kidney before and shortly after birth. Significant expression of aquaporin-1 does not occur until after birth.[184] Peak levels appear around 3 weeks of postnatal age. It is present in both apical and basolateral membranes in an equivalent staining intensity, suggesting that this protein appears simultaneously in both membranes.[185,186] Betamethasone stimulates a significant, modest induction of aquaporin-1 in neonatal rat kidneys 4 days of age. Adult rats showed no such response.[43] By contrast, aquaporin-1 was detected by immunohistochemical staining techniques in the human fetus at 14 weeks gestation in a newly developing proximal tubule, and, by 17 weeks, staining

was evident in both developing proximal tubules and in newly forming thin limbs from the outer cortex. By 24 weeks, staining was noted in thin limbs of the medulla. At 1 month of postnatal age, aquaporin-1 immunostaining was prominent over apical and basolateral membranes of proximal tubules and thin descending limbs of Henle.[187] This finding correlates with differences in concentrating ability; the human kidney concentrates urine at birth, and concentrating ability approaches that of the adult by 1 to 2 months (see earlier discussion), whereas the rat concentrates urine primarily at the time of weaning.[169]

In other studies, aquaporin-1 was observed as early as 12 weeks gestation in the human fetus, although not at 8 weeks, and was shown to increase steadily to reach 47% of adult levels at birth and 79% of adult levels by 15 months of postnatal age.[188] This coincides approximately to the age when urine concentrating ability reaches adult values (see Table 133-2). In this study, aquaporin-1 was initially localized to newly forming proximal tubular structures in the inner cortex. S-shaped bodies, glomeruli, and tubular structures of ureteric bud origin were not stained for aquaporin-1. By 15 to 20 weeks, proximal tubular structures in the cortex were better differentiated, and staining could be seen to localize mainly in the apical membrane of polarized epithelial cells to include proximal tubules and thin descending limbs of Henle. Basolateral staining, evident in proximal tubules and thin descending limbs in adult kidneys, was not clearly defined in the fetal kidneys, even at 24 weeks[188] (Figure 133-9).

As mentioned earlier, aquaporin-1 is poised to facilitate reabsorption of large amounts of water in proximal tubules and development of the countercurrent multiplication process by allowing rapid movement of water across thin descending limbs. Whereas osmotic water permeability of neonatal proximal tubules is higher than that of adults, osmotic permeability of brush-border membrane vesicles in neonates is lower. Quigley and colleagues[189] reported that in rabbit proximal tubular basolateral membrane vesicles, both expression of aquaporin-1 protein and osmotic water permeability were lower in neonates than in adults, although solute permeability (NaCl, $NaHCO_3$) was similar. They concluded that the higher neonatal transepithelial osmotic permeability in the neonate is not related to increased water movement across water channels.

Aquaporin-1 is also present in descending vasa rectae of adult kidneys. However, an interesting pattern of distribution of this protein is seen in the developing rat kidney.[190] Aquaporin-1 was found throughout the arterial vascular tree of fetal and neonatal kidneys from 17 days gestation to 7 days of postnatal age. During the next week expression regressed, becoming limited to descending vasa rectae. After about 3 weeks of age, aquaporin-1 also appeared in lymphatic vessels. This persisted in adults. The investigators suggest that the transient developmental expression may relate to fluid equilibrium in the developing kidney, or perhaps to a role in regulating fetal growth or branching of the vascular tree.

Aquaporin-2 protein and aquaporin-2 messenger RNA have been reported in rats to increase during 10 to 40 days of life and particularly over the weaning period from 15 to 20 days of life, a period of time when urinary concentrating ability dramatically increases. When rats were exposed to a single injection of betamethasone, known to accelerate maturation in other organs, an increase in renal medullary aquaporin-2 protein and messenger RNA as well as in urine osmolality occurred.[191] In studies of aquaporin expression during human nephrogenesis, aquaporin-2 was seen by week 12 of gestation, but not at 8 weeks. In contrast to aquaporin-1, aquaporin-2 was found only in structures derived from the ureteric bud. For example, in a 13-week fetal human kidney, aquaporin-2 stained in apical cell membranes in the collecting system and branching ureteric bud as it extended from the medulla toward the cortex. Comma- and S-shaped bodies, glomeruli, and proximal tubular structures did not stain for aquaporin-2, and this pattern continued throughout development. Therefore, it was evident from early in gestation that aquaporin-1 staining was limited to proximal segments and thin descending limbs, whereas aquaporin-2 was exclusively found in ureteric bud-derived cortical and medullary collecting duct segments[188] (see Figure 133-9).

Bonilla-Felix and Jiang[192] found that aquaporin-2 expression was reduced in immature rats compared with adults. The relation between aquaporin-2 expression and urinary osmolality on ad libitum fluid intake was close. However, whereas immature rats increased their aquaporin-2 content after stimulation with both dehydration and ADH, concentrating ability was not concurrent or equal. Immature rats did show an ability to translocate aquaporin-2 from intracellular vesicles (during water loading) to plasma membranes (during dehydration or treatment with ADH). Thus, although aquaporin-2 expression and trafficking were present in immature kidneys, concentrating ability was still limited.

Baum and colleagues[193] found expression of aquaporin-2 (apical membrane) and aquaporin-3 (basolateral membrane) in rat epithelial cells derived from ureteric bud and collecting ducts. Expression was detected as early as 16 to 18 days gestation. They also found expression of both aquaporins in comparable membranes of collecting ducts in human 3-day-old and adult kidneys. The rat kidneys showed little change in aquaporin expression over the first 3 days of life, but showed a dramatic (2.5-fold) increase in aquaporin-2 messenger RNA by 10 to 14 days of life. Given the presence of fetal and neonatal aquaporins, delay in maturation of maximal concentrating ability in neonates is not related to a simple lack of water channels. However, the importance of functional aquaporin-2 protein in neonates was illustrated in an aquaporin-2 knockin mouse model of recessive diabetes insipidus. Gene replacement caused a mutant protein leading to death within approximately 6 days of life without supplemental fluid.[194]

In one study, aquaporin-3 and aquaporin-4 expression was demonstrated in rat kidney basolateral membrane collecting duct cells after birth, but staining intensity was stable throughout life. By contrast, aquaporin-2 staining increased with age.[184] In another study, aquaporin-4 was detected early in the postnatal period of mice and rats, and there was a gradual increase in immunostaining during development.[195] Although the correlation between aquaporin expression and increasing concentrating ability during maturation may not be unequivocal, a general increase in aquaporin expression that occurs as concentrating ability matures is clearly seen.[196]

Prostaglandins

Prostaglandins, synthesized in renal medullary interstitial and collecting duct cells, are thought to regulate medullary functions such as blood flow, sodium chloride transport, and water reabsorption. Accordingly, prostaglandins could play a role in urinary concentration.[197,198] In experimental animals, prostaglandin E_2 (PGE_2) was found to inhibit sodium chloride transport in the medullary thick ascending limb.[199] PGE_2 antagonizes ADH-mediated water flow across the collecting tubule and bladder,[4] inhibits urea flux across toad bladder epithelium, and decreases urea reabsorption in the rat collecting tubule.[200,201] PGE_2 also can induce renal vasodilation and increase medullary blood flow.[53] These effects tend to wash out a medullary osmolar gradient and decrease maximal concentrating capacity. In fact, prostaglandins reduce the corticomedullary osmotic gradient and the medullary solute (salt and urea) content,[202] thereby reducing the driving force for water reabsorption. It has been suggested that increased prostaglandin synthesis in fetal and neonatal kidneys and blood vessels may interfere with neonatal concentrating ability.[203-205] However, the relationship between prostaglandin excretion and maturation of renal function is not firmly established,[206] and a role of prostaglandins becomes clouded when one recognizes that prostaglandin excretion increases with age. Benzoni and colleagues[207] noted that urinary excretion of PGE increased

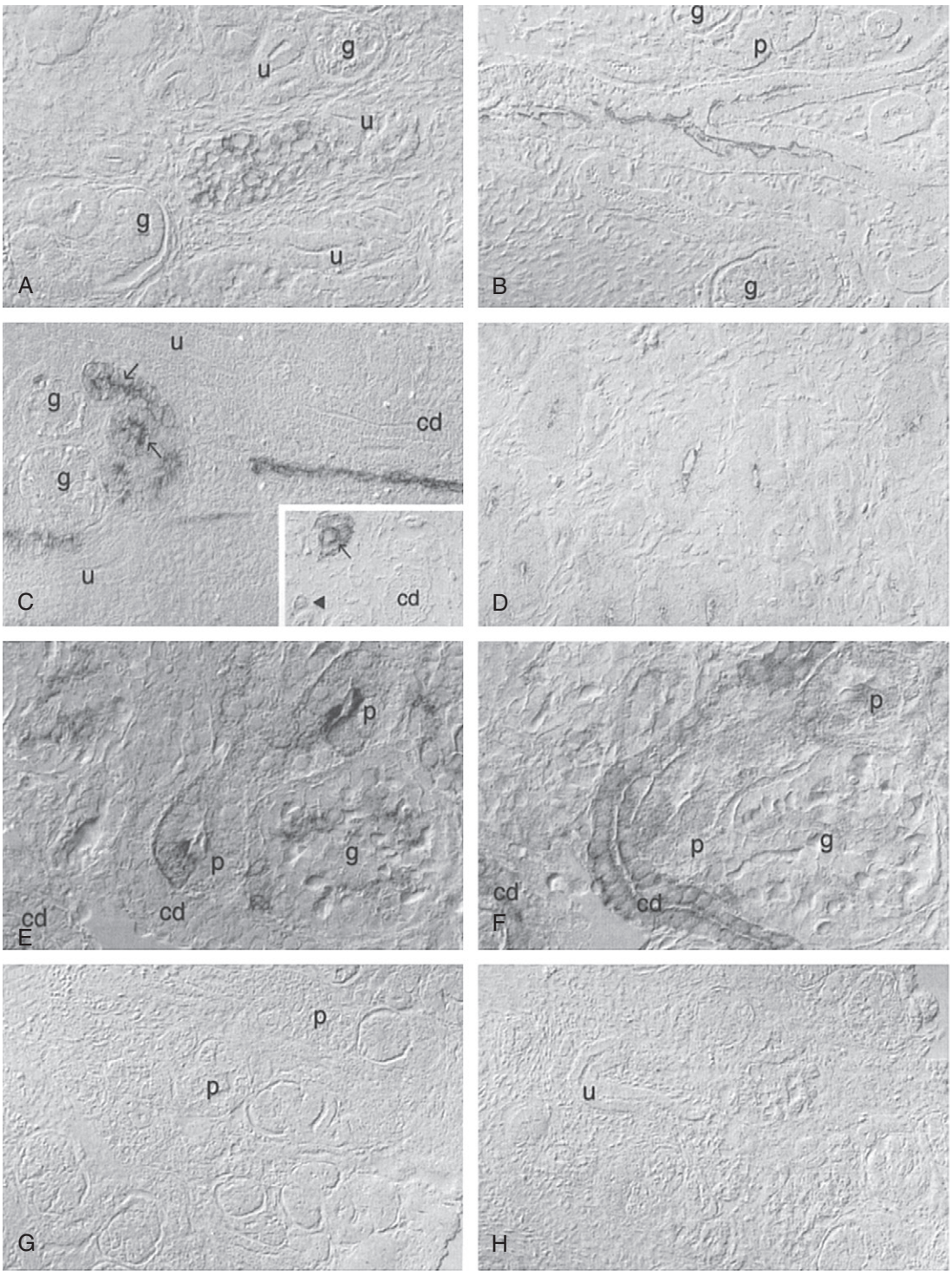


Figure 133-9 Immunohistochemical staining to localize aquaporin-1 and aquaporin-2 during human nephrogenesis. **A,** Positive staining for aquaporin-1 is restricted to a developing proximal tubule in the inner cortex of a 13-week human fetal kidney. Adjacent ureteric buds *(u)* and immature glomeruli *(g)* are unstained. No membrane polarity is localized. **B,** Staining for aquaporin-2 is positive in a branching ureteric bud and localized to the apical membrane in the inner cortex of a 13-week human fetal kidney. Glomeruli *(g)* and immature proximal tubules *(p)* are unstained. **C,** Positive staining for aquaporin-1 in a 15-week human fetal kidney. Staining is present for juxtaglomerular proximal tubules extending to the medulla, where an early descending thin limb of Henle is stained *(inset);* apical membranes are stained in proximal tubules *(arrows)* and thin descending limbs *(inset arrowhead).* Glomeruli *(g),* ureteric buds *(u),* and collecting ducts *(cd)* are unstained. **D,** Positive staining for aquaporin-2 is restricted to the apical membrane region of medullary collecting ducts in an 18-week human fetal kidney. **E** and **F**, Sections from the cortex of a 24-week human fetal kidney. In **E**, staining for aquaporin-1 is positive in the apical membrane of proximal tubule cells *(p).* Collecting ducts *(cd)* are unstained. In **F,** staining for aquaporin-2 is positive in collecting ducts *(cd)* and not in proximal tubule cells *(p).* Minimal glomerular *(g)* staining for aquaporin-1 and basolateral collecting duct staining for aquaporin-2 were nonspecific. **G,** Cortex of an 18-week human fetal kidney shows no aquaporin-1 staining in proximal tubules *(p)* when tissue is incubated with preimmune serum for aquaporin-1. **H**, Inner cortex of a 14-week human fetal kidney shows no ureteric bud *(u)* staining when tissue is incubated with preimmune serum for aquaporin-2. See reference 188 for details. *(From Devuyst O, Burrow CR, Smith BL, et al: Expression of aquaporins-1 and -2 during nephrogenesis and in autosomal dominant polycystic kidney disease.* Am J Physiol *271:F169, 1996.)*

during the first 24 months of life and correlated linearly with urine osmolality. Although this period is precisely when urinary concentrating ability shows the most dramatic degree of maturation, a clear cause and effect relationship was not established.

Perhaps any limiting effect of prostaglandins on the neonatal concentrating ability is largely masked by the relentless maturation of the many other factors that come to bear on the concentrating process. Nevertheless, studies using molecular biologic techniques are of interest. At least three different receptors for PGE_2 have been identified. These activate different intracellular signaling mechanisms, and the EP3 receptor, which is coupled to an inhibitory guanine nucleotide-binding G protein, inhibits generation of cAMP when stimulated by PGE_2. Immature collecting ducts have decreased generation of cAMP when stimulated by ADH. This response is mediated by prostaglandin, probably by activating the inhibitory G protein.[208] Receptor mRNA expression was found primarily in the distal nephron, (i.e., in medullary thick ascending limbs) as well as cortical and inner medullary collecting ducts. During development, rabbit kidney expression for the EP3 receptor mRNA increased to a maximum at 2 weeks of postnatal age and then decreased to reach adult levels by 8 to 10 weeks of postnatal age.[209] This finding lends credence to the idea that part of the blunted concentrating ability in maturation relates to increased expression of an inhibitory receptor stimulated by PGE_2 that blunts the ability of ADH to stimulate cAMP in collecting ducts.

Renin-Angiotensin System

It has become clear that the renin-angiotensin axis must be intact during nephrogenesis for normal development of urinary concentration. Profound adverse effects on adult renal function occur when the angiotensin-converting enzyme inhibitor enalapril or the angiotensin II type 1 receptor antagonist losartan is given early in neonatal life during nephrogenesis. For example, use of these agents in neonatal rats and piglets causes irreversible histologic changes, including chronic interstitial inflammation and fibrosis, renal vascular changes involving interlobular arteries, and, most particularly, profound papillary necrosis. Functionally, as adults, such animals show polyuria and a decreased urinary concentrating ability. The effect on other functions is variable. Proximal tubular fluid reabsorption is reduced, whereas glucose reabsorption, urinary acid excretion, acidifying ability, glomerular filtration, and renal blood flow are largely unaffected.[210-214] The most critical period for renal vulnerability for adverse effects appears to be the first 13 days of life in the rat, coinciding with the duration of continued postnatal nephrogenesis.[211] Of interest, histologic and functional abnormalities were normalized in enalapril-treated rats when they were concomitantly treated with insulin-like growth factor-I, another renal growth-promoting factor.[215] In functional studies, decreased urine concentrating ability was associated with a decrease in negative water clearance (T_cH_2O), medullary tissue osmolality, Na and urea concentration, and density of inner medullary aquaporin-2. Because density of aquaporin-2 is higher in the proximal one third than the more distal two thirds of the inner medullary collecting duct located mainly in the papilla, it would seem that the decreased concentrating ability is not wholly due to papillary atrophy.[213]

Anatomic Considerations

Loops of Henle of Deep Nephrons

Maximal concentrating ability directly correlates with the lengths of long loops of Henle as they descend into the medulla. Species able to develop highly concentrated urine tend to have both longer loops of Henle and a greater proportion of nephrons that send their loops deep into the medulla.[13] In the newborn infant, the length of loops and the renal papillae are relatively short, but they increase during maturation.[136] In the newborn rabbit, the inner medulla and papilla are underdeveloped, but during the first week of postnatal life the medulla becomes larger and the papillae become longer. Maximal concentrating ability in the rabbit increases from an osmolality of 600 mOsm/kg at birth to approximately 2000 mOsm/kg by 3 weeks of age. When osmotic solute gradients in the papilla are measured during development, sodium and urea concentrations are seen to increase gradually and roughly in parallel to approach adult levels by 6 weeks of age[155] (Table 133-7). Improved concentrating ability during development, therefore, might reflect the development of these anatomic parameters. However, Rane and colleagues,[169] evaluating the role of steroid hormones in the development of the concentrating mechanism in rats, observed an increase in urine osmolality and papillary tissue sodium and urea content from day 16 to day 20 that was temporally related to an increase in serum corticosterone. The increased concentrating ability was augmented by treating rats with exogenous glucocorticoids (betamethasone) and prevented by adrenalectomy. However, the length of the papilla (i.e., length of long loops of Henle) was not affected by adrenalectomy, indicating that the growth of long loops of deep nephrons contributed little to maturation of concentrating ability during this time. Perhaps glucocorticoids can modulate enzyme systems that help augment maturation of the hypertonic medullary gradient and the urinary concentrating mechanism.

TABLE 133-7

Comparison of Sodium and Urea Content in the Cortex and Papilla of Maturing Rabbits

	SODIUM (mEq/L TISSUE WATER)			UREA (mm/L TISSUE WATER)		
Age	Cortex	Papilla	P/C	Cortex	Papilla	P/C
Newborn (9)	61	137	2.2	24	115	4.8
5 days (3)	63	211	3.3	45	319	7.1
13 days (4)	57	345	6.1	25	345	13.8
28 days (3)	64	340	5.3	22	367	16.7
42 days (2)	68	477	7.0	23	488	26.6
Adult (1)	64	560	8.7	14	512	36.6

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Animals were food- and fluid-deprived and given intramuscular pitressin 18 hours before sacrifice. By 6 weeks, values approach those of the adult. Number in parentheses is number of animals.

P/C, Papilla/cortex ratio.

Loops of Henle of Short Nephrons

Long loops of juxtamedullary nephrons help determine the degree to which urine can be concentrated, but the shorter loops of superficial nephrons also play a pivotal role.[13,167] These nephrons allow accumulation of urea in the inner medulla by increasing the load and concentration of urea delivered to medullary collecting ducts and recycling urea to minimize its loss from the medulla. Were interstitial urea to enter long loops and vasa rectae and leave the medulla, the medullary urea gradient would become depleted. By entering descending limbs of superficial nephrons, urea can recycle to the medulla and maintain the gradient. In fact, the descending limbs of short loops of superficial nephrons, not long loops of deep nephrons, are the ones that accompany vascular bundles of the inner stripe of the outer medulla.

Edwards and colleagues[167] clarified the role of short-looped nephrons in maturation of the concentrating mechanism (Figure 133-10). In the rat, 40% to 50% of total solute in the adult papillary interstitium is urea. During nephrogenesis, superficial nephrons are the least mature and the last to develop. Their entrance or elongation into the deeper cortical and outer medullary portion of the kidney coincides with improvement in concentrating ability. During the first 16 days of life, the length of the

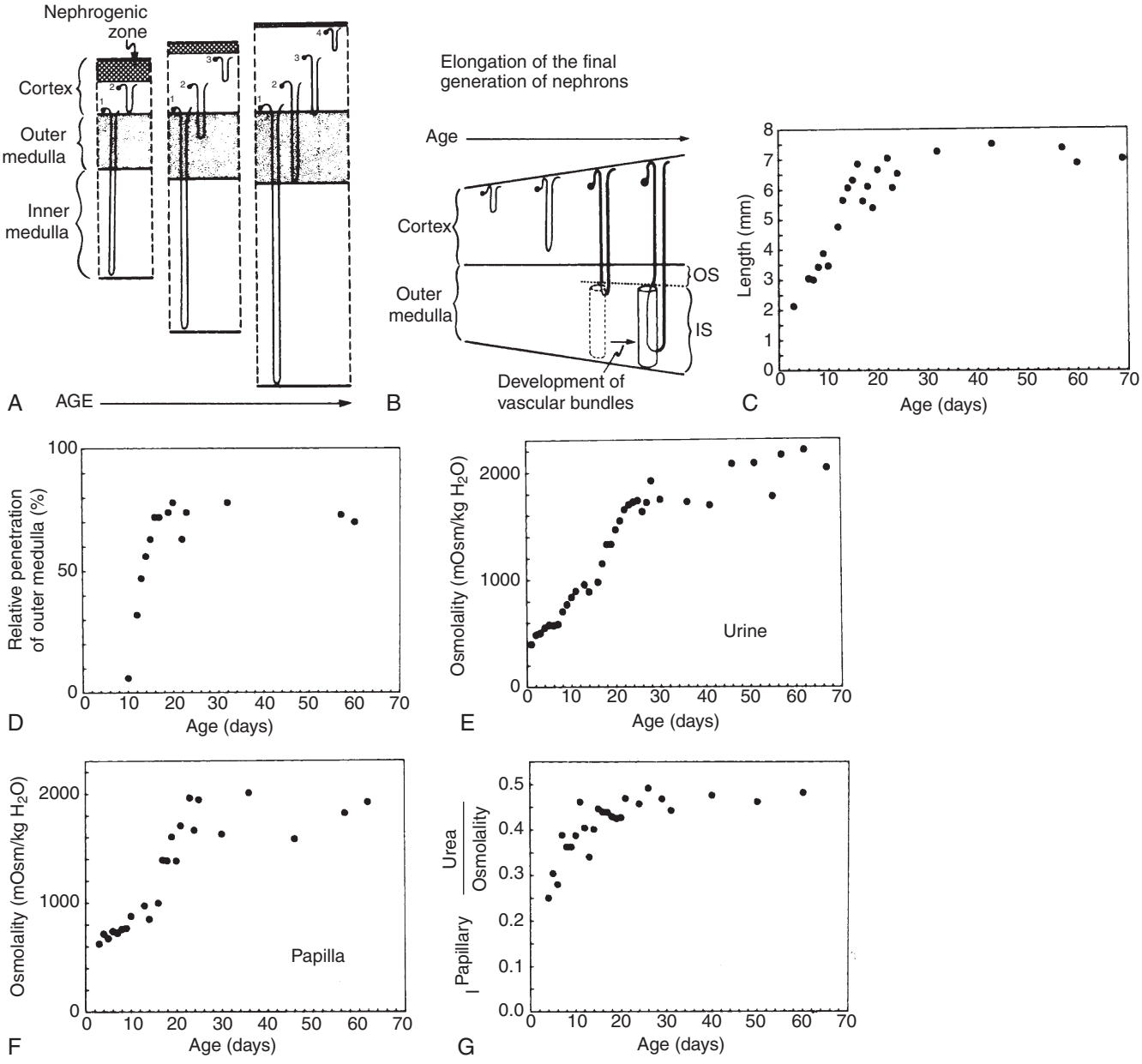


Figure 133-10 These figures depict anatomic maturation of superficial and deep nephrons, penetration of short-looped nephrons toward outer medulla, and maturation of concentrating ability. **A,** Simplified scheme of nephrogenesis in the rat. Numbers refer to successive generations of nephrons. **B,** Diagrammatic representation of elongation of the loop of Henle belonging to a surface nephron. In the rat, the vascular bundles are recognizable at 20 days of age but not at 10 days. The descending limbs of superficial loops become incorporated with these structures. *IS,* Inner stripe; *OS,* outer stripe;. **C,** Length of the corticopapillary gradient as a function of age in the rat ($N = 28$). Each point represents a single animal except for days 13, 15, and 18 (two rats each) and day 14 (three rats), for which averages are shown. **D**, Penetration of outer medulla by superficial loops of Henle. Each point represents a single animal except for day 13 (two rats) and day 14 (three rats). An average of three loops per rat was examined. **E,** Urinary osmolality, after 8 hours of dehydration, as a function of age in the rat ($N = 138$). Each point represents the average value for one to eight rats (mean rats). **F,** Osmolality of the papillary tip, after 8 hours of dehydration, as a function of age in the rat ($N = 56$). Each point represents the average value for one to five rats (mean = 2.3 rats). **G,** Fraction of total papillary osmolality contributed by urea as a function of age in 67 rats dehydrated for 18 hours. Length of corticopapillary gradient increases with age, particularly in the first 16 days, corresponding to time of most marked elongation of superficial loops. Urine and papillary tip osmolality increase primarily in the first 3.5 weeks and most steeply after approximately day 16. The proportion of urea composing papillary osmolality increases until adult proportions are reached by approximately 15 to 20 days. *(From Edwards BR, et al: In Spitzer A [ed]:* The Kidney During Development: Morphology and Function. *New York, Masson, 1982, pp 223-240.)*

TABLE 133-8

Urine Concentrating Capacity in Rats 10 to 20 Days of Age

	MAXIMAL URINARY OSMOLALITY (mOsm/kg)		URINE (UREA) (mm)		URINE/PLASMA OSMOLAR RATIO	
	10 day	20 day	10 day	20 day	10 day	20 day
Control	938 ± 28	1449 ± 71	469 ± 23	774 ± 49	3.3 ± 0.1	4.8 ± 0.2
Urea load	907 ± 29	1716 ± 59	482 ± 27	1049 ± 70	3.1 ± 0.1	5.5 ± 0.3

Modified from Trimble ME: Renal response to solute loading in infant rats: Relation to anatomical development. *Am J Physiol* 219:1089, 1970.

corticopapillary gradient increased 3.4-fold and the superficial nephrons elongated markedly so that they came to cross more than 70% of the outer medulla. During this time, urinary osmolality increased following a period of water deprivation. After maximal penetration of superficial loops to the outer medulla had occurred, urinary concentrating ability increased even more sharply, coincident with a marked increase in papillary tip osmolality and an increase in the proportion of the papillary solute composed of urea. It should be re-emphasized that the bulk of filtered fluid is reabsorbed in the cortex.This decreases the amount of fluid delivered to the collecting ducts.Therefore deeper tissues are not faced with the need to reabsorb so much water that the medullary solute gradient would be diluted and dissipated. The arcade segments in the renal cortex appear to play an important role in this regard (see later discussion).

The ability to use urea to enhance urinary concentration matures over the first 3 weeks of life in rabbits and rats.[216,217] For example, Trimble[217] found that an acute exogenous nondiuretic urea load given subcutaneously had no effect on urinary concentrating ability in rats 10 days of age, but it increased the medullary urea content and maximal concentrating ability in rats 20 days of age (Table 133-8). During this time, developmental changes occur in the length of the corticopapillary gradient and the length of superficial loops so that they penetrate well into the outer medulla. In addition, complex vascular bundles appear in the outer medulla, and the diluting capacity of superficial loops of Henle improves.

Zink and Horster[138] found that in young rats up to 15 days of age on a high-salt diet, fluid from the early distal tubule was isotonic with plasma and contained primarily NaCl as the solute. However, in rats 4 to 5 weeks of age, the early distal tubular fluid became hypotonic and contained a much smaller proportion of total solute as NaCl. Therefore the loop more efficiently reabsorbed NaCl without water. In other studies, Horster[157,158] reported a maturational increase in NaCl reabsorption in rabbit cortical thick ascending limbs. These factors help accumulate urea in the medulla. Edwards and colleagues,[167] in fact, found that the urea contribution to rat papillary osmolality increased sharply over the first 2 to 3 weeks of life, but thereafter further increases in papillary osmolality were associated with proportional increases in urea and nonurea solute.[167] Therefore urea accumulated in the papilla coincident with an increased activity of sodium absorption in the loop of Henle and a rearrangement of nephrons so that superficial nephron loops came to penetrate the outer medulla.

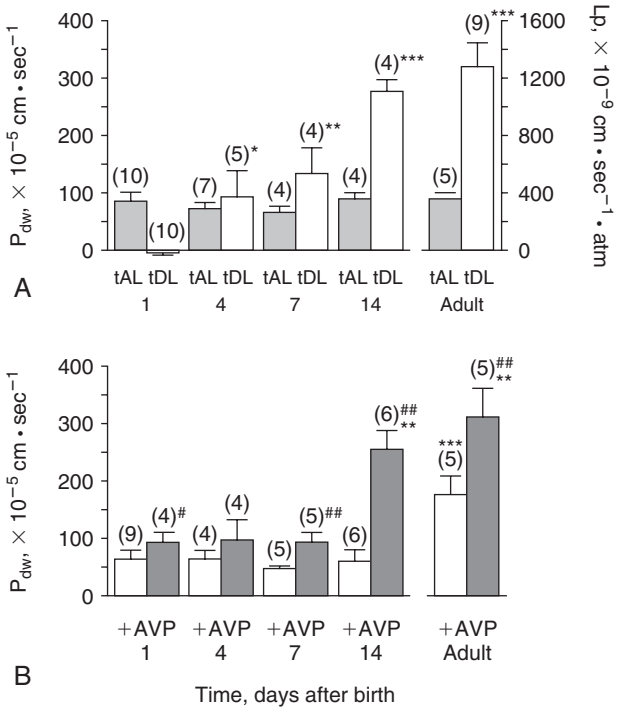


Figure 133-11 Maturational changes in water transport. **A,** Water permeability *(P_{dw})* was constant and low through adulthood in thin ascending limb *(tAL),* emphasizing its impermeability, and remained low in thin descending limb *(tDL)* until day 14, indicating low water permeability during the neonatal period. **B,** In the inner medullary collecting duct, basal water permeability *(clear bar)* remained low until adulthood. Vasopressin 1 nmol/L *(hatched bar)* stimulated water transport only modestly in the early neonatal period, but did not reach adult levels until day 14. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .0001$ versus day 1; $\#p < .05$; $\#\#p < .01$ versus baseline. Number in parentheses indicates number of tubules examined. *(From Liu W, Morimoto T, Kondo Y, et al: "Avian-type" renal medullary tubule organization causes immaturity of urine-concentrating ability in neonates.* Kidney Int *60:680, 2001.)*

Renal Inner Medullary Tubule Organization

Liu and colleagues[218] analyzed organization and function of inner medullary tubules during development in isolated perfused tubules from rat kidneys. In thin descending limbs, hydraulic water conductivity was absent on day 1, appeared by day 4, but remained low until day 14 of life. This segment was impermeable to water early in life. Diffusional water permeability of thin ascending limbs was low from day 1 to adulthood, emphasizing low water permeability of this segment. Basal water permeability remained low in inner medullary collecting ducts until adulthood. Water permeability was mildly enhanced by vasopressin in this segment early in life, but remained low and only reached adult levels by day 14 (Figure 133-11).Therefore little water reabsorption occurred from these segments early in life.

Urea permeability was low in thin descending limbs throughout development and adulthood. In thin ascending limbs, urea permeability was low, but reached two thirds of adult value by day 14. In the inner medullary collecting duct, basal urea permeability was negligible until day 7 and remained low into adulthood. Vasopressin induced little increase in urea permeability in this segment until day 14, although this value was still less than a third that of the adult (Figure 133-12). Expression of the urea transporter UT-A1 was not apparent until the late neonatal period. Therefore urea transport contributed little to neonatal urinary

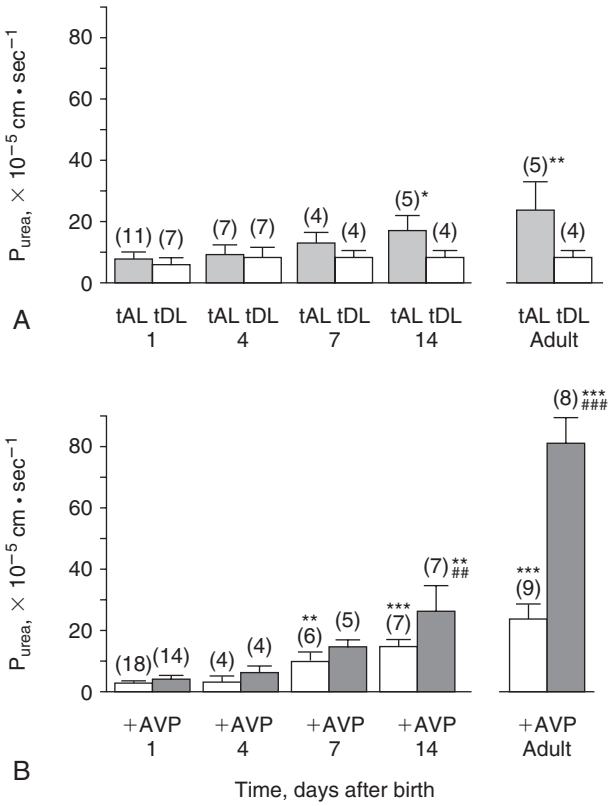


Figure 133-12 Maturational changes in urea permeability.
A, Urea permeability *(P_{urea})* was low through adulthood in thin descending limb *(tDL)* and gradually increased in thin ascending limb *(tAL),* approaching adult values only by day 14.
B, In the inner medullary collecting duct, basal urea permeability increased by day 7 and increased only mildly thereafter. Vasopressin *(AVP)* stimulated urea transport by day 14, but the value was considerably less than that of the adult. Number in parentheses indicates number of tubules examined. *$p < .05$; **$p < .01$; ***$p < .0001$ versus day 1; ##$p < .01$; ###$p < .0001$ versus baseline. *(From Liu W, Morimoto T, Kondo Y, et al: "Avian-type" renal medullary tubule organization causes immaturity of urine-concentrating ability in neonates.* Kidney Int *60:680, 2001.)*

concentration. The chloride channel ClC-K1 was expressed in thin ascending limbs from day 1 through adulthood, but it was functionally absent early in life because chloride permeability via this channel was not appreciable. The Na-K-Cl2 co-transporter CCC2 was prominent in the thin ascending limb early in life, similar to that in the thick ascending limb, but it regressed in the thin limb by adulthood to be replaced functionally by the chloride channel. In addition, early in life, transepithelial voltages in the thin ascending limb could be inhibited by bumetanide (inhibitor of the Na-K-Cl2 cotransporter in the thick ascending limb) and ouabain (inhibitor of the Na pump), and transepithelial voltages in the inner medullary collecting duct could be inhibited by amiloride (inhibitor of Na channel and active electrogenic Na reabsorption). These results indicate capability for active NaCl reabsorption in the thin ascending limb early in life, in contrast to its passive reabsorption in this segment in adults. Aquaporin-1 was observed by day 4 in thin descending limbs, persisting into adulthood, and aquaporin-2 was present throughout development. These findings, taken with others, indicate that water channels became poised to support water transfer once other medullary relationships are in place.

The investigators concluded that there is a fundamentally different organization of the inner medulla between neonates and adults (Figure 133-13). Transiently early in life, the inner medulla is characterized by active reabsorption and accumulation of NaCl. Compared with that of the adult, the thin descending limb is much less permeant to water. Because the thin ascending limb remains impermeant to water, the neonatal inner medulla is poised to reabsorb NaCl actively without water transfer, allowing urinary dilution early in life. At the same time, the inner medullary collecting duct is not geared to recycle urea. Subsequently, tubular transport properties appear that include increased thin descending limb water permeability, passive NaCl reabsorption in the thin ascending limb with functional maturation of a chloride channel, and increased urea permeability and responsiveness to vasopressin. Efficient urinary concentration requires that these properties be in place.

Renal Blood Flow

In the adult kidney, 90% of blood flows through the cortex only, 10% perfuses the outer medulla, and 1% to 2% reaches the inner medulla and papilla. This arrangement helps preserve the osmotic gradient established by the countercurrent mechanism. Rapid flow through the medulla tends to dissipate it. Neonatal renal blood flow is proportionately greater to deeper medullary regions of the kidney, and glomerular blood flow is greater in deep than in superficial nephrons.[139-141] After birth, renal vascular resistance drops more than resistance in systemic vessels, cardiac output and surface area increase briskly, and renal blood flow rises. Increases in single-nephron blood flow are proportionally greater in outer than in deep cortical nephrons. A relatively greater medullary flow in neonatal kidneys may delay establishing the corticomedullary gradient.

Renal Pelvis

The renal pelvis in the newborn animal is relatively narrow and does not extend too far into the body of the kidney. As the animal matures, secondary fornices and outpouchings of the renal pelvis come to extend well toward the renal cortex (Figure 133-14) so that urine in the pelvis will bathe a greater part of the papilla.[155,217] As mentioned previously, this pelvic maturation may facilitate recycling of urea from pelvic urine back to medullary interstitium to further increase both interstitial solute concentration and final urine osmolality. It is of interest that ADH appears to have some impact on the growth of the renal medulla and renal pelvis. In young growing lambs, treatment with ADH (DDAVP) for 13 weeks starting at 2 weeks of life induced an increase in the size of the renal medulla, especially the outer medulla, and the surface area of the renal pelvis. Ruminants have particularly well-developed renal pelvic fornices in their outer medulla. This is the area where dimensions were most impressively enlarged by vasopressin. Therefore the outer medulla may play a particularly important role in the reabsorption and recycling of urea in these animals.[219] Nevertheless, the role of this whole process in maturation of the urinary concentrating mechanism remains speculative at this point.

In summary, the low urinary concentrating capacity of newborn mammals is related to a variety of anatomic and functional factors. The loops of Henle are relatively short, the transport properties of the thick ascending limb of Henle are immature, and the number of loops of Henle from more superficial nephrons that penetrate deeper into the cortex and outer medulla is insufficient. Renal blood flow to deeper juxtamedullary nephrons is proportionally greater in the newborn than in the adult. Enhanced prostaglandin production has the potential to reduce salt, water, and urea transport at critical sites and to increase medullary blood flow. These factors may all contribute to the smaller medullary gradient and the lower medullary urea content found in the newborn. Neonatal collecting duct cells have decreased responsiveness to ADH and limited ability to maintain a transepithelial sodium (osmolar) gradient. Pelvic spaces surrounding

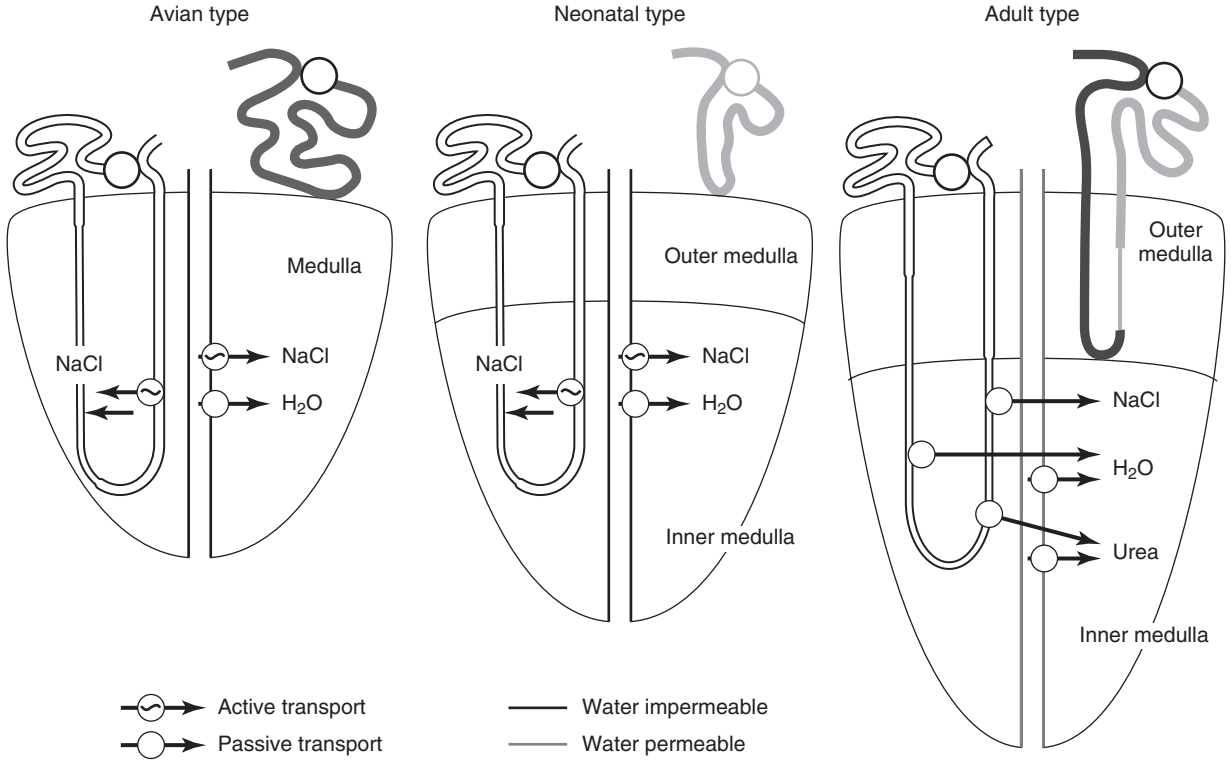


Figure 133-13 Comparison of neonatal and adult renal medullary tubule organization. In the neonatal kidney, only long-looped nephrons penetrate the medulla, and the medulla has essentially no water movement in loops of Henle. Medullary NaCl accumulates by active transport in the thin ascending limb, and there is amiloride-sensitive Na reabsorption in the inner medullary collecting duct. During maturation, cortical loops come to penetrate the outer medulla. In addition, NaCl reabsorption becomes passive in thin ascending limb, collecting duct amiloride-sensitive Na reabsorption disappears, the thin descending limb becomes water permeant, and urea permeability in the inner medullary collecting duct becomes highly sensitive to vasopressin. Neonatal tubule organization is likened to the avian kidney. Delayed maturation of neonatal concentrating ability is related to a more "primitive" tubule organization early in life. *(From Liu W, Morimoto T, Kondo Y, et al: "Avian-type" renal medullary tubule organization causes immaturity of urine-concentrating ability in neonates.* Kidney Int *60:680, 2001.)*

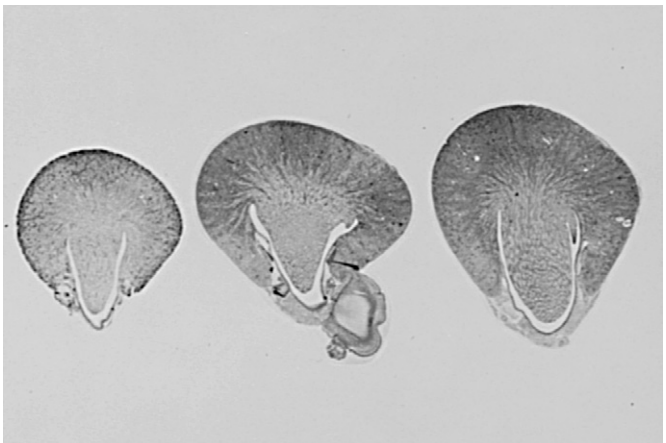


Figure 133-14 Cross-section showing gross structure of kidney and renal pelvis. In a 3-day-old rat *(left),* specialized fornices, evaginations of pelvic space, are absent and the pelvis is of the simple type. In a 10-day-old rat *(center),* specialized fornices are just beginning to appear, and by 20 days of age *(right),* fornices are well developed and extend into kidney tissue between the cortex and outer zone (× 7). *(From Trimble ME: Renal response to solute loading in infant rats: Relation to anatomical development.* Am J Physiol *219:1089, 1970.)*

papillae are narrower and more extensive in adults. This latter anatomic difference may contribute to a low concentrating ability during maturation because urine bathing the papillae may enhance urine and papillary osmolality. Aquaporin-1 may also limit neonatal concentrating ability because the amount of this protein is decreased in the human kidney at birth and increases gradually to approach adult levels at a time when the young kidney concentrates urine nearly as well as the adult. Less likely to play a role is aquaporin-2 because this protein is not only present in utero at 20 weeks gestation but also must be functional as evidenced by the response of the isolated human fetal medullary collecting tubule of similar gestational age to vasopressin.

PHYSIOLOGY OF THE URINARY DILUTING MECHANISM

Urinary dilution is thought to have developed when our primitive ancestors left the briny seas for more freshwater habitats. In so doing, they were forced to evolve mechanisms that could separate water from solute to preserve needed solute, eliminate imbibed excess water, and avoid fatal dilution of body fluids. It was considerably later in evolution when creatures had to develop a renal concentrating system to retain water as they traveled from one water locale to another.[220] Diluting capacity is considerable in the human adult, who can excrete 10% to 12% of the filtered load, or approximately 14 to 17 liters of water/day if the GFR is 100 mL/minute. More water can be excreted with higher rates of filtration. In terms of absolute volume, this

capacity is considerably more limited in the infant, although the young subject can excrete 15%, and perhaps more, of the filtered load.

Our ability to dilute urine and remove excess water is more efficient than our ability to concentrate urine and retain needed water. For example, suppose a 70-kg subject has a serum osmolality of 300 mOsm/kg and a daily renal solute excretory load of 600 mOsm. If urine osmolality is 300 mOsm/kg and the 600 mOsm solute load is excreted in 2 liters of water, the subject is in water balance because the urine is isotonic to plasma and the kidney has neither to concentrate nor dilute the urine to maintain a normal serum sodium level. If the subject is water deprived, urine osmolality can increase to a maximum of approximately 1200 mOsm/kg. At this urine concentration, 500 mL of water is needed to excrete the 600-mOsm solute load. Stated another way, of the 2 liters of water/day needed to excrete this solute isotonically, a maximum of approximately 1500 mL of water/day can be retained to maintain normal serum osmolality; however, 600 mOsm cannot be concentrated in a volume of less than 500 mL. If the subject is water loaded, excess water can be excreted by reducing urine osmolality to a minimum of approximately 50 mOsm/kg. In this case, the kidney can excrete the 600 mOsm of solute in a urine volume of 12 liters and remove 10 liters of "pure" water in the process to maintain a normal serum sodium level.

In this example, renal diluting capacity (10 liters water) is nearly seven-fold the concentrating capacity (1.5 liters water) in terms of actual volume of water handled to maintain osmolar balance. This translates to a 24-fold increase in urine osmolality and urine volume when the urine changes from maximally dilute (osmolality, 50 mOsm/kg; volume, 12,000 mL) to maximally concentrated (osmolality, 1200 mOsm/kg; volume, 500 mL). Renal excretory solute loads in children usually range from 10 to 40 mOsm/100 calories expended. Urine volume required to excrete this solute varies with diet and availability of water.[221] Ingested water enters the total body water compartment where it is partly incorporated into new cells during growth. Extra water is eliminated by insensible losses and urine output, the latter allowing removal of the renal excretory solute load. A net excess or deficit of water leads to alterations in electrolyte balance.

When water intake is sufficient to lower serum osmolality by 1% (approximately 3 mOsm/kg), ADH secretion is suppressed. Solute reabsorption continues in the thick ascending limb, distal nephron, collecting tubule, and collecting duct. The latter segments become less permeable to water when ADH is absent. As a consequence, fluid traversing the distal nephron and entering the medulla becomes progressively dilute. Because water remains in the tubule lumen, urea concentration is reduced, and a major urea concentration difference between collecting duct fluid and interstitium will fail to develop. Because urea permeability in the medullary collecting duct is also decreased in the absence of ADH, urea entering this segment will be more easily excreted. The overall effect is that urea tends to be eliminated from, rather than recycled in, the medullary system. Interstitial as well as urinary urea concentrations decrease, whereas total urea excretion actually increases, but in a larger volume of water.

Reduced tubule permeability to water and continued reabsorption of solute in more distal parts of the nephron might be expected to increase interstitial solute concentrations. However, interstitial solute concentrations are actually reduced compared with the antidiuretic state. It is likely that the water gradient from lumen to interstitium at the medullary collecting duct is high enough to overcome the low water permeability induced by lack of ADH. As a result, reabsorption of water in medullary collecting ducts is actually greater, not less, during water diuresis. Perhaps as much as half the water delivered to collecting ducts backdiffuses into the interstitium, but NaCl is sufficiently reabsorbed along this segment so that the urine actually becomes more dilute.[4,222]

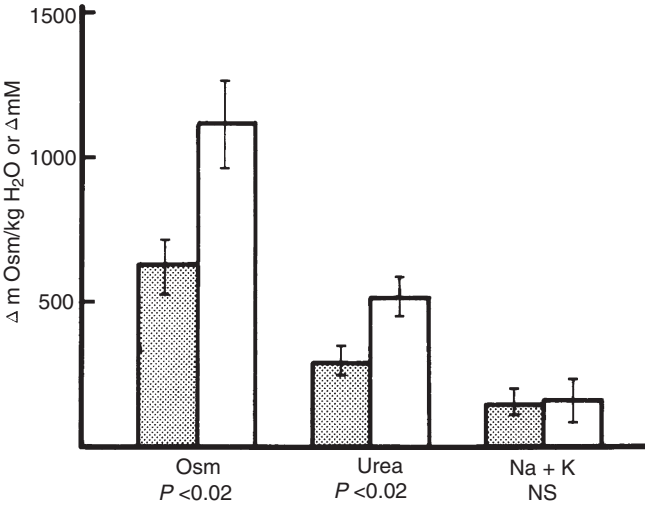


Figure 133-15 Effect of full pelvic refluxes during rising urine flow on solute concentrations in hamsters. Urine flow was increased to a rate just below that needed to cause full pelvic reflux. Refluxes were then mechanically induced for 20 minutes in experimental kidney *(filled bar)*. The other kidney served as a control. Values (mean ± SE) are difference in osmolality (mOsm/kg H_2O), urea (mM), and Na + K (mM) concentrations between renal papilla and refluxing or nonrefluxing kidney. *(From Schmidt-Nielsen B: The renal pelvis.* Kidney Int *31:621, 1987.)*

Another factor is of potential importance to urinary dilution and water excretion.[35] The renal pelvis has contractile properties and can induce reflux of urine around the renal papilla and medulla. Full retrograde pelvic reflux is not typical of the antidiuretic state, but exposure of medullary structures to urine occurs much more often with the high flow rates associated with dilute urine. Urine refluxing into the fornices may actually reduce the amount of urea in the renal medulla (Figure 133-15). Accordingly, full pelvic reflux may help eliminate a fluid load. Investigators have noted a lack of clear correlation between concentrating ability and size and complexity of pelvic extensions, but a correlation exists between the size of pelvic extensions and the need to excrete a water load. Animals living in arid areas and requiring no drinking water exhibit a high urinary concentrating ability but have difficulty excreting a water load rapidly enough to avoid water intoxication and possible death. Such animals do not have large pelvic fornices. Animals that tolerate dehydration but periodically need water must be able to eliminate water reasonably rapidly and tend to have large pelvic extensions. Experimental evidence shows that during antidiuresis water exchanges across the pelvic epithelium and hypotonic fluid can be added to pelvic urine from the medulla to enhance papillary osmolality.[223] Although it has not been directly studied, one might similarly predict that during water diuresis, water from dilute urine could enter the medulla from pelvic extensions and dilute the medullary interstitium, facilitating excretion of water.

Fluid along the proximal tubule is reabsorbed isotonically and does not directly add to urinary dilution. However, the amount of fluid reabsorbed at this site determines, to a major extent, the volume of dilute urine that can later be formed. A drop in filtration rate or an increase in proximal tubular reabsorption allows less fluid to arrive at the diluting site and therefore will limit the actual amount of water excretion. Some segment must be capable of reabsorbing solute in excess of water to allow luminal fluid to become dilute. This process occurs along the entire length of the ascending limb of Henle, especially along its thick portion. Any problem in solute reabsorption along this site will effectively prevent excretion of dilute urine. After urine passes

into the distal nephron, in which solute reabsorption continues, water must remain largely in the lumen. Vasopressin determines the degree to which water will be reabsorbed because the water permeability of collecting duct epithelium depends on the presence of this hormone.

It becomes apparent, then, that to dilute urine, three fundamental conditions must be met. First, a sufficient amount of filtrate must escape reabsorption along the proximal tubule and reach the diluting segment (ascending loop of Henle) to deliver a sufficient amount of potential pure water. Second, this segment must be able to reclaim NaCl and separate reabsorbed solute from water so that urine becomes dilute (water stays in the lumen for excretion). Third, as solute continues to be reabsorbed from more distal sites, further diluting the urine, the ADH-sensitive nephron segments must be able to eliminate the excess water (these segments must not be exposed to secreted ADH and thereby forced to reclaim free water). Accordingly, urinary dilution results from the active reabsorption of NaCl along the thick ascending limb of Henle and the continued reabsorption of solute along more distal nephron segments in concert with a disproportionate reduction in reabsorption of water. Absence of any of these factors may lead to a defect in urinary dilution.

Urinary Dilution in the Fetus

The human kidney can dilute urine early in fetal life.[155] Urine voided at birth (i.e., formed in utero) is hypotonic compared with the infant's serum. In contrast, maternal urine osmolality is five-fold more concentrated than that of the infant. Urine voided during the first 24 hours after birth becomes more concentrated than serum but still has an osmolality less than half that of the mother's urine.[224] Therefore the fetal kidney behaves as though it is trying to excrete a water load, whereas the newborn kidney seems to be stimulated to retain water. Amniotic fluid is hypotonic, particularly toward the end of pregnancy. A term fetus ingests substantial amounts of dilute amniotic fluid (approximately 5 mL/kg/hour).[225] This value is similar to the estimated urine flow rate of 5 mL/kg/hour in primates[104] and is close to the amount of breast milk ingested per day by a healthy newborn infant. A need to excrete this fluid load would account for dilution of fetal urine. Because infants often are not given oral fluid immediately after birth, subsequent concentration of urine represents another appropriate response.[155]

Urinary Dilution in the Neonate

One might expect that limitations in concentrating ability would enable an infant to excrete water more easily and protect itself from water intoxication. However, despite effective urinary dilution, limitations to water excretion exist early in life.[226,227] Low GFR limits the volume of water that can be excreted. A reduced ability to separate solute from water along the immature ascending limb may further limit the amount of free water cleared from this segment,[138,157,158] but this factor is probably of limited importance beyond a few weeks of age. In fact, urine from infants can be maximally diluted to an osmolality of approximately 50 mOsm/kg,[228-230] a value comparable with that in older children and adults. Although data were not strictly limited to neonates, Rodriquez-Soriano and colleagues[229] found that at periods of maximal free water clearance, urine osmolality in infants dropped to 52 mOsm/kg on average, and urine flow rates reached nearly 23 mL/dL of glomerular filtration. These infants and children effectively eliminated a water load with minimal impact on serum sodium and osmolality. In this study, fractional delivery of sodium to the distal nephron was high, indicating decreased proximal tubular salt reabsorption. However, reabsorption of salt along diluting segments of the distal nephron increased, indicating that enhanced distal reabsorption compensated for decreased proximal reabsorption. In general, the youngest infants tended to have high rates of delivery of sodium to the distal nephron, but they also had high rates of distal tubular sodium reabsorption. Clearly, at a young age, diluting segments have the potential to reabsorb solute avidly, and ADH-sensitive segments can be inhibited effectively from reabsorbing excess water. The primary limitation in neonatal water excretion, therefore, falls largely on the low rates of filtration.

The rapidity of maturation of an infant's ability to excrete an acute water load was illustrated by Ames.[231] In this study, premature and term infants of different postnatal ages received 30 mL/kg of either an oral water load or an intravenous 2.5% dextrose solution load. Water excretion was initially low (Figure 133-16). On average, infants in the first 24 hours of life excreted only 10% to 15% of the fluid load in 2 to 3 hours, with only a mild increase by 3 days of age. However, the ability to excrete the water load improved quickly. Infants 8 to 14 days of age excreted 60% of the load, and most infants older than 15 days (and all infants older than 30 days) excreted the total fluid load within 2 hours. One premature infant of birth weight 1.2 kg excreted 50% of an infused fluid load by the fourth day of life and excreted 100% by 12 days of age. Infants older than 7 days of age effectively reduced urine specific gravity to 1.002. The increasing ability to excrete the water was associated with a higher GFR. This finding is consistent with the notion that the low GFR limits the absolute amount of water that can be excreted.

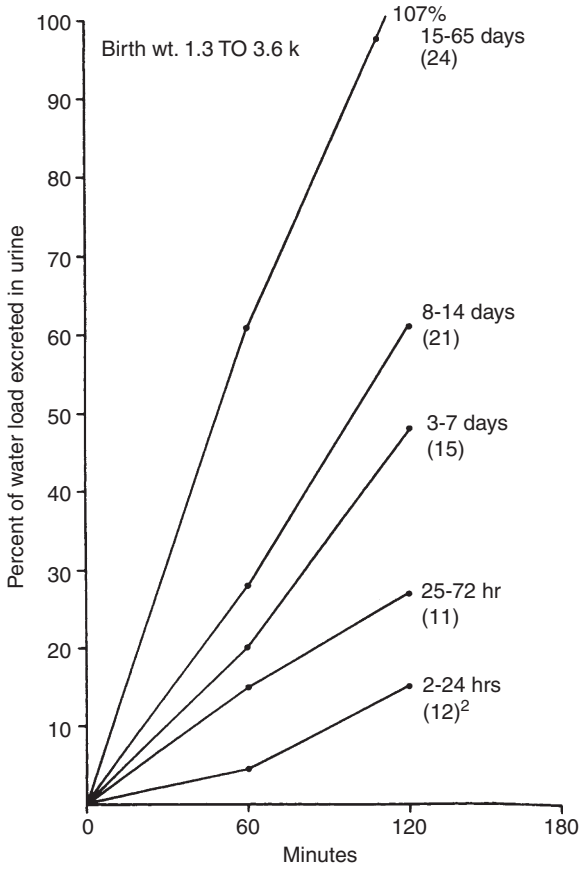


Figure 133-16 Comparison of ability to excrete water load by infants of various ages after intravenous administration of 2.5% glucose, 30 mL/kg body weight given at -10 to 0 minutes. Number in parentheses is number of infants per group. *(Reproduced by permission of* Pediatrics: *Ames RG: Urinary water excretion and neurohypophysial function in full term and premature infants shortly after birth.* Pediatrics *12:272, 1953.)*

Calcagno and colleagues[149] found that 60 minutes after an acute water load, a 1.6-kg, 23-day-old premature infant was able to increase urine flow to 8.3 mL/minute/1.73 m^2 (compared with 12 to 14 m:/minute/1.73 m^2 in the adult) and to decrease urine osmolality to 50 mOsm/kg. In the study by Barnett and coworkers,[228] five premature infants were given a 40-mL/kg oral water load. Urine osmolality dropped to 41 to 63 mOsm/kg. Although their urine flow rates after an hour were, in general, only about half that of adults, one 36-day-old premature infant not only diluted the urine maximally, but also had a urine flow rate fully comparable with that of the adult. Clearly, the postnatal maturation of this particular function is fairly rapid.

It should be noted that although ability to excrete an acute water load matures quickly, a sustained water load is more difficult to excrete in infants who have a low GFR and a limited ability to reabsorb solute along the distal nephron.[230] Fortunately, GFR matures relatively early in life and allows efficient elimination of a sustained water load. This is illustrated by a 6.5-kg, 2-month-old infant who had been given water whenever he became fretful. He ingested up to 2100 mL/day, developed polyuria, excreted urine with an osmolality of 42 mOsm/kg, and had only mild hyponatremia. His free water clearance was nearly 11 mL/minute/dL of glomerular filtration.[232] In the acutely or chronically water-loaded state, ADH is suppressed. Aquaporin-2 staining is more prominent in cytoplasmic vesicles and less so in the apical membrane of collecting ducts. Because aquaporin-2 is thought to be the actual ADH-sensitive water channel, excretion of a water load is likely made possible largely by acutely removing or more chronically down-regulating water channels from the membrane, thereby reducing apical membrane water reabsorption.[70,71,88] Recovery from the down-regulation of apical membrane water channels will take time because this protein up-regulates its production and membrane content in response to subsequent water restriction.

Torres and colleagues[233] provide a compelling example of potential clinical utility of such observations. In patients with autosomal dominant polycystic kidney disease, cAMP stimulates epithelial cell proliferation and fluid secretion into cysts, leading to cyst enlargement and renal dysfunction. Cyclic AMP is promoted by ADH acting through V2 receptors. Decreasing cyclic AMP may delay cyst/renal enlargement. Water loading can suppress ADH. The authors suggest that in patients with this disease and normal renal function, continuous water intake evenly throughout the day may mitigate the effect of cAMP and delay progression toward renal failure. This should also apply to children in whom diluting capacity matures rather quickly. Frequent voiding and attention to volume would reduce the risk of bladder dysfunction and hyponatremia.

CONCLUSION

In summary, diluting function depends on the ability to deliver adequate quantities of filtrate to nephron segments that can separate solute from water. More distal segments must be relatively water impermeant. Infants can maximally dilute their urine at an early age. Their ability to excrete an acute water load matures within a few weeks of birth. Limited excretion of a sustained water load likely relates to the low GFR typical of this age. However, moderately large amounts of ingested water can be effectively eliminated even in relatively young infants. Diluting capacity is quantitatively greater than concentrating capacity and tends to mature at an earlier age. The range of these functions is such that infants, even those of very low birth weight, tolerate a large range of fluid intake without having to sustain major alterations to their fluid and electrolyte stability. It is interesting that the staple infant food, breast milk, is dilute with a low renal solute load. The infant kidney must soon become adept at excreting a water load, an important adaptation if extra "free" water is ingested. The delay in maturation of urinary concentration for several months becomes less critical in the healthy infant because generally introducing foods of greater solute content is delayed until later in infancy.

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134 Urinary Acidification

Eileen D. Brewer

The mature kidney plays an important role in the regulation of normal acid-base balance and provides secondary compensation for primary respiratory acidosis or alkalosis. The kidney accomplishes these functions through reabsorption of filtered bicarbonate (HCO_3^-) and excretion of hydrogen ion (H^+) as ammonium (NH_4^+) and titratable acids, all of which are mediated by renal tubular H^+ secretion.[1-3] The resultant urinary pH is usually acidic (<7.4) with respect to blood pH.

Urinary acidification has been studied infrequently in human neonates. Some of the current knowledge has come from actual studies in newborn infants, but much more information has come from studies of fetal and newborn animals, which may not be directly applicable to human infants. Studies in preterm infants, especially those of extremely low birth weight (gestational age <29 weeks), have helped to clarify our knowledge,[4,5] but more studies are needed. Understanding developmental changes in the fetus and neonate first requires an understanding of current concepts of acid-base physiology in the mature kidney.

TUBULAR REABSORPTION OF FILTERED BICARBONATE IN THE MATURE KIDNEY

Buffering of strong acids is divided approximately equally between HCO_3^- and non-HCO_3^- buffers (hemoglobin, plasma and intracellular proteins, and phosphate).[6] The most important extracellular fluid (ECF) buffer is HCO_3^-, which is filtered from the plasma almost completely by the glomerulus and is reclaimed by reabsorption along the renal tubule to maintain a normal ECF HCO_3^- concentration. The majority (90%) of reabsorption occurs in the proximal tubule and the thick ascending limb of Henle of the renal tubule. As much as 20% of filtered HCO_3^- may be reabsorbed passively along paracellular pathways from proximal tubular lumen to peritubular blood.[6] Reabsorption of the rest of the filtered HCO_3^- is mediated by equimolar secretion of H^+ into the tubular lumen. H^+ secretion occurs mostly via an electroneutral sodium (Na^+)-H^+ exchanger (NHE), specifically isoform NHE-3 in both segments of the tubule and to some extent NHE-2 in the thick ascending limb.[1,2,6] Na^+ exchange for H^+ is driven by a downhill Na^+ concentration gradient, higher in the tubular lumen than in tubular cell cytoplasm, and maintained by active cellular extrusion of Na^+ at the basolateral cell surface mediated by a Na^+, potassium (K^+)–adenosine triphosphatase (ATPase) pump within that membrane. In the proximal tubule, a vacuolar H^+-ATPase pump also participates in H^+ secretion, but to a lesser extent, contributing less than one third of the H^+ secretion.[1-3,6] Both the NHE and H^+-ATPase pump are located in the apical (luminal) membrane of the cells of these renal tubular segments.

The distal nephron accounts for the other 10% of tubular HCO_3^- reabsorption and also depends on H^+ secretion to facilitate HCO_3^- reabsorption. However, H^+ secretion in the distal tubule occurs by a different mechanism and occurs within specialized collecting duct cells called intercalated cells, which are interspersed among principal cells.[1,3,6] In the mature kidney intercalated cells account for approximately one third of the tubular cells in the cortical and outer medullary collecting duct but fewer than 10% in the inner medullary collecting duct. In α-intercalated cells, H^+ secretion is mediated by a translocating H^+-ATPase and H^+,K^+-ATPase pumps in the apical membrane.[1,6] Vacuolar H^+-ATPase is also present in a specialized tubulovesicular system in the apical pole cytoplasm and may be important for acute response to acidosis.[7]

A second type of intercalated cells, the β-intercalated cells, located only in the cortical collecting duct, do not secrete H^+ but secrete small amounts of HCO_3^- in exchange for chloride (Cl^-) at the apical membrane and then extrude H^+ across the basolateral membrane via an H^+-ATPase pump.[1,3,6] β-Intercalated cells may be more important for secreting HCO_3^- in the process of renal compensation during chronic metabolic alkalosis.[1,6] Acid loading stimulates functional and morphologic changes in β-type intercalated cells[1] and can lead to a redistribution of vacuolar H^+-ATPase from the tubulovesicular system to the apical membrane itself in α-type cells.[7] Whether acid loading stimulates a change from β-type to α-type intercalated cells as a compensatory mechanism for chronic metabolic acidosis remains controversial.[1]

In the cortical collecting duct, principal cells adjacent to α-intercalated cells mediate Na^+ reabsorption, which creates negativity in the tubular lumen favorable for H^+ secretion out of the intercalated cells.[1,6] The rate of H^+ secretion is influenced by a variety of factors that allow the kidney to respond to changes in acid-base homeostasis. Factors favoring H^+ secretion include avid renal tubular Na^+ reabsorption, increased HCO_3^- concentration and fluid flow rate in the tubular lumen, angiotensin II, aldosterone, and K^+ depletion (hypokalemia); the rate may be decreased by ECF volume expansion and parathyroid hormone.[1-3,6]

Once H^+ is secreted into the tubular lumen, it combines with HCO_3^- filtered from the plasma by the glomerulus to form carbonic acid (H_2CO_3).[6] H_2CO_3 dissociates to water (H_2O) and carbon dioxide (CO_2). The reaction is driven in that direction, partially because CO_2 diffuses easily through apical membrane aquaporin 1 (AQP1) gas channels into the renal tubular cell for further metabolism[8] and, in the proximal tubule only, because of the presence of carbonic anhydrase isoenzyme IV attached to the apical membrane brush border to catalyze the reaction.[1,6,8] Once CO_2 is inside the cell, cytosolic carbonic anhydrase isoenzyme II catalyzes the conversion of CO_2 and H_2O back to H_2CO_3 to yield H^+ and HCO_3^-.[3,6] HCO_3^- then exits the cell across the basolateral membrane by mass action or via either of two transport systems, an electrogenic Na^+-$3HCO_3^-$ (NBC-1) cotransporter (primarily in the proximal tubule and loop of Henle) or a Cl^--HCO_3^- (AE1) exchanger (primarily in the distal collecting duct).[1,6,8] HCO_3^- leaving the tubular cell then returns to the circulation via the peritubular capillaries. H^+ is secreted back into the tubular lumen to help reclaim filtered HCO_3^- all over again by the same process.

In the proximal tubule, where 80% of HCO_3^- reabsorption occurs, carbonic anhydrase isoenzyme IV attached to the apical brush border membrane greatly increases the capacity for HCO_3^- reabsorption by catalyzing the rapid dissociation of luminal H_2CO_3 to H_2O and CO_2 to minimize luminal concentrations of H_2CO_3 and H^+ and facilitate more H^+ secretion.[1,3,6] The proximal tubule thus has a high capacity for H^+ secretion and HCO_3^- reabsorption. When luminal carbonic anhydrase IV is inhibited by acetazolamide, H^+ secretion and HCO_3^- reabsorption are

notably diminished.[3] Proximal tubular reabsorption of HCO_3^- is also decreased by expansion of ECF or effective arterial blood volume.[9]

The filtered HCO_3^- not reabsorbed proximally is reclaimed partially or completely as needed in the distal tubule, primarily in the collecting duct as noted earlier.[1,3,6] When distally delivered HCO_3^- is entirely reabsorbed and luminal fluid pH falls to less than 6.4, H^+ buffers in the lumen can facilitate further H^+ excretion and regeneration of HCO_3^-. This mechanism allows for the excess acid produced daily by normal metabolism to be excreted in the urine (see Urinary Excretion of Hydrogen Ion by the Mature Kidney later). The primary urinary buffers are ammonia (NH_3) and sodium phosphate (Na_2HPO_4), which combine with H^+ in the tubular lumen to be excreted in the urine as ammonium (NH_4^+) or titratable acid (NaH_2PO_4).[1,3] Aldosterone directly enhances distal tubular secretion of H^+, both by direct stimulation of H^+-ATPase in α-intercalated cells and by stimulation of Na^+ reabsorption by the adjacent principal cells.[3,6] Because carbonic anhydrase is not present in the brush border membranes of the distal tubule, the capacity of the distal tubule to reabsorb HCO_3^- is much less than that of the proximal tubule.[1,6] Hence, a relatively small increase in distal delivery of HCO_3^-, such as that occurring with ECF or effective arterial blood volume expansion, may exceed the HCO_3^- reabsorptive capacity of the distal tubule, increase distal tubular fluid pH to greater than 6.4, and thereby limit formation of NH_4^+ or titratable acids for net H^+ excretion.

RENAL BICARBONATE THRESHOLD

The renal HCO_3^- threshold, which has been measured frequently in studies of renal acidification in neonates,[10-13] is defined as the plasma HCO_3^- concentration at which HCO_3^- is no longer completely reabsorbed in the renal tubule and begins to appear in significant amounts in the urine. The HCO_3^- threshold does not necessarily characterize the intrinsic renal tubular capacity for HCO_3^- reabsorption, because it is significantly altered by ECF volume status.[8,14] When ECF volume contraction is maintained during HCO_3^- loading, the HCO_3^- threshold is increased, even in neonatal animals.[15] When ECF volume is expanded during HCO_3^- loading, the HCO_3^- threshold becomes apparent at a plasma HCO_3^- concentration of less than 24 mEq/L, but when ECF volume expansion is minimized or prevented, the threshold may not be reached until the plasma HCO_3^- concentration exceeds 27 mEq/L. Thus alterations in the renal HCO_3^- threshold often reflect the effects of ECF volume status on renal tubular HCO_3^- reabsorption rather than the intrinsic properties or maturity of the renal tubular cells. This phenomenon has clinical consequences in both preterm and term infants (see Developmental Aspects of Bicarbonate Reabsorption section later).[14]

URINARY EXCRETION OF HYDROGEN ION BY THE MATURE KIDNEY

Normal metabolism generates H^+ in the form of volatile and fixed acids, which must be excreted to prevent acidosis.[16] Volatile acid, primarily H_2CO_3, is excreted by the lungs as CO_2. Because the fixed acids, which include lactic acid, ketoacids, sulfuric acid, and phosphoric acid, are initially buffered by extracellular buffers, including HCO_3^-, HCO_3^- must be regenerated by the kidneys, a process that occurs through excretion of H^+ in the urine as NH_4^+ and titratable acids. NH_3 is produced in the kidney principally in the proximal tubule by metabolism of glutamine, which is catalyzed by glutaminase and phosphoenolpyruvate carboxykinase.[1,3,6] The NH_3 generated is secreted into the luminal fluid of the proximal tubule both by diffusion and by exchange of NH_4^+ (NH_4^+ substituted for H^+) with Na^+ via the NHE exchanger in the apical membrane. NH_3 and NH_4^+ traverse through the tubular lumen to the thick ascending limb of Henle, where NH_4^+ is subsequently actively reabsorbed from the relatively alkaline luminal fluid via an apical cell membrane Na-K-2Cl cotransporter (NH_4^+ substituted for K^+), thereby greatly increasing the concentration of NH_3 in the medullary interstitium.[1] NH_3 from the medullary interstitium then diffuses into the acidic luminal fluid (pH <6.4) along the length of the collecting duct, where it buffers secreted H^+ and is entrapped as NH_4^+ for excretion in the urine.[1,3,6] Substantial trapping of H^+ by NH_3 occurs in the lumen of the inner medullary collecting duct, where urine pH is lowest and NH_3 concentration is greatest.[3,6] The other major buffer system is the titratable acids, of which NaH_2PO_4 is the most abundant. NaH_2PO_4 is derived from filtered Na_2HPO_4, which escapes proximal tubular reabsorption and is delivered to the distal nephron, where it reacts with secreted H^+ to be excreted as NaH_2PO_4.[6]

NET ACID EXCRETION

The quantity of H^+ excreted as NH_4^+ and titratable acids minus any excreted HCO_3^- (normally a negligible amount) is called the net acid excretion.[3,6,16,17] Net acid excretion accounts for 3% or less of the H^+ secretion by the renal tubule and leads to HCO_3^- regeneration back to the blood. Urinary pH itself is not a good indicator of net acid excretion, because the pH does not reflect the amount of buffering capacity available as NH_3 and titratable acids. More H^+ can be excreted in urine with excess buffers and a slightly higher pH that is nearer the pK_a of the buffer (e.g., pK_a 5.75 for NH_3).

DEVELOPMENTAL ASPECTS OF URINARY ACIDIFICATION

In the human fetus, nephronogenesis is not complete until 34 weeks gestation.[14] Functioning renal tubules can be identified by 9 weeks gestation.[14] During fetal life, because H^+ is removed primarily through the placenta for excretion by maternal organs,[14,18] renal acidification is not important until birth. Exactly when renal acidification mechanisms become functionally active and reach maturity in the developing human kidney is not known. In fetal lambs, urinary pH is always lower than plasma pH, a finding indicating the ability of the developing fetal renal tubule to establish a concentration gradient for H^+.[18] Carbonic anhydrase is present in the human fetal kidney in late gestation[18] and increases with gestational age,[19] perhaps explaining in part the observed increase of renal HCO_3^- reabsorption with gestational age. However, after birth human neonates have similar renal carbonic anhydrase activity compared with adults.[20]

DEVELOPMENTAL ASPECTS OF URINARY EXCRETION OF HYDROGENION

The renal tubular mechanisms for net acid excretion are already mature in the last-trimester fetal kidney. Although net acid excretion per unit body weight is reduced in near-term fetal lambs compared with nonpregnant ewes, the fetus responds appropriately to acid loading by increasing urinary excretion of both NH_4^+ and NaH_2PO_4 and by lowering urinary pH.[18]

Term infants are able to respond appropriately to an NH_4Cl acid load within the first month of life,[10,12] although it is uncertain whether preterm infants have the same capacity. Svenningsen[12] reported that net acid excretion in NH_4Cl-loaded preterm infants (gestational ages 29 to 36 weeks, birth weights 1300 to 2470 g) was not comparable with that of term infants until after the third week of life. Schwartz and co-workers[11] found that preterm infants (birth weights 1630 ± 250 g) were able to acidify their urine to less than 5.5 and to excrete NH_4^+ and NaH_2PO_4 appropriately during an NH_4Cl-loading test performed just before hospital discharge at least 3 weeks after

birth. Unfortunately, Schwartz and colleagues[11] gave no estimates of gestational age at birth or postconceptional age at the time of the NH_4Cl loading to allow comparison with the data of Svenningsen.[12] Kalhoff and associates[4] evaluated urinary pH in a large number of premature infants (83 weighing 1.5 to 2.4 kg and 87 weighing 1.0 to 1.4 kg; no gestational ages given). In approximately one third of infants in each group, urinary pH values spontaneously decreased to less than 5.4 by the third to fourth weeks of postnatal life, a finding indicating maximal stimulation of tubular H^+ secretion. At the same time that urinary pH was low, however, capillary blood pH and base excess showed mild metabolic acidosis, a finding suggesting inadequate net acid excretion, possibly as a result of immaturity of the renal tubule for ammoniagenesis or decreased availability of phosphate (especially low in breast-fed infants).[17] Unfortunately, net acid excretion was not measured. Studies of neonatal rats have shown that renal ammonia production is reduced and the capacity to increase ammoniagenesis during acidosis is less than in adult rats.[20] More studies in human preterm infants of different gestational ages, especially those less than 29 weeks gestation and at different postconceptual ages, are needed to evaluate the postnatal development of renal tubular H^+excretion and the capacity for net acid excretion.

DEVELOPMENTAL ASPECTS OF BICARBONATE REABSORPTION

The normal range for plasma HCO_3^- concentration has been reported to be lower for preterm infants (16 to 20 mEq/L) and term infants (19 to 21 mEq/L) than for children and adults (24 to 28 mEq/L).[10,11] This relative acidosis likely results from both physiologic factors and immaturity of the renal tubule.[3,6,14,21,22] During the first few weeks of life, the wide range of low but normal values of plasma HCO_3^- concentration is the most likely explanation for the designation in the past of late metabolic acidosis in otherwise healthy preterm infants with values of plasma HCO_3^- concentration in the acidotic range for children or adults.[11,17,23,24]

The renal HCO_3^- threshold measured in term and preterm infants in studies reported before 1980 was found to be low compared with that in older children and adults.[10-12] These studies were done before our current understanding of the obligatory need for neonatal diuresis of excess ECF in the first week after birth.[5,14] Excess fluid and Na^+ administered during obligatory diuresis may perpetuate ECF volume expansion. ECF volume expansion, which is a major determinant of the HCO_3^- threshold regardless of the acid-base status of the infant, accounts at least in part for the reduced HCO_3^- threshold measured in the classic studies. The likelihood that ECF volume status contributes importantly to the apparently low renal HCO_3^- threshold is suggested by studies of fetal lambs[25] and neonatal puppies.[15] During HCO_3^- loading in fetal and neonatal animals in which the ECF space is volume contracted, the HCO_3^- threshold and tubular reabsorption of HCO_3^- increase, and the urinary pH becomes more acidic. Conversely, in neonatal puppies with ECF volume expansion, the HCO_3^- threshold and tubular reabsorption of HCO_3^- decrease. These findings suggest that the renal tubule in the fetus and neonate can increase its HCO_3^- reabsorption appropriately when stimulated by volume contraction, and, conversely, a reduced HCO_3^- threshold is at least in part, if not all, a physiologic phenomenon caused by an expanded state of ECF volume.

The ECF volume of the human fetus and newborn at birth is normally 40% to 60% of body weight, and it decreases to less than 30% after birth.[14] The usual ECF volume expansion of the fetus and newborn probably results in depressed proximal tubular HCO_3^- reabsorption, increased delivery of HCO_3^- to the distal nephron, and an alkaline urinary pH. After birth, when obligatory diuresis of excess ECF is complete and ECF volume is contracted, renal tubular HCO_3^- reabsorption increases, and urinary pH can become more acidic. These phenomena were confirmed in studies of extremely low-birth-weight infants. Ramiro-Tolentino and colleagues[5] studied 22 preterm infants (23 to 29 weeks gestation; birth weight 540 to 982 g) during the first 5 days of life. Intravenous fluid and electrolyte therapy were carefully controlled to allow body weight loss between 1% and 4% per day during obligatory diuresis and to maintain serum Na^+ concentrations between 130 and 145 mEq/L, serum K^+ concentrations between 4 and 6 mEq/L, and plasma HCO_3^- concentrations between 18 and 22 mEq/L. When diuresis began at approximately 24 hours after birth, measured glomerular filtration rate and filtered load of HCO_3^- increased, and so did urinary HCO_3^- excretion. After diuresis, when ECF was contracted and glomerular filtration rate was only slightly decreased, urinary HCO_3^- excretion markedly dropped, and cumulative HCO_3^- balance became positive. If prescribed fluid therapy had continued to supply an excess of salt and water during the period of diuresis, and if ECF volume expansion had persisted, relative metabolic acidosis caused by continued depression of renal tubular reabsorption of HCO_3^- might have developed in these infants.[14]

Immaturity of tubular function may also play a role in the expression of a lowered plasma HCO_3^- threshold in the neonate. Histochemical and biochemical analyses of human fetal kidney tissue suggest that carbonic anhydrase is present in sufficient amounts to allow normal reabsorption of HCO_3^- at 24 to 26 weeks gestation in fully formed juxtamedullary nephrons but not in immature nephrons in the more cortical nephrogenic areas.[26] In preterm infants born at less than 35 weeks gestation, when nephrogenesis is not yet complete, heterogeneity of nephron development could contribute to a reduced overall capacity to reabsorb HCO_3^-. In studies of renal tubule segments of infant rats, Karashima and associates[27] found carbonic anhydrase II activity of 1-week-old rats to be only 14% of adult values, increasing to 40% at 3 weeks and 97% by 7 weeks.

Direct studies of tubular HCO_3^- reabsorption[28] and NHE activity[21,22] in isolated proximal tubular segments of neonatal rabbits suggest that postnatal maturation of the proximal tubule occurs both quantitatively and qualitatively. HCO_3^- reabsorptive capacity abruptly and markedly increases after the fourth week of life in this rabbit model. A similar abrupt and marked increase in tubular reabsorptive capacity occurs 3 weeks after birth in the proximal tubule of newborn guinea pigs.[29] Nephronogenesis is complete before birth in guinea pigs, but not until 4 weeks after birth in rabbits; neonatal guinea pig kidneys therefore are more comparable with those of term infants, and neonatal rabbit kidneys compare more favorably with those of preterm infants. In both experimental models, the abrupt increase in reabsorption has been associated with a concomitant marked increase in the basolateral membrane surface area of the proximal tubule. Studies of rat proximal tubules also demonstrate maturational changes from neonate to adult in NHE activity, which is associated with an increase in NHE-3 gene expression,[30] and a conversion from the predominance of isoform NHE-8 present in brush border membrane vesicles of neonatal rats to isoform NHE-3 in adult rats.[22]

Increased production of glucocorticoids and thyroid hormone may play an important role in promoting postnatal maturation of proximal tubule acidification.[21,22] Administration of exogenous glucocorticoids or thyroid hormone to neonatal rabbits or directly to opossum kidney cells in culture caused a marked increase in proximal tubular NHE and Na^+,K^+-ATPase activity measured in vitro.[21,22] In studies in preterm extremely low-birth-weight infants, however, urinary HCO_3^- excretion in the first week of life was the same whether infants had received antenatal glucocorticoids or not.[5] More studies in human infants are needed to determine the importance of postnatal renal tubular maturation for HCO_3^- reabsorption during the first few weeks of life.

INHERITED DISORDERS OF RENAL ACID-BASE BALANCE

A variety of genes encode for the protein transporters, exchangers, and enzymes required to carry out normal urinary acidification. Mutations in any of these genes can lead to inherited clinical diseases. Rare forms of proximal renal tubular acidosis (RTA) have been associated with mutations in genes encoding for the Na^+-HCO_3^- exchanger NBC-1 (also known as SLC4A4), carbonic anhydrase II, and pyruvate carboxylase deficiency.[3,31] Several types of distal RTA have been associated with mutations, including the most common autosomal dominant distal RTA caused by mutations in the Cl^-/HCO_3^- exchanger AE1 (SLC4A1) and a rare autosomal recessive distal RTA with sensorineural deafness caused by mutations in the vacuolar H^+-ATPase pump (ATP6V1-B1 subunit).[31] As knowledge of the molecular basis of urinary acidification increases, more diseases of abnormal renal acid-base balance may be identified, and our knowledge of the basis of known forms of RTA will be increased.

NEONATAL METABOLIC COMPENSATION FOR RESPIRATORY ACIDOSIS OR ALKALOSIS

The ventilatory response to retained CO_2 increases with gestational age and postnatal age,[32] so is relatively impaired in preterm compared with term neonates and adults and may predispose preterm infants to bouts of respiratory acidosis. In addition, if the lungs are diseased and alveolar ventilation is ineffective, as occurs in infants with respiratory distress syndrome or bronchopulmonary dysplasia, CO_2 is retained, blood CO_2 tension rises, and respiratory acidosis occurs. Retained CO_2 is buffered initially by intracellular proteins, phosphates, and plasma hemoglobin.[33] Within 12 to 24 hours of the onset of respiratory acidosis in adults, renal metabolic compensation begins to occur and is not complete for 3 to 5 days.[6,33,34] The kidneys attempt to return blood pH toward normal by excreting H^+ in the urine as NH_4^+ and titratable acids and by reclaiming filtered HCO_3^-. As blood HCO_3^- concentration rises, serum chloride concentration decreases because of loss of chloride in the urine and a shift of chloride into red blood cells; hypochloremia therefore is a consistent finding in compensated respiratory acidosis.[34]

Hyperventilation whether disease related or iatrogenic leads to respiratory alkalosis. The initial reduction in blood CO_2 tension is acutely titrated by intracellular buffers.[33] In adults, renal metabolic compensation for respiratory alkalosis begins within hours and is complete within 1 to 3 days. Renal compensation is characterized by suppression of renal acidification with excretion of HCO_3^- in the urine, alkaline urinary pH, and decreased excretion of NH_4^+ and titratable acids.[6,33,35] Reduced renal tubular HCO_3^- reabsorption enhances tubular chloride reabsorption and leads to hyperchloremia as a consistent finding in compensated respiratory alkalosis.[35]

Maturation of the ability to compensate for respiratory acid-base disorders has not been carefully studied in the developing kidney. A study in 5- to 12-day-old rabbits[36] suggested that metabolic compensation for both acute hypercapnia and hypocapnia is intact. Studies in human infants are needed to elucidate further the compensatory response and capacity of renal acidification.

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135 Response to Nephron Loss in Early Development

Robert L. Chevalier

OVERVIEW

The developing kidney depends on a complex program of morphogenesis resulting in a delicate balance of growth and differentiation. A number of factors can interfere with this process in fetal, perinatal, or neonatal life. The resulting nephron deficit, in turn, leads to adaptive responses that have short- and long-term consequences. This chapter reviews the causes and consequences of nephron loss with emphasis on the unique features of the developing kidney that increase its vulnerability.

EXPERIMENTAL STUDIES

Renal Mass and Number of Nephrons

Renal growth occurs as a result of normal development or in response to a reduction in renal mass. It is therefore not surprising that the compensatory increase in renal mass following nephron loss is proportionately greater in the newborn than in the adult.[1-3] Despite a significant increase in glomerular volume, both normal and compensatory renal growth are primarily caused by an increase in proximal tubular mass.[4,5] It is now recognized that there is considerable variation in the number of nephrons in normal subjects, and there is a linear relationship between the number of glomeruli and birth weight in humans.[6,7] Moreover, there is an overall negative correlation between the number of nephrons at birth and blood pressure in adulthood, suggesting that conservation of nephrons is particularly important in early life.[8-10] In a particularly striking report of autopsies of middle-aged hypertensive patients killed in accidents, the median number of glomeruli was 50% of that in matched normotensive subjects.[11] These observations have spurred greater interest in the determinants of nephron number at birth.

There are at least three broad categories of factors contributing to the number of nephrons in the metanephric kidney: developmental factors, nutritional factors, and toxic/iatrogenic influences (Table 135-1). Reduced nephron number may result from excessive apoptosis in the developing ureteral bud, the result of loss of an endogenous survival factor such as PAX2.[12] Administration of specific growth factors may also alter nephrogenesis in developing kidneys in vivo or in vitro—transforming growth factor-β1 impairs nephrogenesis whereas insulin-like growth factor 1 stimulates nephrogenesis.[13,14] Early human fetal urinary tract obstruction results in a reduced number of nephrons, and the number of glomeruli can be related to the time of developmental arrest.[15] In animal models, ureteral obstruction also results in reduced glomerular number, and ureteral obstruction impairs nephrogenesis in direct proportion to the duration of obstruction.[16-19] Fetal rats of mothers whose uterine artery has been ligated, or rats with spontaneous intrauterine growth restriction, have reduced nephron number.[20,21] Infants with intrauterine growth restriction are born with fewer nephrons than those whose birth weight is appropriate for gestational age.[22] The timing of intrauterine growth restriction may determine whether nephron number is reduced.[23] Infants of diabetic mothers have an increased incidence of congenital malformations, and maternal hyperglycemia in rats leads to nephron deficit.[24] Reduced maternal protein intake leads to reduced nephron number in experimental animals and may program the individual for salt-sensitive adult hypertension.[20,25] Intrauterine malnutrition in aborigines is associated with low birth weight and impaired nephrogenesis, suggesting that optimizing fetal growth may reduce renal disease, hypertension, and cardiovascular disease in this population.[26] In a provocative study, mild vitamin A deficiency was shown to lead to inborn nephron deficit in the rat—there was a linear relationship between maternal plasma retinol levels and the number of glomeruli in the fetus.[27] This study suggests that small variations in maternal nutritional status may have significant implications for the ultimate complement of nephrons. The electrolyte environment may also play a significant role—fetal mouse kidneys grown in vitro exhibited impaired nephrogenesis if potassium concentration was reduced.[28] Perinatal asphyxia or severe circulatory disturbances can cause irreversible nephron loss.[29] Exposure of the fetus to maternal administration of angiotensin-converting enzyme inhibitors or to prostaglandin inhibitors impairs nephrogenesis and renal maturation.[30,31] Maternal exposure to glucocorticoids, aminoglycosides, beta lactam antibiotics, or cyclosporine may impair nephrogenesis and may lead to hypertension.[32-36] Fetal exposure to toxins such as maternal smoking or alcohol consumption can impair kidney development.[37] Finally, emerging reports suggest that extremely preterm birth itself can curtail nephrogenesis, with serious long-term implications (see later: Postnatal Adaptation to Nephron Loss).[38,39]

Renal Hemodynamics

The hemodynamic response to nephron loss is proportional to the amount of renal mass removed. Thus uninephrectomy in the rat doubles glomerular blood flow, but removal of 75% of renal mass increases flow more than threefold 2 to 3 weeks later.[40] In 8-week-old dogs subjected to 75% nephrectomy at birth, renal plasma flow was not different from that of control littermates; however, flow in 14-week-old dogs studied 6 weeks after 75% renal ablation was 50% less than that in controls.[41] This study indicates that hemodynamic adaptation to nephron loss is more complete in early development than in later life.

A potentially important difference in the hemodynamic response to nephron loss in early compared to later development has been shown for autoregulation of renal blood flow (RBF), whereby flow is kept constant during changes in renal perfusion pressure. Uninephrectomy in the adult rat causes renal vasodilation at reduced as well as normal perfusion pressure; however, uninephrectomy in the neonatal rat increases RBF at normal, but not at reduced, arterial pressure.[42] Thus autoregulation is reset to higher flows in the uninephrectomized adult but is impaired in the newborn rat with a single kidney.[42] The neonate with reduced renal mass may therefore be more susceptible than the adult to ischemic renal damage. Another difference between the response to uninephrectomy in neonates compared to adults relates to the development of hypertension— uninephrectomy in the neonatal rat leads to salt-sensitive hypertension in adulthood, which in turn leads to glomerular damage.[43,44]

Because of the critical dependence of the fetal and neonatal glomerular capillary pressure on angiotensin-mediated efferent arteriolar constriction, the fetus and neonate are particularly susceptible

TABLE 135-1

Causes of Reduced Nephron Number

1. Developmental factors
- Genetic determinants
- Renal maldevelopment, including obstructive nephropathy
- Intrauterine growth restriction
- Maternal diabetes

2. Nutritional factors
- Reduced maternal protein intake
- Reduced maternal vitamin A intake
- Reduced fetal potassium intake

3. Toxic/iatrogenic influences
- Fetal hypoxia-perinatal asphyxia
- Severe fetal-perinatal circulatory disorders
- Maternal angiotensin-converting enzyme or angiotensin receptor inhibitors
- Maternal prostaglandin inhibitors
- Maternal glucocorticoids
- Maternal aminoglycosides
- Maternal beta lactam antibiotics
- Maternal cyclosporine
- Extremely preterm birth

to a marked reduction in glomerular filtration rate (GFR) after exposure to angiotensin-converting enzyme (ACE) inhibitors.[45] Both the potency and duration of action of captopril are significantly greater in neonates than in older children.[46] Following a reduction in renal mass, the remaining hyperfiltering immature nephrons are even more susceptible to the action of ACE inhibitors, which can induce renal failure and additional nephron injury.

Stimuli for Compensatory Renal Growth

Numerous studies indicate that reduction in renal mass results in release of renotropic factors and/or suppression of inhibitors of renal growth.[47] Although blood-borne renotropins are probably not released from the kidneys themselves, renal tissue factors may be necessary to activate the humoral compounds.[48,49] The enhanced compensatory renal hypertrophy observed in the neonate may relate to differences in tissue factors. In this regard, kidneys of adult rats and mice have been found to contain a renal growth inhibitory factor, although none was found in neonatal kidneys.[50] The initial phase of compensatory renal growth following unilateral nephrectomy in immature rats is independent of growth hormone secretion, but is associated with an increase in insulin-like growth factor-1 (IGF-1) and IGF-1–receptor gene expression.[51,52]

In addition to reduction in functional renal mass, numerous compounds have been shown to stimulate renal growth, including sodium, ammonium chloride, folic acid, thyroxine, growth hormone, and mineralocorticoids.[53] One stimulus to renal hypertrophy and hyperfunction that has generated much interest is increased dietary protein intake. Increased protein intake can enhance compensatory renal hypertrophy.[54,55] The ribonucleic acid (RNA)-protein ratio and DNA content of the remaining kidney are increased as a result of uninephrectomy, but not with increased dietary protein.[56] Thus the additive effect of dietary protein is probably caused by a separate mechanism rather than being an amplification of the normal hypertrophic response. Although angiotensin II has been shown to act as a renal growth factor, compensatory renal growth in neonatal mice subjected to unilateral ureteral obstruction is not impaired in animals lacking functional angiotensinogen genes.[57] Perhaps as important as what stimulates compensatory renal growth is what limits compensatory renal growth. Mice lacking the gene for cyclin-dependent kinase inhibitor p21 do not develop progressive renal insufficiency following renal ablation.[58] A shift from renal cellular hypertrophy to proliferation in these mutants may underlie the protective effect.[59]

Adaptation Versus Maladaptation

As described earlier, structural and functional adaptation appear to be more "complete" after reduction of renal mass in the neonate compared to adaptation that follows nephron loss later in life. However, increased intraglomerular pressure and blood flow, and consequent hyperfiltration, may lead to progressive glomerular injury, a maladaptive response.[60,61] The mechanisms responsible for this process are not completely understood, but they include increased filtration of protein and other macromolecules that are taken up by mesangial cells, which are thereby damaged.[61,62] Proteinuria also leads to injury to the renal tubules and interstitium. Proteinuria can lead to apoptosis of tubular cells and the development of interstitial fibrosis.[63,64] Uninephrectomy in the neonatal rat has been shown to result in greater proteinuria and glomerular sclerosis of the remaining kidney than occur following uninephrectomy in the adult.[65,66] Increased protein intake in the young rat can stimulate kidney growth by promoting cell proliferation as well as by increasing the GFR.[67] Studies of young rats undergoing unilateral nephrectomy reveal that survival was significantly reduced in animals receiving a high-protein compared to a low-protein diet.[68] Moreover, this was preceded by an increase in urinary protein excretion.[69] These data have disturbing implications for neonates with nephron loss, as a more vigorous early adaptive response in remaining renal tissue may lead to progressive renal insufficiency during development. However, attempts to slow the progression by restricting protein in infancy carry the risks of protein malnutrition and impairment of normal somatic growth.

Compensatory Renal Adaptation in the Fetus

Until recently, experimental evidence for compensatory renal growth in the fetus had not been clearly established. Peters and colleagues[70] induced unilateral ureteral occlusion in the fetal lamb at midtrimester and observed a significant increase in contralateral kidney weight within 2 weeks, which became maximal by 1 month (50% increase). Although the increase in renal mass in this study was not associated with an increase in the total number of nephrons, ovine fetal uninephrectomy results in a 45% increase in the number of nephrons but a lower glomerular volume.[71] Because the placenta provides the excretory function for the fetus, the results of this study indicate that an increased excretory or homeostatic burden on the kidney is not required to initiate compensatory growth. Rather, alterations in growth factors or inhibitors presumably modulate the prenatal changes.

CLINICAL ASPECTS OF RENAL RESPONSE TO NEPHRON LOSS

Because of the relatively small numbers and heterogeneity of patients with lesions resulting in nephron loss during early development, there remains a paucity of clinical data. However, in general, the results of studies addressing this problem are consistent with the experimental findings discussed earlier. Thus compensatory renal hypertrophy following uninephrectomy appears to be proportionately greater the younger the age at the time of nephrectomy.[72] If nephrectomy is delayed beyond the first year of life, however, a significant effect of age on the compensatory response may be difficult to demonstrate.[73,74]

Prenatal Nephron Loss

Unilateral Multicystic Kidney and Renal Agenesis

Most nephron loss in the neonate results from fetal renal maldevelopment. Unilateral multicystic renal dysplasia results in a nonfunctional kidney early in gestation and therefore affords an opportunity to study the adaptation by the remaining kidney.

An autopsy study of 20 human fetuses with a unilateral kidney revealed a significant increase in the proportional weight of the single kidney.[75] These findings are corroborated by two prenatal ultrasound studies of fetuses with unilateral renal agenesis or multicystic kidney. In both reports, the single functioning kidneys were significantly longer than those in the control patients.[76,77] Sonography permits serial measurement of renal size in the fetus such that renal growth can be compared with normal ranges and followed beyond birth. Such tracking of renal size may be clinically useful in monitoring the function of an abnormal contralateral kidney in infants with two functioning kidneys. An exaggerated rate of increase in renal size has been correlated with contralateral renal function that contributes less than 15% of total function.[78]

In infants born with unilateral renal agenesis, renal volume increases to 188% of that of a single normal kidney during postnatal development.[73] Consistent with the hypothesis that hypertrophy and prolonged hyperfiltration by remnant glomeruli lead to progressive renal injury, patients with unilateral renal agenesis may develop focal glomerular sclerosis and renal insufficiency in adulthood.[79-81]

Renal Hypoplasia and Dysplasia

Because of the wide variation in functional nephron loss resulting from renal hypoplasia and dysplasia, it has been difficult to systematically examine the adaptation of remaining nephrons in these disorders. However, in oligomeganephronia, a rare form of congenital renal hypoplasia, infants are born with less than 25% of the normal nephron number.[82] Presumably, because of the severity of nephron loss, compensatory hypertrophy is pushed to its limits, resulting in glomerular volumes that are several fold greater than normal.[79] This disorder is associated with the eventual development of focal glomerular sclerosis.[79,82]

Congenital Hydronephrosis

Along with renal hypoplasia-dysplasia, congenital urinary tract obstruction accounts for most cases of nephron loss in the neonate. Complete ureteral obstruction (atresia) early in gestation results in multicystic dysplasia and a nonfunctional kidney.[83] However, most forms of hydronephrosis in the neonate result from incomplete obstruction of the urinary tract or vesicoureteral reflux. Severe bladder obstruction resulting from posterior urethral valves can cause renal maldevelopment in utero such that adaptive renal growth is impaired despite either prenatal or postnatal relief of obstruction.[84,85] Unilateral ureteropelvic junction obstruction, on the other hand, may be relatively mild in utero such that most renal damage occurs postnatally unless the obstruction is relieved.[86] Severe vesicoureteral reflux can eventually lead to glomerulosclerosis, proteinuria, and renal insufficiency if both kidneys are involved.[87]

Postnatal Adaptation to Nephron Loss

In cases of unilateral loss of nephrons (or more severe impairment of one kidney than the other), the contralateral kidney generally compensates accordingly. This phenomenon, first called *renal counterbalance* by Hinman,[88] appears to be exaggerated in early development.[89,90] The postnatal hypertrophic response of the less impaired kidney can be further compromised by the presence of vesicoureteral reflux or infection.[91,92] As in experimental studies, the hypertrophic response by the intact kidney is dependent on the proportion of nephron loss that is caused by scarring of the affected kidney.[40,93] Adaptation by remaining nephrons can also be influenced by iatrogenic factors such as radiation or chemotherapy, which may impair compensatory hypertrophy to a greater extent in patients younger than in those older than 2 years of age.[94] Although compensatory growth of the contralateral kidney has been suggested as an index of functional impairment in children with unilateral ureteropelvic junction obstruction, biologic variability and limitations of clinical ultrasonographic imaging limit the early detection of compensatory renal growth.[95,96]

It is disturbing that children born prematurely have smaller kidneys than term children, without catch-up with age through 18 months.[97] It appears that nephrogenesis continues after birth in extremely preterm infants, but ceases after 40 days.[38] Surviving infants develop glomerular hypertrophy and mesangial proliferation, which may predispose them to vasomotor or nephrotoxic injury in the postnatal period or in later life.[39] When these infants develop acute renal failure, long-term outcome is poor. Despite improvement of GFR by 1 year of age, nearly 50% of one cohort of such patients over a period of years developed decreased renal function and increased urine protein excretion.[98]

Prediction of Ultimate Renal Function

Progression of Renal Insufficiency

A major problem confronting the physician caring for the infant with reduced functioning renal mass is the lack of a reliable means of predicting the ultimate compensatory renal adaptation. Will the infant require dialysis or renal transplantation, and if so, when? As described earlier, normal and compensatory renal development are interdependent and significantly influenced by the etiology of nephron loss, the proportion of intact nephrons remaining, and the presence of factors that impair the adaptive process. It is becoming increasingly clear that many of the data currently available are marginalized because of an insufficient follow-up period. Although unilateral renal agenesis was previously thought to represent a benign condition, in fact the deleterious consequences may not appear until late adulthood. Nevertheless, it is still reasonable to predict that an infant with a single normal kidney can have a relatively normal life expectancy, particularly if dietary protein intake is not excessive. In the neonatal period, it may be difficult to demonstrate that a single functioning kidney is entirely normal, particularly if the contralateral kidney is multicystic.[99] Because nephrogenesis is not complete until the thirty-fourth week, preterm infants with nephron loss may have a slower increase in renal mass and function than term infants. Moreover, because even an abnormal kidney undergoes accelerated growth at the time of the pubertal growth spurt, the patient with limited "renal reserve" may not manifest significant renal insufficiency until adolescence.[93,100] As shown in Figure 135-1, maldevelopment of the kidneys or urinary tract, intrauterine or perinatal insults, or extreme prematurity can reduce renal function at birth. In general, the better the initial renal function, the better the outcome (trajectory 1, Fig. 135-1). Infants with compromised renal function at birth can subsequently develop a gradual decline in function (trajectory 2), or a more rapid decline (trajectory 3). Every attempt should be made to slow the progression of renal insufficiency.

Adults subjected to partial removal of a solitary kidney (after contralateral nephrectomy for renal malignancy) are at increased risk for proteinuria, glomerulopathy, and progressive renal failure.[101] Twenty-five to 30% of patients undergoing unilateral nephrectomy in childhood (mean age 7 years) developed proteinuria and renal insufficiency after a median follow-up of 25 years.[102] The infant with a GFR of less than 50% of normal for age at the time of diagnosis is even more likely to experience progressive renal insufficiency.[100,103] Although infants with posterior urethral valves and a serum creatinine concentration less than 0.8 mg/dL during the first 12 months of life are likely to maintain adequate renal function for a number of years, acceleration of the rate of renal deterioration may increase at adolescence or during adulthood.[104] As discussed above, a number of conditions are associated with impairment of nephrogenesis (Table 135-1), which may predispose patients to hypertension, proteinuria, and renal insufficiency in adulthood.[105]

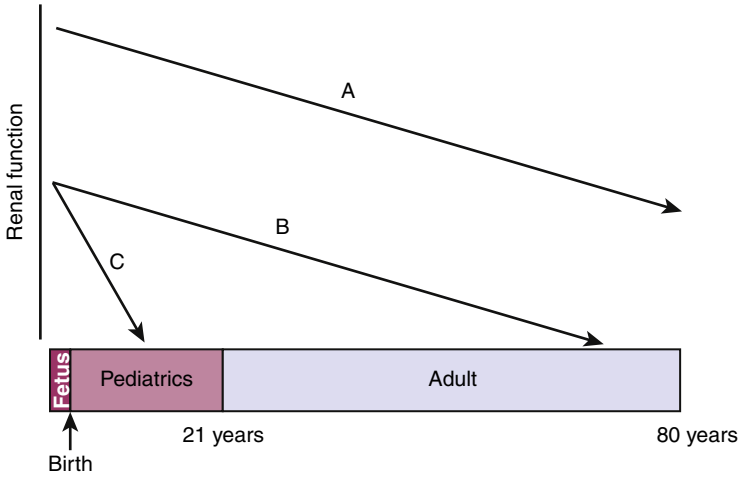


Figure 135-1 Scheme showing long-term impact of congenital renal disorders. The urinary tract abnormality develops in embryonic and fetal life, but if mild, **A,** the consequences of the condition may become apparent only later in adulthood, if at all. If moderate, **B,** progression of renal insufficiency may develop earlier in adulthood. If severe, **C,** renal failure develops in infancy or childhood. Long-term follow-up of blood pressure and urine protein excretion is desirable in virtually all patients discharged from a neonatal intensive care unit. *(Modified from Chevalier RL: Obstructive Uropathy: Assessment of Renal Function in the Fetus. In Oh W, Guignard JP, Baumgart S [eds]:* Nephrology and Fluid/Electrolyte Physiology: Neonatology Questions and Controversies. *Elsevier, 2008, Philadelphia, pp 226.)*

Long-term Follow-up

Until more comprehensive predictive data are available, the most prudent approach to follow-up of the neonate with possible nephron loss from any cause is periodic measurement of somatic and renal growth (by ultrasonography), blood pressure, GFR (by serum creatinine concentration), and urine protein excretion (by urine protein-creatinine ratio). In addition, urine culture; urinalysis; and serum calcium, phosphorus, and total carbon dioxide concentration should be monitored in selected high-risk patients. By continual tracking of these parameters, ultimate renal adaptation can be optimized for each patient. It appears that the progression of renal disease may be slowed by the judicious inhibition of intrarenal angiotensin II by using either an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker.[106] In addition to controlling hypertension, a number of experimental studies have shown a salutary effect of angiotensin inhibition on proteinuria and the development of interstitial fibrosis. As noted above, because angiotensin II is necessary for normal renal development and for maintaining renal hemodynamics perinatally, inhibition of angiotensin should be avoided if possible during the perinatal and neonatal periods. Recent studies in neonatal rats with partial ureteral obstruction show that angiotensin-converting enzyme inhibitors or angiotensin receptor blockers can actually exacerbate the renal lesions if administered too early.[107,108] However, if given after the period of nephron maturation (after weaning in the rat), the effects are salutary.[109,110] In view of their vasoconstrictor effects, patients with a reduced number of nephrons should also avoid the use of nonsteroidal antiinflammatory drugs. The combination of improved perinatal/neonatal evaluation and management, as well as seamless longitudinal follow-up by the pediatrician and internist, should lead to optimal preservation of renal function in infants born with a reduction in renal mass.

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Pathophysiology of Acute Renal Failure in the Neonatal Period

Acute renal failure (ARF) is the precipitous development of azotemia resulting from a depressed rate of glomerular filtration. Specific causes of ARF have conventionally been classified into prerenal, renal, or postrenal, thereby localizing the primary site of the disorder. *Prerenal failure* describes a process whereby a disorder in the systemic circulation causes renal hypoperfusion, and it reflects contraction of the intravascular volume or cardiac pump failure, or both. The implication of this disorder is that correction of the underlying disturbance, with volume repletion or improvement in cardiac function, or both, will restore glomerular filtration. When correction of the circulatory impairment does not restore a normal level of filtration, development of renal parenchymal damage and transition to *intrinsic renal failure* are implied. An important subset of intrinsic renal failure is *acute tubular necrosis*. Although necrosis is not always evident histologically, the term connotes a clinical syndrome that results from ischemia and excludes prerenal and postrenal failure, as well as intrinsic renal damage resulting from vascular, glomerular, or interstitial lesions. *Postrenal failure* refers to obstruction of urine flow by a bilateral lesion or a site within the bladder or urethra. An important example of the latter in the neonate is posterior urethral valves.

The final common pathway that underlies ARF, regardless of the cause or primary site of injury, is a fall in the glomerular filtration rate (GFR), which becomes inadequate to eliminate metabolic waste products. The total GFR in both kidneys depends on the number of filtering nephrons and the GFR in each individual nephron. Thus reduction in the total kidney GFR may result from a loss of a number of filtering nephron units or a decrease in the rate of filtration in individual nephrons. However, unless the cause of ARF is trauma, surgery, or thrombus involving circumscribed areas of the kidney, a reduction in nephron numbers ordinarily does not contribute to the decreased GFR in ARF in neonates. This finding is in contrast to the pattern in chronic renal failure, in which, because functional internephron heterogeneity predominates, some nephrons are severely damaged or even completely nonfunctional, whereas others attain normal (or increased) function. Some studies have revealed considerable variability in nephron number among physiologically normal individuals.[1] Although it is not known whether such variability in the complement of nephrons affects the occurrence or severity of acute injury, it appears to have profound impact on development of hypertension and progressive renal damage including chronic renal failure after acute injury (see later).

Certain nephron populations may be at greater risk for damage. In experimental studies of adult animals, deeper nephrons sustain greater injury after ischemia than do superficial cortical nephrons, and the deeper medullary region of the kidney appears metabolically at greater risk for ischemic damage.[2,3] These observations may be relevant in the premature infant in whom glomerulogenesis proceeds in a centrifugal manner and continues through the 36th week of gestation.[4,5] It is possible, therefore, that renal injury in an extremely premature baby would occur in the deeper, perhaps more vulnerable, areas of the kidney before

the relatively more resistant cortical nephrons have developed. This is an especially pivotal locus with respect to nephron generation, and injury at this site may have additional adverse implications in limiting the number and integrity of future nephrons. It is important to point out that recovery of renal function in a premature baby after renal insult likely represents healing of some injured nephrons and acquisition of new nephrons, at least through 34 to 36 weeks gestation, after which no further nephrogenesis occurs, no matter how extensive the loss of renal parenchyma. In this connection, a recent study in neonates in whom an autopsy was performed at 30 to 105 weeks postconceptual age found that extremely premature infants who sustained maximum creatinine above 2.0 mg/dL had radial glomerular count of only 60% of normal. Of interest, extremely preterm infants without a history of such creatinine increase had radial glomerular count of 80% of normal, indicating the possibility that even subtle degrees of renal insult may contribute to reduction in the complement of nephrons in survivors.[6]

Not only are certain nephron populations at greater risk of damage, but also certain nephron segments, particularly those located in the outer medulla, appear especially susceptible to injury. Thus the straight segment of the proximal tubule (S3 segment) and the medullary thick ascending limb demonstrate heightened susceptibility to ischemic and nephrotoxic injuries.[7] This susceptibility relates to low oxygen tension in this region, limited capacity to achieve anaerobic respiration, and high workload in these segments.[7,8] Impairment of oxygen delivery to the susceptible outer medullary region has been linked to endothelial injury of the peritubular capillaries, together with leukocyte activation that physically impedes blood flow.[9] The endothelial injury, in turn, is related to reactive oxygen species, whereas leukocyte infiltration has been related to adhesion molecules, such as selectins and integrins, as well as inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor α (TNFα).[9-13] Medullary vascular congestion appears pivotal to the central pathologic lesion of ARF, namely, tubular injury.[11-13] Intravital videomicroscopy after renal artery occlusion and release demonstrates an immediate recovery of erythrocyte velocity in peritubular capillaries, which is followed by a profound and sustained deceleration in blood flow.[14] Morphologically, disruption of peritubular endothelial cell actin cytoskeleton and junctional complexes has recently been documented in peritubular endothelial cells as well, allowing extravasation of capillary contents into interstitial space.[15] Functional importance of the peritubular capillary endothelium has been demonstrated by intraaortic injection of endothelial cells at the time of renal reperfusion (after 45 minutes of renal ischemia). which prevented the four-fold increase in creatinine observed in control ischemic rats and normalized the renal histologic injury scores.[14] Injection of HEK-293 cells, stably transfected with endothelial nitric oxide synthase, also ameliorated the postischemic rise in creatinine and fall in peritubular capillary blood flow.[14] These same investigations subsequently demonstrated that adult skeletal muscle stem cells differentiated in vitro into endothelial lineage cells and infused at the time of ischemia-reperfusion not only were incorporated into renal vascular structures including peritubular capillaries but also ameliorated the subsequent rise in creatinine.[16] Persistent abnormalities in peritubular capillaries may also link acute renal dysfunction with persistent or progressive renal injury later. Thus, in a rat model of renal ischemia-reperfusion, although renal tubular morphology had normalized by 4 and 8 weeks after injury, significant reduction in the density of peritubular capillaries persisted to 40 weeks.[17] Moreover, chronic reduction of renal interstitial oxygen delivery was documented in postischemic rats with reduced density of peritubular capillaries. Thus chronic tissue hypoxia may be among the mechanisms leading to renal interstitial scarring and progressive deterioration in renal function seen after ARF in the neonatal period (see later).[18]

As noted earlier, regardless of the origin or primary site of injury, the decrease in whole-kidney GFR in ARF reflects a decreased rate of glomerular filtration in individual nephrons. It is useful, therefore, to review the conceptual factors determining single-nephron GFR, along with pertinent clinical examples and molecular studies that illustrate their individual roles in decreasing the GFR. Reduction in the single-nephron GFR in ARF can be attributed to changes in one or more of the following parameters (Figure 136-1)[19]: (1) the rate of glomerular plasma flow; (2) glomerular transcapillary hydraulic pressure difference (the pressure within the glomerular capillary bed favoring ultrafiltration of fluid minus the pressure in the proximal tubule opposing ultrafiltration); (3) systemic plasma colloid osmotic pressure; and (4) permeability properties of glomerular capillaries. Although perturbations in these parameters can describe any type of ARF,[19-21] this discussion focuses primarily on the pathophysiologic mechanisms in intrinsic ARF.

PLASMA FLOW RATE

A decreased glomerular plasma flow rate is seen in systemic circulatory impairment (prerenal failure) and may continue even after systemic and circulatory impairments are apparently restored (intrinsic renal failure). This hypoperfusion reflects enhanced

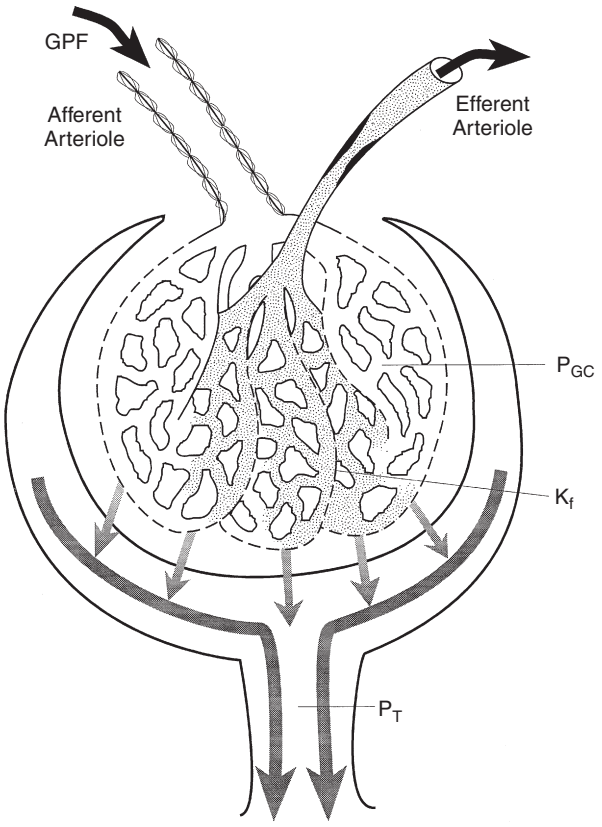


Figure 136-1 Schematic presentation of the glomerular filtration process. Glomerular filtration is determined by the rate of glomerular plasma flow (GPF) entering the afferent arteriole; hydraulic pressure within the glomerular capillary (P_{GC}) minus hydraulic pressure in proximal tubule (P_T); and glomerular capillary ultrafiltration coefficient (K_f), which describes the permeability properties of the glomerular capillary membrane. *(Modified from Winkler D, Elger M, Sakai T, Kriz W: Branching and confluence pattern of glomerular arterioles in the rat,* Kidney Int *39[Suppl 32]:S2, 1991.)*

vasoconstriction of the renal vasculature.[22,23] Increasing renal blood flow by volume expansion or administration of vasodilators before or soon after the onset of renal failure can prevent or ameliorate the development of certain human and experimental ARF. Most treatments designed to increase filtration by increasing renal blood flow failed once renal failure was established. Several agents, however, have been shown to increase renal blood flow with resultant improvement in GFR, even in well-established ARF in animal models and in humans. These agents include atrial natriuretic peptide, insulin-like growth factor I, hepatic growth factor, α-melanocyte–stimulating hormone, osteogenic protein-1, and antagonists of adenosine or endothelin.[24-31] Theophylline, which blocks binding of the vasoconstrictor adenosine to its receptor, has recently been shown to reduce renal dysfunction after perinatal asphyxia.[32,33] Two different studies found that, among those who received treatment, the rates of renal dysfunction were less, peak creatinine was less, and urine output was increased, whereas death rates and neurologic outcomes were similar to those in the untreated group. However, caution has been urged in interpreting these data as death in babies given treatment with theophylline was linked to persistent pulmonary hypertension from both studies (with no persistent pulmonary hypertension deaths in the placebo groups).[34] It should be noted that decreased blood flow does not typify all cases of ARF, and attempts to increase renal blood flow in this setting would not be expected to be universally successful. The clinical implications of these observations are that trials of volume infusion and vasodilators may be useful in the prevention and treatment of ARF, especially in the early stages. However, these maneuvers carry the risks of systemic vasodilation and hypotension that could worsen renal perfusion, as well as volume overload and congestive heart failure once the transition has been made to established renal failure.

Despite the near-normal renal plasma flow observed in many forms of clinical and experimental ARF, vascular responsiveness of the kidney is impaired and may contribute to hypofiltration. The ability of the renal vasculature of healthy persons to vasodilate and vasoconstrict is the basis for maintaining remarkably constant levels of renal blood flow and glomerular filtration in the presence of sudden changes in systemic pressure, that is, renal autoregulation.[35] For example, decreasing renal perfusion pressure causes the preglomerular, afferent arteriole to vasodilate and the postglomerular, efferent arteriole to constrict (see Figure 136-1). The sum total consists of a decrease in total renal vascular resistance and preservation of renal blood flow. In contrast, because blood flow autoregulation appears to be impaired in ARF, decreasing renal perfusion pressure increases renal vascular resistance and decreases renal blood flow, and this may be sufficient to cause hypofiltration.[36-40] This is important because even minimal (not hypotensive) decreases in systemic blood pressure may cause additional renal ischemia and may perpetuate hypofiltration or even parenchymal renal damage in the already injured kidney.[37] These observations underscore that seemingly normal values of renal blood flow belie the abnormal autoregulation of injured kidneys. Abnormal vasomotor response in ARF is multifactorial and includes increased responsiveness to renal nerve stimulation, norepinephrine, endothelin, and increased afferent arteriolar cytosolic calcium (Figure 136-2).[12,41,42] Decreased renal microvascular responsiveness to vasodilators such as prostaglandins, acetylcholine, bradykinin, and nitric oxide also appears to exist.[43,44] In this connection, endothelial nitric oxide synthase knockout mice, despite higher systemic blood pressure, have lower baseline renal blood flow and higher renal vascular resistance than wild-type mice.[45] When administered a dose of endotoxin not associated with a fall in mean arterial pressure, endothelial nitric oxide synthase knockout mice demonstrated a fall in GFR and renal blood flow not seen in wild-type mice. This was accompanied by acute tubular injury in approximately 10% of renal tubules versus none in wild-type mice.

At first glance, newborn babies seem to be at risk for hypoperfusion. When compared with adults, normal newborns have a low level of renal plasma flow, and their systemic blood pressure is less than the lower limits for autoregulation in adults (80 to 90 mm Hg). However, normal young animals autoregulate blood flow efficiently at the lower prevailing systemic blood pressure.[39,40,46] By contrast, young animals that undergo removal of one kidney demonstrate impairment in renal blood flow autoregulation immediately after uninephrectomy, and this impairment persists for at least 4 to 5 weeks.[46] Furthermore, young uninephrectomized animals that are also volume contracted have a higher renal vascular resistance and lower renal blood flow over a range of decreasing renal perfusion pressures when compared with saline-infused animals. Thus renal injury and neonatal volume status determine the effectiveness of renal blood flow autoregulation in the setting of ARF. The clinical implication is that volume depletion negatively affects this protective autoregulatory mechanism and makes the infant more susceptible to the development and propagation of ARF.

GLOMERULAR TRANSCAPILLARY HYDRAULIC PRESSURE DIFFERENCE

Glomerular capillary hydraulic pressure favors glomerular filtration. It is opposed by pressure in the proximal tubule (see Figure 136-1). Although both these pressures are fairly stable under

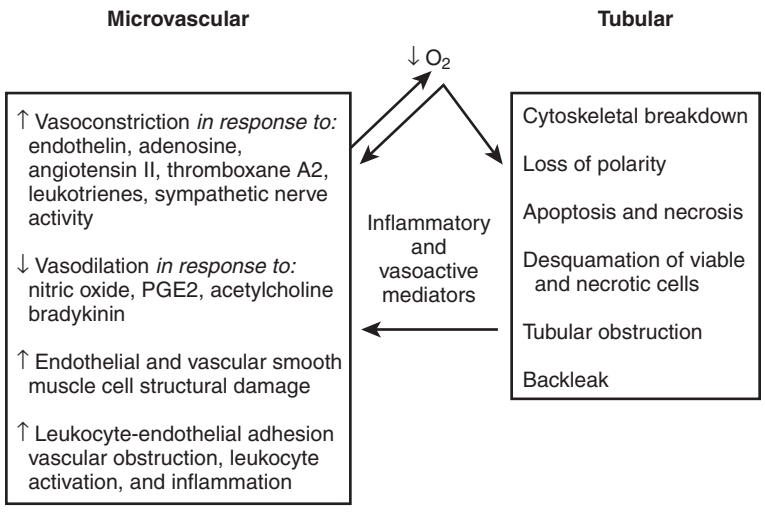


Figure 136-2 Interacting microvascular and tubular events contributing to the pathophysiology of ischemic ARF. *(Modified from Bonventre JV, Weinberg JM: Recent advances in the pathophysiology of ischemic acute renal failure,* J Am Soc Nephrol *14:2200, 2003.)*

normal circumstances, the pathophysiologic processes of ARF can affect either or both by several mechanisms. Decreased renal perfusion pressure normally causes a selective increase in the postglomerular, efferent arteriolar resistance, thereby maintaining intraglomerular capillary pressure, which contributes to constancy in GFR. When the systemic blood pressure falls to less than the autoregulatory range, glomerular capillary pressure, renal plasma flow, and the GFR follow the fall in systemic blood pressure.[35] Thus, in circulatory collapse, as may precede the development of ARF, the autoregulatory response of the renal vasculature is exceeded, and severe hypofiltration may result. In addition to profound hypotension, which overwhelms the normal autoregulatory mechanisms, under certain conditions even relatively modest systemic hypotension is associated with apparent ineffective autoregulation and a decrease in the GFR. As noted earlier, in animals with volume depletion or congestive heart failure, a 30% decrease in renal perfusion pressure (which did not affect GFR in normal rats) caused a more than 25% fall in the GFR.[47] The impaired ability to preserve the GFR in the presence of reduced renal perfusion pressure in both conditions was, at least in part, the result of a fall in the glomerular capillary pressure, which, in turn, reflected an inadequate vasoconstrictive response within the efferent arterioles.

As noted earlier, afferent arteriolar dilation and renal blood flow adjust to decreasing renal perfusion pressure in adult and immature animals. However, one study found that although decreasing the renal perfusion pressure by approximately 30% from baseline was accompanied by a minimal fall in the GFR in adult rats, in young rats the GFR plummeted by more than 80%.[39] Micropuncture experiments revealed that the profound hypofiltration in young rats largely reflected decreased glomerular capillary pressure. Because angiotensin II (AII) is known to vasoconstrict the efferent arteriole and to maintain glomerular capillary pressure, it is thought to have an important role in autoregulation of the GFR. A similar degree of water deprivation causes a greater increase in plasma renin activity in adult animals than in immature ones, and a higher dose of exogenous AII is required in immature than in adult animals to cause a similar increase in glomerular capillary pressure.[39] With these factors taken together, young animals have limited ability to activate at least one vasoconstrictor system, namely, AII, and the immature efferent arteriole may have limited responsiveness to AII. Thus, even in the presence of afferent vasodilation after decreasing renal perfusion pressure, young animals may develop hypofiltration. These observations provide a mechanism for dissociation between renal blood flow and the GFR in that dilation in the afferent arteriole without sufficient vasoconstriction in the efferent arteriole is insufficient to maintain a transcapillary pressure that promotes glomerular filtration. Of the two currently recognized receptors for AII, AT1 and AT2, the AT1 is most abundantly expressed and transduces the bulk of the recognized actions of AII including efferent arteriolar constriction.[48,49] Glomerular AII hyporesponsiveness in the neonatal kidney does not appear to reflect inadequate AT1 receptor density, because kidney AT1 expression peaks after birth at twice the adult level.[50] Further, AII availability is also maximized, reflecting an abundance of renal angiotensin-converting enzyme that increases after birth such that within 2 weeks of birth it surpasses adult levels, as does circulating angiotensin-converting enzyme.[51] Moreover, renin production in neonates is robust and extends beyond the juxtaglomerular apparatus to include more proximal segments of the renal arterial tree.[52] Angiotensinogen also undergoes a dramatic postnatal increase in liver expression before decreasing and settling to the adult level.[53] Overall, the receptors and the enzymes required for production of the mature AII ligand are exuberantly expressed in the neonatal kidney. The observed hyporesponsiveness of the neonatal kidney therefore appears to reflect inadequate postnatal maturation of postreceptor processes. It is possible, however, that the blunted vasoconstriction of the neonatal efferent arteriole in response to AII reflects the vasodilatory contribution of the AT2 receptor. This may occur though a direct effect of the AT2 receptor or though AT2-mediated stimulation of nitric oxide and bradykinin.[54]

The transcapillary hydraulic pressure difference includes not only the hydraulic pressure within the glomerular capillaries favoring filtration but also the opposing pressure in the tubules. Tubular injury is an important component in the pathophysiology of ARF.[7-13,19-22] Studies have clarified the cellular and molecular events that underlie the tubular injury.[55,56] Under normal conditions, tubular epithelial cell function depends on maintenance of defined apical and basolateral membranes with distinct protein populations. One such class of protein consists of the integrins, which are membrane-spanning glycoproteins located on the basolateral surface. Integrins interact with the intracellular cytoskeleton, as well as the extracellular matrix, and they mediate tubular cell adhesion.[57] Renal ischemia and subsequent adenosine triphosphate depletion induce redistribution of integrins to the apical surface and cause the actin cytoskeleton to move from its normal circumferential position to a perinuclear location (Figure 136-3). These injury-induced changes culminate in cellular rounding, retraction, and detachment from the basement membrane. Moreover, redistribution of integrins to the apical surface in the detached cells may contribute to aggregation of detached tubular cells within the lumen. This may, in turn, contribute to tubule obstruction, increased intratubular pressure, and decreasing transcapillary pressure. Indeed, infusion of the tripeptide arginine–glycine–aspartic acid that interferes with integrin adhesion mechanisms has been shown to abolish tubule obstruction and to improve renal function.[58] By contrast, in patients who underwent renal transplantation, persistence of increased tubular pressure and obstruction were documented in those patients with delayed graft function, characterized by mean GFR of 8 ± 8 mL/minute at 7 days after transplantation.[59]

Noteworthy are the observations that intratubular material contains not only cellular debris but also viable cells. Thus viable cells have been recovered from the urine of patients with ARF, a finding implying lethal cellular injury, as well as disturbance in mechanisms of normal cellular adhesion as underlying tubule pathophysiology in ARF.[60] It is also important to point out that nonviable exfoliated tubular cells are not uniformly necrotic. In fact, apoptosis appears to predominate as the mechanism of tubular cell death after ischemia, though necrosis and apoptosis appear to constitute a spectrum with apoptosis favored in the distal nephron.[61] A variety of apoptosis-instigating pathways are stimulated by tubular injury, but caspase activation appears to be the final common step.[13] In this connection, tubular injury by myoglobin (in experimental glycerol-induced ARF) was shown to be ameliorated by concomitant pan-caspase inhibition. Not only was apoptosis lessened by pan-caspase inhibition, but activation of IL-1β and IL-18 was blocked, limiting subsequent macrophage infiltration.[62]

Emerging data also support an important role for inflammatory cells and cytokines in the response after tubular injury.[63] Experimental animals demonstrate a prompt increase in serum IL-6 after renal ischemia. Immunohistochemistry demonstrates IL-6 colocalization with infiltrating macrophages in the outer renal medulla. Supernatant from S3 segment renal tubular cells injured by reactive oxygen species in vitro increases IL-6 production by macrophages in culture by six-fold.[64] IL-6 knockout mice had lower creatinine levels and histologic injury scores than wild-type at 24 hours after ischemic injury. In vivo depletion of monocytes-macrophages in experimental rats resulted in decreased tubular necrosis and renal tubular apoptosis after renal ischemia.[65] Histologic improvement in macrophage-depleted animals was associated with reduction in levels of renal messenger RNA for IL-6, TNFα, macrophage chemoattractant protein 1, and IL-1β within 23 hours after ischemia. In adults with ARF, elevation in serum

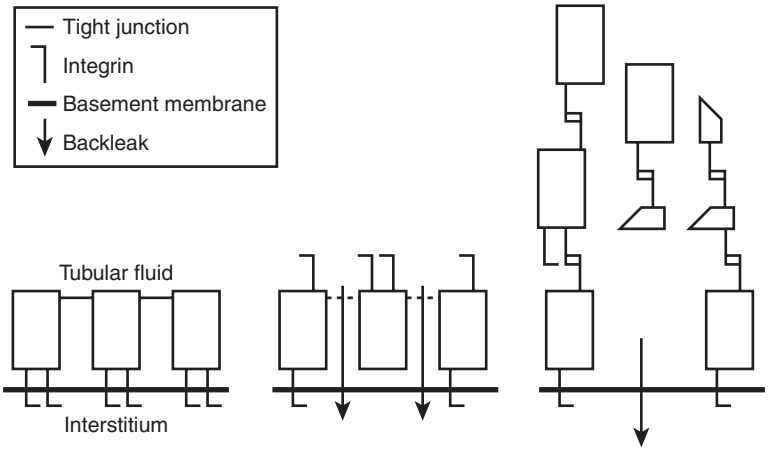


Figure 136-3 Schematic representation of the role of intratubular obstruction and back leak in ARF. *Left,* Normal tubular cells linked by tight junctions into an epithelial sheet that forms a selective barrier between the tubular fluid compartment and the interstitium. The tight junction regulates cell polarity, segregating molecules such as the integrins to the basal plasma membrane, forming the adhesive anchorage of the cell basement membrane junction. *Middle,* Ischemia-induced tight junction dysfunction leads to a loss of barrier function contributing to loss of polarity and integrin migration to the apical surface, as well as leakage of glomerular filtrate back into the interstitium. *Right,* Loss of basal anchorage molecules allows cells or cell fragments to exfoliate into the tubular lumen and adhere and aggregate with other exfoliated cells or fragments, contributing to increase in tubular pressure proximal to the obstruction. Tubular obstruction will oppose glomerular filtration, as well as accelerate back leak of tubular fluid across the denuded basement membrane. *(Modified from Lee DBN, Huang E, Ward HJ: Tight junction biology and kidney dysfunction,* Am J Physiol Renal Physiol *290:F26, 2006.)*

IL-6 at baseline and continuing for 2 weeks after injury has been observed in nonsurvivors versus survivors.[66] In fact, serum levels of IL-6, IL-8, and IL-10 all correlated with risk of death after ARF, though the association with IL-6 was the strongest. Polymorphisms of TNFα, IL-1β, IL-6, and IL-10 have been investigated in neonates in whom ARF developed after infection.[67] The allele frequency of variants of individual genes did not differ between those with and without ARF. However, the combination of variants TNFα AG and IL-6 GC or CC was found in 26% of those with ARF versus only 6% of those without ARF. What makes this somewhat unclear, however, is that IL-6 CC has been shown to be associated with lower serum IL-6 levels in healthy adults than in those with GC or GG alleles.

It is at the level of tubule that the ubiquitously expressed heat shock protein (HSP) system acts to promote stabilization of the ischemic renal tubular cell.[68-70] Thus transfection of both HSPs *HSP70* and *HSP27* has been shown to ameliorate facets of tubular cell derangement after ischemia. *HSP70* transfection lessens Na^+,K^+–adenosine triphosphatase detachment from cytoskeleton and may actually chaperone the ectopic protein because colocalization of HSP70 at the apical membrane with Na^+,K^+–adenosine triphosphatase has been observed. HSP27 is thought to maintain cellular integrity after ischemia by stabilization of the actin cytoskeleton. Genetic polymorphism of the *HSP70* gene has been studied in neonates. ARF developed in very-low-birth-weight infants homozygous for a less-active allele of HSP70 (A→G[1267]) substantially more often than in those with other genotypes, even when adjusted for established factors of renal failure in neonates.[71]

Ischemia induces redistribution of another protein—Na^+,K^+–adenosine triphosphatase, which is normally localized to the basolateral side of the epithelial cells.[72,73] This may, at least in part, be responsible for the increased urinary sodium excretion observed in ARF, a finding frequently used in establishing the diagnosis of acute tubular necrosis. In addition, increased distal sodium delivery may stimulate vasoactive substances that decrease renal blood flow through the tubuloglomerular feedback mechanism. Of interest, increased tubular sodium concentration has recently been shown to contribute to polymerization and gel formation by Tamm-Horsfall protein (secreted in the thick ascending limb), providing another link between dysregulated tubular Na^+ delivery, decreased tubular flow, and increased intratubular pressure.[74]

Significant tubular cell metabolic derangements occur with hypoxia, which further compound injury.[13,75-79] Adenosine triphosphate depletion leads to xanthine dehydrogenase conversion through increased protease action to xanthine oxidase. During reperfusion, the resultant conversion of accumulated tubular cell hypoxanthine to xanthine produces hydrogen peroxide and superoxide. Further oxidative stress in ARF may originate from mitochondrial respiratory electron transport chain and neutrophil-associated respiratory burst via the activation of membrane-bound reduced nicotinamide adenine dinucleotide phosphate oxidase, contributing to accumulation of superoxide and hydrogen peroxide. Oxygen radicals readily combine with nitric oxide to yield peroxynitrite, capable of lipid peroxidation and protein nitrosylation, leading to loss of cell viability. Production of nitric oxide in the renal tubule dramatically increases with hypoxia through tubular inducible nitric oxide synthase. Pharmacologic inhibition of inducible nitric oxide synthase or lessening of oxidative stress (through exogenous superoxide dismutase) lessened nitrotyrosine, a marker of peroxynitrite action, and decreased the peak creatinine after ischemia.[76] Moreover, a specific scavenger of peroxynitrite, ebselen, dramatically lessened peak creatinine after ischemia. *N*-acetylcysteine is a reactive oxygen scavenger currently in use for reduction of the occurrence of contrast nephropathy.[77] Edaravone, a scavenger of free radicals and inhibitor of lipid peroxidation currently used in humans for cerebral ischemia, has also been shown benefit in ischemic ARF in animals.[78] In this connection, a single nucleotide polymorphism in the coding region of reduced nicotinamide adenine dinucleotide phosphate oxidase subunit p22phox has recently been shown to impact response to renal injury.[79] Among 200 adults with ARF, those carrying the T allele (associated with higher plasma nitrotyrosine levels—a byproduct of superoxide and nitric oxide generation) showed an increased likelihood of dialysis requirement or hospital death.

In addition to direct feedback mechanisms that affect the glomerular hemodynamics, tubular injury also interrupts structural integrity, and this allows creatinine and other markers of glomerular filtration to permeate the tubules and return into the circulation (i.e., back leak) (see Figure 136-3). As noted earlier, disruption of the junctional complexes affects not only the epithelial cell polarity but also tubular permeability. Histologic and functional data in human and experimental ARF support a disruption in the integrity of tubular epithelium and the presence of back leak.[80-84] Loss of junctional complexes that encompass tight junctions, adherent junctions, desmosomes, and gap junctions are especially relevant to back leak. At least in part, tyrosine phosphorylation of proteins within adherent junctions alters paracellular permeability and likely contributes to back leak.[83] By reducing the clearance of GFR markers, as well as metabolic waste products, back leak contributes to the high levels of creatinine in the circulation and maintenance of azotemia in ARF. Persistent derangements of tight junction and adherens junction proteins (zonula occludens and beta, alpha catenins respectively) predominated in patients with sustained ARF with transtubular back leak after renal transplantation but was not observed in those with recovery of renal function.[84] Studies in adults with ARF have estimated that back leak constitutes 10% of the reduction in GFR.[12,81] In preterm neonates, however, back leak has recently been proposed as one of the explanations of transiently increased serum creatinine even without ARF, attributed to immaturity of the renal tubule.[85] Therefore it is reasonable to postulate that an immature renal tubule with back leak at baseline would be more prone to increase back diffusion of creatinine and other constituents of plasma ultrafiltrate in the setting of renal tubular structural disintegrity or functional disruption, though quantitation of this phenomenon has yet to be determined.

Repopulation of renal tubular segments by proliferating was part of the recovery process. Although tubule regeneration after injury is well recognized, mechanisms that underlie the cellular repopulation remain unsettled and include (1) proliferation of surviving dedifferentiating tubular cells that reenter the cell cycle, (2) proliferation and differentiation of resident stem or progenitor cells from within the kidney, and (3) transdifferentiation of cells that originate outside the kidney, notably, bone marrow–derived stem cells.[86-100] These observations have generated considerable excitement regarding mechanistic and therapeutic possibilities. Thus, 24 to 48 hours after injury, the presence of mitotic cells containing the mesenchymal marker vimentin and the renal developmental gene *PAX-2* suggests that dedifferentiation and proliferation of surviving epithelial cells participate in this process.[89,96] Experimental, as well as human, data confirm the ability of bone marrow–derived cells to become renal epithelial cells.[91-98] Bone marrow–derived cells engraft tubules and can differentiate into tubular epithelial cells and replace the damaged cells.[91,92,99] Although this finding is exciting, it is now believed that such replacement from the bone marrow contributes only a small percentage to regenerating tubules.[98-100] Furthermore, the observed beneficial effects of bone marrow–derived cells may relate to modulation of nonepithelial, for example, endothelial cells, especially within the peritubular vasculature,[16] as well as cell replacement–independent effects such as direct inhibition of cell death or inflammatory cell infiltration.[101] Intrarenal cells, as opposed to bone marrow–derived cells, have recently been identified as a source of epithelial cell regeneration after injury. In particular, the renal papilla appears to be a source of adult kidney stem cells.[88,102,103] A recent study that addressed the relative importance of intrinsic renal versus bone marrow–derived cells in tubular epithelial cell proliferation after ischemia-reperfusion injury found that the overwhelming majority (89%) of the proliferating epithelial cells were of intrarenal origin; only 11% of these cells originated from bone marrow–derived cells.[99] It is of interest that the study also noted that a large number of interstitial cells within the injured kidney were bone marrow derived. This finding was recently expanded by a study that traced the fate of bone marrow–derived cells and showed that approximately 32% of myofibroblasts present in the postischemic kidney originated from the bone marrow cells.[104] The study further showed that these cells have increased production of extracellular matrix and contribute significantly to fibrosis after renal ischemia. Overall, it is currently believed that marrow-derived cells may be an important source for regenerating tubular cells. However, caution is in order in considering cell-based therapies to encourage renal tubule regeneration as stem cells. Bone marrow–derived cells may take on unexpected phenotypes that may participate in progressive renal destruction observed after acute renal injury.

PLASMA COLLOID OSMOTIC PRESSURE

Theoretically, an increase in systemic plasma colloid osmotic pressure, which opposes filtration, will decrease the GFR. However, newborn infants normally have lower levels of plasma protein (6.5 mg/dL in term babies vs. 7.5 mg/dL in adults), which tends to increase the GFR. Furthermore, hyperoncotic states (e.g., dysproteinemia) are almost unheard of in neonates, and even severe dehydration-induced hemoconcentration is unlikely to be accompanied by changes in plasma colloid osmotic pressure sufficient to change the GFR.[105]

GLOMERULAR CAPILLARY ULTRAFILTRATION COEFFICIENT

In addition to alterations in the pressures and flows already described, changes in the glomerular membrane properties themselves can contribute to the depressed level of glomerular filtration in ARF. The glomerular capillary ultrafiltration coefficient (K_f) encompasses the capillary surface area available for filtration and the permeability property of the capillaries themselves. Although technical advances have permitted study of this parameter in numerous experimental diseases, difficulties relating to the size and accessibility for direct glomerular measurements in immature animals are evidenced by the paucity of information on K_f in ARF in neonates. It is known that K_f is low in immature animals and increases with maturation.[5] One experimental study also suggested that a decrease in K_f may lower the single-nephron GFR to a greater extent in immature than in adult animals.[106] Entities causing inflammatory, proliferative, and microangiopathic changes in glomerular capillaries, which are expected to decrease glomerular capillary permeability, are distinctly unusual causes of ARF in the neonate. Renal ischemia and nephrotoxins are the most frequent pathogenic factors. In these conditions, glomeruli are, morphologically (relatively), spared.[12,13] However, because of our lack of knowledge regarding the primary site determining the impedance to the filtrate, it remains unclear whether subtle morphologic changes noted in some forms of human and experimental ARF, that is, reduction in endothelial fenestrae, cause decreased glomerular capillary permeability. Moreover, changes in the permeability of glomerular capillaries do not necessarily require fixed structural alterations in glomerular anatomy. Instead, a decrease in K_f may occur by hormonal autacoid (AII, vasopressin, prostaglandins, leukotrienes, endothelins) regulation of contractile cells within the glomerulus, which modulates the capillary area available for filtration.[105] In this regard, both gentamicin and amphotericin B are well-known contributors to ARF in neonates, yet remain in common use because of their excellent antimicrobial activity. As a result optimal dosing in neonates continues to be a therapeutic goal.[107,108] Both antimicrobials cause a decrease in GFR in part through reduction in K_f, which appears to be mediated in part by mesangial cell contraction.[109-111]

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SECTION XVII

Fluid and Electrolyte Metabolism

137 Fluid Distribution in the Fetus and Neonate

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The most dramatic changes in body fluid compartments occur during intrauterine growth when approximately 4000 mL of water accumulates in the human uterus (2800 mL in the fetus, 800 mL amniotic fluid, and 400 mL in the placenta) and during the postnatal adaptation of the neonate from the "aquatic" intrauterine to the "terrestric" extrauterine environment. In relationship to body weight, the amount of total body water (TBW) and its distribution between the body fluid compartments are markedly different in the fetus and newborn than in the adult. This implies that, during the perinatal period, the associated volume-regulatory mechanisms are functioning at a level that is unique in comparison with that in later life. In addition, the amniotic fluid that surrounds the fetus is often considered to be an extension of the fetal extracellular space, again suggesting unique volume regulatory mechanisms before birth. This chapter starts with a review about TBW content and the regulation of fluid distribution during fetal life including the role of the lymphatic system and of the capillary membrane in mediating fluid homeostasis. The postnatal adaptation of the neonatal body fluid compartments is then described with special emphasis on the clinical implications of these physiologic changes. The last section focuses on the effects of perinatal events on the regulation of blood volume in the immediate postnatal period.

DEVELOPMENTAL CHANGES OF BODY FLUID VOLUMES DURING FETAL LIFE

Total Body Water

In 1961, Friis-Hansen[1] reported the developmental changes in the relative fluid volumes in humans from the early fetal period through adulthood. As shown in Figure 137-1, in the early fetal period, approximately 95% of the fetus is water. The proportion of total body weight that is water gradually decreases throughout the fetal period to reach 80% at 8 months gestation and 75% water at term. This reduction of TBW is caused by the accumulation of body solids during growth. In the first two thirds of gestation body solids increase because of the accretion of protein and minerals, whereas little fat deposition occurs. We know from postmortem chemical analyses that at 27 weeks gestation 86% of body weight is water, 12% is fat-free dry solids, and only 2% is fat.[2] Measurements in appropriate–for–gestational age (AGA) preterm infants with birth weights below 1500 g showed TBW contents of 83%[3] and no detectable fat accumulation in preterm infants when measured by dual photon absorptiometry.[4] During the last trimester of gestation the proportion of body solids increases from 14% to 24% of body weight because of the deposition of body fat, which is 2% of body weight at 27 weeks gestation and 10% to 15% of body weight at term.[2]

In human neonates born after intrauterine growth restrictions, the TBW content is increased compared with normal intrauterine growth. In small–for–gestational age (SGA) preterm neonates, the mean TBW content was 62 mL/kg higher than in AGA preterm neonates,[5] and in term SGA neonates mean TBW content was increased by 76 mL/kg[6] to 102 mL/kg.[7] The higher body water content in preterm SGA infants reflects the reduced deposition of fat-free dry solids.[8] In contrast, the increase in body water content in term SGA neonates reflects primarily the reduced deposition of fat, which usually occurs during the third trimester of gestation in fetuses with normal growth. Several lines of evidence indicate that adipose tissue is indeed reduced in SGA term neonates. Intrauterine growth restriction late in gestation resulted in fetuses with reduced abdominal wall fat thickness measured by ultrasound.[9] The percentage of adipose tissue estimated from dual photon absorptiometry with use of ^{153}Gd magnetic resonance tomography was 2% in term SGA neonates compared with 13% in term AGA neonates.[4] Moreover, a smaller skinfold thickness in SGA term neonates indicated less subcutaneous fat.[10] One recent study

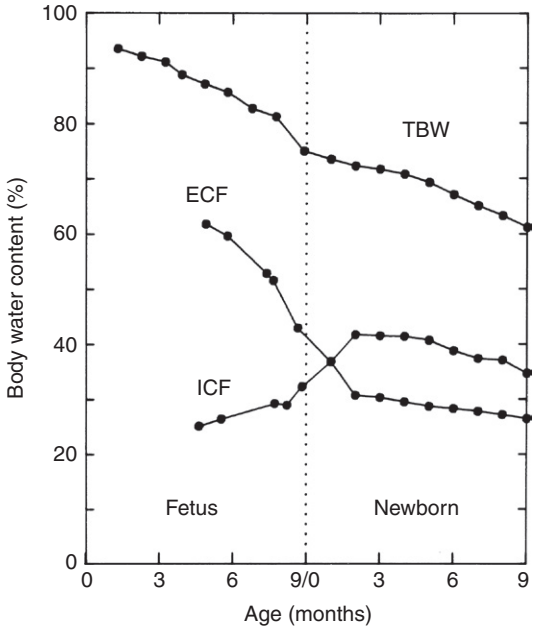


Figure 137-1 Total body water *(TBW)* content and fluid distribution between intracellular fluid *(ICF)* and extracellular fluid *(ECF)* compartments in humans during the fetal and neonatal periods and during the first 9 months after birth. *(Data from Friis-Hansen B: Body water compartments in children: changes during growth and related changes in body composition,* Pediatrics *28:169, 1961.)*

† Deceased

using dual-energy X-ray absorptiometry analysis also found reduced fat contents in near-term SGA compared with normal term infants, although the differences did not reach statistical significance.[11]

Taken together these finding suggest that the decrease in TBW with gestation is due to the deposition of protein, minerals, and body fat during normal intrauterine growth. The increased TBW after intrauterine growth restriction reflects reduced protein and mineral deposition in preterm SGA neonates and primarily reduced fat deposition in term SGA neonates.

Extracellular and Intracellular Fluid Compartments

Concurrent with the decline in TBW content during fetal life, major changes occur in the distribution of fluid. The TBW compartment can be subdivided into the extracellular volume and the intracellular volume. Early in gestation, the extracellular volume is extremely large, accounting for 62% of body weight, and is more than twice the size of the intracellular volume. The relatively large extracellular fluid space during fetal life is because growth occurs by cell division rather than by cell growth and produces tissues with small cells surrounded by a broad layer of extracellular fluid. By enlargement of cell size during normal intrauterine growth in the second half of gestation, the intracellular space increases from 25% of body weight at midgestation to 32% at term, whereas at the same time the extracellular volume decreases from 62% to 43% of body weight.[1]

Intrauterine growth restriction, unless severe, seems to have little influence on the distribution of TBW between the intracellular and extracellular volumes. A disproportionate expansion of extracellular volume of 73 mL/kg was found only in a subgroup of neonates with severe growth restriction indicated by a reduced weight and length compared with neonates with moderate growth restriction indicated by reduced weight but normal length.[12]

Another factor that reduces the extracellular water content in the fetus is the decrease of hyaluronan in the extracellular matrix with advancing gestation. Hyaluronan has a high water-binding capacity and is the most abundant component of the fetal extracellular matrix during the early phase of rapid cell multiplication and migration. Proteoglycan and collagen have lower water-binding capacities, and their concentrations increase with the subsequent tissue condensation.[13]

The extracellular fluid compartment is functionally divided into the plasma and the interstitial fluid space. Little is known about factors that regulate the distribution of fluid between the plasma and interstitial space in human fetuses because in vivo measurements have been made only in animals. In late-gestation fetal sheep, the best estimate for plasma volume is 76 mL/kg.[14] Interstitial volume has been estimated to average three times this volume, or 235 to 240 mL/kg of body weight.[15,16] It should be noted that the ratio of interstitial fluid volume to plasma volume in the sheep fetus (3:1) is similar to that which occurs in the adult. But when this ratio is corrected for the roughly 30% of the fetal plasma that circulates outside the fetal body (i.e., in the umbilical cord and placenta) the ratio of interstitial-to-plasma volume becomes 4.4:1 in the fetus. This clearly indicates that the interstitial space of the fetus is expanded relative to that of the adult. The elevated interstitial volume is consistent with the observation that interstitial compliance in the ovine fetus is roughly 10 times that observed in the adult.[15] Because the weight-normalized fetal plasma volume (76 mL/kg) does not vary with fetal weight over the range of 1 to 4 kg fetal weight,[14] the large decrease in fetal extracellular fluid volume with advancing gestational age must be a reduction in interstitial fluid volume rather than plasma volume.

Blood Volume

The volume of blood circulating in the fetus is considerably greater than that in the newborn, because roughly one third of the fetal blood volume is contained in the cord and fetal side of the placenta.[17] Reported values for fetuses of several species are shown in Table 137-1. These data should be interpreted cautiously because the potential exists for major methodologic errors. Human data are derived from fetuses with anemia receiving intrauterine blood transfusions. The fetoplacental blood volume in the human fetus increases from 100 to 150 mL (130-200 mL/kg fetal body weight) at 25 weeks gestation to 300 to 350 mL (130-160 mL/kg fetal body weight) at 35 weeks gestation.[18,19] Total placentofetal blood volume in human neonates at term is approximately 120 mL/kg of which 70 mL/kg is in the fetus and 50 mL/kg in the placenta.[20] Changes in fetal blood volume can occur by fetal-to-maternal hemorrhage and by fetal-to-fetal transfusion in monochorionic twins. Large fetomaternal hemorrhages can induce acute hypovolemia and fetal death. Fetuses that survive a large fetomaternal hemorrhage are born with anemia. The more profound the anemia at birth the higher the risk for adverse neonatal outcome.[21] Fetofetal transfusion develops in monochorionic twins via placental vascular anastomoses. If a pressure imbalance exists between the twin circulations, for example via arteriovenous anastomoses, a net transfusion of blood occurs from one twin (donor) to the other (recipient). This results in hypovolemia, a decrease in interstitial volume, and oligohydramnios in the donor twin and hypervolemia, increased interstitial volume (hydrops), and polyhydramnios in the recipient twin.[22] Thus abnormalities in circulating fetal blood volume in the form of large fetomaternal hemorrhage and twin-twin transfusions result in considerable morbidity and mortality.

Lymph and Lymph Flow

The volume of fluid within the lymphatic system is small and averages only 1 mL/kg body weight in adult dogs as estimated from lymph flow dynamics.[23] An estimate of lymphatic volume in the fetus or neonate has not been made. However, it is reasonable to speculate that developmental changes exist in lymphatic volume.[24]

A few estimates of lymph flow in developing animals allow comparison with adult values. In studies in unanesthetized fetal sheep, thoracic duct lymph flow averaged 0.25 mL/minute/kg when low-resistance catheters were used and the lymph was returned to the fetal circulation.[25] Basal lymphatic flow rates in the fetus are substantially higher than those in the adult and may

TABLE 137-1

Blood, Plasma, and Red Blood Cell Volumes in Fetuses

Blood Volume (mL/kg)	Plasma Volume (mL/kg)	RBC Volume (mL/kg)	Species	Age	Reference
162*			Human	16-22 wk	118
160†			Human	35 wk	19
105‡			Human	Term	53
101†			Human	18-31 wk	18
110*	87*	34‡	Sheep	125-137 days	14
134§	90‡	45‡	Sheep	112-146 days	81

*Determined with plasma label.
†Determined from hematocrit changes during packed cell transfusions.
‡Determined with RBC label.
§Determined with RBC plus plasma label.

be considerably higher than in the newborn. Thus the lymphatic system of the fetus must handle the increased interstitial fluid and the increased fetal lymphatic flow. In addition, a slight increase in central venous pressure can result in a dramatic decrease in the lymphatic flow rate. Therefore the fetus is particularly susceptible to the development of edema.

Even though lymphatic flow rates in the fetus and neonate are higher than those in the adult, the concentration of protein in the thoracic duct lymph displays an apparently uniform relationship to the plasma protein concentration. Under resting conditions, the thoracic duct lymph protein concentration averages 50% to 75% of the plasma protein concentration in the fetus, newborn, and adult. This does not mean that the absolute protein concentrations are the same, because fetal plasma protein concentrations increase during gestation and average a little more than half of adult values at term (although species-related differences exist).[23,25]

Both fetal and newborn sheep undergo an increase in left thoracic duct lymph flow after vascular volume expansion.[26,27] Extensive vascular volume loading increases the lymph flow rate to a maximum of 3.5 times normal, and draining the thoracic duct externally causes a rapid fall in fetal blood volume of 10% within 2 to 3 hours. Studies in the ovine fetus[28-30] have found that angiotensin II augments left thoracic duct lymph flow and atrial natriuretic factor suppresses lymph flow.

Maldevelopment of the lymphatic system can lead to lymphedema, congenital chylothorax, ascites, or hydrops fetalis. The dysplastic lymphatics obstruct the lymph flow and increase lymphatic pressure until lymph starts to leak into the surrounding tissues. The dysplastic lymphatics can be hypoplastic, hyperplastic, or lymphangiomatous. The pathogenesis is not completely understood, but members of the vascular endothelial growth factor and angiopoietin families seem to have an important role in lymphatic development.[31]

Amniotic Fluid Volume

The fetal membranes surround amniotic fluid. Amniotic fluid volume (AFV) is largely dependent on fetal organ function in the second half of gestation and is regulated within a narrow range to maintain fetal fluid status.[32] Therefore it can be regarded as one of the fetal body fluid compartments. During an uncomplicated pregnancy AFV shows a slow increase from 350 mL at 20 weeks gestation to 700 mL at term.[33] The chemical composition of amniotic fluid also changes with gestation. Up to around the 20th week it reflects that of fetal extracellular fluid because the skin of the fetus is permeable to the free exchange of substances. When fetal urine begins to enter the amniotic sac, amniotic fluid osmolarity decreases slightly compared with fetal blood and decreases further after keratinization of the fetal skin until it reaches values of 250 to 260 mOsm/kg water near term.

With progress of gestation, fetal organ function becomes more and more important for the regulation of AFV. After midgestation, the two primary sources of amniotic fluid are fetal urine and tracheal and pulmonary fluid secretion. After midgestation the fetus produces 300 mL/kg of hypotonic urine per day. This reduces amniotic fluid osmolarity and provides a large potential force for the outward flow of water across the intramembranous pathway. The fetus secretes 60 to 100 mL/kg of tracheal and pulmonary fluid each day, which serves to expand the airways and promote normal lung growth. This fluid is formed by an active secretion of chloride ions into the alveolar spaces, resulting in a progressive accumulation of fluid in the lungs as gestation progresses. Because the secretion of lung fluid is far in excess of that needed for volume expansion of the lungs, the excess lung fluid exits through the trachea. Rather than a gradual egress, fluid appears to flow out of the trachea in bouts and either is swallowed or enters the amniotic fluid.[34] In fetal lambs, the tracheal flow rate averages 4.4 mL/kg/hour during the latter half of gestation,[34-36] and, in a 24-hour period, this volume represents approximately 10% to 15% of body weight. The volume of fluid that resides in the airways during the fetal period depends on the developmental stage of the lungs. Volume is very low early in gestation and increases rapidly with the accelerated lung growth that occurs late in gestation. At term gestation in fetal lambs, lung liquid volume averages 45 mL/kg of body weight,[34] or slightly more than half of the plasma volume. This large volume suggests that lung liquid may be a potential source of fluid to the fetus; however, it is not certain how easily fetal lung fluid can be transferred to the circulation.

Removal of amniotic fluid occurs by fetal swallowing of approximately 200 to 250 mL/kg/day and by intramembranous absorption of amniotic fluid into the fetal blood, which perfuses the fetal surface of the placenta. Swallowing during the perinatal period is episodic, and the volume of fluid swallowed in one event may be as large as 100 to 200 mL, which is roughly equal to 3% to 5% of body weight. Increasing or decreasing intramembranous absorption seems to be the main way by which AFV is altered, although the precise mechanism of regulation remains elusive. The primary mechanism that drives intramembranous volume flow is not passive osmosis. Instead it has been suggested that amniotic fluid with all its dissolved solutes is transported across the amniotic membrane via bulk flow transfer by a yet unspecified vesicular transport mechanism.[37] Aquaporins may also take part in amniotic fluid absorption,[38] but it is not known what role they play or whether abnormalities in either their type or number contribute to fetal water-related disorders.

Near term the daily amniotic fluid turnover of 1000 mL is higher than the absolute AFV of 700 to 800 mL. Therefore AFV must be highly regulated to avoid oligohydramnios or polyhydramnios. Regulatory mechanisms for AFV act at three levels: (1) *Transfer of water between mother and fetus across the placenta*. Transplacental transfer is dependent on the existence of hydrostatic and/or colloid osmotic pressure differences between fetal and maternal vessels.[39] Placental water transfer between mother and fetus can serve as a reservoir to stabilize fetal extracellular volume and blood pressure. The intravascular infusion of large amounts of concentrated NaCl into fetal sheep did not result in fetal water accumulation as predicted but instead resulted in the transfer of all of the infused sodium and chloride across the placenta to the mother either against or in the absence of chemical concentration gradients.[40] The intravascular infusion of large volumes of physiologic crystalloid solutions in fetal sheep similarly resulted in the transfer of large amounts of water and solute across the placenta to the mother.[25] These observations suggest that a normal fetus can protect itself against both volume and salt overloads by rapid transfer of excess to the mother. Reduced fetal blood pressure releases fetal angiotensin. This increases the resistance in fetal placental precapillary vessels, reduces fetal placental capillary pressure, and thereby promotes water transfer from mother to fetus to restore fetal blood pressure.[41] (2) *Regulation of flow between the amniotic cavity and the fetus*. Increasing or decreasing intramembranous absorption appears to be the main mechanism to regulate flow between amniotic cavity and fetus. When fetal urine output is increased by intravascular infusion of physiologic saline solution, amniotic fluid volume increases by only a fraction of the infused volume because the excess amniotic fluid is absorbed through the intramembranous pathway.[25] (3) *Changes of maternal hydration*. Maternal hydration with water significantly increases AFV in oligohydramnios,[42] but this effect is seen only when hypotonic solutions are infused and maternal osmolality is reduced.[43]

CHANGES IN BODY FLUID COMPARTMENTS IN THE IMMEDIATE NEONATAL PERIOD

Prelabor Changes

Several observations collectively suggest that the fetus undergoes significant changes in its fluid status during the last few days, hours, and minutes before delivery. Studies have shown that the lungs begin to dry out several days before delivery,[34,44] in large part because the amount of fluid secreted by the lungs gradually decreases.[35] The reduction in fetal lung liquid secretion is mediated by an increased expression of epithelial sodium channels and sodium-potassium adenosine triphosphatase near birth, which changes epithelial cell ion transport from predominantly chloride secretion to predominantly sodium absorption. Epinephrine and other hormones (vasopressin, cortisol, and aldosterone), which are released just before and during labor, have a critical role in triggering this switch from liquid secretion to absorption.[45] Therefore infants born after elective cesarean section, who did not have the labor-induced clearance of lung liquid, have an increased risk for postnatal respiratory disease.[46] Relatively large increases in arterial pressure (20%) also occur in fetal lambs during the last few days before delivery.[47] If this pressure were transmitted to the capillaries, it would exert a major effect on the transcapillary distribution of fluid and perhaps protein.

Changes During Labor and Delivery

Although it was once thought that the amount of water in the newborn infant varied with mode of delivery, studies suggest that normal infants born vaginally or by cesarean section have an average water content of 75%, irrespective of mode of delivery.[48]

Blood volume decreases in both human and animal fetuses during labor and delivery. A decrease in circulating fetal plasma and blood volumes during labor and delivery by 18% and 12%, respectively, was found in fetal sheep.[47] These results are consistent with studies in humans. The umbilical vein hematocrit averages 44% in infants delivered by elective cesarean section without labor[49] and 51% immediately after delivery if labor has occurred.[20,50-53] Assuming that red blood cells (RBCs) are not released into the circulation with labor, this ratio of 44:51 demonstrates that the circulating blood volume is reduced by 14%. This corresponds to a reduction in plasma volume of 25%.

The mechanisms that mediate the loss of plasma during labor and delivery are multifactorial. Uterine contractions cause a direct compression or transformational change of the fetus.[54] As during a Valsalva maneuver, this elevates fetal vascular pressures, reducing circulating blood volume by 2% to 4%.[14,55] Furthermore, hypoxia reduces circulating blood volume in fetal sheep, resulting from a loss of plasma volume into the interstitial space.[56,57] The placenta may also contribute to the reduction in fetal blood volume during labor and delivery in that severe hypoxia may produce an in utero translocation of fetal blood out of the placenta and into the fetal body.[58] This leads to a rise in capillary pressure and the transcapillary filtration of fluid out of the fetal circulation. In addition, major changes in the plasma concentrations of many vasoactive hormones occur during labor and delivery. Although the effects of only a few hormones have been investigated, arginine vasopressin,[59] norepinephrine, cortisol,[60] and atrial natriuretic factor[16,61-63] have the ability to reduce the circulating blood volume and plasma volume when acutely infused into the circulation of fetal sheep at low to moderate rates. The umbilical cord concentrations of each of these hormones are elevated after vaginal delivery in humans, suggesting that these hormones may indeed play a role in mediating the shift of fluid from the plasma into the interstitial compartment at the time of labor and delivery.

Neonatal Weight Loss

Adaptation to extrauterine life represents a transition from aquatic to terrestric life and encompasses marked changes in body water content and fluid homeostasis. During the first postnatal days a weight loss of approximately 10% of birth weight occurs in healthy term neonates caused by a reduction of the extracellular volume.[6] A postnatal weight loss of up to 15% of birth weight occurs in low-birth-weight infants[64] and in extremely low-birth-weight infants.[65] Measurements of body water compartments demonstrated that this weight loss reflects a decrease in TBW. In low-birth-weight neonates with respiratory distress syndrome (mean birth weight 1558 g) the mean postnatal weight loss was 181 g (7.7% of birth weight). A concurrent reduction occurred in TBW by 183 mL and in extracellular volume by 204 mL.[66] These findings were confirmed in a more recent study in preterm neonates after normal intrauterine growth.[3] The postnatal weight loss of 7.8% of birth weight was caused by a reduction of the extracellular volume accompanied by a negative fluid and sodium balance. No evidence of tissue catabolism was found because body solids remained unchanged. Furthermore, nitrogen retention and energy intake was sufficient to meet energy expenditure by day 2.

Postnatal weight loss in SGA neonates differs somewhat from that of AGA infants. Postnatal weight loss in seven SGA preterm neonates (birth weight <5th percentile) with a mean gestational age of 35 weeks was only 5% and resulted from a proportionate reduction of body water and body solids.[67] This study included no information about fluid intake or diuresis and no AGA control group. In another study comparing five SGA preterm neonates (mean gestational age 35 weeks) with 14 weight-matched AGA neonates (mean gestational age 31 weeks) the SGA neonates had a maximal postnatal weight loss of only 2% compared with a maximal postnatal weight loss of 8% in the AGA control infants. On days 4 to 6 of life the SGA neonates had already regained birth weight, and no change was detectable in TBW or body solids, whereas, at the same postnatal age, body weight and TBW in the AGA neonates was significantly lower than at birth.[68] No differences were found in day-to-day fluid and energy intake during the first week of life between the SGA and AGA groups; however, the AGA infants had a higher urine output during this time interval. A possible reason for the attenuated postnatal increase in urine output in SGA preterm neonates is their altered hemodynamic adaptation. SGA preterm neonates did not exhibit the postnatal increase in cardiac output observed in the AGA neonates.[69]

It is important to emphasize that, in the management of fluids and electrolytes in low-birth-weight infants, the appropriate amount of weight loss needs to be anticipated and facilitated. When the normal contraction of the extracellular fluid volume was limited by liberal administration of IV fluids, the incidence of untoward outcomes such as patent ductus arteriosus, bronchopulmonary dysplasia, and necrotizing enterocolitis was increased in low-birth-weight infants.[70-73]

CHANGES IN BLOOD VOLUME IN THE IMMEDIATE NEONATAL PERIOD

Measurement of Red Cell Mass, Plasma Volume, Blood Volume, and Interstitial Fluid Volume

It is difficult to measure red cell mass, plasma, blood, or interstitial fluid volumes in the fetus and newborn (as well as in the adult) accurately because the methods are technically complex and time consuming. In the following paragraphs methodologic issues of body fluid measurements relevant for the interpretation of data are discussed.

Red Cell Mass

Red cell mass is determined as the dilution space of a known quantity of labeled erythrocytes. For a measurement blood is taken from the study subject, mixed with the erythrocyte label,

and then retransfused into the subject. The classic red cell label radioactive ^{51}Cr[74] is no longer acceptable for use in infants. More recently biotin has been validated as red cell label in adults[75] and in neonates.[76]

Plasma Volume

Plasma volume is measured as the dilution space of labeled high-molecular-weight substances (such as albumin or plasma proteins) after injection of a labeling substance into the circulation. For measurements in neonates radioactively labeled albumin[77] or albumin labeled with Evans Blue[78] or with indocyanine green[79] is used. These measurements frequently are corrected for loss of the label from the intravascular space by extrapolating the concentration-time curve backward to the time of injection. This requires several blood samples. Even with this correction, plasma volume tends to be overestimated for two major reasons: (1) All plasma labels are rapidly lost from the circulation through the capillary membranes of organs, such as the liver, which have high permeabilities even to high-molecular-weight substances. Extrapolating back to the time of injection does not correct for this loss because it is too rapid to be detected. (2) Most labels such as radioisotopes or dyes are not completely bound to the plasma proteins when injected into the circulation. The unbound labels are rapidly lost from the circulation and again lead to an overestimation of plasma volume. The extent of this overestimation has been explored in the unanesthetized sheep fetus. Fetal blood volume determined with radiolabeled albumin and fibrinogen was 11 mL/kg higher than blood volume determined with labeled RBCs (121 mL/kg vs. 110 mL/kg).[14]

Blood Volume

When blood volume is calculated from measured plasma volume or red cell mass and hematocrit, the hematocrit used is a potential source for error. It is well established that, because of rheologic factors, the hematocrit of the blood in the capillaries of the body (or in small-diameter glass tubes) is only 75% of the hematocrit in larger blood vessels. The net effect is to lower the average hematocrit within the body as a whole in relationship to that measured in blood sampled from large vessels such as the veins. To overcome this problem, investigators have advocated the use of a correction factor between 0.70 and 0.90 in the neonate, as well as in the adult. However, from a mechanistic viewpoint, it has been estimated that less than 10% of the blood in the body is in the systemic capillaries. If the hematocrit in capillaries is reduced by 25% compared with that of the large vessels as noted earlier, the whole body–to–large vessel hematocrit ratio should average 0.975. The mean body–venous hematocrit ratio measured in critically ill children was approximately 0.90.[80]

When blood volume is calculated from plasma volume and hematocrit, the errors in plasma volume measurement produce errors in blood volume determinations as well. Creasy and colleagues[81] have demonstrated that blood volume as determined with ^{51}Cr-labeled RBCs averaged 113.3 mL/kg in fetal sheep. In contrast, when ^{125}I-labeled albumin was used to estimate blood volume, it averaged 156.1 mL/kg. Thus, given the present state of the art, a RBC label provides a better estimate of blood volume provided that errors caused by unbound labels are minimized.

Measurement of Interstitial Volume

Interstitial fluid volume can be calculated as the difference between extracellular fluid volume and plasma volume. However, measurement of each of these volumes is subject to error. Extracellular volume has been measured as the volume of distribution of a variety of labels injected into the circulation. Each of these labels has a unique distribution space that does not exactly match the biologic extracellular volume. Therefore the comparison of extracellular volumes measured with different methods is difficult. Some of the labels (such as radioactive sodium ion) enter the cells or are metabolized and thus overestimate volume, but others (e.g., sucrose) do not penetrate the dense connective tissue water and thus measure only the readily exchangeable extracellular volume. Bromide and sucrose are the more widely used extracellular fluid markers in the newborn.[82,83] Bromide may not be adequate in the fetus because of transplacental losses,[82] and it has a considerable intracellular distribution during the fetal and neonatal periods.[83] Sucrose has been validated in neonates and children[84]; it does not enter cells and is not metabolized.

Fluid Distribution Between Intravascular and Interstitial Compartment

Few studies have simultaneously measured plasma and interstitial fluid volumes. In term baboon neonates, interstitial volume calculated from an extracellular volume of 392 mL/kg (corrected bromide space) minus a plasma volume of 50 mL/kg (Evans Blue) was 342 mL/kg. Thus the interstitial-plasma volume ratio was 6.8:1.[85] In human term neonates interstitial volume calculated from an extracellular volume of 376 mL/kg (corrected bromide space) minus a plasma volume of 43 mL/kg (Evans Blue) was 333 mL/kg; the interstitial-plasma volume ratio was 7.7:1.[7] In human preterm neonates (<1500 g) interstitial volume calculated from an extracellular volume of 444 mL/kg (sucrose space) minus a plasma volume of 46 mL/kg (Evans Blue) was 398 mL/kg. The interstitial-plasma volume ratio was 8.6:1.[3]

In contrast, the interstitial and plasma volumes in 1- to 3-week-old lambs have been reported to be 283 and 54 mL/kg, respectively.[82] The interstitial-plasma volume ratio of 5.2:1 is significantly lower than that calculated for the human newborn infant. Furthermore, in anesthetized newborn puppies, the interstitial volume is 172 mL/kg and the plasma volume is 82 mL/kg.[86] The interstitial-plasma volume ratio of 2.1:1 is considerably lower than the values for the human neonate and even lower than the ratio observed for the ovine neonate, suggesting that considerable differences may exist in the ratio of interstitial volume to plasma volumes in the newborns of different species. Some of these discrepancies appear to be related to differences in hematocrit at birth. For example, the high hematocrit of human neonates is associated with a low plasma volume, which, in turn, elevates the ratio of interstitial-to-plasma volume. In comparison, the much lower hematocrit in lambs produces a lower interstitial fluid–to–plasma volume ratio. Furthermore, methodologic differences may contribute to interstudy variability.

In principle, it is clear that the distribution of fluid between the intravascular and interstitial compartments depends on the equilibrium across the capillary membrane and the balance between capillary permeability and lymphatic function. Several important differences exist in the fluid homeostasis across the capillary membrane between the human fetus or neonate and adult. The fetus and neonate operate at low intravascular hydrostatic and oncotic pressures, and a high permeability of the capillary wall and a high lymphatic flow exist.

1. The intravascular hydrostatic pressure, which favors fluid movement out of the intravascular space, is low in the fetus and neonate. Mean blood pressure in the first 12 hours of life is 37 mm Hg for a 1000-g neonate and increases to 50 mm Hg for a term neonate.[87] The lower limit of mean blood pressure for extremely low-birth-weight infants is lower than that of more mature neonates and likewise increases over the first hours to days of life.[88]
2. The intravascular oncotic pressure is low because of low plasma albumin concentrations in the fetus and neonate, which increases from 2 g/dL at 24 weeks gestation to just above 3 g/dL at term.[89] Nonetheless, routinely increasing albumin concentrations in preterm infants has no therapeutic benefit.[90]

3. The capillary wall is much more permeable to albumin in the human neonate than in the adult. The transcapillary escape rate of albumin in term neonates is 18% to 20% per hour, which is approximately three to four times greater than in adults.[91] Studies in the sheep support this hypothesis in that the capillaries of the ovine fetus have a filtration coefficient five times that of adult values[15] and a permeability for plasma proteins 15 times that in the adult.[92] Capillary permeability to plasma proteins in the lungs may be the same in near-term fetal and neonatal sheep.[93]
4. It has been known for more than 60 years that lymph flow from subcutaneous tissue in anesthetized puppies is approximately twice that observed in adult dogs when expressed in relation to body weight.[94] Furthermore, lymph flow from the lungs is higher in anesthetized newborn lambs and puppies than in their adult counterparts.[61,95] Other studies in unanesthetized newborn lambs have also demonstrated higher pulmonary lymph flow rates.[92,93] These observations support the concept that the local, as well as whole body, lymph flow rates (in relation to body weight) are significantly greater in the neonatal period than later in life. The increased lymphatic flow during the neonatal period is probably indicative of the elevated interstitial volume in the neonate in relation to the adult and the higher capillary permeability.

Volumes Under Steady-state Conditions

Reported blood volumes in neonates at birth vary considerably for methodologic reasons. Blood volumes calculated from plasma labels tend to be higher than blood volumes calculated with red cell labels. In addition, with "late" cord clamping, neonates receive a placental transfusion and have higher blood volumes (Table 137-2).

No consistent differences exist in blood volumes at birth between term and preterm neonates, and blood volume in preterm neonates remains stable despite the postnatal reduction of extracellular volume during the first days of life.[3]

Intrauterine growth restriction is not necessarily associated with an increased plasma volume or red cell mass. Plasma volume measured by Evans Blue dilution in 14 term SGA neonates (37 ± 4 mL/kg) did not differ from that in AGA neonates (37 ± 6 mL/kg), and also no difference was observed in RBC volume (SGA: 69 ± 17 mL/kg; AGA: 63 ± 12 mL/kg) or blood volume (SGA: 105 ± 18 mL/kg; AGA: 101 ± 16 mL/kg) calculated from plasma volume and hematocrit.[96] Yet, in the subgroup of SGA neonates who had polycythemia after birth, blood volume was increased. Blood volume in SGA neonates with polycythemia calculated from hematocrit changes during exchange transfusion was 106 mL/kg compared with 86 mL/kg in AGA neonates with polycythemia.[97] The increased RBC volume can be the result of an adaptation to chronic intrauterine hypoxemia (as indicated by increased erythropoietin levels in SGA neonates) or the consequence of an acute placentofetal transfusion.

TABLE 137-2

Blood, Plasma, and Red Blood Cell Volumes in Newborns

Blood Volume (mL/kg)	Plasma Volume (mL/kg)	RBC Volume (mL/kg)	Neonate	Age (days)	Cord Clamping	Reference
85*	41†	41‡	Term	0-1	Variable	50
77†			Term	0	Early	51
103†					Late	
100†	62†	37.7†	Term	0-1	Late	96
62‡			Preterm	0	Early	100
74‡			Preterm	0	Late	
89†	49†		Preterm	0	Early	3
78†	40†	37†	Preterm	0	Early	116

*Determined with RBC plus plasma label.
†Determined with plasma label.
‡Determined with RBC label.

The relationship between blood and plasma volumes in the neonate has been explored in several studies. Neonates with high blood volumes also have high RBC volumes but decreased plasma volumes.[50,96] The weight-normalized RBC volume varies directly with the venous hematocrit, whereas plasma volume varies inversely with hematocrit (see Table 137-2).

The relationship between hematocrit and true circulating red cell mass has received considerable attention because RBC volume measurements are not available in daily clinical practice and the hematocrit is used routinely to estimate RBC volume to guide transfusion practices. Correlations vary from weak ($r = 0.5$)[98] to strong ($r = 0.907$)[99] between venous hematocrit and RBC volume in neonates. However, RBC volumes for individual neonates with a hematocrit of 30 ranged from 15 to 28 mL/kg even when a strong correlation was present. This range is of considerable clinical significance[99] and demonstrates that hematocrit is a relatively poor indicator of RBC volume.

Effects of Placental Transfusion

The single most important factor that affects blood volume and hence plasma volume in the newborn during the first few days of life is the extent of the placental transfusion that occurs at the time of delivery.[20,50,52,53] Total placentofetal blood volume decreases from 180 mL/kg fetal weight at 18 weeks gestation to 120 mL/kg at term of which 70 mL/kg is in the fetus and 50 mL/kg in the placenta.[20] The distribution of blood volume between neonate and placenta after delivery is influenced by a number of perinatal events. A fetoplacental transfusion that displaces blood from the fetus to the placenta occurs when the diameter of the umbilical arteries or the pressure gradient between the arteries and the umbilical vein increases. A fetoplacental transfusion is augmented by maternal hypotension, cord compression, holding of the infant above the placenta, or cesarean section. A placentofetal transfusion, which displaces blood from the placenta to the fetus, occurs when the diameter of the umbilical vein or the pressure gradient between umbilical vein and central veins in the fetus increases. Placentofetal transfusion is augmented by intrauterine asphyxia, uterine contractions, and increases in maternal uterine blood flow.[20]

The timing of cord clamping markedly influences the placental transfusion and neonatal blood volume. If the umbilical cord is clamped immediately after delivery, the hematocrit in the newborn infant averages 48% to 51% and is stable over a period of days.[20,50,52,53] However, if the cord is not clamped immediately and the newborn infant is positioned at or below the level of the placenta, the placenta gradually transfuses the neonate with blood, which reaches a maximum at 3 to 4 minutes after delivery.[20,52,53] This placental transfusion of 25 to 50 mL/kg represents an increase in RBC and blood volumes within the neonate of 50%.[52] With delayed cord clamping, the hematocrit gradually increases over 30 minutes to 2 hours to average 60% to 65%, or higher if the cords are stripped.[20,52,53] This increase in hematocrit is due to a loss of plasma from the circulation. Further increases in hematocrit occur over the next 2 hours, as a total of 30 mL/kg of plasma is lost from the circulation during the first 4 hours of life in infants with late cord clamping. This reduction in plasma volume is due largely to a shift of fluid from the intravascular into the interstitial space, although urine flow is elevated as well.[53] In a recent randomized controlled trial, blood volume in preterm infants (24-32 weeks gestation) after delayed cord clamping (74 ± 11 mL/kg) was significantly greater than after early cord clamping (63 ± 8 mL/kg)). Late cord clamping was more effective in increasing neonatal blood volume after vaginal

delivery than after cesarean section.[100] Placental transfusion has significant circulatory effects and increases neonatal blood pressure, central venous pressure, renal blood flow, and urine flow.[20] Therefore enhancing placental transfusion by late cord clamping has been used as a clinical therapy in term and preterm neonates. In term infants delayed cord clamping reduced the incidence of anemia in early infancy.[101] A reduction in the severity of respiratory distress syndrome,[102] less need for blood transfusions,[103] and a reduction in the incidence of intraventricular hemorrhage and late-onset sepsis[104] have been reported in randomized controlled trials after delayed cord clamping in preterm infants. In a meta-analysis, delayed cord clamping in preterm infants reduced the need for transfusion and the incidence of intraventricular hemorrhage. With delayed cord clamping, infants had higher hematocrit values and serum bilirubin levels but not to an extent that necessitated more therapeutic interventions.[105]

Responses to Hemorrhage

Perinatal blood loss of the fetus or neonate can occur after rupture of umbilical vessels, during premature placental separation, by bleeding of the fetus into the maternal circulation (fetomaternal transfusion) or into a twin fetus (fetofetal transfusion), or by internal bleeding, for example, cerebral or intraabdominal hemorrhage.

In adults of several species, including humans, dogs, rats, cats, and sheep, 24 to 48 hours is required for blood volume to return to normal after a loss of blood.[106] This restoration of volume occurs as plasma volume returns to or rises above normal, whereas RBC volume remains reduced. The time required for full volume restoration after hemorrhage in the fetus or neonate is shorter than in the adult. Fetal sheep restore twice the volume within 30 minutes after rapid hemorrhage as in the adult.[107] The ovine fetus also restores its blood volume to normal within 3 to 4 hours after a 30% hemorrhage over 2 hours[108]; that is in one tenth the time required in the adult.[106] This rapid restoration is mediated by a translocation of fluid and protein from the interstitial space into the vascular space but does not appear to be attributable to a net movement of fluid across the placenta.[62] Neonatal kittens and rabbits[109,110] were better able to tolerate blood loss than adults were, in that more blood had to be removed before arterial pressure decreased. This was attributed to a more rapid mobilization of interstitial fluid in the young animals in the first week of life. Neonatal lambs also rapidly restore their blood volume to normal after hemorrhage.[111]

Responses to Volume Loading

Intravascular Infusion of Isotonic Solutions

Rapid intravascular infusions of isotonic solutions, such as saline or Ringer's lactate, expand blood volume by only a fraction of the infused volume largely because of a loss of the infused fluid into the interstitial spaces. When adults of several species received intravascular infusions of isotonic crystalloid solutions, average intravascular retention was 20% to 50% of the infused volumes 30 to 60 minutes after rapid infusion. This is quite similar to the response in anesthetized newborn sheep, in that intravascular retention averaged only 30% to 40% after intravascular saline infusions.[26] Yet, in the unanesthetized ovine fetus, average intravascular retention after similar infusions was only 6% to 7% of the infused volume.[112] The reduced intravascular retention of crystalloid during fetal life is due largely to a high interstitial compliance–vascular compliance ratio, as well as a higher capillary filtration coefficient.[15] The increased capillary filtration coefficient permits very rapid fluid movements across the capillary membrane, and the high interstitial-to-vascular compliance ratio allows for extensive fluid shifts.

The reduced intravascular retention of fluid in the fetus compared with that of the adult may also be a primary factor in determining the urine flow responses to volume loading. It is well established that, after volume loading, normal adults will excrete the entire volume load through their kidneys over a period of several hours. Newborns of several species, including human and ovine, have a reduced capacity to excrete volume loads. This reduced excretory capacity can, in part, be attributed to the reduced intravascular retention.[113] With high intravascular retention in the adult, plasma renin activity and plasma concentrations of arginine vasopressin and atrial natriuretic factor all change in a direction appropriate for elevating urine flow. In the ovine fetus with a low intravascular retention, plasma arginine vasopressin and renin activity are unchanged, and atrial natriuretic factor undergoes only a transient increase.[113] Thus fetal urine flow rapidly returns to normal after rapid vascular volume expansion because the hormonal stimuli that promote urinary output are not maintained.[113] It has yet to be documented whether this is indeed the mechanism responsible for the diminished response to volume loading in human newborn infants.

Blood Transfusion

Similarly, after intravascular transfusions of blood only a percentage of the transfused volume remains intravascular. In human fetuses with anemia receiving intravascular transfusions, 31% of the transfused volume was lost from the circulation.[19] After late clamping of the umbilical cord in human neonates, plasma volume decreased from 50 mL/kg to 35 mL/kg 2 hours after receiving the placental transfusion.[51] The transcapillary escape rate of albumin increased with the magnitude of the placental transfusion.[114]

Responses to Hypoxia

Newborns with a history of fetal distress and birth asphyxia have elevated blood and plasma volumes (similar to those of normal infants with late cord clamping, even though the cord is clamped immediately after birth[115]). These observations suggest that prenatal hypoxia or asphyxia induces a placental transfusion before delivery. This is consistent with the observation that acute hypoxia promotes a translocation of fetal blood out of the placenta in sheep.[58] In addition, Yao and colleagues[115] have shown that 10 to 20 minutes of hypoxia immediately before delivery in sheep elevates plasma, RBC, and blood volumes in the newborn compared with lambs with early cord clamping. Linderkamp and co-workers[116] also observed that intrauterine hypoxia is associated with an elevated RBC volume in the newborn but that acute, intrapartum asphyxia is not associated with a predelivery placental transfusion. Thus evidence is ample supporting the concept that prenatal hypoxia causes a partial placental transfusion in utero, but its time course, exact extent, and reversibility are unclear.

It has been known for several years that postnatal hypoxia reduces blood volume in the human fetus because of a loss of plasma and proteins from the circulation,[117] but the causes of the reduced volume have not been explained. In the ovine fetus, this reduction in blood volume occurs rapidly and is linearly related to the reduction in arterial oxygen tension[56,57] (a 1% decrease occurs in blood volume for each 1 mm Hg decrease in arterial Po_2). The loss of plasma is undoubtedly due to an elevation of capillary pressure within the fetal body. This phenomenon occurs with no changes in fetal arterial or venous pressure during mild hypoxia (presumably because of vasodilation in selected organs) and is accompanied by increases in arterial and venous pressures with moderate to severe hypoxia.[57]

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John M. Lorenz

Fetal and Neonatal Body Water Compartment Volumes With Reference to Growth and Development

Water is the most abundant component of the body and is the solvent in which all metabolic reactions occur. Body water exists in compartments with diverse solute compositions that are separated by semipermeable lipid membranes. Thus, although recognizable anatomic boundaries exist, physiologically these compartments are interconnected.

COMPARTMENTALIZATION OF BODY WATER

Total body water (TBW) includes water both inside and outside of cells and water normally present in the nasorespiratory, gastrointestinal, and genitourinary systems. TBW can be theoretically divided into two main compartments (Figure 138-1). The *anatomic extracellular water* (ECW) includes all water external to cell membranes. *Intracellular water* (ICW) includes all water within cell membranes and constitutes the medium in which chemical reactions of cell metabolism occur. This compartment is heterogeneous and discontinuous; the interior of each cell is separated from the ECW and from the interior of other cells by the semipermeable cell membrane.

The anatomic ECW is functionally subdivided into *physiologic ECW* and *transcellular water.* The physiologic ECW constitutes the medium through which all metabolic exchange occurs; it includes *plasma* (intravascular water) and *interstitial fluid* (ISF). The ISF is the fluid that directly bathes the cells of the body. It is the ECW into which ions and small molecules diffuse freely from plasma. The composition of the physiologic ECF is fairly uniform throughout the body. In addition, potential spaces in the body (pericardial, pleural, peritoneal, and synovial) exist that are normally empty except for a few milliliters of viscous lubricating fluid and are considered to be part of the ISF compartment. Transcellular water includes water in extracellular compartments enclosed by an epithelial membrane, the volume and composition of which are determined by the cellular activity of that membrane. These heterogeneous compartments include the aqueous humor in the eye; the cerebrospinal fluid; and water in the nasorespiratory, gastrointestinal, and genitourinary systems.

ESTIMATION OF BODY WATER COMPARTMENT MASSES AND VOLUMES

All in vivo methods used to measure the mass or volume of body water compartments are of limited accuracy and precision. Accuracy of measurement is limited by the degree to which the assumptions on which methods are based are true. Precision is limited by biologic variability (body water compartment volumes may vary during the course of a day) and analytic error. Body water compartment volumes cannot be measured with a precision much greater than 2% to 5% in the healthy adult.[1-6] Therefore these techniques are better suited to estimate average body water compartment volumes in groups of subjects rather those of individual persons. Sutcliff[6] and Ellis[7] have published in-depth reviews of the techniques discussed in the following subsections.

Total Body Water

Desiccation

With this method, TBW is calculated as the difference in body mass before and after whole body desiccation to a constant mass at 100°C. Although it is most accurate, this technique is obviously not applicable to in vivo studies.

Densitometry

Fat has a much lower density (0.92) than lean body mass (LBM; 1.10). Therefore the proportion of body mass that is fat can be determined from whole body density by underwater weighing, as follows:

$$(4.570 / \text{Whole body density}) - 4.142$$

Whole body density must be corrected for air in the lungs and the gastrointestinal tract. The error in measurement of the former is the major source of analytic error with this method. LBM is calculated as the difference between total body mass and fat mass. This technique assumes that the density of fat and LBM are constant. This is more true for fat than for LBM, even in adults.[7-12] TBW mass can then be calculated from LBM, assuming that TBW is a constant 73.2% of the latter. This is a reasonable assumption for healthy young white adults. Still this proportion is not a constant in this population, only an average value. Moreover, for samples of very young or old persons or of persons of different ethnicity or with disease states, the proportion is different and population specific.

This technique is not applicable to the fetus, newborn, and infant because the density of LBM increases during development, the proportion of LBM that is water decreases during development, and substantial subject cooperation is required. Determination of body density by air-displacement plethysmography[13] obviates the last limitation, but not the others.

Indicator Dilution Method

This method is based on *Fick's principle:* if a known amount of a solute (indicator) is administered to a subject and limited to and distributed uniformly throughout a body water compartment, then the volume of that compartment can be calculated as the quotient of the amount administered and the concentration of the indicator in body water within the compartment of interest. The ideal indicator (1) is nontoxic; (2) is limited to and uniformly distributed throughout the body water compartment; (3) is not lost from the body during the time necessary for the indicator to distribute throughout the compartment evenly; (4) is metabolically inert; and (5) has a concentration that can be easily, precisely, and accurately measurable in small volumes of body fluid. No ideal indicators exist for measurement of any body water compartment volumes. Nevertheless, the indicator dilution method is the most reliable method of determining TBW and is the method used to validate other methods.

Figure 138-2 is a schematic diagram of the volumes of distribution of indicators used to estimate body water compartment volumes. Most are excreted in the urine, are metabolized, or exchange with components of body solids. All these properties will lead to overestimation of the volume of the body compartment, if

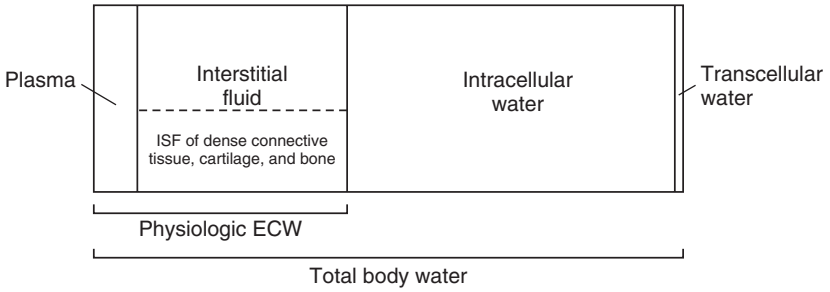


Figure 138-1 Body water compartments. The anatomic extracellular water (ECW) compartment includes the physiologic extracellular water compartment and the transcellular water compartment. *ISF,* Interstitial fluid. *(From Lorenz JM, Kleinman LI: Physiology and pathophysiology of body water and electrolytes. In Kaplan L, Pesce A, editors:* Clinical chemistry, *4th ed, St. Louis, 2003, Mosby.)*

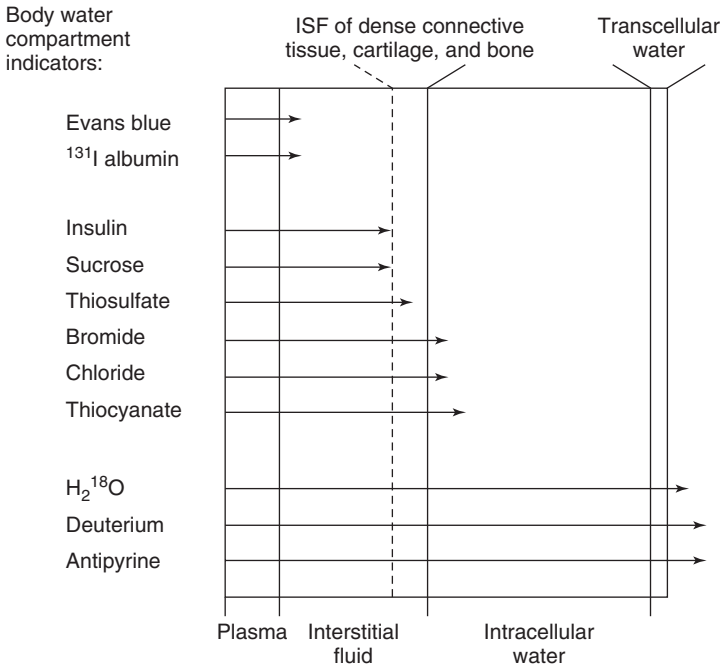


Figure 138-2 Apparent volumes of distribution of indicators used to estimate body water compartment volumes. *ISF,* Interstitial fluid.

corrections are not made. Depending on how the indicator is "lost" from the compartment, the rate of this loss, and the time required for the indicator to distribute uniformly throughout a body water compartment, two methods are used to determine an indicator's volume of distribution: the plateau method and the back-extrapolation method. Assumptions implicit in both methods are not strictly valid. Theoretically volume of distribution of a TBW indicator with the former method is greater than the true volume of the body water compartment, and it is less than the true volume of the body water compartment with the latter method.[14,15] In practice, however, both methods yield similar results in infants[16] and adults.[17]

Antipyrine, deuterium, and ^{18}O-labeled water are used as indicators of TBW volume. Antipyrine is less difficult to analyze than isotopes of water, but it is relatively rapidly metabolized. In addition, approximately 10% is protein bound. With the use of the back-extrapolation method to correct for metabolism, the antipyrine results in an apparent volume of distribution less than true TBW volume.[18] Measurement of isotopes of water requires elaborate equipment. Moreover, the volume of distribution of hydrogen water isotopes is approximately 4% higher than TBW measured by desiccation, as a result of exchange of nonaqueous exchangeable hydrogen atoms, mainly on carboxyl and hydroxyl groups.[19] $H_2{}^{18}O$ also exchanges with nonaqueous exchangeable oxygen atoms, but to a lesser extent—its volume of distribution is approximately 1% greater than TBW volume.[20,21]

As the result of the larger extracellular space in the term infant, the overestimation of TBW volume with deuterium, compared with that by desiccation and $H_2{}^{18}O$, is less than in the adult, that is, approximately 2%. This is because the ratio of TBW to protein is lower when a greater proportion of TBW is ECW and because protein is the primary nonaqueous source of exchangeable hydrogen atoms. This demonstrates the pitfalls of "correcting" indicator spaces in infants on the basis of data from healthy adults.

Bioelectrical Impedance Analysis, Single Frequency

Total body single-frequency bioelectrical impedance analysis (SF-BIA) is a frequently used bedside method of estimating TBW mass.[22,23] The advantages of this technique are that it is noninvasive, safe, simple, and rapid and the equipment is portable and inexpensive. It is based on the assumptions that only water can conduct electricity within the body and that the body is a homogeneous, perfect cylinder with a uniform cross-sectional area. Bioelectrical impedance is determined by measuring the voltage drop when a small electrical current (800 μA) is applied across the skin of the subject at a frequency capable of penetrating cell membranes (usually 50 kHz). The impedance is composed of two components: the resistance and the reactance. The magnitude of the resistance component seems to be well correlated with the volumes of distribution of tritiated water and deuterium.[5,24-26] TBW is calculated from resistance with a regression equation derived for the population of interest by using TBW volume determined

by a reference standard to correct for the previous assumptions. A major limitation of this technique is that the regression equations do not provide accurate estimates in populations other than those in which they were derived. Therefore prediction equations must be validated in or derived for each population in which they are used. Validation consists of comparison with a reference method. Another major limitation is that equations developed in normally hydrated patients are not valid for overhydrated or underhydrated individuals.[27,28] Apparently, electrolyte imbalances influence SF-BMI independently of fluid changes.[29] Furthermore, SF-BMI cannot accurately assess acute changes in TBW in individuals.[30]

Measurement of TBW using SF-BIA has been reported to compare favorably with the volume of distribution of $H_2{}^{18}O$ in neonates.[31-34] However, considerable variation exists in the regression equations used to predict TBW among these studies. It is unclear whether this variation is the result of differences in electrode position,[34,35] postnatal age,[31] gestational age,[34] birth weight, or possibly other factors. Moreover, derived regression equations incorporating body weight alone correlated as well as or better than (compared with those incorporating resistance measured with BIA) the volume of distribution of $H_2{}^{18}O$.[31,33,34]

Total Body Electrical Conductivity

Total body electrical conductivity (TOBEC) is an alternative bioelectrical technique for estimating TBW. Eddy currents are induced in the conductive tissues of the body by placing the patient in a coil that generates a time-varying low-energy electromagnetic field. These eddy currents, in turn, change the impedance of the coil. The difference in the impedance with the coil empty and the coil with the body in place is the TOBEC number. The latter is proportional to the conducting volume of the body, that is, to TBW. Estimation of TBW volume requires a regression equation, which (as with BIA) must be derived by using a reference standard. An instrument is available for infants. The TOBEC number has been shown to correlate well with the volume of distribution of $H_2{}^{18}O$ in healthy infants,[36] and a regression equation has been derived for healthy infants 2 to 12 weeks of age.[37] The major limitations of this technique are that the regression equation may not provide accurate estimates in populations other than that from which it was derived and the technique is applicable only to neonates who do not require support that would preclude safely placing them in the instrument. Moreover, the instrument is expensive and not portable.

Extracellular Water

Total Body Chloride

Total body chloride content, measured by neutron activation in vivo[38] or analysis of total body ash content, has been used to estimate the ECW volume. ECW is calculated as the quotient of total body chloride content and ECF chloride concentration, assuming that all body chloride is extracellular and is uniformly distributed throughout the ECW compartment. However, intracellular chloride exists, principally in red blood cells. Moreover, the apparatus required for neutron activation analysis is expensive, is difficult to calibrate, and requires irradiation. The last feature precludes the use of this method in infants and children.

Indicator Dilution

Inulin, sucrose, thiosulfate, and bromide are commonly used indicators to estimate ECW volume in neonates, infants, children, and adults. As illustrated in Figure 138-2, the volume measured depends on the degree to which the indicator leaks into cells or penetrates the ISF of dense connective tissue. Under normal circumstances, ISF does not behave as a simple aqueous solution. Solutes do not penetrate as easily into the ISF of dense connective tissue, cartilage, or bone. Therefore, when indicators are used to estimate ECW volume, it is more correct to refer to the value of the indicator *volume of distribution*. Correction of the volume of distribution of ECW indicators for volume of protein in plasma and (in the case of indicators that exist in solution as ions) for the Donnan equilibrium is required when the concentration of the indicator is measured in the plasma.[39]

The proportion of ECW contained in the ISF of dense connective tissue is lower in infants than in children and lower in children than in adults. Therefore differences between the physiologic ECW volume and the volumes of distribution of indicators for which diffusion into connective tissue ISF is limited (inulin, sucrose, thiosulfate) are less in the neonate than in the adult; these differences increase during development. Bromide penetrates connective tissue ISF well, but it is not completely limited to the ECW compartment; it diffuses to some extent into ICW, predominantly that of red blood cells. In disease states, bromide penetration into ICW may be even greater.[40] On the other hand, because bromide does not cross the blood-brain barrier, the bromide space does not include ECW of the brain. Because the brain ECW is 6% of total ECW volume in newborns compared with 1% in adults, the underestimation of the anatomic ECW volume resulting from bromide's failure to cross the blood-brain barrier is more pronounced in the newborn than the adult.

Bioelectrical Impedance Analysis, Multiple Frequency

Multiple-frequency bioelectrical impedance analysis (MF-BIA) can be used to estimate ECW volume. Theoretically, the most appropriate measure of ECW volume is given by impedance measure at zero frequency, because at this frequency current does not penetrate cell membranes.[41] However, measurement of impedance at zero frequency is not practical. Measurement of impedance over a range of frequencies allows resistance and reactance to be determined at each frequency. Reactance is then plotted against resistance, and the theoretic value of resistance at zero frequency is obtained by extrapolation of the curve to zero frequency.[42]

Measurement of TBW with use of MF-BIA analysis has been reported to compare favorably with the corrected bromide space in adults[5] and in preterm and term neonates.[43]

Plasma Water

Plasma water volume can be more accurately measured than ECW volume. Evans Blue and iodine-131–labeled serum albumin are commonly used indicators to estimate plasma volume. Both are uniformly distributed throughout the plasma water compartment, but both leak from the plasma water into the ISF compartment during the time required for distribution to occur. The rate of loss of these two indicators and their volumes of distributions are similar in the adult[44,45] and in the newborn.[46] However, the rate of loss is three- to four-fold greater in the newborn in the first day of life than in the adult—0.2% to 0.5% of the injected dose per minute.[46-48] Failure to correct for loss of the injected dose during the period necessary for uniform distribution throughout the plasma water compartment with use of the back-extrapolation method results in an apparent volume distribution greater than true plasma volume.

Interstitial Fluid

ISF volume cannot be directly measured. It is calculated as the difference between ECW volume and plasma water volume. Therefore its value depends on techniques used to estimate ECW and plasma water volumes and is subject to the errors of both techniques.

Intracellular Water

The volume of the ICW compartment is usually calculated as the difference between TBW volume and ECW volume. Therefore its value depends on techniques used to estimate TBW and ECW

volumes and is subject to the errors of both techniques. ICW volume calculated in this manner includes transcellular water.

MATURATIONAL CHANGES IN BODY WATER COMPARTMENT VOLUMES

TBW is 60% of body weight in average men and 50% of body weight in women. This difference between men and women is largely the result of differences in body fat. As a percentage of total body weight, TBW varies inversely with body fat content, from approximately 70% in very thin persons to less than 50% in very obese persons.

When the volume of distribution of thiosulfate is used to estimate the physiologic ECW volume, it is approximately 25% of body mass and one third of TBW volume in the average adult. Plasma volume is 5% of body mass. ISF volume is approximately 15% of body weight and one fourth of TBW. ICW water, calculated as the difference between the TBW and ECW mass, is equal to 35% of body weight and almost two thirds of TBW in the average adult. ICW mass calculated in this manner includes transcellular water, which has been estimated to be 1% to 3% of body weight.

The fraction of body mass that is water and the distribution of body water between the ECW and ICW do not remain constant during growth. Figure 138-3 is a compilation of body water compartment estimations from the literature.[49] Different methods were used to estimate body water compartment volume, and the data are cross-sectional. TBW expressed as a percentage of body mass gradually decreases during gestation and early childhood from approximately 90% during the first trimester to approximately 80% at 26 weeks of gestation to approximately 75% at term. It reaches a value approximating that in the adult at approximately 1 year of age. This change results from an increased accretion of body solids (protein, fat, and minerals) relative to water during development.[50-52] During this time, ECW (expressed as a percentage of body weight) decreases and ICW (expressed as a percentage of body weight) increases. Thus ECW becomes a lesser and ICW a greater proportion of TBW. ECW decreases from more than 50% of body mass during the first trimester to approximately 45% at 26 weeks to 40% at term. It reaches a value approximating that in the adult after 1 year of age. This decrease is the result of decrease in the proportion of body mass that is ISF during development, because plasma volume remains constant at 4% to 5% of body weight throughout life. This relative decrease in ECW and the increase in ICW during development are the result of changes in the proportions that various tissues contribute to total body weight (the proportion of water that is extracellular and intracellular differs among tissues) and changes in the proportion of water that is extracellular and intracellular within given tissues during development.[51] Of course, the absolute volumes of TBW, ECW, ICW, and plasma all increase with growth.

More recent longitudinal measurements of TBW (the volume of distribution of deuterium corrected for exchange of nonaqueous exchangeable hydrogen atoms) in healthy term infants (33 males and 43 females) at 0.5, 3, 6, 9, 12, 18, and 24 months of age obviate many of the problems inherent in the construction of the curve for TBW in Figure 138-3 from composite data. These data (Figure 138-4) show a more rapid decrease in the percent of body weight that is water than in Figure 138-3, largely as the result of larger increment in the percent of body weight that is fat, such that TBW per unit body weight is similar to that of the adult by 3 months.[15]

POSTNATAL CHANGES IN BODY FLUID COMPARTMENTS

Independent of the gradual changes in the proportion of body weight that is water and the changes in the proportions of TBW contributed by the ECW and ICW compartments during development, an abrupt and absolute decrease in TBW volume occurs in the first few days after birth.[34,53-58] It is controversial what proportion of this decrease in TBW is ECW or ICW, but the best evidence is that a disproportionate loss of water occurs from the ECW compartment in appropriate-for-gestational-age (AGA) very-low-birth-weight infants (Figure 138-5).[53-57] This decrease in ECW volume is the result of decrease in ISF volume, without change in plasma volume.[56,57] Older studies demonstrating an increase in plasma volume during the first hours of life in term and preterm newborns[48,59-63] describe a physiologic response to placental transfusion, the result of delayed cord clamping.

Although physiologic correlates of the postnatal diuresis and natriuresis have been described,[64-70] the *reason* for these is unknown. The disproportionate decrease in ISF precludes ascribing this decrease solely to catabolism. That it is physiologic is suggested by certain observations. First, relatively large differences in water and caloric intake are required to moderate this weight loss.[71-73] Furthermore, higher caloric intake has been correlated with less postnatal weight loss but no difference in the magnitude of ECW contraction.[47] Moreover, increases in ICW and body solids *per kilogram of body weight*, but not in ECW *per kilogram of body weight*, occur with subsequent weight gain.[53,57,74] Finally, fluid and sodium intakes high enough to prevent this decrease in ECW volume[75] have been associated with increased morbidity in premature newborns.[73,76,77]

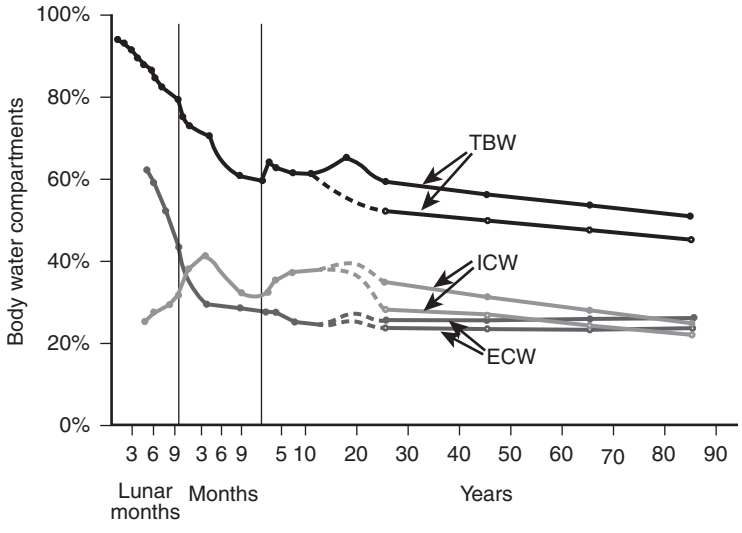


Figure 138-3 Change in body water and distribution with age. *(From Friis-Hansen B: Hydrometry during growth and aging. In Brozek J, editor:* Human body composition. Approaches and applications, *Oxford, United Kingdom, 1965, Pergamon Press).*

Data are more limited for term and small-for-gestational-age (SGA) infants. No studies exist of ECW volume before and after postnatal weight loss in term AGA infants. In the single study of the change in TBW with postnatal weight loss in AGA term infants, no change in the proportion of body weight that was water was associated with postnatal weight loss. These data are consistent with proportionate decreases in TBW and total body solids.[78] Bauer and colleagues[79] observed a decrease in body weight and TBW during the first week of life in preterm AGA infants (mean gestational age 31 weeks) but no change in body weight or TBW in weight-matched SGA infants (mean gestational age 35 weeks). Van der Wagen and associates[80] found that the proportion of TBW and total body solids remained constant during the period of postnatal weight loss and subsequent weight gain in SGA infants with a mean gestational age of 35.7 weeks. These studies suggest that postnatal weight loss, if it occurs, is the result of catabolism rather than reduction in ECW volume in the term and SGA infant. Conversely, Singhi and colleagues[81] found that nearly 90% of the decrease in TBW was the result of a decrease in ECW in both preterm AGA and gestational age–matched SGA infants. The reason for this difference between the studies of Bauer and associates[79] and van der Wagen and colleagues[80] and that of Singhi and associates[81] may be that the SGA infants in the first two studies were closer to term, whereas the SGA infants in the latter study were more preterm. Taken together, these data could be interpreted to be consistent with postnatal weight loss as the result of decrease in ECW in more premature infants and the result of catabolism in near-term infants, regardless of appropriateness for growth.

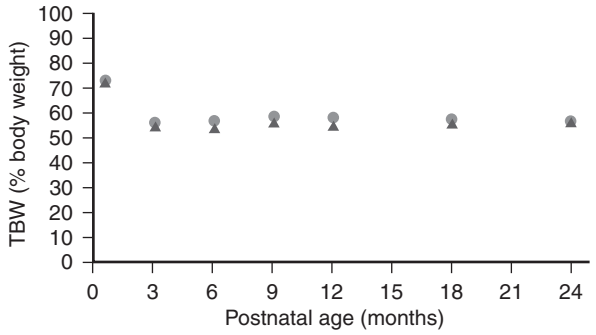


Figure 138-4 Longitudinal measurements of TBW, the volume of distribution of deuterium corrected for exchange of nonaqueous exchangeable hydrogen atoms, at 0.5, 3, 6, 9, 12, 18, and 24 months of age. *Open circles* depict males and *open triangles* females. *(Data from Butte NF, Hopkinson JM, Wong WW, et al. Body composition during the first 2 years of life: an updated reference,* Pediatr Res *47:578-585, 2000).*

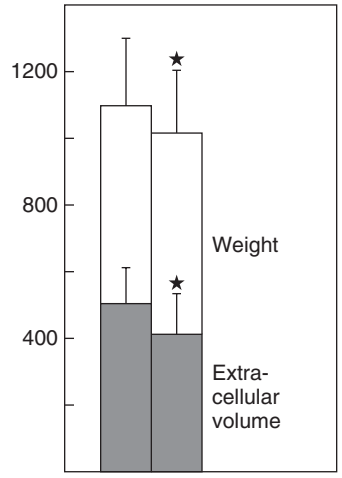


Figure 138-5 Body weight (in grams) and ECW volume (volume of distribution of sucrose, in milliliters) in 13 preterm AGA infants within the first 12 hours of life *(left bars)* and when postnatal weight loss exceeded 5% (*right bars;* average age, 84 hours). Error bars are SDs. $^{*}p < .001$ versus respective values within the first 12 hours of life. Mean weight loss was the same as the mean decrease in ECW volume. *(Modified from Bauer K, Versmold H: Postnatal weight loss in preterm neonates less than 1500 g is due to isotonic dehydration of the extracellular volume,* Acta Paediatr Scand Suppl *360:37-42, 1989).*

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139 Pathophysiology of Edema

David P. Carlton

GENERAL CONSIDERATIONS

Edema is the clinical term used to describe excessive fluid accumulation in the adventitial tissue spaces of the body. Excess fluid accumulation results when the net rate of transvascular fluid filtration from the microcirculation is exceeded by the rate of fluid clearance from the interstitial space by lymphatics. Postnatally, edema occurs in the neonate in association with a variety of conditions, including respiratory failure, sepsis, and renal failure. Underlying all circumstances in which there is edema, a disturbance in the normal balance of total body salt and water is present, whether this occurs as a primary (e.g., anuria) or secondary (e.g., retention of fluid to preserve circulating volume) event.[1] Although renal or hormonal abnormalities may not be directly responsible for edema formation, nearly all conditions associated with edema have disturbances in these systems, and the resolution of edema ultimately occurs when normal renal and hormonal control of fluid and salt balance is established.

Total body water balance is a dynamic process during fetal development.[2] Early in gestation, total body water content is approximately 95% of total body weight, decreasing to approximately 75% of body weight at term, and then to near 60% of body weight by the end of the first year after birth. The fraction of total body water that comprises interstitial fluid declines during fetal development.

TRANSVASCULAR FLUID FILTRATION

Fluid movement across the endothelium is driven by forces that regulate fluid flux across a semipermeable membrane.[3] These forces are the hydrostatic and protein osmotic, or oncotic, pressures of the intravascular and interstitial spaces. The ostensibly quantitative expression of how these forces influence fluid filtration is shown in the equation.

$$J_v = K\{(P_{mv}Pi) - \sigma(\pi_{mv} - \pi_i)\} \qquad \text{(Equation A)}$$

where J_v represents net transvascular fluid flow, K is a coefficient that accounts for the permeability of the barrier and surface area for filtration, σ is the reflection coefficient of the barrier to protein, P_{mv} and P_i are the hydrostatic pressures of the microvascular compartment and interstitium, respectively, and π_{mv} and π_i are the osmotic pressures generated by the protein in the microvascular and interstitial spaces, respectively. For a barrier that is completely impermeable to protein, σ would assume the value of 1, and for a barrier across which protein moves without restriction, σ would assume the value of 0. The most complete analysis of fluid filtration across the endothelium would include values for σ that are specific for each plasma protein.

Countless attempts have been made to quantify each of the variables in Equation A for different organs in animals and in man. All experimental approaches involve assumptions about the validity of the values measured and thus all values so measured lack a sense of finality, particularly in the fetus and newborn.[4,5]

In most tissue spaces in the body, under most circumstances J_v is greater than 0. That is, the sum of forces regulating fluid filtration result in a net movement of fluid out of the microcirculation.[6] It is important to recognize, however, that fluid filtration processes are dynamic and at any one time, in any specific section of the microcirculation, filtration forces may not result in fluid moving into the interstitium, even though the net J_v of the tissue or organ is greater than 0. For instance, the clearance of alveolar liquid from the lung that occurs after birth takes place predominantly across the microcirculation and not by lymphatic channels.[7]

Intravascular pressure in the microcirculation, P_{mv}, can be measured by direct techniques using micropuncture and by indirect techniques using isogravimetric approaches.[8] It is unlikely that intravascular pressure remains constant across the entire fluid-exchanging surface because resistance to flow along the vessel results in a drop in pressure over the length of the vessel. Therefore P_{mv} is understood to be the net hydrostatic force for the surface area involved in fluid exchange, even if the hydrostatic pressure at the arterial end of the vessel might result in fluid filtration and hydrostatic pressure at the venous end might result in fluid reabsorption.

P_{mv} is influenced by the relative vascular resistances in the circulation before and after the fluid-exchanging regions.[9] An increase in upstream resistance or a decrease in downstream resistance reduces P_{mv} and fluid filtration. A decrease in upstream resistance or an increase in downstream resistance has the opposite effect on fluid filtration. The importance of considering the profile of vascular resistance distribution is that the effect of a change in arterial pressure on fluid filtration cannot be predicted with certainty. The redistribution of vascular resistance in response to an intervention or a change in condition ultimately determines whether transvascular fluid filtration is affected, and because resistance cannot be assessed clinically, the ability to predict whether a change in transvascular filtration will occur is difficult. For instance, alveolar hypoxia increases pulmonary arterial pressure in both adults and neonates, but alveolar hypoxia affects transvascular fluid filtration only in the newborn.[10-12]

Interstitial pressure, P_i, has been assessed by several different techniques, including porous capsule embedment, direct micropuncture, and cotton wick insertion into the tissue space.[8] It is not possible to assign a general value to P_i because it assumes different values depending on the tissue bed under study. Values that exceed atmospheric pressure and those that are subatmospheric have been measured.[13] Although P_i usually assumes a value near 0 mm Hg, changes in interstitial pressure in response to physiologic disturbances can be dramatic. For example, in tissue that has suffered thermal trauma, P_i may decrease manyfold over baseline values, thus, in part, accounting for the rapid accumulation of interstitial fluid seen with burn injuries.[14] The molecular mechanisms underlying control of interstitial pressure are not clear, but the binding of collagen molecules on the surface of adventitial cells may play a role, because antibodies to the β_1-integrin lowers interstitial pressure and prompts edema formation.[15]

Microvascular protein osmotic pressure, π_{mv}, has been the focus of investigation and clinical study in order to understand better whether changing this value can improve total body fluid balance.[16,17] In the strictest analysis, π_{mv} should be measured for each plasma protein constituent along with the corresponding σ, but like the aforementioned P_{mv}, a net value of osmotic pressure

generated by the sum of plasma proteins suffices to account qualitatively for the relative importance of plasma oncotic pressure to transvascular fluid filtration. The value of transvascular differences in protein osmotic pressure as an important feature of transvascular fluid filtration is highlighted by recognizing that the osmotic force of all components of the extracellular fluid is on the order of 5000 to 6000 mm Hg, but because these components pass unimpeded across the endothelial barrier of the microcirculation (that is, they have a reflection coefficient of 0), they exert no influence on fluid flux.[18] Because plasma proteins do not move with complete freedom across the circulation, they have a significant effect on fluid filtration, despite an osmotic pressure in plasma in the range of 10 to 20 mm Hg.

Interstitial protein osmotic pressure, π_i, has been measured by direct micropuncture techniques, implanted tissue capsules, and absorbent materials placed within the tissue space.[8,18] In vivo measurements of π_i have relied on collection of lymph from organs of interest, with the assumption that protein concentration of lymph is equivalent to that of interstitial fluid (likely a safe assumption under steady-state conditions when lymph is collected from afferent lymphatics).

The membrane parameter, σ, represents the sieving ability of a semipermeable membrane for protein. High values of σ imply that osmotic pressure differences across the vascular barrier will exert a greater effect on fluid flux than will lower values of σ. One can measure σ in vitro as the ratio between the measured and expected osmotic pressure generated by the protein of interest. Furthermore, the value of this coefficient can be estimated in vivo from experiments in which transvascular fluid filtration is maximized (i.e., under conditions in which protein flow is nearly all convective).

Equation A describes the driving forces for transvascular fluid movement, but a different mathematical relationship exists for describing transvascular protein movement. This relationship contains two components, one describing flow of protein as a result of convective movement:

$$J_s = (1-\sigma)\, P\, J_v$$

where Js is transvascular protein flow, σ is the protein reflection coefficient, P is the concentration of protein in the vasculature, and J_v represents net transvascular fluid movement. The second relationship describes the flow of protein as a result of diffusion:

$$J_s = K\,(P-L)$$

where K is the product of the permeability and surface area of the microcirculation, and P and L represent the concentration of protein in the vasculature and lymph (interstitium), respectively. Combining these two equations yields the equation that describes net total transvascular protein movement:

$$J_s = (1-\sigma)\, P\, J_v + K\,(P-L)$$

Under steady-state conditions, lymph contains all the transvascular protein filtered if no metabolism occurs in the interstitium, and thus J_s reduces to LJ_v. In this analysis, J_v is equal to lymph flow and L is equal to lymph protein concentration. Rearranging the above equation, simplifying it, and setting up experimental conditions in which lymph flow is maximized yields the equation $\sigma = 1 - L/P$.[19] When measured experimentally, σ is well over 0.5 in most tissue beds and usually in the range of 0.75 to 0.90, even in the neonate.[20] Capillary beds that contain fenestrations, as in the liver, represent little restriction to transvascular protein movement, and under such conditions σ would be low. On the other hand, capillaries in other vascular beds contain few if any discontinuous regions, and σ under these circumstances would be closer to 1. The closer σ is to unity, the greater the influence of plasma proteins on fluid filtration.[19]

The lower the value of σ, the less able are proteins to generate a force counteracting fluid filtration. This arises for two reasons. First, with a less restrictive barrier, π_i increases numerically toward π_{mv} because the sieving quality of the membrane is diminished. Second, any difference between π_{mv} and π_i is minimized as σ decreases. Thus under conditions in which the vascular barrier is injured, allowing a greater degree of protein leak, administration of protein intravenously to augment vascular protein osmotic pressure and reduce edema formation theoretically should have little, if any, effect. The administration of protein to affect fluid balance in some other fashion (for example, on barrier function per se or to provide other favorable effects unrelated to fluid balance [e.g., to maintain plasma drug binding]) may subserve a clinical benefit, but such a rationale would be independent of the presumed advantage of an increase in microvascular protein osmotic pressure on transvascular fluid filtration.

Finally, the coefficient K represents the product of barrier hydraulic conductivity and the surface area available for fluid filtration.[16] These two components of K are difficult to separate experimentally. Hydraulic conductivity itself is a function predominantly of the density of the pathways for liquid and solute movement and is not necessarily a measure of protein permeability. That is, more pathways, or pores, for solute and liquid exchange might exist under different conditions without the individual pathways being more permeable. Thus changes in K may occur with true alterations in liquid and solute permeability (that is, pathways that allow passage of larger proteins), but changes in K may occur simply with an alteration in the density of pathways for liquid and solute movement or with changes in surface area for filtration, as would occur when unperfused capillaries are filled.

Consideration of Equation A allows several general statements about fluid filtration under normal conditions during steady-state.[19] First, because there is always some degree of sieving across the microvascular barrier, the protein concentration in the interstitium is less than that in the vascular space. This difference in protein concentration yields a difference in protein osmotic pressures that is subtracted from the hydrostatic pressure difference term. In this sense, protein osmotic pressure attempts to balance the "edema-promoting" effect of microvascular hydrostatic pressure. Second, because there is net transvascular fluid filtration out of the circulation, the hydrostatic pressure difference term in Equation A must exceed the difference in osmotic pressure term when total body fluid balance is under consideration. There is no experimental evidence indicating the presence of net "active" transport of water and solute across the endothelium that would influence transvascular fluid flux.

Although the concepts expressed in Equation A serve physiologists and clinicians well when considering fluid balance in a general sense, the equation does not always allow a precise calculation of the change in fluid filtration (J_v) when one variable in the equation changes. This arises because a change in one of the variables in Equation A usually results in a change in one of the other variables, even if such a change is unexpected. For instance, under conditions in which microvascular hydrostatic pressure is increased, one might assume that transvascular flow will increase by an amount that can be arrived at arithmetically from Equation A. When P_{mv} increases in the presence of a stable vascular barrier, J_v increases, but as it does, interstitial protein concentration decreases. This occurs because the driving force for liquid exceeds the bulk flow of protein across the barrier because σ is greater than 0 for protein; there is no sieving of water. When interstitial protein concentration is reduced, the protein osmotic pressure difference between the vascular and interstitial spaces increases. Thus from Equation A, J_v will increase in response to an increase in P_{mv}, but as $(\pi_{mv} - \pi_i)$ becomes larger, J_v assumes a new steady-state value that is less than that predicted by the increase in P_{mv} alone. Additionally, if excess fluid expands the interstitium, interstitial pressure increases to some extent, although the magnitude of this change is not predictable because tissue space

compliance is not linear in the presence of increasing interstitial edema. This increase in tissue hydrostatic pressure also acts to slow transvascular fluid movement. Changes that occur in driving forces for filtration that counteract the change in the "edema-promoting" variable are referred to as the "edema safety factors." The implication of the edema safety factors is that increases in transvascular filtration are blunted because of the countervailing changes seen in other variables in Equation A.[8,16,18,19]

LYMPH FLOW MODULATION OF EDEMA

An increase in interstitial fluid volume can be conceptually understood as an inability of the lymphatic system to remove fluid at the same rate as has been filtered across the microcirculation. Lymphatics are present in most but not all tissue beds, and the brain, specific areas of the eye, and bone marrow are examples of organs that do not contain lymphatics.[19] With regard to fluid balance, lymphatics serve to transport not only water but also proteins back into the circulation. Without the return of protein to the circulation, protein concentration differences between the interstitium and circulation narrow. Lymphatics are located primarily in loose connective tissue spaces and appear to end bluntly. Like blood vessel capillaries, the smallest terminal lymphatics do not contain smooth muscle, but the presence of actin filaments in lymphatic endothelium suggests that the initial lymphatics may possess some limited contractile properties. As the lymphatic vasculature is traced centrally, valves appear, and smooth muscle cells surrounding the lymphatics are more consistently observed. Lymphatics ultimately drain into the central circulation through the thoracic and right lymphatic ducts. The movement of interstitial fluid from the tissue space into the terminal lymphatics requires a driving force. Experimental evidence points to the importance of both interstitial pressure and interstitial volume in modulating lymph flow. However, an increase in interstitial pressure alone, in the absence of a change in interstitial volume, would be unlikely to result in a hydrostatic pressure gradient sufficient to account for lymphatic filling. It is the combination of anchoring filaments attached to the external wall of the lymphatics and an increase in interstitial volume that results in a pressure gradient sufficient to account for initial lymphatic filling.[18,19]

Lymph drainage is not constant but varies with changes in filtration driving force.[16] Thus lymph drainage can also be considered an edema safety factor. Interstitial pressure is closely associated with lymph flow, but the relationship between interstitial pressure and lymph flow is not linear over all tissue pressures. Although an association exists between interstitial pressure and lymph flow, other factors also influence lymphatic clearance. One of the most important is the presence of valves within the lymphatics that provide directionality to flow.[19] Valves also reduce the direct influence of downstream outflow pressure on transendothelial fluid movement into the terminal lymphatics. Lymph is propelled centrally by extrinsic and intrinsic factors. Extrinsic forces include both passive and active muscular movement. Intrinsic forces are those associated with spontaneous lymph vessel contraction and relaxation. Motion associated with respiration and with blood vessel pulsation also contributes to lymphatic movement.

Effect of Outflow Pressure on Lymphatic Drainage

Lymph from large collecting vessels ultimately drains into the central circulation. If lymph drainage is obstructed, interstitial tissue volume increases and edema becomes obvious, implying that outflow pressure is an important variable influencing the effectiveness of lymph drainage.[21] Because the outflow pressure for the thoracic duct is the central venous circulation, an increase in central venous pressure should be an important factor influencing lymph drainage. Experimental evidence from fetal and adult animals confirms this supposition.[22-24]

MOVEMENT OF PROTEIN ACROSS THE MICROVASCULAR BARRIER

The specific site of fluid and protein transfer along the circulation is likely variable within an organ and among tissues. Venules, arterioles, and capillaries may each contribute to transvascular fluid filtration. Molecular size plays a role in transvascular filtration, with ease of transport into the interstitium being inversely proportional to size.[17] Proteins cross the endothelium through channels or pores and by transcytosis, but the magnitude of each to total transvascular protein movement is debated.[25] The importance of protein movement through pores of various sizes in the vascular barrier is highlighted by studies that show that protein moves into the interstitium closely linked to the flow of liquid, that is, moving by convection or bulk flow. In the lung, inhibitors of transcytosis do not significantly decrease transvascular protein transport, casting doubt on the importance of transcytosis as an important pathway for transvascular protein movement involved in fluid balance.[26]

The morphologic basis for an increase in microvascular permeability is not completely resolved. Under some conditions, gaps between endothelial cells can be seen microscopically, but this is not a consistent observation.[25] Intercellular proteins between endothelial cells are linked to actin and other intracellular structures that are thought to regulate microvascular protein permeability by rearrangement of the cytoskeleton. Cellular contraction is thought to be the means by which intercellular gaps are formed and thus permeability regulated.[27]

Like studies examining the morphologic basis for permeability changes, the molecular basis for changes in permeability is incompletely understood.[28-30] Clinically, inflammatory processes are frequently associated with changes in permeability. The molecular initiation of permeability occurs, at least in a number of circumstances, as a result of calcium-dependent events. Calcium entry appears to be regulated, at least in part, by potassium modulation of transmembrane potential and not through voltage-regulated calcium channels. An increase in intracellular calcium is thought to promote myosin light-chain phosphorylation by kinases and thus initiate contraction. Although mediator-induced changes in cytosolic calcium concentration are common, the fact that intracellular calcium may be influenced by novel means is shown by experiments in which surface molecules not thought to be receptors for the typical inflammatory ligands influence vascular permeability. A well-studied example is the ligation of the luminal surface integrin, $\alpha_v\beta_3$, on the microvasculature.[31] Another effect of an increase in intracellular calcium is the modulation of nitric oxide synthesis and the generation of cyclic guanosine monophosphate (cGMP). These mediators are known to affect vascular permeability, but the mechanism by which this occurs is unclear. Their effect may be indirect, perhaps by modulating the concentration of intracellular cyclic adenosine monophosphate (cAMP); cAMP blunts cell contraction and myosin light-chain phosphorylation, but it also may influence the expression of molecules important in intercellular binding and thus limit the ability of the cytoskeleton to influence permeability.[25]

SPECIFIC CLINICAL CONSIDERATIONS: CHANGES IN TRANSVASCULAR FLUID FILTRATION WITH INTRAVENOUS PROTEIN INFUSIONS

Intravenous infusions of solutions containing protein or dextran are frequently administered to patients with edema to increase plasma protein osmotic pressure, in the expectation that transvascular fluid filtration will be less after the plasma protein concentration is increased. Experiments that address this issue show

that infusing albumin in sufficient quantities to increase plasma albumin concentration by as much as 20%, or infusing dextran in sufficient quantity to increase plasma osmotic pressure by more than two-fold, have little effect on steady-state lymph flow.[32,33] However, if microvascular hydrostatic pressure is then increased, the additional plasma osmotic pressure blunts the expected increase in transvascular fluid filtration. The explanation for this response is not completely forthcoming, but likely results from changes in hydraulic conductivity and from changes in the distribution of vascular resistance, resulting in changes in effective P_{mv}.

Experiments evaluating hypoproteinemia in the neonate predictably show that lymph flow increases when protein is removed from the circulation.[20,34] The explanations for this response are conflicting, but at least in the newborn lamb increases appear to result from a decrease in transvascular protein osmotic pressure difference and an increase in vessel hydraulic conductivity, but not from an increase in protein permeability. Whether albumin plays some role in maintaining the health of the barrier wall is uncertain. In vitro information suggests that albumin per se favorably influences the endothelial barrier.[35]

The preceding discussion examines the issue of plasma proteins and fluid balance in the context of a normally permeable microvascular barrier. When barrier integrity is compromised, the movement of protein is less restricted, the reflection coefficient is reduced, and any effect of protein osmotic pressure on fluid movement is minimized. Consistent with this theoretic analysis, experiments in which the lung microvasculature has been injured show no change in fluid movement out of the pulmonary circulation with the infusion of albumin.[36] Thus any favorable effect of plasma protein osmotic pressure on transvascular fluid balance depends on the extent to which the endothelial barrier remains intact.

SPECIAL CONSIDERATIONS OF EDEMA IN THE FETUS

An edematous fetus, regardless of the etiology of the edema, is labeled with the diagnosis of hydrops fetalis. More than 100 different medical illnesses or conditions are associated with hydrops, but the pathophysiologic link between the condition and edema formation is not clear in many of these patients.[37] Approximately 75% of the cases of nonimmune hydrops in the United States occur in association with an identifiable disorder, and in the remainder the condition is labeled idiopathic.[38]

PROPOSED MECHANISMS OF EDEMA FORMATION IN PATIENTS WITH HYDROPS

From the foregoing discussion it is seen that edema may form if the driving forces for fluid filtration or vascular permeability increase without a concomitant increase in lymphatic drainage. Although disturbances in microvascular permeability could be relevant in explaining some cases of fetal hydrops, there is no evidence at this time that vascular protein permeability is significantly different, at least in the lung; in the fetus, neonate, or adult; or between hydropic and nonhydropic fetuses.[7,39,40]

A variety of hypotheses might explain the pathophysiology of hydrops, but one that is most compatible with the current experimental evidence is that elevation of central venous pressure is a critical element in the pathogenesis of fetal edema. Conditions that elevate central venous pressure are common in hydropic infants and thus provide a compelling clinical link for this hypothesis. An elevation in central venous pressure increases microvascular pressure upstream and thus enhances transvascular fluid movement. An increase in central venous pressure also impairs lymphatic drainage into the central circulation and thus increases interstitial fluid volume. If lymphatic drainage is completely interrupted (a limiting-case example of increased central venous pressure), the fetus becomes hydropic.[21] In experiments designed to evaluate more modest effects of disturbances in central venous pressure on lymph drainage, thoracic duct lymph flow was found to be inversely proportional and linearly related to outflow pressure. In these studies, increasing central venous pressure by only 5 mm Hg was impaired by nearly 50% the rate of lymph drainage.[24]

A helpful model that clarifies total body fluid dynamics in the presence of elevated central venous pressure is the fetal lamb that undergoes rapid atrial pacing. When fetal sheep are paced at rates of 300 to 320/minute, central venous pressure nearly doubles, without changes in systemic arterial pressure, plasma albumin concentration, or vascular protein permeability.[41,42] Fetal edema occurs quickly, sometimes within 12 to 24 hours, indicating excessive transvascular fluid filtration, impaired lymphatic clearance, or both. Thoracic duct lymph flow increases by nearly 50% when measured at an outflow pressure equivalent to central venous pressure under baseline conditions, but when measured at the central venous pressure induced by pacing, lymph flow is significantly reduced. Thus in conditions that increase central venous pressure, hydrops may be caused, or at least aggravated, by two distinct mechanisms: increased transvascular fluid entry and impaired interstitial fluid clearance.

Anemia is another human fetal condition often associated with hydrops. Exchange transfusion in fetal lambs sufficient to reduce hematocrit from 32% to 12% produces hydrops, but only if the anemia is associated with an increase in central venous pressure.[43] Whether edema develops may depend to a large extent on whether lymph drainage can accommodate the increase in fluid filtration.

In newborn sheep made hypoproteinemic, plasma protein osmotic pressure is reduced and transvascular fluid filtration increases, but no increase in interstitial fluid volume occurs, at least in the lung, because lymph flow increases to match transvascular filtration.[20,34] Likewise, in fetal sheep, simply lowering plasma protein oncotic pressure by removing protein from the circulation has no effect on total body water.[44] The lack of effect of hypoproteinemia on edema formation is likely a result of increased lymphatic drainage back into the central circulation. In isolated fetal hypoproteinemia, central venous pressure does not increase, and thus there is no impediment to lymph drainage back into the central circulation. The increase in fetal lymph flow is likely on the order of approximately 40%, similar to that seen in newborn lambs made hypoproteinemic.[20,34] Because fetal lymph flow can increase two- to four-fold over baseline values, increases in transvascular filtration prompted by hypoproteinemia can be easily accommodated by increases in fetal lymph flow.

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Philippe S. Friedlich and Istvan Seri

Regulation of Acid-Base Balance in the Fetus and Neonate

The majority of the existing information on the regulation of acid-base homeostasis in mammals and humans was obtained from studies in adult subjects. With the current revo1ution in micromethodology and advances in understanding of developmental physiology and molecular biology, however, data have been accumulating on fetal and neonatal regulation of acid-base balance.

In general, acid-base homeostasis is tightly regulated by extracellular and intracellular buffer systems and respiratory and renal compensatory mechanisms of the organism.The normal range of H^+ ion concentration in the extracellular fluid is 35 to 45 mEq/L corresponding to a pH of 7.35 to 7.45. Under physiologic circumstances, volatile and fixed acids generated by normal metabolism are excreted and the pH remains stable.[1] Volatile carbonic acid is produced in the largest amounts, and it is readily excreted by the lungs in the form of carbon dioxide. Fixed acids, which include lactic acid, ketoacids, phosphoric acid, and sulfuric acid, are buffered principally by extracellular bicarbonate. The bicarbonate used in this process is then regenerated by the kidneys in a series of transmembrane transport processes resulting in the excretion of H^+ ions in the form of titratable acids and ammonium.

EXTRACELLULAR BUFFER SYSTEM

Using various acid-base pairs, the extracellular buffer system responds immediately to alterations in pH in a fashion represented by the Henderson-Hasselbalch equation. The carbonic acid–bicarbonate system is the most important component of this buffer system. Because the isohydric principle[2] allows one to follow the changes in the concentrations of a single acid-base pair as an indicator of acid-base homeostasis for the entire system, serial measurements of the carbonic acid-bicarbonate buffer system have been used to describe accurately the changes in both the experimental and the clinical settings.

INTRACELLULAR BUFFER SYSTEM

The most important components of the intracellular buffer system are the hemoglobin and intracellular proteins and phosphates acting as an intracellular H^+ sink and reservoir attached to the extracellular buffers. This system provides buffering at a slower rate compared to extracellular buffers and requires several hours to reach maximum capacity.[3]

RESPIRATORY COMPENSATORY MECHANISM

Because of the open nature of the carbonic acid–bicarbonate system, normal gas exchange in the lungs serves as an immediate regulator of acid-base homeostasis by maintaining a normal $PaCO_2$, thus eliminating the excess carbon dioxide generated by an acid load. Activation of the respiratory compensatory mechanism is necessary, however, to return pH further toward normal. Because the movement of bicarbonate across the blood-brain barrier is regulated by active transport mechanisms,[4] and, because the central respiratory drive is triggered by low steady-state values of the cerebrospinal fluid and not the plasma bicarbonate,[5]

a full activation of this system only occurs a few hours after the development of metabolic acidosis. Because carbon dioxide moves freely across the blood-brain barrier,[5] cerebrospinal fluid and cerebral interstitial fluid H^+ ion concentrations are altered more rapidly by respiratory acidosis, leading to an immediate activation of the respiratory compensatory mechanism in such cases.

RENAL COMPENSATORY MECHANISM

By altering renal H^+ excretion, renal compensation is the ultimate mechanism to adjust H^+ content in the body.[6] This system, however, requires hours to 2 to 3 days for a full response.

REGULATION OF ACID-BASE BALANCE IN THE FETUS

Fetal Extracellular Buffer System

The fetus has an intact extracellular buffer system. The carbonic acid–bicarbonate buffer system is especially important because it contains a volatile element, carbon dioxide gas. For the fetus, the placenta is the organ of respiration, and it quickly eliminates excess carbon dioxide generated by the development of fetal metabolic acidosis, provided that placental function, uterine and umbilical blood flows, and maternal respiratory status are uncompromised.[7]

Fetal Intracellular Buffer System

Although the fetus has a significantly smaller intracellular compartment compared with the child or adult, its intracellular buffering capacity is still considerably larger than the extracellular one.[7]

Fetal Respiratory and Renal Compensatory Mechanisms

The respiratory and renal compensatory mechanisms of the fetus are limited by its level of maturity and the surrounding maternal environment. The placentomaternal unit performs most of the effective compensatory functions.[7] However, the fetal kidney has the ability to contribute to the maintenance of fetal acid-base balance. For instance, ammonium excretion and thus generation of bicarbonate as well as sodium excretion has been documented to increase during the recovery period from hypocapnic hypoxia in the fetal sheep.[8]

Fetal Metabolic Acidosis

The most frequent cause of fetal metabolic acidosis is fetal hypoxemia due to abnormalities of uteroplacental function, blood flow, or both. Primary maternal hypoxemia or maternal metabolic acidosis secondary to maternal diabetes mellitus, sepsis, or renal tubular abnormalities is an unusual cause of fetal metabolic acidosis. During the course of fetal hypoxemia, metabolism becomes anaerobic, and large quantities of lactic acid accumulate. H^+ ions are buffered by the extracellular and intracellular buffering systems, and pH drops as plasma bicarbonate decreases. Because of the unhindered diffusion of carbon dioxide through the placenta,[9] restoration of fetal pH toward normal initially occurs through elimination of the volatile element of the carbonic acid-bicarbonate system via the maternal lungs. Lactate and other fixed acids cross the placenta more slowly,[7] however, so that effective maternal renal compensation of fetal metabolic acidosis is delayed. If fetal oxygenation improves, the products of anaerobic metabolism are also metabolized by the fetus.

Although metabolic acidosis stimulates fetal breathing movements,[10] the respiratory control system in the fetus is much less sensitive to changes in pH than after birth.[11] No physiologic significance, however, to this compensatory mechanism in utero is apparent.

Several lines of evidence indicate that the fetal kidney is able to excrete acid[12-14] and organic acids[15] as well as to generate more bicarbonate.[16] Studies in fetal sheep have found age-dependent increases in glomerular filtration rate (GFR), urinary titratable acid, ammonium, and net acid excretion.[12] Furthermore, a positive relationship has been demonstrated between changes in GFR and bicarbonate, sodium, and chloride excretions.[12,13] The fetal kidney, however, has a developmentally regulated limited ability to adapt to changes in fetal acid-base balance. In fetal sheep, in response to metabolic acidosis induced by the infusion of hydrochloric acid, blood pressure increases but the GFR does not change. Urinary titratable acid, ammonium, and net acid excretion increase without significant changes in renal bicarbonate absorption.[14] Evidence also indicates that the fetal kidney has the ability to increase bicarbonate reabsorption, at least during periods of volume depletion.[16] With regard to the human fetus, only limited information is available concerning renal acidification.[17] The physiologic importance of these adaptive fetal renal responses, however, is limited when compared with those in the postnatal period because the acid load excreted in the fetal urine remains within the immediate fetal environment and still has to be eliminated by the placenta or metabolized by the fetus.

Fetal Respiratory Acidosis

Fetal respiratory acidosis develops when prolonged maternal hypoventilation occurs with maternal asthma, airway obstruction, narcotic overdosing, maternal anesthesia, and magnesium sulfate toxicity. Fetal breathing movements increase, and the fetal kidney exerts a maturation-dependent limited response by reclaiming more bicarbonate in an attempt to restore the 20:1 ratio of bicarbonate to carbonic acid and thus increase the pH toward normal.[7] Obviously, only the renal compensation has some limited physiologic significance for the fetus in cases of respiratory acidosis.

Fetal Metabolic Alkalosis

Metabolic alkalosis rarely affects the fetus, but it may occur in hyperemesis gravidarum. As a result of the significant hydrogen chloride losses in this condition, bicarbonate is retained by the mother to maintain anionic balance with sodium in the serum. Because bicarbonate is transported slowly across the placenta, the development of fetal metabolic alkalosis lags behind that of the mother. Maternal hypoventilation then also tends to restore normal pH in the fetus as a result of the rapid movement of carbon dioxide across the placenta.

Fetal Respiratory Alkalosis

The *physiologic hyperventilation* of the pregnant woman causes a compensatory decrease in her serum bicarbonate concentration to approximately 22 mM.[7] Acute hyperventilation, however, may lead to the development of fetal metabolic acidosis because it induces uterine vasoconstriction.[18] In such cases, restoration of maternal carbon dioxide levels rapidly corrects both the abnormal uterine blood flow and the acid-base abnormality in the fetus.

REGULATION OF ACID-BASE BALANCE IN THE NEONATE

The neonate continues to undergo maturation of the overall adaptive responses to changes in acid-base homeostasis. Available information suggests that an abrupt increase in the sensitivity of the central respiratory control system to pH changes occurs at the time of delivery and that regulation of acid-base balance becomes tighter postnatally compared with that in the fetus.[11] The cellular mechanism responsible for the increased sensitivity of the acid-base balance regulation system is not known.

Neonatal Extracellular and Intracellular Buffer Systems

The neonate has well-functioning extracellular and intracellular buffering systems. Postnatally, the gradual increase in the intracellular compartment further enhances the overall buffering capacity.

Neonatal Respiratory Compensatory Mechanism

With postpartum establishment of the functional residual capacity, the neonate's lungs become the end organ of the respiratory compensatory mechanism to changes in acid-base balance. Respiratory acidosis induces immediate increases in ventilation because carbon dioxide diffuses freely across the blood-brain barrier.[5] In the case of metabolic acidosis, however, full respiratory compensation is delayed for a few hours until cerebrospinal fluid and cerebral interstitial bicarbonate completely equilibrate with plasma bicarbonate.[4]

The effectiveness of respiratory compensation depends on the maturity of the central respiratory control system and pulmonary function. Because central respiratory control is sensitive to changes in cerebral interstitial pH in the neonate,[4,11] the effectiveness of the postnatal respiratory response depends mainly on pulmonary function. Therefore neonates with parenchymal lung disease, especially if they are born prematurely, have a limited ability to increase ventilation in response to metabolic acidosis.

Neonatal Renal Compensatory Mechanism

As mentioned earlier, by regulating bicarbonate and acid secretion in response to changes in extracellular pH, renal compensation is the ultimate mechanism to adjust the H^+ content of the body. Although full activation of this system usually requires 2 to 3 days, alterations in renal acidification may be seen as early as a few hours after the development of acid-base disturbance.

The renal compensatory mechanisms of the neonate are immature, resulting in a developmentally regulated decreased ability to maintain acid-base balance.[19,20] Both renal microhemodynamic and tubular epithelial factors play a role in the limited renal compensatory capacity of the newborn.

Renal blood flow significantly increases after the immediate postnatal period, and the vasodilatory mechanisms appear to be functionally mature as early as 24 weeks gestation.[21] GFR is also low in the immediate postnatal period, and it increases as a function of both gestational and postnatal age.[22,23] The low GFR is one of the most important factors limiting the ability of the preterm and term infant to handle an acid load adequately.[19,20] Net renal acid excretion also depends on several gestational and postnatal age-dependent tubular epithelial functions.[20,24] Under physiologic conditions in the proximal tubule, four transport mechanisms regulate active acid extrusion and transepithelial bicarbonate reabsorption: the H^+-ATPase, the electrogenic $Na^+/3HCO_3^-$ cotransporter, the Na,K^+-ATPase-driven secondary active Na^+/H^+ antiporter, and the Na^+,K^+-ATPase-driven tertiary active Na^+-coupled organic ion transporter.[24] Because approximately 85% to 90% of the filtered bicarbonate is reabsorbed in the proximal tubule,[20,24] the function of these proximal tubular transporters essentially determines the renal threshold for bicarbonate reabsorption. The bicarbonate threshold is 18 mEq/L in the premature and 21 mEq/L in the full-term infant, and it reaches adult levels (24 to 26 mEq/L) only after the first year of life.[25,26] In the extremely premature neonate, however, the renal bicarbonate threshold may be as low as 14 mEq/L. Because renal carbonic anhydrase is present and active during fetal life,[27] and because its activity is similar in the 26-week-old, extremely immature neonate to that of the adult,[28] a developmentally regulated immaturity of the expression, molecular structure, membrane-assembly, or second-messenger and third-messenger function of the described proximal tubular transporters is most likely responsible for the low bicarbonate threshold during early development. Indeed, both the activity and the hormonal responsiveness of the proximal tubular Na^+,K^+-ATPase have been found to be decreased in younger rather than older animals.[29]

Medications used in the treatment of critically ill neonates may also affect proximal tubular bicarbonate reabsorption. For example, dopamine administration may potentially decrease the low bicarbonate threshold of the neonate[30] because the drug inhibits activity of the proximal tubular Na^+/H^+ antiporter.[31] Carbonic anhydrase inhibitors also decrease proximal tubular bicarbonate reabsorption by limiting bicarbonate formation and H^+ ion availability for the Na^+/H^+ countertransporter. Furosemide, acting on several transport proteins along the nephron, directly increases urinary excretion of titratable acids and ammonium.[32]

Under physiologic circumstances, the distal nephron reabsorbs the remaining 10% to 15% of filtered bicarbonate via transport mechanisms similar to those of the proximal tubule.[24] An important difference between proximal and distal tubular bicarbonate reabsorption is the absence of the carbonic anhydrase enzyme in the distal tubule.[24] Net H^+ ion secretion in the distal nephron continues even after the reabsorption of virtually all bicarbonate via active H^+ secretion and the ability of the distal tubular epithelium to maintain large transepithelial concentration gradients for H^+ and bicarbonate.[24] Among the endocrine factors influencing distal tubular acidification, aldosterone is one of the most important hormones.[24] By affecting the function of several different transport mechanisms, aldosterone stimulates net H^+ ion excretion in the distal nephron. The distal nephron of the premature neonate, however, has a developmentally regulated relative insensitivity to aldosterone.[26,33]

The secreted H^+ ions are excreted in the urine in the form of titratable acids (phosphate and sulfate salts) and as ammonium salts, which are formed by the combination of H^+ with ammonia.[24] Because the major constituent of titratable acid in the urine is $H_2PO_4^-$, drugs that decrease proximal tubular phosphate reabsorption and thus increase the delivery of phosphate to the distal nephron may increase the renal acidification capacity of the neonate. Indeed, by inhibiting proximal tubular phosphate reabsorption, dopamine has been shown to increase the excretion of titratable acids in preterm infants.[34]

Urinary excretion of titratable acid and ammonium increases as a function of gestational and postnatal age.[20] Because the ability to acidify the urine effectively is acquired by the age of 1 month even in very premature infants, however, distal tubular H^+ ion secretion appears to be inducible independent of the gestational age of the infant.[35]

Neonatal Metabolic Acidosis

The most frequent causes of increased anion-gap metabolic acidosis in the neonate are hypoxemia or ischemia secondary to perinatal asphyxia; severe lung disease; volume depletion; vasoregulatory disturbances; or myocardial dysfunction caused by immaturity, sepsis, or asphyxia. Severe metabolic acidosis caused by a neonatal metabolic disorder is rare but should always be considered. Interestingly, the type of anesthesia during caesarean section has also been suggested to influence neonatal acid base status. Indeed, according to a meta-analysis of the findings of 27 studies, cord pH was lower and base deficit was higher in newborns whose mothers received spinal anesthesia compared with those receiving general and epidural anesthesia.[36] Therefore, when assessing a neonate born via caesarian section that presents with metabolic acidosis in the immediate postnatal period, the type of anesthesia needs to be considered when searching for the etiology of the acid-base disturbance.

As discussed earlier, preterm neonates frequently present with a mild to moderate normal anion-gap acidosis, which is almost always the consequence of the low renal bicarbonate threshold of the premature kidney.[20,25,26] However, the use of carbonic anhydrase

inhibitors and parenteral alimentation, as well as the maturation related decreased sensitivity to aldosterone, may also contribute to the development of normal anion-gap acidosis in the neonate.[2,26,33]

In metabolic acidosis caused by the accumulation of lactic acid, H^+ ions are buffered by the extracellular and intracellular buffering systems; pH drops as plasma bicarbonate concentration decreases. Restoration of pH toward normal initially occurs through elimination of the volatile element of the carbonic acid-bicarbonate system via the lungs. This process, however, may be severely compromised in the sick preterm and term neonate with parenchymal lung disease. As described earlier, the renal compensatory mechanisms are also less effective because of the developmentally regulated immaturity of neonatal renal function. The main elements of the response of the neonatal kidney to metabolic acidosis in the immediate postnatal period are the attenuated increases in GFR, proximal tubular bicarbonate reabsorption, and distal tubular net acid secretion. A significant improvement in the overall renal response, however, occurs after the first month of life even in the premature infant.[20]

Neonatal Respiratory Acidosis

In the clinical setting, neonatal respiratory acidosis develops most frequently in preterm infants with respiratory distress syndrome. Although stimulation of the central respiratory center by the elevated interstitial carbon dioxide immediately increases respiratory rate and depth, carbon dioxide elimination by the lungs is usually limited because of immaturity and parenchymal disease. The kidneys reclaim more bicarbonate in response to respiratory acidosis. Especially during the first few weeks of life, however, renal compensation is limited by the developmentally regulated immaturity of proximal and distal tubular functions.

Neonatal Metabolic Alkalosis

Metabolic alkalosis most frequently develops in the preterm neonate who receives prolonged diuretic treatment for bronchopulmonary dysplasia. The respiratory response is a decrease in the rate and depth of breathing to increase carbon dioxide retention. This response may not be effective if the intubated neonate is relatively overventilated with the mechanical ventilator. In response to a metabolic alkalosis, urinary bicarbonate reabsorption and distal tubular net acid excretion decrease, resulting in a return of the extracellular pH toward normal.

Neonatal Respiratory Alkalosis

Neonatal respiratory alkalosis occurs most frequently secondary to fever and iatrogenic hyperventilation of the intubated preterm and term infant. Rarely respiratory alkalosis may be the presenting sign of a urea cycle disorder during the first days of life because rising ammonia levels may initially stimulate the central respiratory center. Renal compensation plays an important although limited role in cases of neonatal respiratory alkalosis.

SUMMARY

The fetus and the neonate exhibit a limited ability to maintain and regulate acid-base homeostasis. Regardless of the state of maturity, however, an abrupt increase in the sensitivity of acid-base regulation occurs with delivery. The mechanisms for this accelerated maturation are unclear.

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SECTION XVIII

Developmental Hematopoiesis

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Mervin C. Yoder

Developmental Biology of Stem Cells: From the Embryo to the Adult

Stem cells are a popular topic at present in both the scientific literature and the lay press. The isolation of human embryonic stem (ES) cells has fueled speculation that these cells may be useful in the repair and regeneration of tissues and organs for a host of patient disorders. Much basic and translational research will be required to achieve the widely publicized therapeutic projections. It has become clear that the controlled differentiation of stem cells into specialized mature cells, the expansion of stem cells in vitro with retention of multipotentiality, and the best source of stem cells for a particular therapeutic application all remain to be determined. The topic of stem cells is further complicated by the realization that the methods used to isolate human ES cells require the sacrifice of a human embryo. Thus spiritual, ethical, and political ideologies have been brought into the ethical debate of when an embryo should be afforded moral status, and this ongoing argument has had major impact on the scientific conduct of human ES cell research in the United States. Some states have conceptually adopted and promoted human ES cell research through research-funding initiatives and political rhetoric, whereas other states have been opposed to the promotion of this area of science. It is unclear whether the controversy of determining how and when or if to assign moral status to the human embryo can be resolved.

This chapter introduces some basic principles and concepts in the broad field of stem cell biology, with emphasis on the important distinction between the two classes of stem cells: embryonic and adult. Stem cell terminology and discussion of the general categories of stem cells are presented first. The remainder of the chapter is organized in an ontologic approach with respect to emergence of cells with stem cell potential, beginning with a description of the totipotent properties of the zygote. Next, a discussion of selected aspects of murine ES cell biology is presented as an introduction to human ES cells, followed by a review of the discovery of human embryonic germ (EG) cells. Finally, several of the more commonly studied adult stem cell populations are discussed—the number of adult stem cell populations that emerge to sustain specified tissues and organs throughout life being too extensive to review in entirety. When appropriate, some discussion of the current use of stem cells in clinical medicine also is provided.

DEFINITION OF STEM CELLS AND STEM CELL TERMINOLOGY

The human body is composed of more than 200 different cell populations that are assimilated into multiple specialized tissues and organs of diverse functions. All of these cells are ultimately derived from the "mother" of all stem cells, the fertilized oocyte (zygote). Only the zygote and blastomere cells within the first few cleavage stage divisions possess the capacity to produce a complete organism. Whereas many tissues and organs, once fully formed, and the cell populations that make up these tissues are stable postnatally and do not require ongoing cellular replacement, other organs (e.g, blood, skin, and intestine) depend on a continuous influx of new cells to maintain tissue homeostasis throughout adult life. Thus not only are stem cells required for the initiation of embryo formation, but they also are critical for homeostasis of the adult organism.

Stem cells are defined as cells possessing the capacity to self-renew (cell division giving rise to at least one cell retaining all the properties of the parent cell) and the ability to proliferate and differentiate into one or all of the more than 200 cell types that make up the fully formed body.[1] The zygote and blastomeres within the first few cleavage stage divisions are *totipotent cells* that can form all of the cells of all tissues and organs that form an embryo. *Pluripotent stem cells* are defined as cells that can give rise to cells developing from all three embryonic germ layers: endoderm, ectoderm, and mesoderm. The primary difference between pluripotent and totipotent cells is the ability of the latter to give rise to trophectoderm cells that form the placenta and some of the extraembryonic membrances. Of importance, defining a stem cell as totipotent or pluripotent generally requires detailed analysis of donor stem cell progeny contribution to tissues and organs on adoptive transfer of cells into a recipient blastocyst or embryo, not just in vitro evidence of cellular gene expression that typifies an expression pattern observed in cells of a particular tissue or organ. For example, induced cell expression of albumin is not equivalent to proof that a cell has become a hepatocyte, even though albumin is predominantly expressed in hepatocytes.

Stem cells may be generally divided into ES and adult stem cell groups. ES cells are derived from the inner cell mass of the mammalian blastocyst.[2,3] Isolation of the inner cell mass cells followed by growth of these cells on preestablished cultures of murine embryonic fibroblasts defines the culture conditions sufficient for emergence of proliferating cells called *ES cells.* ES cells do not exist in vivo, but they develop in vitro from the cultured inner cell mass cells of the blastocyst. ES cells are pluripotent and display unlimited proliferative potential in vitro in the presence of embryonic fibroblasts. Murine ES cells also can be maintained in the absence of the feeder layers when they are fed high concentrations of leukemia inhibitory factor (LIF), the lone growth factor sufficient to maintain murine ES cell self-renewal divisions. Murine ES cells maintained under these conditions in vitro retain the potential to form a complete embryo when they are injected into a recipient blastocyst and are implanted in the womb of a pseudopregnant dam.[4]

A second form of stem cells derived from the embryo can be isolated from the developing gonads of fetal mammals.[5,6] By recovery of developing gonads, dispersion of the germ cells onto a layer of embryonic fibroblasts in vitro, and passage of the resulting colonies, the isolation of *embryonic germ (EG) cells* is readily accomplished. EG cells also are pluripotent, exhibit high proliferative potential, and must be maintained in culture similar to that for ES cells. ES and EG cells display similar capacities for differentiation into a variety of differentiated cell types in vitro.

Stem cells are said to differentiate when they divide, and at least one of the progeny commits to the activation of a new gene expression pattern or inactivation of the genes maintaining the stem cell state. These genetic changes cause the cells to take on new properties characteristic of mature cells of a particular tissue or organ. Just a few years ago, it was believed that once the ES or EG cells began the commitment process to differentiate into a mature cell type, these cells were no longer capable of reacquiring the pluripotentiality that is characteristic of a self-renewing ES or EG cell. As discussed later in the chapter, however, additional evidence has emerged showing that coexpression of a certain group of transcription factors is sufficient to permit even fully differentiated cells to regain pluripotent properties similar to ES or EG cells in vitro and in vivo.

Adult stem cells are cells residing in tissues and organs that maintain homeostasis by repleting cells lost to cellular senescence, disease, or injury. The most widely recognized organs with stem cells include bone marrow, skin, intestine, and liver. However, the brain, cornea, retina, skeletal muscle, and dental pulp also contain renewable stem cells, as may several other organs and tissues such as heart, skeletal muscle, bone, and lung. Adult stem cells also are called *somatic stem cells* to distinguish them from the germ cells of the adult organism. Adult stem cells in general fail to display the in vitro proliferative potential of ES or EG cells, although these cells obviously self-renew for the life of the organism (which otherwise would run out of blood, intestinal villi, skin, and so on). In general, adult stem cells display tissue-specific characteristics and differentiate into progeny specialized for the tissue or organ within which they reside. This fundamental tenet of biology is reflected in the clinical use of stem cells. Thus, when a patient needs a bone marrow transplant for a blood cell disorder, hematopoietic stem cells are transplanted as the donor tissue and not stem cells derived from another organ.

Some adult stem cell populations have been demonstrated to give rise to progeny in vitro and in vivo that normally are not derived from that type of stem cell. For example, some bone marrow cells have been found to differentiate into cardiomyocytes in mice after experimentally induced myocardial ischemia.[7] This ability of an adult stem cell of one tissue to generate mature cells of another tissue type has been called *stem cell plasticity.* This is a relatively new concept and is not yet mechanistically understood, although a growing body of evidence suggests that in many cases, the donor cells may fuse with injured host cells, thus giving the *appearance* of change to the host cell type.[8] Other evidence suggests that stem cell plasticity is a rare event, and it is unclear whether this phenomenon normally occurs to maintain homeostasis.[9]

A feature of most adult stem cells is the production of a variety of progenitor cell populations with varying levels of proliferative potential. For example, hematopoietic stem cell proliferation may cause one daughter cell to retain stem cell potential (self-renewal) but the other daughter cell to become a progenitor cell. The progenitor cell may retain the ability to proliferate into additional progenitor daughter cells, but it cannot regain the potential to recreate itself (self-renew). In a stepwise hierarchical fashion, progenitor cells become more committed to mature cell differentiation with each subsequent division, progressively losing their proliferative potential.

ES, EG, or adult stem cells may or may not be clonally derived, that is, a state when all of the cells in a population can be demonstrated to be the progeny of a single initiating stem cell. Several clonal human ES cell lines have been created.[10] Obtaining a clone of adult stem cells has been more difficult. Methods to isolate adult stem cells generally result in a mixture of stem and progenitor cells. However, hematopoietic stem cells are one adult stem cell type that have clearly been demonstrated to clonally reconstitute the entire hematopoietic system in lethally irradiated host animals.[9] This is an important point in considering the question of stem cell plasticity. As discussed further on, the term *stem cell plasticity* should be reserved for those instances in which it is clear that a single stem cell derived from one tissue can be demonstrated to give rise to a clone of progeny of another tissue, not just to a few scattered cells within that tissue.

EMBRYONIC STEM CELLS

Murine Cells

Isolation

Efforts to isolate murine cells with pluripotent properties began in the 1950s. Leroy Stevens observed that male mice of the 129 strain developed testicular teratomas, and cells from these tumors could be transplanted with recurrence of the teratoma.[11] Further studies revealed that fetal germ cells (derived from the 129 strain of mice) implanted in the testicle also could precipitate teratocarcinoma formation. The most profound insight provided by these studies, however, was that cells isolated from the inner cell mass of murine blastocysts also gave rise to teratocarcinomas on transplantation. Stevens called these cells *pluripotent ES cells,* the first such designation of inner cell mass–derived cells as ES cells.[12] However, the injected embryonic cells gave rise to carcinomas as well as teratomas in this model and later became known as *embryo carcinoma (EC) cells.*

Two independent groups of investigators reported in 1981 that cells from the inner cell mass of the murine blastocyst could be maintained in vitro in a pluripotent state under defined conditions that in some cases included addition of conditioned medium from cultured teratocarcinoma cells.[3,13] Growth of the cells from the inner cell mass required a co-culture system using preestablished murine embryonic fibroblast feeder monolayers. LIF was identified as the factor secreted by the embryonic fibroblast that was required to retain ES cells with high proliferative potential and to maintain pluripotency in vitro.[3] ES cells can now be maintained indefinitely without chromosomal aberrations under these culture conditions and contribute to the formation of extraembryonic, fetal, and germ cell lineages on reintroduction into donor blastocysts and transfer into the fallopian tubes of pseudopregnant female mice. In fact, if the murine ES cells are cultured with both LIF and bone morphogenetic protein-4 (BMP-4), serum-free defined culture medium conditions can be used.[14] This is a great advance for the field, because fetal calf sera are known to fluctuate in trace molecule concentrations from batch to batch, and such variability can lead to vast changes in gene expression and ES cell function.

Maintenance of Self-Renewal

The molecular mechanisms that maintain ES cells in a self-renewal state are not clearly delineated. It is clear, however, that signals generated during LIF binding to the cell surface LIF receptor are sufficient to maintain pluripotency.[15] LIF binding to a cell surface heterodimeric receptor results in activation of numerous molecules including nonreceptor tyrosine kinases. These kinases subsequently activate nuclear transcription factors including STAT3 (*s*ignal *t*ransducer and *a*ctivator of *t*ranscription-*3*). STAT3 activation alone is sufficient to maintain murine ES cells

in a self-renewal state with inhibition of cellular differentiation.[16] STAT3 proliferative effects on ES cells can be blocked by activation of the Erk (*e*xtracellular signal–*r*egulated *k*inase) pathway. A balance between the differentiating effects of Erk kinase activation and the proliferative effects of STAT3 activation appears to play a key role in determining whether ES cells are maintained in a pluripotent state.[17]

Another hallmark of pluripotent ES cell homeostasis is expression of the nuclear transcription factor Oct-4 (*oct*amer-binding transcription factor-*4*).This factor is present in the mouse zygote and is required for normal formation of the blastocyst and maintenance of the pluripotent state of the inner cell mass cells. Oct-4 also is highly expressed in germ cells. Evidence to date implicates specific control of the level of Oct-4 expression for maintaining the pluripotent state.[18] Overexpression of Oct-4 in ES cells leads to differentiation of the cells along the endoderm and mesoderm lineages, and diminished expression inhibits trophectoderm formation.To date, only a few of the target genes regulated by Oct-4 have been identified; transcription of some is inhibited and of others is increased by Oct-4 binding to target sequences in these genes. In general, the current theory is that Oct-4 may prevent expression of those genes required for ES cell differentiation.

In Vitro Differentiation

The ability to culture pluripotent cells in vitro has permitted novel insights into and greater accessibility of gene expression and cellular differentiation pathways than had been previously possible during attempts to study the murine post-implantation embryo in situ. Removal of LIF from the culture medium initiates murine ES cell differentiation. Plating LIF-deprived ES cells onto nonadhesive culture plates or semisolid medium (methylcellulose) or placing ES cells into suspended drops of culture medium induces the cells to form spherical structures called *embryoid bodies* (EBs).[19] Within the EB, cells derived from the embryonic germ layers develop with relationships similar to those of the developing embryo—embryonic endoderm on the exterior of the EB and mesoderm and ectoderm on the interior surrounding a fluid-filled cavity (yolk sac–like). ES cells present in the EBs differentiate into multiple mature cell types including neurons, pancreatic cells, blood cells, endothelial cells, cardiomyocytes, skeletal muscle cells, adipocytes, and numerous other cells.[20] By extrapolation, the ability of murine ES cells to proliferate extensively in vitro and to differentiate into specific cell types underlies the current enthusiasm for studying human ES cells as a future means to create mature cells to replace aged or damaged cells in human patients.What remains to be determined are the specific cell-to-cell interactions within the EBs that result in the emergence of all three germ layers and resulting progeny, because ordered positional information is lacking in the spherical EBs (no obvious anterior-posterior axis, Hensen's node, or primitive streak is present).[14]

Human Cells

Isolation and Characterization

The isolation and characterization of human ES cells were reported in 1998.[2] In this landmark study, infertile couples donated cleavage-stage human embryos, after informed consent and institutional review board approval of the study. From the 36 donor embryos, 20 were successfully grown to the blastocyst stage (day 5 after fertilization), immunosurgery was then performed to remove the trophoblast cells, and inner cell mass cells were plated on murine fibroblast feeder cell layers (similar to derivation of murine ES cells). Five ES cell lines from five separate embryos were derived, and each continuously proliferated for 6 months; all were successfully cryopreserved.The karyotype of the ES cells was normal; three lines were female and two were male.A murine fibroblast monolayer was required to prevent the ES cells from differentiating on the tissue culture plates. However, when the ES cells were allowed to overgrow and "pile up" on the feeder layers, the cells underwent differentiation. Addition of human LIF to the human ES cells plated in the absence of mouse feeder layers failed to maintain ES cell pluripotency and growth.Therefore one early notable difference between murine and human ES cells was the inability of LIF to maintain ES cell pluripotency in the human cells.

The morphology of human ES cells is somewhat similar to that of murine ES cells. Both demonstrate a high nuclear-cytoplasmic ratio with prominent nucleoli. Human ES cells form colonies in vitro that are not as compact as murine ES cells but are similar to rhesus monkey ES cells. Human ES cells express the cell surface markers SSEA-3 (*s*tage-specific *e*mbryonic *a*ntigen-*4*) and alkaline phosphatase. Oct-4 expression is high, consistent with the self-renewal potential of these cells. Like murine ES cells, human ES cells express high levels of telomerase, an enzyme that lengthens telomeres that normally shorten with each cell division.Telomere length maintenance is a property of self-renewing stem cells and some cancer cells.

The most favored test of human ES cell pluripotency consists of injection of the cells into immunocompromised mice followed by observation for development of subcutaneous teratomas.[2] Analysis of the tissue in the teratomas has demonstrated that derivatives of all three embryonic germ layers are present. Two cloned human ES cell lines were derived from one of the original ES cell lines.[10] The cloned human ES cells formed teratomas in vivo similar to the original ES cell population.This result confirms that the progeny of a single human ES cell retains pluripotency, even though these cells have been "passaged" extensively in vitro.

In Vitro Differentiation

Advances in the in vitro differentiation of human ES cells into specific lineages have been reported.[21,22]The addition of retinoic acid and nerve growth factor to cultured human ES cells was reported to enhance neuronal cell differentiation significantly. The differentiated cells demonstrated extensive outgrowth of neuronal processes and expression of neuron-specific molecules. Co-culture of human ES cells with murine bone marrow or yolk sac cell lines induced hematopoietic differentiation in vitro.[23] The ES cell–derived hematopoietic cells expressed known human hematopoietic cell surface antigens and transcription factors.When they were plated in colony-forming cell assay cultures containing hematopoietic growth factors, numerous hematopoietic progenitor populations were identified. Hematopoietic cell maturation appeared to be normal. Finally, plating of human ES cells in conditions that promote EB formation resulted in spontaneous contractions in 8.1% of the EBs.[24] Cells from the areas of spontaneous contractions expressed cardiac-specific proteins and messenger RNA (mRNA) for cardiac-specific transcription factors, and they displayed morphologic evidence of sarcomeric organization and intercalated disk formation connecting adjacent cells. In addition, the ES cell–derived cells exhibited cardiomyocyte-like calcium ion transients that were synchronous with the recorded contractions and responded to both positive and negative chronotropic agents.These data suggest that human ES cells can be differentiated into neurons, hematopoietic cells, and cardiomyocytes in vitro. At present, it is unknown whether these cells would function in vivo, a critical experimental hurdle for which new transplantation models may be required.

Potential Uses

ES cells may hold the potential for replacing cells and tissues lost to a variety of devastating illnesses. Neurologic diseases, diabetes, heart failure, end-stage kidney disease, and liver failure are a few areas in which availability of a replenishable source of cells and tissues may provide new therapeutic alternatives. In addition, ES cells differentiated into certain tissues may serve as a powerful

in vitro screening method for new pharmacologic agents, teratogens, and environmental toxins. To achieve these breakthroughs, however, much research is required to address the following questions[25]:

- What mechanisms maintain human ES cells in a pluripotent state?[26]
- What cellular and molecular signals are required to activate human ES cells to begin differentiating into a single specialized cell type?
- Are human ES cells homogeneous or heterogeneous?
- Do all human ES cells pass through a stem or progenitor stage during the process of cellular differentiation?
- What stage of differentiation of human ES cells will be most optimal for transplantation?
- How will differences in transplantation antigen expression between the human ES cell–derived transplantable tissue and the host be overcome?

ADULT STEM CELLS

Hematopoietic Stem Cells: Murine Cells

Isolation

More is known about the hematopoietic stem cell than about any other stem cell population. In fact, hematopoietic stem cells currently represent the only stem cell population used in standard therapeutic regimens in human patients. The mouse model has been an invaluable tool in the search for the hematopoietic stem cell that began more than 50 years ago.

Whole-body irradiation was noted to depress hematopoiesis and cause life-threatening pancytopenia (conditions in which all blood types are diminished in number in the bloodstream) in exposed mice. However, the mice could be protected from radiation-induced hematopoietic impairment if the spleen of the irradiated animal was shielded from the radiation beam or if hematopoietic cells from another mouse were infused into the radiation-exposed animal.[27,28] Confirmation that blood cells—and not plasma—conferred radiation protection was reported soon after.[29] In 1961, Till and McCulloch[30] provided evidence that a single blood cell precursor (colony-forming unit–spleen cell) present in the bone marrow could be identified in vivo by intravenously injecting the donor marrow cells into a lethally irradiated recipient animal and examining the host spleen for blood cell–containing colonies 8 to 12 days later. This experimental method advanced the study of blood cell production by supporting the concept of a transplantable hematopoietic precursor population that could produce numerous progeny in vivo and led the way to identification of hematopoietic stem cells—a landmark feat recognized by awarding of the Lasker Award to Drs. Till and McCulloch in 2005.

Several assays of stem cell function have subsequently been developed. The current standard is to measure stem cell function by transplanting donor test cells into a lethally irradiated mouse and then documenting that donor-derived cells have replaced host blood cell lineages over a prolonged period (4 months).[31] Analysis for donor-derived cells in the host requires distinction of the exogenous and endogenous cells. Male-to-female mismatch transplants, use of congenic mouse strains that differ at one or two genetic loci, use of gene transfer to mark the donor cells, and application of fluorescent labeling dyes to the donor cells all have been successfully used to identify donor cells in the host. As a surrogate for the in vivo analysis of stem cell function, several in vitro assays whereby hematopoietic stem cells are cocultured with stromal cells and the co-cultures are examined for prolonged production of colony-forming cells in vitro have been reported. A good correlation between the in vitro assays and in vivo repopulating activity has been reported in studies identifying stem cell activity in adult murine bone marrow cells.[32]

In mice, hematopoietic stem cell repopulating activity appears to be conferred by two populations of cells.[33] Some transplanted cells demonstrate the capacity to reconstitute the bloodstream of the lethally irradiated host for several months and rescue the animal from pancytopenia (the irradiation kills the cycling hematopoietic progenitor cells, so that on loss of mature platelets and neutrophils in the postirradiation period, hemostasis capability and host defense cannot be maintained). However, these short-term repopulating cells do not continue to produce circulating blood cell progeny after 3 to 4 months subsequent to transplantation, and the bone marrow of animals reconstituted with these cells is devoid of donor stem cells that are capable of repopulating the hematopoietic system of a secondary recipient mouse on transplantation. By contrast, long-term repopulating hematopoietic stem cells do not display the ability to acutely rescue the host animal from the sudden deprivation of hematopoietic progenitor cells, but if the animal is given some short-term radioprotective cells, the stem cells produce hematopoietic progeny for the life of the primary recipient, and bone marrow from the primary recipient animal is capable of repopulating secondary recipient mice. Only recently have these two populations and even more committed progenitor cell populations been identifiable and amenable to isolation and study.[34]

Various techniques have been used to isolate hematopoietic stem cells. Stem cells can be separated from more committed cells using velocity sedimentation, adhesion, or exposure to chemotherapeutic agents. One useful method for isolating hematopoietic stem cells from mice takes advantage of the finding that stem cells express a variety of proteins and carbohydrates on their cell surface, some which appear to be hematopoietic-specific. If monoclonal antibodies to these cell surface proteins are labeled with fluorescence-emitting molecules, the stem cells with bound antibodies may be analyzed and isolated by flow cytometry.[35] In the mouse, expression of antigens such as stem cell antigen-1 (Sca-1), c-Kit, CD150, CD34, CD38, CD43, CD44, CD45, Thy-1, and AC133, along with high expression of the ABCG2 transporter protein and low expression of RNA- or DNA-binding dyes, enriches for hematopoietic stem cells.[36,37] Stem cells do not express markers that commonly are expressed by myeloid and lymphoid lineages (lin) and therefore are described as lin^-. In studies using multiparameter sorting, murine hematopoietic stem cells have been reportedly purified to homogeneity.[38,39]

Characterization

The site of hematopoietic stem cell residence changes during murine ontogeny.[40] Hematopoietic cells first appear in the yolk sac on embryonic day 7.0 (E7.0). Although the immediate precursor of the first hematopoietic progenitor cells has not yet been identified, hematopoietic cells are known to emerge from the mesoderm cells that emigrate into the yolk sac after migrating away from the primitive streak during gastrulation. Stem cell activity has been identifiable in the yolk sac as early as E9.0 but only if the donor cells are infused into sublethally myeloablated newborn mice.[41] The first stem cells to repopulate lethally irradiated adult mice appear in the region of the developing gonads, mesonephros, and aorta on E10.[42] More recent evidence demonstrates that all of the hematopoietic progenitor cells that initially seed the fetal liver (before hematopoietic stem cell seeding) and give rise to circulating red and white blood cells and platelets are derived from the yolk sac. Stem cell activity is present in the fetal liver on E11.0, although more recent studies indicate that substantially greater stem cell expansion occurs in the placenta from E11 to 13 than in the fetal liver.[43] By E14, however, the fetal liver is the sole organ with both hematopoietic stem and progenitor cell potential, and subsequently this activity is sequentially found in the blood, spleen, and marrow just before and after birth. The liver becomes significantly depleted of hematopoietic stem cells after 2 weeks of life, although some stem cell activity

can be identified in circulating blood and thus in all organs in the adult mouse; an estimated 100 to 200 stem cells continuously circulate in the mouse systemic circulation.[44] The spleen and marrow compartments are lifelong sites of hematopoietic stem cell production in the mouse. A direct comparison of hematopoietic cell–repopulating ability of stem cells from each of these sites has not been conducted; however, fetal liver hematopoietic stem cell repopulating ability is nearly two-fold that of marrow-derived hematopoietic stem cells.

Murine hematopoietic stem cells are estimated to be present at a frequency of 1 per 10,000 to 1 per 100,000 bone marrow cells. The hematopoietic stem cells with the greatest repopulating potential in adult mice are characterized by preferential existence in a quiescent state.[45] Most of these cells are arrested in the G_0 stage of the cell cycle. Two estimates of the turnover of the stem cell pool have been reported. In young adult mice, 8% of the stem cell pool in one study randomly entered the cell cycle daily.[46] Using another method of analysis, hematopoietic stem cells were estimated to display a half-life (the time required for 50% of the cells to divide) of approximately 19 days.[47] Of interest, commitment of hematopoietic stem cells to the progenitor compartment results in an increase in the frequency of cells in cycle. One estimate of the total number of cell divisions required to produce a mature blood cell from an initiating hematopoietic stem cell ranges from 17 to 19.5, for an overall net amplification of approximately 170,000- to 720,000-fold.[48]

The behavior of hematopoietic stem cells can be allocated into four general activities in the marrow environment: self-renewal, differentiation, migration, and apoptosis. Each of these activities is briefly discussed next. The mechanisms that regulate the overall activity state of the marrow stem cell population, however, remain elusive.

Self-Renewal Ability

Self-renewal divisions are difficult to prove in vitro. Such events clearly occur in vivo with bone marrow transplantation, when the stem cell must not only repopulate all the mature blood cell lineages but also expand the stem cell pool to normal levels. Co-culture of hematopoietic stem cells with certain hematopoietic stromal cells in vitro has permitted documentation of self-renewal divisions, as evidenced by maintenance or slight expansion of repopulating activity on transplantation.[49,50] Certain growth factors—stem cell factor, LIF, and thrombopoietin—appear capable of stimulating hematopoietic stem cells to undergo limited self-renewal division in vitro.[51] Overexpression of the transcription factor HOXB4 permits extensive proliferation of hematopoietic stem cells in vivo and in vitro, with retention of repopulating ability implying numerous self-renewal divisions.[52] Novel approaches such as identifying specific genes that are expressed in different populations of stem cells with differing repopulating potentials may provide new insights into the mechanisms regulating hematopoietic stem cell self-renewal.[53]

Differentiation

The stem cell theory of hematopoiesis states that the hematopoietic system can be viewed as being composed of a continuum of functionally distinct hematopoietic cell compartments. Once the commitment decision is made by a dividing stem cell, the progenitor cell gives rise to progressively more lineage committed hematopoietic progenitor cells and eventually mature blood cells. It remains unclear whether the commitment process occurs randomly (stochastic theory) or is deterministic (controlled by growth factors or other factors released into each specific microenvironment in which the stem cells reside). Nevertheless, evidence suggests that progenitor cells in each functional compartment express distinct combinations of cell surface markers that permit isolation and determination of the stage of hematopoietic commitment of these cells.[34]

Migration

Migration of hematopoietic stem cells through the circulation into developing organs is a hallmark of early fetal and neonatal hematopoiesis. Evidence confirms that hematopoietic stem cell migration throughout the systemic circulation is a consistent feature even in adult mice.[44] At present, it is unclear whether certain organ vascular beds sequester the circulating stem cells or whether the stem cells are randomly distributed throughout the systemic circulation. Progress has been made in identifying strategies to mobilize large concentrations of stem cells into the circulation using growth factors and chemotherapeutic agents; however, the mechanisms regulating the constitutive release, circulation, and reentry of stem cells into the marrow compartment remain elusive.[54] Recent use of transgenic and knockout mice models with hematopoietic specific targeting strategies has permitted new insights into identification of the role of specific molecules in hematopoietic stem cell homing during development in the mouse.[43]

Apoptosis

The size of the hematopoietic stem cell pool remains relatively constant. One mechanism to regulate pool size is to control the rate of stem cell division tightly (see earlier). A backup mechanism is to stimulate apoptosis of stem cells if excessive numbers are produced. Evidence that apoptosis is an active component of normal hematopoietic stem cell homeostasis is derived from experiments in which overexpression of the anti-apoptotic protein Bcl-2 results in significant expansion of the total hematopoietic stem cell pool in transgenic mice.[55] Hematopoietic stem cells readily undergo apoptosis in vitro if they are not supplied with appropriate growth factors, serum, or hematopoietic stromal cells for co-culture.

Hematopoietic Stem Cells: Human Cells

Isolation

Identification of the human hematopoietic stem cell has relied heavily on correlations between murine and human in vitro hematopoietic colony-forming cell assays and murine transplantation experiments. Correlative data also have been obtained from canine and nonhuman primate transplantation models. Several xenotransplantation models in which human hematopoietic stem cells are infused into immunodeficient mice or preimmune fetal sheep in utero have permitted identification of cells with properties of human hematopoietic stem cells.[56,57] However, the current standard for defining stem cell activity ultimately must rely on effective engraftment and repopulation with the test cells in a human patient.

The history of hematopoietic stem cell transplantation in human patients spans less than 50 years.[58] E. Donnall Thomas and colleagues[59] reported that careful filtering of human marrow to remove fat and bone particles allowed large amounts of marrow to be infused into patients, although with little clinical improvement. Many unsuccessful reports followed these early transplantation attempts and led to several decades of intense research in animal models aimed at elucidating the complex biologic and immunologic barriers that need to be overcome, including graft-versus-host disease (GVHD), histocompatibility mismatch, and alternatives to total-body irradiation as a pretransplant conditioning method.[58] Improvements in all aspects of clinical transplantation have resulted in a growing number of transplant recipients; in 2000, more than 18,000 patients received a bone marrow transplant.

While the scientific foundation for effective clinical transplantation was being investigated, advances in the culture of marrow cells led to identification of human hematopoietic progenitor cells.[36] As in the mouse, human hematopoietic stem and progenitor cells can be isolated using monoclonal antibodies and flow

cytometry. CD34 is an antigen expressed by most hematopoietic stem and progenitor cells, although endothelial cells also express this antigen. Some hematopoietic stem cells do not express CD34, and it is unclear whether these cells are the precursors for the CD34-expressing population. Furthermore, some patients who receive CD34-expressing bone marrow cell transplants have demonstrated multilineage engraftment more than 7 years after the procedure, indicating that the $CD34^+$ cells possess long-term repopulating ability. Other antigens expressed by the human hematopoietic stem cell population include c-Kit, Thy-1/CD90, CD133, and the ABCG2 transporter protein.[60] The presence of this transporter protein results in rapid efflux of many different molecules including certain DNA dyes, allowing for selection of DNA dye low-staining stem cells referred to as a *side population profile* on the flow cytometer.

When bone marrow cells are first depleted of all cells expressing mature blood cell lineage markers (lin) and are then selected for CD34 expression ($CD34^+lin^-$), the frequency of hematopoietic stem cells in the enriched population ranges from 1 per 200 to 1 per 500.[61] As noted earlier, analysis of human hematopoietic stem cell number and function is often inferred from experiments performed in vitro or from in vivo xenotransplantation assays. Plating of human marrow cells in colony-forming assays (to enumerate progenitor cell activity) or in co-culture with stromal cells to evaluate the proliferative potential of the cells may reflect the population of cells that retains the most potential. Intravenous administration of human hematopoietic cells into immunocompromised mice or the preimmune sheep fetus permitted assay of the ability of the human hematopoietic stem and progenitor cells to home to the marrow compartment and to produce committed progenitor and mature cells in vivo.[61] Although these types of stem cell transplantation models clearly have limitations, no better surrogate for human clinical transplantation currently exists.

Characterization

The site of human hematopoietic cell production also varies during in utero development. Although the exact site of origin of human hematopoietic stem cells remains elusive, evidence suggests that the intraaortic clusters of cells identified in the month-old embryo may be one of the first sites composed of hematopoietic stem and progenitor cells.[62] The human fetal liver is a prominent site of hematopoiesis for much of the gestational period. Circulating hematopoietic cells that engraft in immunocompromised mice have been identified in the second trimester. Umbilical cord blood is enriched in hematopoietic stem and progenitor cells; preterm infant cord blood is more enriched for these cells than cord blood derived from full-term infants. Umbilical cord blood cells engraft 10 to 50 times better than adult marrow hematopoietic cells on transplantation into immunocompromised mice.[63] Proliferative potential in vitro is higher in cord blood hematopoietic cells than in adult marrow progenitor cells. No direct comparison of the hematopoietic repopulating ability of marrow cells isolated from young versus old subjects has been performed to date.

Administration of granulocyte colony-stimulating factor to human subjects results in mobilization of hematopoietic stem and progenitor cells into the systemic circulation. Combining granulocyte colony-stimulating factor or AMD3100 (a small-molecule inhibitor of CXCR4 ligand binding) administration with leukapheresis permits isolation of an enriched population of peripheral blood transplantable cells.[64] Stem cell mobilization has become a preferred method for isolating cells for clinical transplants because of the increased numbers of cells recovered and the earlier neutrophil and platelet recovery in the conditioned patient.[36]

Human hematopoietic stem cells are difficult to maintain in culture. Most methods developed for expansion of hematopoietic cells ex vivo have resulted in large increases in the number of committed progenitor cells, with loss of the stem cells. This is particularly true for culture conditions relying solely on combinations of recombinant growth factors to induce the cellular proliferation. The addition of extracellular proteins or certain endothelial or stromal cell lines appears to improve the ex vivo maintenance of the most primitive hematopoietic progenitor and stem cells while inducing overall cellular expansion.[65,66]

Therapy for Human Clinical Disorders

Bone marrow transplantation is a potentially useful therapy for various cancers of the blood including acute lymphoblastic and myeloblastic leukemia, chronic myelogenous leukemia, Hodgkin disease, multiple myeloma, and non-Hodgkin lymphoma[36] Transplantation can be performed using autologous (the patient's own) cells or allogeneic (another person's) cells. Cells from the bone marrow or mobilized cells in the peripheral blood can be used for either type of transplant. Although autologous cells can be transplanted without concern for whether the cells will be rejected or induce GVHD, these cells may harbor the very transformed cells that the patient is receiving chemotherapy to eradicate. Application of flow cytometry or other cell-sorting strategies (e.g., immunomagnetic beads) to isolate hematopoietic stem cells can provide graft cells free of detectable tumor cells.

The use of allogeneic grafts for hematopoietic reconstitution in patients with cancer is rational because these donor cells are not contaminated with the cancer cells. However, both bone marrow and mobilized peripheral blood grafts contain T lymphocytes that will recognize host tissues as foreign on transplantation and will induce GVHD unless the host and the donor share the same histocompatibility antigens (human leukocyte antigens [HLAs]). HLA typing of the donor and the host is routine, to obtain the best possible match. Because of the polymorphic nature of HLA genes, the probability of a match between a host and a sibling is only 25%. Of interest, recent studies suggest that transplantation of mesenchymal stem cells provide some amelioration of GVHD in transplant recipients.

In certain circumstances, the provision of donor-derived T cells in an HLA-matched allogeneic graft may cause a "graft-versus-leukemia" (GVL) response and may actually help to kill the transformed cells in the patient. In fact, once transplanted cells have engrafted and the patient has recovered from any GVHD, peripheral blood lymphocytes from the graft donor can be recovered and infused into the patient to provide a stronger GVL effect. This strategy has been used successfully in patients with chronic myelogenous leukemia.[58] More recent data indicate that cord blood–derived donor cells may give rise to less severe GVHD disease but also provide less GVL effect.

Allogeneic grafts are currently useful in the treatment of inherited blood disorders. Aplastic anemia, β-thalassemia, Blackfan-Diamond syndrome, sickle cell anemia, severe combined immunodeficiency, X-linked lymphoproliferative syndrome, and Wiskott-Aldrich syndrome are some of the candidate diseases for bone marrow or cord blood hematopoietic stem cell transplantation.[58] Single-gene metabolic defects such as Hunter syndrome and Hurler syndrome and certain forms of osteopetrosis also are amenable to bone marrow transplantation.

Umbilical cord blood is becoming a favored graft source when a suitable HLA-matched donor is not available. Mismatched umbilical cord blood appears to engraft with less GVHD in pediatric or adult patients when compared with partial mismatched marrow or peripheral blood grafts. The primary concern with using umbilical cord blood as a source of donor cells is that the number of hematopoietic stem cells in the cord blood sample may be limiting for large adult patients. Efforts to expand the hematopoietic stem and progenitor cells in cord blood samples remain experimental (see earlier). Recent success has been achieved with coadministration of two separate units of allogeneic cord blood

cells in adult patients with leukemia, after use of less aggressive myelosuppressive regimens.[67]

Neural Stem Cells

The central nervous system (CNS) is composed of hundreds of different types of neurons, astrocytes, and oligodendrocytes. Although the CNS is susceptible to the same environmental, genetic, metabolic, and infectious stressors as for other tissues in the body, the neuronal cell types were thought to differ from cells in other organs in being completely postmitotic. Numerous studies since the early 1990s, however, provided compelling evidence that neurogenesis occurs in the adult brain, and cells are being continuously generated in some portions of the adult CNS.[68] Furthermore, more recent techniques have permitted isolation of the neural stem cells (NSCs), and methods to expand, differentiate, and transplant these cells are being developed. Such rapid progress has been exceeded only by the growing hope of anticipated benefits generated by families of patients and patients suffering from CNS disorders.

Neurogenesis in the adult brain occurs in primarily two areas: the dentate gyrus of the hippocampus and the olfactory bulb in humans, rodents, and nonhuman primates.[69] The *neural progenitor cells* (NPCs) that give rise to the new neurons in the dentate gyrus of the hippocampus are derived from the subgranular zone of the dentate gyrus. The neuronal progeny and glial progeny of the NPC are formed in the granular layer of the dentate gyrus after migration of the NPC into this layer. Functional connections with other neurons in the hippocampus are established in as few as 4 to 10 days after neuronal differentiation is initiated in the NPC progeny. NPCs that seed the olfactory bulb are derived from the anterior part of the subventricular zone (layer of cells beneath the ependymal lining cells of the lateral ventricles).[70] Migration of the NPCs from the subventricular zone to the olfactory bulb occurs through a rostromigratory stream composed of specialized astrocytes. Cell-to-cell communication between the NPC and the astrocytes is mediated by secreted glycoproteins on the surface of the NPC. Disruption of these important cellular interactions results in diminished NPC migration and a reduction in the size and organization of the olfactory bulb.

Like the hematopoietic system, the neuropoietic system is a hierarchy with fully mature cells composing the base and NSC composing the apex of the pyramid. NSCs give rise to NPCs, which give rise to unipotent committed neural or glial progenitors. Unlike with the hematopoietic system, little is known of the kinetics of neuronal turnover. Young rats appear to generate 9000 new neurons daily in the dentate gyrus, or approximately 0.1% of the total granule cell population.[71] Another estimate suggests that in the mouse, one new neuron is generated daily for every 2000 existing neurons. Whereas neurogenesis (and the presence of NSCs) persists throughout adulthood in mammals in the dentate gyrus and olfactory bulb, the rate of neurogenesis diminishes with age.

Like other adult stem cells, NSCs are defined as CNS cells that can give rise to themselves in a self-replicating way but also can give rise to neurons, astrocytes, and oligodendrocytes. In vitro assays of neurosphere formation have proved useful in identifying the clonal proliferative behavior consistent with NSC. The standard method for isolating NSCs begins with dissecting tissue from a region in a fetal or adult brain known to harbor NSCs.[68] The tissue is disaggregated, and cells are plated in the presence of growth factors, such as epidermal growth factor or fibroblast growth factor-2. NSCs present in the cultured tissue spontaneously form spherical structures. These neurospheres are highly proliferative and can be expanded into large numbers of cells. By withdrawing epidermal growth factor or fibroblast growth factor-2 or adding other morphogens, NSCs can be differentiated into specific cell types in vitro or can be transplanted in vivo, whereupon the NSCs become incorporated into various central and peripheral nervous system cell types.[69]

NSCs have been difficult to isolate directly from CNS tissue. Successes in using specific monoclonal antibodies and flow cytometry have led to reports of NSC isolation from human fetal spinal cord and CNS tissue. These human NSCs express the cell surface AC133 antigen but not CD34 or CD45.[72] Single-sorted cells were capable of initiating neurosphere cultures and could be recloned with retained neurosphere potential. Other similar enrichments of NSCs have been reported in rats and mice using both positive and negative selection strategies.[70] These techniques will permit rapid progress in understanding the growth and expansion requirements of NSCs and will permit comparisons of the molecular regulation of NSC self-renewal with other adult stem cell populations.

Although a detailed study of the biology of NSCs will require considerable time, use of neural tissue for human clinical transplantation has already proved feasible. What types of grafts will be the most desirable? Human fetal CNS tissue has proved effective in providing symptomatic relief in patients with Parkinson disease; however, technical and ethical issues may preclude provision of sufficient numbers of cells for wide application.[70] NSCs cultured to large cell concentrations would appear to be an ideal source of cells. Whether the NSCs isolated from fetal CNS tissue or adult tissue display similar in vivo repopulating ability has not been directly tested. The report of isolation of NPCs from postmortem human olfactory bulb, dentate gyrus, and the subventricular zone of the forebrain suggests another potential source of transplantable NPCs.[73] Finally, ES cells can be differentiated into numerous neuronal cell types and may serve as a source of cells for repair of CNS damage; particular interest in the research community centers on the use of these cells to treat spinal cord injury.

Hepatic Stem Cells

The murine liver is formed by migration of foregut endoderm cells into the septum transversum on E8.5. Interactions between the endoderm cells and both cardiac mesenchymal and endothelial cells are critical for liver formation.[74] The foregut-derived cells begin expressing alpha fetoprotein on E9.0 and albumin soon thereafter. Further differentiation into hepatoblast cells occurs over the next 24 hours. Several days later (E12), the bipotent hepatoblast cells begin to proliferate rapidly as they differentiate into hepatocytes and cholangiocytes (biliary tract precursors). Distinct patterns of gene expression along the primary cellular lineages in the liver are demonstrable from E16 onward.[75] The basic lobular structure of the liver is not fully formed until several weeks after birth. One interesting feature of liver development is the inverse relationship between the level of hematopoiesis in the rodent fetal liver and the synthetic, metabolic, and enzymatic functions of the organ. As the liver function rapidly increases over the first 2 weeks of postnatal life, the hematopoietic function of the liver diminishes.

In terms of regenerative capacity, the mature liver is unique among solid organs.[76] Up to two thirds of the fully mature rodent liver may be resected, and within 1 to 2 weeks thereafter, compensatory growth and restoration of cellular numbers and function by the remaining cells will have occurred. Technically, the lost structures in the resected liver are not replaced or regenerated; instead, the remaining liver simply expands to the original mass of the organ. Surprisingly, this liver expansion results from proliferation of 70% to 90% of the hepatocytes in the unresected portion undergoing proliferation. It has been estimated that the proliferative capacity of adult hepatocytes is sufficient for repopulation after up to two-thirds liver resection without the need to recruit progeny from liver stem or progenitor cell populations. To date, the mechanisms regulating the overall mass and turnover of liver parenchymal cells remain obscure.

Under certain experimental circumstances, recruitment of hepatocytes from precursors can be demonstrated. In such instances, proliferation and differentiation of the hepatic stem and progenitor cells can be induced only with a concomitant block in the proliferative ability of mature hepatocytes[76] Thus treatment of rodents with irradiation, carcinogens (ethionine, 2-acetylaminofluorene, and 3-methyl-4-dimethylaminobenzene), RNA and protein synthesis inhibitors (D-galactosamine), or DNA-alkylating agents (retrosine) has been used to generate a proliferative population of epithelial cells called *oval cells* in the periportal region.[76] Oval cells initially proliferate in the canals of Hering (luminal channels linking the hepatocyte canalicular system to the biliary tree) and then differentiate into hepatocytes and bile duct cells in mice and rats treated with the aforementioned injurious agents.[77] The finding that differentiating oval cells recapitulate hepatocyte and bile duct gene expression patterns in a sequence similar to that seen during embryonic liver development strengthens the evidence that oval cells serve as a stem cell population for the liver.

Proof that oval cells repopulate the liver requires in vivo transplantation data. Limited hepatocyte and bile duct repopulation occurs in normal adult mice transplanted with oval cells isolated from D-galactosamine–treated rodents.[76] When the recipient animals underwent two-thirds partial hepatectomy alone, oval cell engraftment was observed, but proliferation was again limited. High levels of engraftment were observed only when the cells were transplanted into mice with ongoing liver injury (incurred as an inherited metabolic defect). By contrast, transplantation of oval cells from E12 to E16 rat fetal livers into normal adult hosts led to long-term engraftment of both hepatocytes and bile duct cells in up to 10% of therecipient livers. Whether this difference in liver repopulation represents a difference in mouse and rat biology, or in young versus older donor oval cells, remains to be determined.

Specific reagents to isolate hepatic stem and progenitor cells remain elusive. Suzuki and associates[78] used monoclonal antibodies and flow cytometry to isolate murine fetal liver progenitor cells expressing the α6 integrin and the β1 integrin. Fetal liver hematopoietic progenitor cells do not express this combination of integrins. In addition, the murine fetal liver progenitor cells did not express c-Kit, a tyrosine kinase receptor that is expressed by fetal liver hematopoietic stem cells. Some evidence has been presented that the pancreas possesses cells capable of differentiating into hepatocytes. Putative hepatic stem and progenitor cell populations from human liver and bone marrow also have been reported.[79] More controversial are several reports that bone marrow or hematopoietic stem cells possess the capacity to differentiate into hepatocytes and bile duct cells in vivo (see later discussion of stem cell plasticity).[39,80] More recently, the contribution of bone marrow–derived cells to liver repopulation has been reported to be due largely to fusion of myelomonocytic cells with injured hepatocytes, at least as measured in one genetic model of liver regeneration.[8]

In sum, the rodent liver possesses remarkable regenerative ability. Mature hepatocytes may be induced to proliferate and compensate for up to a loss of two thirds of a mature liver. When the mature hepatocytes are inhibited in their proliferation, oval cells proliferate and differentiate to produce hepatocytes and bile duct cells. Whether oval cells or other stem cell populations participate in maintaining the hepatic parenchyma during homeostasis or participate in restoring normal hepatocyte numbers during liver regeneration remains to be determined.

Although human liver transplantation has been used in clinical medicine for many years, basic information on potential liver stem cell participation in liver regeneration after transplantation is limited. This paucity of data may be a result of the immunosuppressive regimens that are required to permit allogeneic liver transplantation and concern for effects of this treatment regimen on the transplanted organ. Alternatively, cellular rejection of the transplanted organ also may complicate interpretation of the homeostatic turnover of hepatocytes and role of liver stem cells in the process. Nonetheless, transplantation of a portion of the liver from living donors has proved to be quite successful, with regrowth of the organ in both donor and recipient suggesting robust regenerative abilities of the human hepatocyte.[81]

Other Epithelial Stem Cells

Skin, hair follicles, and intestinal villi are all examples of tissues that must undergo rapid and continual self-renewal. The outer layer of human epidermis is sloughed, with continued replacement by differentiating cells moving upward from a basal layer in the dermis.[82] Cells in the basal layer display slow but persistent cell division. Within this layer are epidermal stem cells and transient amplifying cells (progenitors). In view of the fact that the human epidermal layer completely turns over every 3 weeks, and that transient amplifying basal cells divide only three to six times before differentiating into epidermal cells, the self-renewal capacity of the epidermal stem cells is astounding.[83]

Hair follicles are composed of an outer root sheath that is contiguous with the epidermis, an inner root sheath, and the hair shaft.[84] Proliferating cells that give rise to the inner root sheath and the hair shaft are located at the base of the follicle and compose the dermal papilla. In human adult skin, the proliferative capacity of the dermal papilla cells is cyclically exhausted, and the lower segment of the hair follicle regresses upward toward the permanent epithelial portion of the follicle called the *bulge.*[85] Follicle stem cells reside in the bulge and can regenerate the dermal papilla on receiving appropriate signals.[84] These follicle stem cells also are capable of regenerating epidermis and play an important role in the repair of wounds and burns to the epidermal layer. In vitro, a single human follicle stem cell can generate an enormous number of progeny (1.7×10^{38})—more epidermal cells than are required to cover the body surface of an adult human.[1]

The architecture of the small and large intestinal lining is unique, with extensive folding of the epithelial cells to form crypts and fingerlike projections called villi.[86] Such an arrangement increases the overall surface area of the intestinal lumen to permit maximal interaction of ingested nutrients with the epithelial cells for efficient absorption. As in the epidermis, newly formed cells move in a polarized fashion as a column of cells from the lower part of the crypt upward to an adjacent villus, where migration occurs along the villus until the cells are shed at the villus tip. The absorptive functions of the cells are operative during the migration from the base to the tip of the villus. The entire migratory passage from newly formed cell in the crypt to sloughed cell at the villus tip is thought to occur within 5 to 7 days.[87] In both the small and large intestine, villi are composed of enterocytes, mucus-secreting goblet cells, and peptide hormone–secreting enteroendocrine cells. An additional cell called the *Paneth cell* is present in small intestinal villi. Paneth cells participate in innate immune defense of the gut by sensing pathogenic microbes and secreting microbicidal peptides.

The unique architecture and migratory route of the epithelial cells permit specific identification of the position of each cell during maturation. In the mouse, each adult intestinal crypt is thought to contain four to six stem cells, each giving rise to dividing transit cells. These cells give rise to up to six generations of daughter cells that form the 250 cells composing the mature villus.[88] During the lifetime of a mouse, intestinal epithelial stem cells in each crypt produce 3.3×10^5 cells, suggesting that each stem cell may undergo a thousand cell divisions. In humans, the number of stem cell divisions may be as high as 5000.[87]

The increasing use of human skin grafts to treat large areas of denuded skin (major trauma or burn injury) has proved to be

quite effective in reducing overall morbidity and mortality for these injuries. Obviously, the ability to expand human skin cells in vitro relies on the stem cell and transient amplifying cell populations recovered from the host to generate enormous numbers of mature keratinocytes.[85] Further modifications to promote accelerated vascularization of the applied tissues will only enhance the use of this cell therapy in human subjects.

Advances in transplantation of human small intestine are steadily emerging and may provide some measure of hope for effective management of newborn infants who have suffered catastrophic losses of intestinal tissue through necrotizing enterocolitis, congenital atresia, or volvulus.[89] Elucidation of how to isolate, expand, and reconstitute injured intestine with intestinal stem cell–derived progeny is eagerly anticipated, although derivation of a functional peristaltic bowel from a completely engineered three-dimensional construct seems many years away from clinical application.[90]

INDUCED PLURIPOTENT STEM CELLS

A recent series of landmark papers may provide a method to generate induced pluripotent stem cells (iPSCs) from somatic cells of male or female mice. Mouse embryonic or adult fibroblast cells were reprogrammed to express ES-like properties by retroviral introduction of Oct-3/4, Sox2 (*S*RY [sex-determining region Y]-b*ox* protein *2*), c-Myc, and Klf4 (*K*rueppel-*l*ike *f*actor *4*) transcription factors and then selection for cells with up-regulated expression of the transcription factor Fbx15.[91] These cells could commit to differentiate into progeny from multiple germ layers, generate chimeric embryos after blastocyst injection, and form teratomas on injection into immunodeficient mice. However, the DNA methylation and gene expression patterns were not similar to those in mouse ES cells, and the iPSCs did not form viable chimeric mice upon injection into recipient blastocysts.

An advance in the procedure with forced expression of the same four fundamental transcription factors, but with selection for up-regulation of Nanog expression, has led to the isolation of reprogrammed murine fibroblasts that now are ES-like in appearance, proliferation, differentiation potential, and germline competency.[92] A precautionary note from these studies was that in nearly 20% of the offspring produced by the chimeric mice, tumors developed that appeared to be related to reactivation of c-Myc expression. Finally, another group of investigators have confirmed that expression of the four transcription factors Oct-3/4, Sox2, c-Myc, and Klf4 in mouse fibroblasts resulted in DNA methylation, gene expression, and chromatin state that were similar to those of ES cells.[93] Of note, the cells derived from mouse fibroblasts could generate live chimeric mice when injected into tetraploid blastocysts.

Although similar studies have not yet been successful in human cells, these data are encouraging that methods are developing that reconstitute an ES-like state from somatic cells. If such methods are shown to be feasible, subsequent studies could permit generation of patient-specific iPSCs for cell-based therapies for genetic or acquired disorders. Challenges in future work will be to develop methods of gene insertion without use of retroviral vectors and to ensure that the introduced genes are under strict inducible control, to avoid the emergence of tumors as reported in some murine studies.[92]

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Kurt R. Schibler

Developmental Biology of the Hematopoietic Growth Factors

HEMATOPOIESIS

Hematopoiesis is defined as the process of blood cell production. Although it is highly variable, all blood cells have a finite life span. For example, red blood cells circulate for 120 days, platelets for nearly 10 days, and neutrophils for only about 6 hours.[1] Under normal conditions, blood cells are replenished at a constant pace that maintains the concentrations of circulating cells within a narrow range and effectively masks the magnitude and complexity of the process. In a 70-kg adult approximately 200 billion red blood cells and 70 billion neutrophils must be released into the circulation and replace the damaged and senescent cells on a daily basis. The mechanisms regulating the precise number of circulating blood cells in each lineage and the turnover of each cell lineage remain elusive, although the concentrations of some hematopoietic growth factors appear to play a direct role in this process.[2]

Development of in vitro methods for the culture of hematopoietic cells has permitted analysis of the effects of soluble and membrane-bound growth factors on the survival, proliferation, and differentiation of hematopoietic stem and progenitor cells. Numerous growth factors have been characterized at the protein and nucleic acid level. The goal of this chapter is to review basic concepts of hematopoiesis and to provide an overview of some of the growth factors and their cognate receptors that are critical for normal hematopoiesis. We review basic concepts of ligand-receptor interactions and initiation of intracellular signaling pathways, and highlight differences in growth factor responsiveness of hematopoietic cells during in utero and postnatal development.

STEM AND PROGENITOR CELLS

Background

The hematopoietic system may be characterized as a hierarchy of cell intermediates progressing from the primitive stem cell to mature cells of specific blood cell lineages (Figure 142-1). A single stem cell can sustain the entire hematopoietic system for the lifetime of a mouse in certain experimental systems.[3,4] Stem cells generally exist in a quiescent state, but 8% of the stem cell pool is renewed daily in young mice.[5] Some of the daughter cells of the stem cell divisions may commit to differentiate and may become more highly proliferative multipotent progenitor cells. These cells, in turn, give rise to more proliferative bipotent progenitor cells that eventually produce unilineage committed progenitor cells. The total cellular amplification that occurs from the time of a single stem cell division to production of a mature blood cell is estimated to be 720,000-fold as a result of at least 17 cell divisions.[6]

Hematopoietic Stem Cells

Analysis of hematopoietic stem cell function has been most reliably understood from the standpoint of functional reconstitution of the hematopoietic system of a lethally irradiated host. In the mouse, multilineage reconstitution of hematopoiesis in a lethally irradiated recipient for more than 4 to 6 months after transplantation constitutes the full definition of a hematopoietic stem cell.[7] In the human system, such experimental proof cannot be undertaken in patients. However, in practical terms, hematopoietic stem cell function has long been demonstrated in patients who have undergone bone marrow transplantation using allogeneic donor bone marrow, umbilical cord blood, autologous marrow, or mobilized peripheral blood.[8] Both in vitro and in vivo experimental systems have been developed to test for human hematopoietic stem cell function.

In vitro systems for culture of hematopoietic stem cells can be divided into stromal-dependent and stromal-independent systems. Preplating of stromal cells onto culture dishes followed by addition of hematopoietic stem cells (with or without growth factors) to establish a co-culture can result in retention of hematopoietic stem cell function ex vivo and, in some cases, expansion of stem cell function.[9-14] Transplantable activity can also be maintained and expanded in stromal-independent cultures in the presence of stem cell factor (SCF), thrombopoietin, insulin-like growth factor-2, and fibroblast growth factor-1.[15] Several xenotransplantation models have been developed to assess human hematopoietic stem cell function in vivo. First, human cells can be transplanted and will engraft in the marrow of immunocompromised mice.[16,17] The extent of human hematopoietic stem cell expansion and differentiation can then be examined in the marrow of the mice with transplantations. Implantation of human fetal thymic and bone fragments into the immunocompromised mice (producing human hematopoietic microenvironments in the mouse) permits a more detailed analysis of human stem cell differentiation into lymphocyte and erythroid populations.[18] Finally, human hematopoietic stem cells engraft in the marrow of fetal sheep in utero and produce human hematopoietic progeny long term in the sheep postnatally.[19,20]

Understanding the innate properties of hematopoietic stem cells has been advanced through the development of techniques to isolate and characterize murine cell populations (using the foregoing assays).[3,4,21] Human hematopoietic stem cells have also been isolated using monoclonal antibodies and flow cytometry and are characterized by expression of the cell-surface antigens AC133, CD34, Thy-1 (low level), rhodamine-123 (low level), and Hoechst 33342 (low level), but not CD33, CD38, human leukocyte antigen-DR, and antigens expressed by mature cells of each lineage.[22-24] Using a combination of antimetabolite treatment and addition of growth factors in vitro will enhance enrichment for human hematopoietic stem cells.[25]

An alternative method for identifying both murine and human hematopoietic stem cells is based on efflux of a DNA-binding dye, Hoechst 33342. A small cell population, termed the *side population,* is identified by a characteristic fluorescence pattern after staining with Hoechst 33342 detected in both far red (>675 nm) and blue (450 nm) emission channels. The side population has been detected in both mouse and human bone marrow and contains the long-term hematopoietic stem cell expressing low or negative levels of CD34.[26] In addition to enriching for hematopoietic stem cells, the side population protocol can be used to identify stem cells in other tissues including skeletal muscle and epidermis.[27,28] Additional specific cell surface markers have been identified in murine systems that refine hematopoietic stem cell identification. These include endoglin, an ancillary transforming growth factor-β receptor, and prion protein, a highly conserved

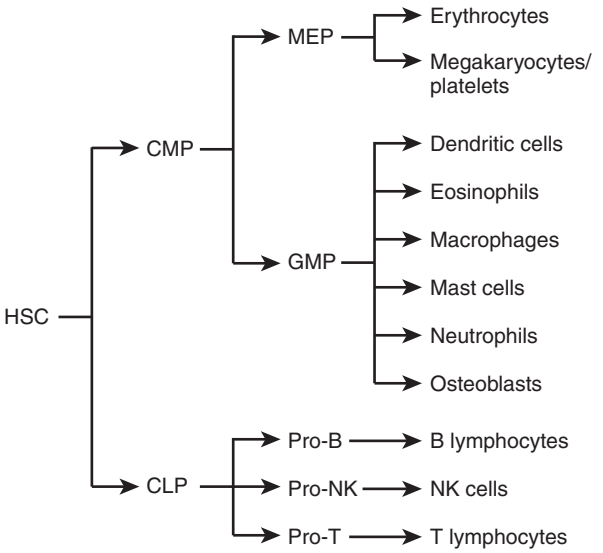


Figure 142-1 Pluripotent hematopoietic stem cells *(HSC)* give rise to committed hematopoietic progenitor cells, common myeloid progenitor *(CMP),* common lymphoid progenitor *(CLP),* granulocyte/macrophage progenitor *(GMP),* and megakaryocyte/erythrocyte progenitor *(MEP),* which undergo clonal expansion and differentiation under the permissive support of hematopoietic growth factors.

glycoprotein associated with the glycosylphosphatidylinositol anchor on hematopoietic cells.[29,30] Although hematopoietic stem cell isolation protocols have advanced significantly, current techniques are enrichment, rather than purification protocols.

Substantial evidence supports the hypothesis that the stem cell population resides in a dormant state with respect to the cell cycle. At any given time, only a few stem cells enter the cell cycle, undergo clonal expansion, and supply all the hematopoietic cells to maintain homeostasis. Prolonged residence in the G_0 or the noncycling state is thought to permit sufficient time to repair damaged DNA that may have accumulated during previous cell divisions and to diminish the chance of a clonal proliferative abnormality.

The concept of defining stem cell identity has undergone change with evidence that adult stem cells derived from one tissue are capable of differentiating into other tissue types.[31] For example, bone marrow can give rise to cells typical of other tissues. These stem cells can be derived from the hematopoietic or stromal compartments of the bone marrow. Indeed, bone marrow stem cells also give rise to cell types typical of other tissues such as muscle, brain, heart, and liver. Likewise, there are examples of nonhematopoietic tissue-specific stem cells reconstituting blood after intravascular injection into irradiated mice.[27,28,32] These and other findings suggest a previously unrecognized degree of plasticity in stem cell function. It appears that cell fate changes are a natural property of stem cells and may be involved in physiologic repair of tissue damage throughout life. In this revised paradigm, stem cell refers to a biologic function that can be induced in many distinct types of cells. Homing signals may result from local damage and influence the migration of stem cells to specific sites. The microenvironment including contact with surrounding cells, extracellular matrix, and growth and differentiation factors is likely to play a key role in determining the function of a stem cell.

Hematopoietic Progenitor Cells

The discovery of in vitro culture systems to examine hematopoietic progenitor cell formation as isolated colonies has enabled study of the categorization, functional analysis, and growth factor requirements of the various hematopoietic progenitor cells.[33-35] Figure 142-1 schematically depicts the hierarchy of hematopoiesis from the stem cell to mature blood cells of each lineage. In general, the hierarchy can be divided into myeloid and lymphoid pathways. Progenitor cells that form colonies containing neutrophils, erythrocytes, macrophages, and megakaryocytes (common myeloid progenitor) reside at the pinnacle of the myeloid pathway. These multipotent progenitors no longer possess the self-renewal properties of the stem cell, but they are considered as a primitive progenitor based on the capacity of these cells to give rise to progeny of multiple lineages. These cells are also highly proliferative and give rise to numerous progeny of more committed progenitors. As the progenitor cells become more committed to a particular lineage, one can begin to identify certain combinations of growth factors that appear to be required for the survival, proliferation, and full maturation of those cells.

The number of hematopoietic growth factors and cytokines that have been identified and characterized and the genes cloned is growing rapidly. Although these factors exhibit either stimulatory or inhibitory functions when they are evaluated in culture as single agents, most exhibit more complex actions when they are tested in combinations with other growth factors. Many hematopoietic growth factors have functions that extend outside the hematopoietic system. Interleukin (IL)-6 regulates certain aspects of immunologic, neuroendocrine, hepatic, and renal functions.[36] Furthermore, several hematopoietic growth factors are known to orchestrate functions affecting cells at different stages of hematopoietic differentiation. For example, injection of granulocyte colony-stimulating factor (G-CSF) into murine and human subjects triggers cycling of stem cells and mobilization into the systemic circulation, but it participates in a specific way to support the proliferation and maturation of lineage-committed neutrophilic progenitor cells.[37]

HEMATOPOIETIC MICROENVIRONMENT

Ontogeny of Hematopoiesis

Hematopoiesis is tissue specific and developmentally regulated. The first blood cells to appear in the murine and human embryo are formed in the extraembryonic yolk sac. Within 72 hours in the mouse, and 4 to 6 weeks in the human, blood cells are beginning to be formed in the liver.[38] The liver predominates as the site of hematopoiesis in the mouse until 3 to 4 days before birth, when circulating cells seed the spleen and bone marrow compartments. After birth, the liver diminishes in hematopoietic cell production and no longer serves as a primary hematopoietic site by 2 to 3 weeks of age, whereas the spleen and marrow serve as lifelong sites of blood cell production.[39]

Remarkably, hematopoiesis in cultured embryonic stem (ES) cells largely recapitulates embryonic events. For example, distinctly timed waves of primitive and definitive erythropoiesis develop in vitro from embryonic stem cells in a fashion that parallels the onset of these developmental stages in early embryos.[40] Keller and colleagues[41-43] discovered a transient progenitor in early embryoid bodies, termed blast-colony-forming cell, which gives rise to primitive erythrocytes, definitive lineages, and endothelial cells. Nishikawa and colleagues[44] identified a developmental hierarchy in ES cell differentiation in which hematopoietic cells arise from a precursor expressing endothelial cell markers. They also identified single progenitor cells capable of hematopoietic and endothelial differentiation. Together these studies established lineage relationships between the first murine hematopoietic precursors and verify the existence of the hemangioblast. Gene-targeting studies have identified numerous transcription factors and signaling molecules important for generation and maintenance of murine ES cell–derived hemangioblasts.[45-48] Recently, Huber and co-workers[49] identified hemangioblasts from the primitive streak of the early mouse embryo.

Importantly, these progenitors have similar properties to ES cell–derived blast-colony-forming cells.

Hematopoietic stem cell development, differentiation, and homeostasis are directed by transcriptional regulators, which control gene expression under the influence of signals from the microenvironment.[50] Commitment steps require the activation of lineage specific genes and concomitant repression of conflicting or unnecessary genes. This balance involves the concerted actions of multiple transcriptional activators, repressors, and epigenetic modifiers. Many of the key hematopoietic transcription factors were first identified in leukemic cells. Subsequently, using knockout studies in animal models, important roles for these protooncogenes were discovered in normal hematopoietic stem cell development of lineage differentiation.[51] Because many of these regulators are required for blood cell development and survival of the embryo, conditional gene targeting strategies made it possible to define the role of these factors at later developmental stages.

Yolk Sac

The first cells to form in the yolk sac are unique products of this organ and are called *primitive erythroblasts.* These are large red blood cells that express embryonic hemoglobin molecules and retain their nucleus even as circulating blood cells.[52] Primitive erythroblast progenitor cells are first identifiable on embryonic day 7 and are no longer detectable in the yolk sac by day 9. The primitive erythroblasts formed retain the ability to proliferate, and a rapid expansion in cell number occurs over a 48-hour period. The only other mature blood cells formed during this time include macrophages and a rare number of megakaryocytes.

Yolk sac hematopoiesis is not restricted to the formation of primitive erythroblasts. Evidence indicates that large numbers of definitive hematopoietic progenitor cells are also formed in the yolk sac, but they do not mature in this organ.[53,54] Circumstantial evidence suggests that the definitive progenitor cells (so called because these cells are like progenitors formed in the liver and marrow) enter the circulation, migrate via the bloodstream to the developing liver, enter the liver, and complete the differentiation process to mature blood cells.

Aorta-Gonad-Mesonephros Region

Hematopoietic cells are also formed in the embryo in sites other than the yolk sac and liver early in embryogenesis. The anatomic site in which the primordial germ cells coalesce to form the nascent gonads and the mesonephros forms is called the *aorta-gonad-mesonephros* region. Along the ventral wall of the aorta in this region, one can observe clusters of hematopoietic cells that appear to emerge from the endothelium.[55] Evidence suggests that this region may be the site of the first hematopoietic stem cells to emerge in the murine and human embryo.[55] Similar clusters of hematopoietic cells have also been identified in the vitelline and umbilical arteries and in the yolk sac, where stem cell activity can also be demonstrated to exist.[56] Apparently, the hematopoietic stem and progenitor cells formed in the aorta-gonad-mesonephros region do not mature in this site but must also migrate to other organs, such as the liver, to differentiate into mature cells.[57]

Placenta

The placenta has also been identified as a major source of hematopoietic stem cells during fetal life.[58] The murine placenta functions as a hematopoietic organ housing a large pool of hematopoietic stem cells during midgestation. The onset of hematopoietic activity parallels that of the aorta-gonad-mesonephros region; however, the placental hematopoietic stem cell pool expands to 15-fold more hematopoietic stem cells than the aorta-gonad-mesonephros region. Expansion of the placental hematopoietic stem cells occurs before and during expansion of hematopoietic stem cells in the fetal liver. Placental hematopoietic stem cells express Sca-1, CD34, CD31, and other hematopoietic stem cells specific markers and transcription factors, and they are found in the embryonic vasculature of the placental labyrinth.[59]

Fetal Liver

As the principal site of hematopoiesis shifts to the fetal liver in week five to eight of human gestation, the total content of hematopoietic progenitor cells increases 10- to 100-fold compared with the yolk sac. As in the yolk sac, erythropoiesis continues to predominate over granulocyte production.[60] In fact, the concentration of granulocyte and macrophage progenitors may even decrease during the transition of hematopoiesis from yolk sac to fetal liver. It remains unclear whether this paucity of granulocytic differentiation is secondary to diminished hepatic production of granulocytic growth factors or results from insensitivity of the fetal liver colony forming units–granulocyte macrophage to granulopoietic factors.

Bone Marrow

The transition of human hematopoiesis from the liver to the bone marrow occurs over the third trimester of intrauterine life. The microenvironment in the bone marrow has been well defined anatomically and is known to consist of osteoblasts, barrier cells, endothelial cells, macrophages, fibroblast cells, mesenchymal stem cells, and adipocytes.[61,62] Barrier cells are the major component of the stroma.[63] These fibroblast-like cells are located around the venous sinuses and form a sheath around the abluminal surface of the endothelium. Some barrier cells are interspersed within the hematopoietic elements in the marrow and extend cell processes that interact with the maturing hematopoietic cells. Macrophages constitute a second major cell type in the microenvironment. These cells are particularly important for erythrocyte maturation because macrophages engulf the extruded nuclei of the maturing red cells. Macrophages also secrete growth factors that promote progenitor cell survival and maturation. Adipocytes and endothelial cells provide supportive roles to the maturing hematopoietic elements, but they may also directly modulate progenitor cell function via secretion of cytokines and growth factors.

Migration of hematopoietic stem cells through the blood and across endothelial vasculature to hematopoietic niches in the bone marrow and other hematopoietic organs is termed *homing.* Homing is required for seeding of the bone marrow by hematopoietic progenitors during fetal development. Similarly, homing is operative in adult bone marrow homeostasis and certain physiological roles are amplified during stress-induced recruitment of leukocytes from the bone marrow and during stem cell mobilization for host defense and repair. Homing is a multistep process that involves signaling by stromal-derived factor 1 (or CXCL12) and SCF, activation of lymphocyte function-associated antigen 1, very late antigens 4 and 5 and CD44, cytoskeletal rearrangement, membrane type 1, matrix metalloproteinase activation, and secretion of matrix metalloproteinase 2 and 9. Rolling and firm adhesion of hematopoietic stem and progenitor cells to endothelial cells in marrow sinusoids under blood flow is followed by migration across the endothelium and extracellular matrix. Stem cells finalize their migration by selective access and anchorage to specialized niches in the extravascular space of the endosteum and in periarterial sites.[64]

The cytokine G-CSF is the most widely used agent for mobilizing hematopoietic stem cells in experimental and clinical settings. CXCL12 and its receptor CXCR4 are known to be a key ligand-receptor pair responsible for retention of hematopoietic stem cells in bone marrow.[65] Work by Katayama and colleagues[66] provides a mechanism for G-CSF–induced mobilization of hematopoietic stem cells and suggested that sympathetic nervous system may regulate stem cell trafficking. This work built on findings by Frenette and colleagues[67] that mice

deficient in the UDP-galactose:ceramide galactosyltransferase (Cgt) exhibited severely impaired mobilization response to G-CSF. Cgt is essential for synthesis of sulfatide by mediating addition of UDP-galactose to ceramide. The mobilization defect could not be linked to the lack of sulfatide and thus involved an indirect mechanism. These investigators speculated that impaired adrenergic nervous system transmission might play a role in the mobilization defect. The sympathetic nervous system has been previously established as a regulator of bone formation. Takeda and co-workers[68] demonstrated that adrenergic signals directly regulate osteoblast function. Katayama and colleagues[66] examined the *Cgt−/−* mouse model and other genetic and pharmacologic models exhibiting impaired nerve conduction. These included mice lacking dopamine β-hydroxylase (*Dbh−/−*) that have deficient synthesis of norepinephrine; mice subjected to pharmacologic disruption of catecholaminergic neurons through postnatal injections of 6-hydroxydopamine and mice treated with β-blockers (Figure 142-2). Each model exhibited decreased hematopoietic stem cell mobilization in response to G-CSF. Osteoblast suppression was not observed and CXCL12 levels remained intact in these models. Involvement of adrenergic activity in stem cell mobilization was further bolstered by observations that norepinephrine levels decreased in bone following G-CSF administration, signifying its release, and that a β_2-adrenergic agonist could restore the mobilization of stem cells in mice lacking dopamine β-hydroxylase.

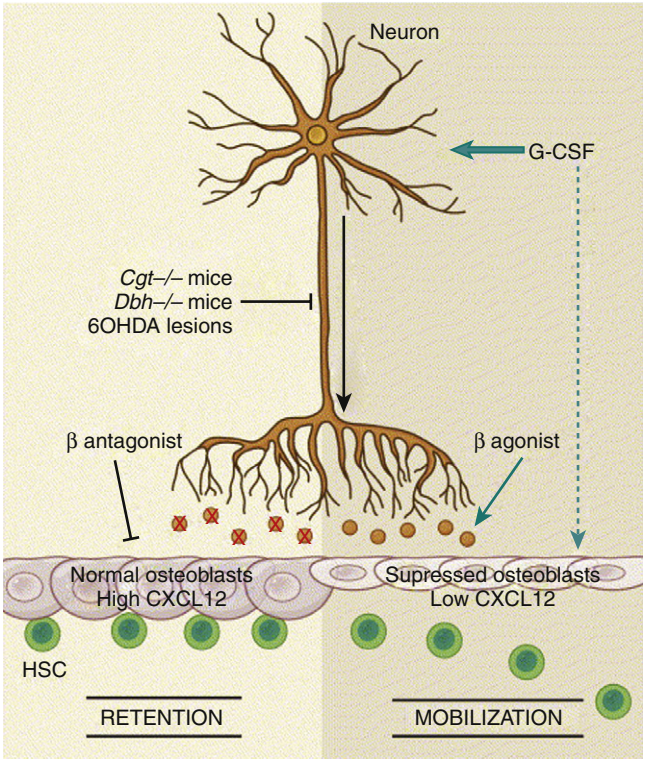


Figure 142-2 Signals from the sympathetic nervous system modulate localization of hematopoietic stem cells to the endosteal niche in bone. Granulocyte colony-stimulating factor *(G-CSF)* activation of peripheral noradrenergic neurons *(thick arrow)* with an unknown mechanism *(dashed arrow)* induces mobilization of hematopoietic stem cells *(HSCs)* through the suppression of osteoblasts and a decrease in CXCL12. HSC mobilization by G-CSF was studied in mouse models with defects in nerve conduction.[66] These models include mice deficient in UDP-galactose:ceramide galactosyltransferase (*Ctg−/−*); mice deficient in dopamine β-hydroxylase (*Dbh−/−*); mice injected postnatally with 6-hydroxydopamine *(6OHDA),* which disrupts catecholaminergic neurons; and mice treated with β blockers (β antagonists). Additionally, a β_2-adrenergic agonist rescues the mobilization defects in mice deficient in dopamine β-hydroxylases and augments G-CSF–induced mobilization in control mice. These studies demonstrate that noradrenergic signals from the sympathetic nervous system are critical mediators of G-CSF–induced mobilization. *(From Larsson J, Scadden D: Nervous activity in a stem cell niche,* Cell *124:253, 2006.)*

GROWTH FACTOR EFFECTS ON STEM AND PROGENITOR CELLS

Factors Influencing Hematopoietic Stem Cells

Certain growth factors are required for primitive hematopoietic stem cells to survive in a quiescent state, whereas others trigger cycling of progenitors once committed to a differentiation pathway. Some data suggest that G-CSF, IL-3, and SCF may act as survival factors for murine hematopoietic stem cells in vitro.[69,70] Other studies, using enriched murine bone marrow progenitor cells, demonstrated that IL-3 and SCF, but not G-CSF, supported survival of G_0 progenitors.[71] Studies by Leary and colleagues[72,73] demonstrated that IL-3 and granulocyte-macrophage CSF (GM-CSF) maintain survival of human primitive progenitors in G_0 phase, whereas IL-6, IL-11, and G-CSF do not promote survival of these dormant progenitors in vitro.[73]

Several factors have been identified that appear to be involved in triggering division of dormant hematopoietic stem cells. IL-1 and IL-3 have been shown to act synergistically in supporting proliferation of murine hematopoietic stem cells.[74,75] Mapping studies of murine blast cell colony formation demonstrated that IL-6, IL-11, G-CSF, and SCF act synergistically in support of colony formation from quiescent progenitors.[76-79] Similarly, synergism between IL-1 and IL-3 has been demonstrated in cultures enriched for human marrow progenitor cells.[80]

Lineage-Specific Factors

Major technologic advances in deriving mature tissues from murine ES cells have advanced our knowledge of hematopoietic growth factor requirements for specific lineage derivation. Two different experimental systems are used to generate blood precursors from ES cells: formation of embryoid bodies (EBs)[41] and induction of hematopoietic differentiation on stromal cells.[81] Upon removal from feeder layers and cytokines that maintain their pluripotency, ES cells form EBs, aggregates of differentiated cells including mesodermal derivatives capable of hematopoietic differentiation. The emergence of hematopoietic progenitors in this culture can be tracked by the appearance of lineage-specific markers through gene expression, immunohistochemistry, flow cytometry or methylcellulose culture assays. In this fashion the effects of specific manipulations such as cytokines, medications, or gene targeting can be analyzed.

Differentiation of ES cells on stromal lines provides a second means for inducing hematopoiesis. The most common line is OP9, a line established from the calvaria of newborn *op/op* mice, which lack functional macrophage colony-stimulating factor (M-CSF).[81] OP9 cells enhance hematopoietic development by providing a supportive microenvironment for differentiation. The absence of M-CSF inhibits survival of monocyte-macrophage cells, which tend to outgrow other lineages in culture systems using wild-type stroma.

Early studies showed that mixtures of hematopoietic cell types developed in ES cell differentiation cultures. More recently, methods were refined to generate relatively pure populations of specific lineages (Table 142-1). The methods were established using murine ES cells, but these are being adapted for use in human cells.

TABLE 142-1

Generation of Specific Hematopoietic Lineages

Hematopoietic Lineage	Cytokines
Erythroid	Major: EPO, KL Other: Insulin, IGF-1, IL-9
Megakaryocytes and platelets	Major: TPO Other: IL-6, IL-11
Neutrophils	Progenitor expansion: bFGF, IL-6 IL-11, KL, LIF, OSM Differentiation: G-CSF, GM-CSF, IL-6
Eosinophils	IL-3, IL-5, GM-CSF, Eotaxin
Mast cells	IL-3, KL
Macrophages	IL-1, IL-3, M-CSF, GM-CSF
Dendritic cells	Progenitor expansion: GM-CSF, IL-3 Differentiation/Maturation: IL-4, TNF-α, LPS, anti-CD40
Osteoclasts	M-CSF, RANKL
B lymphocytes	Flt-3 ligand, IL-7
T lymphocytes	Flt-3 ligand, IL-7
NK cells	Flt-3 ligand, IL-6, IL-7, IL-15, KL

bFGF, Basic fibroblast growth factor; *EPO,* erythropoietin; *G-CSF,* granulocyte colony-stimulating factor; *GM-CSF,* granulocyte-macrophage colony-stimulating factor; *IGF,* insulin-like growth factor; *IL,* interleukin; *KL,* Kit ligand; *LIF,* leukemia inhibitory factor; *LPS,* lipopolysaccharide; *M-CSF,* macrophage colony-stimulating factor; *NK,* natural killer; *OSM,* oncostatin; *RANKL,* receptor activator of nuclear factor κB ligand; *TNF,* tumor necrosis factor; *TPO,* thrombopoietin.

Erythroid cells

When 7- to 8-day-old EBs are disaggregated and plated into methylcellulose culture with erythropoietin and Kit ligand (also called SCF), the majority of resultant cultures are erythroid.[41] Methods to derive definitive erythrocytes from ES cells were advance by Carotta and colleagues[82] who developed conditions in which EB-derived immature erythroid progenies can be expanded 10^7-fold. This approach relies on dexamethasone to promote erythroid progenitor self-renewal. Removal of dexamethasone from culture and adjustment of cytokines results in terminal differentiation.

Megakaryocytes

Culturing ES cells on OP9 stroma in the presence of thrombopoietin enriched for a population of megakaryocytes with functional features characteristics including proplatelet production and fibrinogen binding after exposure to platelet agonists.[83,84]

Neutrophils

Greater than 75% enrichment of neutrophils from cultured ES cells was achieved by inducing maturation of EB-derived hematopoietic progenitors on OP9 cells with added cytokines.[85,86] The resultant neutrophils exhibited signature markers, chemotactic responses, and superoxide production.

Eosinophils

Eosinophils were generated by Hamaguchi-Tsuru and colleagues[87] by differentiation of EB cells on OP9 stroma with IL-5 in the presence of either IL-3 or GM-CSF. The capability to generate and study eosinophil-enriched populations at defined stages of development and function may be useful in understanding human conditions such as allergy and asthma.

Mast Cells

EB-derived hematopoietic progenitors cultured with IL-3 and Kit ligand generate pure populations of mast cells that can be expanded in vitro.[41,88] Upon transplantation into mast cell deficient mice ($Kit^w/Kit^{w\text{-}v}$), ES-cell derived mast cells proliferate, mature, and mediate immunoglobulin (Ig) E-dependent immune responses.

Macrophages

Macrophages can be produced from ES cells using either EB or OP9 systems. ES-derived macrophages express Mac-1 and F4/80, a macrophage specific antibody.[81]

Dendritic Cells

Dendritic cells (DCs) are potent antigen-presenting cells that can promote either immune responses or self-tolerance. ES-cell–derived DCs expand in the presence of IL-3 and GM-CSF and are induced to mature by tumor necrosis factor-α and IL-4 plus lipopolysaccharide or by anti-CD40.[89,90] ES-derived DCs, like bone marrow-derived DCs, express characteristic markers, process antigens, and stimulate T-cell responses in mixed leukocyte reactions.

Osteoclasts

Osteoclasts, which are derived from hematopoietic stem cells, are involved in bone resorption and remodeling. Cytokines that promote osteoclast maturation from ES cells include M-CSF and receptor activator of nuclear factor κB (NFκB) ligand. Osteoclast production is augmented by ascorbic acid, dexamethasone, and 1α,25-dihydroxyvitamin D_3.[91-93]

B and T Lymphocytes

Nakano and colleagues[81] cultured ES cells on OP9 stroma with IL-7 to produce early B lymphocytes. These cells were predominantly IgM− and had completed diversity-joining gene rearrangement; however, a small proportion differentiated into IgM+ cells that expressed complete μ chain mRNA. B cell maturation progressed further when ES cells were exposed to IL-7 in combination with Flt-3 ligand.[94]

Differentiation of T lymphocytes from ES cells is complicated by developmental requirement of thymic stromal interactions in vitro. de Pooter and colleagues[95] differentiate ES cells on OP9 stroma then reseed FLK1+CD45− cells into fetal thymic organ cultures. Although the fraction of T cells is low, normal CD4 and CD8 thymic subsets were generated.[95] Recognizing the importance of Notch signaling in T-cell development, the same group of investigators developed OP9 cells that express delta-like-1 notch ligand. Differentiation of T cells on this modified stroma allowed more complete T-cell maturation including γδ and αβ T-cell receptor bearing cells.[96]

Natural Killer Lymphocytes

Natural killer (NK) cells represent an important arm of the innate immune system involved in destruction of cells that fail to express correct major histocompatibility complex class I receptors. NK cells derived from ES cells exhibit the capacity to kill certain tumor lines and major histocompatibility complex class I–deficient lymphoblasts.[97]

RECEPTORS

Cytokine Receptor Families

Hematopoietic growth factor receptors have been categorized (based on similarities in extracellular genetic characteristics) into several families of receptors (Figure 142-3).[98-100] Some receptors contain structural domains characteristic of two or three subgroups within the cytokine superfamily of receptors. For instance,

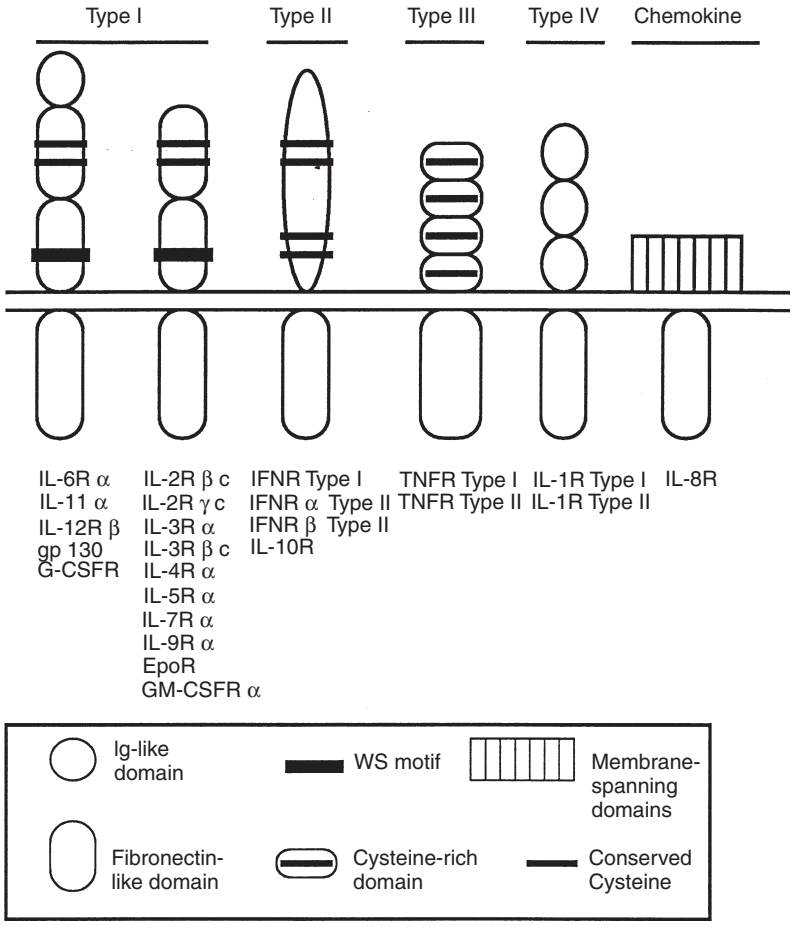


Figure 142-3 Cytokine receptors have been grouped into related families based on presence of conserved structural elements in their extracellular domains. Type I receptors (including interleukin [IL]-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage CSF [GM-CSF], and erythropoietin [EPO] receptor chains) are characterized by the presence of fibronectin-like domains, conserved cysteine residues, and the WS motif (a highly conserved region containing a tryptophan-serine-1 amino acid–tryptophan-serine motif). Type II receptors, which possess several conserved cysteine residues, include the interferon (IFN)-α, IFNβ, IFNγ, and interleukin (IL)-10 receptors. Type III receptors include two varieties of tumor necrosis factor (TNF) receptors (types I and II), which are characterized by repeated cysteine-rich domains. The IL-1 receptors are characteristic of type IV class of receptors (immunoglobulin superfamily receptors). Several members of the type I family also include immunoglobulin-like domains (IL-6Rα, IL-11Rα, IL-12Rβ, G-CSFR, and gp130). The IL-8 receptor differs from other receptors because it includes seven transmembrane domains. This structure is characteristic of the chemokine receptors.

the receptors for IL-1, IL-6, SCF (c-kit), and M-CSF (c-fms) contain extracellular immunoglobulin-like domains, placing them in the immunoglobulin superfamily. IL-1 receptors are distinct from other type I cytokine receptors such as IL-6R because they lack the tryptophan serine (WS) motif, fibronectin-like domains, and conserved cysteine residues. The receptors for SCF and M-CSF differ from other hematopoietic growth factor receptors in that they contain intrinsic intracellular protein tyrosine kinase motifs that are involved in signal transduction. The IL-2Rβc, IL-3Rα, IL-3Rβc, IL-4Rα, Il-5Rα, IL-6Rα, IL-6Rβc (gp130), IL-7Rα, IL-9Rα, IL-12Rβ, EpoR, G-CSFR, and GM-CSFR belong to the type I family of receptors and share several highly conserved protein motifs.[101-107] The structural similarities among these conserved regions suggest that members of this receptor family evolved from a common ancestral gene. Other cytokine receptors include the interferon (IFN) group (IFNR type I, IFNRα type II, IFNβ type II, and IL-10R), which possess a common binding domain characterized by cysteine pairs at both the amino-terminal and the carboxyl-terminal, and the tumor necrosis factor (TNF) group (TNFR type I and type II), which contain several cysteine-rich domains.[108] The IL-8 receptor belongs to still another family of receptors, the chemokine family. Structurally, it differs markedly from other cytokines because it contains seven transmembrane domains.[109,110]

Although cytokine receptors are clearly evolutionarily related, their intracellular domains exhibit little homology.[111] Furthermore, a lack of protein kinase–related sequences is conspicuous in most family members even though ligand-receptor interaction triggers a cascade of tyrosine phosphorylation within target cells.[112] Moreover, although cytokine receptor family members do not belong to the protein kinase family, they use protein kinase activity to propagate their intracellular signals. The finding that intracellular domains of cytokine receptors lack sequences known to be important in signal transduction suggested that associated molecules must be responsible for propagating the signal intracellularly. Cytokine receptors can be subdivided into two distinct groups based on the mechanism by which they associate to transduce their signal. One group of receptors forms homodimers for signal transduction (e.g., EPO), and the second group consists of receptors that require two distinct subunits to produce effective high-affinity ligand binding and signal

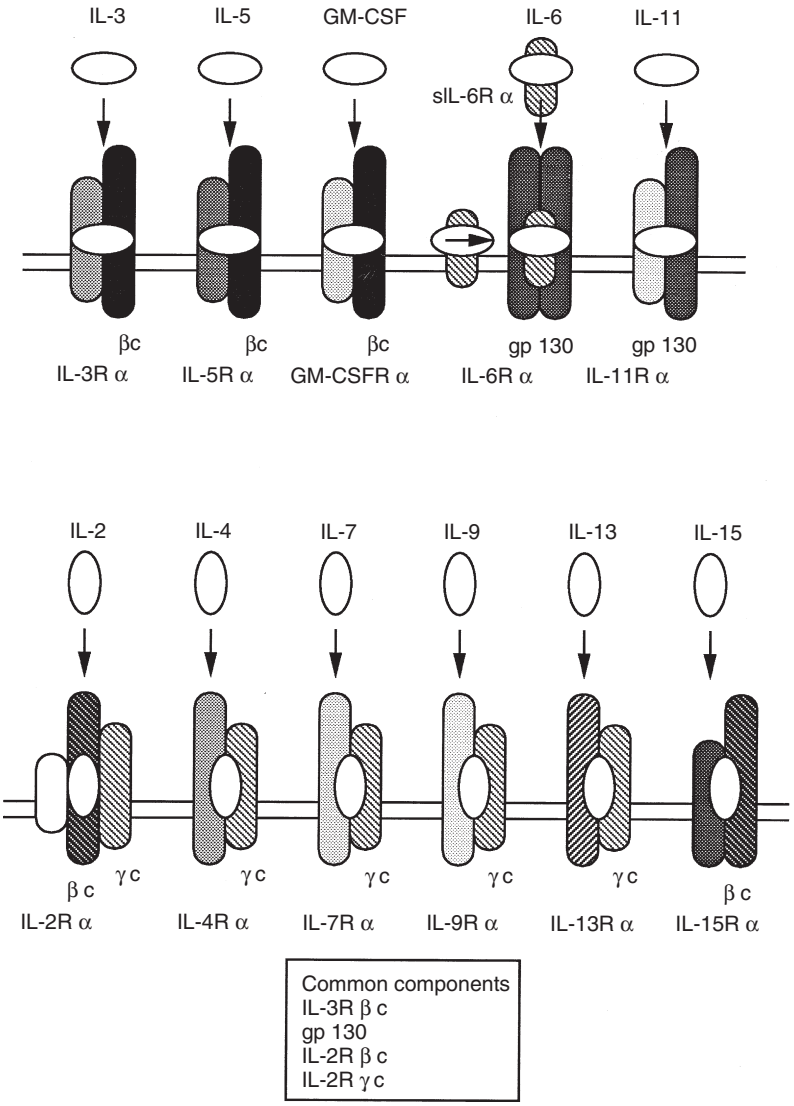


Figure 142-4 Cytokines and hematopoietic growth factors bind to their specific α-chain receptors to form low-affinity complexes that do not initiate signal transduction. Subsequently, they form high-affinity complexes capable of signal transduction by interacting with other α-chains to form homodimers as with erythropoietin (EPO) *(not shown)* or by interacting with common chains (interleukin-2Rβc [IL-2Rβc], IL-2Rγc, IL-3Rβc, and gp130) to form heterodimeric complexes. *GM-CSFR,* Granulocyte-macrophage CSF receptor.

transduction.[113] The βc chains are shared by several receptors in this family. For instance, the IL-3Rβc forms heterodimers with IL-5R and GM-CSFR after ligand binding to form a high-affinity interaction that is capable of propagating intracellular signals. Similarly, the IL-6Rβc (gp130) chain is shared with IL-11R, and the IL-2γc chain is shared with IL-4R, IL-7R, IL-9R, and IL-15R[114] (Figure 142-4).

Specificity of Cytokine Action at the Receptor Level

Cytokine action can be regulated at two major levels: outside and inside the target cell (Figure 142-5).[115] Outside the target cells, cytokine effects are determined by the amount of cytokine produced and the duration of its availability in the microenvironment. The half-life of a cytokine and the presence or absence of a matrix that concentrates or sequesters the cytokine affect the duration of its effect. The expression of cytokine receptors on the surface of cells determines what cells surrounding cytokine-producing cells can respond to the cytokine signal. Cell-cell interactions between cytokine-producing cells and responding cells also affect the duration and magnitude of the cytokine's effect.

Soluble Growth Factors and Growth Factor Receptors

Growth factor regulation at the level of the receptor occurs by several distinct mechanisms. First, receptors may be restricted to specific cell types. Second, receptor number on the membrane surface may differ because of varying receptor mRNA expression, receptor degradation, or internalization. Third, receptor affinity may be modified by other membrane proteins or by availability of shared common receptor chains. Finally, membrane-bound receptors may be cleaved to soluble forms or, alternatively, secreted directly as soluble receptors. Soluble receptors found in serum include sIL-1RII, sIL-4R, sIL-6Rα, sIL-6Rβ, TNFR I, TNFR II.[116] Soluble IL-1RII is believed to act as a scavenger of circulating IL-1. Complexes of IL-6 and it soluble receptors can function as agonists or antagonists.[117] Soluble TNF receptors, TNFR I and TNFR II, protect against proteolysis.

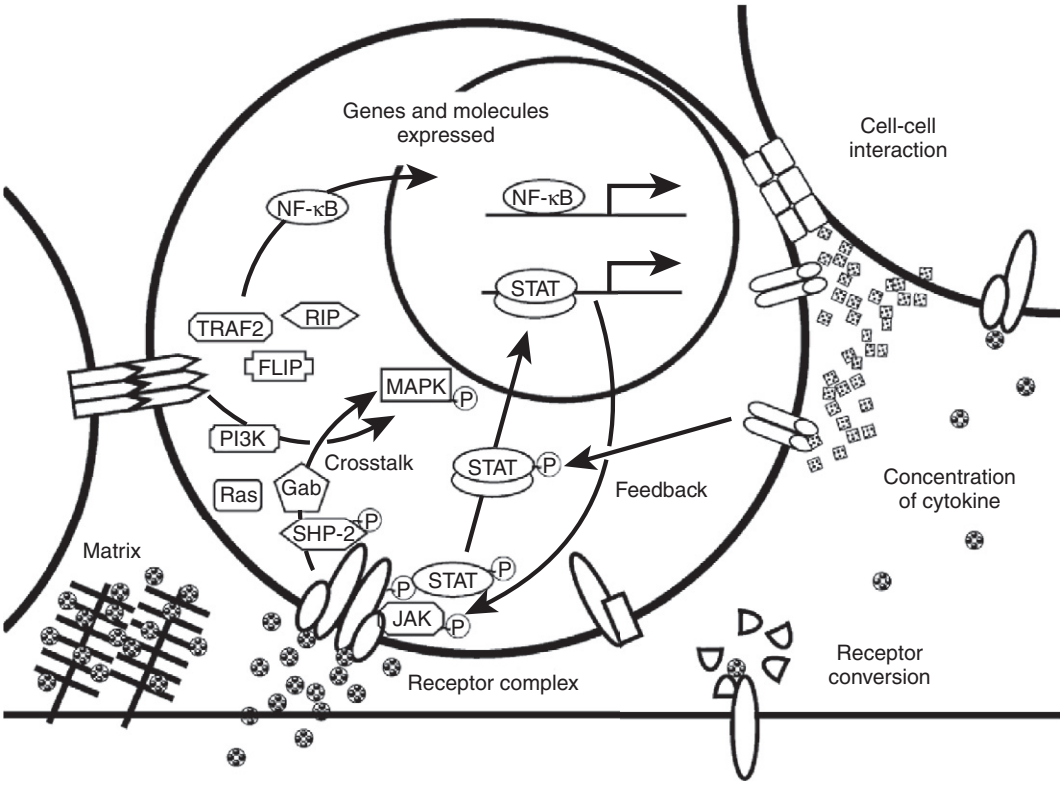


Figure 142-5 Extracellular (receptor-mediated) and intracellular (signal transduction) factors involved in determination of cell specificity of a cytokine. *(From Ishibara K, Hirano T: Molecular basis of the cell specificity of cytokine action,* Biochim Biophys Acta *1592:281, 2002.).*

Two mechanisms responsible for generation of soluble receptors are differential splicing of the receptor pre-mRNA (which results in formation of receptors lacking the transmembrane portion of the receptor molecule) and proteolytic cleavage of the membrane-bound form (releasing the extracellular portion of the molecule).[118]

The actual physiologic role of the soluble receptors depends on the relative concentrations of growth factor–receptor complexes, the individual components, and their dissociation constants. In the case of IL-6, circulating levels are generally 10 pg/mL or less in normal individuals, whereas the soluble receptor concentrations are approximately 70 ng/mL.[119,120] With a dissociation constant of 1×10^{-9} mol, all IL-6 in the serum should be present in complexes with soluble receptors. Therefore it would be anticipated that this interaction would antagonize the effects of IL-6. In fact studies by Narazaki and colleagues[121] demonstrated antagonist activity in complexes of IL-6, IL-6R, and gp130. In contrast, in vitro studies suggest that soluble IL-6 receptor complexes may propagate an agonist IL-6 effect on cells not expressing the IL-6R subunit but expressing the gp130 chain.[122,123] Thus IL-6 and IL-6 receptors shed from one cell type could alter ligand binding and act on a cell type not expressing IL-6R but expressing gp130. Such interactions have been documented in hepatoma cell lines and in hematopoietic cells.[117,124]

SIGNAL TRANSDUCTION

Inside cells, the cytokine signals are regulated at the plasma membrane, in the cytoplasm, and in the nucleus. Upon binding of a cytokine to its specific receptor, a given set of transduction pathways is activated based on the kinases and phosphatases constitutively associated with the given receptor. The cytoplasmic tail of each receptor has multiple structures that determine the signaling pathways by recruiting kinases, phosphatases, and transcription factors. Adapter molecules play pivotal roles in providing positive or negative regulation of certain pathways and integration of multiple pathways. The gene set transcribed by a specific transcription factor helps determine the biologic output. Additionally, a given target cells receives multiple signals through independent receptors and these signals converge or engage in cross-talk inside the cell.

Tyrosine kinase–based signaling can be initiated both by receptors possessing intrinsic tyrosine kinase and by receptors lacking an intrinsic tyrosine kinase. Hematopoietic growth factors possessing intrinsic tyrosine kinase activity include SCF and M-CSF. Factors such as GM-CSF and the interleukins lack intrinsic catalytic activity, but they trigger signaling activity through cytoplasmic tyrosine kinases. The carboxyl-terminal portion of these receptors, although lacking motifs involved in known catalytic activity such as the protein tyrosine kinase domains, contains highly conserved motifs in the intracellular membrane proximal region.[112,125,126] These structures are instrumental in triggering downstream signaling events.

Common features of the intracellular signal transduction mechanism of cytokine receptors are noteworthy. First, ligand binding to monomeric forms of these receptors alone is generally insufficient to initiate signal transduction; however, once specific ligand binding has occurred, the receptors are capable of forming homodimers or heterodimers that initiate signal transduction.[111] Second, several receptor subunits are shared with other hematopoietic growth factors.[127] Third, on binding of the specific ligand and dimerization of the receptor subunits, autophosphorylation and transphosphorylation can occur, and they initiate a cascade of tyrosine phosphorylation of sites intrinsic to the intracellular portions of the receptors and also on nonreceptor protein tyrosine kinase molecules associated with the intracellular domains

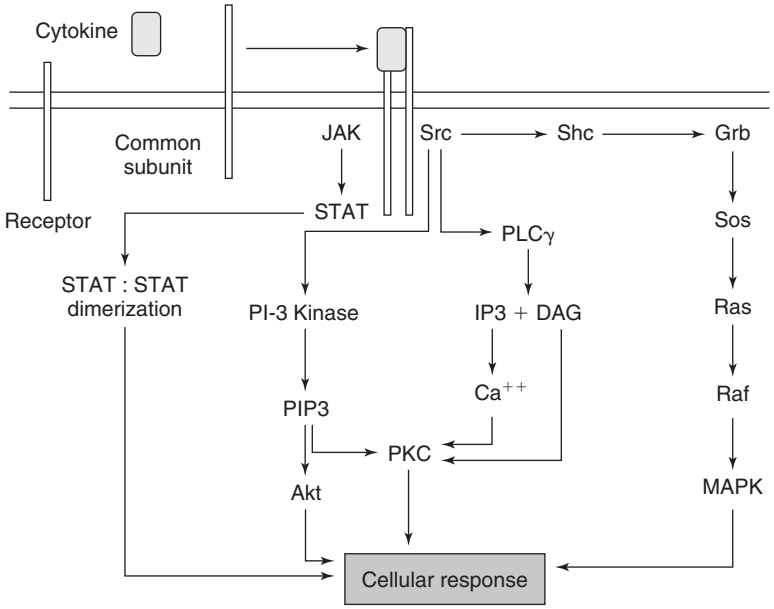


Figure 142-6 Transduction of external signals across the cell membrane is accomplished by binding of a cytokine with its specific receptor. The receptor then forms dimers with a common receptor chain to form a high-affinity complex. This association initiates a cascade of tyrosine phosphorylation both on the intracellular domains on the receptor itself and on receptor-associated tyrosine kinase molecules. One family of nonreceptor tyrosine kinases is the JAK proteins. The phosphorylation events lead to docking of STAT proteins. These interactions lead to phosphorylation and dimerization of STAT proteins, nuclear translocation, and transcription of cellular response genes. Receptor phosphorylation can activate other receptor-associated tyrosine protein kinases such as Src. Activation of Src triggers activation of phosphatidylinositol 3-kinase *(PI-3 kinase)* and phospholipase Cγ *(PLCγ)*. Tyrosine kinases also trigger activation of the serine threonine kinase Raf through the coupled proteins Shc and Grb, the nucleotide exchange factor Sos, and the small G-protein Ras. Raf activates mitogen-activated protein kinase *(MAPK)* to regulate cellular responses.

of the cytokine receptors. Signal transducing proteins containing Src homology 2 (SH2) domains associate with the phosphotyrosine residues on the activated receptor and activated nonreceptor protein tyrosine kinases and, in turn, are activated and propagate the signal downstream.[128] This interaction affords another level of specificity on biologic actions of cytokines as each particular receptor interacts with its own specific subset of SH2-containing proteins.[129] The key features of the signal transduction process are illustrated in Figure 142-6.

Several families of nonreceptor protein tyrosine kinases have been isolated and have been implicated in cytokine signal transduction. These include molecules in the Src family, the Syk-ZAP70 family, the Btk-Tec family, and the Janus kinase (JAK) family.[112] The mechanisms by which these factors mediate downstream events have not been fully defined; however, the interactions between the JAK and Src proteins and several downstream factors have been partially characterized.

JAK Protein Tyrosine Kinases

One family of protein tyrosine kinases recognized to be involved in cytokine signaling is the JAK family. Early clues implicating the involvement of a receptor-associated tyrosine kinase was drawn from the observation that purified growth hormone receptor exhibited kinase activity.[130] Members of the JAK family were isolated by polymerase chain reaction technology using degenerate primers based on a conserved catalytic domain.[131] Evidence pointing to the role of this family of proteins in cytokine signaling was provided by genetic complementation studies in a cell line defective in type I IFN signaling.[132] This mutant line was rescued by transfection of these cells with genomic DNA encoding Tyk2, a member of this kinase family. Similar studies on IFNγ provided evidence for Jak1 and Tyk2 in type I receptor signaling and Jak1 and Jak2 in the IFN type II signaling mechanism.[133] The JAK family of protein tyrosine kinases has been found to be a critical component in signaling through certain cytokine receptors including IL-2R, IL-3R, IL-4R, IL-5R, IL-6Rβc (gp130), IL-7R, IL-12R, and EPOR.[134] Each receptor appears to associate with distinct members of the JAK family. For instance, IL-2R associates with JAK1 and JAK3, whereas the EPOR associates only with JAK2.[134] A restrictive interaction of the cytokine receptor intracellular domains with specific signal transduction elements, such as Jak protein kinases, is one mechanism regulating the specificity of growth factor action.

Dimerization of the cytokine receptor induced by ligand brings JAK proteins in proximity with each other, resulting in cross-phosphorylation.[134] This interaction is somewhat restrictive because JAK1 and JAK3 always bind in conjunction with other family members, whereas JAK2 binds to receptors alone.[112] Thus JAK2 molecules are capable of cross-phosphorylating each other, whereas the other JAK proteins appear to require interaction with related family members to effect optimal activation.[112] The observation that patterns of JAK protein phosphorylation are distinct in different cell lines may provide insight into the functional pleiotropism of cytokines.[134,135]

The JAK proteins appear to associate with the box 1 motif in the membrane proximal portion of cytokine receptors. This region exhibits a high degree of sequence conservation.[136] Presence of the box 1 motif is critical in the mitogenic response to several cytokines.[137] Although the downstream signal propagating events that follow JAK protein phosphorylation are not fully understood, its role in activation of one family of latent transcription factors, called STATs (signal transducers and activators of transcription), has been characterized. In addition, the phosphorylation of cytokine receptors by JAK proteins potentiates the interaction between the receptor complex and other signal

transducing molecules such as those containing SH2 regions.[138] STAT proteins, present in the cytoplasm, become activated on interaction with activated Jak proteins. The phosphorylated STAT proteins form homodimers or heterodimers with peptides containing SH2 domains; these dimers are subsequently transported to the nucleus, where they function as transcription factors.[135-138]

SRC Protein Tyrosine Kinases

Src is the prototype for a family of tyrosine kinases that includes at least nine members: Src, Fyn, Yes, Lyn, Hck, Fgr, Lck, Blh, and Yrk.[139] All Src members contain a site near the N-terminus signaling for myristoylation and, in many cases, for palmitoylation, which direct the association of these proteins with cellular membranes. C-terminal to this region is the Src homology 3 domain that promotes protein-protein interactions with proteins exhibiting left-handed polyproline type II helices.[140,141] The next adjacent domain toward the C-terminus is the SH2 domain. The SH2 domain is the binding site for phosphotyrosine-containing proteins. This domain is frequently found in phospholipase Cγ, phosphatidylinositol-3-kinase, Shc, and STAT proteins, which are involved in tyrosine kinase–based signal transduction.[129,140] The SH2 domains of Src kinases mediate interactions of these proteins with receptor-type protein tyrosine kinases and with protein substrates. C-terminal to the SH2 domain is the catalytic domain. Phosphorylation of Tyr416 in the catalytic domain is required for optimal activity. In addition, the presence of a conserved tyrosine (Tyr527) in the C-terminal tail region is essential for the phosphorylation-based activity characteristic of Src tyrosine kinases. Multiple Src family members have been observed in hematopoietic cells; however, it is not known to what extent selective roles exist for each Src family member or whether redundancy exists in Src-based signal transduction.

Ras GTPase Superfamily

Members of the Ras guanosine triphosphatase (GTPase) superfamily modulate many signal transduction pathways. These proteins are low-molecular-weight GTP-binding proteins that act as molecular switches in regulating many different signaling pathways in virtually all cells.[141] Approximately 60 Ras family members have been identified to date in mammalian cells. GTPases exist in an inactive (guanosine diphosphate–bound) and an active (GTP-bound) conformation. Guanine nucleotide exchange factors catalyze the release of guanosine diphosphate and allow GTP to bind. In the active, GTP-bound state, Ras interacts with target proteins to promote a cellular response. An intrinsic GTPase activity, catalyzed further by GTPase-activating proteins, returns Ras GTPase to its inactive, guanosine diphosphate-bound state (Figure 142-7).

Striking features of the Ras superfamily include the diversity of membrane receptors and upstream regulators that can activate these GTPases, the diversity of cellular targets that can interact with an individual GTPase, and the extensive cross-talk that exists among GTPase-regulated signal transduction pathways.[141] Numerous examples of the coordinated activation and functional cooperation between Ras superfamily members at the molecular and cellular level have been described in animal cells. The best characterized of these functions are cell proliferation, gene expression, and actin-based cell motility.

Cell-cycle progression depends on cyclin-dependent protein kinases in which activity vacillates periodically during growth and division of cells.[142] The critical role for Ras in cell-cycle progression was suggested by experiments in which Ras activity was disrupted by microinjection of anti-Ras neutralizing antibodies or by expression of a dominant mutant interfering with endogenous Ras expression. These manipulations prevented mitogen-induced progression through G_1 and entry into S phase of the cell cycle.

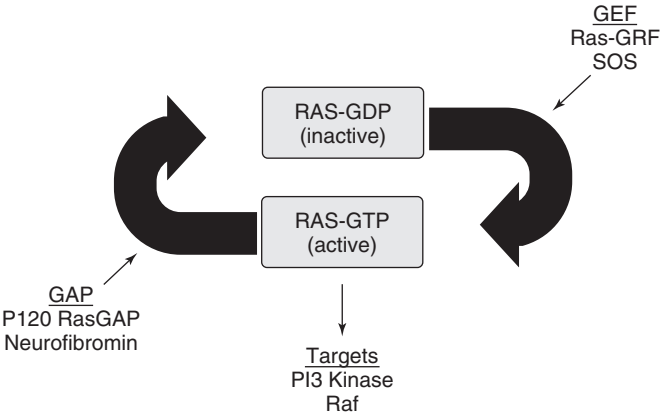


Figure 142-7 Ras interacts with multiple guanine nucleotide exchange factors *(GEF),* guanosine triphosphatase–activating proteins *(GAP),* and target proteins. The figure shows only a few examples of many interacting proteins identified to date. *GDP,* Guanosine diphosphate.

More recent studies have determined that the proliferative effects of Ras are dependent on signaling input from other Ras family members, Rho and Rac (Figure 142-8).[136,143]

The link between Ras superfamily GTPase activity and hematopoietic growth factor–induced proliferation and differentiation has been elucidated through in vitro and in vivo experiments. Overexpression of Ras-related GTPase, *Rac1*, enhanced the proliferative signal provided by GM-CSF in cultured cells.[144] Expression of a dominant negative Ras mutant (*ras*T17N) partially suppressed proliferation of cultured cells in response to GM-CSF. Studies to delineate M-CSF and GM-CSF receptor activation revealed differential modulation of the macrophage scavenger receptor A through the Ras signaling pathway[145] (Figure 142-9).

The role of neurofibromin, the protein encoded by the *NF1* tumor suppressor gene, in Ras signaling and hematopoietic cell proliferation has been examined in vitro and in vivo using a genetic approach. Humans and animals with mutations in *NF1* are predisposed to juvenile myelomonocytic leukemia. A hallmark of both human juvenile myelomonocytic leukemia cells and murine Nf1-deficient myeloid progenitors is a selective hypersensitivity to GM-CSF.[146] Whereas homozygous NF1-mutant ($NF1^{-/-}$) embryos die in utero, adoptively transferred $Nf1^{-/-}$ fetal liver cells consistently induce juvenile myelomonocytic leukemia–like disorder in irradiated recipients.[147] GM-CSF appears to play a central role in establishing and maintaining this phenotype in vivo.[148] Primary $NF1^{-/-}$ hematopoietic cells exhibit constitutive activation of the Ras-Raf-MAP kinase signaling pathway and enhanced cell proliferation in response to SCF and IL-3 or GM-CSF.[147]

DOWNSTREAM SIGNALING MECHANISMS AND SPECIFICITY

Down-stream from cytokine receptor-mediated events complex signal transduction interactions act to modify the ultimate cellular response (see Figure 142-5). Variable sets of signaling molecules, balancing of contradictory signals, feedback, and cross-talk lead to specific downstream gene expression events and cellular response.

Set of Signaling Molecules Determines Specificity

Different cell types express different sets of signaling molecules associated with the same cytokine receptor. For example, in cord blood hematopoietic progenitors the activation pattern of IL-15–dependent transcription factors is dependent on the maturation

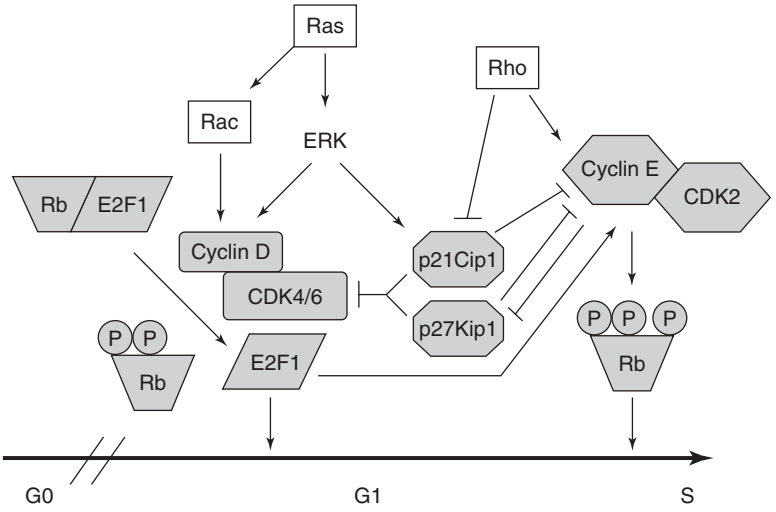


Figure 142-8 Influence of Ras and Ras family members Rho and Rac on cell-cycle progression. Cell-cycle regulatory proteins targeted by Ras, Rho, and Rac signal transduction pathways are shown. Cyclin D is a major target for Ras and Rac, whereas Rho acts primarily downstream of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. Rac and Ras act synergistically to activate cyclin-dependent kinase 4 (*CDK4*) and CDK6 through mechanisms to induce cyclin D transcription and assembly of cyclinD-CDK4 and 6 complexes at an early stage of G_1. The principal activity of these complexes is to phosphorylate Rb protein and thus allow activation of early-region-2 (E2F) transcription factors that regulate genes required for the G_1/S transition. Under circumstances in which Ras expression leads to cell-cycle arrest caused by induction of $p21^{Cip1}$, Rho can suppress the induction of $p21^{Cip1}$, thus enabling Ras to stimulate cell-cycle progression. Rho also regulates cell-cycle progression by inducing degradation of the CDK inhibitor $p27^{Kip1}$ through cyclin E/CDK2 activity. *ERK,* Extracellular regulated kinase.

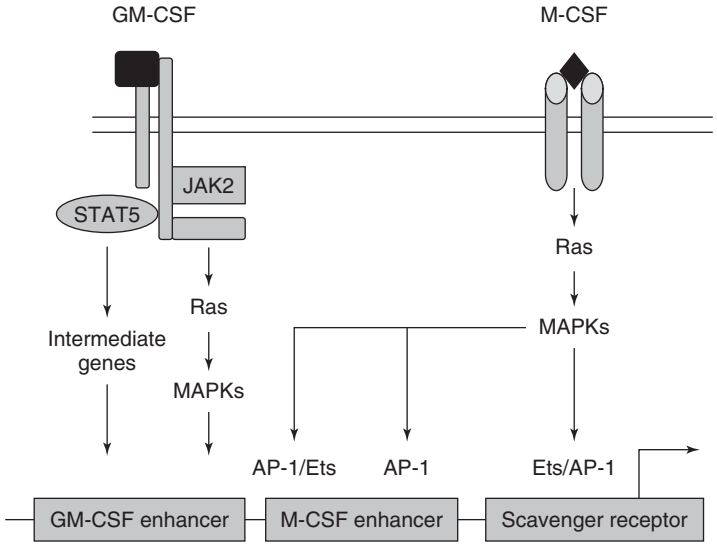


Figure 142-9 Mechanism for transcriptional control of scavenger receptor A *(SR-A)* by granulocyte-macrophage colony-stimulating factor *(GM-CSF)* and macrophage-CSF *(M-CSF).* Activation of SR-A by GM-CSF is dependent on a distinct enhancer element located upstream of the M-CSF–dependent enhancer. The GM-CSF enhancer is occupied by constitutively expressed transcription factors that, on regulation by Ras, activate immediate to early transcriptional responses. Sustained expression of SR-A in response to GM-CSF may be directed in part by transcription factors that are targets of STAT5 SR-A activation by M-CSF occurs through AP1- and Ets-mediated pathways. These transcription factors are targets of a Ras-dependent mitogen-activated protein kinase *(MAPK)* cascade that controls their level of expression and activities. *AP-1,* Activator protein-1; *Ets,* e twenty-six specific.

status of the progenitor cells and the form of IL-15. In freshly isolated unprimed cord blood progenitor cells, exposure to soluble IL-15 only activates NFκB.[149] In these cells nuclear translocation of NFκB is increased by short-term contact with a membrane-bound form of IL-15 (mb-IL-15). In the same cells cultured for 5 days in vitro in the presence of SCF and Flt-3L and therefore committed to the pro-NK pathway, soluble IL-15 triggers activation of STAT3. In contrast, short-term contact with mb-IL-15 activates STAT5 and STAT6.

Balance of Contradictory Signals Determines the Cell Specificity

Simultaneous activation of multiple pathways that produce contradictory signals is common in the signal transduction of cytokine receptors. For example, IL-6 can simultaneously induce growth promoting and growth suppressing signals through gp130 in the murine leukemia M1 cell line. When STAT3 activation

is suppressed, gp130 stimulation favors growth-promoting signals rather than growth-suppressing signals.[150] Similarly, signal transduction through gp130 can activate cell cycle transition from G_1 to S phase in a STAT3 dependent manner, and simultaneously, can induce a p21 cyclin-dependent kinase inhibitor that opposes these STAT3 dependent signals.[151] These contradictory signals arise from distinct regions of gp130. The balance of these contradictory signals determines the specificity of the biologic effect.

Modulation by Feedback or Cross-talk Determines the Specificity

Modulation of signals by feedback can be positive or negative. A cytokine signal conducted by activation of gp130, JAK, and STAT3 by tyrosine phosphorylation can be rapidly terminated by dephosphorylation. This occurs by a negative feedback circuit through recruitment of SH2-containing phosphatase, SHP-2, which is recruited to tyrosine 759 of gp130.

Feedback

Two families of proteins, the SOCS and protein inhibitor of activate STAT (PIAS) families are involved in less direct downregulation of cytokine signals. The SOCS family is comprised of eight members, SOCS-1 through SOCS-7 and cytokine-inducible SH2-containing protein (CIS). [152] Each of these proteins contains two regions of homology: a central SH2 domain and a SOCS box and a C-terminal 40 amino acid motif. SOCS-1 binds all four JAK kinases through its central SH2 domain and inhibits their kinase activity. In vitro studies have shown that SOCS-1 inhibits the STAT activation induced by IL-4, IL-6, interferon, and leukemia inhibitory factor.[153,154] SOCS-1 also inhibits Tec kinase and Vav function. SOCS-3 mRNA expression is induced by leukemia inhibitory factor, IL-2, IL-6, and IL-11.[154-157] Overexpression of SOCS-3 inhibits signaling of each of these cytokines.[115] Cytokine-inducible SH2-containing protein is a negative regulator of STAT5 activation in response to several cytokines including erythropoietin (EPO), IL-2, and IL-3. SOCS-1 deficient mice die perinatally due to excessive interferon responses.[158,159] SOCS-3–deficient mice die embryonically, likely due to excessive erythropoiesis.[160]

The binding of STAT to DNA also negatively regulates cytokine signaling. PIAS bind to STAT molecules blocking the DNA-binding activity and inhibiting STAT-mediated gene activation. PIAS1 blocks STAT1-mediated gene activation in response to interferon.[161] Similarly PIAS3 blocks DNA binding of STAT3 and its downstream effects.[162]

Cross-Talk at Receptor Level

Cross-talk between the IFNγ receptor and the IFNα/β receptor, which are localized to the calveolar membrane domain, is important for maximal antiviral response by IFNγ.[163] In mouse embryonic fibroblasts lacking IFNγ receptor, the IFNγ-induced antiviral response is impaired. Downstream IFNγ-I–induced STAT1 activity is diminished and formation of interferon-stimulated gene factor 3 is undetectable. In a similar manner, IFNβ null mouse embryonic fibroblasts lack IFNγ-induced STAT1 activation and antiviral response. Provision of exogenous IFNβ at a low concentration rescued the IFNγ antiviral response without activation of STAT1. This indicates the importance of constitutive subthreshold IFNα/β signaling at the receptor level for generation of a full IFNγ antiviral response.[163]

Cross-Talk Between Cytosolic Transcription Factors

Distinct cytokine signaling pathways are enacted through cytosolic interactions involving of several types of signaling molecules, such as adaptor proteins, kinases, phosphatases, and transcription factors. One example of cytosolic cross-talk between signaling molecules is IL-11 signaling in activated macrophages. IL-11 is a cytokine that displays antiinflammatory properties. IL-11 reduces lipopolysaccharide-induced TNF-α, IL-1β, IL-6, and IL-12p40 mRNA levels in activated macrophages. IL-11 acts to maintain levels of cytosolic NFκB inhibitors—IκB-α and IκB-β—thus inhibiting NFκB transcriptional activation.[164]

PLEIOTROPY AND REDUNDANCY OF CYTOKINE FUNCTION

Regulation of hematopoietic growth factors clearly occurs on many levels, including production (quantity and location), interaction with other factors, receptors (location, cell type, soluble versus membrane bound), and signal transduction mechanisms. *Pleiotropy* describes the property in which cytokines exhibit many different effects on various tissue and cell types. *Redundancy* refers to the property of cytokines in which two or more factors mediate similar biologic effects on the same cell type.

A cytokine designated IL-11 was cloned in 1990 based on its capacity to produce a mitogenic activity for the IL-6–dependent cell line T1165.[165] Even though IL-11 is not similar in structure to IL-6, the two cytokines exhibit certain similarities in their spectrum of biologic activities.[166,167] IL-6 and IL-11 have been shown to share the common signal transduction element, gp130.[168] Moreover, several cytokines share common chains to form heterodimers to effect signal transduction. The redundancy in function of several of these cytokines may, in part, be attributed to downstream actions of shared receptors.

Redundancy of cytokine function is not solely the result of shared receptor signal transduction elements. M-CSF and GM-CSF are both responsible for various aspects of macrophage differentiation and function. Although they do not share receptors, activation of M-CSF and GM-CSF receptors results in expression of the macrophage scavenger receptor A. Both receptors use components of the Ras signal transduction pathway to effect this response. However, M-CSF exerts its action by engaging the M-CSF–dependent enhancer recognized by activator protein-1 and cooperating Ets domain transcription factors, whereas activation of scavenger receptor A by GM-CSF is dependent on a distinct enhancer located immediately upstream of the M-CSF enhancer (see Figure 142-9).[145]

MATERNAL AND PLACENTAL PRODUCTION OF GROWTH FACTORS

Placental Production of Hematopoietic Growth Factors

The precise role and significance of growth factor production by placental tissues in maintenance of viable pregnancy and in the labor process are far from complete; however, it is likely that they are involved in two distinct regulatory processes: the neutralization of the immune response against the fetus and the provision of an environment conducive to the maturation and differentiation of the fetus.[169] From the immunologic standpoint, pregnancy has been likened (in the borrowed terms of transplant immunology) to the presence of a fetal allograft. Furthermore, the aggressive nature of the implantation event has appeared to some investigators to be similar to tumor invasion. Although certain similarities exist, the immunology of pregnancy exhibits unique features.

The components required to mount an immune response against the fetopaternal antigens are present in the maternal decidua. In addition to foreign antigen, antigen-presenting cells and effector cell are present. Despite this milieu, several mechanisms seem to abrogate the T-cell–mediated immune response at the fetomaternal interface. Although the mature T cells and NK cells are capable of migrating to the decidua and recognizing foreign antigen, they do not express their cytotoxic effects in this microenvironment. Certain mechanisms appear to act in concert to inhibit T-cell proliferation and cytotoxicity. First, the decidua produces large quantities of prostaglandin E_2 during

the first trimester.[170] Prostaglandin E_2 has been shown to down-regulate T-cell activation and IL-2 production. Second, the steroid hormones, which provide functions essential for maintenance of pregnancy, also exert significant immunologic functions. For instance, dehydroepiandrosterone (DHEA) dramatically enhances the production of IL-2 by stimulated lymphocytes. During pregnancy, DHEA levels decrease by approximately 50% because of rapid conversion of DHEA to estriol and other estrogen metabolites. Estrogens indirectly inhibit IL-2 production and accentuate IL-4 production through potentiation of the activity of 1,25-dihydroxyvitamin D_3. IL-4 effects a T-helper 2–type response that down-regulates cell-mediated cytotoxicity and phagocytic functions and favors humoral immunity.

Although the adaptive immune system appears to be inhibited by the physiologic alterations in the uterus during pregnancy, cells within the fetomaternal interface produce several hematopoietic growth factors. Among these are IL-1, IL-4, IL-6, IL-8, GM-CSF, M-CSF, tumor growth factor-β, and TNF. Production of these factors during gestation appears to be tightly regulated and to follow a specific sequence of expression. These cytokines can exhibit dichotomous functions. For instance, expressed at the proper time, concentration, and location, growth factors may promote trophoblast growth, whereas if they are expressed in abnormal amounts or sites, they may have pathophysiologic actions such as facilitating the onset of preterm labor.

Transport of Factors Across the Placenta

Studies in sheep have demonstrated that no transplacental transfer of erythropoietin (EPO) occurs.[171] Moreover, in vitro studies of EPO transport across the perfused human placenta provide evidence that Epo does not cross the placenta. However, no studies have been published to address whether the transplacental transfer of EPO occurs in vivo in humans.[172]

Studies in rats demonstrated that G-CSF (at least in small quantities) can cross from the maternal to the fetal circulation.[173] Although transfer appears to be meager, in one animal study it was sufficient to improve survival of rat pups subsequently exposed to group B streptococci.[174] Administration of recombinant G-CSF to humans resulted in transplacental passage of G-CSF in an amount capable of stimulating fetal granulopoiesis.[175] The mechanism underlying this transfer is uncertain, but it does not require an intact G-CSF receptor.[176] The physiologic relevance of transplacental passage of maternally derived hematopoietic cytokines remains to be delineated.

DEVELOPMENTAL ASPECTS OF INTERACTION BETWEEN HEMATOPOIETIC GROWTH FACTORS AND HEMATOPOIETIC PROGENITORS

Developmental Differences Between Adult and Fetal Progenitors

Developmental differences have been identified among hematopoietic progenitors from various sources. For instance, differences exist between fetal liver–derived and adult bone marrow–derived progenitors. Fetal liver progenitors exhibit a rapid cycling rate, rapidly expanding pool size, accelerated in vitro maturation time, increased sensitivity to Epo, and decreased sensitivity to GM-CSF compared with adult marrow progenitors.[60] Furthermore, circulating hematopoietic progenitor cells derived from umbilical cord blood differ from those derived from adult human bone marrow in that a subset of cord blood progenitors grows in methylcellulose cultures in the absence of added growth factors. Hematopoietic progenitors obtained from fetal subjects also differ from those obtained from adults. For instance, Roodman and colleagues[177] observed that by including dexamethasone with cultures of human fetal progenitors, fewer erythroid colonies and fewer normoblasts per colony developed. These reductions were not observed when progenitors from adults were subjected to the same conditions.[177] In addition, Christensen and colleagues[178] observed that high concentrations of EPO in vitro resulted in a dose-dependent down-modulation of neutrophil production from fetal, but not from adult, colony forming units–granulocyte macrophage.[178] A third difference between fetal and adult progenitors was observed by Emerson and associates[179] and by Valtieri and colleagues,[180] who reported that EPO alone induced erythroid burst formation from embryonic and fetal burst-forming units-erythroid, whereas burst-forming units-erythroid of adult origin required GM-CSF or IL-3 in addition to EPO. A fourth difference is that some fetal progenitors undergo clonogenic maturation in the absence of added growth factors or when they are stimulated with factors such as IL-11 that, as single agents, do not support maturation of progenitors from adults.[167,181] Other studies suggest that the progenitor cells or their progeny express growth factors, IL-3, and GM-CSF, and thus support clonal expansion in an autocrine or paracrine fashion.[182]

Experiments to delineate the effects of hematopoietic growth factors on fetal hematopoietic progenitor cells have demonstrated differences in the actions of these factors on fetal versus adult progenitors. For instance, the presence of early-acting factors (IL-6 and SCF) initiates clonal expansion in vitro in the absence of other growth factors added to the culture. In addition, IL-11 enhances cycling of both committed multipotential and lineage-specific colonies from cord blood, but it does not induce cycling of adult progenitors derived from bone marrow.[167,181-183]

Effects of Hematopoietic Growth Factors on Nonhematopoietic Tissues During Embryonic and Fetal Development

SCF appears to have a critical role in embryonic and fetal hematopoietic development. Schmitt and colleagues[184] observed that SCF and c-*kit* were among the factors expressed in undifferentiated embryonic murine stem cells. The importance of SCF and its interaction with the c-*kit* receptor during early development are illustrated by murine strains with mutations at the W locus encoding the c-*kit* product, and mice with the mutation of the SL locus encoding SCF protein.[185,186] Both strains exhibit pleiotropic developmental defects, not only in hematopoiesis, but also in gametogenesis and melanogenesis.

The utilization of homologous recombination to generate transgenic mouse models with deletion of specific growth factors has shed light on the role of these factors during development and their relative importance in normal hematopoietic homeostasis. The GM-CSF knock-out mouse illustrates this point well.[187] It was expected that a mouse homozygous for defective GM-CSF alleles would exhibit defects in hematopoietic development. Such is not the case, because these mice do not exhibit detectable hematopoietic defects. Rather unexpectedly, they develop a form of alveolar proteinosis. Whether the pathophysiology observed in this defect results from defective alveolar macrophage function, pulmonary epithelial function, or a combination of both is unclear.

Mice deficient in both GM-CSF and M-CSF gene products (genotype $GM^{-/-}$ $M^{-/-}$) have been generated by interbreeding GM-CSF–deficient mice with M-CSF–deficient osteopetrotic mice.[188] These mice exhibit coexistent features of both deficiency states, yet they still have circulating monocytes and phagocytic tissue macrophages. This would indicate that factors other than GM-CSF and M-CSF are capable of supporting macrophage lineage differentiation. These mice (like the GM-CSF–deficient mice) exhibit a form of chronic lung disease that is more severe than that of GM-CSF deficiency alone, a finding implicating M-CSF in modulating the pulmonary disease associated with GM-CSF deficiency in vivo.

Several hematopoietic growth factors including EPO, G-CSF, GM-CSF, IL-6, IL-8, IL-11, TPO, and their respective receptors have

been identified in nonhematopoietic tissues of the human fetus and neonate. EPO receptor has been identified in murine cell lines of neuronal origin, in cultured murine embryonic neurons, and in the brain and spinal cord of rodents.[189,190] The presence of EPO was also identified in the cerebrospinal fluid of normal and preterm infants.[191] Subsequently, EPO and its receptor were identified in the central nervous system of human fetuses as early as 5 to 6 weeks after conception.[192] The expression of EPO and EPO receptor mRNA in the spinal cord remained relatively constant from 7 to 16 weeks gestation, whereas they both increased in the brain as gestation progressed. By immunohistochemical analysis, *EPO* and *EPO* receptor are localized to the periventricular germinal zone at 5 to 6 weeks gestation.[193] By late gestation, EPO and EPO receptor localization has diverged; EPO is localized primarily in neurons, and EPO receptor reactivity is localized most prominently in astrocytes. In vitro studies of neuronal cell lines subjected to hypoxic conditions and glutamate-induced injury demonstrated a protective effect of EPO.[190,192] In vivo studies in animals also demonstrated a protective effect of EPO in ischemic brain injury models.[194,195] The role these hematopoietic factors play in neurodevelopment and homeostasis remains to be identified.

G-CSF and G-CSF receptor are present in virtually all organs between 8 and 24 weeks gestation.[196,197] Their distribution in the intestine and kidney changes with progression of gestation. In preterm and term neonates, G-CSF protein concentration has been examined. GM-CSF protein and mRNA are detected during human fetal development in the lung, spleen, adrenal gland, and neuronal tissue.[198] The cell types expressing GM-CSF include macrophages, neurons, and glial cells.

IL-11 is a hematopoietic growth factor that also affects nonhematopoietic tissues. IL-11 is produced by alveolar and bronchial epithelial cells in large amounts and may play a significant role in pulmonary inflammation. Additionally, both IL-11 and its receptor are expressed in epithelial cells of the gastrointestinal tract.[199] IL-11 interacts with gastrointestinal epithelial cells and reversibly inhibits its proliferation of intestinal crypt stem cell lines. This activity may play a role in normal intestinal growth and in resistance to injury. IL-11 has been evaluated for its protective effects in several models of gastrointestinal disease, including acute chemical-induced colitis and chronic inflammatory bowel disease in transgenic animals expressing human leukocyte antigen-B27 and β_2-microglobulin.[200] IL-11 has also been used to ameliorate damage in a model of ischemic bowel injury, a murine burn model, and a rat model of short bowel syndrome.[201-203] IL-11 administration increased survival rates and decreased bacterial translocation in these models.

DEVELOPMENTAL DIFFERENCES IN PRODUCTION OF CYTOKINES BY MATURE EFFECTOR CELLS

Some cytokines regulating pivotal functions required for activation of immune effector cells are synthesized in diminished quantities by human neonates compared with adults. Production of IFNγ and of IL-6 is decreased by circulating leukocytes derived from neonates compared with adults.[204-206] The role of these factors in the immune response to certain bacterial and viral pathogens is underscored by experiments involving IFNγ– and IL-6–deficient mice generated by homologous recombination and by studies using neutralizing antibodies to these factors.[206-209] IFNγ–deficient mice manifest severely impaired defenses against both bacterial and viral pathogens. IL-6–deficient mice exhibit defective cytolytic activity of T cells in response to viral infections and diminished capacity to recruit immune cells to sites of infection after experimental bacterial inoculation. The mechanism underlying these developmental defects has not been fully defined; however, diminished production of IL-12 by antigen-presenting cells may contribute to diminished IFNγ responses. Investigations of neonatal monocyte-derived dendritic cells revealed a selective defect in IL-12 synthesis at the protein and mRNA level in response to lipopolysaccharide, CD40 ligation, or polynucleotides.[210] Moreover, these investigators found that neonatal dendritic cells were less effective than adult dendritic cells in stimulating IFNγ production by allogeneic adult T cells. This deficit was corrected with addition of recombinant IL-12. Deficiencies in production of these factors could account for impairment in certain aspects of host defenses observed in the newborn period.

Two hematopoietic growth factors, GM-CSF and G-CSF, which exhibit significant effects on myeloid clonal expansion and differentiation, are also produced in diminished quantities by neonatal blood mononuclear cells compared with adult cells.[211,212] Although GM-CSF receptor numbers and binding affinity appear to be similar between adult and neonatal blood mononuclear cells, GM-CSF gene expression by these cells is clearly lower in cells derived from term neonates. Similarly, monocytes from term human neonates produce significantly lower quantities of G-CSF in vitro than cells derived from adults. Furthermore, developmental dysregulation of G-CSF expression appears to be more pronounced in cells of preterm neonates. Studies in mice demonstrate similar decreases in expression of IL-6 and G-CSF in vivo.[206] Diminished production of GM-CSF by phytohemagglutinin- and phorbol myristate acetate–stimulated newborn mononuclear cells compared with adult mononuclear cells is secondary to posttranslational regulation of mRNA stability. The precise molecular mechanisms responsible for the differences in regulation of G-CSF between neonates and adults have not been delineated; however, currently available data support a posttranscriptional mechanism.[213]

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Developmental Granulocytopoiesis

FETAL GRANULOCYTE PROGENITORS

The first circulating blood cells during embryonic development are derived from the extra embryonic mesoderm of the yolk sac (YS), and as the embryo develops, the anatomic site of hematopoiesis subsequently switches to the liver and then to the marrow.[1] The hematopoietic stem cells (HSC) seeding these organs include YS-derived HSC, which appear to be a transient embryonic population, and definitive stem cells arising from the aorta-gonad-mesonephros (AGM) region within the embryonic splanchnopleuric mesoderm. The origin of the AGM-HSC is not clear, but they are located in the ventral aspect of the dorsal aorta in the preumbilical region near the anterior limb bud level and are observed between 30 and 37 days gestation.

Hematopoiesis from the YS continues from the 3rd through the 8th to 10th week. Before liver colonization with HCS, the placenta appears to be a substantial reservoir of HCS.[2] The origin and fate of these placental HCS are under investigation. There is overlap with hematopoiesis in the YS, placenta, and liver, with the latter beginning about the 5th week and continuing through 20 to 24 weeks. Hematopoiesis is observed in the marrow by the 11th week,[3] when committed granulocytic progenitors are first seen in the clavicular marrow followed by mature neutrophils in the marrow from the 14th week onward. In the fetal liver, although neutrophil progenitors are abundant, mature neutrophils are not identifiable.[4]

Pluznick and Sachs[5] and Bradley and Metcalf[6] first cultured hematopoietic progenitor cells in semisolid media and termed this variety of progenitor a *colony-forming unit* (CFU). They observed that when grown in appropriate conditions, some CFUs gave rise to clones containing mixtures of blood cells, including macrophages, monocytes, neutrophils, and erythrocytes. These CFUs were termed *CFU-MIX* or, if the colony contained granulocytes, erythrocytes, megakaryocytes, and macrophages, *CFU-GEMM*. Other CFUs gave rise to colonies containing only one or two cell types. Those developing neutrophils only were termed *CFU-G* (granulocyte), those with only macrophages or monocytes were termed *CFU-M*, those with neutrophils plus macrophages or monocytes were termed *CFU-GM*, and those with only megakaryocytes were termed *CFU-Meg*.[7] Other progenitors develop into colonies of normoblasts and have been divided conceptually into two classes. The more primitive variety give rise to a large burst of normoblasts and are termed *BFU-E* (burst-forming units—erythroid) and the more mature variety "e" give rise to a smaller clone of normoblasts and are termed *CFU-E* (colony-forming units—erythroid). The mechanisms underlying this process of lineage commitment are now beginning to be understood.[8] The effect of biomechanical forces as critical regulators of hematopoiesis is a new field of investigation,[9] as is the prevention of apoptosis as a mechanism for hematopoietic progenitor cell growth and development.[10]

Umbilical cord blood and fetal blood have a 10- to 50-fold higher concentration of CFU-GM than does the blood of adults.[11] However, this may not indicate that the mass of CFU-GM per kilogram body weight is higher in the fetus than in the adult. In fact, animal studies indicate that the opposite is true; namely, compared with adults, the fetus has relatively few granulocytopoietic progenitors but has a much larger proportion of progenitor pool in the blood.[12] Similar findings are reported for CFU-MIX.[13] Further evidence of a small granulocytopoietic pool in the fetus comes from studies of soluble FcRIII (sCD16), which is derived from apoptotic neutrophils. Its low concentration in plasma of preterm neonates may reflect a low total body neutrophil mass. The low sCD16 levels of premature infants reach adult values by the fourth postnatal week.[14]

Although relatively fewer in number per kilogram body weight, a higher proportion of the CFU-GM in the fetus are actively cycling, compared with CFU-GM of adults.[11-13] This high proportion in cycle may be a mechanism for increasing neutrophil production needed to build a sizable neutrophil reserve pool in preparation for birth and to keep pace with the rapid rate of somatic growth in the second and third trimesters.

Basic differences have been postulated in hematopoietic progenitors of fetal versus adult origin. Emerson,[15] Valtieri,[16] and Holbrook[17] and their colleagues observed that fetal BFU-E undergo clonal maturation in the presence of erythropoietin alone, whereas BFU-E of adult origin require other factors (such as granulocyte-macrophage colony-stimulating factor [GM-CSF], interleukin-3 [IL-3], or IL-9) in addition to erythropoietin. Gardner and co-workers[18] reported a similar phenomenon for fetal CFU-GM; namely, a fraction of fetal CFU-GM undergoes clonal maturation when IL-6 alone is included in the tissue culture, whereas CFU-GM from adults require other factors as well. Schibler and associates[19] reported a similar finding with IL-11. Some of this apparent difference between fetal and adult progenitors might be explained by environmental differences rather than intrinsic differences in progenitors. Evidence for this was provided by Schibler and colleagues,[20] who observed transcripts for GM-CSF and IL-3 in fetal but not adult $CD34^+$ and noticed that the "factor independence" of fetal progenitors was ablated by adding neutralizing anti-GM-CSF and anti-IL-3 antibodies to the cultures. On this basis, they speculated that the factor independence ascribed to fetal hematopoietic progenitors was the result of self-generation of factors in the culture dish.

FETAL HEMATOPOIETIC MICROENVIRONMENT

Hematopoiesis in the fetal liver is predominantly erythropoietic, but in the fetal bone marrow, it is predominantly granulocytopoietic.[21] However, when progenitors from either organ are cultured in vitro, they are capable of producing both erythrocyte and granulocytes.[22,23] Thus the local microenvironment appears to determine the fate of fetal hematopoietic progenitors (Figure 143-1). Clearly, the microenvironment changes with maturation, which likely explains differences in the pattern of developmental hematopoiesis.[24] The microenvironment includes a complex amalgam of cellular elements, growth factors, extracellular matrix, and biomechanical factors. The cellular elements consist of macrophages, reticular cells, adipocytes, and endothelial cells, whereas the extracellular matrix includes collagen and a variety of glycoproteins such as hemonectin and fibronectin.[25]

Roles of cellular elements of the microenvironment are being defined (Figure 143-2). Mesenchymal stem cells are multipotent and can generate various microenvironmental components, and these cells have a role in the switch of hematopoiesis between YS,

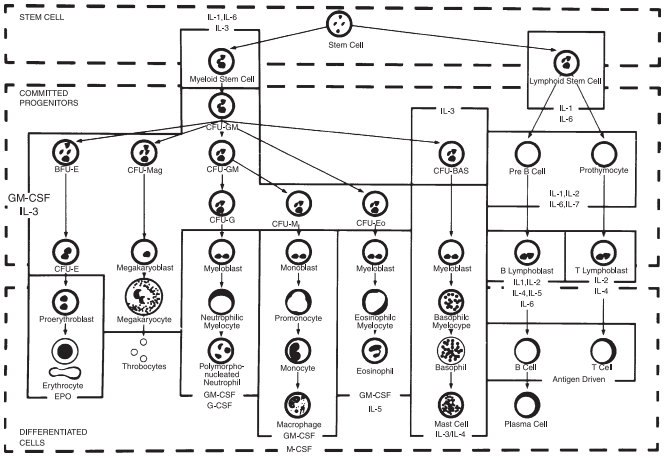


Figure 143-1 Hematopoietic development and regulation of myeloid committed progenitors.

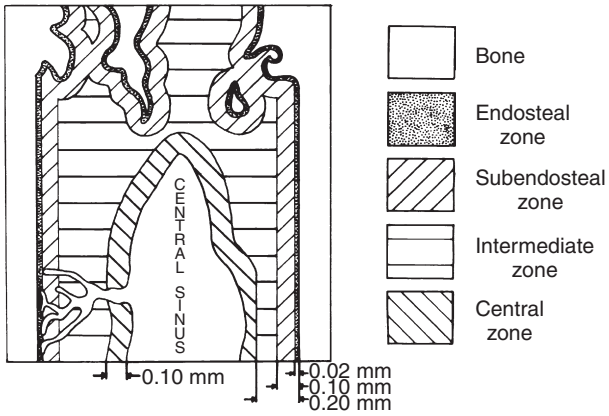


Figure 143-2 Scale drawing of the stereologic zones used for the determination of hematopoietic colony cell distribution. *(From Lambersen RH, Weiss L:A model of intermedullary hematopoietic microenvironments based on stereologic studies of the distribution of endocloned marrow colonies,* Blood *63:287, 1984.)*

placenta, liver, and marrow.[26] Among the factors generated by mesenchymal cells are cytokines and growth factors.[27] For instance, clones of normoblasts associate physically with macrophages that have long branching processes encircling the developing normoblasts.[28] Similarly, in vitro erythroid colonies generally develop around a nesting macrophage.[25] In contrast, clones of granulocytes associate with reticular cells that have fibroblast-like appearance and function.[28] These reticular cells not only serve to hold the cells in proximity but also bind specific glycoproteins needed for granulocytic development. As granulocytic cells mature in the matrix, they lose their binding sites for adhesive molecules and are released into circulation.

Differences in stromal cell populations were postulated as a mechanism explaining the difference between fetal and adult hematopoietic microenvironments. However, Bethel and colleagues[29] did not find a difference in stromal cellular elements comparing fetal liver, fetal marrow, and adult marrow and speculated that the cellular portion of the microenvironment was not the relevant feature.

Extracellular matrix components have also been postulated as a mechanism explaining differences between fetal and adult hematopoietic microenvironments. Heparan-sulfated proteoglycans can bind to tumor growth factor-β, basic fibroblast growth factor, IL-3, GM-CSF, and IL-7.[30] Osteonectin can immobilize platelet-derived growth factor. In addition, ECM can bind to cell surface glycoproteins. Fibronectin, collagens, and laminin are ligands for integrins, which not only control anchorage, spreading, and migration of hematopoietic cells but are also involved in various intracellular signaling cascades. Hyaluronan is a ligand of CD44 and, in several systems, facilitates cell-cell adhesion and cell migration.[31]

Collagens I, III, and IV have been found in the adherent cell layer of long-term marrow cultures.[32]Their specific roles have not been defined, but Zuckerman and colleagues,[33] using a murine long-term bone marrow culture system and a proline analogue *cis*-4-hydroxyproline (CHP) to inhibit collagen synthesis, demonstrated that collagen is important in long-term cultures. Using various concentrations of CHP, they documented decreased cellularity, decreased synthesis of collagen, and decreased hematopoietic growth.

Proteoglycans are among the regulators of the hematopoietic microenvironment. For instance, erythropoietic suppression induced by polycythemia involves an increase in marrow content of sulfated glycosaminoglycans.[34] Chondroitin sulfate and hyaluronic acid are increased in the marrow of SI/SI^d mice, who have a defective marrow microenvironment.[35] In contrast, stimulating glycosaminoglycan synthesis promotes hematopoietic proliferation.[36]

Proteoglycans and glycosaminoglycans are involved in cell adhesion to the extracellular matrix. Del Rosso and co-workers[37] showed that mature granulocytes did not normally adhere to the extracellular matrix, but if treated with hyaluronidase, most did. Treatment of stroma with heparitinase reduced adhesion of immature marrow granulocytes to the stroma. Glycosaminoglycans may function indirectly to stimulate hematopoiesis. Gordon and colleagues[38] showed that glycosaminoglycans from the bone marrow stromal layer were capable of trapping GM-CSF. Fibronectin did not trap GM-CSF, and glycosaminoglycans from fetal liver adherent cells were not able to trap GM-CSF. Thus different proteoglycans or glycosaminoglycans may have different roles in hematopoiesis.

Hemonectin and fibronectin are extracellular matrix molecules with hematopoietic functions. Hemonectin is associated with granulocytic development,[39] whereas fibronectin is involved in erythroid development. Developing erythroid progenitors express fibronectin receptors, which they lose as they mature, suggesting that fibronectin may be involved in the release of erythroid cells from the bone marrow.[40] Mechanisms whereby the granulocytopoietic microenvironment operate, including the genes involved and the role of oxygen, are being studied.[41,42]

NEUTROPHIL MITOTIC AND MATURATION COMPARTMENTS

The life cycle of a neutrophil has been conceptually divided into three phases, representing time spent in the (1) marrow, (2) blood, and (3) tissues. It is assumed that neutrophils move through this system approximately in order, as if in a pipeline. The initial part of the pipeline involves commitment and clonal maturation of progenitors within the marrow. The earliest neutrophilic precursors are capable of cell division and thus are referred to as the "mitotic compartment," also known as the *neutrophil proliferative pool* (NPP). This compartment is composed of myeloblasts, promyelocytes, and myelocytes.

As neutrophils mature, their capacity to undergo cell division is lost. Neutrophils that can no longer divide are housed in the marrow for the purposes of maturation and storage as a ready reserve of cells to be released from the marrow into the blood on demand. This stage in neutrophil development is termed the "maturation compartment," also known as the *neutrophil storage pool* (NSP). This compartment consists of the metamyelocytes,

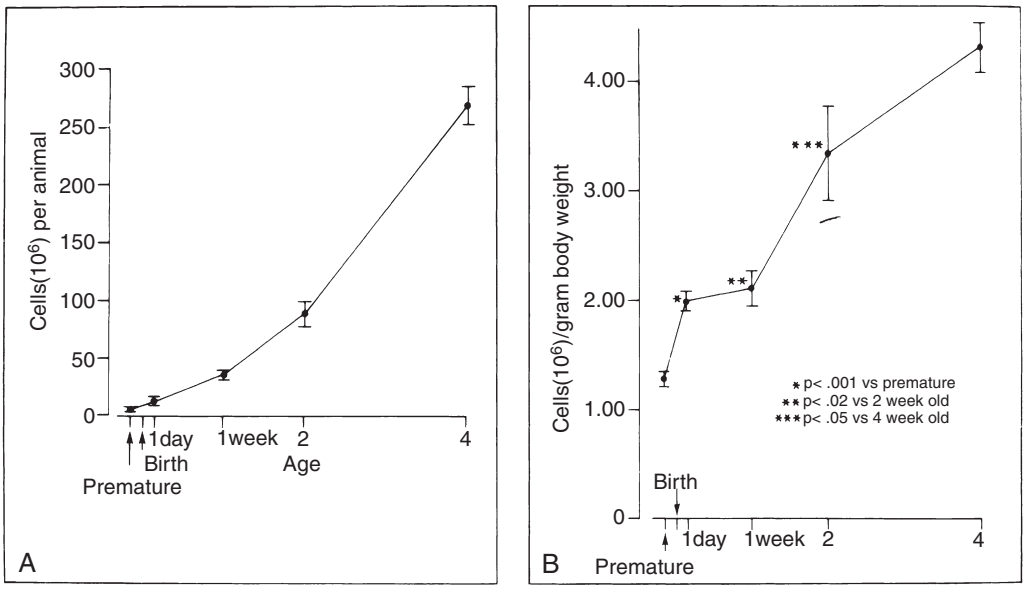


Figure 143-3 A, Neutrophil storage pool size at various ages in the developing rat. Each point represents the mean of 8 to 12 animals ± SE. **B,** Neutrophil storage pool size per gram of body weight at various ages in the developing rat. Each point represents the mean of 8 to 12 animals ± SE. *(From Erdmann SH, Christensen RD, Bradley PP, et al: Supply and release of storage neutrophils: A development study,* Biol Neonate *41:132, 1982.)*

band neutrophils, and segmented neutrophils in the marrow. Studies in fetal and newborn rodents indicate that the liver and spleen house a significant fraction of the NPP and NSP,[43] but in human neonates, only the marrow, not the liver and spleen, houses neutrophil production and storage.[44]

Studies in adults indicate that under steady-state conditions, four to five cell divisions occur among cells in the NPP.[45] Similar studies have not been reported for neonates. Studies using tritiated thymidine incorporation and $DF^{32}P$ labeling indicate that the NPP contains about 2×10^9 cells/kg body weight and the NSP about 6×10^9 cells/kg.[46] Adult rats also have an NSP that contains about 6×10^9 cells/kg; however, no neutrophils at all are observed before about 14 days gestation. By 19 days gestation (term = 21 days), rats have a NSP of 0.9×10^9 cells/kg, and by term, the NSP has increased to 1.2×10^9 cells/kg (Figure 143-3).[43] Although the size of the NSP has not been quantified in human neonates, it appears from studies of human abortuses that no neutrophils are present before 14 weeks gestation and the number per kilogram body weight at 22 to 24 weeks gestation is far fewer that at term.[44] It also appears that, in contrast to rodents, granulocytopoiesis in the human fetus takes place almost exclusively in the bone marrow.[47] Although the spleen contains CFU-GM, these appear to be within the blood circulating through. Histologic sections of fetal spleen fail to show any nests of granulocytopoietic activity, and RNA extracted from fetal spleen fails to reveal transcripts for neutrophil-specific growth factors. Similarly, differential cell counts, histologic sections, and RNA studies of human fetal liver reveal little, if any, granulocytopoiesis in that organ, and the fetal liver does not exhibit nests of granulocytopoiesis. Thus the fetal spleen and liver contain granulocyte progenitors but are probably not normally active sites of granulocytopoiesis.[44]

CIRCULATING AND MARGINATED BLOOD NEUTROPHIL POOLS

Neutrophils released from the marrow enter the blood where they distribute into two compartments of approximately equal size: the circulating and the marginal pools. Neutrophils in the former compartment are freely circulating, whereas those in the marginal compartment are temporarily adherent to endothelial cells, generally in the postcapillary venules. In normal adult humans, the total of these two compartments (termed the *total blood neutrophil pool*) contains about 0.6×10^9 cells/kg body weight; with about one half of the cells (0.3×10^9 cells/kg) in each (circulating and marginal) pool.[46]

In the marrow, the proportion of band to segmented neutrophils is much higher than in the blood. On that basis, it is likely that a selective release of segmented neutrophils occurs from the marrow, whereas band neutrophils generally remain behind to mature. The mechanisms controlling release are only partly understood. G-CSF can induce such release,[48] as illustrated by G-CSF-deficient mice,[48] and so can hydrocortisone and related corticosteroids.[46] Some evidence exists that a factor derived from the activation of C3 can induce NSP release as well.[49]

After epinephrine administration, neutrophils are rapidly released from the marginated compartment and enter the circulating pool.[46] The neutrophilia thus produced persists only for 30 to 45 minutes. No change in differential cell count accompanies this demargination; thus the proportion of segmented neutrophils is the same in the marginated and circulating neutrophil pools. The opposite movement of neutrophils, from the circulating to the marginated pool, occurs for a period of 1 to 2 hours after experimental intravenous administration of endotoxin. Newborn infants with strenuous crying can experience neutrophil demargination and can double their blood neutrophil concentration within 3 to 5 minutes.[50,51] A mild demarginating response is also reported to occur in preterm infants after erythrocyte transfusion.[51]

The half-life of neutrophils in the blood of adult humans, measured by autologous $DF^{32}P$ labeling, is about 6.3 hours,[52] and this agrees closely with studies using ^{3}H-TdR (7.4 hours).[53] Similar studies on neutrophils from human fetal subjects have not been reported. However, studies on cord blood neutrophils suggest a lower rate of spontaneous apoptosis compared with adult cells. The significance of these findings remains to be determined. It is likely that neutrophils leave the blood and enter the tissues randomly, rather than according to their age as is the case for

TABLE 143-1

Fetal White Blood Cell and Neutrophil Counts

Gestational Age (wk)	White Blood Cells ($\times 10^3/mm^3$)	Neutrophils (%)
18-20	4.2	5 ± 2
21-22	4.19	5.5 ± 3.5
23-25	3.95	7.5 ± 4.5
26-30	4.44	8.5 ± 2.5

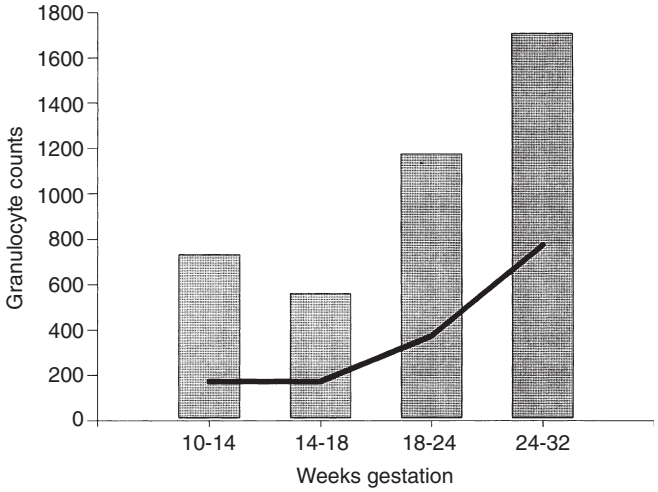


Figure 143-4 Mean and range of neutrophil counts at 10 to 14, 14 to 18, 18 to 24, and 24 to 32 weeks gestational age. *(From Thomas DB, Yoffey JM: Human foetal haemopoiesis. I. The cellular composition of foetal blood,* Br J Haematol *8:290, 1962.)*

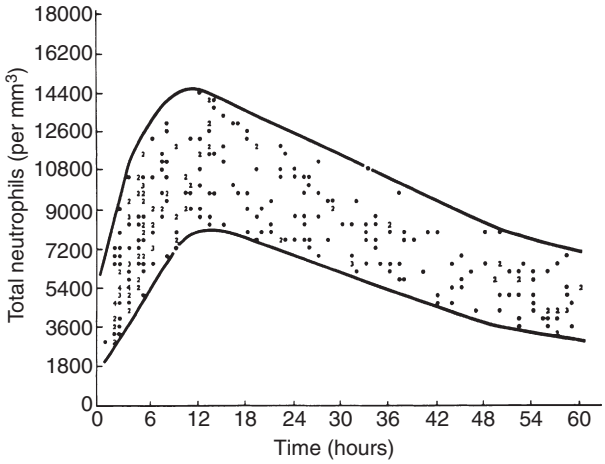


Figure 143-5 The total neutrophil count reference range in the first 60 hours of life. *(From Manroe BL, Weinberg AG, Rosenfeld CR, et al: The neonatal blood count in health and disease,* J Pediatr *95:89, 1979.)*

erythrocytes and platelets. This theory is supported by the observation that transfused, labeled neutrophils appear in the blood and saliva simultaneously.[54]

After their short time in the blood, neutrophils move through vessel walls to enter the tissues. Within minutes of developing a local site of tissue damage or infection, adherence of neutrophils to the endothelium and their subsequent migration into the tissues can be observed. Studies on fetal sheep show that after intraamniotic exposure to endotoxin, the number of circulating neutrophils initially decreases by 4 hours owing to egress into the tissues, followed by an increase over the next 6 days.[55] After the initial adherence of the neutrophil to an endothelial cell, it projects pseudopodia and forces a passageway between endothelial cells. The length of time that neutrophils spend in the tissues and their fate there are not completely clear. Labeled neutrophils are found in saliva, although they are not found in the salivary ducts.[54] This is presumably due to migration of neutrophils into the gingiva. Loss of neutrophils has been observed into the urine, and through the lungs, liver, spleen, and gastrointestinal tract.[55] Not all neutrophils are used by encountering infectious organisms because the size of the marrow and blood neutrophil pools is essentially the same in germ-free and normal mice.[54]

Reference ranges for circulating neutrophil varies with gestation. The proportion of neutrophils in early gestation is on the order of 5% (Table 143-1). In samples obtained by hysterotomy at 26 to 30 weeks gestation, neutrophils constitute 8.5% of the leukocytes, or only about 350/μL.[56] Playfair and associates[57] studied blood counts from fetuses obtained from pregnancies terminated by either hysterotomies or vaginal abortion. Those delivered vaginally had much higher neutrophil counts than did those delivered operatively. Neutrophil counts can be higher for up to 3 days in preterm infants after antenatal maternal administration of betamethasone when the drug is administered close to delivery.[58]

There is a doubling of the neutrophil count from 14 to 18 weeks gestation and a fourfold increase by 24 to 32 weeks gestation (Figure 143-4)[59] At birth, the neutrophil count is much higher still. Manroe and co-workers[60] published neutrophil reference ranges in term neonates during the first 28 days after birth. Peak neutrophil counts occurred between 12 and 24 hours postnatally (Figure 143-5), with the 95% confidence limit ranging from a low of 7800 to a high of 14,500/μL. Counts then decreased, achieving a stable lower value of 1750 by 72 hours of life. The stable upper limit of the next 28 days was achieved at about 6 days.

Mouzinho and colleagues[61] revised the reference ranges for blood neutrophil concentrations in very-low-birth-weight infants (see Figure 143-5). Serial counts were obtained between birth and 28 days of age from 63 infants of 29.9 ± 2.3 weeks gestation. No difference in the upper limit of normal was observed between those very-low-birth-weight infants and the more mature infants reported by Manroe. Concentrations of greater than 14,000/μL at 12 hours and greater than 9000/μL after 48 hours defined neutrophilia. However, the very-low-birth-weight infants had a wider range of counts, encompassing substantially lower values compared with mature infants; neutropenia was defined as a concentration less than 2000/μL at 12 hours of life and less than 100/μL after 48 hours. Lower counts in healthy very-low-birth-weight infants were also reported by Prober and colleagues.[62]

Altitude (>4800 feet above sea level) appears to have an elevating effect on neonatal neutrophil counts of term (Figure 143-6) and preterm (Figure 143-7) infants.[63] If the effect of altitude is not taken into account, neutrophilia can be overdiagnosed, and, erroneously, neonates with a normal complete blood count can be labeled as having neutrophilia (Figure 143-8).[64]

NEUTROPHIL ANTIGENS IN THE FETUS

The expression of specific granulocyte antigens has been reviewed by Bux.[65] In 1926, Doan[66] observed that serum from some patients agglutinated leukocytes. Although techniques for measuring leukoagglutinins have improved over the years, the field of recognition of granulocyte antigens has been plagued by an inherent tendency of granulocytes to agglutinate. Neutrophil-specific antigens were initially discovered by Lalezari and Radel[67] while studying neonatal neutropenia. Numerous other antigens

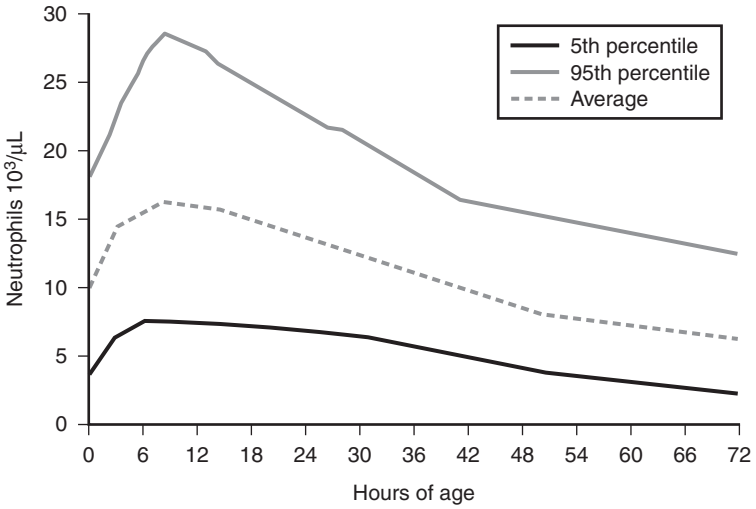


Figure 143-6 Reference ranges for blood neutrophil concentrations at altitude (>4800 ft) during the first 72 hours after the birth of neonates of more than 36 weeks gestation. *(Modified from Schmutz N et al:* J Perinatol *28:275, 2008.)*

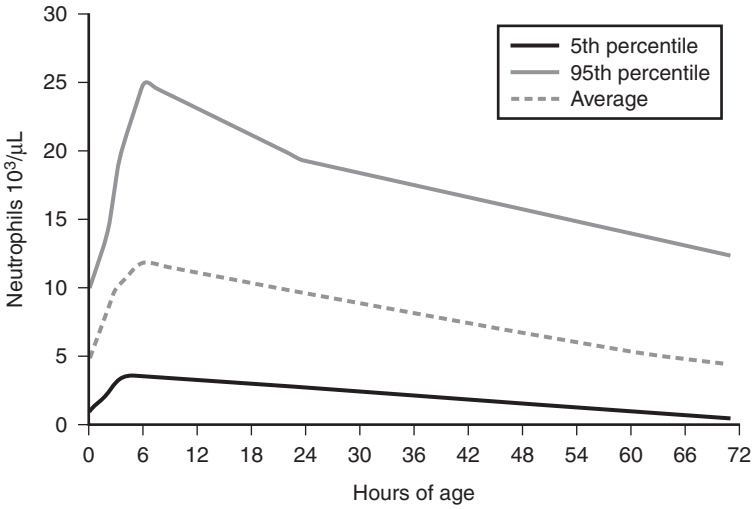


Figure 143-7 Reference ranges for blood neutrophil concentrations at altitude (>4800 ft) during the first 72 hours after the birth of preterm neonates (28 to 36 weeks gestation). *(Modified from Schmutz N Henry E, Jopling J, et al: Expected ranges for blood neutrophil concentrations of neonates: the Manroe and Mouzinho charts revisited,* J Perinatol *28:275, 2008.)*

have been added subsequently and can be broadly classified into three subgroups: (1) antigens with expression limited to granulocytes (such as NA, NB, NC, ND, NE, HGA-3, and LAN), (2) antigens that can also be detected on other cell types (such as HGA-1, Mart, and Ond), and (3) antigens distributed throughout the body (such as the HLA system and antigen 5b). In the original nomenclature, the letter *N* stood for neutrophil-specific antigen, followed by a capital letter designating a specific gene locus and a number designating a certain allele at that locus. As is apparent from the previous list, a number of antigens described later did not follow this convention, and the subsequent molecular characterization of the antigens showed that some of these were actually identical to each other.

A new nomenclature proposed by the Granulocyte Antigen Working Party of the International Society of Blood Transfusion (ISBT) attempted to address these anomalies.[68] It named the well-defined granulocyte alloantigens as HNA, which stands for "human neutrophil alloantigen." Glycoprotein location of the antigen is coded by a number (e.g., location on FcγRIIIb is denoted as HNA-1). Different polymorphisms of the same glycoprotein are designated alphabetically, in order of the date of publication (HNA-1a, -1b, and so on). The genes coding for glycoproteins are named according to the guidelines of the International Workshop on Human Gene Mapping (e.g., Fcγ receptor IIIb is abbreviated to FCGR3B). The allelic variants of the gene are then designated with Arabic numerals and are separated from the gene designation by an asterisk (e.g., FCGR3B*1). For the naming of newly detected antigens, the use of acronyms is permitted until their eventual inclusion in this system.

The HNA system currently comprises seven antigens that are assigned to five glycoproteins (Table 143-2). This classification does not include NB2, NC1, ND1, NE1, SAR, LAN, LEA, CN1, and RED antigens. NB2 is not the antithetical antigen to NB1 and may in fact be identical to 9a. NC1 seems to be identical to NA2. ND1 and NE1 are solitary representatives of other presumed systems that have been associated with autoimmune neutropenia, but specific antisera against these two antigens are no longer available, and hence testing is not possible. The other antigens are still being characterized. A detailed description of individual antigens can be found elsewhere.[65]

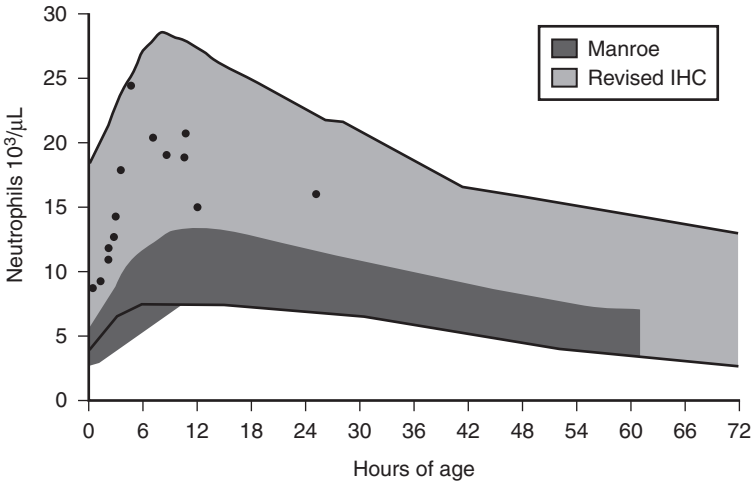


Figure 143-8 The blood neutrophil concentration and the age (hours) after birth when the complete blood count was drawn are shown by *black squares*, one for each of 14 patients admitted to the neonatal intensive care unit (NICU) for antibiotic treatment. Superimposed on these 14 black squares are the Manroe reference range (Texas) and the Intermountain Healthcare reference range (Utah). The 14 neonates were mistakenly thought to have a elevated neutrophil count and on that basis were admitted to the NICU for intravenous antibiotics. *(Modified from Lambert RM, Baer VL, Wiedmeier SE, et al: Isolated elevated blood neutrophil concentration at altitude does not require NICU admission if appropriate reference ranges are used,* J Perinatol *29:822-825, 2009.)*

TABLE 143-2

Human Neutrophil Alloantigens

Antigen Groups	Carrier Glycoproteins	Antigens	Older Names	Alleles
HNA-1	Fcγ receptor IIIb; CD16b	HNA-1a HNA-1b HNA-1c	NA1 NA2 SH	FCGR3B*01 FCGR3B*02 FCGR3B*03
HNA-2	NB1 glycoprotein; CD177	HNA-2	NB1	—
HNA-3	Unknown (GP 70-95)	HNA-3a	5b	Unknown
HNA-4	MAC-1; CR3; αMβ2 integrin; CD11b	HNA-4a	MART	ITGAM*01 (230G)
HNA-5	LFA-1; αLβ2 integrin; CD11a	HNA-5a	OND	ITGAL*01 (2372G)

Modified from Bux J: Molecular genetics of granulocyte polymorphisms, *Vox Sang* 78(Suppl 2):125, 2000.

Neutrophil-specific antigens have been identified on mature adult neutrophils, myeloid precursors, and cord blood neutrophils. The other antigens seen on the neutrophils include those of the HLA system and the red cell antigens. Class I HLA antigens (HLA-A, -B, and -C) are seen on neutrophils and their precursors in the bone marrow, although it is not known whether these are intrinsic or are passively absorbed from the plasma. Class II HLA antigens have not been described on neutrophils but are present on immature granulocytes. Red cell antigen groups expressed on neutrophils include the polylactosaminyl glycolipids bearing Ii, Le^x, and P systems. Although the antigens of the ABO systems were initially reported to be present, subsequent investigations have provided evidence to the contrary. Similarly, the MN, Rh, Kidd, Duffy, Kell, and Kx antigen systems are not present on the surface of granulocytes.

Neutrophil antigens inherited from fathers but not present in mothers may cause an antibody response during gestation. Antineutrophil antibody can cross the placenta to the fetal circulation and cause severe neutropenia in the fetus and newborn infant. A wide variety of antigenic targets, including HNA-1, HNA-2, and HNA-3, NC1, SH, SAR, LAN, LEA, CN1, and possibly HLA have been identified in ANN. In the United States, one half of all the cases of ANN are mediated by antibodies that bind to HNA-1a, -1b, or -2a antigens. Estimates of the frequency of ANN vary from 0.2% to 20%, although the incidence of granulocyte-specific antibodies is likely to be higher. Although self-limited, alloimmune neutropenia can be associated with serious infections and death.[69] A mortality rate as high as 5% has been reported. Antineutrophil antibodies may also develop as an isolated autoimmune phenomenon and may be seen even in premature neonates. The etiologic reasons for autoimmune neutropenia of infancy (AINI) are not exactly known, but associations with parvovirus B19 infection and β-lactam antibiotics have been reported. Suggested mechanisms include development of cross-reacting antibodies due to molecular mimicry, changes in endogenous antigens, enhanced HLA expression, or loss of suppression of clones of self-reacting lymphocytes. HNA-1a (NA1) autoimmunization has been linked with HLA-DR2, indicating the involvement of immune response genes. However, the occurrence of AINI in only one of monozygotic twins suggests that genetics may not be the sole responsible factor. Like ANN, these infants also present with minor infections such as otitis media, respiratory tract infection, gastroenteritis, or cellulitis, or the AINI may be diagnosed only by chance after detection of neutropenia. AINI is a self-limited disorder, with a median duration of about 30 months (range, 6 to 60 months), and 95% recover by 4 years of age. Neutropenia, in rare instances, may also be seen in newborn infants born to mothers with autoimmune neutropenia, owing to transplacental passage of her antineutrophil autoantibodies. A review of these disorders can be found elsewhere.[70]

The therapy for immune-mediated neutropenia in neonates is supportive, and a prompt clinical response can usually be obtained with the use of recombinant hematopoietic growth factors such as the granulocyte colony-stimulating factor (G-CSF). Antibiotic prophylaxis has not been shown to be effective. Exchange transfusion has been suggested as a way to lower the antibody titer, but this therapy has not been subjected to adequate testing. Steroid therapy has not been shown to be effective. These disorders are self-limited, but infection must be treated promptly.

ACCELERATING NEUTROPHIL PRODUCTION IN THE FETUS AND NEONATE

In the near-term and term rat, neutrophil production occurs at such a marked pace that significant further increases in neutrophil production might not be possible.[71,72] At 14 days gestation (term = approximately 21 days), no neutrophils whatsoever are observed in the fetal rat liver, marrow, spleen, or blood. The remarkably rapid development of neutrophils just before and after birth result in a total body sum of neutrophils of 1.2×10^6/g at birth and 5×10^6/g by 4 weeks of life.[53] To achieve this, granulocyte progenitors appear to be cycling at nearly maximal capacity.[71,72] Indeed, during the experimental infection of newborn rats, cycling of granulocyte progenitors does not appear to increase beyond that observed in the baseline, noninfected state.

In adult mice, inoculation with *Escherichia coli* results in a rapid, marked acceleration of neutrophil production.[73] This increase is preceded by an increase in G-CSF transcripts within hematopoietic and nonhematopoietic organs (particularly in the lungs). Mice who lack the G-CSF gene (because of homologous recombination knockout experiments) fail to accelerate neutrophil production during bacterial infection and have an extremely high mortality rate from infection.[74] In contrast to adult mice, newborn mice inoculated with *E. coli* fail to develop a marked increase in G-CSF transcripts in hematopoietic or nonhematopoietic organs, fail to accelerate neutrophil production, and have a high mortality rate from infection.[73] Thus the capacity of newborn mice to accelerate neutrophil production during infection appears to be limited at least in two ways: by an already rapid rate of production in the noninfected state and by lack of prompt production of G-CSF after bacterial inoculation.

It is not clear to what extent the observations on neutrophil production during infection made in rodents apply to human neonates.[75] Although certain parallels are likely, it does not appear that neutrophil production in the noninfected human fetus is maximal, and human neonates do appear capable of generating G-CSF during an infectious challenge, although perhaps not as responsively as do adults (Table 143-3). For example, during chorioamnionitis, maternal and fetal blood concentrations of G-CSF increase from their baseline low levels (generally <5 pg/mL) to 2000 to 4000 g/mL.[76] The concordance of maternal and fetal G-CSF blood concentrations during chorioamnionitis has led to the speculation that G-CSF produced in tissues of maternal origin (such as decidual macrophages) crosses from the maternal to the fetal circulation.[76] This hypothesis is supported by the finding that during chorioamnionitis, low concentrations of G-CSF messenger RNA transcripts are found in the fetal blood, but high concentrations are present in the mother's blood.[76] Stallmach and Karolyi[77] observed that during chorioamnionitis, granulocyte production increases in the fetus, in fact, expanding to sites in the liver and spleen that do not normally contain granulocytopoietic activity. Thus granulocytopoiesis in the human fetus is clearly capable of increasing above the baseline, noninfected rate. However, in neonates with sepsis-induced neutropenia, serum and urine G-CSF concentrations are significantly higher than those with low neutrophil counts from other causes. These findings suggest that the G-CSF receptors might already be saturated in neonates with sepsis-induced neutropenia and raise the possibility that recombinant G-CSF (rG-CSF) therapy may not be highly effective in these patients.[78]

If transplacental passage of G-CSF from the mother to the fetus occurs during chorioamnionitis, it is likely that rG-CSF administered before delivery would also cross from the maternal to the fetal circulation. This possibility has been tested in pregnant rat model by Medlock and colleagues[79] and Novales and colleagues.[80] They observed that a single dose (50 μg/kg) of G-CSF given to pregnant rats 1 day before delivery resulted in maternal peak G-CSF serum concentrations of 1200 ng/mL and fetal peak serum concentrations of 1.5 ng/mL. Despite the relatively low apparent transplacental passage, this concentration of G-CSF in the fetal rats constituted a significant increase above their normally undetectable baseline G-CSF concentrations. Moreover, the increase in G-CSF concentrations was sufficient to stimulate fetal neutrophil production, elevate blood neutrophil concentrations, and improve the outcome of pups that were subsequently inoculated with group B streptococci. Calhoun and associates[81] performed a human counterpart study, by administering rG-CSF (one dose of 25 μg/kg) to 11 women about to deliver an extremely preterm neonate. As in the rat model, transplacental passage of a small quantity of G-CSF was detected, and in those infants born 24 to 72 hours after the maternal treatment, neutrophilia of 5 to 7 days' duration was observed. It is not clear, however, whether human fetuses might derive benefit from antenatal rG-CSF treatment in the same manner as was shown in the pregnant rat model.

Several clinical trials have tested the hypothesis that rG-CSF administration to neonates with sepsis will alleviate neutropenia and reduce mortality. Gillan and colleagues[82] reported a dose-dependent biologic effect of rG-CSF in human neonates. They studied 42 neonates who had presumed bacterial infection during their first 3 days of life and were randomized to receive a placebo or 1.0, 5.0, 10.0, or 20.0 μg/kg body weight/day of rG-CSF for 3 consecutive days. A significant, dose-dependent increase in blood neutrophil concentrations was observed, and a dose-dependent increase in the size of the marrow neutrophil reserve was also seen. Neutrophil C3bi expression was significantly increased 24 hours after starting at least 10 μg/kg. The rG-CSF doses were well tolerated in all infants. Since then, the efficacy of rG-CSF in neonatal sepsis has also been examined by Schibler,[83] Bedford Russell,[84] Miura,[85] Barak,[86] and Kocherlakota[87] and their colleagues. In a meta-analysis based on these studies, 73 rG-CSF recipients had a lower mortality than their 82 controls.[88] However, when the nonrandomized studies were excluded, the analysis did not remain statistically adequate. Makhlouf[89] and La Gamma[90] and their co-workers have

TABLE 145-3

Peripartum Complications Found to Have a Significant Effect on Neutrophil Values

Complications	Total Infants	Normal Infants	Neutropenia	Neutrophilia
Maternal hypertension	70	12 (17%)*	53 (76%)	0
Periventricular hemorrhage	13	0	8 (62%)	3 (23%)
Asphyxia	7	0	1 (14%)	2 (10%)
Reticulocytosis	20	3 (15%)	5 (25%)	2 (10%)
Hemolytic disease	86	23 (27%)	0	40 (47%)
Asymptomatic hypoglycemia	16	0	0	7 (44%)
Intrapartum oxytocin	22	0	0	6 (27%)
Maternal fever only	48	0	0	22 (46%)
Postoperative	10	0	0	10 (100%)
Stressful labor†	21	1	0	14 (67%)
Seizures‡	7	0	0	5 (71%)
Pneumothorax	5	0	0	4 (80%)
Meconium aspiration syndrome	9	0	0	7 (78%)

*The percentage of affected infants shown in parentheses. With each complication, $p < .01$ by one-tail test.

†Included are labor ≥18 hr, midforceps rotation, breech extraction, and ≤10-minute second stage of labor.

‡This is not associated with hypoglycemia, intracranial hemorrhage, or asphyxia.

Adapted from Stockman JA, III, Graeber JE, Clark DA, et al: Anemia of prematurity: Determinants of the erythropoietin response. J Pediatr 105:786, 1984.

similarly reported successful use of rG-CSF to treat neutropenia in neonates delivered to women with pregnancy-induced hypertension. rG-CSF therapy is generally well tolerated in neonates, although reductions in platelet counts have been reported. However, it is unclear whether the thrombocytopenia is caused by rG-CSF administration or the underlying sepsis. With longer-term rG-CSF therapy, greater vigilance is needed in view of reports of leukemic transformation (described in a child with Kostmann syndrome),[91] anti-G-CSF antibodies, and osteopenia.

Recombinant GM-CSF (rGM-CSF) has been given to a limited number of human neonates. Cairo and associates[92] administered either placebo or 5.0 or 10.0 μg/kg/day of rGM-CSF to 20 neonates. Very-low-birth-weight infants (<1500 g) began treatment in their first 72 hours of life and received daily treatments for 7 days. Within 48 hours of beginning the drug, significant dose-dependent increases in circulating neutrophil and monocyte concentrations were observed. Tibial marrow aspirates revealed an increase in neutrophil reserves, and neutrophil C3bi receptor expression was increased within 24 hours of beginning treatment. Bilgin and co-workers,[93] in a prospective, randomized study, administered rGM-CSF to 30 patients for 7 consecutive days.

Twenty-five patients from the rGM-CSF group and 24 from the conventionally treated group had early-onset sepsis, and the remaining 11 patients had late-onset sepsis. The rGM-CSF-treated group showed significantly higher absolute neutrophil counts and a lower mortality rate. In a more recent multicenter randomized trial (the PROGRAMS trial), prophylactic rGM-CSF corrected neutropenia but did not reduce sepsis or improve survival and short-term outcomes in extremely preterm, small-for-gestation neonates.[94]

rGM-CSF has also been evaluated as prophylaxis against nosocomial infections in very-low-birth-weight infants. Carr and colleagues[95] administered 10 μg/kg of rGM-CSF to 75 noninfected newborn infants with less than 32 weeks gestation, initiated within the first 72 hours, for 5 days. Prophylactic GM-CSF therapy abolished neutropenia in treated infants, including those with and without sepsis, during a 4-week period after study entry. Although the rGM-CSF recipients had fewer symptomatic, blood culture–positive septic episodes than controls, the difference did not reach statistical significance. Similar results were obtained in a large, randomized, placebo-controlled trial in 264 very-low-birth-weight neonates weighing less than 1000 g. rGM-CSF was administered prophylactically at a daily dosage of 8 μg/kg for the first 7 days and then every other day for 21 days. The treated infants had higher absolute neutrophil counts, but the incidence of confirmed nosocomial infection did not differ from controls.[96]

In a systematic review of the efficacy of rG-CSF and rGM-CSF in the treatment or prevention of neonatal infections, Carr and colleagues[97] analyzed seven treatment studies of 257 infants with suspected systemic bacterial infection and three prophylaxis studies comprising 359 neonates. The addition of rG-CSF or rGM-CSF to antibiotic therapy in preterm infants with suspected systemic infection did appear to reduce immediate all-cause mortality. However, in a subgroup analysis, the 97 infants in the three treatment studies who had clinically significant neutropenia ($<1.7 \times 10^9$/L) in addition to systemic infection at trial entry showed a significant reduction in mortality by day 14 (relative risk, 0.34 [95% confidence interval (CI) 0.12, 0.92)]; rate difference, −0.18 [95% CI, −0.33, −0.03]; number needed to treat, 6 [95% CI, 3 to 33]). In these prophylaxis studies, however, rGM-CSF did not significantly reduce mortality.

The benefits and risks of recombinant myeloid growth factor therapy for conditions in the fetus and newborn infant are still being defined. Despite evidence of benefit from animal studies and promising clinical trials, the evidence currently available is insufficient to support the routine use of rG-CSF or rGM-CSF in neonatal sepsis.[98] Further studies are needed in neonatal subgroups with a particularly higher risk for sepsis because preliminary evidence suggests that such an approach may be more effective. The newer, longer-acting polyethylene glycol–conjugated or glycosylated forms of G-CSF also hold promise as viable therapeutic alternatives.

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ERYTHROCYTE KINETICS

Sites and Stages of Fetal Red Blood Cell Production

Extraembryonic Erythropoiesis

Extraembryonic erythropoiesis begins in the fetal yolk sac by 14 days gestation.[1,2] Small nests of nucleated blood cells are present in the mesenchymal and endodermal layers of the yolk sac.[3] These red blood cells, called hematocytoblasts, are the product of primitive megaloblastic erythropoiesis, and they differ from erythrocytes formed later in gestation when definitive normoblastic erythropoiesis occurs. Primitive erythroblasts are nucleated, macrocytic, and 20 to 25 μm in diameter. They are characterized by a mean cell volume (MCV) of more than 180 fL and on histopathologic examination demonstrate a characteristic fine nuclear chromatin pattern and a polychromatophilic cytoplasm containing abundant hemoglobin.[1,3] Red blood cells enter the embryonic circulation at 3 to 4 weeks gestation, coincident with joining of the vitelline and umbilical circulations.[4]

Intraembryonic Erythropoiesis

Definitive normoblastic erythropoiesis of the fetus begins in the liver during the early first trimester.[5] By 6 to 8 weeks gestation, the liver replaces the yolk sac as the primary site of red blood cell production, and by 10 to 12 weeks, extraembryonic erythropoiesis has essentially ceased. Red blood cell production occurs in the liver throughout the remainder of gestation, although production begins to diminish during the second trimester as bone marrow erythropoiesis increases (Figure 144-1). Erythroblasts are first noted in the marrow at 8 to 9 weeks gestation.[5] By the end of the third trimester, almost all erythropoiesis is taking place in the bone marrow, although residual erythropoiesis may continue in the liver, and it may be found in other sites. In rodents, the spleen also is a site of erythropoiesis before the onset of marrow red blood cell production. As with the liver, this site ceases production shortly after birth.[6] It is unclear whether the spleen contributes to erythropoiesis in the human fetus. Although erythroid precursors have been identified as early as 6 to 7 weeks gestation in human splenic tissue,[7] it is not clear whether such cells are actually developing within splenic tissue or whether they are simply part of the circulation.[8]

Ontogeny of Stem Cells

Red blood cell precursors in the yolk sac are extremely primitive cells, and they may either disappear or seed other areas in which erythropoiesis later becomes prominent. The *unicentric theory* proposes that all hematopoietic stem cells originate from the yolk sac and then migrate from one hematopoietic site to another. Moore and Metcalf[9] demonstrated circulating pluripotent stem cells in the peripheral blood just before the development of liver erythropoiesis. These workers suggested that the development of intraembryonic hematopoiesis requires an intact yolk sac, and that migration of stem cells from the yolk sac to hematopoietic tissue is necessary for the development of intraembryonic hematopoiesis. The development of other organ systems through migration of cells (such as neural crest tissue) gives credence to this theory.

The *multicentric theory* suggests that formation of a new clone of hematopoietic stem cells occurs at different sites of hematopoiesis during fetal development. Yolk sac cells are capable of producing granulocytic, megakaryocytic, and erythroid colonies when transplanted into adult irradiated recipients and in conditioned newborn recipients[9,10]; this observation supports the view that fetal stem cells possess the capacity to be pluripotent and that their differentiation is controlled by microenvironmental factors.

Marrow, Liver, and Blood Differential Counts

Table 144-1 presents the differential counts for fetal liver, marrow, and circulating blood according to gestational age.[11-14] The liver of the midtrimester human fetus is the primary organ of erythropoiesis, with myeloid-to-erythroid ratios ranging from 0.07 at 14 to 17 weeks to 0.2 at 21 to 24 weeks gestation.[11] By 24 weeks, the composition of the fetal bone marrow begins to resemble that of adult marrow, differing by the presence of a large number

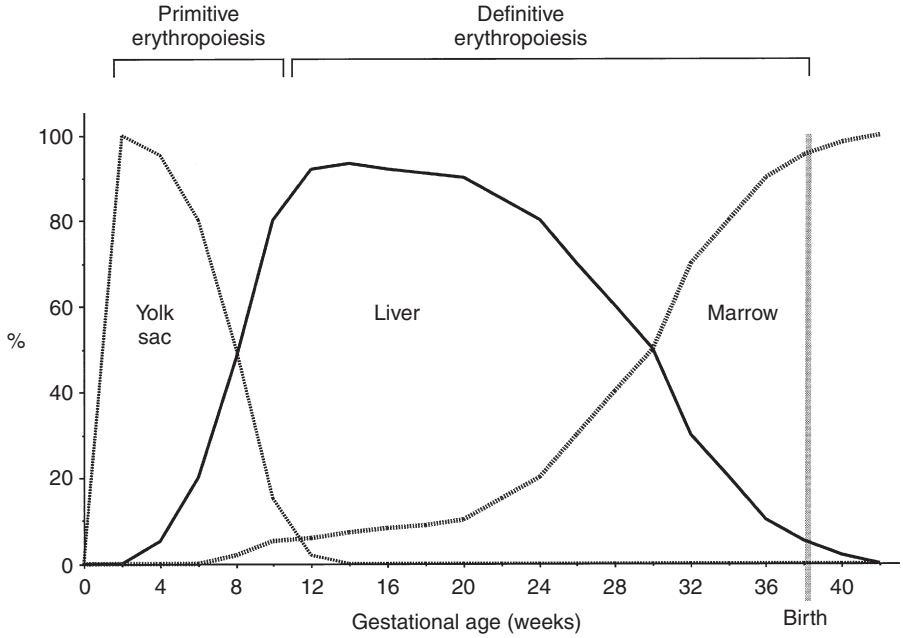


Figure 144-1 Sites and stages of fetal erythropoiesis. Primitive erythropoiesis begins in the yolk sac at 2 to 3 weeks after conception. By 12 weeks gestation, the liver has become the primary erythroid organ. The liver is the primary source of red blood cells from 12 to 24 weeks gestation, and the bone marrow is the primary source of red blood cells after 24 weeks gestation.

of stromal elements, the absence of plasma cells and lymph follicles, and an overall increased cellularity in the fetal marrow. Unlike in adult marrow, large fat cells are not present in fetal bone marrow. Between 18 and 22 weeks gestation, the mitotic index of the fetal bone marrow becomes virtually identical to that of adult bone marrow. The fetal bone marrow myeloid-erythroid ratio starts to exceed the normal adult bone marrow ratio (1.5 ± 0.4) early in midgestation and remains elevated even at the time of birth.

Progenitor Cell Concentrations

Studies of bone marrow cells in tissue culture have identified specific committed red blood cell precursors, termed *erythroid progenitors,*[15-18] on the basis of their characteristic growth in vitro. When bone marrow cells are placed in semisolid media culture systems for 5 to 7 days, an erythropoietin (Epo)-sensitive erythroid progenitor cell, termed a *colony-forming unit–erythroid* (CFU-E), clonally matures into a single cluster containing 30 to 100 normoblasts (Figure 144-2). An erythroid-specific progenitor that is less well differentiated than a CFU-E (and therefore a more primitive cell) is termed a *burst-forming unit–erythroid* (BFU-E). At 12 to 14 days after bone marrow cells are placed in culture, a BFU-E develops into a large, multicentered colony of normoblasts, in which each center contains 200 to 10,000 normoblasts. Finally, the most primitive erythroid progenitor cell identifiable through in vitro culture is termed a *colony-forming unit–granulocyte, erythrocyte, macrophage, megakaryocyte* (CFU-GEMM, or CFU-mix). As with the BFU-E, at 12 to 14 days after marrow cells are placed in culture, this multipotent progenitor develops into a mixed colony of both normoblast clusters and granulocyte-macrophage clusters.

The ability of an organ to produce red blood cells is based on the number of progenitor cells it contains, as well as the growth factors stimulating those cells to proliferate. Determination of erythroid progenitor numbers obtained from cell suspensions of liver, marrow, spleen, and blood of midtrimester human fetuses (Table 144-2) showed twice the number of multipotent progenitors and erythroid progenitors in the fetal liver as in marrow.[8,11,12] Erythroid progenitors from fetal liver also appeared to be more sensitive to Epo (the primary erythroid growth factor) than those from fetal marrow. Progenitor cell concentrations in the spleen were nearly identical to concentrations in the circulation.[8,12] Whether the progenitors isolated from liver and from marrow represent different subpopulations of progenitors (e.g., populations that express different erythroid growth factor receptors, different receptor numbers, different receptor affinity, or different cycling rates) remains to be determined.

Regulation of Erythropoiesis

Erythropoietic regulation in the human fetus differs markedly from that in the adult. In the adult, erythropoietic regulation primarily involves maintaining the red blood cell mass. By contrast, constant and dramatic changes characterize erythropoiesis in the embryo and fetus. The astonishing rate of somatic growth and the resultant need for continual increase in the fetal red blood cell mass necessitate an extraordinary erythropoietic effort. Moreover, as a consequence of its relatively low oxygen tensions and high metabolic rates, fetal tissue requires a system of oxygen delivery that differs significantly from the adult system.

Erythroid Growth Factors

The production of erythrocytes, from pluripotent stem cell to mature red blood cell, is governed by various growth factors. These erythropoietic growth factors are produced by accessory cells such as liver macrophages and marrow stromal cells, and they stimulate maturation, growth, and differentiation at various stages of red blood cell production. The progenitors involved in red blood cell production and the factors that stimulate their cellular maturation are depicted in Figure 144-2. Although these growth factors all facilitate production of red blood cells, none plays a more important regulatory role than that of Epo. Epo is a 30- to 39-kDa glycoprotein that binds to specific receptors on the surface of erythroid precursors and

TABLE 144-1

Differential Counts in Liver, Marrow, and Blood During Gestation

A. DIFFERENTIAL COUNTS FOR LIVER CELL SUSPENSIONS FROM FETUSES AT 14 TO 24 WEEKS GESTATION

	% ± SD		
Cellular Element	**14-17 Weeks (*n* = 5)**	**18-20 Weeks (*n* = 7)**	**21-24 Weeks (*n* = 8)**
Normoblast			
Pronormoblast	3.1 ± 1.4	3.4 ± 1.0	2.9 ± 1.8
Basophilic	18.4 ± 7.6	13.7 ± 1.6	13.7 ± 4.7
Polychromatophilic	57.5 ± 12.6	55.3 ± 7.5	51.1 ± 5.5
Orthochromic	13.9 ± 5.2	15.9 ± 3.7	14.2 ± 5.0
Total erythroid	9.3 ± 6.3	87.8 ± 4.8	81.9 ± 5.0
Neutrophil			
Promyelocyte	0 ± 0	0.2 ± 0.2*	1.2 ± 0.4*†
Myelocyte	0 ± 0	0 ± 0	0.2 ± 0.2*†
Metamyelocyte	0 ± 0	0 ± 0	0 ± 0
Band	0 ± 0	0 ± 0	0 ± 0
Segmented	0 ± 0	0 ± 0	0 ± 0
Total neutrophils	0 ± 0	0.2 ± 0.2	1.4 ± 0.5*†
Undifferentiated blast	0.5 ± 0.6	3.1 ± 1.8*	2.2 ± 0.7*
Macrophage	0.5 ± 0.6	1.2 ± 4.0*	1.3 ± 5.0
Lymphocyte	5.4 ± 2.6	3.9 ± 3.6	11.3 ± 4.2*†
Eosinophil	0 ± 0	0 ± 0	0 ± 0
Other‡	0.8 ± 0.8	3.4 ± 2.4	1.9 ± 2.4

*$P < .05$ versus 14-17 weeks.
†$P < .05$ versus 18-20 weeks.
‡Hepatocyte, megakaryocyte, or cell of undetermined origin.

B. DIFFERENTIAL COUNTS FOR MARROW CELL SUSPENSIONS FROM FETUSES AT 14 TO 24 WEEKS GESTATION

	% ± SD		
Cellular Element	**14-17 Weeks (*n* = 6)**	**18-20 Weeks (*n* = 6)**	**21-24 Weeks (*n* = 8)**
Normoblast			
Pronormoblast	0.3 ± 0.2*	1.1 ± 0.4*	0.5 ± 0.2*
Basophilic	1.4 ± 0.6*	3.1 ± 0.8*	1.7 ± 1.0*
Polychromatophilic	9.2 ± 5.0*	12.9 ± 6.2*	12.3 ± 5.2*
Orthochromic	12.1 ± 11.1	20.7 ± 12.2	8.1 ± 4.4
Total erythroid	23.0 ± 13.3*	37.8 ± 16.8*	22.6 ± 9.0*
Neutrophil			
Promyelocyte	5.9 ± 2.9*	5.8 ± 2.6*	5.0 ± 1.7*
Myelocyte	2.7 ± 1.4*	3.4 ± 1.6*	2.1 ± 1.0*
Metamyelocyte	2.1 ± 1.0*	2.5 ± 1.8*	1.7 ± 1.0*
Band	3.1 ± 1.8*	3.2 ± 2.7*	1.6 ± 1.2*
Segmented	1.2 ± 1.3*	1.1 ± 1.3*	0.4 ± 0.2*
Total neutrophils	13.8 ± 6.1*	16.1 ± 8.0*	10.7 ± 5.0*
Undifferentiated blast	8.9 ± 12.4*	11.4 ± 5.4*	11.6 ± 3.6*
Macrophage	5.4 ± 3.8*	4.2 ± 2.4*	3.1 ± 1.6*
Lymphocyte	35.3 ± 15.8*	28.8 ± 8.0*	50.3 ± 6.8*
Eosinophil	0.5 ± 0.6	1.1 ± 1.2	1.2 ± 0.8*
Other†	0.8 ± 0.4	0.5 ± 0.4	0.4 ± 0.2

*$P < .05$ versus fetal liver.
†Megakaryocyte or cell of undetermined origin.

C. DIFFERENTIAL COUNTS FOR CORD BLOOD SAMPLES FROM FETUSES AT 18 TO 29 WEEKS GESTATION

	% ± SD		
Cellular Element	**18-21 Weeks (*n* = 186)**	**22-25 Weeks (*n* = 230)**	**26-29 Weeks (*n* = 144)**
Normoblast (% WBCs)	45.0 ± 86.0	21.0 ± 23.0	21.0 ± 67.0
Basophilic	0.5 ± 1.0	0.5 ± 1.0	0.5 ± 1.0
Neutrophil	6.0 ± 4.0	6.5 ± 3.5	8.5 ± 4.0
Lymphocyte	88.0 ± 7.0	87.0 ± 6.0	85.0 ± 6.0
Eosinophil	2.0 ± 3.0	3.0 ± 3.0	4.0 ± 3.0
Monocyte	3.5 ± 2.0	3.0 ± 2.5	3.0 ± 2.5

WBCs, White blood cells.

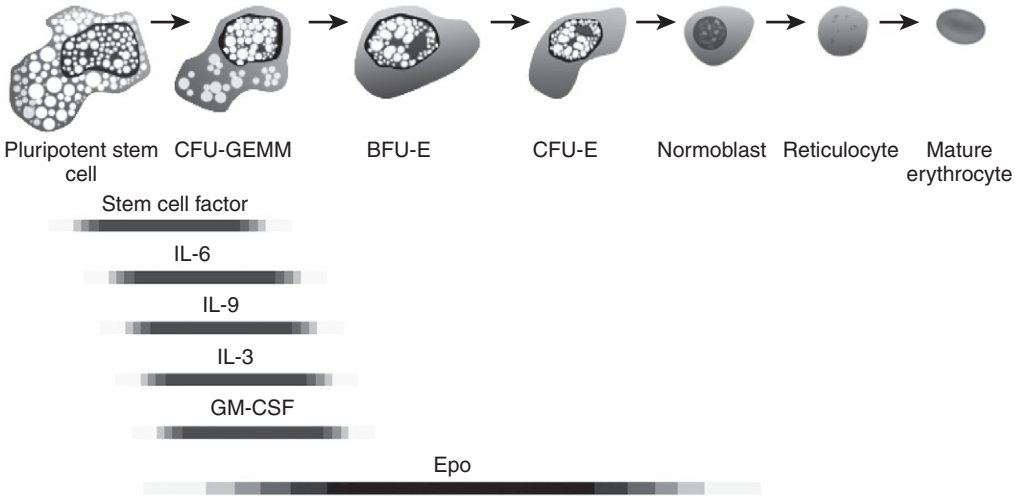


Figure 144-2 Erythropoietic progenitors and the growth factors influencing erythropoiesis. *BFU-E,* Erythroid burst-forming unit; *CFU-E,* erythroid colony-forming unit; *CFU-GEMM,* colony-forming unit–granulocyte, erythrocyte, macrophage, and megakaryocyte; *Epo,* erythropoietin; *GM-CSF,* granulocyte-macrophage colony-stimulating factor; *IL-3,* interleukin-3; *IL-6,* interleukin-6; *IL-9,* interleukin-9.

TABLE 144-2

Progenitor Cell Concentrations per 5×10^3 Plated Cells

Source	CFU-Mix	BFU-E
Liver	12.7 ± 2.1	20.7 ± 3.1
Marrow	6.7 ± 1.4*	9.3 ± 2.7*
Spleen	4.2 ± 2.7*	5.9 ± 3.7*
Blood	—	8.0 ± 5.4*

*P <.05 versus liver.

BFU-E, Burst-forming unit–erythroid; *CFU-mix,* colony-forming unit–granulocyte, erythrocyte, macrophage, megakaryocyte.

stimulates their differentiation and clonal maturation into mature erythrocytes.[19,20] The gene for Epo is located on chromosomal region 7q21-22.[21] In the human fetus, Epo is produced principally by cells of monocyte-macrophage origin residing in the liver. Postnatally, Epo is produced almost exclusively by peritubular cells of the kidney.

Other growth factors besides Epo play a role in the differentiation and clonal expansion of erythroid progenitors. These include granulocyte-macrophage colony-stimulating factor (GM-CSF)[15]; stem cell factor (SCF) (also known as c-Kit ligand)[22]; and the interleukins IL-3, IL-6, and IL-9.[23-25] Thrombopoietin, a growth factor involved in platelet production whose receptor is similar in structure to the Epo receptor, also stimulates erythroid colony formation.[26] IL-3 and GM-CSF were the first growth factors noted to have erythroid-stimulating properties, initially described as "burst-promoting activity." In combination with Epo, these factors synergistically stimulate differentiation and proliferation of BFUs-E and CFUs-GEMM.

SCF is a multipotent growth factor that, in combination with other factors, supports clonal maturation of hematopoietic progenitors. Murine studies indicate that SCF may be expressed during embryogenesis, thereby affecting embryonic and fetal erythropoiesis. In vitro studies show that SCF alone stimulates clonal maturation of fetal (but not adult) multipotent progenitors. Erythroid progenitors isolated from term cord blood are more responsive to SCF, alone and in synergism with GM-CSF and IL-3, than adult marrow progenitors.[22]

Term circulating erythroid progenitors also are more sensitive to IL-6 and IL-9 than are adult progenitors.[24] IL-6 is a multifunctional, 22- to 26-kDa glycoprotein cytokine involved in B cell stimulation and immunoglobulin production, acute phase reactions, and induction of hematopoietic progenitors from a noncycling (G_0) phase into an active cycling (S) phase. IL-6 alone supports clonogenic maturation of newborn cord blood BFUs-E and CFUs-GEMM and induces progenitor cell cycling.[24] IL-9 also is a multipotent cytokine and is similar to IL-6 in its ability to stimulate primitive erythroid progenitors and multipotent progenitors isolated from cord blood.[25] Insulin-like growth factor-I (IGF-I) has been shown to cause clonal expansion of erythroid progenitors isolated from adult marrow,[27,28] but its effects on fetal progenitors are still being determined.

Unique Features of Fetal Progenitors

Fetal erythroid progenitors respond in a slightly different fashion from that observed for adult erythroid progenitors. In addition to the features noted earlier, fetal progenitors appear to be more sensitive than adult erythroid progenitors to Epo.[29] Specifically, BFUs-E of fetal origin develop more rapidly into erythroid colonies, and the colonies generally contain significantly more normoblasts. In addition, BFUs-E from adult bone marrow require a combination of Epo plus another factor, such as IL-3 or GM-CSF, to mature clonally; however, many fetal BFUs-E mature in the presence of Epo alone.[30] Studies have shown that fetal clones produce GM-CSF and IL-3, which may explain their unique capability for growth factor independence and autostimulation.[31]

Control of Erythropoietin Production

Erythropoiesis in utero is controlled by erythroid growth factors produced by the fetus, not the mother. Epo is the primary regulator of erythropoiesis in adults and appears to be the controlling factor for fetal erythropoiesis, especially during late gestation. Epo does not cross the placenta in humans,[32,33] monkeys, or sheep,[34] although it has been reported to do so in mice.[35] In the mouse, suppression of maternal erythropoiesis by hypertransfusion does not suppress fetal erythropoiesis.[36] In humans, stimulation of maternal Epo production does not result in stimulation of fetal red cell production.[37]

The exact mechanism regulating the expression of Epo is unknown, but it is thought to be controlled by an oxygen-sensing mechanism in the liver and kidney.[38] Both hypoxia and anemia stimulate erythropoiesis by stimulating messenger RNA

TABLE 144-3

Red Blood Cell Indices During Gestation

Weeks Gestation	WBCs* ($\times 10^9$/L)	Total WBC Count ($\times 10^9$/L)	Platelets ($\times 10^9$/L)	RBCs ($\times 10^{12}$/L)	Hemoglobin (g/dL)	Hematocrit (%)	Mean Corpuscular Volume (fL)
18-21 (n = 760)	4.68 ± 2.96	2.57 ± 0.42	234 ± 57	2.85 ± 0.36	11.69 ± 1.27	37.3 ± 4.3	131.1 ± 11.0
22-25 (n = 1200)	4.72 ± 2.82	3.73 ± 2.17	247 ± 59	3.09 ± 0.34	12.2 ± 1.6	38.6 ± 3.9	125.1 ± 7.8
26-29 (n = 460)	5.16 ± 2.53	4.08 ± 0.84	242 ± 69	3.46 ± 0.41	12.91 ± 1.38	40.9 ± 4.4	118.5 ± 8.0
>30 (n = 440)	7.71 ± 4.99	6.40 ± 2.99	232 ± 87	3.82 ± 0.64	13.64 ± 2.21	43.6 ± 7.2	114.4 ± 9.3

From Forestier F, et al: Developmental hematopoiesis in normal human fetal blood, *Blood* 77:2360, 1991.
*Including normoblasts.
RBCs, Red blood cells; *WBCs,* white blood cells.

(mRNA) transcription and Epo protein production.[39] Promoter and enhancer elements within the Epo gene are responsive to hypoxia, as well as to cobalt exposure in vitro.[40] In animal models, the liver-sensing mechanism has a decreased sensitivity to hypoxia, producing one-tenth the amount of Epo in response to comparable stimuli in the kidney.[41,42] The liver also appears to require more prolonged hypoxia to achieve an Epo response.[43,44] Other hormones or factors have been shown to enhance Epo production or its effects, either in vivo or in vitro.[45,46] These factors include testosterone,[47] estrogen,[48] thyroid hormone,[49] prostaglandins,[50] vitamin E,[51] and lipoproteins.[52]

It is not known what factors regulate the switch of Epo production from the liver to the kidney. Renal production of Epo is not necessary for normal fetal erythropoiesis.[53] The lack of renal contribution to Epo production is illustrated by the normal serum Epo concentrations and normal hematocrits in anephric fetuses.[54] In the sheep, Epo production in both the liver and the kidney is highest at 60 days gestation (term being 140 days in this species).[55] Epo production in the fetal liver decreases by 90 days gestation; however, increased renal production of Epo continues until 130 days gestation, when production falls to levels seen in adult sheep.

Studies in human fetal and neonatal kidney obtained from postmortem specimens also report measurable quantities of Epo mRNA,[56] and quantitative mRNA studies in 10 to 24 weeks gestation human fetuses reveal that the fetal kidney produces approximately 5% of the amount of Epo mRNA that the fetal liver produces during the second trimester.[57] Thus it appears that regulation of Epo gene transcription differs between liver and kidney in utero.

Mechanisms by which gene expression can be differentially regulated are through methylation of promoter and enhancer regions of a gene, and through deacetylation of histones.[58-62] Increased methylation generally results in decreased gene expression, while histone deacetylation causes relaxation of DNA structures and allows gene expression to occur.These two mechanisms may also be involved in developmental Epo gene expression, in that methylation of the enhancer region (leading to decreased expression) of the Epo gene in developing human kidney during the second trimester was much greater than in the liver.[63]

Epo levels have been measured in cord blood during the third trimester, and these levels gradually increase throughout later development.[64-66] From Epo measurements made in cord blood from infants of laboring and nonlaboring mothers[67] and from infants undergoing labor stress,[68-72] it seems that individual cord Epo levels primarily reflect hypoxic stress during labor and delivery. Serum Epo concentrations at birth normally range from 5 to 100 mU/mL. For comparison, serum Epo concentrations in anemic, nonuremic adults may be as high as 300 to 400 mU/mL.[73]

Red Blood Cell Indices in the Fetus and Neonate

Red Blood Cell Concentrations and Hematocrit

Red blood cell indices change during gestation and continue to change through the first year of life. Circulating red blood cell concentrations gradually increase during the second trimester, from 2.85 ± 0.36 × 10^6/μL at 18 to 21 weeks to 3.82 ± 0.64 × 10^6/μL at 30 weeks[12] (Table 144-3). At term, circulating red blood cell concentrations range from 5.0 to 5.5 × 10^6/μL.[74] In parallel with increasing red blood cell concentrations, hematocrit values increase from 30 to 40% during the second trimester and continue to increase to term values over the latter part of the third trimester. Term hematocrit values range from 50% to 63%, with some variability noted because of delayed clamping of the umbilical cord.[75] Values also are dependent on the sampling site. Capillary hemoglobin concentrations measure as much as 3.5 g/dL higher than venous samples.[76]

Hemoglobin

The hemoglobin concentration gradually rises during gestation.[77] At 10 weeks gestation, the average hemoglobin concentration is approximately 9 g/dL.[78] By 22 to 24 weeks, fetal hemoglobin values reach 11 to 12 g/dL, and by 30 weeks, the hemoglobin concentrations are 13 to 14 g/dL.[12] Premature male infants reach term cord hemoglobin values earlier than premature female infants,[79] possibly because of the erythropoietic effects of testosterone.[47]

Hemoglobin concentrations are relatively constant over the last 6 to 8 weeks gestation, and at term the average hemoglobin concentration is approximately 16 to 17 g/dL.[80-82] At birth, a 1- to 2-g/dL rise in hemoglobin may result from transfusion of placental blood at delivery.[75,83] An increase in hemoglobin by 2 hours of postnatal life occurs in most infants, resulting from a decrease in plasma volume. By 8 to 12 hours of life, the hemoglobin concentration achieves a relatively constant level. Red blood cell production decreases significantly at birth, so by the end of the first postnatal week, hemoglobin concentrations gradually decline.[75] The decrease in red cell production after birth is predominantly the result of the increased availability of oxygen in the extrauterine environment, which greatly reduces Epo production and endogenous erythropoiesis. The continued fall in hemoglobin concentration over the next several weeks results from (1) decreased red blood cell production, (2) a shortened red blood cell life span of the fetal erythrocyte, and (3) plasma dilution and an increase in blood volume related to growth. The nadir of hemoglobin concentration in full-term infants is seen at approximately 8 weeks, with an average hemoglobin concentration of 11.2 g/dL.[74,78] This value gradually rises, so that by 6 months, the average term infant has a hemoglobin concentration of 12.1 g/dL. Altitude may have a modest effect on the postnatal changes in hemoglobin concentration. Infants living at an elevation of 1600 m had higher hemoglobin concentrations (by 0.4 g/dL) by 6 months of age than infants living at sea level.[84,85]

The average decline in hemoglobin concentration in preterm infants weighing less than 1500 g is remarkably different from that in term infants. This is partly because of phlebotomy losses that invariably occur in preterm infants, as well as the effects of transfusions on endogenous erythropoiesis. Such infants reach a nadir of hemoglobin, which averages 8 g/dL at 4 to 8 weeks of age.[86] Tables 144-4 and 144-5 demonstrate relationships among

TABLE 144-4

Expected Hemoglobin Values for Postnatal Age in Low-Birth-Weight Infants

	HEMOGLOBIN LEVEL (g/dL)				
Birth Weight (g)	2 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks
800-1000	16.0 (14.8-17.2)	10.0 (6.8-13.2)	8.7 (7.0-10.2)	8.0 (7.1-9.8)	8.0 (6.9-10.2)
1001-1200	16.4 (14.1-18.7)	12.8 (7.8-15.3)	10.5 (7.2-12.3)	9.1 (7.8-10.4)	8.5 (7.0-10.0)
1201-1400	16.2 (13.6-18.8)	13.4 (8.8-16.2)	10.9 (8.5-13.3)	9.9 (8.0-11.8)	9.8 (8.4-11.3)
1401-1500	15.6 (13.4-17.8)	11.7 (9.7-13.7)	10.5 (9.1-11.9)	9.8 (8.4-12.0)	9.9 (8.4-11.4)
1501-2000	15.6 (13.5-17.7)	11.0 (9.6-14.0)	9.6 (8.8-11.5)	9.8 (8.4-12.1)	10.1 (8.6-11.8)

From Williams ML, et al: Role of dietary iron and fat in vitamin E deficiency anemia of infancy, *N Engl J Med* 292:887, 1975. Reprinted by permission of *The New England Journal of Medicine*.

TABLE 144-5

Red Blood Cell Indices in Term Infants: Postnatal Changes With Age*

	REPORTED VALUE											
	DAYS			WEEKS			MONTHS					
Feature	Birth†	1	3	1	2	4	2	3	4	6	9	12
Hemoglobin (g/dL)	16.5 (13.0)	18.5 (14.5)	18.6 (16.5)	17.5 (13.5)	16.6 (13.4)	13.9 (10.7)	11.2 (9.4)	11.5 (9.5)	12.2 (10.3)	12.6 (11.1)	12.7 (11.4)	12.7 (11.3)
Hematocrit (%)	51 (42)	56 (45)	55 (42)	54 (41)	53 (33)	44 (28)	35 (29)	35 (32)	38 (31)	36 (32)	36 (33)	37
Red blood cells ($\times 10^{12}$/L)	4.7 (3.9)	5.3 (4.0)	5.6 (3.9)	5.1 (3.9)	4.9 (3.3)	4.3 (3.1)	3.7 (3.1)	3.8 (3.5)	4.3 (3.9)	4.7 (4.0)	4.7 (4.1)	4.7
Mean corpuscular volume (fL)	108 (98)	108 (95)	110 (104)	107 (88)	105 (88)	101 (91)	95 (84)	91 (74)	87 (76)	76 (68)	78 (70)	78 (71)
MCH (pg)	34 (31)	34 (31)	36.7	34 (28)	33.6 (30.0)	32.5 (29)	30.4 (27)	30 (25)	28.6 (25)	26.8 (24)	27.3 (25)	26.8 (24)
MCHC (g/dL)	33 (30)	33 (29)	33.1	33 (28)	31.4 (28.1)	31.8 (28.1)	31.8 (28.3)	33 (30)	32.7 (28.8)	35 (32.7)	34.9 (32.4)	34.3 (32.1)

Data from Saarinen UM, Siimes MA: Developmental changes in red blood cell counts and indices of infants after exclusion of iron deficiency by laboratory criteria and continuous iron supplementation, *J Pediatr* 92:412, 1978; and Dallman PR: Blood and blood forming tissues. In Rudolph A, editor: *Pediatrics,* ed 16, New York, 1977, Appleton-Century-Crofts, p 1111.

*Values represent means (values in parentheses are ±2 SD).

†Measured in cord blood.

MCH, Mean corpuscular hemoglobin; *MCHC,* MCH concentration.

birth weight, chronologic age, and red blood cell indices in term and preterm infants.[74,78,85-89]

These data do not always apply to infants born small for gestational age, in whom placental insufficiency and secondary polycythemia are common.[83,90] Infants of diabetic mothers, infants of smoking mothers, and infants born at higher altitudes also tend to have higher hemoglobin concentrations at birth.[84,91,92] In growth-restricted infants born to hypertensive mothers, the blood supply to the placenta and the capacity to deliver oxygen to the fetus both are diminished. Accelerated erythropoiesis is thought to be part of a compensating mechanism designed to raise oxygen-carrying capacity to maintain an adequate supply to the fetus. In infants of diabetic mothers, increased metabolic demands of the fetus (resulting from increased glucose availability) may account for the higher fetal oxygen needs and the compensatory increase in hemoglobin concentration by the fetus. The increased red blood cell mass in infants of diabetic mothers is not thought to result from higher maternal levels of hemoglobin A_{1c} (a high-affinity hemoglobin capable of decreasing the oxygen transferred to the fetus). In smoking mothers, the increase in fetal carbon monoxide and the subsequent decrease in oxygen available from hemoglobin constitute the likely cause of the compensatory increase in hemoglobin levels in the fetus.

Mean Cell Volume

The size of the red blood cell gradually decreases during development. The MCV is more than 180 fL in the embryo, falls to 130 fL by midgestation, and decreases to 115 fL by the end of pregnancy. By 1 year of age, the MCV reaches an average of 82 fL.[88] Similar to cells of other organs in the infant born prematurely, the MCV of preterm infants declines quickly after birth, and the postnatal changes in MCV appear to be related to chronologic age rather than postconceptual age.[87] The mean corpuscular hemoglobin concentrations remain relatively constant, and the value for mean corpuscular hemoglobin decreases slightly.[92]

Blood Volume

The placenta and umbilical cord contain 75 to 125 mL of blood at term, or approximately one fourth to one third of the fetal blood volume.[74] Umbilical arteries constrict shortly after birth, but the umbilical vein remains dilated, and blood flows in the direction of gravity. Infants held below the level of the placenta can receive half of the placental blood volume (30 to 50 mL) in 1 minute. Conversely, infants held above the placenta can lose 20 to 30 mL of blood back into the placenta in 1 minute.[93] The blood volume of infants with early cord clamping averages 72 mL/kg, and the volume of infants with delayed cord clamping averages 93 mL/kg. Preterm infants have slightly larger blood volumes (89 to 105 mL/kg), owing to an increased plasma volume. By the age of 1 month, blood volumes in term infants average 73 to 77 mL/kg.

Fetomaternal Red Blood Cell Transfer

Maternal and fetal circulating cells may, at various times, cross the placental barrier. Fetal contamination of the maternal circulation can occur before delivery, as evidenced by studies of maternal blood group immunization. Approximately 50% to 75%

of pregnancies are associated with some degree of fetomaternal transfer of blood. This event is uncommon in the first trimester (3%). Volumes of fetal transplacental transfer are relatively small, usually on the order of 0.01 to 0.1 mL, but on occasion they may be much greater. Approximately 1 pregnancy in 400 is associated with fetal transplacental bleeding of 30 mL or greater, and 1 pregnancy in 2000 is associated with a potential fetal transplacental hemorrhage of 100 mL or more.[94] The overall risk for occurrence of Rh immunization in an Rh-incompatible pregnancy is 16% if the fetus is Rh-positive and ABO-compatible with its mother. This risk is 1.5% if the fetus is Rh-positive and ABO-incompatible. Fetal transfer of cells to the mother occurs during abortions as well (an approximately 2% incidence of such transfer with spontaneous abortion and a 4%+ to 5% rate if induced).[95] Because fetal hemoglobin is resistant to acid elution, cells containing fetal hemoglobin can be distinguished from cells containing hemoglobin A. The Kleihauer-Betke stain of peripheral maternal blood uses this characteristic of fetal hemoglobin to detect fetal cells in the maternal circulation,[96] although results with blood smears from mothers with diseases that result in increased fetal hemoglobin synthesis (such as maternal sickle cell disease, maternal thalassemia, and maternal hereditary persistence of fetal hemoglobin) are not reliable. Diagnosis of fetomaternal hemorrhage also may be missed when both mother and infant are ABO-incompatible. In such instances, the fetal cells are rapidly cleared from the maternal circulation by maternal anti-A or anti-B antibodies.

ERYTHROCYTE BIOCHEMISTRY

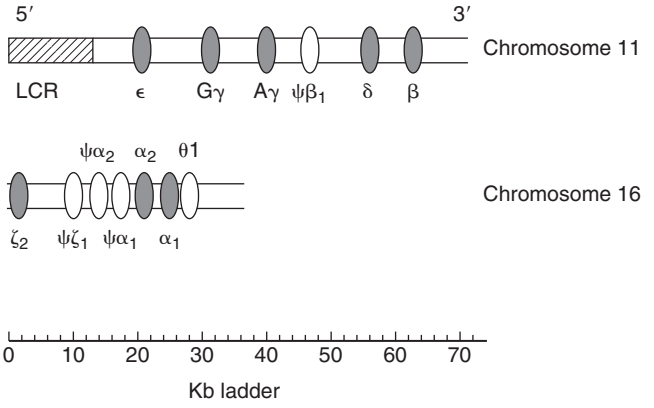
Hemoglobin Synthesis

During fetal erythropoiesis, an orderly evolution of the production of different hemoglobins occurs. Eight globin genes direct the synthesis of six different polypeptide chains, designated α, β, γ, δ, ε, and ζ. These globin chains combine in the developing erythroblast to form seven different hemoglobin tetramers: hemoglobin Gower 1 (ζ_2-ε_2), hemoglobin Gower 2 (α_2-ε_2), hemoglobin Portland (ζ_2-γ_2), fetal hemoglobin (α_2-γ_2), and two types of adult hemoglobin (α_2-β_2), known as hemoglobin A_1, and α_2-δ_2, known as hemoglobin A_2.

Globin Genes

The globin genes are organized into two clusters (Figure 144-3). The α-like genes are located along a 20-kB distal segment of the short arm of chromosome 16. The cluster contains three functional genes (α_1, α_2, and ζ_2), three pseudogenes (evolutionary remnants of genes that are not expressed because of inactivating mutations that prevent production of a functional globin protein), and one gene of undetermined function (a globin-like gene without inactivating mutations). The β-like gene cluster is located along a 60-kB segment of the short arm of chromosome 11, and it contains five functional genes—β, δ, $^{A}\gamma$, $^{G}\gamma$, and ε—and one pseudogene. Within each complex, the genes are all in the same 5′-3′ orientation, and they are arranged in the order in which they are expressed during development.[97]

The α genes are duplicated in humans. Each α gene is approximately 4 kB long, interrupted by two small nonhomologous regions. Within each gene are intervening sequences (introns) that do not code for any protein structure, although the entire gene is transcribed into mRNA. The exons and first introns of the two α-globin genes have identical sequences, but the second intron of α_1 is nine bases longer and differs by three bases from that of the α_2 gene. Despite the high degree of similarity or *homology* between these two genes, the sequences diverge in the 3′ untranslated regions, 13 bases beyond the TAA stop codon. Because of these sequence differences, the relative output of the two genes can be measured. α_2 mRNA is produced two to three times more abundantly than α_1 in fetal liver and marrow and in fetal and adult erythrocytes.[97]



Embryonic:	Hemoglobin Gower 1	$\zeta_2\varepsilon_2$
	Hemoglobin Gower 2	$\alpha_2\varepsilon_2$
	Hemoglobin Portland	$\zeta_2\gamma_2$
Fetal:	Hemoglobin F	$\alpha_2\gamma_2$
Adult:	Hemoglobin A	$\alpha_2\beta_2$
	Hemoglobin A_2	$\alpha_2\delta_2$

Figure 144-3 Organization of the globin genes. The *bottom line* reflects the scale in kilobases. Transcription of mRNA takes place from the 5′ to the 3′ end, and for both chromosomes the genes are arranged in order of their developmental activation. The *upper segment* represents the beta-like globin genes on the short arm of chromosome 11, and the *lower segment,* the alpha-like genes on the distal short arm of chromosome 16. Regions of the gene that code for primary globin proteins are shown as *shaded ovals,* and regions that code for pseudogenes (ψ-non-expressed remnants possessing a number of inactivating mutations that prevent transcription and translation into functional globin protein) are shown as *open ovals.* θ1 is a globin–like gene without inactivating mutations. The locus control region *(LCR)* is shown as a *hatched segment.* The composition of embryonic, fetal, and adult hemoglobins also is provided.

The ζ gene appears to be critical to normal fetal development. In a murine model, both α and ζ gene expression can be measured from the onset of erythropoiesis in the yolk sac.[98] In addition, expression of both genes occurs concomitantly within the same cells. Expression of ζ gene occurs predominantly through the first 6 to 8 weeks of gestation, although minute quantities of ζ-globin can be measured in fetal and neonatal red cells.[99]

Like the α_1 and α_2 genes, the $^{G}\gamma$ and $^{A}\gamma$ genes appear to be virtually identical over a span of 1.5 kb, suggesting a mechanism for gene matching during evolution. Amino acid sequencing of normal cord blood has shown that either glycine ($^{G}\gamma$) or alanine ($^{A}\gamma$) is present at the 136 position in the γ-chain.[100] Short-chain organic acids, such as butyrate and acetate, increase γ gene promoter activity. This activity can be enhanced even further by incorporation of sections of the β gene locus control region.[101,102]

The β-globin gene cluster occupies a region of approximately 17 kb on the short arm of chromosome 11.[103] Each of its constituent genes, their flanking regions, and large stretches of the regions between them have been sequenced. Studies suggest that the transcription factor NF-E1 (nuclear factor–erythroid 1) regulates increased β-globin gene expression during erythroid maturation. Deletion of the NF-E1 binding site in the upstream

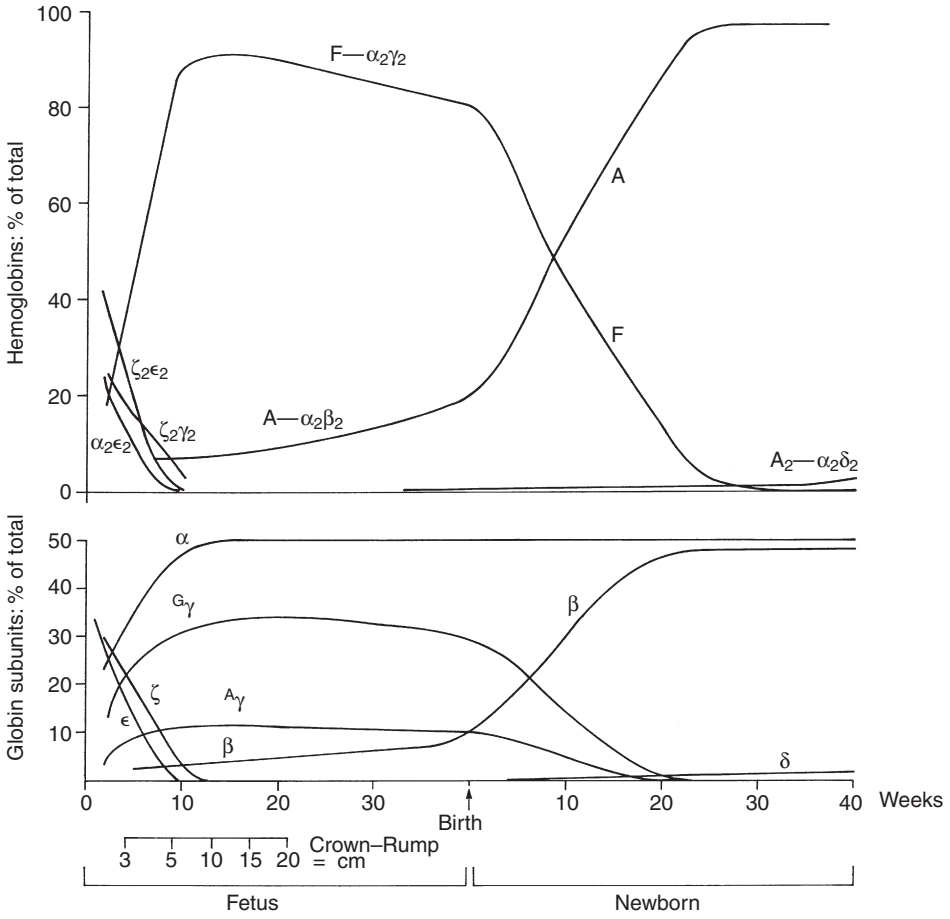


Figure 144-4 Changes in hemoglobin tetramers *(top)* and in globin subunits *(bottom)* during human development from embryo to early infancy. *(From Bunn HF, Forget BG:* Hemoglobin: Molecular, Genetic and Clinical Aspects, *Philadelphia, 1986, WB Saunders, p 68.)*

segment of the β-globin promoter blocks induction of transcription in murine leukemia cells.[104]

δ-Globin gene expression occurs early at the erythroid progenitor stage. By the reticulocyte stage, no δ-globin mRNA synthesis can be detected. The δ-globin gene promoter functions at a much lower level than the β-globin gene promoter, resulting in significantly lower levels of δ-globin mRNA production. Evidence suggests the presence of a globin promoter–specific silencer element located upstream of the δ-globin gene.[105] Moreover, δ-globin mRNA is less stable than β-globin mRNA, which probably accounts for the early cessation of δ-globin synthesis and the low levels of hemoglobin A_2 compared with hemoglobin A_1.

The ε gene is located 5′ of the other β-like genes, and it is expressed during embryonic development. The ε gene is similar in sequence to the γ genes. A silencing element of the promoter region may be responsible for inactivating gene expression after the embryonic stage.[106] The only known mutation that results in persistent expression of this globin gene occurs in trisomy 13, in which the switch from ε- to γ-globin production is delayed.[107]

Globin Chain Synthesis

It has been possible to analyze the patterns of globin chain production at early ages of embryonic development during the transition from yolk sac (primitive) to hepatic (definitive) erythropoiesis. Still to be elucidated is why primitive erythroid progenitors programmed to produce one type of hemoglobin, such as Gower 1, give way to definitive progenitors programmed to produce a different type of hemoglobin, such as fetal hemoglobin. Quantification of globin gene synthesis reflects production by numerous red blood cells, and production of a specific hemoglobin usually is reported as a percentage of the total hemoglobin measured. Studies evaluating hemoglobin production by erythroid colonies in culture, however, show that individual cells in a colony produce predominantly one type of hemoglobin.[108]

During weeks 4 and 5, the main globin chains synthesized are ζ, δ, and ε (Figure 144-4). During weeks 6 and 7, α-, δ-, ε-, $^{G}\gamma$-, and $^{A}\gamma$-chains are produced in the remaining primitive erythroblasts, and α-, ε-, $^{G}\gamma$-, and $^{A}\gamma$-chains are produced in definitive erythrocytes. By week 7 to 8, εε- and ζ-chain synthesis is no longer detectable, and the main globin chains produced are α, $^{G}\gamma$, and $^{A}\gamma$. Production of β-chain is just barely detectable at this time and gradually increases so that by 10 weeks, it makes up 10% of total non–α-chain production.[109] As soon as β-chain production occurs, genetic disorders associated with β-chain synthetic or structural abnormalities may be detected in utero, a finding suggesting an asynchronous transition from ζ- to α- as compared with εε- to γ-chain production, with the ζ-to-α switch occurring slightly earlier.[110]

From week 10 to approximately week 33 of gestation, the main globin chains synthesized are α, $^{G}\gamma$, $^{A}\gamma$, and β. Assessment of the output of the two linked α-globin genes by mRNA analysis suggests that they are expressed in the ratio of α_2 to α_1, ranging from 1.5:1 to 3.0:1 throughout fetal life. This ratio does not appear to change during development and is the same as that observed in normal adults. The relative rates of $^{G}\gamma$-chain and $^{A}\gamma$-chain production also are constant throughout fetal life at a $^{G}\gamma/^{G}\gamma$ ratio of approximately 3:1.[100] It is not known whether these pairs of genes are initially activated at this ratio; however, this production ratio is reached early in development.

Somewhere between 32 and 36 weeks gestation, the relative rate of β-chain synthesis increases and that of γ-chain production declines, so at birth, β-chain synthesis makes up approximately 50% of non–α-chain synthesis. It generally is held that the transition from fetal to adult erythropoiesis starts at 30 to 36 weeks after conception, but the rate of the transition has been controversial.[109] A gradual transition from fetal to adult hemoglobin synthesis occurs, starting in the first trimester.[110,111] Considerable variation among infants is usual, however, because many infants show prolonged dependence on fetal hemoglobin. After birth, the level of γ-chain production steadily declines and that of β-chain production increases, so by the end of the first year, γ-chain synthesis reaches the low level characteristic of adult life. The normal range of postnatal fetal hemoglobin production is shown in Figure 144-5. Over the first few months of life, the $^{G}\gamma/^{A}\gamma$ ratio changes from 3:1 to 2:3, although this ratio is variable in adults.[112-114]

δ-Chain production has been observed as early as 32 weeks. Activation of δ gene lags behind that of β gene, so the adult β/δ synthesis ratio is not attained until 4 to 6 months after birth.

Hemoglobin Production

Developmental changes in the production of the various hemoglobins are depicted in Figure 144-4. Before the onset of other chain formation, unpaired globin chains may form tetramers, resulting in the presence of ε4.[115] Almost immediately thereafter, α- and ζ-chain production begins, and hemoglobins Gower 1 (ζ_2-ε_2), Gower 2 (α_2-ε_2), and Portland (ζ_2-γ_2) are formed.[116] By 5 to 6 weeks of gestation, hemoglobins Gower 1 and Gower 2 constitute 42% and 24% of the total hemoglobin, respectively, with fetal hemoglobin (α_2-γ_2) making up the remainder. By 14 to 16 weeks, hemoglobin F constitutes 50% of the total hemoglobin, and by 20 weeks, it forms more than 90% of the total.[117,118] Small quantities of hemoglobin A (α_2-β_2) are found beginning at 6 to 8 weeks of gestation. The increase in β-chain production occurring between 12 and 20 weeks gestation accounts for the sudden rise in hemoglobin A found at the end of the first trimester of pregnancy. Tetramers of γ chains (γ4, or hemoglobin Barts) and β-chains (β4, or hemoglobin H) can be found in conditions in which α-chain synthesis is impaired or absent, such as the α-thalassemia syndromes.

Fetal hemoglobin is easily distinguished immunologically and biochemically from adult hemoglobin. The most significant physiologic characteristic of fetal hemoglobin is the decreased interaction with 2,3-diphosphoglycerate (2,3-DPG). 2,3-DPG binds to deoxyhemoglobin in a cavity between the β-chains and stabilizes the deoxy form of hemoglobin, resulting in a reduced hemoglobin oxygen affinity. 2,3-DPG binds less effectively to the γ-globin chains, because of the differing amino acid sequence in the non–α-chain. Consequently, 2,3-DPG does not reduce the oxygen affinity of hemoglobin F as much as that of hemoglobin A.

Other differences in physical properties exist between fetal and adult hemoglobin. Hemoglobin F is more soluble in strong phosphate buffers than hemoglobin A.[96] Hemoglobin F is oxidized to methemoglobin more easily than hemoglobin A, and it has a considerably greater affinity for oxygen than adult hemoglobin as a result of differences in binding to 2,3-DPG. Fetal hemoglobin is resistant to acid elution, which allows differentiation of cells containing fetal hemoglobin from cells containing hemoglobin A.[96]

$^{G}\gamma$-Chains represent 70% to 80% of the total γ-chains in the blood of the fetus and newborn. This fraction falls to approximately 40% by the age of 5 months. This unique difference in $^{G}\gamma$-chain production found in the fetus helps to distinguish fetal hematopoiesis from that found in later life. Under stress, the older infant and adult revert to this intrauterine form of fetal hemoglobin structure. This reversion often occurs in leukemic states in children and adults, and in other conditions as well.[119,120] The delay in the switch of fetal hemoglobin to hemoglobin A has been noted in conditions of maternal hypoxia,[121] in infants small for gestational age,[122] and in infants of diabetic mothers.[123,124] Elevated levels of fetal hemoglobin may have protective effects in some disease states: One study suggests that a high fetal hemoglobin level in sickle cell patients may be a predictor of increased adult life expectancy.[125]

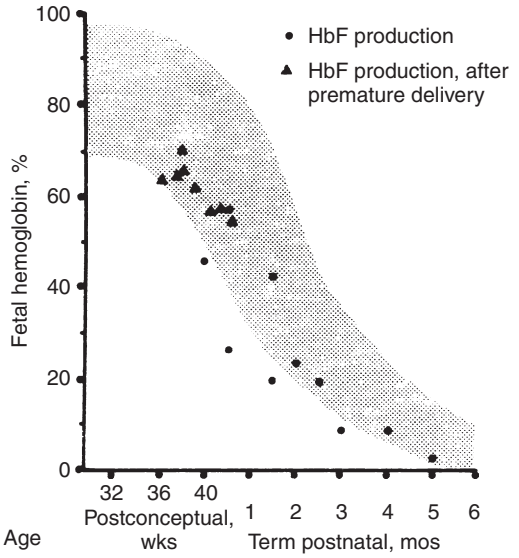


Figure 144-5 Postnatal changes in percentage fetal hemoglobin (Hb F) *(shaded area).*[60-64] *Triangles* represent postnatal production by reticulocytes in premature infants, and *dots* represent cord and postnatal reticulocyte production in term infants. Percentage Hb F present reflects Hb F production over the previous weeks, whereas rate of Hb F production is a result of the current proportion of Hb F produced by reticulocytes present and is thus lower as the Hb F–to–Hb A switch progresses. *(From Brown MS: Fetal and neonatal erythropoiesis. In Stockman JA, Pochedly C, editors:* Developmental and Neonatal Hematology, *New York, 1988, Raven Press, p 258.)*

The postnatal decline of fetal hemoglobin production and of the intercellular distribution of fetal and adult hemoglobins has been extensively examined over the first few months of life. Immediately after birth, a brief rise in hemoglobin F occurs, followed by a steady decline (see Figure 144-5). Studies of the intercellular distribution of hemoglobin F, using the relatively insensitive acid elution technique, have shown that over the first few months of life the distribution of hemoglobin F is quite heterogeneous. At 3 months, the distribution of hemoglobin F becomes bimodal, with populations of cells that contain acid-resistant hemoglobin F and populations of adult "ghost" cells. These observations suggest that fetal hemoglobin-containing cells are replaced by a population of cells containing adult hemoglobin during the early postnatal period.

Profound changes occur in the rates of red blood cell production immediately before birth and during the first few months after birth. On a body weight basis, red blood cell production during the latter months of gestation is significantly greater than that in adult life. Immediately after birth, erythropoiesis is considerably reduced, presumably as an adaptation to the extrauterine environment, and red blood cell production occurs at a low level for the first few weeks of life. It is clear from globin chain synthesis studies that a steady and linear decline in γ-chain synthesis occurs during the period of reduced neonatal erythropoiesis. Newly synthesized red blood cells appearing in the circulation when erythropoiesis resumes contain predominantly adult hemoglobin. These observations may explain the short plateau in the proportion of fetal hemoglobin (but not absolute levels) after

birth and the appearance of predominantly adult hemoglobin–containing cells during the second and third months of life. These findings, together with the results of analyses of the intercellular distribution of fetal and adult hemoglobin by sensitive immunologic methods, suggest, although they do not prove, that the transition from fetal to adult hemoglobin production occurs in the same erythrocyte population. This conclusion also is consistent with the patterns of fetal and β-chain production in red cell colonies grown from neonatal blood.[126]

Studies show that the type of globin chains produced at different stages of development are not closely related to the site of erythropoiesis. It appears that ζ- and ε-chains are synthesized in both primitive and definitive cell lines. Moreover, the switch from γ- to β-chain production occurs synchronously throughout the liver and bone marrow during the later stages of fetal development. The transition from γ- to β-chain synthesis is most closely related to postconceptional age and not chronologic age.[119] Thus premature infants continue to synthesize relatively large quantities of γ-chains (and fetal hemoglobin) until 40 weeks gestation.

Erythrocyte Metabolism

The mature red blood cell differs from other cells in the body in that it has no nucleus; it consequently lacks the ability to engage in de novo protein synthesis. It has no mitochondria or ribosomes, no cellular machinery for nucleic acid or deoxyribonucleic acid synthesis or for the Krebs cycle of intermediary metabolism, and no electron transport system for oxidative phosphorylation. Thus cellular metabolism is dependent on a limited supply of preexisting enzymes. These enzymes, coenzymes, and the substrates of glucose metabolism interact with hemoglobin and the red blood cell membrane to perform all the primary functions of the red blood cell, the most important being oxygen transport.

Important functions of red blood cell metabolism include maintaining adequate amounts of energy in the form of adenosine triphosphate (ATP), producing reducing substances to act as antioxidants, and maintaining appropriate amounts of red blood cell 2,3-DPG to assist in the modulation of hemoglobin's oxygen affinity. Energy metabolism is largely a function of the Embden-Meyerhof pathway. This pathway also regulates the quantity of red blood cell 2,3-DPG within the cell. The pentose phosphate pathway has, among other functions, a vital role in the production of reducing substances such as reduced nicotinamide adenine dinucleotide phosphate (NADPH). Box 144-1 lists the various metabolic characteristics of neonatal erythrocytes. A few of the variations in these cells from adult cells are clinically significant in that they affect the life span of the neonatal cell.

Glycolysis

Red blood cells need a constant supply of carbohydrate to maintain adequate levels of ATP. Although glucose is the preferred carbohydrate, the red blood cell metabolizes fructose or mannose almost as readily. Galactose is metabolized more slowly. Intracellular glucose concentrations equilibrate immediately with changes in plasma glucose concentrations. Glucose enters the human erythrocyte by facilitated transfer, and it is either converted to glucose-6-phosphate or reduced to its polyol derivative, sorbitol, which is then converted to fructose[127] (Figure 144-6). Once formed, glucose-6-phosphate is metabolized by one of three pathways. The *Embden-Meyerhof pathway* converts glucose-6-phosphate to lactate or pyruvate and in the process generates ATP. Metabolism by way of the *pentose phosphate pathway* produces reduced intermediates and a phosphorylated pentose sugar (ribulose-5-phosphate). This sugar ultimately returns to the Embden-Meyerhof pathway. Finally, *glucose-6-phosphate* may be converted to glucose-1-phosphate and then to glycogen, although less than 1% of glucose is metabolized to glycogen within the red blood cell.[128]

BOX 144-1

Metabolic Characteristics of Erythrocytes in the Newborn

Carbohydrate Metabolism
- Glucose consumption increased
- Galactose more completely utilized as substrate both under normal circumstances and for methemoglobin reduction*
- Decreased activity of sorbitol pathway*
- Decreased triokinase activity*

Glycolytic Enzymes
- Increased activity of hexokinase, phosphoglucose isomerase,* aldolase, glyceraldehyde-3-phosphate dehydrogenase,* phosphoglycerate kinase,* phosphoglycerate mutase, enolase,* pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, 6 phosphogluconic dehydrogenase, galactokinase, and galactose 1-phosphate uridyltransferase
- Decreased activity of phosphofructokinase*
- Distribution of hexokinase isoenzymes differs from that of adults*

Nonglycolytic Enzymes
- Increased activity of glutamic oxaloacetic transaminase and glutathione reductase
- Decreased activity of NADP-dependent methemoglobin reductase,* catalase,* glutathione peroxidase, carbonic anhydrase,* adenylate kinase,* and glutathione synthetase
- Presence of α-glycerol-3-phosphate dehydrogenase*

Adenosine Triphosphate (ATP) and Phosphate Metabolism
- Decreased phosphate uptake,* slower incorporation into ATP and 2,3-diphosphoglycerate*
- Accelerated decline of 2,3-diphosphoglycerate during red blood cell incubation*
- Increased ATP levels
- Accelerated decline of ATP during brief incubation

Storage Characteristics
- Increased potassium efflux and greater degree of hemolysis during short periods of storage
- More rapid assumption of altered morphologic forms on storage or incubation*

Membrane
- Decreased ouabain-sensitive ATPase*
- Decreased potassium influx*
- Decreased permeability to glycerol and thiourea*
- Decreased membrane filterability*
- Increased sphingomyelin and decreased lecithin content of stromal phospholipids*
- Decreased content of linoleic acid*
- Increase in lipid phosphorus and cholesterol per cell
- Greater affinity for glucose*

Other
- Increased methemoglobin content*
- Increased affinity of hemoglobin for oxygen*
- Glutathione instability*
- Increased tendency for Heinz body formation in presence of oxidant compounds*

From Oski FA, Naiman JL: *Hematologic Problems in the Newborn,* ed 3, Philadelphia, 1982, WB Saunders, p 107.
*Appears to be a unique characteristic of the newborn's erythrocytes and not merely a function of the presence of young red cells.
NADP, Nicotinamide adenine dinucleotide phosphate.

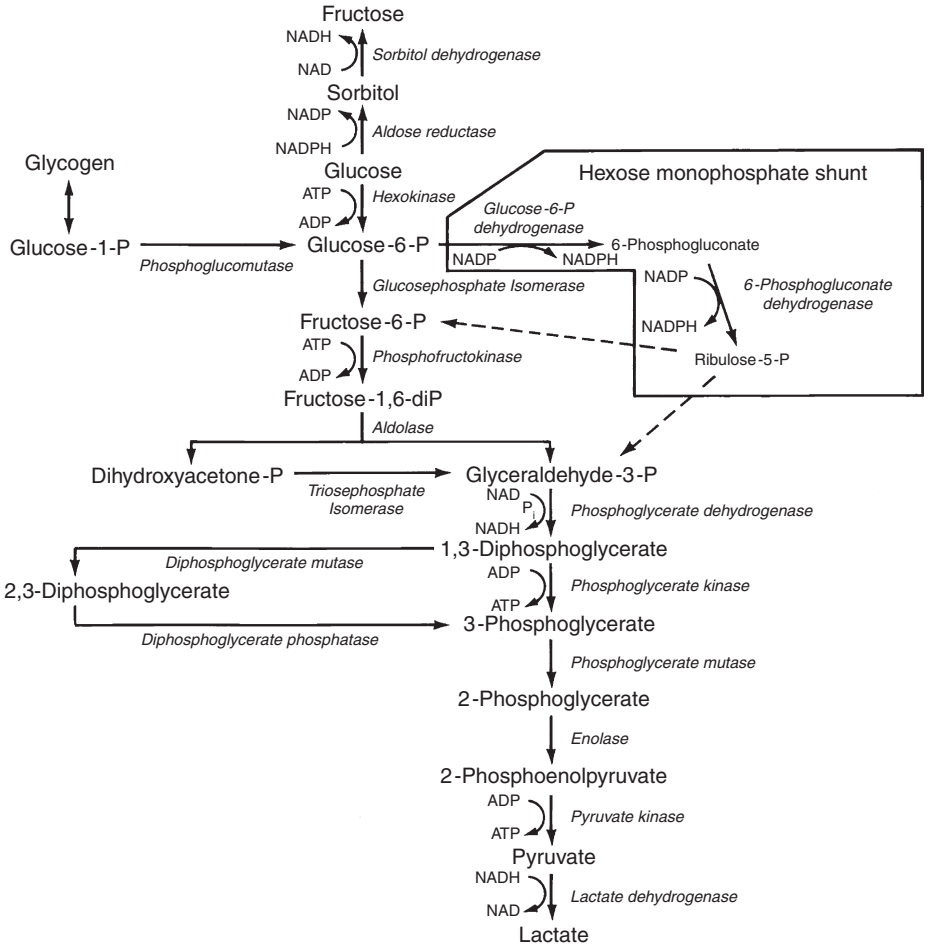


Figure 144-6 Pathways of red blood cell glucose metabolism.

Embden-Meyerhof Pathway

At least 90% of glucose is metabolized by way of the Embden-Meyerhof pathway.[129,130] Two moles of ATP are produced for every mole of glucose catabolized, yielding two moles of lactic acid. This potential for the production of ATP is not fully achieved, because approximately 20% of metabolized glucose traverses the 2,3-DPG cycle, thereby bypassing one of the kinase steps, which is a site of ATP generation.[131]

The Embden-Meyerhof pathway has several unique characteristics with respect to fetal and newborn cells. These cells consume greater quantities of glucose than do the red blood cells of adults.[132] Galactose metabolism in the newborn red blood cell also differs.[133] Galactokinase activity is three times greater in the erythrocytes of newborns, and these cells consume galactose more rapidly than do those of the adult. The glycolytic enzymes phosphoglycerate kinase and enolase are much more active in the cells of the fetus and newborn infant than would be anticipated from their young cell age.[134,135] By contrast, the activity of phosphofructokinase (a rate-controlling enzyme in glycolysis) is lower than normal in the erythrocytes from newborn infants.[134,136,137] Developmental changes in the activities of these three enzymes toward normal adult values during the first year of life appear to be independent of red blood cell age and reflect a transition from fetal to adult erythropoiesis. The decreased phosphofructokinase activity of fetal cells may be a consequence of accelerated decay of an unstable enzyme. The relative deficiency of this enzyme appears to result in alterations in glucose metabolism, and it could be functionally significant. Several of the other enzymes of the Embden-Meyerhof pathway have shown differences in the staining intensity of certain isoenzyme zones as compared with adult controls, although the significance of this observation is not known.

The ATP generated by the Embden-Meyerhof pathway is necessary for the maintenance of the normal biconcave shape of the erythrocyte.[138] It also is necessary for pyrimidine nucleotide synthesis, completion of purine nucleotide synthesis and glutathione synthesis,[139] incorporation of fatty acids into membrane phospholipids,[140] active cation transport,[137] and the initial step in the phosphorylation of glucose by the hexokinase enzyme. Not all of these synthesizing functions are important in the mature red blood cell, which lacks a nucleus. However, loss of red cell ATP produces a marked decrease in cell deformability. As the cell ages, ATP levels fall, and older cells exhibit lower glucose utilization, greater osmotic fragility on incubation, lower membrane lipid content, lower potassium concentration, and greater sodium concentration. They are less deformable and have a shorter life span. The red blood cells of term and preterm infants, when studied in the first several days of life, contain higher levels of ATP than do cells from adults.[141,142] The small premature infant has even higher ATP levels.[141]

Newborn red blood cells seem to demonstrate a transient immaturity in their metabolism. This results in a slower uptake of phosphorus, a delayed incorporation into 2,3-DPG, and a marked decline in 2,3-DPG, as well as ATP, during short periods of incubation in vitro. The precise reason for this relatively transient immaturity is not known, but it may explain why the newborn red blood cell loses potassium at an accelerated rate and undergoes marked morphologic alteration during short incubations in vitro.

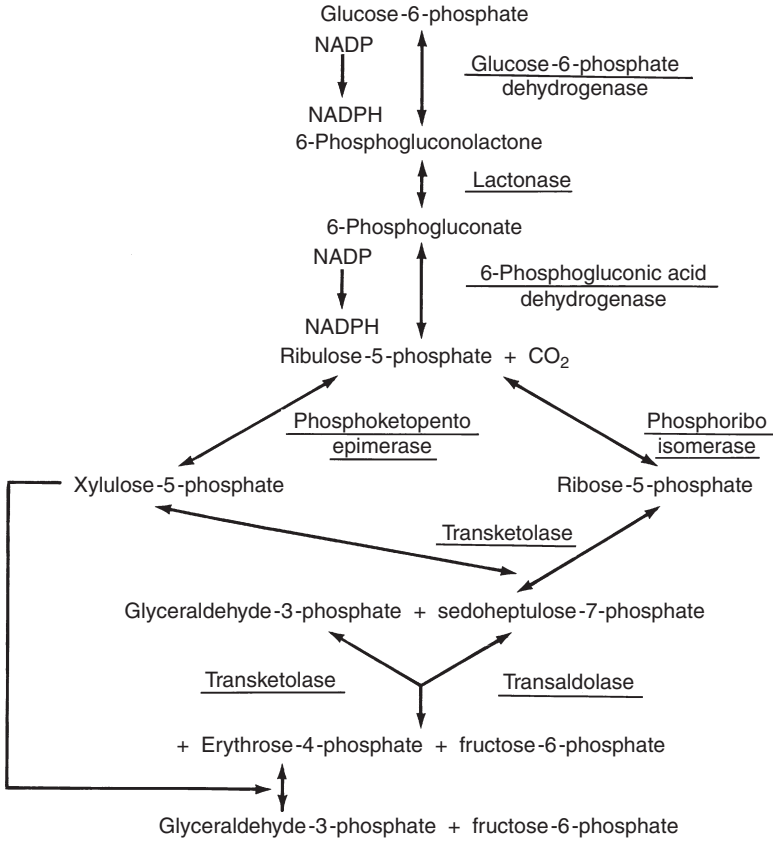


Figure 144-7 The pentose phosphate pathway.

Pentose Phosphate Pathway

Glucose-6-phosphate undergoes oxidative decarboxylation through the pentose phosphate pathway (Figure 144-7), consuming oxygen and producing carbon dioxide. The pentose pathway requires NADP as a cofactor. In the first step, oxidation of glucose-6-phosphate to 6-phosphogluconolactone is catabolized by glucose-6-phosphate dehydrogenase, generating $NADPH_2$. This step is followed by enzymatic hydrolysis of 6-phosphogluconolactone to 6-phosphogluconate, which is then oxidized in the presence of 6-phosphogluconic dehydrogenase to ribulose-5-phosphate with the production of carbon dioxide. Approximately 3% to 10% of all glucose metabolized by the cell is cycled through the pentose pathway. Hypoxia and acidosis increase the proportion of glucose metabolism shunted through this pathway.

The pentose phosphate shunt results in the production of two important products, ribose-5-phosphate and $NADPH_2$. Ribose-5-phosphate is a vital constituent of the pyridine nucleotides, NAD and NADP, and the purine nucleotides adenosine diphosphate (ADP) and ATP. No pathway exists for de novo purine formation inside the red blood cell; however, the mature red blood cell does retain the ability for pyridine nucleotide formation. $NADPH_2$ is critical for preservation of cell integrity because it is necessary for methemoglobin reduction, the reduction of glutathione, and the stabilization of certain enzymes.[143] $NADPH_2$ serves as a hydrogen donor in the presence of the enzyme glutathione reductase, resulting in reduction of glutathione. Reduced glutathione ultimately serves as a substrate for the enzyme glutathione peroxidase, which is responsible for the detoxification of hydrogen peroxide. Hydrogen peroxide is a byproduct of the conversion of oxyhemoglobin to methemoglobin, which is a naturally occurring reaction inside the red blood cell in the presence of oxidative stress. The absence of $NADPH_2$ (or anything that interferes with the production of reduced glutathione), the synthesis of glutathione, or the inability to detoxify hydrogen peroxide will severely impair the viability of the red blood cell.

The pentose phosphate pathway in the newborn red cell differs from that in the adult red blood cell. Two enzymes of the pentose pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase, are active at levels higher than those seen in adult red blood cells.[144] Carbon dioxide production by erythrocytes of term and preterm infants is equal to or greater than that seen in red blood cells of adults.

Although some studies suggest that the pentose phosphate pathway activity in the newborn may be normal, additional evidence indicates that newborn infants are more susceptible to oxidant-induced injury, leading to glutathione instability, Heinz body formation, and the development of methemoglobinemia.[145,146] This oxidant vulnerability may be caused by factors unrelated to the pentose phosphate pathway. For example, the red blood cell membrane in the fetus and newborn may have a decreased number of membrane sulfhydryl groups, making these cells more susceptible than mature red cells to Heinz body formation.[146] Additionally, NADPH-methemoglobin reductase activity is decreased in neonatal red cells, and the plasma of newborn infants appears to have a diminished antioxidant capacity.[147]

The precise mechanisms underlying the newborn red blood cell vulnerability to oxidant injury are not known. Fetal and newborn red blood cells have diminished glutathione peroxidase, which may render the cells more vulnerable to hydrogen peroxide–induced oxidant injury. In addition, the newborn red blood cell may have a diminished capacity for handling other activated oxygen radicals, such as singlet oxygen and the superoxide radical.[148] The latter is converted to hydrogen peroxide in the presence of the enzyme superoxide dismutase. Superoxide dismutase levels vary widely between infants. Diminished activity of the superoxide dismutase enzyme could result in

accumulation of superoxide radicals. Free radicals generally are detoxified by antioxidants such as α-tocopherol (vitamin E). If, however, superoxide dismutase levels are increased (as has been described in some infants), the hydrogen peroxide presented to reduced glutathione may not be adequately detoxified.[149] Therefore a delicate balance appears to exist between enzymes involved in production and detoxification of free radicals and oxidative intermediates. The use of inhaled nitric oxide for treatment of pulmonary hypertension in sick neonates may affect this balance, and further studies are required to determine its impact on oxidant injury beyond an increase in methemoglobin formation.

2,3-Diphosphoglycerate Metabolism

The affinity of a hemoglobin solution for oxygen can be decreased by interaction with certain organic phosphates.[150] Among the organic phosphates tested, 2,3-DPG and ATP are the most effective in lowering oxygen affinity. The highly charged anion 2,3-DPG binds to deoxyhemoglobin but not to oxyhemoglobin. Various conditions increase the amount of 2,3-DPG present within the red blood cell, as regulated in the Embden-Meyerhof pathway. Once formed, 2,3-DPG binds reversibly to 1 mole of deoxyhemoglobin tetramer in a 1:1 ratio under physiologic conditions of solute concentration and pH. Fetal deoxyhemoglobin does not possess as great an affinity for 2,3-DPG as that of adult deoxyhemoglobin, so it cannot bind 2,3-DPG to the same degree as for adult hemoglobin. Thus the fetal leftward-shifted hemoglobin oxygen dissociation curve is not easily modulated in the presence of 2,3-DPG. The half-saturation pressure (P_{50}) value for fetal blood is 19 to 21 mm Hg, some 6 to 8 mm Hg lower than that of adult blood. As the fetal hemoglobin concentration declines, however, a marked rightward shift is seen in the hemoglobin oxygen equilibrium curve (Figure 144-8). The percentage of adult hemoglobin and the red cell 2,3-DPG content alter the position of the hemoglobin oxygen dissociation curve. Infants with a greater proportion of adult hemoglobin but less 2,3-DPG may have the same P_{50} as that for infants with increased quantities of fetal hemoglobin but a high red cell 2,3-DPG content.

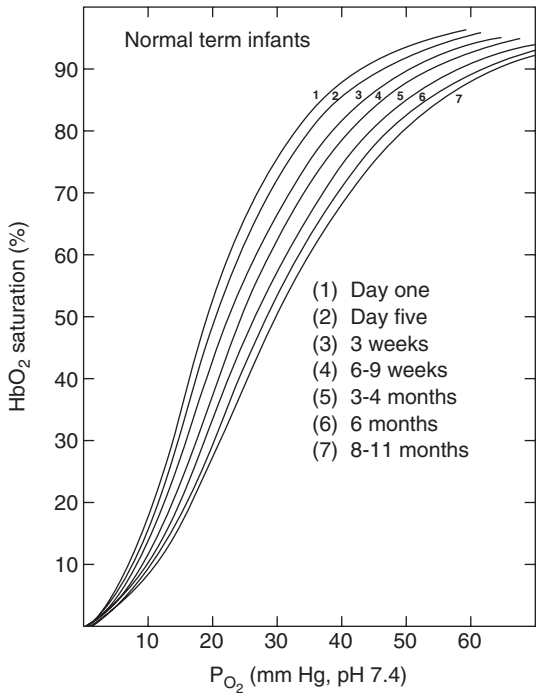


Figure 144-8 Oxygen equilibrium curve of blood from full-term infants at different postnatal ages. The oxygen half-saturation pressure of hemoglobin (P_{50}) on day 1 is 19.4 ± 1.8 mm Hg and has shifted to 30.3. ± 0.7 at age 11 months (normal adult value, 27.0 ± 1.1 mm Hg). *(From Oski FA, Naiman JL:* Hematologic Problems in the Newborn, *ed 3, Philadelphia, 1989, WB Saunders, p 250.)*

ERYTHROCYTE PHYSIOLOGY

Physical Properties of Neonatal Erythrocytes

The fetal and neonatal red blood cell differs from the mature red blood cell of the older infant, child, and adult in various ways. Specific characteristics of fetal red blood cells include a shortened life span, macrocytosis, high fetal hemoglobin content with a $^{G}\gamma/^{A}\gamma$ ratio of 3:1, the presence of i antigen,[151] and low carbonic anhydrase enzyme activity.[152]

Life Span

The differences in physical properties of red blood cells derived from term and preterm infants may in part account for the decreased life span of neonatal red blood cells within the circulation. The average life span for a neonatal red blood cell is 60 to 90 days,[153] approximately one-half to two-thirds that for an adult red blood cell. When neonatal red blood cells are transfused into adults, they exhibit a shortened life span, owing to alterations intrinsic to the neonatal red blood cell.[154] By contrast, cells transfused from adult donors appear to survive normally in newborns.[154] With increasing degrees of prematurity, remarkably shorter red cell life spans (35 to 50 days) are found. Fetal studies using [^{14}C]cyanate-labeled red cells in sheep revealed an average red cell life span of 63.6 ± 5.8 days.[155] The mean red cell life span increased linearly from 35 to 107 days as the fetal age increased from 97 days (midgestation) to 136 days (term).

The shortened red blood cell life span of the preterm and term neonate may be explained by some of the characteristics specific to newborn cells—namely, a rapid decline in intracellular enzyme activity and ATP,[156] loss of membrane surface area by internalization of membrane lipids, decreased levels of intracellular carnitine,[157] increased susceptibility of membrane lipids and protein to peroxidation,[158] and increased mechanical fragility resulting from increased membrane deformability.[159]

Size

Red blood cell dimensions change markedly during fetal and neonatal development. Early in embryogenesis, cell diameters range from 20 to 25 μm, and the MCV averages 150 to 180 fL.[1] During fetal development, red cells gradually decrease in size and volume (see Table 144-3); at birth, the average cell is 8 to 10 μm in diameter, with an MCV between 108 and 118 fL. Over the first year of life, red blood cells continue to diminish in size, and at 1 year of life, they resemble adult red blood cells (see Table 144-5).

Shape and Deformability

Just as with variation in the size of newborn red blood cells, variation in shape occurs as well. Irregularly shaped cells are present in much greater numbers in the peripheral blood of newborn infants than in that of adults.[160] Target cells, acanthocytes, puckered immature erythrocytes, and other irregular projections normally may be found. For example, a greater percentage of neonatal red blood cells have membrane surface pits, which probably are the sites of formation of endocytic vacuoles. In normal adults, 2.6% of erythrocytes appear to have surface pits or craters ranging in size from 0.2 to 0.5 μm in diameter, demonstrated by interference contrast microscopy.[161] By contrast, pits can be found in almost half of the erythrocytes of preterm infants and in one fourth of the erythrocytes of term infants.

Red blood cell deformability is governed principally by three factors: the surface area–volume relationship of the red blood

cell, the viscosity of the cytoplasm of the cell, and intrinsic red blood cell membrane rigidity.[159] The deformability of erythrocytes is important for several reasons. First, this deformability appears to be an important determinant of red blood cell life span in vivo. The removal of a red blood cell from the circulation is thought to be a consequence of declining deformability, making the cell susceptible to sequestration in the spleen and other organs, where it must negotiate extraordinarily narrow passages. Second, red blood cell deformability directly influences blood flow in the peripheral circulation. Third, red blood cell deformability affects whole blood viscosity, which in turn affects peripheral vascular resistance and cardiac workload.[162]

Neonatal red blood cells with the greatest density (representing the oldest cells in the circulation) lose more volume in comparison with adult red blood cells, have a higher mean corpuscular hemoglobin concentration, and are less deformable than the oldest red blood cells seen in adults. These observations suggest an accelerated decrease in deformability of aging red blood cells related to a more pronounced increase in the mean corpuscular hemoglobin concentration, the principal determinant of the internal viscosity of the red blood cell. Neonatal red blood cell membranes deform more readily to a given shear force than do adult red blood cell membranes, resulting in greater susceptibility of neonatal cell membranes to fragment.[162] These mechanical properties may lead to accelerated membrane loss and a decreased life span. A study evaluating the red cell deformability of preterm infants receiving recombinant Epo reported that erythrocyte deformability was significantly related to the reticulocyte count, indicating that the improvement of erythrocyte deformability with recombinant Epo therapy was due to the formation of well-deformable young erythrocytes.[163]

Surface Charge

The surface charge of newborn red blood cells is more negative than that of adult red blood cells. The negative charge at the red blood cell surface is largely responsible for the electrophoretic mobility of the cell, and it appears to reflect the sialic acid content of the red blood cell membrane.[164,165] Proteases expose more negative sites on neonatal red blood cells than on adult red blood cells and increase the electrophoretic mobility to a greater degree.[166] The sialic acid content of the newborn infant's erythrocyte membrane shows a gradual but significant decrease in the first several weeks of life.[167] Most studies, however, have shown that the electrophoretic mobility of the neonatal cell is similar to that of the adult cell.[165] The more negative surface charge of the neonatal red blood cell membrane is one of the many characteristics that results in a decreased sedimentation rate in newborns.[168]

Osmotic Fragility

The osmotic fragility of red blood cells is a composite index of their shape, hydration, and, within certain limitations, vulnerability to in vivo destruction.[159,169] Preterm and term infants have an increased osmotic resistance.[170,171] The osmotic fragility of neonatal cells begins to revert toward adult values shortly after birth, and osmotic resistance reaches adult values by 4 to 6 weeks of age. No advantage has been recognized for the increased osmotic resistance seen in the neonatal red blood cell. However, studies of osmotic fragility in neonates have practical implications. It has been suggested that the diagnostic criteria for hereditary spherocytosis used in adults and older children are unreliable in newborn infants. For example, spherocytes are not regularly seen in all infants with hereditary spherocytosis. Indeed, spherocytes may be a frequent finding in ABO incompatibility. In performing an osmotic fragility test, a neonatal osmotic fragility curve must be used, rather than an adult curve.[172] This underscores the need for calculating an osmotic fragility curve for newborn infants in whom a diagnosis of hereditary spherocytosis is considered.

Surface Receptors and Antigens

The neonatal red blood cell differs from the adult red blood cell in its ability to bind various substances. For example, neonatal red blood cells bind more insulin than that bound by adult red blood cells because of the presence of greater numbers of insulin receptors per cell.[173,174] Newborn red blood cells also have approximately 2.5 times the number of digoxin receptors in adult red blood cells,[175] and as a consequence neonates exhibit erythrocyte-to-plasma digoxin ratios three times those in adults. This finding may explain the greater tolerance of newborns who are receiving maintenance digoxin therapy.

Another unique characteristic of the fetal red blood cell is the expression of the i antigen on the cell surface (adult red blood cells express I antigen). Membrane i antigen is a carbohydrate moiety located on protein membrane band 3, which, during development, is converted from a linear polylactosamine to a branched carbohydrate chain of *N*-acetyl lactosamine units.[151] Red blood cells bearing the i antigen usually are not detectable by the first year of age. It has been suggested that the switch from fetal to adult hemoglobin and the transformation of i antigen expression that occur during the first year of life are governed by a common control mechanism; therefore the presence of i antigen can serve as a marker of fetal hematopoiesis.

Red blood cell antigens in the ABO, MN, Rh, Kell, Duffy, and Vel systems are well developed in early intrauterine life.[176] Antigen expression on the fetal red cell can be detected by 5 to 7 weeks gestation, and expression remains constant for the remainder of intrauterine development. Other antigens, such as the Lutheran and XgA systems, develop more slowly but are present at birth. Lewis antigens are lacking in the newborn. By approximately 2 years of age, the child's red blood cell and plasma antigens have developed a pattern that is seen throughout the remainder of life.[177]

Although A and B antigens are present early in utero, production of A and B isoagglutinins occurs much later.[178] By 30 to 34 weeks gestation, however, approximately 50% of infants have some measurable anti-A or anti-B antibodies. The fetal production of such antibodies is not related to maternal ABO blood type. Intrauterine exposure to gram-negative organisms, whose antigens are chemically related to those of blood groups A and B, is a potent stimulus for the development of these antibodies. Isohemagglutinin antibodies ultimately are demonstrable in normal infants by 6 months of age and approach adult values at 2 years of age. Low to absent titers after this time are suggestive of immune deficiency.[179]

Oxygen Transport

At no other time of life are the mechanisms controlling oxygen transport more complicated than during development in utero and in the immediate postnatal period. During prenatal life, the fetal arterial oxygen tension (Po_2) is approximately 30 mm Hg, and the venous Po_2 is approximately 15 mm Hg (Figure 144-9). This low Po_2 contributes to the development of relative polycythemia in the fetus. After birth, numerous factors affect oxygenation, including the inspired gas mixture, pulmonary function, the arterial oxygen dissociation curve, and the ability to extract oxygen at the tissue level.[180,181] It has been speculated that the actual amount of oxygen released to tissues may be greater in utero, in keeping with the characteristics of the hemoglobin oxygen dissociation curve (Figure 144-10).

If pulmonary function is normal, a rise in the Po_2 of pulmonary blood in adults and neonates, from the 40 mm Hg of pulmonary arterial blood to the 100 mm Hg of pulmonary venous blood, will occur. Because of the shape of the hemoglobin oxygen dissociation curve, these Po_2 values permit 95% saturation of hemoglobin by oxygen. Further increases in Po_2 produce little additional rise in saturation. In the normal adult (see Figure 144-9), approximately 50% of hemoglobin will be saturated with oxygen when the Po_2 has fallen to 27 mm Hg (P_{50} = 27). In situations in which the hemoglobin-oxygen dissociation curve has shifted to the

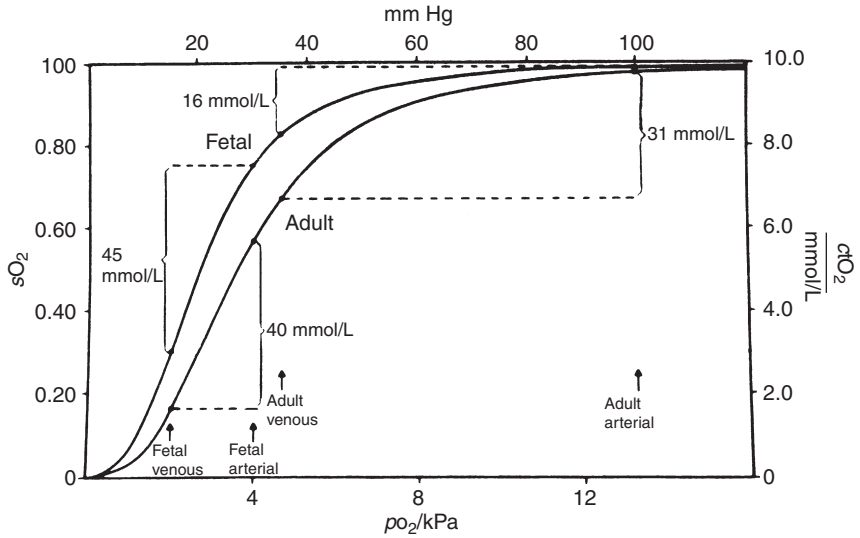


Figure 144-9 Differences in the hemoglobin oxygen dissociation curve between the newborn and the adult. This diagram demonstrates the effects of these differences on oxygen tension (ctO_2) and oxygen saturation (sO_2). *(From Stockman JA: Fetal hematology. In Eden RD, Boehm J, editors:* Assessment and Care of the Fetus, *Norwalk, Conn., 1989, Appleton & Lange, p 124.)*

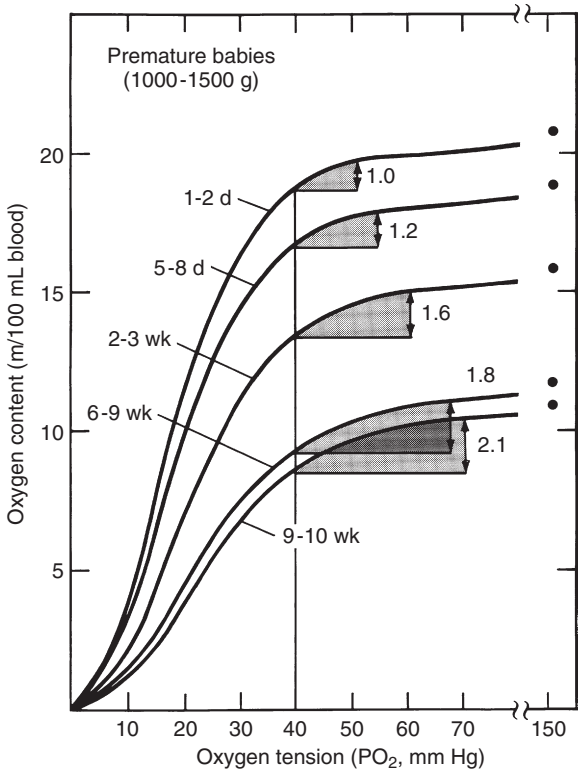


Figure 144-10 Oxygen equilibrium curves for blood from premature infants (1001 to 1500 g) at different postnatal ages. *Double arrows* represent the oxygen unloading capacity between a given arterial and venous Po_2. Points corresponding to 150 mm Hg on the *abscissa* are the oxygen capacities. Each *curve* represents the mean value for the infants studied in each group. *(From Delivoria-Papadopoulos M, et al: Postnatal changes in oxygen transport of term, preterm and sick infants: the role of red cell 2,3 diphosphoglycerate in adult hemoglobin,* Pediatr Res *5:235, 1971.)*

right, the affinity of hemoglobin for oxygen is reduced. Thus, at any given Po_2, more oxygen is released to tissues. Conversely, if the curve is shifted to the left, the affinity of hemoglobin for oxygen is increased, so that at any given Po_2, less oxygen is released to the tissues.

Certain factors are known to alter hemoglobin's affinity for oxygen (Box 144-2). The most important of these are the fetal hemoglobin concentration and the red cell 2,3-DPG content. The level of red cell 2,3-DPG gradually increases with gestation. At term, the concentration of red cell 2,3-DPG is similar to that in adults. By the end of the first week of life, the 2,3-DPG levels are considerably higher than they are at birth. After the first week, red cell 2,3-DPG levels remain relatively unchanged for the next 6 months. In term infants, the hemoglobin oxygen dissociation curve gradually shifts to the right, and by 4 to 6 months of age, the P_{50} values approximate those in the adult.

The situation is somewhat different in preterm infants. Fetal hemoglobin synthesis is still quite active; therefore increases in P_{50}, as seen in term infants consequent to the switch from fetal to adult hemoglobin, do not occur. The red cell 2,3-DPG concentrations are slightly lower in preterm infants as well.[89] These concentrations can be increased with the use of Epo, thereby shifting the oxygen dissociation curve to the right.[182]

A precise relationship between the decrease in oxygen affinity of a neonate's blood and the progressive decline in the concentration of fetal hemoglobin does not exist.[181] Rather, changes in P_{50} reflect the interplay between the levels of red cell 2,3-DPG, the decline in fetal hemoglobin, and the subsequent increase in hemoglobin A. A unifying concept has emerged consisting of a "functioning 2,3-DPG fraction,"[89] which determines how well hemoglobin is able to release oxygen to tissues. The functioning 2,3-DPG fraction (expressed as mμmol/mL of red blood cells) is calculated by multiplying the red cell 2,3-DPG content by the percent adult hemoglobin[86]:

$$[2,3\text{-DPG}]_{\text{functional}} = [2,3\text{-DPG}]_{\text{observed}} \times [100 - \%\ \text{hemoglobin F}]$$

If P_{50} cannot be measured directly, it may be calculated from the following equation[86] after the functioning 2,3-DPG fraction has been determined:

$$P_{50} = 18.4 + 0.0016\,[2,3\text{-DPG}]_{\text{functional}}$$

BOX 144-2

Factors Affecting Hemoglobin-Oxygen Affinity

Increased Red Cell 2,3-DPG, Increased P_{50}
- Adaptation to high altitude
- Hypoxemia associated with chronic pulmonary disease
- Hypoxemia associated with cyanotic heart disease
- Anemia
  - Secondary to iron deficiency
  - Secondary to chronic renal disease
  - Associated with sickle cell anemia
- Decreased red blood cell mass
- Chronic liver disease
- Hyperthyroidism
- Red cell pyruvate kinase deficiency

Decreased Red Cell 2,3-DPG, Decreased P_{50}
- Septic shock
- Severe acidosis
- After massive transfusions of stored blood
- Neonatal respiratory distress syndrome

Increased P_{50}, No Consistent Alteration in Red Cell 2,3-DPG
- Abnormal hemoglobins (Kansas, Seattle, Hammersmith, Tacoma, E)
- Vigorous exercise

Decreased P_{50}, No Consistent Alteration in Red Cell 2,3-DPG
- Abnormal hemoglobins (Kempsey, Chesapeake, J. Capetown, Yakima, Rainier)

2,3-DPG, 2,3-Diphosphoglycerate; *P_{50},* oxygen half-saturation pressure of hemoglobin.

Table 144-6 illustrates the expected changes in hemoglobin concentration, 2,3-DPG, functional 2,3-DPG fraction, and P_{50} during the first 3 months of life in low-birth-weight infants.[89] In low-birth-weight infants, the amount of oxygen released by hemoglobin can be determined on the basis of unloading from a normal arterial Po_2 adjusted for age to an arbitrary central venous Po_2 of 40 mm Hg.[86] Although oxygen-carrying capacity (hemoglobin concentration × percent oxygen saturation × 1.36 mL of oxygen/g of hemoglobin) decreases over the first few months of life as a consequence of a decline in hemoglobin concentration, the amount of oxygen capable of being delivered to tissues actually increases. For example, in a newborn weighing 1000 g with a hemoglobin concentration of 15 g/dL, a P_{50} of 19, and a central venous Po_2 of 40 mm Hg, 1 mL of oxygen will be unloaded to tissues for every 100 mL of blood that passes through the capillary bed. By 10 weeks of age, the P_{50} has shifted to the right and is now 24 mm Hg. In this same infant, 2.1 mL of oxygen will now be delivered per 100 mL of blood, even though the hemoglobin has declined to 8 g/dL (see Figure 144-10).

These calculations emphasize the importance of understanding a particular infant's ability to deliver oxygen to tissues in determining whether to administer an erythrocyte transfusion. The decision to transfuse should not be based on hemoglobin concentration alone. Transfusions will significantly affect the infant's endogenous erythropoiesis. For infants who undergo exchange transfusion or multiple transfusions, both Epo concentrations and reticulocyte counts are lower at any given hemoglobin concentration[86,183] (Figure 144-11).

It often is assumed that oxygen delivery is decreased in newborns because of the presence of high-affinity hemoglobin. In fact, a leftward shift in the hemoglobin-oxygen dissociation curve resulting from high levels of fetal hemoglobin may better maintain oxygen delivery during episodes of severe hypoxemia. Figure 144-12 illustrates the arterial Po_2 below which shifts to the right in the neonatal oxygen dissociation curve are no longer advantageous. The gas tension at which this occurs is known as the *cross-over Po_2*.[184] The cross-over Po_2 is dependent on how low the venous Po_2 falls before oxygen delivery ceases. Wimberley[185] calculated that if the arterial Po_2 fell to less than 32 mm Hg and if the venous Po_2 fell to 10 mm Hg (a value found in the cerebral venous blood of some sick newborns), the infant would achieve better oxygen delivery with a fetal oxygen dissociation curve than with an adult curve. Conversely, if arterial Po_2 can be maintained at a higher value, better oxygen delivery would exist if the oxygen dissociation curve were shifted to the right.

In neonates with hypoxemia (e.g., infants with cyanotic congenital heart disease), a shift to the right (a higher P_{50}) in the hemoglobin-oxygen dissociation curve tends to lower the arterial and venous oxygen saturation at any given Po_2. It has been documented that if arterial hypoxemia results from right-to-left shunting, shifts to the right in the oxygen dissociation curve always tend to increase the arterial-venous oxygen difference, thereby improving oxygen delivery.[186]

After intrauterine transfusion, oxygen-unloading properties of blood in the newborn are characteristic of those of adult blood. Despite the decrease in oxygen affinity that accompanies intrauterine transfusion, no deleterious effects of this procedure with respect to oxygen uptake by the fetus have been documented.[187] The physiologic significance of manipulating the hemoglobin oxygen affinity of fetuses and extremely preterm infants remains to be determined.

HYPOPROLIFERATIVE ANEMIAS

Physiologic and Pathologic Anemia in Neonates

A gradual decrease in hemoglobin concentration occurs over the first 2 to 3 months of life in term and preterm infants. The hemoglobin concentration remains stable over the next several weeks and then slowly rises. In term infants, the fall in red cell production after birth is the result of improved oxygenation and occurs as a natural adaptation to extrauterine life. Accordingly, the decrease in hemoglobin concentration has been recognized as a *physiologic nadir,* rather than representing true anemia. In preterm infants, however, adaptive mechanisms may not be complete. Preterm infants generally exhibit a drop in hemoglobin to lower values than those seen in term infants, and the nadir varies with the degree of prematurity. Table 144-4 illustrates the decline in hemoglobin concentration based on birth weight and gestational age. Hemoglobin values as low as 7 g/dL are common in preterm infants who have not undergone phlebotomies.[87,188]

Assessing the factors that characterize oxygen supply and oxygen demand has provided insight into the adaptive changes occurring in preterm infants in response to low hemoglobin concentrations. In preterm infants, a low central venous Po_2 correlates with a mild increase in Epo production[189] (Figure 144-13). The decline in central venous Po_2 appears to be a sensitive indicator of the presence of anemia, representing the integration of variables that determine oxygen supply and demand: hemoglobin concentration, red cell oxygen affinity, intravascular volume, oxygen consumption, heart rate, cardiac stroke volume, and arterial oxygenation. The hemoglobin concentration is only one of many important variables ensuring adequate oxygen delivery in both term and preterm infants.[181]

Anemia of Prematurity

Characteristics

Despite the slight increase in Epo production that occurs in response to declining central venous Po_2 values, Epo concentrations in preterm infants are still significantly low, in view of the

TABLE 144-6

Changes in Hemoglobin Concentration, Hematocrit, and Other Markers of Oxygen Delivery During the First 3 Months of Life in Low-Birth-Weight Infants

Age	Total Hemoglobin (g/dL)	Hematocrit (%)	MCHC (%)	O_2 Capacity of Blood (mL/dL)	P_{50} at pH 7.40 (mm Hg)	2,3-DPG (μmol/mL of RBCs)	Fetal Hemoglobin (% of Total)	FFDPG (μmol/mL)
Group I: Birth Weight <1000 g*								
2 weeks	17.2	47.0	36.6	23.9	18.0	6255	83.0	1002
4 weeks	8.5	26.0	32.7	11.8	15.0	3923	81.0	761
9 weeks	7.2	22.0	32.7	10.0	15.0	4636	87.1	974
11 weeks	7.7	22.5	34.2	10.7	17.0	5867	78.0	1290
Group II: Birth Weight 1001-1500 g								
1-2 days	15.1 ± 1.3†	45.7 ± 3.7	33.0 ± 0.7	21.0 ± 1.8	180 ± 1.7	4124 ± 1562	86.6 ± 3.1	580 ± 287
5-8 days	13.4 ± 1.1	41.4 ± 3.2	33.5 ±2.9	18.7 ±1.5	18.9 ± 3.0	4501 ± 1919	84.4 ± 3.8	903 ± 689
2-3 weeks	12.6 ± 3.1	33.6 ± 6.0	34.2 ± 1.1	15.9 ± 3.1	21.2 ± 1.9	5721 ± 1375	83.3 ± 5.1	1119 ± 557
4-5 weeks	8.8 ± 0.9	25.3 ± 1.8	34.9 ± 1.7	12.3 ± 1.3	20.5 ± 1.7	6095 ± 2081	85.2 ± 2.3	931 ± 456
6-9 weeks	9.1 ± 1.7	24.5 ± 5.8	35.1 ± 2.2	11.8 ± 2.4	23.4 ± 1.1	8734 ± 1834	77.2 ± 1.9	1995 ± 480
9-10 weeks‡	8.2	24.0	34.0	11.1	24.0	9000	77.0	2070
Group III: Birth Weight 1501-2000 g								
1-2 days	16.1 ± 0.9	47.8 ± 1.9	33.7 ± 1.9	22.4 ± 1.2	19.3 ± 0.9	4475 ± 1174	87.2 ± 3.6	703 ± 331
5-8 days	16.8 ± 3.3	48.5 ± 10.0	34.7 ± 0.5	25.3 ± 4.7	19.8 ± 1.3	5489 ± 1428	79.4 ± 5.0	1056 ± 590
2-3 weeks	13.6 ± 3.0	40.4 ± 9.8	34.4 ± 1.5	18.8 ± 4.0	21.3 ± 1.8	6002 ± 998	80.6 ± 5.8	1184 ± 329
4-5 weeks	11.2 ± 2.8	31.9 ± 9.9	35.5 ± 2.2	15.5 ± 3.8	20.8 ± 1.6	5841 ± 839	75.8 ± 7.8	1569 ± 577
6-9 weeks	8.0 ± 0.7	22.1 ± 1.7	35.9 ± 0.7	11.1 ± 1.0	24.0 ± 0.9	7290 ± 634	67.5 ± 6.2	2457 ± 575
Group IV: Birth Weight 2001-2500 g								
1-2 days	15.9 ± 0.9	46.2 ± 5.8	35.8 ± 1.9	21.9 ± 1.5	20.2 ± 1.6	5306 ± 1075	76.8 ± 5.43	1258 ± 392
5-8 days	15.6 ± 1.7	47.0 ± 5.0	34.2 ± 1.1	21.5 ± 2.4	21.3 ± 3.3	6417 ± 1527	77.7 ± 6.3	1457 ± 603
2-3 weeks	12.3 ± 1.1	35.1 ± 3.2	34.9 ± 0.5	17.1 ± 1.5	22.0 ± 1.3	7145 ± 1737	76.9 ± 4.7	1666 ± 472
6-9 weeks‡	14.0	44.0	34.0	19.5	25.5	7100	43.0	3212

From Delivoria-Papadopoulos M, et al: Postnatal changes in oxygen transport of term, preterm and sick infants: the role of red cell 2,3 diphosphoglycerate in adult hemoglobin, *Pediatr Res* 5:235, 1971.

*Only one patient.

†Values are given as means ± SD.

‡Fewer than five infants.

2,3-DPG, 2,3-Diphosphoglycerate; *FFDPG,* functioning fraction of 2,3-DPG; *MCHC,* mean corpuscular hemoglobin concentration; *RBCs,* red blood cells.

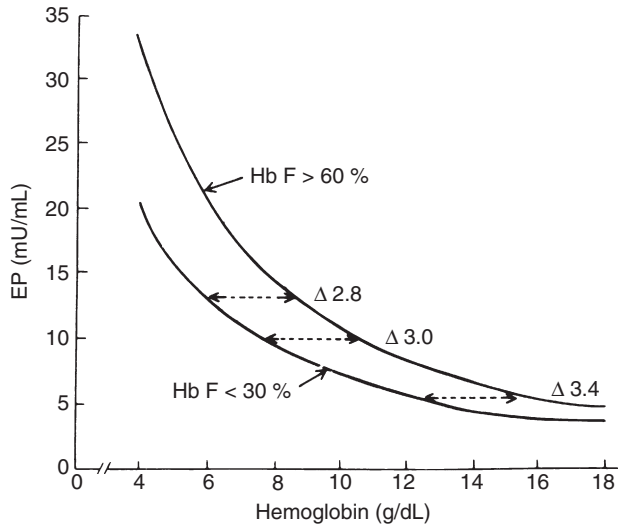


Figure 144-11 In infants born prematurely who have been exchange-transfused or multiply transfused (fetal hemoglobin [*Hb F*] <30%), the hemoglobin concentration may fall by several g/dL units before erythropoietin production *(EP)* resumes. *(From Stockman JA, et al: The anemia of prematurity: factors governing the erythropoietin response,* N Engl J Med *296:647, 1977. Reprinted by permission of* The New England Journal of Medicine.*)*

degree of anemia.[73,189-191] This anemia, termed the *anemia of prematurity,* affects infants born at less than 32 weeks gestation, and it is the most common anemia encountered in the neonatal period. It is a normocytic, normochromic anemia, generally associated with hemoglobin concentrations less than 10 g/dL and low reticulocyte counts. Some infants may be asymptomatic, whereas others demonstrate signs of anemia that are alleviated by transfusion. These signs traditionally include tachycardia, increased episodes of apnea and bradycardia, poor weight gain, an increased oxygen requirement, and elevated serum lactate concentrations that decrease after transfusion.[192-195]

Anemia of prematurity was first described by Schulman,[196] who divided the anemia into three phases. *Early anemia* was marked by an initial fall in hemoglobin concentration. The second, or *intermediate*, phase was characterized by maintenance of low hemoglobin concentrations. The third phase, *late anemia of prematurity,* resulted in hemoglobin concentrations that continued to fall despite unequivocal evidence of anemia, which failed to induce appropriate adaptive changes. The anemia of prematurity usually resolved spontaneously by 3 to 6 months of life.

Although much information has accumulated since the 1970s, the mechanisms responsible for the anemia of prematurity remain poorly defined. Shortened erythrocyte survival,[197] hemodilution associated with a rapidly increasing body mass,[198] and the transition from fetal to adult hemoglobin[89] all have been implicated. Despite diminished available oxygen to tissues[86] and the appearance of signs of anemia,[191] serum Epo concentrations remain low.[191] However, erythroid progenitors are highly sensitive to

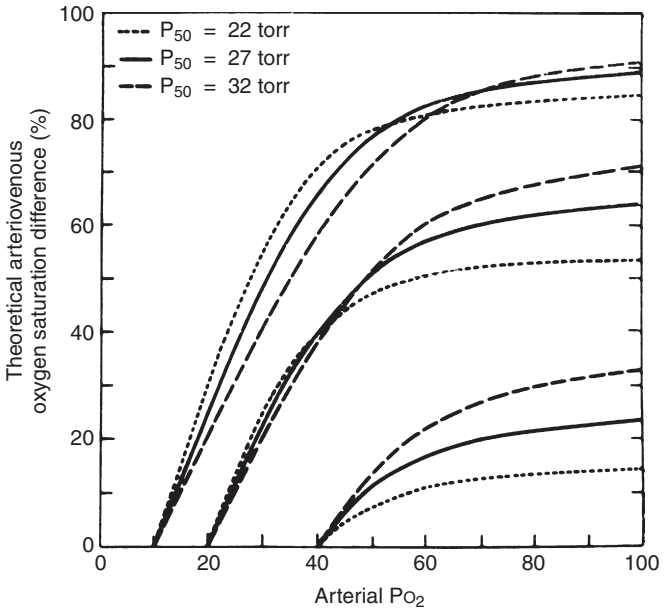


Figure 144-12 The effect of arterial partial pressure of oxygen (Po_2) on theoretical arteriovenous oxygen content difference when venous Po_2 is 40 mm Hg *(lower set of curves),* 20 mm Hg *(middle set of curves),* or 10 mm Hg *(upper set of curves)* at various P_{50} values. *P_{50},* Oxygen half-saturation pressure of hemoglobin. *(From Woodson RD: Physiological significance of oxygen dissociation curve shifts,* Crit Care Med *7:368, 1979. © by Williams & Wilkins, 1979.)*

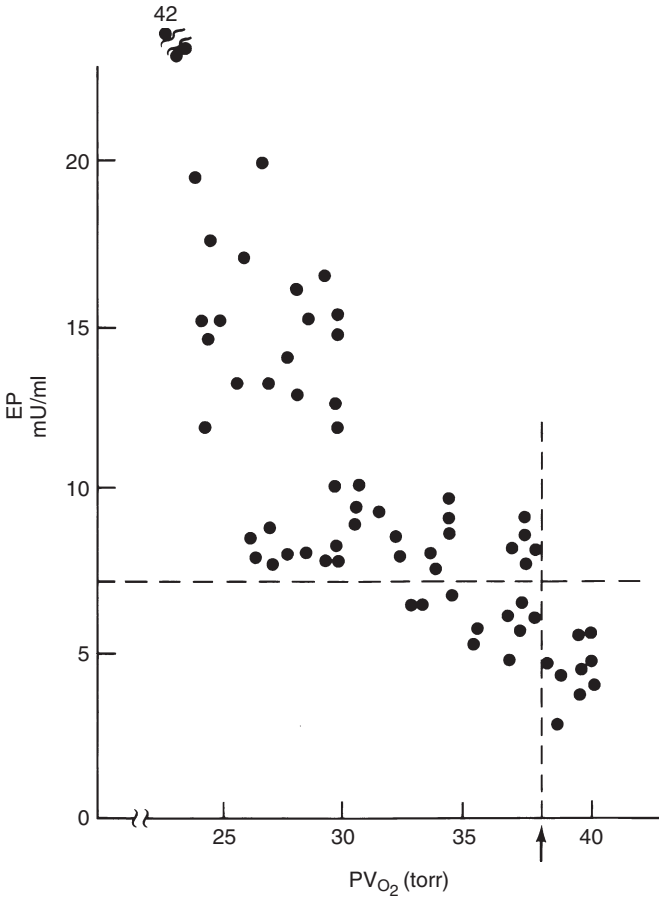


Figure 144-13 Changes in plasma erythropoietin concentrations in response to declines in central venous oxygen tension (PVo_2) in preterm neonates. *Arrow* indicates the position of 38 mm Hg, which for the purposes of this figure is the lower limit of normal for PVo_2. The *horizontal dashed line* is the upper limit of normal for erythropoietin taking PVo_2 ≥38 mm Hg as normal. *EP,* Erythropoietin production. *(From Stockman JA, et al: Anemia of prematurity: determinants of the erythropoietin response,* J Pediatr *105:786, 1984.)*

Epo,[199,200] and concentrations of other erythropoietic growth factors, including IL-3 and GM-CSF, appear to be normal.[201]

Infants born prematurely lack a normal response to anemia, so that Epo production does not increase despite an apparent need for improved tissue oxygenation.[202] This failure to respond has been documented regardless of the cause of anemia, whether it is the early anemia of phlebotomy loss or the later anemia of prematurity. Possible molecular and cellular mechanisms responsible for this lack of responsiveness include defects in transferring the hypoxic signal to the nucleus, diminished or defective binding of transcription factors to the promoter or enhancer regions of the gene, decreased production or stability of the transcriptional factors, decreased production or stability of Epo mRNA, diminished or unstable protein production, and increased production of counterregulatory proteins such as IL-1 or tumor necrosis factor. The inability of the kidney or liver in the premature infant to produce Epo mRNA with magnitude equal to that seen in the fetus also may involve a developmental delay in expression of transcriptional factors responsible for increasing Epo production, or in persistence of fetal Epo gene methylation patterns. Because Epo produced by the liver is indistinguishable from that produced by the kidney, it is unknown whether preterm infants rely on Epo produced by the liver or the kidney, or a combination of the two. However, macrophages from preterm infants generate Epo mRNA and protein as effectively as do those from term infants and adults.[203] The anemia of prematurity thus may involve a delay in activation of Epo gene expression, from prenatal (liver) to postnatal (kidney) sites of production.

Clinical Trials

Multiple clinical studies have been performed evaluating Epo administration administered to preterm infants.[204] Randomized, controlled studies using weekly Epo doses of more than 500 U/kg are summarized in Table 144-7.[205-219] In the first randomized multicenter trial published, Maier and the European Epo Study Group[205] evaluated the effect of Epo on the need for transfusions in preterm infants weighing 750 to 1499 g. Infants who were not receiving mechanical ventilation were randomized on day 3 of life to receive Epo or no treatment. A total of 241 infants were evaluated, 177 of whom completed the study according to protocol. Epo recipients received fewer transfusions (1.2 versus 0.8) and demonstrated a greater success rate (defined as maintaining hematocrit of more than 32% without transfusions) compared with placebo recipients.

Shannon and the United States Study Group[207] evaluated Epo to treat the anemia of prematurity. Infants born at a weight of 1250 g or less and before 31 weeks gestation were randomized to receive Epo (100 U/kg) or placebo, administered subcutaneously (SC), 5 days a week, for a total of 6 weeks. All infants were receiving enteral feedings of at least 60 kcal/kg/day; at the time of study entry, hematocrit values were lower than 40%, and phlebotomy losses were minimal. Infants in both groups received oral iron and vitamin E supplementation, and uniform transfusion criteria were followed by all participating centers. Infants were 24 days of age at the beginning of the study, and they had

TABLE 144-7

Randomized Studies of Erythropoietin and Vitamin/Iron Supplements in Preterm Infants*

Study	Population	Erythropoietin Dose and Schedule	Iron ±Vitamin Supplementation	No. of Transfusions/ Patient (% Success)	Volume Transfused
Haiden et al., 2006[219]	40 infants ≤800 g and ≤32 wk *Treatment*: 21 infants; *control*: 19 infants	300 U/kg/d IV OR 700 U/kg 3×/wk SC to 40 wk PCA	Both groups (with exceptions noted): *Iron dextran*: 1.5 mg/kg/d IV OR *iron polymaltose*: 9 mg/kg/d *Vitamin E*: 5 mg/d PO *Folate*: 100 µg/kg/d (60 µg/kg/d for controls) PO *Vitamin B_{12}*: 3 µg/kg/d IV OR 21 µg/kg/wk SC (0 for controls)	2 (38% not transfused)	28 mL
		Control		4.5 (5% not transfused)	75 mL
Haiden et al., 2006[218]	64 infants 801-1300 g and ≤32 wk *Treatment*: 31 infants; *control*: 33 infants	300 U/kg/d IV OR 700 U/kg 3×/wk SC administered to 40 wk PCA	Both groups (with exceptions noted): *Iron dextran*: 1.5 mg/kg/d OR *iron polymaltose*: 9 mg/kg/d *Vitamin E*: 5 mg/d PO *Folate*: 100 µg/kg/d (60 µg/kg/d for controls) PO *Vitamin B_{12}*: 3 µg/kg/d IV OR 21 µg/kg/wk SC (0 for controls)	1.13	15.8 mL
		Control		1.12	17.6 mL
Pollak et al., 2006[217]	29 infants <1300 g and <31 wk *Treatment*: two groups of 10 infants each; *control*: 9 infants	300 U/kg/IV every 3 days × 6	*Iron*: 2 mg/kg/d IV + 9 mg/kg/d PO	0.4 ± 0.3	12 ± 9 mL
		300 U/kg/IV every 3 days × 6	*Iron*: 9 mg/kg/d PO	0.8 ± 0.4	15 ± 7 mL
		Control	*Iron*: 9 mg/kg/d PO	0.7 ± 0.3	14 ± 6 mL
Turker et al., 2005[216]	30 infants <1000 g (subset of study) *Treatment*: 15 infants; *control*: 15 infants	750 U/kg/wk SC DOL5-40	Both groups: *Iron*: 6 mg/kg/d PO	2	28 mL
		Control		5	63 mL
Meyer et al., 2003[215]	33 infants <1700 g and <33 wk *Treatment*: 22 infants; *placebo*: 21 infants	1200 U/kg/wk SC × 3 wk, then 600 U/kg/wk	*Iron*: 6 mg/kg/d PO	2.5 ± 2.3	—
		Placebo	*Iron*: 2 mg/kg/d PO	3.2 ± 3.4	—
Pathak et al., 2003[214]	30 infants ≤1250 g and ≤32 wk, at 2 wk of age *Treatment*: two groups of 15 infants each	100 U/kg/d SC 5 d/wk (all infants)	*Vitamin E*: 55 IU/d PO	0.9 ± 0.3	16 ± 6 mL
			Vitamin E: 5 IU/d PO	1.1 ± 0.4	18 ± 6 mL
Maier et al., 2002[213]	219 infants 500-999 g *Treatment*: two groups of 74 infants each; *control*: 71 infants	Group 1: 250 U/kg 3×/wk IV/SC starting wk 1	*Iron*: 3 mg/kg/d PO	13%†	0.4 mL/kg/d†
		Group 2: 250 U/kg 3×/wk IV/SC starting wk 4	Adjusted to transferrin saturation	11%	0.5 mL/kg/d
		Control		4%	0.7 mL/kg/d
Ohls et al., 2001[212]	172 infants 401-1000 g *Treatment*: 87 infants; *placebo*: 85 infants	400 U/kg/3×/wk IV/SC to 36 wk PCA	*Iron*: 5 mg/kg/wk IV OR 6 mg/kg/d PO *Vitamin E*: 15-21 IU/d PO	4.3 ± 3.6	80 ± 53 mL/kg†
		Placebo	*Iron*: 1 mg/kg/wk IV OR 6 mg/kg/d PO *Vitamin E*: 15-21 IU/d PO	5.2 ± 4.2	95 ± 63 mL/kg
	118 infants 1001-1250 g *Treatment*: 59 infants; *placebo*: 59 infants	400 U/kg/3×/wk IV/SC to 36 wk PCA	Both groups: *Iron*: 6 mg/kg/d PO *Vitamin E*: 15-21 IU/d PO	1.0±1.6	39 ± 26 mL/kg
		Placebo	Adjusted to ferritin	1.1 ± 1.6	35 ± 24 mL/kg
Donato et al., 2000[211]	114 infants ≤1250 g *Treatment*: 57 infants; *placebo*: 57 infants	1250 U/kg/wk × 2 wk, then 750 U/kg/wk	Both groups: *Iron*: 6 mg/kg/d PO	3.4 ± 1.1	51 ± 35 mL/kg
		Placebo × 2 wk, then 750 U/kg/wk		5.4 ± 3.7	84 ± 54 mL/kg
Maier et al., 1998[210]	184 infants <1000 g *Treatment*: two groups: 93 infants and 91 infants	250 U/kg SC 3×/wk, to 38 wk PCA (in 93 infants)	*Iron*: 3 mg/kg/d PO	30%	12.9 mL/patient
		500 U/kg SC 3×/wk, to 38 wk PCA (in 91 infants)	Adjusted to transferrin saturation	28%	10.5 mL/patient
Ohls et al., 1997[209]	28 infants ≤750 g *Treatment*: 15 infants; *placebo*: 13 infants	200 U/kg/d IV for 14 d	*Iron*: 1 mg/kg/d IV OR	4.7 ± 0.7†	70 ± 11 mL/kg†
		Placebo	*Iron*: 6 mg/kg/d PO	7.5 ± 1.1	112 ± 17 mL/kg

Continued

TABLE 144-7

Randomized Studies of Erythropoietin and Vitamin/Iron Supplements in Preterm Infants*—cont'd

Study	Population	Erythropoietin Dose and Schedule	Iron ±Vitamin Supplementation	No. of Transfusions/ Patient (% Success)	Volume Transfused
Ohls et al., 1995[208]	20 infants ≤1500 g *Treatment*: 10 infants; *placebo*: 10 infants	200 U/kg IV d for 14 d Placebo	Both groups: *Iron*: 3-6 mg/kg/d PO	0.2 ± 0.1† 1.4 ± 0.4	2.7 mL/kg† 22.3 mL/kg
Shannon et al., 1995[207]	157 infants ≤1250 g *Treatment*: 77 infants; *placebo*: 80 infants	100 U/kg SC 5×/wk for 6 wk Placebo	Both groups: *Iron*: 3-6 mg/kg/d PO Adjusted to ferritin	1.1 ± 1.5† 1.6 ± 1.7	17 ± 23 mL/pt 24 ± 26 mL/pt
Meyer et al., 1994[206]	80 infants <32 wk	200 U/kg SC 3×/wk for 6 wk (40) Placebo (40)	*Iron*: 3 mg/kg/d PO Adjusted to ferritin	7 total† 21 total	148 mL total† 438 mL total
Maier et al., 1994[205]	241 infants 750-1499 g *Treatment*: 121 infants; *control*: 120 infants	250 U/kg SC 3×/wk, DOL 3-42 Control	Both groups: *Iron*: 2-6 mg/kg/d PO Adjusted to ferritin	0.87† 1.25	0.09 mL/kg/d† 0.41 mL/kg/d

*Measured in cord blood.
†Using ≥500 U of erythropoietin/kg once a week for 2 weeks or longer.
DOL, Day of life; *IV,* intravenous administration; *PCA,* postconceptual age; *PO,* by mouth (enteral administration); *SC,* subcutaneous administration; *U,* units.

received an average of 3.5 transfusions before study entry. Epo recipients required a lower number (1.1 versus 1.6) and smaller volume of transfusions (16 mL/patient versus 24 mL/patient) compared with placebo recipients, and no adverse effects were noted.

Our own laboratory evaluated the efficacy and cost-effectiveness of treating very low-birth-weight infants with Epo for 2 weeks, starting in the first 2 days of life.[208] Epo recipients received a lower number of transfusions than that required by the placebo recipients (0.2 versus 1.4 transfusions per patient), as well as smaller-volume transfusions. When the analysis was extended to include other infants in the intensive care unit who met all study eligibility criteria, the transfusion rate for the placebo recipients was lower (at 2.8 transfusions per patient during the same period) than for infants who were not in the study. This difference in frequency of transfusion also was noted by Bifano and colleagues,[220] who observed that instituting standardized transfusion guidelines decreased the transfusion requirements of very low-birth-weight infants.

In the Argentina multicenter trial, Donato and colleagues[211] randomized infants weighing less than 1250 g to receive either early (250 U/kg five times a week intravenously [IV] for 2 weeks, then 250 U/kg three times a week SC for 6 weeks) or late Epo (placebo for 2 weeks, then 250 U/kg three times a week SC for 6 weeks). These investigators concluded that despite the lack of difference in total number of transfusions between the early and late treatment groups, infants weighing less than 800 g with a phlebotomy loss of more than 30 mL/kg (the early Epo group) appeared to benefit; however, the benefit was noted only after 2 weeks of therapy. A similar delay in response was seen in extremely low-birth-weight infants in the National Institute of Child Health and Human Development Network study,[212] which showed a benefit for Epo treatment after 8 weeks.

Maier and colleagues[213] also compared early administration of Epo (from the first week for 9 weeks) with late Epo (from the fourth week for 6 weeks) in infants weighing less than 1000 g. A control group of infants did not receive Epo. As in their previous studies, these investigators measured the rate of "success," defined by no transfusions and a hematocrit value that never fell to less than 30%. The success rate in the early Epo group was three times greater than that in control subjects: 13% in the early Epo group, 11% in the late Epo group, and 4% in control infants. The investigators reported more than twice the "success" rate (27.6%) for that in same-size infants obtained using a similar dose, dosing schedule, transfusion criteria, and study protocol in one of their previous studies.[210]

Pharmacokinetics

Pharmacokinetic studies in newborn monkeys and sheep indicate that neonates have a larger volume of distribution and a faster elimination of Epo,[221,222] necessitating the use of higher doses than those required for adults.[223] These pharmacokinetic differences apply to preterm infants as well.[224,225] Although various Epo doses and dosing schedules have been evaluated in preterm infants (see Table 144-7), the erythrokinetics of Epo (the interaction between erythroid progenitors and their growth factors) in preterm infants are unknown. Brown and associates[226] compared two dosing schedules in preterm infants: 100 U/kg five times a week versus 250 U/kg twice a week. These workers reported that the more frequent dosing schedule appeared to be more effective in stimulating erythropoiesis, although no differences in transfusion requirements were observed between the two dosing schedules. In a study during which Epo was given continuously over 24 hours to preterm infants for 10 days (versus a daily subcutaneous injection), Epo concentrations and reticulocyte responses were similar in both groups.[225] Peak concentrations in both groups declined from day 3 to day 10.

The route of administration also influences the effectiveness of Epo. Rapid intravenous administration of Epo generates peak serum concentrations that far exceed physiologic concentrations.[227] This may result in wasting of drug through increased renal excretion or binding to nonerythropoietic receptors. Dosing strategies that achieve lower peak serum concentrations over a more prolonged period may be more effective. It appears that doses in the range of 500 to 1400 U/kg/week, administered SC every day or every other day, result in an adequate clinical response. Newer, long-acting erythropoiesis–stimulating agents (ESAs) such as darbepoetin are currently being studied.[228-230] A similar pharmacokinetic relationship in preterm infants is likely in that half-life, clearance, and volume of distribution of these agents suggest that higher doses will be required; however, a decrease in dosing frequency to only once every 2 to 3 weeks may be possible—a great benefit over thrice-weekly subcutaneous dosing.

Side Effects of Treatment

Side effects in neonates receiving Epo have been rare. A side effect unique to neonates, transient neutropenia, was noted in early studies.[231-233] The neutropenia was not associated with depletion of either neutrophil reserves or colony-forming unit–granulocyte-macrophage (CFU-GM) concentrations, but

it appeared to involve reduced production of neutrophils from granulocytic progenitors.[234,235] This disturbance resolved after discontinuation of the Epo. The pathomechanism may involve depletion of the total number of progenitors in slightly older preterm infants or possibly down-modulation of granulocyte-CSF receptors on pluripotent progenitors. In animal studies, neutropenia was documented to occur in newborns after administration of very high doses of recombinant Epo as a result of decreased production of neutrophils from progenitors.[234]

Neutropenia was not reported in any of the trials evaluating administration of Epo to preterm infants starting in the first days of life. In addition, Epo recipients did not experience any increase in morbidity or adverse outcome measures when compared with the placebo recipients. Early studies reported a decreased incidence of symptomatic patent ductus arteriosus,[206,208] possibly as a result of interactions between Epo and endothelin production.[236] Later studies have not shown a statistical effect of Epo on the incidence of symptomatic patent ductus arteriosus.[211-213] In the clinical trial by Emmerson and colleagues,[237] two infants in the Epo-treated group died of sudden infant death syndrome. Meyer and associates[206] also reported one late death ascribed to this syndrome. No other studies reported this association, and follow-up studies did not reveal an association between sudden infant death syndrome and Epo administration.[238]

Overall, the side effects of Epo administration in preterm infants have been minimal compared with those reported in adults—specifically, hypertension, bone pain, rash, and, rarely, seizures. None of these effects were reported in preterm infants involved in clinical trials. Moreover, no studies have reported differences between placebo and Epo recipients in the incidence of neonatal comorbid illnesses such as chronic lung disease, intraventricular hemorrhage, necrotizing enterocolitis, retinopathy of prematurity (ROP), or late-onset sepsis. In a summary report, Romagnoli and colleagues[239] reported an increased incidence of ROP in preterm infants receiving Epo and supplemental iron, compared with a group of control infants. These findings have not been duplicated in any randomized trial, and Epo is still administered to preterm infants cared for by this group. Owing to the inclusion of this report in a recent meta-analysis, the authors of the meta-analysis suggested a relationship between Epo administration and increased incidence of ROP[240]; however, this relationship has not been reported in any of the randomized placebo-controlled trials published in peer-reviewed journals.

Although long-term follow-up studies are few, it appears that the administration of Epo has no adverse effect on developmental outcome or growth, measured at 18 to 22 months.[238] As with many new therapies, however, reports of adverse effects invariably surface as use of the drug increases. Initial concerns regarding red cell aplasia[242] resolved when use of a specific brand of Epo in prefilled syringes was determined to be the primary cause. A report from the U.S. Food and Drug Administration (FDA) raised other concerns over the use of ESAs in adults with cancer.[241] Because of the increased incidence of adverse events documented in cancer patients in remission, new hemoglobin limits have been set for use of ESAs in adults. None of those side effects (stroke, increased thrombosis) have been reported in preterm infants, but careful observation continues.

Nutritional Supplementation

Iron has been administered both parenterally and enterally in published studies. Carnielli and colleagues reported no adverse effects of intravenous iron[243] given at doses of 20 mg/kg once a week. The infants in their study also received vitamin E, 10 mg/kg, intramuscularly twice a week. This iron supplementation regimen, although not using the standard dose or typical route of administration in the United States, may be associated with improved iron availability; it resulted in a greater and more prolonged erythropoietic response than that seen in other studies. Friel and colleagues[244] reported iron accretion rates of preterm infants (not receiving Epo) of 1000 μg/kg/day. Multicenter trials using intravenous iron at doses of 1 to 5 mg/kg/week have shown no adverse effects while producing adequate iron status,[209,212,245] and no long-term oxidant effects have been reported after low-dose parenteral iron infusion.[246]

Oral iron supplementation rates have ranged from 2 to 40 mg/kg/day. Studies evaluating the oxidant effects of infant formulas and oral iron[188] showed increased erythrocyte lysis associated with formulas containing greater amounts of iron and polyunsaturated fatty acids. This effect resolved with the addition of vitamin E. Functional iron deficiency frequently has been reported in pediatric and adult patients receiving Epo, and such deficiency probably limited the success of some of the early Epo trials in preterm infants. Studies evaluating iron stores in Epo recipients[205,206] noted increased iron requirements in infants receiving Epo, as evidenced by diminished ferritin concentrations and elevated numbers of hypochromic red blood cells, despite iron doses of 6 mg/kg/day. Infants receiving Epo probably are at greater risk for iron deficiency than for iron overload and increased oxidant stress. Further evaluation is required to determine the optimal dose and most effective route of administration of iron in preterm infants receiving Epo.

Vitamin E is an antioxidant, inhibiting peroxidation of polyunsaturated fatty acids in the lipid bilayers of all cell membranes. Vitamin E requirements may be increased as a consequence of iron supplementation, because iron promotes oxidation of polyunsaturated fatty acids. Pathak and colleagues[214] evaluated high-dose vitamin E in preterm infants receiving Epo and showed no benefit from doses of 50 IU/kg/day. Further study is needed to determine the optimal dose of vitamin E in preterm infants receiving Epo.

More recently, folate and vitamin B_{12} have been evaluated in combination with Epo and iron, and results have shown significant promise.[218,219] In a study in preterm infants with birth weights less than 800 g, Haiden and colleagues[223] achieved significantly greater success (38% of infants not transfused) when vitamin B_{12} at a dose of 21 mg/kg/week SC was added to a regimen of Epo, iron, vitamin E, and folate. When combined with limited phlebotomy losses, this therapy shows great promise in extremely low-birth-weight infants.

Other Erythropoietin-Responsive Anemias in the Newborn Period

Late Anemia of Rh Disease

Infants with Rh hemolytic disease often exhibit an "early" (congenital) anemia caused by ongoing, antibody-mediated hemolysis, and late-onset anemia (at the age of 1 to 3 months) may develop as a consequence of diminished erythrocyte production. The incidence of late anemia appears to be much higher in infants who receive intrauterine transfusions.[247,248] This anemia is characterized by low serum concentrations of Epo, but erythroid progenitors remain highly responsive to recombinant Epo in vitro.[249] Infants with the late anemia of Rh hemolytic disease routinely receive transfusions until their anemia spontaneously resolves, generally by the third or fourth month of life. During the period of decreased circulating antibody synthesis and delayed endogenous Epo production, administration of exogenous Epo may serve as an alternative to erythrocyte transfusion. In centers in which infants requiring transfusions are hospitalized, Epo administration also may diminish or eliminate the need for hospitalization, because this agent can be given in an outpatient setting. Preliminary studies evaluating the use of recombinant Epo for treatment of the late anemia of Rh hemolytic disease have shown it to be effective in stimulating reticulocyte production and increasing hematocrit.[249,250]

Anemia of Bronchopulmonary Dysplasia

Alverson and associates[251] observed the development of anemia in patients with bronchopulmonary dysplasia (BPD) and reported that repeat transfusions generally are required for management of anemia. Christensen and colleagues[252] subsequently characterized the anemia of BPD as normocytic, normochromic, hyporegenerative anemia, with marrow normoblast iron stains that are distinct from those observed in both the anemia of chronic disorders and the anemia of prematurity. Patients with the anemia of BPD differed from infants with the anemia of prematurity in that not all of the patients with the anemia of BPD were born prematurely, and their average age was older than that typical for infants with anemia of prematurity. Preliminary results show Epo to be effective for treatment for the anemia of BPD.[253] Further study is ongoing to determine the explanation for reduced Epo production in patients with BPD, including the evaluation of factors that could create a relatively Epo-resistant environment, such as IL-1, tumor necrosis factor, and interferon-γ.

Anemia in Neonates with Congenital Heart Disease

In an attempt to decrease transfusion requirements and possibly decrease the incidence of graft rejection, infants with congenital heart disease awaiting transplantation received daily Epo (200 U/kg/day).[254] These infants showed a significant increase in hematocrit and decreased transfusion requirements. Although case reports of infants undergoing open heart surgery have been published,[255] randomized controlled trials evaluating the use of Epo to decrease transfusion requirements of newborns with congenital heart disease should be completed before its widespread use in this population.

Anemia in Neonates Requiring Surgery

Many studies in adults have been performed evaluating the use of Epo as an adjuvant to transfusion before surgery. Pediatric studies are limited. Neonates who are born with problems that require surgical repair, such as omphalocele, gastroschisis, meningomyelocele, congenital diaphragmatic hernias, and craniofacial abnormalities, often undergo surgery during the period of physiologic anemia. These infants are hospitalized and sustain blood loss through phlebotomy, the surgery itself, and postoperative care. As a result, the physiologic anemia is exacerbated, and hemoglobin concentrations may drop to such a low level that transfusions are given. Pilot studies on the use of Epo in these populations have shown promise,[256] but randomized studies are required to determine whether these infants will respond to Epo administration by increasing erythropoiesis.

In addition to neonates who require surgery, other neonatal populations may benefit from Epo therapy. As in adults, Epo has been used successfully to treat anemia in newborns with end-stage renal disease.[257] In addition, infants with ABO incompatibility,[258] hemolytic disease from Kell antibody,[259] and hereditary spherocytosis[260] have shown increased erythropoiesis when treated with Epo. Further studies are required in these populations to determine whether treatment with Epo is beneficial and does not cause further harm through increased hemolysis and hyperbilirubinemia.

In view of transfusion-associated risks such as transmission of hepatitis or *Trypanosoma cruzi,* West Nile virus, or human immunodeficiency virus infection,[261-263] as well as the possible development of graft-versus-host disease,[264] treatment of anemic neonates using recombinant Epo may potentially be cost-effective. Thus Epo administration, in combination with the development of more rigorous and standardized transfusion criteria and the decrease in blood loss through phlebotomy, is likely to have the greatest impact in decreasing transfusion requirements in term and preterm infants. Regardless of treatment strategy, a critical understanding of the physiologic influences affecting oxygen delivery in term and preterm infants is required before attempts at altering the hematocrit through an erythrocyte transfusion or by the administration of Epo or another ESA.

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145

Martha Sola-Visner and Brandon S. Poterjoy

Developmental Megakaryocytopoiesis

BACKGROUND

Megakaryocytes, among the rarest and most unusual hematopoietic cells in the human bone marrow, comprise 0.02% to 0.1% of the total nucleated marrow cells.[1,2] Over the past decades, the study of these cells lagged behind that of other hematopoietic lineages, partly because of the rarity and fragility of megakaryocytes in the bone marrow, and partly because of the lack of a potent thrombopoietic factor to stimulate the growth of these cells in culture.The cloning of thrombopoietin (Tpo) in 1994, the development of better culture systems, and the ability to employ flow cytometry to study megakaryocytes broke many of these barriers and led to major advances in our understanding of megakaryocytopoiesis. This chapter first summarizes these advances and our current knowledge about the biologic processes leading to platelet production and then focuses on what is known about fetal and neonatal megakaryocytopoiesis. Although many questions in this field remain unanswered, a mounting body of evidence suggests that fetal and neonatal megakaryocytes represent

a distinct lineage, with biologic features clearly different from those of their adult counterparts. The implications of these differences are widespread and expand beyond neonatal hematology into the field of cord blood transplantation, a therapy frequently complicated by delayed platelet engraftment.

MEGAKARYOCYTOPOIESIS

In a very schematic fashion, the complex process of platelet production can be represented as consisting of four main steps (Figure 145-1): (1) the production of thrombopoietic factors (mainly Tpo), (2) the proliferation of megakaryocyte progenitors, (3) the differentiation and maturation of megakaryocytes through a unique process of endomitosis, and finally (4) the production and release of platelets into the circulation. The first part of this chapter is dedicated to reviewing the biology of megakaryocyte progenitors, the differentiation and maturation of megakaryocytes, and the effects of thrombopoietic cytokines (particularly Tpo) on the different steps of the process. The second part summarizes our current understanding of neonatal thrombocytopenia, with an emphasis on the factors underlying the predisposition of ill neonates to develop severe and prolonged thrombocytopenia.

Megakaryocyte Progenitors

The development of the megakaryocytic lineage from pluripotent hematopoietic stem cells is not clearly understood. The classical model states that hematopoietic stem cells give rise to a common lymphoid progenitor, which produces lymphocytes, and a common myeloid progenitor, which gives rise to the myeloid, macrophage, eosinophil, erythroid, and megakaryocytic lineages.[3] In adults, erythroid and megakaryocyte lineages arise from a common megakaryocyte-erythroid progenitor (MEP),[4] and recent evidence indicates that the same is true in embryonic and fetal development.[5] However, this classical pathway of hematopoiesis has been questioned by recent findings suggesting that MEPs can arise directly from hematopoietic stem cells.[6] The specific signals that regulate the separation into the different lineages are not well understood and are the subject of active research efforts.

The first cells fully committed to the megakaryocyte lineage are the megakaryocyte progenitors. By definition, these cells have proliferative potential and are characterized by their ability to form clusters of pure megakaryocytes (megakaryocyte colonies) when cultured. Two different types of megakaryocyte progenitors have been identified. The most primitive megakaryocyte progenitor is the burst-forming unit–megakaryocyte (BFU-MK). A later, more mature progenitor has been designated colony-forming unit–megakaryocyte (CFU-MK). These two progenitor types can be differentiated on the basis of their immunologic markers and the characteristics of the colonies that they form in vitro (Table 145-1). Overall, CFU-MK-derived colonies appear in vitro sooner than BFU-MK-derived colonies. They are also smaller (usually <50 cells) and unifocal, compared with the large and multifocal appearance of BFU-MK colonies. CFU-MK progenitors are also the predominant MK progenitor type in the bone marrow, whereas BFU-MK progenitors predominate in the blood. Finally, although both progenitors express CD34, only CFU-MKs express the human leukocyte antigen (HLA)-DR.

Unlike megakaryocyte progenitors, which possess a high proliferative potential, megakaryocytes are cells that have lost the ability to proliferate but instead undergo a unique process known as *endomitosis*. The transition from megakaryocyte progenitors to mature megakaryocytes is characterized by a significant level of overlap, which gives rise to a population of cells known as *transitional cells*, *megakaryocyte precursors*, or *promegakaryoblasts*, which have increasing ploidy (4 to 8 N) and decreasing proliferative potential. These cells are thought to represent the intermediate step between progenitors and megakaryocytes and are difficult to identify morphologically. Immunologic

TABLE 145-1

Comparison of Primitive Megakaryocyte Progenitors (BFU-MK) and Differentiated Megakaryocyte Progenitors (CFU-MK)

Characteristic	BFU-MK	CFU-MK
Appearance in culture	Later	Earlier
Cells/colony	>50	3-50
Foci of development	≥2	1
Immunologic phenotype	$CD34^+$, $HLA\text{-}DR^-$	$CD34^+$, $HLA\text{-}DR^+$
Site of predominance	Peripheral blood and bone marrow	Bone marrow

From Sola MC. Fetal megakaryocytopoiesis. In Christensen R (ed): *Hematologic Problems of the Neonate,* Philadelphia, 2000, WB Saunders.

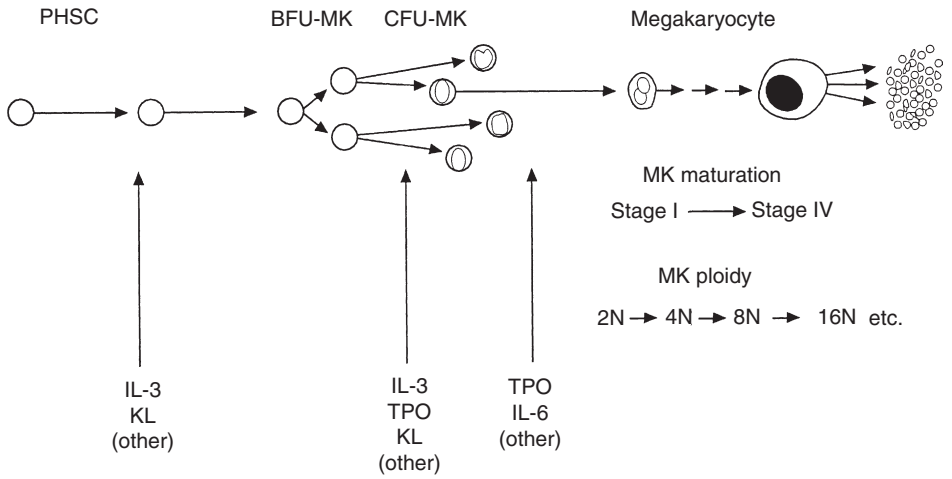


Figure 145-1 Simplified scheme of megakaryocyte (MK) development. Many growth factors probably influence megakaryocytopoiesis at each stage. In some studies, the synergistic effect of interleukin-3 (IL-3) and thrombopoietin (TPO) was demonstrated. IL-3 seems to be essential for early megakaryocyte proliferation (especially fetal) from pluripotent hematopoietic stem cell (PHSC) through colony-forming unit, megakaryocyte (CFU-MK). Although many factors affect megakaryocyte maturation, TPO is probably the primary stimulant. *BFU,* Burst-forming unit.

studies have suggested that transitional cells express both CD34 and CD61 (GPIIIa).[7] One transitional cell may also be the megakaryoblast, the earliest cell of the megakaryocyte lineage that can be identified with the platelet peroxidase reaction.[8] During this phase, the cells begin to accumulate various α-granule proteins: platelet factor 4 (PF4), thrombospondin, β-thromboglobulin, von Willebrand factor, and surface markers such as platelet GPIb and the von Willebrand factor receptor,[9] which can later be detected on megakaryocytes (Figure 145-2).[10-14]

Megakaryocytes

Megakaryocytes are cells that have lost their proliferative abilities and undergo a complex process of maturation. Through this process, they evolve from small, mononuclear cells that are indistinguishable from those of other lineages, to very large, polyploid cells that are easily recognized as megakaryocytes. The process of maturation involves both nuclear and cytoplasmic changes as well as a corresponding increase in size. At the nuclear level, maturation involves a process known as *endoreduplication* or *endomitosis*, by which the ploidy of the cells increases. The ploidy level of megakaryocytes can be measured on stained individual cells in a cytospin, in a colony, or by flow cytometry. Using the latter technique, the modal ploidy in adult marrow has been shown to be 16 N. Recently, it has also been demonstrated that increasing ploidy levels directly correlate with increased platelet production by megakaryocytes.[15] Eventually, as platelets are released, the megakaryocytes become smaller, and multiple nuclear lobes coalesce into a compact nucleus.[16]

Based on a combination of size and nuclear and cytoplasmic features, the maturational stages of the megakaryocytes have been classified as stages I to IV (Table 145-2). This morphologic classification is often difficult, however, particularly because megakaryoblasts and stage I and II megakaryocytes can easily be missed on microscopic examination. For these reasons, other criteria have been established for megakaryocyte differentiation and to define various maturational stages, including the development of surface glycoproteins and the appearance of specific granules.[10,12] For example, GPIIIa and GPIIb/IIIa are present in very early megakaryocytes, whereas GPIb (CD42b) and GPIV (CD36) appear later during megakaryocyte maturation (see Figure 145-2).[11,17,18] The GPIb-V-IX (CD42) complex has also been identified on late megakaryocytes.[19] The development of the demarcation system and of α-granules has been documented by electron microscopy. Contents of the α-granules, such as fibrinogen, thrombospondin, and von Willebrand factor, also correlate with megakaryocyte maturation.[11,12,14] Fibrinogen appears late in megakaryocyte maturation.

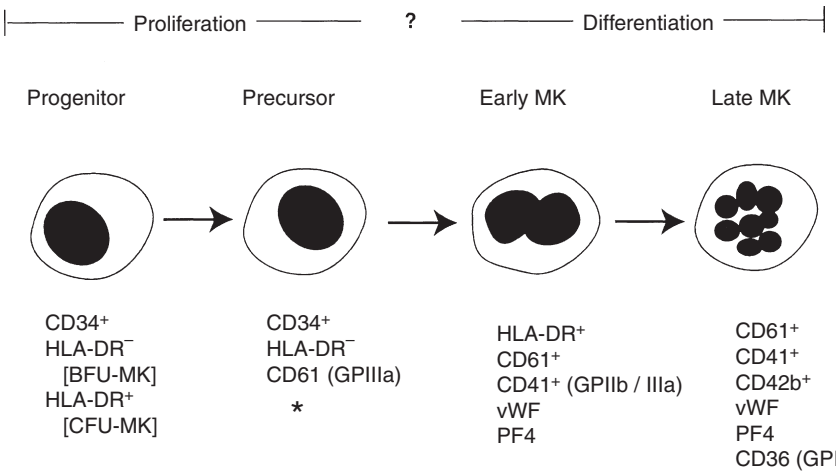


Figure 145-2 Immunologic classification of megakaryocyte (MK) development. Burst-forming unit, megakaryocyte (BFU-MK), and colony-forming unit, megakaryocyte (CFU-MK), are considered progenitor cells. CFU-MK may transition into a precursor cell that still retains proliferative potential. Fragmentary data on various stages of megakaryocyte differentiation suggest a chronologic appearance of specific platelet glycoproteins (GP) and other platelet antigens such as von Willebrand factor (vWf) and platelet factor 4 (PF4). The *asterisk* indicates that megakaryocyte antigens CD41 (GPIIb/IIIa) and CD41a (GPIIb) may be expressed on an earlier cell of the megakaryocyte lineage that retains proliferative capacity.

TABLE 145-2

Cytologic Characteristics of Megakaryocyte Maturation States and Nuclear-Cytoplasmic Ratios at Each State*

Stage	Nuclear Morphology	Cytoplasmic Staining (Wright-Giemsa)	Approximate Size Range	Demarcation Membranes	Granules	Suggested Name
I	Compact (lobed)	Basophilic	6-24 μm	Present by electron microscopy	Few present by electron microscopy	Megakaryoblast
II	Horseshoe	Pink center	10-30 μm	Proliferating to center of cell	Starting to increase	Promegakaryocyte
III	Multilobed	Increasingly more pink than blue	16-56 μm	Extensive but asymmetric	Great numbers	Granular megakaryocyte
IV	Compact but highly lobulated	Eosinophilic	20-50 μm	Evenly distributed	Organized into platelet fields	Mature megakaryocyte

Modified from Williams N, Levine RF: The origin, development and regulation of megakaryocytes. *Br J Haematol* 52:173-180, 1982.
*Much of the size variation for each stage is related to ploidy level.

Transcriptional Regulation of Megakaryocytopoiesis

The process of megakaryocytopoiesis is carefully coordinated by a number of transcription factors responsible for regulating the expression of megakaryocyte-specific genes. Some of the best understood transcription factors that play a role in megakaryocytopoiesis are summarized here.

The GATA family of transcription factors plays key roles in normal hematopoiesis. GATA-1, a 47-kDa protein with the corresponding DNA sequence mapped to the X-chromosome, is required for terminal differentiation of both erythroid and megakaryocytic cell lines. GATA-1 contains three functional domains: two zinc fingers (N- and C-) and an N-terminal activating domain. The C-finger binds to GATA-1 binding sites.The N-finger provides stability during DNA binding, and recruits the cofactor Friend of GATA-1 (FOG-1). Interaction with FOG-1 is necessary for normal hematopoiesis, as demonstrated by the presence of anemia and thrombocytopenia in individuals with *GATA1* mutations that interfere with the binding of FOG-1.[20] The N-terminal activating domain confers transcriptional activity.[21]

Specific GATA1 knockout mice models have demonstrated the necessity of GATA1 in normal hematopoiesis. GATA1-null mice die during gestation from severe anemia. GATA1 mice have normal GATA1 expression in erythroid cells but decreased expression in megakaryocytes. These mutants have thrombocytopenia without anemia, with platelet counts approximately 15% of normal. Morphologically, GATA1-deficient megakaryocytes are also abnormal: small size, large segmented nuclei, scant cytoplasm, and decreased ploidy compared with wild-type cells.[21-22] Several *GATA1* mutations have been described in patients with thrombocytopenia, usually characterized by the presence of large platelets (macrothrombocytopenia).

Calligaris and colleagues first described a short isoform of GATA-1, termed GATA-1 short (GATA-1s).[23] This isoform is 40 kDa in size, compared with the 47-kDa full-length protein, and is produced by the initiation of translation of the GATA-1 transcript at amino acid 84.The resultant protein lacks the N-terminal transactivation domain.The authors demonstrated both transcripts in K-562 cells, a human erythroleukemia cell line with the ability to differentiate into erythroid and megakaryocytic cells, and in murine fetal liver. GATA-1s plays an important role in the pathogenesis of transient myeloproliferative disorder (TMD) and acute megakaryoblastic leukemia (AMKL) seen in Down syndrome. Multiple mutations in the *GATA1* gene have been described in these patients, all of which reside within exon 2 of the GATA-1 transcript and result in the exclusive production of GATA-1s. The megakaryocyte phenotype of these patients is characterized by increased proliferation and maturational arrest of erythromegakaryocytic cells.[24]

FOG-1 is an essential cofactor for proper megakaryocytopoiesis. Single point mutations (V205M, D218G D218Y, G208S) have been described, which result in reduced GATA-1 and FOG-1 interaction and a phenotype of macrothrombocytopenia with anemia.[21] Mice engineered with complete absence of FOG-1 have a complete absence of megakaryocyte progenitors. PF4 and GPIIB (early megakaryocyte transcripts) are detectable in these models, implying an impaired differentiation and replication of early megakaryocyte lineage cells. These data taken together suggest that FOG-1 plays multiple roles in megakaryopoiesis at both early and later stages.[21,25]

NF-E2 is a transcription factor found in erythroid, megakaryocytic, and mast cells. In megakaryocytes, NF-E2 regulates B-tubulin, a major component of microtubulin. Microtubulin formation is required for proplatelet formation and subsequent release of platelets from the megakaryocyte. Loss of NF-E2 results in increased numbers of megakaryocytes with a disorganized internal membrane and impaired release of proplatelets, leading to thrombocytopenia and a lethal coagulopathy in the neonatal period with only a mild decrease in erythropoiesis.[25,26]

Friend Leukemia Integration-1 (Fli-1) is a member of the Ets family of transcription factors. It is expressed in hematopoietic cell lines as well as vascular endothelial cell lines and regulates expression of multiple megakaryocyte proteins, including GATA-1, GPIb, IIb, VI, and XI, and c-mpl. In K-562 cells, expression of Fli-1 results in increased CD-41 and CD-61 expression. In murine knockout models, loss of Fli-1 results in embryonic death due to hemorrhage (related to a state of defective vasculature) as well as dysmegakaryocytopoiesis with immature-appearing megakaryocytes that express early genes (αIIB and cMpl) but diminished expression of late genes (GPIX). Disruption of Fli-1 expression caused by a terminal deletion in chromosome 11 underlies Paris-Trousseau syndrome, which is characterized by thrombocytopenia with platelets that display large α-granules. This clinical phenotype can occur independently or as part of Jacobsen syndrome (platelet defects with cardiac anomalies, facial anomalies, mental retardation). In both instances, megakaryocytes are increased in number but have a diminished number of α-granules as well as a disorganized internal membrane.[25,27]

The Ets family of transcription factors is characterized by the presence of a highly conserved winged helix-turn-helix DNA binding domain (ETS domain) that allows recognition of purine-rich DNA sequences with a core GGA(A/T) consensus, designated EBS (Ets binding sequence). Ets-1 is up-regulated in megakaryocytopoiesis, and Ets binding sites (EBS) have been identified in many megakaryocyte-specific gene promoters. Overexpression of Ets-1 in cultured megakaryocyte cells results in a state of increased proliferation with larger megakaryocytes that have an increased number of nuclear lobes. Gene expression studies in these same cells reveals increased GATA-2, GPIIb, and PF4, indicating that Ets-1 promotes megakaryocyte differentiation.[28,29]

Cytokine Effects on Megakaryocytopoiesis

Before the cloning of Tpo, the prevalent theory was that two different growth factors were responsible for the proliferation of megakaryocyte progenitors and for the maturation of megakaryocytes. These putative factors, although not isolated, were called megakaryocyte colony-stimulating factor (MK-CSF) and Tpo. MK-CSF was thought to be the primary stimulant of proliferation, whereas Tpo was thought to stimulate the maturation of megakaryocytes, leading to increased platelet production.

CFU-MK proliferation was noted in the presence of serum from patients or animals with aplastic anemia, or when hematopoietic progenitor cells were cultured in media from human embryonic kidney cell cultures.[30,31] These therefore became widely used biologic sources of thrombopoietic factors. However, all attempts to isolate and purify MK-CSF or Tpo from these sources failed, until the fortuitous discovery in the early 1990s of a viral myeloproliferative leukemia virus oncogene *(v-mpl)*, whose structure suggested that it was a member of the hematopoietic growth factor superfamily.[32] Surprisingly, the cellular homologue of this viral oncogene (termed cellular myeloproliferative leukemia virus, or *c-mpl*) was found to be expressed almost exclusively in $CD34^+$ cells, megakaryocytes, and platelets.[33-36] These discoveries led to the hypothesis that *c-mpl* was likely the orphan receptor for Tpo, and triggered an active search for its ligand. The search culminated in 1994 with the almost simultaneous isolation of the new thrombopoietic growth factor by four different groups, each one of which assigned the protein a different name: MPL ligand,[37] Tpo,[38] megakaryocyte growth and development factor (MGDF),[39] and megapoietin.[40] Except for species-related differences, all groups sequenced the same protein: the elusive Tpo had finally been found.

Using fluorescence in situ hybridization, the gene that encodes Tpo was localized to the long arm of human chromosome 3,[41,42] and specifically to 3q26-27, an area that has been linked with thrombocytosis in acute nonlymphocytic leukemia.[43-45] The *THPO* gene

is composed of five coding exons, similar to the five erythropoietin exons.[46] The protein is composed of 332 amino acids, divided into two domains: an active amino-terminal half (153 amino acids) with marked homology to erythropoietin, and a carboxyl-terminal domain with 179 aminoacids.[41] At all stages of development (i.e., during fetal, neonatal, and adult life), the main site of Tpo production is the liver,[38,47,48] although Tpo expression has also been identified in the kidneys, spleen, and bone marrow.[47,49] In response to severe thrombocytopenia, however, the usually low level of Tpo messenger RNA (mRNA) expression in bone marrow stromal cells increases greatly.[49] One study has shown that platelet-derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2), both platelet α-granule proteins, might mediate this increase.[50]

The regulation of Tpo production has also been the object of extensive research. Early studies by several investigators demonstrated that, in steady-state and in noninflammatory conditions (i.e., idiopathic thrombocytopenia [ITP], aplastic anemia), hepatic Tpo production was constitutive; circulating Tpo levels were inversely related to the concentration of available c-mpl receptors and therefore to the platelet and megakaryocyte mass.[51-54] Although this model seems to apply to most conditions, it was challenged by the finding of elevated Tpo levels in patients with thrombocytosis associated with inflammatory conditions, such as infections or Kawasaki disease.[55] Indeed, more recent studies demonstrated that hepatic Tpo expression is up-regulated in response to interleukin-6 (IL-6) both in vitro and in vivo.[56,57] Because an anti-Tpo antibody neutralizes the thrombopoietic effect of IL-6, it is now evident that this is the pathway responsible for inflammation-induced thrombocytosis[55] (Figure 145-3).

Defects in the Tpo/c-mpl axis have also been recognized as the etiology of several human disorders. Specifically, c-mpl mutations causing frameshifts and early termination cause congenital amegakaryocytic thrombocytopenia, a disorder typically characterized by severe thrombocytopenia and absence of megakaryocytes beginning in infancy.[58,59] In contrast, activating mutations in the Tpo gene promoter[60] or in the c-mpl receptor[61] have been found in a subset of patients with familial essential thrombocythemia.

In the years following the isolation of Tpo, multiple in vitro and in vivo studies were conducted to characterize the role of this cytokine. It quickly became evident that Tpo acted both as a potent proliferative factor (MK-CSF) and as a maturational factor,[62,63] at least on murine and *adult* human megakaryocytes (see later). Initial reports that Tpo did not stimulate the maturation of human megakaryocytes likely were due to the use of *cord blood* CD34$^+$ cells in a number of studies,[64] which (unlike *adult* progenitor cells) do not generate high-ploidy megakaryocytes in response to Tpo.[65] The role of Tpo on the terminal stages of megakaryocyte maturation and proplatelet formation has been less clearly established, and it is currently believed that this cytokine does not play a major role on those processes.[66,67] In vitro studies also have suggested that Tpo plays a role in platelet activation, mostly priming the platelets to the effects of other agonists.[68] However, these effects have not been confirmed in vivo.[69,70] Tpo and *c-mpl* knockout mice exhibit platelet counts equivalent to 10% to 15% of wild-type mice and greatly decreased marrow megakaryocytes,[71,72] but the mice are otherwise healthy and viable. This suggests that Tpo is the primary regulator of platelet production, but that alternative pathways exist for megakaryocytopoiesis. Further studies also have shown that the Tpo/c-mpl axis functions in early hematopoietic progenitors (including hematopoietic stem cells), disclosing an important role for Tpo in hematopoiesis in general, in addition to its megakaryopoietic functions.[73,74] These findings have significant clinical implications for patients with disorders affecting this axis, such as individuals with congenital amegakaryocytic thrombocytopenia, who frequently progress to aplastic anemia.

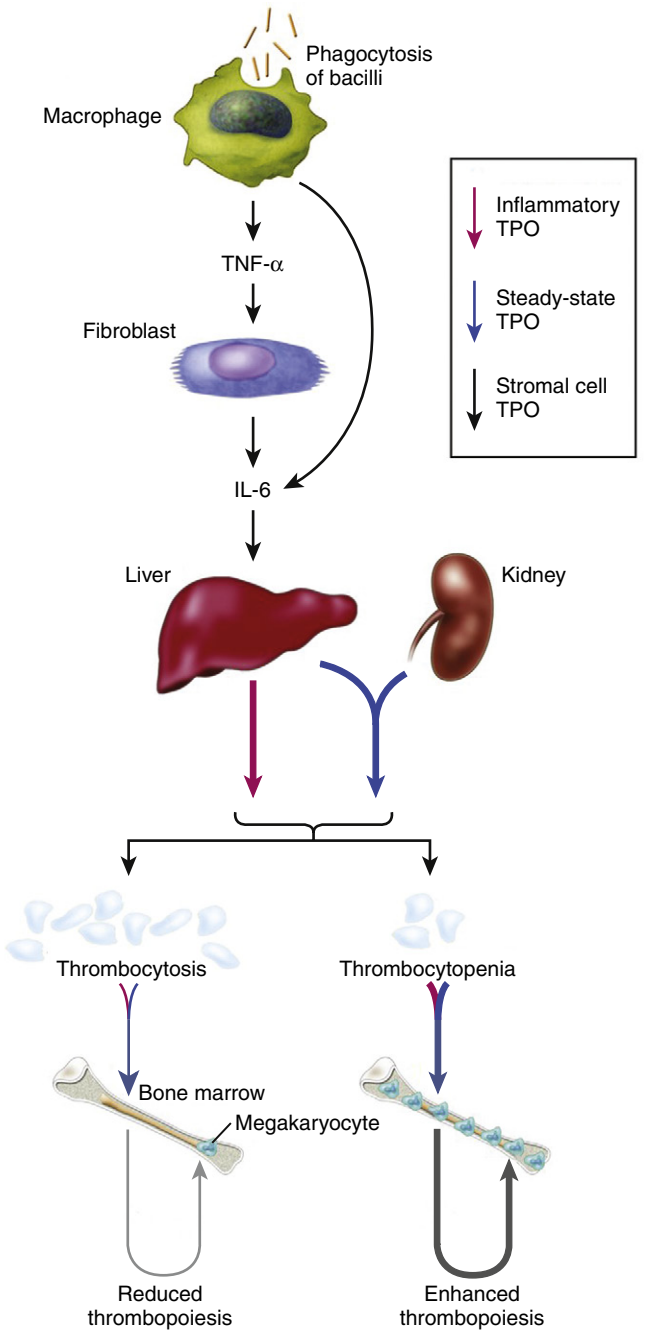


Figure 145-3 The regulation of thrombopoietin levels. A steady-state amount of hepatic thrombopoietin (TPO) is regulated by platelet c-Mpl receptor-mediated uptake and destruction of the hormone. Hepatic production of the hormone is depicted. Upon binding to platelet c-Mpl receptors, the hormone is removed from the circulation and destroyed, which reduces blood levels. In the presence of inflammation, interleukin-6 (IL-6) is released from macrophages and, through tumor necrosis factor-α (TNF-α) stimulation, from fibroblasts and circulates to the liver to enhance TPO production. Thrombocytopenia also leads to enhanced marrow stromal cell production of TPO, although the molecular mediator of this effect is not yet completely understood. *(From Kaushansky K: The molecular mechanisms that control thrombopoiesis,* J Clin Invest *115:3339-3347, 2005.)*

The cloning of Tpo in 1996 led to a flurry of studies that quickly progressed from bench research to animal studies and then to clinical trials, mostly using the full-length recombinant Tpo (rTpo) molecule or a recombinant human polypeptide that contains the receptor-binding N-terminal domain of Tpo (termed *megakaryocyte growth and development factor*, or rhMGDF). When injected into normal animals, rTpo or rhMGDF induced significant thrombocytosis, with peak mean platelet counts up to 670% of baseline.[69,75-78] When administered to animals exposed to myelosuppressive chemotherapy, rhMGDF ameliorated or completely prevented the associated thrombocytopenia[75] and also accelerated red blood cell and neutrophil recovery.[79,80] Most human studies were conducted using a pegylated form of rhMGDF (PEG-rhMGDF, a molecule 5 to 10 times more potent in vivo than the unconjugated polypeptide).[69] In phase I and II clinical trials, rTpo and PEG-rhMGDF potently stimulated platelet production in nonthrombocytopenic adults[81,82] and in patients with chemotherapy-induced thrombocytopenia.[83,84] However, trials showed limited efficacy in the setting of thrombocytopenia associated with myeloablation and stem cell transplantation.[85] In addition, a number of subjects treated with PEG-rhMGDF developed neutralizing antibodies against endogenous Tpo, which resulted in severe thrombocytopenia and aplastic anemia.[86] Ultimately, these complications led to the discontinuation of clinical trials involving any of these forms of recombinant Tpo.

As an alternative, much interest was directed to the development of thrombopoietin-mimetic molecules. These are small molecules that have no sequence homology to Tpo but bind to the Tpo receptor and have biologically comparable effects. The lack of homology represents a significant advantage over recombinant forms of Tpo because it should preclude the development of cross-reactive neutralizing antibodies against endogenous Tpo. At least five different thrombopoietin receptor agonists have been described,[85-87] and three have been studied in human subjects.[88-91] In 2006, Bussel and colleagues published a phases I and II trial of AMG 531 for the treatment of patients with ITP.[89] AMG 531 is an engineered peptibody composed of a recombinant protein carrier FC domain linked to multiple c-mpl binding domains. Forty-five thrombocytopenic patients who had received at least one previous treatment for ITP were enrolled in a phase I or II trial of AMG 531 therapy (24 and 21 patients, respectively). The phase I study demonstrated a dose-dependent increase in platelet counts in response to AMG 531. Overall, a platelet count of at least 50×10^9/L was achieved in 7 of 12 patients given two doses of 3, 6, or 10 μg of AMG 531. In the phase II study, 12 of 16 patients treated with six weekly doses of 1 or 3 μg/kg of AMG 531 reached or exceeded the targeted platelet range (50 to 450×10^9/L). None of the patients had neutralizing antibodies to AMG 531 or thrombopoietin, and the only adverse events attributable to the study drug were headache and transient posttreatment worsening of the thrombocytopenia. Similar results were reported by Newland and co-workers,[90] who performed a phases I and II multicenter trial in Europe in which 16 patients received escalating doses of AMG 531 (30, 100, 300, or 500 μg administered subcutaneously on days 1 and 15 of study). Platelet counts increased to or above the targeted range in 8 of 11 patients who received a dose equivalent to 1 μg/kg or more. The most frequently reported adverse event was headache, and one patient experienced a transient increase in lactic dehydrogenase. No anti–AMG 531 or anti-Tpo antibodies were detected. In 2007, Jenkins and associates[88] also published a phase I placebo-controlled clinical study involving Eltrombopag (SB-497115), an oral, nonpeptide thrombopoietin receptor agonist. In this trial, 73 healthy male subjects received eltrombopag as once-daily oral capsules for 10 days, at doses of 5, 10, 25, 30, 50, and 75 mg/day. Eltrombopag increased platelet counts in a dose-dependent manner, without adverse events attributable to the study drug. In 2008, both AMG 531 (romiplostim) and eltrombopag were approved by the Food and Drug Administration for the treatment of adults with chronic ITP.

Many other growth factors affect megakaryocyte colony growth in vitro. IL-11 also increases megakaryocyte proliferation in vitro, but not in the absence of other thrombopoietic stimulants.[92] Recombinant IL-11 (rIL-11) has been released for clinical use and is approved by the U.S. Food and Drug Administration as a thrombopoietic agent. Studies have shown that rIL-11 stimulates megakaryocytopoiesis in adults and children.[93,94] However, it is also associated with significant flulike symptoms and fluid retention. In addition, at least in one study involving patients with refractory ITP, rIL11 administration resulted in significant side effects combined with a lack of efficacy, leading to early termination of the trial.[95]

Granulocyte-macrophage CSF (GM-CSF) and IL-3 stimulate megakaryocytopoiesis, alone or in combination.[96,97] A synergistic effect of IL-3 and GM-CSF has also been documented.[97] IL-6 affects megakaryocytopoiesis *in vitro*, and megakaryocyte maturation in particular.[98-100] IL-6 also promotes CFU-MK proliferation of in vitro human marrow cultures, although the effect is less than that seen with IL-3 or GM-CSF.[101] The IL-6 receptor has been identified on megakaryocytes by in situ hybridization techniques, and the administration of IL-6 to suspension cultures increases ploidy.[102] In nonhuman primates, recombinant human IL-6 has been shown to affect megakaryocytopoiesis in vivo.[103-105] Recombinant IL-6 (5 to 80 μg/kg/day), injected twice daily for 14 days, caused dose-related increases in platelets and a shift to larger megakaryocytes in the bone marrow, indicating a maturational effect.[103] In a clinical phases I and II study, recombinant human IL-6 was administered to 20 patients with cancer.[106] Doses of 0.5 to 20 μg/kg/day were used. Increases in platelet counts were noted at doses higher than 1 μg/kg/day. Side effects included fever, headache, myalgia, and erythema. At doses higher than 2.5 μg/kg, nausea, elevated liver enzymes, and anemia were noted.

Numerous additional growth factors participate in the process of megakaryocytopoiesis, although their roles have been less extensively studied. These include erythropoietin,[107] stem cell factor,[108-109] vascular endothelial growth factor (VEGF),[110,111] IL-1,[112] stromal cell–derived factor (SDF),[113,114] basic FGF,[114,115] interferon-γ,[116] PDGF,[117] and nicotinamide.[118] Some factors have been shown to be potent inhibitors of thrombopoiesis, including transforming growth factor-TGF-β[119] and platelet factor 4 (PF4).[120,121]

FETAL AND NEONATAL MEGAKARYOCYTOPOIESIS

Fetal Megakaryocyte Progenitors

The study of fetal megakaryocytopoiesis is intrinsically difficult because of the low frequency of megakaryocytes in fetal bone marrow and the limited availability of neonatal bone marrow specimens. Because of that, most studies have been performed using umbilical cord blood, from which large quantities of megakaryocyte progenitors can be readily obtained. Olson and associates[122] found a significantly higher concentration of CFU-MK progenitors in term and preterm cord blood than in adult peripheral blood. Furthermore, when stimulated with previously irradiated aplastic canine serum, cord blood CFU-MK-derived colonies contained significantly more megakaryocytes than adult colonies. Zauli and co-workers[123] compared fetal blood– and adult bone marrow–derived megakaryocyte colonies. Hematopoietic progenitors ($CD34^+$ cells) were obtained from the blood of fetuses between 18 and 22 weeks gestation or from adult bone marrow and were cultured in a fibrin clot assay. They found that, as in adult blood, BFU-MKs predominate in fetal blood, with a CFU-MK–BFU-MK ratio of 0.4:1. Fetal and adult megakaryocyte progenitors had similar immunologic profiles with regard to the expression of CD34 and HLA-DR. However, there were significant differences in the morphology and size of the BFU-MK-derived colonies: the fetal BFU-MK-derived colonies were significantly larger than the adult colonies and were

usually composed of only one or two foci of development, whereas the adult BFU-MK-derived colonies were always multifocal.

Other investigators also reported the existence of a megakaryocyte progenitor with an unusually high proliferative potential in the human fetal bone marrow. Bruno and colleagues[124] described a high proliferative potential cell–megakaryocyte (HPPC-MK) in fetal bone marrow that gives rise to large, unifocal colonies with more than 300 cells. This cell, not observed in adult bone marrow cultures, may represent a more primitive megakaryocyte progenitor. Nishihira and associates[125] cultured cord blood hematopoietic progenitors (26 to 41 weeks gestation) with optimal concentrations of rTpo and found that all colonies consisted of more than 50 cells and that 50% had more than 500 cells. It is unclear whether these colonies should be classified as BFU-MK- or HPPC-MK-derived colonies.

The development of miniaturized assay systems to study megakaryocyte progenitors made it possible to study these cells in the peripheral blood of neonates. Using such a technique, Murray and collaborators[126] showed that preterm neonates (24 to 36 weeks gestation) in the first day of life had higher circulating concentrations of all megakaryocyte progenitors than term neonates. This finding was in concordance with the previously described gestational age-related decrease in the concentration of other committed hematopoietic progenitors.[127] Saxonhouse and associates[128] also observed an inverse relationship between the concentration of circulating megakaryocyte progenitors and the postconceptional age (gestational age + weeks of life) of growing preterm infants being cared for in the neonatal intensive care unit, suggesting that the gestational age–dependent decrease in circulating megakaryocyte progenitors is a process that continues after birth in infants born prematurely.

Based on the premise that circulating megakaryocyte progenitors are a reflection of bone marrow megakaryocytopoiesis, investigators applied these assays to the evaluation of thrombocytopenic neonates. Murray and Roberts[129] quantified circulating megakaryocyte progenitors in preterm neonates with early thrombocytopenia secondary to maternal preeclampsia or intrauterine growth restriction. They observed that the concentration of circulating progenitors in those neonates was significantly lower than in nonthrombocytopenic preterm neonates, and increased as the thrombocytopenia resolved, suggesting that decreased platelet production underlies this variety of thrombocytopenia. Other investigators have used the concentration of circulating megakaryocyte progenitors in combination with other tests to evaluate the mechanisms of thrombocytopenia in neonates with congenital HIV infection[130] and congenital amegakaryocytic thrombocytopenia.[131]

Fetal and Neonatal Megakaryocytes

The yolk sac appears to be the first hematopoietic site.[132] Megakaryocytes have been observed in the yolk sac by 5 weeks gestation,[133] and the first platelets appear in the circulation at 8 to 9 weeks.[134] The transition to hepatic hematopoiesis is thought to involve migration of stem cells from the yolk sac to the liver.[135] Bleyer and colleagues documented that megakaryocytes were present in liver and spleen tissue by 10 weeks gestation.[136] Every stage of megakaryocyte development could be found in the liver phase, although megakaryocytes at every stage were smaller than their adult counterparts.[137-139] Fetal bone marrow hematopoiesis has been documented as early as 11 weeks gestation, when megakaryocytes constitute 0.58% of the nucleated cells (this percentage increases to 1.2% by week 22).[140] By 18 weeks gestation, hematopoiesis is well established in the fetal bone marrow.[139,141-143]

Using different cell sources and techniques, several investigators evaluated the size and ploidy of megakaryocytes in fetuses and neonates. Allen Graeve and de Alarcon[143] studied the size and maturational stage of fetal bone marrow megakaryocytes from 20 human fetuses with 12 to 21 weeks gestation. They reported that, at all stages of maturation, fetal megakaryocytes were substantially smaller than adult megakaryocytes (Table 145-3). There were also less stage IV megakaryocytes in fetal bone marrow compared with adult marrow, reflecting a shift toward less mature megakaryocytes during fetal life (Table 145-4). These investigators[143] also reported lower megakaryocyte ploidy levels in fetal bone marrow biopsy specimens compared with adult bone marrow megakaryocytes.

TABLE 145-3

Mean (SD) Diameter of Megakaryocytes* in Biopsy Specimens

Stage	Fetal (μm)	Adult (μm)	*p* Value
I	12.2 (1.2)	15.2 (3.9)	.2
II	15.8 (1.1)	22.4 (2.4)	.003
III	20.7 (4.9)	29.2 (1.3)	<.001
IV	22.6 (5.4)	29.3 (2.4)	<.001
Total	15.2 (1.4)	20.6 (4.1)	.04

Modified from Allen Graeve JL, de Alarcon PA: Megakaryocytopoiesis in the human fetus. *Arch Dis Child* 64:483, 1989.

*Megakaryocytes identified by staining with anti-glycoprotein IIb antibody.

TABLE 145-4

Percentage Distribution of Megakaryocytes* by Age

Stage	Fetal (%)	Adult (%)	*p* Value
I	50	49	.7
II	28	22	.004
III	14	13	.3
IV	8	16	<.001
Total	100	100	<.001

*Megakaryocytes identified by staining with antiglycoprotein IIb antibody.

Hegyi and co-workers[144] cultured megakaryocytes from livers obtained from 8- to 20-week fetuses and from cord blood and compared their size, maturity, and ploidy with that of cultured adult bone marrow megakaryocytes. These investigators found a progressive shift to higher ploidy classes and to larger megakaryocytes during development. Using ultrastructural techniques, they also evaluated the maturity stage of the very small liver megakaryocytes and found that they had all the cytoplasmic components of a mature cell, with multiple granules and a well-developed demarcation system. These findings supported previous observations by Kelemen and associates, who stated that "megakaryocytes with only one to four nuclei produce the platelets of the fetus."[145] Later studies by Ma and co-workers[146] also reported a developmentally regulated shift toward larger and more mature megakaryocytes during gestation. Despite this shift, however, 78% of megakaryocytes in the bone marrow of fetuses with 7 to 8 months gestation had a ploidy of 8 N or less, compared with only 33% of the megakaryocytes in adult bone marrow.

Similar results to those evaluating cultured megakaryocytes or fetal necropsy specimens (which can be associated with artifacts) were also obtained when megakaryocytes elutriated from term cord blood were examined. Levine and associates[147] reported that cord blood contained significantly larger numbers of circulating megakaryocytes than adult blood. As in the fetus, cord blood megakaryocytes were significantly smaller and had less nuclear lobulations than adult megakaryocytes but were otherwise mature cells. These investigators suggested that these small but mature cells were the result of a left shift in the megakaryocyte ploidy.

In summary, megakaryocytes of fetuses and neonates are smaller and of lower ploidy than those of adults. In 2002, Mattia

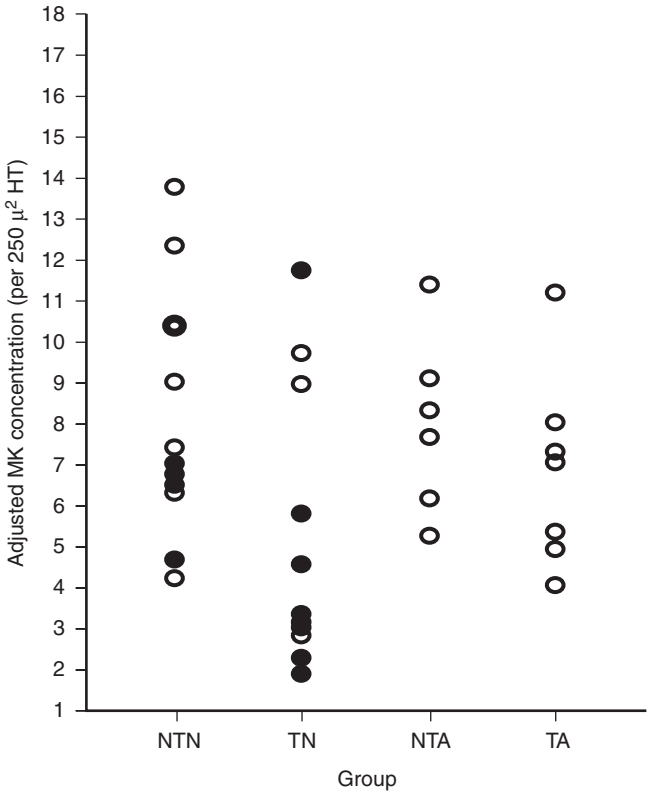


Figure 145-4 Megakaryocyte concentration (per 250 μm² of hematopoietic tissue), after correcting for differences in cellularity, in nonthrombocytopenic neonates (NTN), thrombocytopenic neonates (TN), nonthrombocytopenic adults (NTA), and thrombocytopenic adults (TA) (chronic idiopathic thrombocytopenia [ITP]). Bone marrow megakaryocytes were immunohistochemically stained with an anti-CD61 antibody and quantified using an eyepiece reticle that measured 250 μm² in nine small squares. After adjusting for differences in cellularity between the samples (particularly between neonatal and adult samples), the megakaryocyte concentration was expressed as megakaryocytes per 250 μm² of hematopoietic tissue. As evident in the figure, thrombocytopenic neonates had decreased, normal, or increased concentrations of megakaryocytes, likely reflecting the different etiologies of the thrombocytopenia. *(Modified from Sola-Visner M, Christensen RD, Hutson AD, Rimsza LM: Megakaryocyte size and concentration in the bone marrow of thrombocytopenic and nonthrombocytopenic neonates,* Pediatr Res *61:481, 2007.)*

and associates demonstrated that decreased ploidy levels are associated with decreased levels of platelet production.[15] Thus it is reasonable to assume that megakaryocytes of neonates produce fewer platelets than their adult counterparts and that the normal platelet counts found in *healthy* fetuses and neonates are maintained by the increased proliferative rate of fetal and neonatal megakaryocyte progenitors. To determine whether (and how) these developmental differences affect the ability of neonates to increase platelet production in response to thrombocytopenia, our group examined the megakaryocyte concentration and size in the bone marrow of 13 thrombocytopenic and 13 nonthrombocytopenic neonates, using a combination of immunohistochemistry and image analysis. We observed significant variability in marrow megakaryocyte concentration among thrombocytopenic neonates, with some neonates having less, some similar, and some increased megakaryocytes compared with their nonthrombocytopenic counterparts (Figure 145-4). In regard to the megakaryocyte diameter, we confirmed that megakaryocytes were smaller in neonates than in adults and observed that thrombocytopenic neonates (unlike adults) did not exhibit an increased percentage of large megakaryocytes, compared with nonthrombocytopenic neonates (Figure 145-5). These findings suggested that adult megakaryocytes respond to thrombocytopenia by increasing cell size, whereas neonatal megakaryocytes do not. The mechanisms underlying the small size of neonatal megakaryocytes are not clearly understood but likely involve a combination of cell-intrinsic factors and factors in the fetal and neonatal microenvironment. This was demonstrated in 2005 by Slayton and associates,[148] who transplanted neonatal liver cells or adult bone marrow cells from green fluorescent protein (GFP) transgenic mice into wild-type adult recipients and evaluated the size and ploidy of the donor-derived (GFP^+) megakaryocytes. Megakaryocytes derived from neonatal stem and progenitor cells, placed in an adult environment, were significantly larger and of higher ploidy than neonatal megakaryocytes in their original environment (fetal liver). However, they were still smaller than posttransplantation megakaryocytes derived from adult bone marrow cells. In vitro studies culturing cord blood $CD34^+$ cells in adult bone marrow stromal cell–conditioned media also yielded megakaryocytes with higher ploidy levels than those cultured in fetal bone marrow stromal cell–conditioned or in unconditioned serum-free media.[65] Taken together, these findings suggest that the environment influences the size and ploidy of megakaryocytes (with the adult environment being more conducive to megakaryocyte maturation) but that cell-intrinsic factors limit the ultimate size and ploidy that neonatal megakaryocytes achieve. Adult-size megakaryocytes have been found by 2 years of age, although insufficient data exist on children from birth to 1 year of age, owing to the lack of normal marrow specimens.

Thrombopoietin in Neonates

After the identification of Tpo as the primary regulator of megakaryocytopoiesis, several investigators evaluated Tpo concentrations in cord blood and in the blood of neonates.[47,149-154] Overall, median Tpo concentrations in neonates ranged between 76 and 191 pg/mL and were slightly higher in serum than in plasma. With the exception of the study by Albert and collaborators,[154] which reported higher Tpo concentration in preterm compared with term neonates, these studies found no obvious gestational age–related changes in plasma or serum Tpo concentrations and no correlation with platelet counts. However, all studies reported Tpo concentrations in neonates that were threefold to fourfold higher than in healthy adults. The reasons underlying this finding are unclear and might involve an increased rate of Tpo production, decreased Tpo clearance, alternative mechanisms of Tpo regulation, or a reduced number of *c-mpl* receptors.[155] In support of the last hypothesis, Kuwaki and associates described a significantly lower number of *c-mpl* receptors in megakaryocytes derived from cord blood cells than in those derived from adult bone marrow cells.[156] To determine when Tpo levels transitioned from normal neonatal to adult concentrations, Ishiguro and collaborators[153] measured Tpo concentrations in healthy neonates, children, and adults. In this study, Tpo concentrations increased shortly after birth, reached a peak on the second day of life, and returned to cord blood levels by the end of the first month. They then decreased gradually until adult levels were reached (Figure 145-6).

Several investigators also evaluated Tpo concentrations in neonates with different varieties of thrombocytopenia,[130,131,154,157-159] and some correlated those with other measures of thrombopoiesis, such as marrow megakaryocytes[130,131,152] or circulating megakaryocyte progenitors.[130,131,149,150] Overall, these investigators found great variability in Tpo concentrations among thrombocytopenic neonates and no strong correlation with platelet counts, probably reflecting the different mechanisms underlying

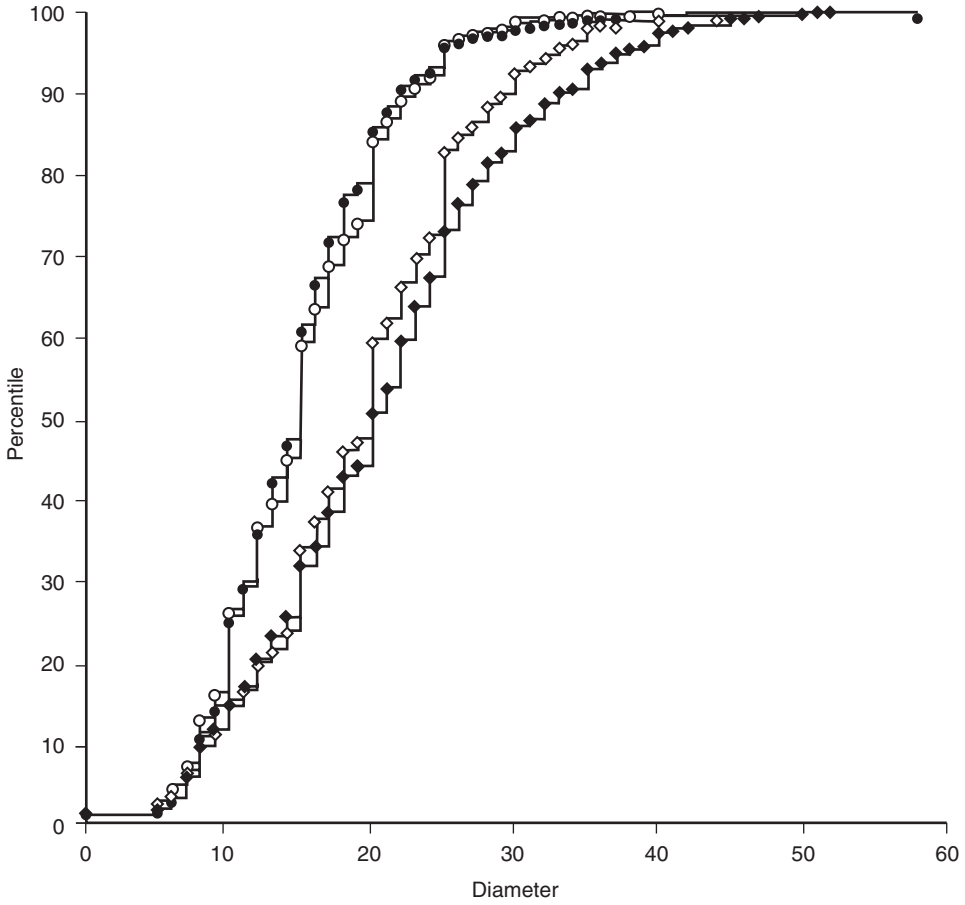


Figure 145-5 Cumulative distribution plots of megakaryocyte diameters in the bone marrow of thrombocytopenic neonates *(filled circles)*, nonthrombocytopenic neonates *(open circles)*, thrombocytopenic adults *(filled diamonds)*, and nonthrombocytopenic adults *(open diamonds)*. Megakaryocytes in marrow histologic sections were immunohistochemically stained with an anti-CD61 antibody and measured with an automated image analysis system. The megakaryocyte diameters are displayed on the *x* axis and their cumulative distribution on the *y* axis. Both adult curves are shifted to the right compared with the neonatal curves, indicating the predominance of larger megakaryocytes in these samples. Furthermore, the curve for the thrombocytopenic adults is also shifted to the right compared with that for the nonthrombocytopenic adults, indicating a higher percentage of large megakaryocytes. In contrast, there is no difference in the megakaryocyte size distribution of thrombocytopenic compared to non-thrombocytopenic neonates. *(Modified from Sola-Visner M, Christensen RD, Hutson AD, Rimsza LM: Megakaryocyte size and concentration in the bone marrow of thrombocytopenic and nonthrombocytopenic neonates,* Pediatr Res *61:482, 2007.)*

the different varieties of thrombocytopenia. In studies in which hyporegenerative thrombocytopenia was suspected on the basis of decreased marrow megakaryocytes or circulating megakaryocyte progenitors, however, Tpo concentrations were less elevated than those reported in adults with thrombocytopenia owing to decreased platelet production.[149,152]

The sites of Tpo production, as detected by reverse transcriptase polymerase chain reaction, were similar in fetuses, neonates, and adults, with the liver accounting for more than 90% of the total Tpo mRNA expression among liver, kidneys, and spleen.[47,48] The response to Tpo, however, has been shown to be substantially different in neonates than in adults. First, studies by several investigators demonstrated that neonatal megakaryocyte progenitors generated significantly larger colonies with more megakaryocytes than adult bone marrow cells.[125,160] Furthermore, when the effects of Tpo on neonatal versus adult bone marrow light-density mononuclear cells were compared, marrow cultures from either thrombocytopenic or nonthrombocytopenic neonates yielded three times more megakaryocyte colonies that adult marrow cultures.[161] These neonatal colonies also contained more megakaryocytes and were more sensitive to Tpo (i.e., the highest number of colonies was reached at a Tpo concentration of 10 ng/mL, compared with 50 ng/mL for adult cultures). These findings were consistent with the pronounced increase in platelet counts observed in newborn rhesus monkeys treated with even low doses of PEG-rHuMGDF.[162]

Pastos and associates[65] also evaluated the effects of Tpo on the ploidy of adult and neonatal megakaryocytes cultured in either serum-free media or adult bone marrow stromal cell–conditioned media with increasing concentrations of Tpo. This study disclosed opposite effects of Tpo on the ploidy of neonatal compared with adult megakaryocytes. Although adult megakaryocytes reached highest ploidy levels when cultured in serum-free medium with maximal concentrations of rTpo, neonatal megakaryocytes reached their highest ploidy when cultured in marrow stromal cell–conditioned media in the absence of rTpo; the addition of supraphysiologic concentrations of rTpo (≥0.1 ng/mL) inhibited their polyploidization (Figure 145-7). This study provided the first evidence that human neonatal and adult megakaryocytes have substantially different biologic responses to Tpo, and potentially to other cytokines. These differences will have to be taken into account when considering the use of thrombopoietic growth factors in the clinical setting of neonatal thrombocytopenia.

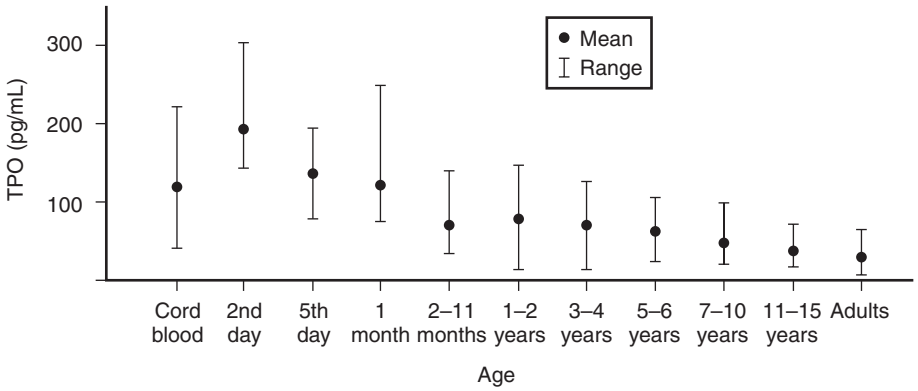


Figure 145-6 Age-related changes in serum thrombopoietin (TPO) levels in disease-free children. TPO concentrations were measured in 254 serum samples, collected from 25 cord blood samples, 200 healthy children, and 29 healthy adults. *Filled circles* indicate the means; *bars* represent the ranges. *(Modified from Ishiguro A, Nakahata T, Matsubara K, et al. Age-related changes in thrombopoietin in children: reference interval for serum thrombopoietin levels.* Br J Haematol *106:884-888, 1999; and from Dame C: Thrombopoiefin in thrombocy to penias of childhood,* Semin Thromb Hemost *27:219, 2001.)*

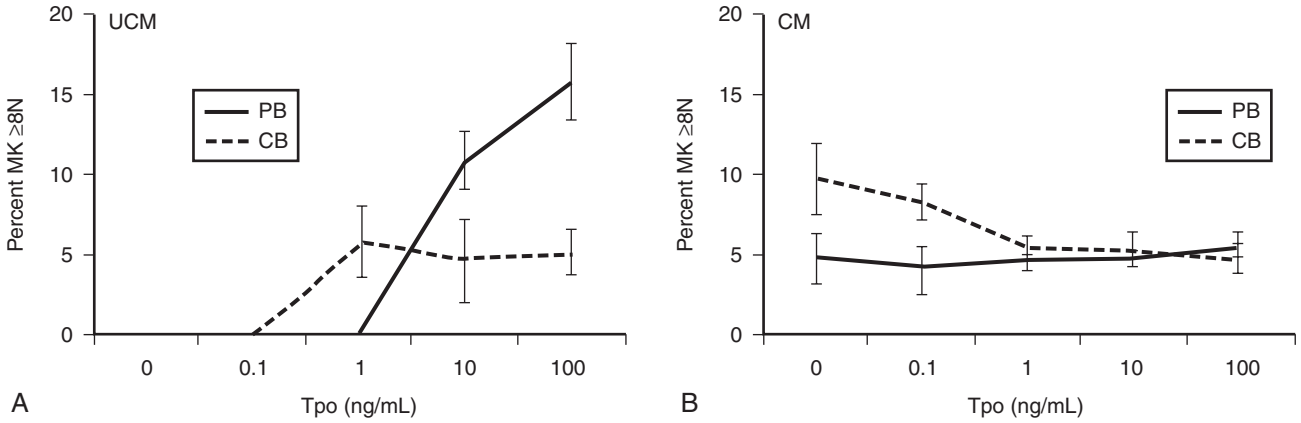


Figure 145-7 The percentage of megakaryocytes (MKs) that were 8 N or greater in PB and CB cultures differed depending on media source and recombinant thrombopoietin (rTpo) concentration. PB- and CB-CD34$^+$ cells were cultured for 14 days in unconditional media (UCM) **(A)** and conditional media (CM) **(B)**, with varying rTpo concentrations. PB-derived MKs *(solid lines)* cultured in UCM **(A)** exhibited a rTpo dose-dependent increase in ploidy levels, an effect inhibited by the presence of CM **(B)**. CB-derived MKs *(dashed lines)* reached highest ploidy levels when cultured in CM with no rTpo, an effect that was reversed by rTpo concentrations of 1 ng/mL or greater **(B)**. Data shown represent the means and standard error of the mean (SEM) of four separate experiments. *(Modified from Pastos, K, et al.,* Blood *108:3361, 2006.)*

More recently, Liu and collaborators[163] demonstrated that neonatal megakaryocytes generated in the presence of Tpo only are fully cytoplasmically mature, despite their small size and ploidy, indicating that neonatal megakaryocytes exhibit diffrent maturational patterns compared to adult magakaryocytes.

NEONATAL THROMBOCYTOPENIA

Platelet Counts

Platelets are produced very early in the fetus, and platelet counts rise in a linear fashion throughout gestation.[164] Premature infants, on average, have a slightly lower platelet count than term infants, but it remains within the same range observed in healthy older children and adults (i.e., 150,000 to 450,000/μL).[165] Appleyard and Brinton[166] evaluated platelet counts in low-birth-weight infants during the first 4 weeks of life (Table 145-5). In this study, platelet counts lower than 100,000/mm^3 were uncommon, and counts less than 50,000/mm^3 were usually associated with some clinical abnormality. As evident in Table 145-5, and described by other investigators, platelet counts rose for several weeks after delivery.[167,168] In term infants, platelet counts are equivalent to those in older children and adults.[166,169]

The incidence of thrombocytopenia in neonates is extremely variable, depending on the population examined. Large studies evaluating platelet counts in thousands of unselected cord blood samples reported an incidence of neonatal thrombocytopenia of 0.7% to 0.9%, depending on the definition of the thrombocytopenia.[170,171] Severe thrombocytopenia (platelet count < 50,000/μL) was present at birth in approximately 0.1% of neonates, but the incidence increased to 0.28% if infants whose platelet counts dropped below that threshold during the first few days of life were included.[171,172]

The relatively low frequency of thrombocytopenia in the general population of neonates is in marked contrast with its high incidence among sick neonates. Mehta and co-workers[173] reported an incidence of 35% among neonates admitted to the neonatal intensive care unit (NICU). Castle and colleagues evaluated platelet counts in 807 consecutive infants admitted to a regional NICU and found thrombocytopenia in 22%. Twenty percent of those infants had platelet counts below 50,000/μL.[174] In

TABLE 145-5

Comparison of Venous Platelet Counts in Low-Birth-Weight Infants According to Birth Weight

	1700-2500 g			<1700 g		
Day	No. of Infants	Mean/mm³	Range (000s/mm³)	No. of Infants	Mean/mm³	Range (000s/mm³)
1	40	202,000	80-356	20	205,000	102-292
3	29	211,000	130-335	18	201,000	61-315
5	9	245,000	100-502	5	211,000	112-345
7	36	319,000	124-678	16	320,000	164-510
10	27	406,000	245-680	13	385,000	172-609
14	33	408,000	262-670	17	343,000	147-460
21	31	403,000	259-720	16	384,000	201-470
28	23	357,000	212-625	17	421,000	278-600

From Appleyard WJ, Brinton A: Venous platelet counts in low birth weight infants. *Biol Neonate* 17:30-34, 1971.

a somewhat smaller study, Oren and associates observed an incidence of 18.2% among preterm neonates and 0.8% among term neonates.[175]

CAUSES OF THROMBOCYTOPENIA IN NEONATES

Thrombocytopenia in the neonatal period can be caused by a large number of disease processes. Several different systems to classify neonatal thrombocytopenia have been proposed, some based on the suspected kinetic mechanism (increased platelet consumption versus decreased production),[176,177] and others based on the time of onset (early versus late thrombocytopenia).[178-180] Our group recently published recommendations for the evaluation of thrombocytopenic neonates based on recognizing typical patterns of thrombocytopenia, which incorporate factors such as time of onset, severity, duration of thrombocytopenia, and suspected mechanism (Table 145-6).[181] These patterns are associated with specific pathophysiologic processes that can cause thrombocytopenia: immune, infectious, genetic, drug-induced, disseminated intravascular coagulation (DIC), placental insufficiency, and miscellaneous causes. Although not all-inclusive, this classification encompasses most causes of neonatal thrombocytopenia.

Immune-mediated Thrombocytopenia

In 1953, Harrington and co-workers[182] described a variety of thrombocytopenia that affects healthy neonates who have no other evidence of disease. The pathogenesis of this type of thrombocytopenia, now known as *neonatal alloimmune thrombocytopenia* (NAIT), resembles that of erythroblastosis fetalis in that it is mediated by maternal antibodies directed against antigens on fetal platelets. When incompatibility between parental platelet antigens exists, the mother can become sensitized to an antigen (inherited from the father) expressed on the fetal platelets. These antibodies can then cross the placenta and coat the fetal platelets, which are then removed from the circulation by the fetal reticuloendothelial system. Unlike neonatal Rh disease, however, the first pregnancy can produce an affected fetus.

The study of NAIT has been complicated by the multiple antigens expressed on platelets and by the fact that the nomenclature for these antigens has undergone multiple changes during the past few decades. To standardize the nomenclature, platelet antigens have been now designated human platelet antigens (HPAs). Different allelic forms are distinguished by an "a" or "b" suffix, with "a" indicating the most common and "b" indicating the uncommon allele[176] (Table 145-7). The most common cause of NAIT is alloantibodies against HPA-1a, formerly known as PLA-1, which accounts for 75% to 90% of all cases of NAIT. However, the frequency with which a particular antigen is responsible for cases of NAIT depends on the relative frequency of this antigen in that population. For example, although HPA-1a is the most common antigen responsible for NAIT in the white population (in which 68.9% of the population has the genotype HPA-1a/HPA-1a), HPA-4 is most frequently implicated among Asian subjects.[176] Cases mediated by antibodies against HPA-5,[183] HPA-3,[184] HPA-2,[185] and HPA-6[186] have also been described, although with much lower frequency. In addition, immunization against "low frequency" or "private" platelet alloantigens might account for a higher percentage of NAIT than previously recognized.[187]

TABLE 145-6

Classification of Etiologies of Thrombocytopenia, Including Severity, Onset, Time to Resolution, and Likely Mechanism

Categories	Subtypes	Severity	On	Time to Resolution	Mechanism
Immune	Alloimmune	Severe	Early	Days to weeks	↑ Consumption
	Autoimmune	Moderate	Early	Weeks to months	↑ Consumption
Infections	Bacterial	Variable	Variable	1-7 days	Mixed
	Viral	Variable	Early	Variable	Mixed
	Fungal	Severe	Late	2-7 days	Mixed
Genetic Disorders	Chromosomal	Moderate	Early	Days to weeks	↓ Production
	Bone marrow failure	Severe	Early*	Variable†	↓ Production
	Familial thrombocytopenias	Mild to moderate	Early	Never	↓ Production
Drugs		Moderate to severe	Late	Median, 8 days‡	Variable
DIC		Severe	Variable	Variable	↑ Consumption
PIH and IUGR		Mild to moderate	Early	7-10 days	↓ Production
NEC		Moderate to severe	Late	7-10 days	↑ Consumption

Sola MC: Evaluation and treatment of severe and prolonged thrombocytopenia in neonates. *Clin Perinatol* 31:1-14, 2004.

*Most congenital bone marrow failure syndromes are present at birth (i.e., congenital amegakaryocytic thrombocytopenia or thrombocytopenia-absent radii). However, the hematologic manifestations of Fanconi anemia usually do not appear until childhood.

†Thrombocytopenia associated with TAR syndrome usually resolves before school age. Other thrombocytopenias associated with bone marrow failure do not improve.

‡After discontinuation of the offending agent.

DIC, Disseminated intravascular coagulation; *IUGR,* intrauterine growth restriction; *NEC,* necrotizing enterocolitis; *PIH,* pregnancy-induced hypertension.

Platelet antigens appear in the fetus early in gestation, and the maternal antibodies can cross the placenta early in the second trimester, thereby inducing severe fetal thrombocytopenia. Bussel and co-workers[188] studied 107 fetuses who had siblings with NAIT, at a mean gestational age of 25 ± 4 weeks. Fifty percent of them had initial platelet counts of 50,000/μL or less, including 21 fetuses evaluated before 24 weeks gestation. The only good predictive factor for severe thrombocytopenia was a history of antenatal intracranial hemorrhage in the sibling.

Overall, NAIT should be suspected in any neonate without obvious clinical illness or malformations, who presents with thrombocytopenia, which is typically severe. Intracranial hemorrhage is the most perilous complication and occurs in 10% to

TABLE 145-7

Classification of Human Platelet Antigens, including Original Name, Associated Glycoprotein, and Cell Surface Marker

System	Antigen	Original Names	Glycoprotein	CD
HPA-1	HPA-1a	Zw^a, PI^{A1}	GPIIIa	CD61
	HPA-1b	Zw^b, PI^{A2}		
HPA-2	HPA-2a	Ko^b	GPIba	CD42b
	HPA-2b	Ko^a, Sib^a		
HPA-3	HPA-3a	Bak^a, Lek^a	GPIIb	CD41
	HPA-3b	Bak^b		
HPA-4	HPA-4a	Yuk^b, Pen^a	GPIa	CD61
	HPA-4b	Yuk^a, Pen^b		
HPA-5	HPA-5a	Br^b, Zav^b	GPIa	CD49b
	HPA-5b	Br^a, Zav^a, Hc^a		
	HPA-6bw	Ca^a,Tu^a	GPIIIa	CD61
	HPA-7bw	Mo^a	GPIIIa	CD61
	HPA-8bw	Sr^a	GPIIIa	CD61
	HPA-9bw	Max^a	GPIIb	CD41
	HPA-10bw	La^a	GPIIIa	CD61
	HPA-11bw	Gro^a	GPIIIa	CD61
	HPA-12bw	Iy^a	GPIbβ	CD42c
	HPA-13bw	Sit^a	GPIa	CD49b
	HPA-14bw	Oe^a	GPIIIa	CD61
HPA-15	HPA-15a	Gov^b	CD109	CD109
	HPA-15b	Gov^a		
	HPA-16bw	Duv^a	GPIIIa	CD61

From Metcalfe P, Watkins NA, Ouwehand WH, et al: Nomenclature of human platelet antigens. *Vox Sang* 85:240-245, 2003.

30% of cases.[189-191] For that reason, neonates with NAIT and platelet counts lower than 50,000/μL should be treated and should have a cranial ultrasonographic examination before hospital discharge. Typically, infants with NAIT do not respond well to random donor platelet transfusions, and the treatment of choice is transfusion with antigen-negative platelets (obtained either from known HPA-1b platelet donors or from the mother). However, recent evidence suggests that a significant percentage of neonates with NAIT respond to random donor platelet concentrates with a substantial increase in platelet counts, suggesting that transfusion of platelet concentrates from random donors is an appropriate strategy in the initial management of severe NAIT, pending the availability of antigen-compatible platelets.[192] High-dose intravenous immune globulin (IVIG, 1 g/kg/day for up to 2 days) has been reported to raise platelet counts to at least twice the baseline level in 48 hours.[193] Methylprednisolone (1 mg/kg/BID for 3 to 5 days) has also been used, although antigen-negative platelet transfusions or IVIG, or both, constitute the first lines of therapy. Retreatment is occasionally required.[194] Subsequent pregnancies can also be managed to limit the risk for fetal bleeding.[195,196] Treatment options include (1) administration of high-dose IVIG or steroids to mothers and (2) intrauterine platelet transfusions, although the latter are associated with high fetal morbidity and mortality.

Neonatal autoimmune thrombocytopenia is similar to NAIT in that it is mediated by the transplacental passage of maternal antiplatelet antibodies. In contrast to NAIT, however, the antibody responsible for these cases binds both maternal and fetal platelets, causing thrombocytopenia in both the mother and neonate. In most cases, the underlying maternal disease is ITP, although other disorders (i.e., systemic lupus erythematosus) can also produce this syndrome. Thrombocytopenia (platelet count <150,000/mm^3) is found in 13% to 64% of infants born to mothers with ITP.[197-203] Unlike in NAIT, however, the incidence of severe neonatal thrombocytopenia (platelet count <50,000/μL) is relatively low, varying from 5% to 20% in different studies. These infants also have a very low rate of significant bleeding complications, particularly intracranial hemorrhages. In an analysis of 474 infants born to mothers with ITP, Cook and colleagues[204] found an incidence of intracranial hemorrhage of 3% among infants with moderate or severe thrombocytopenia. Several subsequent studies have confirmed a very low fetal morbidity associated with maternal ITP.

Infection

Most of the infants with immune platelet disorders appear well. Sick infants should be investigated for an underlying cause. Infections constitute the most common cause of thrombocytopenia among sick NICU patients. Platelet counts less than 150,000/mm^3 were noted in 77% of patients with gram-positive septicemia and in 55% of the patients with gram-negative septicemia.[205] In a small series of neonates with septicemia, 16 of 21 culture-positive neonates had platelet counts lower than 100,000/mm^3.[206] In a larger retrospective review, Guida and associates evaluated neonates with culture-proven sepsis.[207] In this cohort, infants with gram-negative or fungal sepsis had a higher incidence of thrombocytopenia (approximately 80%) compared with gram-positive sepsis (approximately 40%). Furthermore, the duration of thrombocytopenia was also longer in the infants with gram-negative and fungal sepsis (2 versus 0.4 days), and they had lower initial platelet counts and lower platelet nadirs.[207] Increased platelet-associated immunoglobulin G (PAIgG) has been observed in both bacterial and viral neonatal infections.[208,209] Although some septic neonates develop thrombocytopenia on the basis of DIC, most have thrombocytopenia without laboratory evidence of DIC. In a study of septic adults, Neame and collaborators[210] found thrombocytopenia in 75% of patients, but only a small minority had DIC. The principal mechanism responsible for thrombocytopenia in infected neonates is thought to be accelerated platelet destruction, probably secondary to endothelial damage with subsequent platelet adhesion and aggregation, or to platelet lysis or removal by the reticuloendothelial system.[205,211] Decreased platelet production has also been proposed as a contributing mechanism.[149,212] In all cases, treatment should be directed at the underlying infection; platelet transfusions should be administered as needed.

Congenital infections are also associated with neonatal thrombocytopenia.[213,214] Typically, thrombocytopenia is present in both congenital and acquired cytomegalovirus (CMV) infections.[215,216] In murine CMV infections, thrombocytopenia may be caused by direct marrow suppression.[217,218] Chesney and associates documented CMV inclusion bodies in the marrow megakaryocytes of a boy congenitally infected with CMV.[217] The megakaryocyte numbers appeared normal, but megakaryocytes were vacuolated, a finding indicating a direct effect on marrow megakaryocytes. Fetal blood sampling has been used to detect severe thrombocytopenia in the fetuses of mothers with active CMV infections.[217] In mothers infected with *Toxoplasmosis gondii* during pregnancy, 12 of 39 infected infants had low platelet counts.[219] Severe thrombocytopenia caused by ineffective platelet production has also been reported in a neonate with congenital HIV infection.[130]

Genetic Causes of Thrombocytopenia

A variety of syndromes and congenital disorders are associated with neonatal thrombocytopenia. In some of these cases, there are associated features that are easily identified in the neonatal period, such as shortened or absent radii in the thrombocytopenia and absent radii (TAR) syndrome, or more subtle physical findings, such as decreased ability to rotate the forearm due to radioulnar synostosis in the syndrome known as amegakaryocytic thrombocytopenia and radioulnar synostosis (ATRUS).[131] In many congenital thrombocytopenias, there are

no physical findings evident in the neonatal period, but the platelets are either abnormally small (microthrombocytopenias; mean platelet volume [MPV] less than 7 fL) or abnormally large (macrothrombocytopenias, MPV greater than 11 fL). Drachman published an excellent review of congenital thrombocytopenias[220] and classified them according to platelet size (Table 145-8), genetic mutations, associated findings (Table 145-9), and inheritance pattern (Table 145-10). In addition, several chromosomal anomalies are associated with fetal and neonatal thrombocytopenia, including trisomies 13, 18, and 21 and Turner syndrome. Certain inherited metabolic disorders also present with thrombocytopenia, including methylmalonic acidemia, isovaleric acidemia, ketoglycinemia, and holocarboxylase synthetase deficiency.

TABLE 145-8

Inherited Thrombocytopenias Classified by Platelet Size

Small Platelets; MPV < 7 fL	Normal Platelets; MPV 7-11 fL	Large/Giant Platelets; MPV > 11 fL
Wiskott-Aldrich syndrome	Familial platelet disorder/acute myeloid leukemia	May-Hegglin anomaly
X-linked thrombocytopenia	Chromosome 10/THC2	Fechtner syndrome
	Congenital amegakaryocytic thrombocytopenia	Epstein syndrome
	Thrombocytopenia and absent radii	Sebastian syndrome
		Mediterranean thrombocytopenia
		Bernard-Soulier syndrome
		Velocardiofacial/ DiGeorge syndrome
		GATA1 mutation
		Gray platelet syndrome
		Paris-Trousseau thrombocytopenia/ Jacobsen syndrome

From Drachman JG: Inherited thrombocytopenia: When a low platelet count does not mean ITP. *Blood* 103:390-398, 2004.

MPV, Mean platelet volume.

Pregnancy-Induced Hypertension and Intrauterine Growth Restriction

Thrombocytopenia is common among neonates delivered to women with pregnancy-induced hypertension (PIH) or intrauterine growth restriction (IUGR).[129,221] The mechanism responsible for this thrombocytopenia is thought to be decreased platelet production, based on the finding of decreased concentrations of circulating megakaryocyte progenitors in affected neonates compared to controls.[129] Interestingly, serum Tpo concentrations in these neonates are not as elevated as those found in adult patients with hyporegenerative thrombocytopenia,[149,152] suggesting that a decreased ability to up-regulate Tpo production might contribute to thrombocytopenia. The thrombocytopenia associated with PIH or IUGR is usually mild to moderate (platelet counts between 50,000 and 150,000/μL) and follows a predictable pattern. It usually presents at birth or in the first few days of life, reaches a nadir on days 2 to 4, and resolves by days 7 to 10. Severe thrombocytopenias or thrombocytopenias that persist beyond 10 days of life deserve further evaluation for other potential causes.

Necrotizing Enterocolitis

Thrombocytopenia occurs frequently in neonates with necrotizing enterocolitis (NEC) and is thought to be caused by increased platelet destruction, based on the short half-life of transfused platelets. The inflammatory mediator platelet-activating factor

TABLE 145-9

Inherited Thrombocytopenias Classified by Genetic Mutations and Associated Findings

Syndrome	Gene Mutation	Chromosome Location	Associated Findings
MYH9-related thrombocytopenia			
May-Hegglin anomaly	MYH9	22q11	Neutrophil inclusions, sensorineural hearing loss, nephritis, cataracts
Fechtner syndrome	MYH9	22q11	
Epstein syndrome	MYH9	22q11	
Sebastian syndrome	MYH9	22q11	
Mediterranean thrombocytopenia/ Bernard-Soulier carrier	GP1BB, ? others	17pter-p12	None
Bernard-Soulier syndrome	GP1BA, GP1BB	17pter-p12	None
Velocardiofacial/DiGeorge syndrome	? GP1BB	22q11	Cardiac, facial, parathyroid, thymus anomalies; cognitive and learning impairment
Familial platelet disorder/ acute myeloid leukemia (AML)	AML1	21q22.2	Myelodysplasia, AML
Chromosome 10/THC2	? FLJ14813	10p12-11.2	None
Paris-Trousseau thrombocytopenia/ Jacobsen syndrome	? FLI1	11q23	Psychomotor retardation, facial anomalies (Jacobsen syndrome)
Gray platelet syndrome	Unknown	Unknown	None
Congenital amegakaryocytic thrombocytopenia	MPL	1p34	Marrow failure during second decade
Thrombocytopenia and absent radii syndrome	Unknown	Unknown	Bilateral short/absent radii
Thrombocytopenia and radioulnar synostosis	HOXA11	7p15-p14.2	Fused radius, incomplete range of motion
Wiskott-Aldrich syndrome	WAS	Xp11.23-p11.22	Immunodeficiency, eczema, lymphoma
X-linked thrombocytopenia	WAS	Xp11.23-p11.22	None
GATA1 mutation	GATA-1	Xp11.23	Anemia, dyserythropoiesis, thalassemia

From Drachman JG: Inherited thrombocytopenia: When a low platelet count does not mean ITP. *Blood* 103:390-398, 2004.

(PAF) has been found by multiple investigators to play a role in the pathogenesis of NEC.[222-224] Administration of a PAF receptor antagonist or of the PAF-degrading enzyme acetylhydrolase can significantly attenuate or prevent NEC in several animal models.[225] PAF has also been causally associated to the thrombocytopenia and neutropenia in dogs with endotoxemia[226] and has been found to be inversely associated with platelet counts in infected patients with DIC,[227] suggesting that it might also contribute to the pathophysiology of the thrombocytopenia in neonates with NEC.

Asphyxia

Several studies have reported that birth asphyxia is significantly more frequent among thrombocytopenic neonates than among nonthrombocytopenic neonates.[174,175,228] The reasons underlying this association are unclear, as is the mechanism responsible for the thrombocytopenia. Saxonhouse and collaborators[229] evaluated the effects of 24 hours of exposure to anoxia on the survival and proliferative potential of cord blood–derived megakaryocyte progenitors. He found that megakaryocyte progenitors did not undergo necrosis or apoptosis under these conditions and subsequently proliferated in a manner similar to normoxic controls. These observations suggest that the thrombocytopenia observed in neonates with asphyxia is not due to a direct deleterious effect of severe hypoxia on the megakaryocyte progenitors.

Miscellaneous Causes of Thrombocytopenia in Neonates and Adults

Numerous drugs have been reported to cause thrombocytopenia in adults. When administered to neonates, these medications are equally capable of causing drug-induced thrombocytopenia through a variety of mechanisms. DIC also complicates several disease processes, usually characterized by severe illness. Finally, thrombocytopenia can also occur in neonates with thrombosis and has been reported in neonates with thrombosis of the sagittal sinus,[230] the right atrium,[231] and the renal vein.

TABLE 145-10

Classification of Congenital Thrombocytopenia Based on Inheritance Pattern

Autosomal Dominant	Autosomal Recessive	X-linked
May-Hegglin anomaly	Congenital amegakaryocytic Thrombocytopenia (CAMT)	Wiskott-Aldrich syndrome
Fechtner syndrome	Thrombocytopenia and absent radii (TAR)	X-linked thrombocytopenia
Epstein syndrome	Bernard-Soulier syndrome	*GATA1* mutation
Sebastian syndrome		
Mediterranean thrombocytopenia/ Bernard-Soulier carrier		
Velocardiofacial/DiGeorge syndrome		
Familial platelet disorder/ acute myeloid leukemia Chromosome 10/THC2		
Paris-Trousseau thrombocytopenia		
Jacobsen syndrome		
Gray platelet syndrome		
Thrombocytopenia and radial synostosis		

From Drachman JG: Inherited thrombocytopenia: When a low platelet count does not mean ITP. *Blood* 103:390-398, 2004.

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SECTION XIX

Hemostasis

146 Developmental Hemostasis

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Hemostasis is a dynamic, evolving process that is age dependent and begins in utero. Although evolving, the hemostatic system in healthy fetuses and infants must be considered physiologic. Because the hemostatic system is dynamic, multiple reference ranges reflecting the gestational age and postnatal age of infants are necessary.[1] The evaluation of newborn infants for hemorrhagic or thrombotic complications presents unique problems that are not encountered in older children and adults.[2,3] For example, physiologic levels of many coagulation proteins in neonates are low, which makes the diagnoses of some inherited and acquired hemostatic problems difficult to establish. An understanding of developmental hemostasis in the broadest sense optimizes the prevention, diagnosis, and treatment of hemostatic problems during childhood and undoubtedly provides new insights into the pathophysiology of hemorrhagic and thrombotic complications for all ages.

THE COAGULATION SYSTEM

The understanding of hemostatic physiology in neonates and infants is deficient compared with knowledge of this subject in adults. This deficit is from several factors: in neonates and infants, multiple reference ranges are required because these patients have rapidly evolving systems[1,4-6]; blood sampling in the young is technically difficult; only small blood samples can be obtained; microtechniques are required[7]; and greater variability in plasma concentrations of coagulation proteins necessitates the use of large patient numbers to establish normative data.

Coagulation proteins are independently synthesized by the fetus and do not cross the placenta.[8-20] By 10 weeks gestational age, plasma concentrations of most coagulation proteins are measurable, and they continue to increase gradually in parallel with the gestational age. Samples obtained during fetoscopy provide the best assessment of normal values for fetuses, and by extrapolation, very premature infants (Tables 146-1 and 146-2).[21] True reference ranges for extremely premature infants are not available because the majority of these infants have postnatal complications. Tables 146-3 to 146-6 provide reference ranges for coagulation proteins, inhibitors of coagulation, and components of the fibrinolytic system for premature (30 to 36 weeks gestational age) and full-term infants on day 1 of life, as well as longitudinally during the first 6 months of life.[1,4,5] Tables 146-7 to 146-9 provide similar data for older children.[22]

The variable results for coagulation screening tests reflect the use of cord blood samples rather than samples from infants or differing ethnic populations or the use of different reagents.[23,24,25] Variation in prothrombin time results can be minimized by reporting the prothrombin time as an international normalized ratio.[26] The international normalized ratio is calculated as the patient prothrombin time divided by control prothrombin time to the power of the international sensitivity index. The international sensitivity index corrects for the large variation in sensitivity of thromboplastin reagents to plasma concentrations of coagulation proteins. Unfortunately, no such standardization exists for activated partial thromboplastin times. Reference ranges for activated partial thromboplastin times will differ with each different reagent and analyzer system, often significantly. Table 146-10 shows the variability in activated partial thromboplastin time reference ranges using the same patient samples, but with different activated partial thromboplastin time reagents.[25,27] The thrombin-clotting time performed in the absence of calcium is prolonged because of the presence of the "fetal" form of fibrinogen at birth.[1,4,5] For Tables 146-3 and 146-4, the thrombin-clotting time was measured in the presence of calcium so that abnormal values secondary to the presence of heparin, as well as low levels of fibrinogen, could be detected.

Coagulant Proteins

The vitamin K–dependent factors are the most extensively studied group of factors in infants, reflecting the clinical significance of hemorrhagic disease of the newborn.[28-30] Physiologically low levels of factors (F) II, FVII, FIX, and FX in Tables 146-3 and 146-4 are similar to those in other reports[1,4,5,31-41] and were measured in infants who received vitamin K prophylaxis at birth. The levels of the vitamin K–dependent factors and the contact factors (FXI, FXII, prekallikrein, and high molecular weight kininogen) gradually increase to values approaching adult levels by 6 months of life.[1,4,5] Recently, low prothrombin levels have been found to be associated with increased rates of intraventricular hemorrhage in very preterm infants. The prolonged activated partial thromboplastin time during the first months of life is in large part due to the low levels of the contact factors.[42]

Plasma levels of fibrinogen, FV, FVIII, and FXIII, and von Willebrand factor (vWf) are not decreased at birth (see Tables 146-3 and 146-4). Fibrinogen levels continue to increase after birth.[43] Plasma levels of FVIII are skewed toward the high measurements, necessitating an adjustment of the lower limit of normal (see Tables 146-3 and 146-4). Levels of both vWf and high molecular weight multimers are increased at birth and for the first 3 months of life.[1]

One potential mechanism for the developmental differences in plasma protein levels is reduced production of coagulation factors. Messenger ribonucleic acid (mRNA) levels have been measured for FVII, FVIII, FIX, and FX, fibrinogen, antithrombin (AT), and protein C in hepatocytes from 5- to 10-week-old human embryos and fetuses and in those from adults. Embryonic-fetal transcripts and adult mRNAs are similar in size; the nucleotide sequences of mRNA for factors IX and X are identical.[44,45] However, the expression of mRNA was variable, with adult values existing for some coagulation proteins, but decreased for others.

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TABLE 146-1

Coagulation Screening Tests and Coagulation Factor Levels in Fetuses, Full-Term Infants, and Adults

	FETUSES (WEEKS GESTATION)			NEONATES	ADULTS
Parameter	**19-23 (n = 20)**	**24-29 (n = 22)**	**30-38 (n = 22)**	**(n = 60)**	**(n = 40)**
PT (sec)	32.5 (19-45)	32.2 (19-44)†	22.6 (16-30)†	16.7 (12.0-23.5)*	13.5 (11.4-14.0)
PT (INR)	6.4 (1.7-11.1)	6.2 (2.1-10.6)†	3.0 (1.5-5.0)*	1.7 (0.9-2.7)*	1.1 (0.8-1.2)
APTT (sec)	168.8 (83-250)	154.0 (87-210)†	104.8 (76-128)†	44.3 (35-52)*	33.0 (25-39)
TCT (sec)	34.2 (24-44)*	26.2 (24-28)	21.4 (17.0-23.3)	20.4 (15.2-25.0)†	14.0 (12-16)
Factor I (g/l) von Clauss	0.85 (0.57-1.50)	1.12 (0.65-1.65)	1.35 (1.25-1.65)	1.68 (0.95-2.45)†	3.0 (1.78-4.50)
I Ag (g/l)	1.08 (0.75-1.50)	1.93 (1.56-2.40)	1.94 (1.30-2.40)	2.65 (1.68-3.60)†	3.5 (2.50-5.20)
IIc (%)	16.9 (10-24)	19.9 (11-30)*	27.9 (15-50)†	43.5 (27-64)†	98.7 (70-125)
VIIc (%)	27.4 (17-37)	33.8 (18-48)*	45.9 (31-62)	52.5 (28-78)†	101.3 (68-130)
IXc (%)	10.1 (6-14)	9.9 (5-15)	12.3 (5-24)†	31.8 (15-50)†	104.8 (70-142)
Xc (%)	20.5 (14-29)	24.9 (16-35)	28.0 (16-36)†	39.6 (21-65)†	99.2 (75-125)
Vc (%)	32.1 (21-44)	36.8 (25-50)	48.9 (23-70)†	89.9 (50-140)	99.8 (65-140)
VIIIc (%)	34.5 (18-50)	35.5 (20-52)	50.1 (27-78)†	94.3 (38-150)	101.8 (55-170)
XIc (%)	13.2 (8-19)	12.1 (6-22)	14.8 (6-26)†	37.2 (13-62)†	100.2 (70-135)
XIIc (%)	14.9 (6-25)	22.7 (6-40)	25.8 (11-50)†	69.8 (25-105)†	101.4 (65-144)
PK (%)	12.8 (8-19)	15.4 (8-26)	18.1 (8-28)†	35.4 (21-53)†	99.8 (65-135)
HMWK (%)	15.4 (10-22)	19.3 (10-26)	23.6 (12-34)†	38.9 (28-53)†	98.8 (68-135)

From Reverdiau-Moalic P, Delahousse B, Body G, et al: Evolution of blood coagulation activators and inhibitors in the healthy human fetus. *Blood* 88:900, 1996.
Values are the mean, followed in parentheses by the lower and upper boundaries, including 95% of the population.
*$p < .05$; †$p < .01$.
Ag, Antigen; *APTT,* activated partial thromboplastin time; *HMWK,* high-molecular-weight kininogen; *INR,* international normalized ratio; *PK,* prekallikrein; *PT,* prothrombin time; *TCT,* thrombin-clotting time.

TABLE 146-2

Blood Coagulation Inhibitor Levels in Fetuses, Full-Term Infants, and Adults

	FETUSES (WEEKS GESTATION)			NEONATES	ADULTS
Parameter	**19-23 (n = 20)‡**	**24-29 (n = 22)**	**30-38 (n = 22)**	**(n = 60)**	**(n = 40)**
AT (%)	20.2 (12-31)*	30.0 (20-39)	37.1 (24-55)†	59.4 (42-80)†	99.8 (65-130)
HCII (%)	10.3 (6-16)	12.9 (5.5-20)	21.1 (11-33)†	52.1 (19-99)†	101.4 (70-128)
TFPI (%)‡	21.0 (16.0-29.2)	20.6 (13.4-33.2)	20.7 (10.4-31.5)†	38.1 (22.7-55.8)†	73.0 (50.9-90.1)
PC Ag (%)	9.5 (6-14)	12.1 (8-16)	15.9 (8-30)†	32.5 (21-47)†	100.8 (68-125)
PC Act (%)	9.6 (7-13)	10.4 (8-13)	14.1 (8-18)*	28.2 (14-42)†	98.8 (68-125)
Total PS (%)	15.1 (11-21)	17.4 (14-25)	21.0 (15-30)†	38.5 (22-55)†	99.6 (72-118)
Free PS (%)	21.7 (13-32)	27.9 (19-40)	27.0 (18-40)†	49.3 (33-67)†	98.7 (72-128)
Ratio of Free PS:Total PS	0.82 (0.75-0.92)	0.83 (0.76-0.95)	0.79 (0.70-0.89)†	0.64 (0.59-0.98)†	0.41 (0.38-0.43)
C4b-BP (%)	1.8 (0-6)	6.1 (0-12.5)	9.3 (5-14)	18.6 (3-40)†	100.3 (70-124)

From Reverdiau-Moalic P, Delahousse B, Body G, et al: Evolution of blood coagulation activators and inhibitors in the healthy human fetus. *Blood* 88:900, 1996.
Values are the mean, followed in parentheses by the lower and upper boundaries, including 95% of the population.
*$p < .05$; †$p < .01$.
‡Twenty samples were assayed for each group but only 10 for 19- to 23-week-old fetuses.
Act, Activity; *Ag,* antigen; *AT,* antithrombin; *HCII,* heparin cofactor II; *PC,* protein C; *PS,* protein S; *TFPI,* tissue factor pathway inhibitor.

Similar concentrations of prothrombin mRNA were found in the livers of newborn and adult rabbits;[46] however, another study reported lower prothrombin mRNA concentrations in sheep. In addition, increased clearance of plasma proteins may account for developmental differences in plasma proteins. Fibrinogen, whether of fetal or adult origin, is cleared more rapidly in newborn lambs than it is in sheep.[47] Similarly, clearance of fibrinogen is accelerated in premature infants with or without respiratory distress syndrome.[48] An increased basal metabolic rate in the young probably contributes to the accelerated clearance of proteins.[49]

Differences in function and structure of fetal versus adult fibrinogen have been recognized for a number of years.[50] These differences have been primarily attributed to an increased sialic acid content of the fetal fibrinogen.[51] Preliminary data suggest that posttranslational modification differences may be present in a number of coagulation proteins in neonates compared with adults. The functional and developmental significance of these changes remain to be determined.

Regulation of Thrombin

Thrombin regulation is both delayed and decreased in newborn plasma compared with adult plasma and is similar to plasmas from adults receiving therapeutic doses of warfarin or heparin.[52] Thrombin generation in newborn plasma is further decreased in the presence of endothelial cell surfaces, but not to the same extent as adult plasma.[53] The amount of thrombin generated is directly proportional to the prothrombin concentration,[54] whereas the rate of thrombin generation reflects the concentration of other procoagulants.

Thrombin is directly inhibited by AT, heparin cofactor II, and α_2-macroglobulin. In addition, a circulating physiologic anticoagulant in cord blood has properties similar to those of dermatan

TABLE 146-3

Reference Values for Coagulation Tests in Healthy Premature Infants (30-36 Weeks Gestation) During the First 6 Months of Life

	DAY 1		DAY 5		DAY 30		DAY 90		DAY 180		ADULTS	
	M	B	M	B	M	B	M	B	M	B	M	B
PT (sec)	13.0 (10.6-16.2)*		12.5 (10.0-15.3)*		11.8 (10.0-13.6)*		12.3 (10.0-14.6)		12.5 (10.0-15.0)*		12.4 (10.8-13.9)	
INR	1.0 (0.61-1.70)		0.91 (0.53-1.48)		0.79 (0.53-1.11)		0.88 (0.53-1.32)		0.91 (0.53-1.48)		0.89 (0.64-1.17)	
APTT (sec)	53.6 (27.5-79.4)†		50.5 (26.9-74.1)		44.7 (26.9-62.5)		39.5 (28.3-50.7)		37.5 (27.2-53.3)		33.5 (26.6-40.3)	
TCT (sec)	24.8 (19.2-30.4)		24.1 (18.8-29.4)*		24.4 (18.8-29.9)		25.1 (19.4-30.8)		25.2 (18.9-31.5)		25.0 (19.7-30.3)	
Fibrinogen (g/L)	2.43 (1.50-3.73)*,†		2.80 (1.60-4.18)*,†		2.54 (1.50-4.14)		2.46 (1.50-3.52)		2.28 (1.50-3.60)		2.78 (1.56-4.00)	
II (U/mL)	0.45 (0.20-0.77)		0.57 (0.29-0.85)†		0.57 (0.36-0.95)		0.68 (0.30-1.06)		0.87 (0.51-1.23)		1.08 (0.70-1.46)	
V (U/mL)	0.88 (0.41-1.44)*,†		1.00 (0.46-1.54)*		1.02 (0.48-1.56)*		0.99 (0.59-1.39)		1.02 (0.58-1.46)*		1.06 (0.62-1.50)	
VII (U/mL)	0.67 (0.21-1.13)		0.84 (0.30-1.38)		0.83 (0.21-1.45)		0.87 (0.31-1.43)		0.99 (0.47-1.51)*		1.05 (0.67-1.43)	
VIII (U/mL)	1.11 (0.50-2.13)		1.15 (0.53-2.05)*,†		1.11 (0.50-1.99)		1.06 (0.58-1.88)*,†		0.99 (0.50-1.87)*,†		0.99 (0.50-1.49)	
vWF (U/mL)	1.36 (0.78-2.10)		1.33 (0.72-2.19)		1.36 (0.66-2.16)		1.12 (0.75-1.84)*,†		0.98 (0.54-1.58)*		0.92 (0.50-1.58)*	
IX (U/mL)	0.35 (0.19-0.65)†		0.42 (0.14-0.74)†		0.44 (0.13-0.80)		0.59 (0.25-0.93)		0.81 (0.50-1.20)		1.09 (0.55-1.63)	
X (U/mL)	0.41 (0.11-0.71)		0.51 (0.19-0.83)		0.56 (0.20-0.92)		0.67 (0.35-0.99)		0.77 (0.35-1.19)		1.06 (0.70-1.52)	
XI (U/mL)	0.30 (0.08-0.52)†		0.41 (0.13-0.69)†		0.43 (0.15-0.71)†		0.59 (0.25-0.93)*		0.78 (0.46-1.10)		0.97 (0.67-1.27)	
XII (U/mL)	0.38 (0.10-0.66)†		0.39 (0.09-0.69)†		0.43 (0.11-0.75)		0.61 (0.15-1.07)		0.82 (0.22-1.42)		1.08 (0.52-1.64)	
PK (U/mL)	0.33 (0.09-0.57)		0.45 (0.25-0.75)		0.59 (0.31-0.87)		0.79 (0.37-1.21)		0.78 (0.40-1.16)		1.12 (0.62-1.62)	
HMWK (U/mL)	0.49 (0.09-0.89)		0.62 (0.24-1.00)†		0.64 (0.16-1.12)†		0.78 (0.32-1.24)		0.83 (0.41-1.25)*		0.92 (0.50-1.36)	
XIIIa (U/mL)	0.70 (0.32-1.08)		1.01 (0.57-1.45)*		0.99 (0.51-1.47)*		1.13 (0.71-1.55)*		1.13 (0.65-1.61)*		1.05 (0.55-1.55)	
XIIIb (U/mL)	0.81 (0.35-1.27)		1.10 (0.68-1.58)*		1.07 (0.57-1.57)*		1.21 (0.75-1.67)		1.15 (0.67-1.63)		0.97 (0.57-1.37)	

From Andrew M, Paes B, Milner R, et al: Development of the human coagulation system in the healthy premature infant. *Blood* 72:1651, 1988.

All factors except fibrinogen are expressed as units per milliliter (U/mL) in which pooled plasma contains 1.0 U/mL. All values are given as a mean *(M)* followed by the lower and upper boundaries encompassing 95% of the population *(B)*. Between 40 and 96 samples were assayed for each value for the neonate. Some measurements were skewed due to a disproportionate number of high values. The lower limit, which excludes the lower 2.5% of the population, has been given (B).

*$p < .05$; †$p < .01$.

APTT, Activated partial thromboplastin time; *HMWK,* high-molecular-weight kininogen; *INR,* international normalized ratio; *PK,* prekallikrein; *PT,* prothrombin time; *TCT,* thrombin-clotting time; *vWF,* von Willebrand factor.

TABLE 146-4

Reference Values for Coagulation Tests in Healthy Full-Term Infants During the First 6 Months of Life

	DAY 1		DAY 5		DAY 30		DAY 90		DAY 180		ADULTS	
	M	B	M	B	M	B	M	B	M	B	M	B
PT (sec)	13.0 (10.1-15.9)*		12.4 (10.0-15.3)*		11.8 (10.0-14.3)*		11.9 (10.0-14.2)*		12.3 (10.7-13.9)*		12.4 (10.8-13.9)	
INR	1.00 (0.53-1.62)		0.89 (0.53-1.48)		0.79 (0.53-1.26)		0.81 (0.53-1.26)		0.88 (0.61-1.17)		0.89 (0.64-1.17)	
APTT (sec)	42.9 (31.3-54.5)		42.6 (25.4-59.8)		40.4 (32.0-55.2)		37.1 (29.0-50.1)*		35.5 (28.1-42.9)*		33.5 (26.6-40.3)	
TCT (sec)	23.5 (19.0-28.3)*		23.1 (18.0-29.2)		24.3 (19.4-29.2)*		25.1 (20.5-29.7)*		25.5 (19.8-31.2)*		25.0 (19.7-30.3)	
Fibrinogen (g/L)	2.83 (1.67-3.99)*		3.12 (1.62-4.62)*		2.70 (1.62-3.78)*		2.43 (1.50-3.79)*		2.51 (1.50-3.87)*		2.78 (1.56-4.00)	
II (U/mL)	0.48 (0.26-0.70)		0.63 (0.33-0.93)		0.68 (0.34-1.02)		0.75 (0.45-1.05)		0.88 (0.60-1.16)		1.08 (0.70-1.46)	
V (U/mL)	0.72 (0.34-1.08)		0.95 (0.45-1.45)		0.98 (0.62-1.34)		0.90 (0.48-1.32)		0.91 (0.55-1.27)		1.06 (0.62-1.50)	
VII (U/mL)	0.66 (0.28-1.04)		0.89 (0.35-1.43)		0.90 (0.42-1.38)		0.91 (0.39-1.43)		0.87 (0.47-1.27)		1.05 (0.67-1.43)	
VIII (U/mL)	1.00 (0.50-1.78)*		0.88 (0.50-1.54)*		0.91 (0.50-1.57)*		0.79 (0.50-1.25)*		0.73 (0.50-1.09)		0.99 (0.50-1.49)	
vWF (U/mL)	1.53 (0.50-2.87)		1.40 (0.50-2.54)		1.28 (0.50-2.46)		1.18 (0.50-2.06)		1.07 (0.50-1.97)		0.92 (0.50-1.58)	
IX (U/mL)	0.53 (0.15-0.91)		0.53 (0.15-0.91)		0.51 (0.21-0.81)		0.67 (0.21-1.13)		0.86 (0.36-1.36)		1.09 (0.55-1.63)	
X (U/mL)	0.40 (0.12-0.68)		0.49 (0.19-0.79)		0.59 (0.31-0.87)		0.71 (0.35-1.07)		0.78 (0.38-1.18)		1.06 (0.70-1.52)	
XI (U/mL)	0.38 (0.10-0.66)		0.55 (0.23-0.87)		0.53 (0.27-0.79)		0.69 (0.41-0.97)		0.86 (0.49-1.34)		0.97 (0.67-1.27)	
XII (U/mL)	0.53 (0.13-0.93)		0.47 (0.11-0.83)		0.49 (0.17-0.81)		0.67 (0.25-1.09)		0.77 (0.39-1.15)		1.08 (0.52-1.64)	
PK (U/mL)	0.37 (0.18-0.69)		0.48 (0.20-0.76)		0.57 (0.23-0.91)		0.73 (0.41-1.05)		0.86 (0.56-1.16)		1.12 (0.62-1.62)	
HMWK (U/mL)	0.54 (0.06-1.02)		0.74 (0.16-1.32)		0.77 (0.33-1.21)		0.82 (0.30-1.46)*		0.82 (0.36-1.28)*		0.92 (0.50-1.36)	
XIIIa (U/mL)	0.79 (0.27-1.31)		0.94 (0.44-1.44)*		0.93 (0.39-1.47)*		1.04 (0.36-1.72)*		1.04 (0.46-1.62)*		1.05 (0.55-1.55)	
XIIIb (U/mL)	0.76 (0.30-1.22)		1.06 (0.32-1.80)		1.11 (0.39-1.73)*		1.16 (0.48-1.84)*		1.10 (0.50-1.70)*		0.97 (0.57-1.37)	

From Andrew M, Paes B, Milner R, et al: Development of the human coagulation system in the full-term infant. *Blood* 70:165, 1987.

All factors except fibrinogen are expressed as units per milliliter (U/mL) where pooled plasma contains 1.0 U/mL. All values are expressed as mean *(M)* followed by the lower and upper boundaries encompassing 95% of the population *(B)*. Between 40 and 77 samples were assayed for each value for the neonate. Some measurements were skewed due to a disproportionate number of high values. The lower limit, which excludes the lower 2.5% of the population, has been given.

*Values that are indistinguishable from those of the adult.

Abbreviations given in Table 146-3.

sulfate.[55] The fetal proteoglycan is present in plasma in concentrations of 0.29 μg/mL, has a molecular weight of 150,000 kd, and catalyzes thrombin inhibition by means of the natural inhibitor heparin cofactor II. The fetal anticoagulant also is present in plasmas from pregnant women and is produced by the placenta.[56] The length of time that the fetal anticoagulant circulates in neonates is not known. α_2-Macroglobulin is a more important inhibitor of thrombin in plasmas from neonates than it is in plasmas from adults.[57,58] α_2-Macroglobulin compensates, in part, for the low levels of AT in neonates, even in the presence of endothelial cell surfaces. The elimination half-life of AT is shorter in healthy infants than in adults.[59] Despite these differences, the

TABLE 146-5

Reference Values for the Inhibitors of Coagulation in Healthy Infants During the First 6 Months of Life

	DAY 1		DAY 5		DAY 30		DAY 90		DAY 180		ADULTS	
	M	B	M	B	M	B	M	B	M	B	M	B
Healthy Full-Term Infants												
AT (U/mL)	0.63 (0.39-0.87)		0.67 (0.41-0.93)		0.78 (0.48-1.08)		0.97 (0.73-1.21)*		1.04 (0.84-1.24)*		1.05 (0.79-1.31)	
2M (U/mL)	1.39 (0.95-1.83)		1.48 (0.98-1.98)		1.50 (1.06-1.94)		1.76 (1.26-2.26)		1.91 (1.49-2.33)		0.86 (0.52-1.20)	
C1E-INH (U/mL)	0.72 (0.36-1.08)		0.90 (0.60-1.20)*		0.89 (0.47-1.31)		1.15 (0.71-1.59)		1.41 (0.89-1.93)		1.01 (0.71-1.31)	
1AT (U/mL)	0.93 (0.49-1.37)*		0.89 (0.49-1.29)*		0.62 (0.36-0.88)		0.72 (0.42-1.02)		0.77 (0.47-1.07)		0.93 (0.55-1.31)	
HCII (U/mL)	0.43 (0.10-0.93)		0.48 (0.00-0.96)		0.47 (0.10-0.87)		0.72 (0.10-1.46)		1.20 (0.50-1.90)		0.96 (0.66-1.26)	
Protein C (U/mL)	0.35 (0.17-0.53)		0.42 (0.20-0.64)		0.43 (0.21-0.65)		0.54 (0.28-0.80)		0.59 (0.37-0.81)		0.96 (0.64-1.28)	
Protein S (U/mL)	0.36 (0.12-0.60)		0.50 (0.22-0.78)		0.63 (0.33-0.93)		0.86 (0.54-1.18)*		0.87 (0.55-1.19)*		0.92 (0.60-1.24)	
Healthy Premature Infants (30 to 36 Weeks Gestation)												
AT (U/mL)	0.38 (0.14-0.62)†		0.56 (0.30-0.82)		0.59 (0.37-0.81)†		0.83 (0.45-1.21)†		0.90 (0.52-1.28)†		1.05 (0.79-1.31)	
2M (U/mL)	1.10 (0.56-1.82)†		1.25 (0.71-1.77)		1.38 (0.72-2.04)		1.80 (1.20-2.66)		2.09 (1.10-3.21)		0.86 (0.52-1.20)	
C1E-INH (U/mL)	0.65 (0.31-0.99)		0.83 (0.45-1.21)		0.74 (0.40-1.24)†		1.14 (0.60-1.68)*		1.40 (0.96-2.04)		1.01 (0.71-1.31)	
1AT (U/mL)	0.90 (0.36-1.44)*		0.94 (0.42-1.46)*		0.76 (0.38-1.12)†		0.81 (0.49-1.13)*,†		0.82 (0.48-1.16)*		0.93 (0.55-1.31)	
HCII (U/mL)	0.32 (0.10-0.60)†		0.34 (0.10-0.69)		0.43 (0.15-0.71)		0.61 (0.20-1.11)		0.89 (0.45-1.40)*,†		0.96 (0.66-1.26)	
Protein C (U/mL)	0.28 (0.12-0.44)†		0.31 (0.11-0.51)		0.37 (0.15-0.59)†		0.45 (0.23-0.67)†		0.57 (0.31-0.83)		0.96 (0.64-1.28)	
Protein S (U/mL)	0.26 (0.14-0.38)†		0.37 (0.13-0.61)		0.56 (0.22-0.90)		0.76 (0.40-1.12)†		0.82 (0.44-1.20)		0.92 (0.60-1.24)	

From Andrew M, Paes B, Johnston M: Development of the hemostatic system in the neonate and young infant. *Am J Pediatr Hematol Oncol* 12:95, 1990.

All values are expressed in units per milliliter (U/mL), in which pooled plasma contains 1.0 U/mL. All values are given as a mean *(M)* followed by the lower and upper boundaries encompassing 95% of the population *(B)*. Between 40 and 75 samples were assayed for each value for the neonate. Some measurements were skewed due to a disproportionate number of high values. The lower limit, which excludes the lower 2.5% of the population, has been given *(B)*.

*Values that are indistinguishable from those of the adult.

†Values different from those of full-term infants.

1AT, Antitrypsin; *2M,* 2-macroglobulin; *AT,* antithrombin; *C1E-INH,* C1-esterase inhibitor; *HCII,* heparin cofactor II.

TABLE 146-6

Reference Values for the Components of the Fibrinolytic System in Healthy Infants During the First 6 Months of Life

	DAY 1		DAY 5		DAY 30		DAY 90		DAY 180		ADULTS	
	M	B	M	B	M	B	M	B	M	B	M	B
Healthy Full-Term Infants												
Plasminogen (U/mL)	1.95 (1.25-2.65)		2.17 (1.41-2.93)		1.98 (1.26-2.70)		2.48 (1.74-3.22)		3.01 (2.21-3.81)		3.36 (2.48-4.24)	
TPA (ng/mL)	9.6 (5.0-18.9)		5.6 (4.0-10.0)*		4.1 (1.0-6.0)*		2.1 (1.0-5.0)*		2.8 (1.0-6.0)*		4.9 (1.4-8.4)	
2AP (U/mL)	0.85 (0.55-1.15)		1.00 (0.70-1.30)*		1.00 (0.76-1.24)*		1.08 (0.76-1.40)*		1.11 (0.83-1.39)*		1.02 (0.68-1.36)	
PAI-1 (U/mL)	6.4 (2.0-15.1)		2.3 (0.0-8.1)*		3.4 (0.0-8.8)*		7.2 (1.0-15.3)		8.1 (6.0-13.0)		3.6 (0.0-11.0)	
Healthy Premature Infants (30 to 36 Weeks Gestation)												
Plasminogen (U/mL)	1.70 (1.12-2.48)†		1.91 (1.21-2.61)+		1.81 (1.09-2.53)		2.38 (1.58-3.18)		2.75 (1.91-3.59)+		3.36 (2.48-4.24)	
TPA (ng/mL)	8.48 (3.00-16.70)		3.97 (2.00-6.93)*		4.13 (2.00-7.79)*		3.31 (2.00-5.07)*		3.48 (2.00-5.85)*		4.96 (1.46-8.46)	
2AP (U/mL)	0.78 (0.40-1.16)		0.81 (0.49-1.13)†		0.89 (0.55-1.23)†		1.06 (0.64-1.48)*		1.15 (0.77-1.53)		1.02 (0.68-1.36)	
PAI-1 (U/mL)	5.4 (0.0-12.2)*,†		2.5 (0.0-7.1)*		4.3 (0.0-10.9)*		4.8 (1.0-11.8)*, †		4.9 (1.0-10.2)*, †		3.6 (0.0-11.0)	

From Andrew M, Paes B, Johnston M: Development of the hemostatic system in the neonate and young infant. *Am J Pediatr Hematol Oncol* 12:95, 1990.

For 2AP, values are expressed as units per milliliter (U/mL) in which pooled plasma contains 1.0 U/mL. Plasminogen units are those recommended by the Committee on Thrombolytic Agents. Values for TPA are given as nanograms per milliliter. Values for PAI-1 are given as units per mL where one unit of PAI-1 activity is defined as the amount of PAI-1 that inhibits one international unit of human single-chain TPA. All values are given as a mean *(M)* followed by the lower and upper boundaries encompassing 95% of the population *(B)*.

*Values that are indistinguishable from those of the adult.

†Values that are different from those of the full-term infant.

2AP, 2-Antiplasmin; *PAI-1,* plasminogen activator inhibitor-1; *TPA,* tissue plasminogen activator.

rate of inhibition of thrombin is still slower in newborn infants than it is in adults.

Whether the overall activity of the protein C/protein S system varies with age is unknown. However, at birth, plasma concentrations of protein C are very low, and they remain decreased during the first 6 months of life.[1,4] Protein C in neonatal plasma has a two-fold increase in single-chain form compared with the double-chain form that is prominent in adults.[60,61] Animal data suggest that fetal protein C has increased glycosylation compared with that of adults. Despite these changes, no evidence that protein C is functionally different in neonates is available. Although total amounts of protein S are decreased at birth, functional activity is similar to that in the adult because protein S is completely present in the free, active form due to the absence of C4 binding protein.[62,63] Furthermore, the interaction of protein S with activated protein C in newborn plasma may be regulated by the increased levels of α_2-macroglobulin.[64] Plasma concentrations of thrombomodulin are increased in early childhood, decreasing to

TABLE 146-7

Reference Values for Coagulation Tests in Healthy Children Ages 1 to 16 Years Compared with Adults

	1 to 5 yr		6 to 10 yr		11 to 16 yr		ADULTS	
	M	B	M	B	M	B	M	B
PT (sec)	11 (10.6-11.4)		11.1 (10.1-12.1)		11.2 (10.2-12.0)		12 (11.0-14.0)	
INR	1.0 (0.96-1.04)		1.01 (0.91-1.11)		1.02 (0.93-1.10)		1.10 (1.0-1.3)	
APTT (sec)	30 (24-36)		31 (26-36)		32 (26-37)		33 (27-40)	
Fibrinogen (g/L)	2.76 (1.70-4.05)		2.79 (1.57-4.0)		3.0 (1.54-4.48)		2.78 (1.56-4.0)	
Bleeding Time (min)	6 (2.5-10)*		7 (2.5-13)*		5 (3-8)*		4 (1-7)	
FII (U/mL)	0.94 (0.71-1.16)*		0.88 (0.67-1.07)		0.83 (0.61-1.04)*		1.08 (0.70-1.46)	
FV (U/mL)	1.03 (0.79-1.27)		0.90 (0.63-1.16)*		0.77 (0.55-0.99)*		1.06 (0.62-1.50)	
FVII (U/mL)	0.82 (0.55-1.16)		0.85 (0.52-1.20)		0.83 (0.58-1.15)*		1.05 (0.67-1.43)	
FVIII (U/mL)	0.90 (0.59-1.42)		0.95 (0.58-1.32)		0.92 (0.53-1.31)		0.99 (0.50-1.49)	
vWF (U/mL)	0.82 (0.60-1.20)		0.95 (0.44-1.44)		1.00 (0.46-1.53)		0.92 (0.50-1.58)	
FIX (U/mL)	0.73 (0.47-1.04)		0.75 (0.63-0.89)*		0.82 (0.59-1.22)*		1.09 (0.55-1.63)	
FX (U/mL)	0.88 (0.58-1.16)		0.75 (0.55-1.01)*		0.79 (0.50-1.17)*		1.06 (0.70-1.52)	
FXI (U/mL)	0.97 (0.56-1.50)		0.86 (0.52-1.20)		0.74 (0.50-0.97)		0.97 (0.67-1.27)	
FXII (U/mL)	0.93 (0.64-1.29)		0.92 (0.60-1.40)		0.81 (0.34-1.37)*		1.08 (0.52-1.64)	
PK (U/mL)	0.95 (0.65-1.30)		0.99 (0.66-1.31)		0.99 (0.53-1.45)		1.12 (0.62-1.62)	
HMWK (U/mL)	0.98 (0.64-1.32)		0.93 (0.60-1.30)		0.91 (0.63-1.19)		0.92 (0.50-1.36)	
FXIIIa (U/mL)	1.08 (0.72-1.43)		1.09 (0.65-1.51)*		0.99 (0.57-1.40)		1.05 (0.55-1.55)	
FXIIIs (U/mL)	1.13 (0.69-1.56)		1.16 (0.77-1.54)*		1.02 (0.60-1.43)		0.97 (0.57-1.37)	

From Andrew M, Vegh P, Johnston M, et al: Maturation of the hemostatic system during childhood. *Blood* 80:1998, 1992.

All factors except fibrinogen are expressed as units/mL (U/mL), in which pooled plasma contains 1.0 U/mL. All data are expressed as the mean *(M)* followed by the upper and lower boundaries encompassing 95% of the population *(B)*. Between 20 and 50 samples were assayed for each value for each age group. Some measurements were skewed due to a disproportionate number of high values. The lower limit, which excludes the lower 2.5% of the population, has been given.

*Values that are significantly different from those of adults.

APTT, activated partial thromboplastin time; *F,* factor; *HMWK,* high molecular weight kininogen; *INR,* international normalized ratio; *PK,* prekallikrein; *PT,* prothrombin time; *vWF,* von Willebrand factor.

TABLE 146-8

Reference Values for the Inhibitors of Coagulation in Healthy Children Ages 1 to 16 Years Compared with Adults

	1 to 5 yr		6 to 10 yr		11 to 16 yr		ADULTS	
	M	B	M	B	M	B	M	B
AT (U/mL)	1.11 (0.82-1.39)		1.11 (0.90-1.31)		1.05 (0.77-1.32)		1.0 (0.74-1.26)	
2M (U/mL)	1.69 (1.14-2.23)		1.69 (1.28-2.09)*		1.56 (0.98-2.12)		0.86 (0.52-1.20)	
C1E-INH (U/mL)	1.35 (0.85-1.83)*		1.14 (0.88-1.54)		1.03 (0.68-1.50)		1.0 (0.71-1.31)	
1AT (U/mL)	0.93 (0.39-1.47)		1.00 (0.69-1.30)		1.01 (0.65-1.37)		0.93 (0.55-1.30)	
HCII (U/mL)	0.88 (0.48-1.28)*		0.86 (0.40-1.32)*		0.91 (0.53-1.29)*		1.08 (0.66-1.26)	
Protein C (U/mL)	0.66 (0.40-0.92)*		0.69 (0.45-0.93)*		0.83 (0.55-1.11)*		0.96 (0.64-1.28)	
Protein S								
Total (U/mL)	0.86 (0.54-1.18)		0.78 (0.41-1.14)		0.72 (0.52-0.92)		0.81 (0.60-1.13)	
Free (U/mL)	0.45 (0.21-0.69)		0.42 (0.22-0.62)		0.38 (0.26-0.55)		0.45 (0.27-0.61)	

From Andrew M, Vegh P, Johnston M, et al: Maturation of the hemostatic system during childhood. *Blood* 80:1998, 1992.

All values are expressed in units per milliliter (U/mL), in which for all factors pooled plasma contains 1.0 U/mL, with the exception of free protein S, which contains a mean of 0.4 U/mL. All values are given as a mean *(M)* followed by the lower and upper boundaries encompassing 95% of the population *(B)*. Between 20 and 30 samples were assayed for each value for each age-group. Some measurements were skewed due to a disproportionate number of high values. The lower limits, which exclude the lower 2.5% of the population, have been given.

*Values that are significantly different from those of adults.

Abbreviations given in Table 146-5.

adult values by the late teenage years; however, the influence of age on endothelial cell expression of thrombomodulin has not been determined.[65-69]

Total tissue factor pathway inhibitor levels in newborn infants are reported as being similar to levels in older children or adults. However, free tissue factor pathway inhibitor is reported as being significantly lower in neonates.[70]

The capacity of newborn fibrin clots to bind thrombin has been assessed through the measurement of fibrinopeptide A production. Cord plasma clots generate significantly less fibrinopeptide A than do adult plasma clots because of the decreased plasma concentrations of prothrombin in cord plasma.[71] This observation suggests that thrombi in newborn infants may not have the same propensity to propagate as do thrombi in adult patients.

THE FIBRINOLYTIC SYSTEM

Although plasmin is generated and inhibited similarly in infants and adults, important differences do exist.[4] In neonates, plasminogen levels are only 50% of adult values, α_2-antiplasmin levels are 80% of adult values, and plasma concentrations of plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (TPA) are significantly greater than adult levels.[1,4-5,31-39,41,72-74] Increased levels of TPA and PAI-1 on day 1 of life are in marked contrast to values from cord blood, in which concentrations of these two proteins are significantly lower than they are in adults.[41,71,72] The discrepancy between newborn and cord plasma concentrations of TPA and PAI-1 can be explained by the enhanced release of TPA and PAI-1 from the endothelium shortly after birth. PAI-2

TABLE 146-9

Reference Values for the Fibrinolytic System in Healthy Children Ages 1 to 16 Years Compared with Adults

	1 to 5 yr		6 to 10 yr		11 to 16 yr		ADULTS	
	M	B	M	B	M	B	M	B
Plasminogen (U/mL)	0.98 (0.78-1.18)		0.92 (0.75-1.08)		0.86 (0.68-1.03)*		0.99 (0.77-1.22)	
TPA (ng/mL)	2.15 (1.0-4.5)*		2.42 (1.0-5.0)*		2.16 (1.0-4.0)*		4.90 (1.40-8.40)	
α_2AP (U/mL)	1.05 (0.93-1.17)		0.99 (0.89-1.10)		0.98 (0.78-1.18)		1.02 (0.68-1.36)	
PAI-1 (U/mL)	5.42 (1.0-10.0)		6.79 (2.0-12.0)*		6.07 (2.0-10.0)		3.60 (0-11.0)	

From Andrew M, Vegh P, Johnston M, et al: Maturation of the hemostatic system during childhood. *Blood* 80:1998, 1992.

For 2AP, values are expressed as units per mL (U/mL), in which pooled plasma contains 1.0 U/mL. Values for TPA are given as nanograms per mL. Values for PAI-1 are given as units/mL, in which 1 unit of PAI-1 activity is defined as the amount of PAI-1 that inhibits one international unit of human single-chain TPA. All values are given as mean *(M)* followed by the lower and upper boundaries encompassing 95% of the population *(B)*.

*Values that are significantly different than adult values.

α_2*AP,* α_2-Antiplasmin; *PAI-1,* plasminogen activator inhibitor-1; *TPA,* tissue plasminogen activator.

TABLE 146-10

APTT Results Obtained Using Different APTT Reagents on a STA-Compact Automated Coagulation Analyzer (Diagnostica STAGO)

	POPULATION TESTED	
Reagent Used	Children 1-10 yr Mean (95% Population)	Adults Mean (95% Population)
PTT-A (Diagnostica STAGO)	38.1 (31.3-44.1)	33.2 (28.1-38.2)
CK-Prest (Diagnostica STAGO)	33.0 (28.7-37.5)	29.1 (24.9-31.5)
Actin FSL (Dade Behring)	37.2 (27.8-43.9)	30.8 (24.1-34.4)
Platelin L (Organon Teknika)	38.0 (28.2-45.0)	31.3 (25.2-35.4)

APTT, activated partial thromboplastin time.

TABLE 146-11

Pre- and Postocclusion Results of Euglobulin Lysis Time

	CHILDREN		ADULTS		
ELT (h)	Female	Male	Female	Male	*P* value
Preocclusion	5.2 (0.30)	4.7 (0.50)	4.2 (0.80)	4.8 (0.60)	NS*
Postocclusion	3.0 (0.50)†	3.2 (0.60)†	1.7 (0.80)	2.0 (0.60)	*P* = .002†

Values expressed as mean ± 95% confidence interval. Analyzed by one-way analysis of variance.

*Not significant.

†Adolescents had significantly longer euglobulin lysis time (ELS) postocclusion results compared with adults. Sex demonstrated no effect.

levels are detectable in cord blood, but are significantly lower than they are in pregnant women.[75] Plasminogen, like fibrinogen, has a fetal form. Fetal plasminogen exists in two glycoforms that have increased amounts of mannose and sialic acid.[76] The enzymatic activity of "fetal plasmin," as well as its binding to cellular receptors for fetal plasminogen, are decreased.

Short whole-blood clotting times, short euglobulin lysis times, and increased plasma concentrations of the Bβ15-42 fibrin-related peptides suggest that the fibrinolytic system is activated at birth.[1,74] At the same time, the capacity of the fetal fibrinolytic system to generate plasmin in response to stimulation by a thrombolytic agent is decreased compared with that of adults; this reflects low levels of plasminogen.[77]

The fibrinolytic response to venous occlusion stress testing in healthy adolescents (13 to 18 years) has been compared with the response in healthy adults.[78,79] Adolescents had significantly decreased TPA antigen levels and increased PAI-1 activity levels after venous occlusion, resulting in significantly prolonged euglobulin lysis times (Table 146-11). The age-related differences in fibrinolytic responses to venous occlusion of younger children, and the significance of these differences on the pathophysiology of clinical hemostatic disorders in children, require further studies.

PLATELETS

Classic platelet studies in newborn infants are inhibited by sample volume requirements. Flow cytometry is useful because of the small sample volume required for extensive platelet function studies. Differences in sample timing, method of collection, and concentrations and compositions of platelet agonists likely contribute to apparently conflicting reports on platelet function in the newborn infant.

Megakaryocytopoiesis has been difficult to study in the fetus and neonate because of the intrinsic low level of megakaryocyte production in the marrow and the lack of availability of marrow samples to study. Using microassay techniques, Murray and Roberts[79] have shown that megakaryocytopoiesis is likely increased at 24 to 36 weeks gestation versus term gestation. Platelets appear at 5 weeks postconception, and megakaryocytes appear in the liver at 8 weeks. Fetal and neonatal megakaryocytes are smaller than adult megakaryocytes.[80]

Megakaryocytes

The cord blood of preterm babies has increased numbers of all megakaryocyte precursors compared with term infants. Furthermore, term infants have increased circulating megakaryocyte

progenitor numbers at birth compared with adults.[81] The magnitude of cord blood megakaryocyte progenitors' proliferative and maturational responses to cytokines is related to developmental age.[79] Reticulated platelet counts are reported as similar to adult levels in healthy neonates greater than 30 weeks gestational age but increased in neonates younger than 30 weeks gestational age.[82] However, a study by Joseph and colleagues[83] reported reduced reticulated platelet counts in neonates of all gestational ages compared with adults. No information on developmental differences of megakaryocyte and platelet precursors during childhood is available.

Platelets in Cord Blood

Platelet counts and mean platelet volumes in neonates are similar to those in adults, with values of 150 to 450 × 10^9/L and 7 to 9 femtolitre (platelet volume), respectively.[84-92] Platelet counts in fetuses between 18 and 30 weeks gestational age also fall within the adult range, with the average value being 250 × 10^9/L.[93] Platelet survival has not been measured in healthy infants. Electron microscopy studies on cord platelets have demonstrated normal numbers of granules; however, serotonin and adenosine diphosphate, which are stored in dense granules, are present at concentrations that are less than 50% of adult values. Flow cytometry studies in whole blood without added agonists show that differences between neonates and adults in platelet binding of monoclonal antibodies for glycoprotein Ib or P-selectin are not significant; however, glycoprotein IIb/IIIa is significantly reduced.[94,95] Platelet adhesion at birth has not been assessed with sensitive and reproducible assays; this may explain the conflicting in vitro results.[96,97] Glycoprotein Ib is present on fetal platelet membranes in adult quantities.[98]

Both the plasma concentrations of vWF and the proportion of high-molecular-weight multimers (and therefore more active forms) of vWF are increased in neonates.[99,100] The cord multimeric pattern of vWF appears similar to the forms released by endothelial cells. This may be explained by the recent finding that newborn plasma has little if any detectable vWF cleaving protease.[99,101] The quantitative and qualitative differences in vWF at birth are likely responsible for the enhanced cord platelet agglutination to low concentrations of ristocetin[100,102] and contribute to the short bleeding time in neonates.[103-105] Glycoprotein IIb/IIIa complexes are expressed on platelet membranes early in gestation;[106] however, the capacity of cord platelets to aggregate following exposure to a variety of agonists has been variable, with some observations being more consistent than others. Recently, differences between the cord blood of premature and full-term infants have been described, with reduced aggregation in preterm infants.[107]

Epinephrine-induced aggregation of cord platelets is consistently decreased compared with that of adult platelets because of the decreased availability of α-adrenergic receptors.[108-115] Ristocetin-induced agglutination of cord platelets is consistently increased compared with that of adult platelets, likely because of quantitative and qualitative increases in the level of vWF. Aggregation of cord platelets induced by adenosine diphosphate, collagen, thrombin, and arachidonic acid is variable and may be moderately decreased or similar to that of adult platelets.[116-118] Thromboxane A_2 production is reduced in neonates, despite normal receptor binding, suggesting a postreceptor signal transduction problem.[119]

Inositol phosphate production and protein phosphorylation are normal, as is production of arachidonic acid and its metabolites.[118] In fact, cord platelets release more arachidonic acid than adult platelets in response to stimulation by thrombin.[120] This increased release may be due to the greater reactivity of platelet membranes induced by low levels of vitamin E.[121,122] Agonist receptors, with the exception of the α-adrenergic receptor discussed previously, do not appear to be decreased in number. Despite a poor response to collagen stimulation, cord platelets have normal numbers of the collagen receptor glycoprotein Ia/IIa present in platelet membranes.[123] Coupling of agonist receptors to phospholipases may be the site of this transient activation defect in response to collagen.[124]

Platelet Function in Neonates

A few studies have assessed aggregation of newborn platelets obtained during the first few days of life; other studies have evaluated platelets of older neonates.[125] Improved platelet aggregation was seen in newborn platelets drawn 2 hours after birth, with normalization of platelet aggregation at 48 hours. Studies using whole-blood flow cytometry show that, compared with adult platelets, neonatal platelets are hyporeactive to thrombin, a combination of adenosine diphosphate and epinephrine, and a thromboxane A_2 analogue.[114,121,126,127] The clinical significance of these observations remains unknown.

Bleeding Time

Measurement of the bleeding time is currently the best in vivo test of platelet interaction with the vessel wall.[128] Bleeding times in infants during the first week of life are significantly shorter than those in adults.[129] Several mechanisms contribute to this enhanced platelet/vessel wall interaction, including higher plasma concentrations of vWF,[1,4,5] enhanced function of vWF due to a disproportional increase in the high molecular weight multimeric forms,[130,131] active multimers, large red cells, and high hematocrits.[132,133] The significance of mild platelet aggregation defects in platelets derived from cord blood is uncertain when bleeding times in neonates are shorter than those in adults. Although automated bleeding time devices modified for newborn infants and children are available and have been standardized, the test remains difficult to reproduce in clinical laboratories.[134]

The platelet function analyzer (PFA)-100 system (Siemens Healthcare Diagnostics, Washington DC) provides an in vitro method of assessing primary platelet-related hemostasis by measuring the time, the closure time, taken for a platelet plug to occlude a microscopic aperture cut into a membrane coated with collagen and either adrenaline or adenosine diphosphate. The PFA-100 system is ideal for neonates because of the small volume required and the rapidity of testing. Normal ranges for the closure time of children are similar to those of adults. Newborn infants have shorter closure times that are not influenced by red or white blood cells.[135-139] The ease of the PFA-100 system and the associated difficulties in performing skin bleeding times have reduced the clinical utility of skin bleeding times. Further studies are required to determine the optimal method of assessing primary hemostasis in neonates and children.

Activation During the Birth Process

There is strong evidence that platelets are activated during the birth process. Cord plasma levels of thromboxane B_2, β-thromboglobulin, and platelet factor-4 are increased, the granular content of cord platelets is decreased, and epinephrine receptor availability is reduced, perhaps secondary to occupation.[140-142] The mechanisms of activation are likely multifactorial and include thermal changes, hypoxia, acidosis, adrenergic stimulation, and the thrombogenic effects of amniotic fluid. Activation of the coagulation system may provide an explanation for the paradox that one-stage coagulation times are prolonged in the neonate, but that various measures of whole-blood clotting duration are shortened compared with those in the adult.[143]

BLOOD VESSEL WALL

The vessel wall profoundly influences hemostasis due to the procoagulant and anticoagulant properties of endothelial cells and extracellular matrix components. Each of these properties is

significantly influenced by age. One of the anticoagulant properties of endothelial cell surfaces is mediated by lipoxygenase and cyclooxygenase metabolites of unsaturated fatty acids. Prostaglandin I_2 production from cord vessels exceeds that of vessels from adults.[144] In a rabbit venous model, increase is significant in glycosaminoglycans by mass in inferior vena cavas from pups compared with adult rabbits. The AT-mediated anticoagulant activity of inferior vena cava glycosaminoglycans, especially heparin sulfate, is increased in pups compared with adult rabbits.[145] In a rabbit arterial model, total proteoglycan, chrondroitin sulfate, and heparin sulfate content are increased in the intima and media of aortas from pups compared with adult rabbits. AT activity in aortas of pups, due to heparin sulfate glycosaminoglycans, is also increased.[146] The increased glycosaminoglycan-mediated vessel wall AT activity in pups compared with adult rabbits suggests that young blood vessels may have greater antithrombotic potential.

When measured directly, thrombin generation in cord plasma is decreased in the presence of human umbilical endothelial cells compared with plastic owing to cell surface promotion of AT inhibition of thrombin.[147] Soluble levels of endothelial cell adhesion molecules and selectins are also age dependent, suggesting developmental differences in endothelial cell expression and secretion of these molecules.[148]

Nitric oxide is a labile humeral agent that modulates vascular tone in fetal and postnatal lungs and contributes to the normal decline in pulmonary vascular resistance at birth. Nitric oxide is a potent inhibitor of platelet adhesion and aggregation and stimulates disaggregation of platelet aggregates. Nitric oxide likely interacts with prostaglandin I_2 and other metabolites of the lipoxygenase pathway to modulate platelet function in a synergistic manner.[149]

It has been observed in sheep that vessel wall thickness increases in the perinatal period.[150] This is likely in response to increases in stress per vessel internal diameter. Contractility also increases with fetal development.[151] The endothelial cells of fetal vessels are larger and less uniform and protrude into the lumen.[152]

SUMMARY

The vascular and hemostatic systems of the fetus and neonate are continually evolving. One must take this into consideration when evaluating these systems for dysfunction. Adult normative data do not apply to the fetus or neonate. Although different in content and structure, these systems should be considered physiologically in the fetus and neonate. This is an important consideration when determining and monitoring therapeutic intervention.

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Thomas G. Diacovo

Platelet–Vessel Wall Interactions

Although an essential step in our evolution as a species, the development of a complex circulatory system has rendered human beings susceptible to physical and biochemical injuries that can result in hemorrhage and ultimately death. To meet these challenges, the body has developed a unique surveillance and repair system that relies in part on the ability of platelets to recognize and adhere to sites of vascular damage. The importance of this interaction is underscored by bleeding sequelae that result from a reduction in platelet numbers or function associated not only with various disease states but also as a complication from advances in critical care medicine. Such is the case with newborns admitted to the neonatal intensive care unit in which a quarter of them will have thrombocytopenia. To complicate matters, previous studies suggest that neonatal platelet function may be impaired, which would put them at further risk for bleeding. To understand both the mechanism(s) that may predispose the sick neonate to bleeding and the clinical relevance of the reported reduction in function of their platelets, it is necessary to have a broad overview of mechanisms that limit, as well as promote, platelet–vessel wall interactions.

ENDOTHELIUM

Anticoagulant Properties

In the absence of injury, platelets do not interact with the blood vessel wall because of the antithrombogenic nature of healthy endothelium. To prevent inappropriate platelet deposition, endothelial cells generate several substances that directly interfere

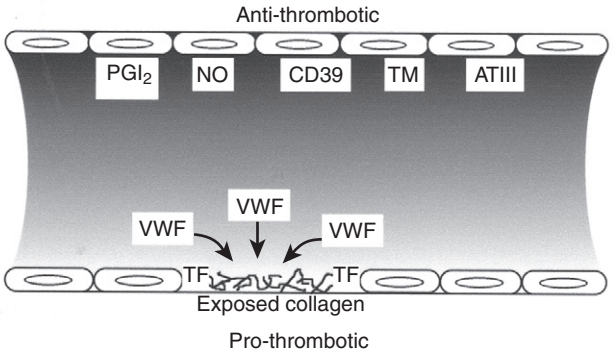


Figure 147-1 Antithrombotic and prothrombotic properties of vascular endothelium. *Top,* Molecules that limit the activation of platelets and the coagulation cascade. *Bottom,* Consequences of disrupting the endothelial cell barrier, which include exposure of tissue factor *(TF)* and collagen, the latter serving as a binding surface for von Willebrand factor*(VWF).*

with platelet function (Figure 147-1).[1] One such product is prostacyclin, which is a major metabolite of arachidonic acid and is produced by the cyclooxygenase system in endothelium.[2] Prostacyclin is released into the circulation where it can bind to a G protein–coupled receptor (GPCR) on platelets, which in turn activates intracellular adenylyl cyclase. GPCRs are transmembrane proteins that interact with the environment both outside and inside the cell. As such, they play a crucial role in receiving chemical signals from other cells and in activating certain cellular responses.[3] Consequently, this results in an increase in levels of cyclic adenosine monophosphate, which ultimately leads to activation of protein kinase A and direct inhibition of Ca^{2+} mobilization and granule release.[4] These events limit the ability of platelets to respond to procoagulant stimuli, thereby preventing interactions with the vessel wall. Because newborns tend to have higher levels of prostacyclin than adults, which are thought to aid in the reduction of pulmonary vascular resistance, these levels may also contribute to the reported hyporesponsive state of their platelets.[5-7]

Nitric oxide (NO) is another important molecule produced by endothelial cells that can prevent platelet activation and aggregation, processes that are part of the sequence of events leading to the formation of a hemostatic plug. It is short-lived (with a half-life of a few seconds), is highly reactive, and is produced by a group of enzymes known as nitric oxide synthases (NOS). Vascular endothelium possesses a type that is constitutively expressed (eNOS). In addition, production of NO can be enhanced by endothelial cell activation or in response to the application of fluid shear stress.[8] Once produced, NO is able to diffuse rapidly into platelets, where it binds to and activates guanylyl cyclase. As a result, levels of 3,5-cyclic guanosine monophosphate are increased, which prevents intracellular calcium release, the critical step in the signal transduction pathway that leads to full platelet activation.[9,10] NO has been shown to blunt the response of neonatal platelets to agonist-induced activation, suggesting that it can play a role in preventing inappropriate platelet–vessel wall interactions in newborns.[11]

Adenosine diphosphate (ADP) is also known to be a potent platelet activator that is released from damaged cells and activated platelets. To limit this possibility, vascular endothelium expresses CD39/ecto-adenosine diphosphatase, a membrane-bound glycoprotein that can hydrolyze ADP.[12] This is supported by the ability of recombinant soluble CD39 to both inhibit ADP-induced platelet activation and significantly reduce the degree of ischemia-induced cerebral infarction in CD39-null mice.[13] Although the role of this ecto–adenosine diphosphatase in contributing to thromboregulation in neonates is not known, human umbilical vein cells do express a functional form of CD39.[14]

Activation of the coagulation cascade in response to vascular injury results in the generation of thrombin, a potent stimulator of platelets. Thrombomodulin (TM) is a membrane-bound protein that modulates the substrate specificity of thrombin so that it preferentially promotes the activation of the protein C anticoagulant system. It is expressed in abundance on all endothelial cells but has no intrinsic enzymatic activity. This pathway is initiated when thrombin binds to the TM, permitting it to convert protein C from an inactive to active state so that it can form a complex with protein S, which leads to inactivation of clotting factors VIIIa and Va. In addition, thrombin bound to TM loses its procoagulant activities such as the ability to convert fibrinogen to fibrin, activates factors V and XIII, inactivates protein S, and mediates platelet aggregation.[15,16] TM can also interact with factor Xa, which prevents the generation of thrombin from prothrombin.[17] Although the TM deficiency in mice is lethal during the early stages of development, it is not known whether differences in function exist between neonates and adults.[18]

Antithrombin III (ATIII) is a protein in blood that prevents activation of the coagulation cascade. It does so by inhibiting thrombin and factors Xa and IXa. ATIII activity is markedly potentiated by heparin and is the principal mechanism by which heparin administration promotes anticoagulation in patients.[19,20] In neonates, ATIII activity has been reported to be 50% to 60% of that found in adults but does not appear to increase their risk of thrombosis.[21] The importance of this protease in preventing inappropriate clot formation is exemplified in patients with congenital ATIII deficiency. It is a rare autosomal disorder that is classified into two major types and results in an increased risk of venous and arterial thrombosis. Type I deficiency is characterized by reduced antithrombin level and function, both at approximately 50% of normal. Type II deficiency results from the presence of a functionally inactive protein with almost normal antigen levels. More than half of all patients with type I disease will likely have a clotting event in their lifetime.[22,23]

Procoagulant Properties

Physical or biochemical disruption of the endothelial cell barrier that exists between extracellular matrix (ECM) components and flowing blood initiates the coagulation cascade and deposition of platelets. Collagen fibers are the most thrombogenic macromolecular components of the ECM.[24] Platelet interaction with collagen occurs as a two-step procedure: (1) indirect contact mediated by von Willebrand factor (VWF) followed by (2) direct contact resulting in platelet activation and firm adhesion to the site of injury (see Figure 147-1).

VWF is a large glycoprotein of complex multimeric structure. It is synthesized exclusively by vascular endothelium and megakaryocytes and is stored and secreted by these cell types, as well as by circulating platelets. In blood, it not only serves as a carrier for factor VIII, protecting it from proteolysis, but also plays a critical role in hemostasis by promoting platelet deposition at sites of endothelial cell injury. To accomplish this task, VWF is able to adhere to components of the subendothelial matrix such as collagen and receptors on the surface of platelets, forming a bridge between them.[25] One remarkable feature of this plasma glycoprotein is its ability to support significant interactions with platelets only on surface immobilization and under specific hemodynamic conditions as encountered on the arterial side of the circulation.[26,27] This avoids the disastrous consequences of platelet–VWF aggregate formation in flowing blood.

VWF production by endothelium occurs through constitutive and inducible mechanisms, with the latter relying on storage

and subsequent release from an intracellular, membrane-bound organelle known as a Weibel-Palade body in response to vascular injury. The inducible pathway is most efficacious in terms of platelet recruitment because it results in the release of the majority of high-molecular-weight multimers of VWF, which possess the greatest hemostatic properties.[28] VWF storage, however, is not limited to endothelial cells because megakaryocytes and platelets also possess this capability (stored in organelles known as α-granules); however, this source of VWF contributes minimally to plasma levels but does play a role in promoting platelet-platelet interactions at sites of vascular injury.

In addition to promoting the deposition of VWF, vascular injury also results in the activation of the coagulation pathway that relies in part on tissue factor (TF).[29] TF is a glycoprotein that usually remains separate from circulating clotting factors in blood and is expressed by multiple cell types including smooth muscle cells in and surrounding blood vessels. On disruption of the endothelial barrier, TF is exposed to blood and can trigger the coagulation cascade, which ultimately results in the generation of thrombin. Thrombin has multiple biologic actions including (1) stimulation of the release of VWF from surrounding endothelium; (2) conversion of fibrinogen to fibrin, the solid component of the clot; and (3) down-regulation of the TM–protein C and S systems. Although deletion of TF in mice is embryonic lethal, no reports of its deficiency in humans have appeared.[30] These results suggest that TF is essential for life, most likely because of its central role in hemostasis.

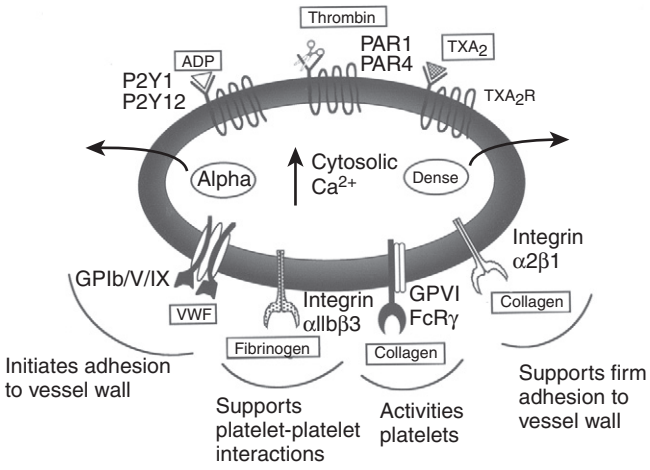


Figure 147-2 Receptors critical for platelet adhesion and activation. The ability of platelets to initiate contact and subsequently adhere with the injured vessel wall relies on the adhesion receptors GPIb-IX-V and α2β1, whereas platelet-platelet interactions are dependent on αIIbβ3. Receptors that support signal transduction include P2Y1/P2Y12, PAR1/PAR4, TxA_2R, and GPVI, which contribute to the mobilization of intracellular calcium stores. Ligands or agonists that bind to platelet receptors are boxed.

PLATELETS

Platelets are small, disk-shaped objects that circulate in the blood for a period of 10 days.[31] They are produced by fragmentation of megakaryocytes located within the bone marrow, and, although platelets do not possess a nucleus, they can synthesize protein products from constitutive or postranscriptionally processed messenger RNAs through a process termed signal-dependent translation.[32,33] In addition, platelets store growth factors, coagulation proteins, adhesion molecules, cytokines, cell-activating agents, and angiogenic factors in secretory organelles known as alpha (α) and dense granules[34]; these play a major role in promoting hemostasis and thrombus formation. In the developing fetus, platelet production by mid–second trimester is sufficient to achieve and maintain counts between 150 and 450 × 10^9/L, which is within the adult range.[35,36] Despite this similarity, it is believed that functional differences exist between newborn and adult platelets that may reduce the capacity of the former to maintain adequate hemostasis especially in the context of increased blood vessel fragility as is the case in premature infants or as a consequence of asphyxia or infection. To better understand the relevance of these observations, it is first necessary to have a basic knowledge of the adhesive and signaling pathways that promote platelet deposition at sites of vascular injury.

Adhesion

Platelets are poised to rapidly adhere to sites where the integrity of the vasculature has been compromised as their unique shape enables them to be pushed against the vessel wall in response to the hydrodynamic forces generated by flowing blood.[37] That said, these same forces would also tend to disrupt thrombus growth, especially on the arterial side of the circulation where the amount of force generated at the vessel wall (termed wall shear stress) is considerable. To overcome this formidable obstacle, platelets rely on several families of cell surface adhesion molecules that have distinct roles in the initiation and stabilization of interactions at sites of vascular injury (Figure 147-2). For instance, the ability of platelets to rapidly attach to the injured vessel wall requires the participation of a constitutively expressed and active glycoprotein complex (GP) known as GPIb-IX-V.[38,39] Each component of the complex is a member of the leucine-rich repeat family and includes GPIbα, GPIbβ, GPIX, and GPV. However, it is GPIbα that plays the predominant role in initiating contact between circulating platelets and the site of injury. The importance of this complex in hemostasis is demonstrated in the autosomal recessive bleeding disorder known as Bernard-Soulier syndrome that results from the absence or decreased expression of these glycoproteins on platelets.[40] The ability of GPIbα to initiate platelet adhesion requires the participation of the plasma protein VWF. As mentioned earlier, VWF serves as a bridge between platelets and components of the damaged vessel wall. It is the role of a specialized region within VWF known as the A1 domain that serves as a ligand-binding site for GPIbα. This interaction, however, is extremely labile in nature and does not permit platelets to remain firmly bound at the site of injury.[26] A role for A1 domain in this process is suggested by the perturbation in hemostasis that can occur in a subclass of patients with von Willebrand disease known as type 2M. Bleeding is believed to occur because of point mutations within this domain that prevent or significantly reduce its interaction with GPIbα.[41] Compared with those of adults, platelets from neonates have been reported to have an enhanced interaction to ECM proteins in the presence of VWF and under flow conditions, which may result from higher amounts of large multimers of VWF in their plasma.[42,43]

For platelets to be retained at sites of vascular injury, a second family of adhesion receptors expressed on their surface must engage counter ligands that are exposed or deposited in such areas. Once such receptor is the integrin $\alpha_2\beta_1$ that permits platelets to firmly adhere to collagen. As with other members of the integrin family, it exists in an inactive form on resting platelets and can only significantly interact with this ECM protein once platelets become activated in response to various agonists. In particular, it is thought that another collagen-binding molecule on platelets known as GPVI plays a supporting role in the activation process. Once engaged, $\alpha_2\beta_1$-collagen interactions are thought to stabilize the formation of the initial layer of platelets covering the injured area. Although an abnormality in collagen-induced platelet activation in solution has been reported in neonates, it

is not clear whether any significant differences in $\alpha_2\beta_1$ function or structure are present.[44] In addition, it appears that absence of this integrin receptor in humans yields only a mild bleeding phenotype, suggesting that it plays a significant but not essential role in hemostasis.[45]

A key step in the formation of a hemostatic plug is the ability of platelets to stick to one another in a process termed aggregation. This is the role of the integrin αIIbβ3, the most abundant glycoprotein expressed on the surface of platelets.[46] The major ligands for this receptor are fibrinogen, fibronectin, and VWF, which on interacting with this adhesion molecule on activated platelets permit aggregation to occur, an event essential for sealing off vascular leaks. Absence or dysfunction of this integrin receptor as occurs in the autosomal recessive disorder known as Glanzmann thrombasthenia results in an inability of platelets to adhere to one another, leading to a severe bleeding phenotype.[40] Alterations in αIIbβ3 function and possibly expression have been implicated as a potential mechanism for neonatal platelet hyporesponsiveness, but results are conflicting. That said, the reported defects in aggregation may have more to do with intracellular signaling events that convert this integrin to an activated state than the actual numbers of receptors present on neonatal platelets.[47-52] However, the mechanisms proposed to contribute to clot strength such as fibrin binding and actin-myosin interactions appear to be intact in neonatal platelets.[53]

Signaling

The formation of a stable hemostatic plug relies not only on adhesion molecules but also on a series of progressive and overlapping biochemical events in platelets. This results from substances generated or exposed at the site of injury and by the platelet itself. Substances that induce platelet activation are called agonists, and each interacts with a specific receptor on the platelet surface, causing a series of intracellular reactions. This process involves both G protein– independent (initial phase of platelet-vessel wall interactions) and –dependent (thrombus growth and stability) signaling.[54] G proteins, short for guanine nucleotide–binding proteins, are a family of proteins involved in second messenger cascades.[3] Regardless of the activating stimulus, a major event that contributes to platelet activation is a rise in cytosolic calcium concentration, which is a widely used mechanism for regulating cell function.[55,56] It appears that the previously documented abnormalities in neonatal platelet function are mainly due to the impaired mobilization of this important intracellular mediator.[57] Ultimately, platelet activation results in changes in the cytoskeleton, release of granule contents, and an increased adhesiveness of integrin receptors, all of which are essential for stabilizing platelet-ECM and platelet-platelet interactions. Platelets express several receptors that convert external environmental cues into biochemical signals in response to interactions with agonists such as collagen, thrombin, ADP, or thromboxane A_2 (TxA_2) (see Figure 147-2). A brief description follows.

The formation of the initial layer of adherent platelets at sites where disruption of the endothelial lining of blood vessels occurs requires activation through the collagen receptor GPVI. It is a member of the immunoglobulin superfamily and is noncovalently associated with a small, immunoreceptor tyrosine-based activation motif (ITAM)–containing subunit, the FcRγ chain.[58] Although GPVI expression relies entirely on its association with the FcRγ chain, signaling can occur by Fc receptor–dependent or –independent pathways. In either case, engagement of GPVI results in increased adhesiveness of the integrins α2β1 and αIIbβ3, enabling platelets to firmly attach to collagen and to one another.[59] It also results in release of storage granule contents that are critical for augmenting and maintaining platelet activation. GPVI deficiency has been reported in humans, causing a mild bleeding diathesis.[60] However, its role in the observed hyporesponsiveness of neonatal platelets remains controversial because of conflicting results concerning the ability of collagen to induce GPVI-mediated platelet aggregation in solution-based assays.[44,61,62] Of interest, neonatal platelets have been reported to have a greater propensity to form aggregates on a surface coated with subendothelial ECM than those from adults, suggesting that this signaling pathway is intact.[42]

ADP also plays an essential role in hemostasis by supporting thrombus growth because of its ability to function in an autocrine and paracrine fashion. It is stored in platelets in organelles known as dense granules and is released in response to agonists such as collagen. ADP binding to the G protein–coupled receptors (GPCR) P2Y1 and P2Y12 on the surface of platelets is required for a full response to this agonist.[54,63] Evidence to support a role for ADP as a positive-feedback mediator required for sustained platelet activation is the reduced response of platelets to thrombin in the absence of these receptors, as well as the observations that patients with deficiencies in either dense granules or P2Y12 have an increased propensity to bleed.[54] Although it is not known whether the number or function of ADP receptors expressed on platelets differs from those of adults, neonatal platelets have been shown to be less responsive to ADP and have impaired dense granule release in response to various agonists.[47,64,65]

As in the case of ADP, TxA_2 is a powerful activator of platelets that also functions in an autocrine and a paracrine manner, permitting the recruitment of platelets into the growing thrombus.[66] TxA_2 is a biologically active lipid mediator that is produced by the metabolism of arachidonic acid through the cyclooxygenase pathway. Its effects on platelets, and on other target cells, are mediated via interaction with G protein–coupled thromboxane–prostanoid receptors. Consistent with the important role of TxA_2 signaling in platelet function and hemostasis is the ability of aspirin, a potent cyclooxygenase inhibitor, to reduce pathologic thrombus formation in patients and the prolonged bleeding times in mice lacking the thromboxane–prostanoid receptor.[67] In the case of neonatal platelets, not only do they have an impaired response to this agonist, but they also produce less of this cyclooxygenase product as compared with their adult counterparts because of a decreased conversion of arachidonic acid to TxA_2.[44,68-70] As no differences in thromboxane–prostanoid receptor binding characteristics have been reported between neonatal and adult platelets, it appears that the reduced responsiveness of the former is mediated by impairment in postreceptor signaling.[69]

As mentioned, thrombin is the major protease of the hemostatic system and is an effective activator of platelets. Although it is not stored in or secreted by platelets, it is generated on the surface of activated platelets where it can further stimulate the coagulation process.[71-73] This includes activating surrounding platelets through GPCRs known as protease-activated receptors (PARs). Of the four known thrombin receptors, only PAR1 and PAR4 are expressed on human platelets, the former shown to mediate platelet activation at low concentrations of thrombin and the latter in the presence of high concentrations of the agonist.[74] As with the majority of platelet agonists, thrombin can trigger shape change, release of granule contents, and increased adhesiveness of integrin receptors. No reported bleeding disorders have been attributed to loss or dysfunction of PAR1 or PAR4 in humans, although inherited disorders in clotting factors demonstrate the requirement for thrombin in promoting effective hemostasis. Reports on neonatal platelet responsiveness to thrombin are conflicting, as well as the ability of plasma from newborns to generate this procoagulant substance.[57,64,75-77]

SUMMARY

The ability of platelets to interact with the vessel wall requires the exposure and generation of agonists that promote adhesion, as well as the activation of the clotting cascade. On the basis of

previous in vitro studies suggesting that neonatal platelets are hyporesponsive to physiologic activators that promote hemostasis, it would appear that they would be at increased risk for bleeding. Despite these findings, the hemostatic system of healthy newborns appears to be effective, because they do not have complications of spontaneous hemorrhage.[78] Thus standardized testing and better methodologies are needed to assess platelet function in neonates before the clinical relevance of these observations can be fully ascertained. What may be of greater importance is the number of platelets in the circulation, as these anucleate cells can release proangiogenic cytokines and growth factors that are essential for enabling vascular endothelium to maintain an antithrombotic barrier in the absence of injury.[79] This is evidenced by the spontaneous extravasation of erythrocytes into surrounding tissues when platelet counts fall below a critical value as a result of the failure of the integrity of the endothelial cell barrier. A better understanding of the role that platelets play in stabilizing the vessel wall is needed to aid clinicians in their decision as to when to transfuse the neonate with thrombocytopenia in the absence of overt bleeding.

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SECTION XX

Developmental Immunobiology

148 Host Defense Mechanisms Against Bacteria

Timothy R. La Pine and Harry R. Hill

Bacterial sepsis is a major contributor to neonatal mortality and morbidity. The incidence of sepsis in the first month of life ranges from 1 to as high as 10 cases per 1000 live births.[1,2] In certain geographic areas, this rate may be significantly higher.[3,4] Bacterial sepsis occurs more frequently in preterm infants and when maternal complications have occurred, such as premature rupture of membranes or intrapartum infections.[1,3] Although advancements in maternal medical management and neonatal intensive care have decreased infant mortality and morbidity, prolonged hospitalization and invasive procedures have contributed to an increased incidence of certain bacterial infections. In spite of the development of potent antimicrobial agents, the mortality rate associated with neonatal bacterial sepsis ranges from 5% to 15%.[1,3,5] Furthermore, the compromised nature of a developing neonate's host defense system allows for bacterial dissemination from intravascular to extravascular sites, including the meninges, lungs, and bones. The morbidity from these secondary infections is high.[6,7]

The unique predisposition of the human neonate to bacterial infections is attributed to defects in both innate and acquired immune responses. The innate, or natural, immune response is nonspecific and not influenced by prior antigen interactions. Various physical (e.g., skin, cilia, mucus) and biochemical barriers (e.g., gastric acid, lysozyme, surfactant proteins), as well as phagocytic cells (e.g., granulocytes and macrophages) and the plasma factors (e.g., acute-phase reactants, the complement system, the coagulation cascade, and pattern recognition receptors) comprise innate immune responses. The acquired, or adaptive, immune response is specific. It is characterized by direct immune responsiveness as a result of an initial antigen exposure, with a memory, or anamnestic, response on antigen reexposure. Acquired immune responses include humoral immunity involving immunoglobulin production by B lymphocytes and cellular immunity mediated by T lymphocytes and associated factors (e.g., natural killer [NK] cells, cytokines, and interleukins). Neonatal immune development is discussed in depth in other chapters in this section; here we highlight deficiencies of this development in host defense against bacterial infections.

THE CHANGING NATURE OF NEONATAL BACTERIAL INFECTIONS

Group A streptococci and other gram-positive organisms often were the etiologic agents of newborn bacterial sepsis before the introduction of antibiotics. Later, after penicillins and sulfonamides were introduced in the 1940s, gram-negative organisms such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* became prominent.[8] Since the mid-1960s, group B streptococcal infections have become the most common infections in neonates throughout the United States and in other developed countries.[1,9] It is interesting that this organism has not played a major role in neonatal sepsis in many developing countries such as Mexico, India, and Thailand, where *Klebsiella* and *E. coli* are still the predominant pathogens.[3] The reasons for this are not clear. Infants generally acquire group B streptococcal infections by vertical transmission from the birth canal of a colonized mother. The low rates of invasive neonatal group B streptococcal disease in developing countries may reflect low maternal colonization rates, less virulent strains of group B streptococci (GBS) bacteria, or high levels of transplacental acquired antibody.

Although GBS and *E. coli* together account for more than 70% of serious bacterial infections in neonates, their distribution is changing.[3] Antibiotics are increasingly used in the perinatal period to reduce the risk for neonatal group B streptococcal infection during premature labor and premature rupture of the membranes. Concern exists that this increased use of antibiotics may result in a change in the spectrum of organisms and their susceptibility to antibiotics. Stoll and associates,[10,11] in a multicenter trial evaluating the etiology of early-onset neonatal sepsis among infants weighing less than 1500 g, found an increased prevalence of *E. coli* sepsis and a marked reduction in group B streptococcal sepsis between 1998 and 2000 when compared with an earlier cohort born between 1991 and 1993. Furthermore, 85% of the *E. coli* isolates were resistant to ampicillin. The mothers who received intrapartum ampicillin for group B streptococcal carriage were more likely to have infants with ampicillin-resistant *E. coli* infections. Antibiotic use can change the spectrum of neonatal bacterial infections and increase the prevalence of antibiotic-resistant organisms.[11]

Staphylococci are a major cause of nosocomial neonatal bacteremia and sepsis in intensive care nurseries. Noel and Edelson[12] identified 23 cases of clinically significant neonatal intensive care unit *Staphylococcus epidermidis* infections during a 17-month period. Ten of these episodes were associated with colonized indwelling catheters. Staphylococci have surface proteins that mediate their attachment to intravascular devices and other foreign bodies in addition to damaged endothelial cells. Once this surface attachment takes place, phagocytosis by neutrophils is inhibited, as is antibiotic penetration, and a nidus for infection is established. Infections with staphylococcal species are of particular concern in the extremely low-birth-weight infants requiring indwelling catheters.

Other organisms such as *Listeria monocytogenes*, *Haemophilus influenzae*, *Neisseria meningitidis*, and groups C, D, and G streptococci also can cause serious neonatal infection.[6] Anaerobic organisms additionally may be isolated alone or in combination with aerobes from septic neonates.

Campylobacter fetus, jejuni, and *coli* also have been implicated in premature delivery and associated with fetal and neonatal infection and mortality.[13]

THE COMPONENTS OF INNATE HOST DEFENSE

The innate, or natural, immune system provides host protection against microbial invasion without the need for prior exposure to that microbe. The innate immune system developed first

phylogenetically, appearing in all multicellular organisms, including plants, insects, and animals, and is the first line of defense against invasive pathogens.[14,15] It is active before the acquired immune responses and does not change or amplify on pathogen reexposure. The activities of innate immunity are both rapid, to prevent early microbial invasion, and nonspecific, to protect against multiple pathogens of diverse nature. Cells of the innate immune system contribute to antigen processing and presentation as well.[14]

Epithelial Cells, Normal Bacterial Flora, and Mucosal Barriers

Integrity of the skin and the epithelial surfaces of the respiratory and gastrointestinal tracts serves to impede microbe ingress physically. Premature infants with incomplete skin epithelization who require indwelling catheters are predisposed to bacterial infections, particularly with staphylococcal species. Damage to the respiratory epithelium with ventilation and oxygenation can predispose neonates to pneumonias. Similarly, immaturity of innate immune responses in the developing gastrointestinal tract predisposes preterm infants to necrotizing enterocolitis and other intestinal infections, all of which can lead to invasive bacterial infections.

Normal microbial flora contribute to innate host defense at epithelial and mucosal surfaces. Humans are colonized with a variety of microorganisms that suppress the overgrowth of pathogenic microbes. Normal flora produce bacteriocins and other antimicrobial agents that suppress the growth of invasive pathogens.[16] Alteration of the flora by antimicrobial therapy may result in the emergence of invasive pathogens.

The moisture content or type of epithelial cell surface may predispose the host to colonization with pathogens. Circumcision decreases the incidence of urinary tract infections.[17,18] Uncircumcised infants are more likely to harbor *E. coli* and *Proteus mirabilis* in the urethral meatus and periurethral area than circumcised boys.[19,20] Fimbriated *E. coli* adhere better to the moist inner surface of the foreskin than to the outer keratinized surface.[21] Vernix caseosa is a product of sebaceous secretions that provides an epidermal barrier and functions to "waterproof" a newborn's skin. Although a direct immunologic effect of vernix has not been described, it possesses growth factors that may promote lung and intestinal development.[22,23]

To produce invasive infection, GBS must first broach the mucosal barrier. Adherence of GBS to mucosal epithelial cells is a critical step for both maternal colonization and invasion of the neonatal respiratory tract. Adhesins are surface molecules on bacteria that facilitate binding to host eukaryotic cells. Lipoteichoic acid and surface proteins facilitate GBS binding to epithelial cells.[24-27] The greater adherence of type III GBS to neonatal respiratory epithelium facilitates their colonization and predominance in early onset neonatal sepsis. The physical disruption of the epithelial cell surface, however, may not be required for GBS invasion. GBS can transgress intact cultured respiratory epithelial cells via actin microfilament–dependent processes.[28]

Innate immunity in the respiratory tract includes the cilia of the nasal mucosa and the passageways of the upper airways, bronchi, and bronchioles. Cilia present on respiratory epithelium serve to impede respiratory invasion of microbial pathogens and remove or expel them.[29] Abnormalities, such as those seen in Kartagener syndrome, underscore the role of cilia in innate host defense. These patients have chronic bronchitis and bronchiectasis because of a defect in ciliary rods that results in immotile cilia. Patients with tracheotomies, or requiring prolonged ventilation, are predisposed to pulmonary infections as a result of the breakdown of epithelial immune integrity in the nasopharynx, glottis, trachea, and lung.

Mucin glycoproteins contribute to airway surface fluid. They provide viscosity and physically impede microbe invasion.[30,31] The genes for the mucin glycoproteins, MUC2 and MUC5A, are up-regulated by lipopolysaccharide (LPS) and by gram-positive and gram-negative bacteria.[32] These glycoproteins do not possess antimicrobial activity but provide a milieu for immune defense. The airway surface fluid contains a variety of antimicrobial proteins, such as lysozyme, lactoferrin, and defensins, as well as reactive oxygen and nitrogen species.[31,33,34] Other inflammatory cells of the respiratory tract include macrophages, neutrophils, mast cells, and NK cells. The cytokines produced by these cells and the respiratory epithelium also contribute to the antimicrobial activity of airway surface fluid.

The salt or fluid content at the airway surface may protect against microbial invasion. The high salt content or the decreased airway fluid observed in patients with cystic fibrosis may inhibit normal airway antibacterial activities and contribute to the persistent bacterial colonization and chronic infections seen in these patients.[33,35]

Growth factors and cytokines within the amniotic fluid contribute to fetal gastric immune development. Pregnancies complicated by oligohydramnios, whether caused by premature membrane rupture or by congenital abnormalities, are at higher risk for intestinal infections.[36] Transplacentally acquired immunoglobulins and hormones also influence the development of the enteric immune system. Immunoglobulins (Ig), particularly IgA, present in breast milk, both promote and provide intestinal mucosal immunity.[37] Within hours after birth, the neonate's intestinal tract is colonized by multiple species of bacteria and some viruses. Premature delivery, systemic infection, diet, and antibiotic exposure can alter enteric flora, resulting in local inflammation or invasive infection.[36]

Cells Involved in Innate Host Defense

The innate immune response involves neutrophils, monocytes, macrophages, and dendritic cells capable of phagocytosis. These cells, along with mast cells, also produce a number of inflammatory mediators, including colony-stimulating factors (CSFs) and a variety of cytokines that have roles in both innate and acquired immune responses.

Neutrophils

Neutrophils are central to neonatal host defense against bacteria.[38] Neutrophils appear after macrophages appear in the human fetus. Scattered neutrophil precursors occur in the liver as early as 5 weeks gestation. Bone marrow neutrophils are first present around 10 to 11 weeks.[39-41] Neutrophils are the first cells to migrate into a local area of microbial invasion and are important components of early innate defense.[42] Activated neutrophils must adhere to and migrate through capillary endothelial cell surfaces, then engulf and destroy invading pathogens.[43] Neonatal neutrophils have profound deficiencies in their ability to perform these functions, which contribute to the neonate's increased susceptibility to infection. Bone marrow neutrophil storage pools are also low in neonates, further predisposing them to bacterial infections.[42,44]

Neutrophils possess individual receptors for complement fragments such as C5a, formylated peptides, leukotriene B_4, and chemokines such as interleukin (IL)-8 (IL-8). These receptors initiate intracellular responses through α, β, and γ and guanosine triphosphate–binding proteins, leading to the activation of protein kinase C and the release of intracellular calcium. Activation of these signaling pathways and the binding of inflammatory mediators induce changes in the neutrophil surface charge, membrane potential, ion flux, and membrane fluidity. This interaction up-regulates the surface expression of adhesive glycoproteins, such as leukocyte selectin, sialyl-Lewis X, and the integrins

CD11/CD18.[42,45] Sialyl-Lewis X promotes neutrophil–endothelial cell selectin interactions, resulting in the rolling along and tethering to activated endothelial cell surfaces. The integrins Mac-1 (CD11b/18) bind to endothelial cell intracellular adhesion molecule 1 (ICAM-1), contributing to tight neutrophil adhesion at the site of microbial inoculation. Stimulated neonatal neutrophils have defective membrane potential changes, decreased fluidity, and an associated decreased intracellular calcium flux when compared with adult neutrophils.[42,45] Their ability to adhere to endothelial cell surfaces in vitro is also reduced when compared with adult cells.[46]

Neutrophil movement is initiated through G-proteins and calcium binding to calmodulin, which activates the contraction of cytoskeleton actin and myosin filaments. This leads to diapedesis through the epithelial cell surface and chemotaxis toward the inflammatory focus. Movement is partially dependent on interactions between Mac-1 (CD11b/18), as well as the other integrins and the extracellular matrix.[47,48] When neutrophils arrive at sites of microbial invasion, the cells spread out and up-regulate several phagocytic cell surface receptors. These include receptors for the Fc fragment of immunoglobulin; the complement receptors, C3b and iC3b; fibronectin; and other nonspecific receptors of innate immunity.[45] Neonatal neutrophils show impaired directed movement. The possible mechanisms for this include decreased complement receptors, defects in signal transduction resulting in low intracellular calcium responses, impaired cytoskeletal regulation, incomplete receptor up-regulation, and reduced energy metabolism.[46]

Neutrophils ingest pathogens opsonized with natural antibodies, specific antibodies, complement fragments, or nonspecific opsonins such as fibronectin.[49] Pathogens are killed by oxygen-dependent or -independent processes and antimicrobial factors. Phagolysosome fusion permits entry of granule contents into phagocytic vacuoles. Myeloperoxidase from primary or azurophilic granules catalyzes the production of hypochlorite ion from chloride and hydrogen peroxide, which can react with amines to produce toxic chloramines.[29] Neutrophils from stressed or infected neonates have decreased respiratory burst activity on exposure to GBS, *E. coli*, and *S. aureus* when compared with adults.[50,51] Other components of azurophilic granules include the antibacterial activities of the defensins, lysozyme, and bacterial permeability–increasing protein.[52] Neutrophils also produce a number of proinflammatory cytokines, including tumor necrosis factor (TNF) α, IL-1β, and IL-6, further activating innate and acquired immune responses.[53]

A newly recognized activity of neutrophils from adults was reported in which neutrophil extracellular traps (NETs) are generated for bacteria.[54] NETs are lattices of DNA, histones, granule enzymes, and antimicrobial proteins that capture and kill extracellular bacteria and fungi.[43] Yost and colleagues[55] found that neutrophils from both term and preterm infants fail to form NETs when activated by inflammatory agonists. This work suggests that the increased susceptibility of human neonates to both gram-negative and gram-positive bacteria may, in part, be related to defective NET production. Extracellular trap function has not been described in mononuclear leukocytes[56] but has been described in mast cells from adults.[57]

Monocytes, Macrophages, and Dendritic Cells

Mononuclear phagocytes, monocytes and macrophages, like neutrophils, are recruited to sites of infection. Neonates have diminished influx of monocytes to areas of inflammation. The adhesion of fetal and newborn monocytes is comparable to that of adult monocytes, but they have reduced chemotactic ability.[58-60] Monocytes exhibit considerably less respiratory burst production of hydrogen peroxide and other toxic reactive molecules compared with neutrophils.[61,62] The ability of fetal and neonatal monocytes to kill pathogens including *S. aureus*, *S. epidermidis, E. coli*, GBS, and *Candida albicans* is equal to that of adult monocytes.[63,64]

Monocytes synthesize and secrete numerous products involved in the inflammatory process. These include polypeptide hormones, complement components, cytokines, coagulation factors, enzymes, extracellular matrix proteins, and bioactive oligopeptides and lipids.[65] Certain products, such as the polypeptides IL-1, interferon (IFN) α, and TNF-α, are synthesized in similar concentrations by newborn and adult monocytes.[66-68] Other molecules, including polypeptides such as IFN-γ, IL-8, IL-10, and granulocyte colony-stimulating factor (G-CSF); extracellular matrix proteins such as fibronectin; and bioreactive lipids, such as leukotriene B_4, are synthesized in reduced concentrations by neonatal monocytes.[69-73] The principal function of monocytes in neonatal host defense may be their production of inflammatory mediators. The significance of these differences in host defense is currently under investigation.

Monocytes from developing bone marrow stores are released into the bloodstream where they may circulate for extended periods of time. These monocytes may then enter into tissues and differentiate into macrophages.[39-41] Macrophages have been described in the developing yolk sac, lung, and liver long before monocyte bone marrow development has been established. Fetal liver and lung macrophages are capable of self-proliferation.[74-76] Macrophages line the vascular sinusoids of the spleen, liver, and lymph nodes. This strategic placement allows for host defense of pathogens just below the epithelial barriers of the skin and respiratory and gastrointestinal tracts, and at blood-filtering sites. Macrophages alert cells of both innate and acquired immunity to microbial invasion through the release of cytokines and other inflammatory mediators.

Macrophages are avidly phagocytic and can readily kill most pathogens such as *S. pneumoniae, H. influenzae,* and *S. aureus*.[29] Some intracellular bacteria, such as *Mycobacteria, Nocardia,* and *Legionella* can survive within macrophages. Cytokine stimulation of infected macrophages (e.g., IFN-γ, IL-12, IL-18, and CSFs), is required to kill these pathogens. Deficiency of these cytokines or their receptors can result in infections with these intracellular bacteria.[29]

Macrophages generate several mediators and proteins that contribute to microbial killing. The ligands include arachidonic acid pathway–derived components such as leukotriene B_4. The proteins include lysozyme, lactoferrin, transferrin, and the defensins.[29] Macrophage-derived mediators initiate the inflammatory response and recruit neutrophils. These include the cytokines, TNF-α, IL-1, IL-12, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF; the chemokines, chemotactic peptides, IL-8, macrophage inflammatory proteins, macrophage chemotactic peptide; and other related chemokines.[29,77,78]

Dendritic cells are macrophage-like cells that are less phagocytic and less adherent than macrophages. They are notable for their irregular shape with numerous membrane processes. These cells may leave the skin and migrate to afferent lymphatics and regional lymph nodes. Dendritic cells are present throughout the lungs and most organs except the brain. These cells, which ultimately initiate acquired immune responses via T cells, have the ability to migrate through tissues and ingest extracellular microbes by endocytosis.[79,80]

Mast Cells

Mast cells can be activated by IgE or by microbial pathogens.[81] Mast cell interaction with microbes such as *Salmonella typhimurium* and *Schistosoma mansoni* may be opsonin dependent or mediated by complement fragments including C3b and iC3b.[82,83] Bacteria such as *E. coli* and *K. pneumoniae* may associate with mast cells in an opsonin-independent manner through mast cell pattern receptors.[84-86] The pattern recognition receptor on mast cells for mannose-binding lectin on *E. coli*

and other enterobacteria appears to be a mannose-containing receptor molecule.[87] In addition, gram-positive bacteria such as *S. aureus* and streptococci are able to induce mast cell–mediated cytokine release.[84]

Mast cell degranulation can initiate leukocyte recruitment and enhance host defense against microbial invasion.[88,89] Leukocyte recruitment is mediated, in part, by TNF-α stimulation of vascular and bronchial ICAM-1 leading to leukocyte adherence. Histamine up-regulates selectins, which promote leukocyte rolling along and tethering to the endothelial cell surface. Platelet-activating factor may enhance CD-18–dependent leukocyte adhesion.[90] Mast cells may release IL-8, which stimulates neutrophil migration. Mast cells can phagocytize and kill both gram-positive and gram-negative bacteria.[84,85,91] Mast cells can present antigen, in the context of class I or II HLA molecules, to cells of the acquired immune system.[92] Mast cells or their products also play an important role in the defense against parasitic infections.[93]

Recent studies on mast cells from adults have shown that they are able to provide extracellular antimicrobial activity through the production of extracellular structures similar to the reported NETs. The major components of mast cell extracellular traps are DNA, histones, and granule proteins such as tryptase and the antimicrobial peptide LL-37.[57] To our knowledge studies on neonatal mast cell extracellular trap formation have not been conducted.

γδ *T Lymphocytes and B-1 Lymphocytes*

The late innate response to bacterial, viral, and parasitic infections includes γδ T-lymphocyte infiltration and B-1–lymphocyte activation.[94] The γδ T-cell receptors recognize glycolipid antigens present on many microbes.[95] Located in the epithelial linings of the skin and gastrointestinal and respiratory tracts, where they proliferate and release cytokines, they are also termed *intraepithelial T lymphocytes*.[96-98] The peritoneal cavity contains B lymphocytes termed B-1 cells.[99] The antigen receptors of these cells, like intraepithelial γδ T cells, have limited diversity.[95-100] B-1–lymphocyte cell receptors bind immunoglobulins, mainly IgM, which are reactive with common microbial antigens, such as LPS and phosphorylcholine. B-1 lymphocytes produce natural antibodies specific for a variety of microbes present at epithelial cell surfaces.

Natural Opsonins

Nonspecific opsonins contribute to the innate immune responses before specific opsonic antibody can be generated. These natural opsonins include natural antibodies, complement fragments, and fibronectin.

Natural Antibodies

Natural antibodies are antibodies present in normal sera; they are not the result of antigen stimulation.[101] They may be self-reactive or autoreactive, or reactive against a variety of bacterial, viral, and fungal pathogens.[102,103] Natural antibodies are mainly the products of B-1 lymphocytes that develop in the fetus and neonate.[99] Natural antibodies serve as a second line of defense, after an organism has breached the epithelial or mucosal barrier. Most natural antibodies are IgM and activate the complement system, but they may be IgG and IgA. They bind to repeating moieties of polysaccharides.[104,105] Because many microorganisms share structural polysaccharides and phospholipids, natural antibodies protect against a wide variety of pathogens. Mice lacking natural antibody have enhanced susceptibility to bacterial peritonitis, reduced cytokine expression, particularly TNF-α, and higher mortality rates from infection.[103,106,107]

Complement

The complement system is part of the innate immune system that defends against initial pathogen invasion. Complement is composed of two pathways: the classic pathway and the alternative pathway. It has three main physiologic effects: (1) bridging the innate and acquired immune responses, (2) defense against pyogenic bacterial infections, and (3) disposing of immune complexes and the products of inflammatory injury. The complement system is made up of a series of more than 30 plasma and regulatory membrane proteins. Genes for several of the complement components are found within the major histocompatibility complex class III region on chromosome 6. Complement functions include (1) amplification of leukocyte-associated inflammatory responses, (2) anaphylactoid responses through stimulation of histamine release, (3) chemoattraction and activation of neutrophils and macrophages, (4) opsonization of foreign microbes and antigens, and (5) cytolysis of target cell membranes.[108]

The classic complement pathway is triggered primarily by antigen-antibody complexes or aggregated IgM or IgG. Natural IgM and IgG antibodies can trigger the complement system, resulting in classic pathway activation and formation of the inflammatory components C3a and C5a, and opsonic fragments C3b and iC3b.[109] Mannose-binding protein activates the classic complement pathway.[110]

The alternative complement pathway is triggered by complex polysaccharides, LPSs, bacterial cell wall components, aggregated IgA and IgE, and yeast. Phagocytic cells have receptors for C3b (CR1) and iC3b (CR3 and CR4) that allow them to ingest, process, and kill pathogens.[95] C1q-coated particles can be ingested by macrophages. The terminal complement components C5 to C9 can lyse some bacteria, notably *Neisseria* species and *E. coli*.[108,111]

The major effects of complement components include anaphylatoxic (C3a and C5a), opsonic (C3bi and C3b), chemotactic (C5a), and cytolytic activity (membrane attack complex, C5 to C9). Deficiency of the early components of the classic pathway results in a high incidence of collagen vascular–like and lupus-like disease, including arthritis, skin rash, and glomerulonephritis (C1q, C1r, C1s, C4, or C2 deficiency), and some patients may have recurrent infection, usually with *S. pneumoniae*.[108,111] Patients with deficiency of C3 usually have pyogenic infections because both the classic and alternative pathways are impaired. Patients with late complement deficiencies have recurrent infections with *N. meningitidis* or *N. gonorrhoeae*.[111]

The production and function of complement proteins are quantitatively and qualitatively different in the neonate compared with adults. Complement protein synthesis in the developing human fetus occurs between 8 and 19 weeks gestational age. The synthesis of C4, C2, and C3 occurs in the liver between 8 and 14 weeks, and functionally active C5 can be detected in fetal lungs and liver as early as 8 to 9 gestational weeks. Complement proteins are produced in decreased quantity with decreased functional activity, depending on the gestational age of the fetus.[108] Few, if any, of the maternal complement components cross the placenta to the developing fetus. Results of studies done in preterm and term infants compared with adults show that preterm and term infants are markedly deficient in complement components, especially C9 and factor B of the alternative pathway.[112,113] Neonatal serum complement opsonic activity and/or chemotactic activity has been studied with strains of *E. coli*, staphylococcus, and group B streptococcus. Neonates and especially preterm infants have deficiencies in complement opsonic activity critical to bacterial host defense.[108] In addition, the cellular and humoral factors required for the activation of complement are also deficient in neonates. Most neonates have low levels of IgM because maternal passage of this immunoglobulin does not occur and little production occurs in the developing fetus. Because many antibodies against bacteria are of the IgM class, opsonization and subsequent bacterial killing are reduced in newborn infants because of insufficient IgM to activate the classic pathway of complement.[108]

Fibronectin

Fibronectin (Fn) is a high-molecular-weight glycoprotein (440 kDa), which participates in a number of complex processes, including hemostasis, wound healing, cell migration and differentiation, and phagocytosis.[114]

Fn binds to organisms such as *S. aureus*, *S. epidermidis*, and *Streptococcus pyogenes* to promote their interactions with phagocytic cells, including macrophages and neutrophils.[115-117] Fn enhances the binding of *S. aureus* to neutrophils and their subsequent ingestion when activated by inflammatory mediators.[118] Fn increases the uptake of antibody-coated GBS by human neutrophils and enhances survival of neonatal rats infected with these organisms.[119] Similar observations have been made with use of macrophages.[120] Yang and associates[117] also found that Fn increases the respiratory burst activity, phagocytosis, and intracellular killing of *S. aureus* and *S. epidermidis* by human neutrophils in the absence of antibody, probably through functional binding of organisms to Fn receptors on phagocytic cells. In contrast, GBS and *E. coli* uptake and killing are enhanced by Fn but only in the presence of opsonic antibody.[119] Binding of Fn to its receptor on neutrophils, monocytes, or macrophages enhances the respiratory burst activity, alters actin polymerization, and promotes the release of TNF-α.[121,122] Reduced levels of plasma Fn are seen in normal newborn infants; neonates with respiratory distress, perinatal asphyxia, and sepsis; and patients with severe trauma, septic shock, or severe burns.[114,119,123-130]

Pathogen-associated Receptors

Pattern recognition receptors are expressed on most effector cells of the innate immune system, including mast cells, macrophages, dendritic cells, and B-1 cells. They recognize specific pathogen-associated molecular patterns present on many microbes. They are secreted into the plasma or tissue fluid or contained intracellularly as cytoplasmic proteins.[14,131]

Toll-like Receptors

Toll-like receptors (TLRs) are a family of type 1 integral membrane glycoproteins that initiate innate immune responses via ligand recognition of pathogen-associated molecular patterns.[132-135] Ligands recognized by TLRs consist of a wide range of microbial products including diacyl and triacyl lipopeptides, lipopolysaccharide (LPS), lipoteichoic acid, peptidoglycan, flagellin, zymosan, and both single- and double-stranded RNA.[132-135] Ligand recognition initiates TLR dimerization, resulting in signal transduction through one of two pathways: the myeloid differentiation protein 88 (MyD88)–dependent pathway or the MyD88–independent pathway.[132-134] TLR signaling through the MyD88–dependent pathway leads to activation of nuclear factor κB (NF-κB) or activator protein 1 (AP-1), and subsequent cytokine and chemokine production.[132-135] MyD88-independent signaling can activate either NF-κB or interferon regulatory factors (IRFs), leading to the production of type 1 interferons.[132-134]

TLRs may contribute to the increased risk of human neonates for bacterial infections. TNF-α production in response to LPS stimulation of TLR-4 has been shown to be diminished in human neonates compared with adults.[136-138] In addition, TLR-4–stimulated IL-10 release is also decreased in neonates when compared with adults.[139] TLR-4 signaling has also been implicated in defective neonatal mouse neutrophil migration and may contribute to the defective chemotaxis observed in human neonatal neutrophils.[140] Diminished concentrations of MyD88 have been associated with decreased LPS-stimulated TNF-α concentrations in human neonates.[137] The deficiency of IL-1 receptor–associated kinase-4 (IRAK-4), a downstream adapter molecule in the MyD88-dependent pathway for activation of NF-κB leading to transcription of inflammatory genes, has been shown to cause severe pyogenic bacterial infections in children.[141] The role of IRAK-4 in human neonates, to our knowledge, has yet to be defined.

Non-TLR pattern-recognition receptors (PRRs) also remain to be fully described in human neonates. Two recently described PRRs include the NOD-1–like receptors NOD-1 and NOD-2. NOD-1 and NOD-2 are cytosolic PRRs that initiate proinflammatory signaling through the NF-κB and mitogen-activated protein kinase pathways, on the detection of bacterial peptidoglycan degradation products.[142] NOD-1 and NOD-2 have been shown to contribute to sepsis and sepsis-related syndromes.[143-145] NOD-1 and/or NOD-2 may play an important role in the neonatal response to bacterial infections. Caron and colleagues[146] have shown a decrease in the production of TNF-α, IL-1β, and TNF-γ by cord blood mononuclear cells compared with cells of adults in response to NOD-1 and NOD-2 stimulation. The role of NOD-1 and NOD-2 in neonatal inflammatory responses is currently under investigation.

Collectins

The collectins are a family of PRRs, including mannose-binding protein and surfactant proteins A and D, which contain collagen and lectin domains and share biologic activities.[14,147,148]

Mannose-binding protein (MBP) is probably the best characterized of the PRR collectins.[149] It binds to carbohydrate structures on gram-positive and gram-negative bacteria, yeast, parasites, and some viruses.[150] MBP is encoded on chromosome 10.[149] Microbial pathogens bound by MBP in the blood or tissue fluid are then opsonized and phagocytized or lysed by the complement system.[14] Deficiency of MBP is a disorder associated with increased susceptibility to bacterial, fungal, and viral infections, usually beginning early in life before the maturation of acquired immune responses.[149,151,152] These patients usually have recurrent upper respiratory tract infections, otitis media, chronic diarrhea of infancy, and failure to thrive within the first year of life.[151,152] Deficiency of MBP also may contribute to the severity and progression of infection beyond infancy because of abnormal interaction of MBP with the complement system.[151]

Surfactant is a mixture of proteins and lipids that promotes normal respiratory function and enhances lung host defense.[147,153] Two surfactant proteins, SP-A and SP-D, belong to the collectin family of pattern recognition receptors. These are products of alveolar type II cells during the latter stages of pregnancy.

LaForce and colleagues[154] initially reported that surfactant improves macrophage killing of *S. aureus*. SP-A and SP-D bind to certain bacteria, viruses, yeast, and fungi to promote their attachment to phagocytic cells via Fc receptors of immunoglobulin and complement receptors, which facilitates their elimination.[147,155] SP-A and SP-D bind to gram-positive and gram-negative bacteria, *Pneumocystis carinii*, fungi such as *Aspergillus*, yeast including *Cryptococcus neoformans*, bacteria such as *H. influenzae*, as well as herpes simplex virus, *Mycobacterium tuberculosis*, and *Mycoplasma pulmonis*.[156-166] SP-A and especially SP-D promote the formation of microbe aggregates that may enhance their mucociliary clearance.[153]

The surfactant collectins stimulate neutrophil and macrophage chemotaxis, actin polymerization, respiratory burst activity, and nitric oxide production. SP-A knockout mice cannot kill mycoplasma via nitric oxide–dependent mechanisms. They also have abnormalities in neutrophil recruitment, production of toxic oxygen and nitrogen intermediates, and defective phagocytosis.[147] These knockout mice develop disseminated GBS infection after intratracheal inoculation and have diminished clearance of *S. aureus* and *P. aeruginosa*.[167-170]

SP-A and SP-D bind LPS from organisms such as *E. coli*, *K. pneumoniae*, and *P. aeruginosa;* SP-A and LPS subsequently bind to macrophages via CD14-dependent and -independent mechanisms, increasing TNF-α release.[156,170] SP-A and SP-D function by clumping pathogens and promoting adherence to inflammatory

cells.[147,171] Furthermore, they may down-regulate T cells and their cytokine responses and suppress the response to inhaled allergens.[172,173]

Deficiencies of surfactant are observed in premature infants and patients with cystic fibrosis, respiratory syncytial virus infection, and adult respiratory distress syndrome. Reduced SP-D opsonization has been observed in diabetic mice because of competition by free glucose for SP-D–binding sites.[147]

Selectins

Selectins are a family of adhesion molecules that facilitate neutrophil rolling along and tethering to the vascular endothelium. Selectins include leukocyte selectin (L-selectin), endothelial selectin (E-selectin), and platelet selectin (P-selectin), which have lectin-like domains.[174] L-selectin binds lipid-linked galactose with sulfate residues. P-selectin binds sulfotyrosine, and E-selectin binds fuco-oligosaccharides present on leukocytes, epithelial cells, and some tumor cells.[148] Deficiency of L-selectin results in leukocyte adhesion deficiency type II and is associated with decreased leukocyte infiltration, resulting in a poor inflammatory response along with associated neurologic and developmental abnormalities caused by impaired fucose metabolism.[175] P-selectin expression by endothelial cells is decreased in human preterm infants.[176]

Bacterial Lipopolysaccharide

Bacterial LPS is a virulence factor of gram-negative bacteria. It is a powerful cellular activator and promotes cytokine release from neutrophils, macrophages, and other cells of the innate immune response. The polysaccharide groups of LPS vary considerably from species to species, and even between strains of the same species, permitting bacteria such as *E. coli* to be serotyped. The lipid moiety of LPS is generally highly conserved.[14,95]

The primary cellular receptor for LPS is CD14, a glycosylphosphatidylinositol-linked protein.[31] CD14 was first identified on phagocytic cells but also occurs on many other cells. Binding of LPS to CD14 is markedly potentiated by LPS-binding protein (LBP), a normal component of the serum. Binding of LPS to LBP enhances effector cell responsiveness to LPS, promoting cellular activation and cytokine release.[14]

Bacterial Permeability–increasing Factor

Bacterial permeability–increasing factor (BPI) is a 55-kDa pattern recognition receptor present in neutrophil granules, which contain two domains, one of which binds with LPS to increase membrane permeability and lysis of gram-negative bacteria, whereas the other promotes opsonization.[14] Neutrophil BPI functions in concert with defensins and the membrane attack complex of complement to cause bacterial lysis. Neonates have reduced release and activity of BPI, perhaps contributing to their enhanced susceptibility to gram-negative bacterial infections.[177]

Natural Antimicrobial Agents

Antimicrobial peptides, first described in insects in 1981,[178] have now been found in all multicellular organisms, including, plants, insects, and humans.[14] Important antimicrobial peptides in humans are the α and β defensins. They are contained in the primary, or azurophilic, granules and in secondary, or specific, granules of phagocytic cells.

Defensins

The defensins are strongly cationic, single-chain peptides with molecular weights of between 3 and 4.5 kDa. Defensins make up 50% of the protein content of the neutrophil primary granules.[29,179] These are divided into α and β defensins. These peptides possess antimicrobial activity against gram-positive and gram-negative bacteria, fungi, mycobacteria, and some viruses. The defensins create voltage-sensitive pores in microbial membranes, resulting in lysis. Humans have six human α defensins (HD1 to 6) and two human β defensins (HBD-1 and HBD-2).[179]

The α defensins, HD 1 to 6, are made primarily by neutrophils and compose 30% to 50% of the primary granule content.[180] Defensins are present after neutrophil degranulation induced by LPS, IL-8, C5a, and other stimuli. They are also found on the epithelial surfaces of the bronchi and in bronchial lavage fluid of patients with various types of inflammatory lung injury. The antimicrobial activity of defensins is inhibited by high salt content, which may contribute to the decreased microbial activity of lung surface fluid in patients with cystic fibrosis.[181]

The β defensins are produced by epithelial cells of the respiratory and gastrointestinal tracts.[16,182] HBD-1 is expressed constitutively by epithelial cells in the bronchi and intestine, whereas HBD-2 synthesis is up-regulated by inflammatory stimuli, including LPS, TNF-α bacterial infection, and injury. Thus HBD-1 acts to kill organisms in the absence of inflammation, whereas HBD-2 acts primarily as part of the inflammatory process. In inflammatory lung disease both α HD 1 to 6 defensins and β HBD-2 may be increased significantly, perhaps contributing to airway inflammation.[29]

Lysozyme and Myeloperoxidase

Lysozyme, present in primary and secondary granules of neutrophils, is an antimicrobial component present in inflammatory exudates, nasal secretions, and alveolar surface fluid.[31] Lysozyme is a small cationic enzyme that hydrolyzes glycosidic bonds that disrupt the peptidoglycan structural components of some gram-positive bacterial cell walls. Lysozyme can be present in airway surface fluid, where it may be an important component of the innate defense system.[33,34]

Myeloperoxidase is the most abundant enzyme in the primary granules of phagocytes. It catalyzes the reaction between chloride and hydrogen peroxide to form hypochlorite ion, a potent microbicidal agent of the respiratory burst. Bacterial killing takes almost twice as long if this enzyme is missing.[42]

THE COMPONENTS OF ADAPTIVE HOST DEFENSE

The hallmark of acquired, or adaptive, immunity is its specificity for structurally distinct antigens and memory of prior exposure. T and B lymphocytes orchestrate the cellular and humoral immune responses of acquired host defense. Acquired immune responses are the last to develop phylogenetically and the last to mature developmentally; they require close cooperation between cellular elements of the innate immune system and the T and B lymphocytes.[14,183]

T Lymphocytes

The major functions of the T-lymphocyte system are in host defense against intracellular pathogens (fungus, viruses, protozoa, and intracellular bacteria such as mycobacteria and *Listeria* species). T lymphocytes play a limited protective role against pyogenic bacteria such as GBS. The T-lymphocyte system also functions in tumor surveillance, delayed hypersensitivity reactions, and graft-versus-host disease.[184-186]

Thymus-dependent T cells are derived from pluripotent lymphopoietic stem cells residing in developing bone marrow stores. As early as the eighth gestational week, immature T cells infiltrate the thymus, where they differentiate and mature under the influences of thymic humoral factors, before migrating to specific lymphoid tissues. During their intrathymic migration, T cells develop specific outer membrane–bound glycoproteins at different stages. Most of the mature T cells express T-cell antigen receptors, as well as CD3 membrane glycoproteins. Nearly 70%

of the T cells also express the CD4 membrane glycoproteins, the helper-inducer T-cell marker. The remaining 30% express the CD8 membrane glycoproteins, the cytotoxic-suppressor T-cell marker. The CD4 and CD8 membrane glycoproteins typically are not presented on the same mature T cell and serve as functional markers of helper-inducer and cytotoxic-suppressor T-cell populations in the peripheral blood or tissues.

The T-lymphocyte system orchestrates pathogen annihilation through antigen-dependent cellular interaction. Antigen-presenting cells, mainly the monocytes and macrophages, produce soluble antigen and present it in combination with HLA class II antigens on their cell surfaces. Thymus-dependent CD4 helper-inducer T cells recognize and bind to this altered antigen HLA class II complex through the antigen-specific T-cell receptors. During this process, the cytokines IL-1 and TNF-α are released from the antigen-presenting cells. The binding of free IL-1 to its receptor on helper-inducer T-cell surfaces initiates T-cell activation. Once activated, the CD4 helper-inducer T cells produce IL-2, which functions as a T-cell promoter causing the T cells to proliferate and release a number of other cytokines. This initiates a cascade of events that serves to amplify and regulate the immune response with the cooperation and recruitment of many other cell types. IL-2 also interacts directly with CD8 cytotoxic-suppressor T cells that bind viral antigens present on the surface of infected cells, resulting in cellular destruction.[184]

At birth, T-cell immunity is immature compared with that of the adult, in part because of the lack of exposure of the fetal immune system to foreign antigens and decreased cytokine production. Neonatal T cells provide ineffective signals to B cells as a result of developmental defects in local cytokine production and cell-cell interaction. Any defect in T-cell immunity is also associated with variable degrees of B-cell deficiency because most of the maturation, differentiation, and activation processes of B cells require T-cell help.[184,185] The most severe form of immunodeficiency is the syndrome of severe combined immunodeficiency. This immunodeficiency category includes a spectrum of X-linked, autosomal recessive, and sporadic genetic defects characterized by the inability to mount a normal T-lymphocyte and B-lymphocyte immunity. Patients usually have failure to thrive, persistent oral candidiasis, recurrent diarrhea, and pneumonia, usually interstitial and often caused by *P. carinii*, in the first months of life.[186]

In response to microbial invasion, most cells of the innate and acquired immune system produce cytokines, which function in autocrine, paracrine, and endocrine fashions. Macrophages, NK cells, neutrophils, mast cells, and γδ T cells produce cytokines critical in the early response to microbial invasion. Antigen-activated T and B lymphocytes also produce cytokines that can activate, or recruit, effector cells of the innate immune system. Lymphocytes, macrophages, and stromal cells of the bone marrow produce CSFs that promote the growth and differentiation of immature leukocytes.[95,187] The placenta also produces cytokines in response to infection. Levels of cytokines, including G-CSF, GM-CSF, TNF-α, IL-1, IL-6, and IL-8, are increased 10-fold in the amniotic fluid of mothers with chorioamnionitis.[188] We will consider select cytokines that mediate host defense to bacteria—notably, TNF, ILs (IL-1, IL-6, IL-8, IL-11, IL-12, and IL-18), IFNs (IFN-α and IFN-γ), and CSFs (G-CSF, macrophage CSF [M-CSF], and GM-CSF).

Tumor Necrosis Factor

TNF-α and TNF-β are structurally related. They bind to the same receptors with equal affinity and elicit similar biologic effects.[95] TNF-α is primarily the product of macrophages and other antigen presenting cells, NK cells, and activated mast cells of the innate immune system, but it can also be produced by antigen-activated T cells. TNF-β is synthesized by T lymphocytes.[189-193]

TNF-α is the cytokine responsible for the host response to gram-negative bacterial infections and specifically to LPS, as well as to gram-positive bacteria.[192,194-196] Along with IL-1, TNF-α is a principal mediator of septic shock. TNF-α activates neutrophils, stimulates their adhesion to vascular endothelial cells, and induces their production of toxic oxygen products.[29,197-200] If TNF-α production is excessive, these toxic oxygen products damage endothelial cells and cause fluid leak, hypovolemia, and depressed cardiac contractility leading to septic shock. TNF-α production is primarily induced by (1) gram-positive bacteria and their products, including peptidoglycans, lipoteichoic acids, and pyogenic exotoxins[201-204]; (2) nonspecific mediators such as fibronectin, C5a, and surfactant proteins[122,205-207]; and (3) lactic acid, which is elevated in septic shock.[208]

TNF-α knockout mice, or normal mice pretreated with anti–TNF-α monoclonal antibody, have increased susceptibility to bacterial infection and a decreased occurrence of septic shock. In vitro, human mononuclear cells stimulated by GBS first generate TNF-α followed by IL-1, IL-6, IL-8, and IFN-γ.[204,209] In experimental models of GBS, anti–TNF-α monoclonal antibody therapy, administered early during infection, improves survival, probably by limiting cytokine-mediated septic shock.[210]

Interleukins

Interleukin-1

IL-1 is produced predominantly by macrophages and macrophage-like cells but also by endothelial and epithelial cells. IL-1 has two forms, IL-α and IL-β, encoded by two separate genes, which bind to the same IL-1 receptors. Two IL-1 receptors bind to the two forms of IL-1 with different affinities. They are distributed on a variety of cells in different concentrations throughout the body.[95]

During development, IL-1 production by fetal macrophages in response to LPS up-regulates G-CSF production by monocytes from the bone marrow and liver of human fetuses.[211] During inflammation, IL-1 up-regulates the expression of endothelial adhesive glycoproteins, such as ICAM-1, promoting neutrophil attachment.[212] IL-1 also promotes IL-6, IL-8, and other chemokine production by macrophages. IL-1, along with TNF-α, induces prostaglandin E_2 production in the hypothalamus, raising the body's temperature. IL-1, TNF-α, and IL-6 cause production and release of acute-phase reactants by the liver, including α_1-antitrypsin, haptoglobin, C-reactive protein, serum amyloid A protein, complement components, and fibrinogen.[213]

Interleukin-6

IL-6 supports hematopoietic progenitor growth, promotes T- and B-cell function, and mediates some acute-phase responses of inflammation. IL-6 is produced mainly by macrophages and mononuclear cells, but also by fibroblasts and endothelial cells. IL-6 is secreted in response to both TNF-α and IL-1, as well as microbes and their products.[95,187] After cellular activation, IL-1 and TNF-α production occurs, followed by IL-6 synthesis. IL-6 does not activate neutrophils or induce cytotoxicity, but it does increase the production of acute-phase reactants. IL-6 promotes the growth of B cells, stimulates antibody production, and enhances T-cell proliferation. IL-6 also enhances the activity of hematopoietic growth factors such as granulocyte and macrophage CSFs, thus playing a role in both innate and acquired immunity.[214] IL-6–deficient mice have increased mortality associated with bacterial infections because of impairment of neutrophil and macrophage responses.[215]

Interleukin-8

IL-8 is a member of the chemokine family that attracts neutrophils to sites of inflammation and plays an important role in host defense against bacterial infections.[216] IL-8 is produced by fetal

thymic epithelial cells, and its in vitro production is increased by IL-1. IL-8 is present in the cord blood of babies born with chorioamnionitis. Its expression is decreased in babies of mothers receiving steroids to induce fetal lung maturation.[217] IL-8 is a major factor in recruitment of neutrophils to the lung in bacterial pneumonia.[77,218]

Interleukin-11

IL-11 was initially characterized as a hematopoietic cytokine with thrombopoietic activity. It is expressed in the central nervous system and the gastrointestinal tract. The hematopoietic actions of IL-11 include the stimulation and proliferation of primitive stem cells. It acts in synergy with other cytokines to support the proliferation and differentiation of all lineages of hematopoietic stem cells.[219-221]

IL-11 has been used effectively in animal models of sepsis and septic shock. Neonatal rats pretreated with IL-11 and then exposed to GBS demonstrated a significant reduction in mortality.[222] Gastrointestinal protective effects of IL-11 exist as well. IL-11 administration in animal models of chemical-induced acute colitis and ischemic bowel disease results in significant increases in survival.[223-225] Decreased translocation of enteric bacteria is observed in the IL-11–treated animals. IL-11 has been used in patients with Crohn disease, and studies of IL-11 administration for the prevention or treatment of necrotizing enterocolitis in neonates is under consideration.[226]

Interleukin-12

Mononuclear cell production of IL-12 is an important component of the early response to microbial infection.[227] Although monocytes and macrophages are the main source of IL-12, other cells, including B cells, also can produce IL-12.[228,229] IL-12 induces IFN-γ production by T cells and NK cells and promotes cellular cytotoxicity and lymphocyte proliferation. It is down-regulated by IL-10.[200] IL-12 can be induced by exposure of mononuclear cells to microbes or their products, including *S. aureus*, GBS, and LPS.[230-234] The IL-12 receptor, composed of β1 and β2 subunits, binds IL-12 with high affinity.[234]

Decreased transcription and secretion of IL-12 and decreased IFN-γ production by newborn mononuclear cells suggest an abnormality within the IL-12–IFN-γ pathway. In vitro studies suggest that IL-12 can partially correct the IFN-γ deficiency.[231] IL-12 administration to neonatal mice with group B streptococcal infection significantly improves their survival.[235] The IL-12–IFN-γ pathway is also suppressed in older patients with critical infections.[236] Patients with hyper-IgE syndrome have defective synthesis of IFN-γ in response to exposure to staphylococci and candida, suggesting an abnormality in the IL-12–IFN-γ pathway in these patients as well.[237]

Interleukin-18

IL-18 is produced by monocytes, macrophages, osteoclasts, and keratinocytes and stimulates the production of IFN-γ. Its structure is similar to that of IL-1, and its receptor is also closely related to the IL-1 receptor.[238] IL-18 stimulates IFN-γ production using a different transcription promoter than that used by IL-12, but IL-12 and IL-18 act synergistically to enhance IFN-γ production in response to microbes and their products. IL-18 knockout mice do not have a complete deficiency of IFN-γ.[239-241]

Stimulation of NK cells with IL-18 markedly enhances their cytotoxic activity.[242,243] IL-18 knockout mice have decreased but not eliminated NK activity.[239] IL-18 stimulates $CD4^+$ T cells and B cells to produce cytokines and antibodies. It also stimulates IL-8 production by mononuclear cells and IFN-γ production by NK cells.[244] Decreased IL-18 production in cord blood mononuclear cells exposed to GBS, along with their deficiency in IL-12 production, contributes to inadequate IFN-γ production and increased susceptibility to GBS and other infections.[245]

Interferons

The IFNs are cytokines between 18 and 20 kDa produced by a variety of cells that function in both innate and acquired immunity. Interferons are divided into type I interferons, which include IFN-α and IFN-β, and type II, or immune, IFN-γ. The type I interferons share a common receptor, induce similar responses, and are encoded on chromosome 9p21 by a cluster of intronless genes. Type II interferon, IFN-γ, has a distinct receptor separate from that of IFN-α and β. IFN-γ is also structurally distinct from the type I interferons and is encoded on chromosome 12p24.[95,187]

IFN-α is the product of mononuclear phagocytes and has been known as leukocyte IFN, whereas IFN-β is produced by fibroblasts and is known as fibroblast IFN. The major stimulus for type I IFN production is a viral infection. In addition, both type I IFNs are stimulated by the acquired immune response to antigens, in response to T cell–derived factors.[95]

Interferon-γ is a 20- to 25-kDa glycoprotein encoded by a single gene on chromosome 12p24.[246,247] It is produced by $CD4^+$ and $CD8^+$ T cells and by NK cells and is a component of both the acquired and innate immune systems. T-cell IFN-γ production occurs after antigen stimulation in the presence of IL-2, IL-12, and IL-18. IFN-γ also has antiviral and antiproliferative activity and up-regulates both class I and class II HLA molecule expression.[247]

Activated macrophages can generate cytokines (IL-12, IL-15, and IL-18) that induce NK cell activation and IFN-γ production, and enhance macrophage antibacterial activity. IFN-γ activates mononuclear phagocytes and neutrophils, resulting in enhanced killing of phagocytized microbes and intracellular pathogens, such as *Mycobacteria* and *Listeria*. Patients with defects in IFN-γ receptors have severe infections with *Mycobacteria* and related pathogens.[248,249]

Neonates have a deficiency in the transcription and production of IFN-γ.[69,231,250] In vitro administration of IFN-γ enhances the chemotactic activity of newborn neutrophils.[246,247] IFN-γ is also decreased in the hyper-IgE syndrome.[251,252] Borges and associates[237] showed that mononuclear cells of these patients have decreased IFN-γ responses to staphylococci. In vitro incubation of their neutrophils with recombinant human IFN-γ significantly improves their chemotactic responsiveness.[247,253] IFN-γ administration to patients with hyper-IgE increased neutrophil responsiveness, improved eczema, and decreased respiratory symptoms.[254] IFN-γ lowers the incidence of serious infections in patients with chronic granulomatous disease without demonstrating an effect on their neutrophil respiratory burst.[255]

Colony-stimulating Factors

The production and function of neutrophils and macrophages are regulated, in part, by CSFs.[256,257] GM-CSF is a 14- to 35-kDa glycoprotein encoded on chromosome 5q23-31 that stimulates production of neutrophils, macrophages, and eosinophils.[187,258] G-CSF is an 18- to 22-kDa glycoprotein encoded on chromosome 17q11-21 that stimulates neutrophil production. M-CSF is a 21-to 40-kDa glycoprotein encoded on chromosome 5q23-31 that stimulates monocyte production.[187]

GM-CSF is produced by activated T cells and mononuclear phagocytes, vascular endothelial cells, and fibroblasts. It promotes the growth and differentiation of monocytes, macrophages, and neutrophils and, to a lesser extent, platelets and progenitor red blood cells. It activates macrophages in much the same fashion as IFN-γ. GM-CSF is produced by macrophages, fibroblasts, and endothelial cells at inflammatory focuses but is not present in the circulation.[187]

G-CSF is also produced by macrophages, fibroblasts, and endothelial cells and is present in low concentrations in the bloodstream. Microbes and their products and cytokines, such as TNF-α and IL-1β, stimulate the production of G-CSF.[29] G-CSF

acts on neutrophil progenitors to produce neutrophil maturation and release from the marrow. It also activates and enhances the function of neutrophils and macrophages.[187,259-261] G-CSF regulates neutrophil adhesion molecule expression, and also neutrophil adhesion, chemotaxis, respiratory burst activity, phagocytosis, and intracellular microbial killing.[262-264] Pulmonary or systemic infection stimulates the production of G-CSF in the blood, with resultant neutrophilia and enhanced inflammatory response.[265,266]

Monocytes and mononuclear cells from human neonates produce G-CSF and GM-CSF in reduced amounts, compared with adults, in response to inflammatory agents in vitro. Preterm infants have even further reduction in G-CSF and GM-CSF production than do term infants. These deficiencies contribute to the diminished neonatal neutrophil storage pool. Plasma concentrations of G-CSF are low in the preterm infant and in newborns with neutropenia and sepsis.[46]

Natural Killer Cells

NK cells are non-B, non-T cells lacking surface immunoglobulins and T-cell antigen receptors that are essential components of the acquired immune system.[267] NK cells appear in the human liver as early as 5 weeks after conception and increase in numbers and functional ability as development progresses. At birth, however, NK function is low compared with that in adult cells.[267,268]

NK cells release a variety of cytokines, including TNF-α, TNF-β, IFN-γ, GM-CSF, and CSF-1.[269] Several cytokines enhance NK activity, notably IL-2, IL-12, IL-15, IFN-α, IFN-β, and IFN-γ. NK cells also appear to be important in protection against intracellular infections such as *L. monocytogenes* and *Toxoplasma gondii*.[270-272] These pathogens induce macrophages to secrete the NK cell–activating cytokines IL-12 and IL-15, which induce NK cells to produce IFN-γ. This NK cell–derived IFN-γ then stimulates macrophages to kill these intracellular pathogens. The newborn infant has depressed NK cell activity, which might contribute to the susceptibility to these pathogens, as well as to other bacteria and viruses.[273,274]

B Lymphocytes

The neonatal ability to produce antibodies is quantitatively and qualitatively different from that of adults. The B-lymphocytic system is derived from stem cells residing in bone marrow stores. These stem cells produce cytoplasmic IgM heavy chains and become pre-B cells as early as 10 weeks gestation. Pre-B cells continue to differentiate to become mature surface IgM, or IgM- and IgD-bearing B cells, which seed peripheral lymphoid tissues via the circulation. On stimulation, IgM-bearing B cells may undergo class switching to IgG-, IgA-, or IgE-bearing B cells. These B cells can then differentiate into immunoglobulin-secreting plasma cells with the help of T cells and T cell–derived cytokines. Some of the B cells further differentiate into small memory B cells.[275]

The major function of B lymphocytes is to produce antibodies to the protein and carbohydrate antigens present on microorganisms, toxins, or other potentially harmful antigenic substances. These antibodies are classified into nine different immunoglobulin isotypes, including IgM, IgG1, IgG2, IgG3, IgG4, and IgA1, IgA2, IgE, and IgD. The IgM antibodies are made first and are the most effective and efficient in activating the classic complement system. IgM antibodies do not cross the placenta; thus the presence of IgM in the newborn infant may suggest congenital infection.[275,276]

The IgG antibodies, which represent 75% of serum immunoglobulin, are the only immunoglobulin class maternal antibodies that are transplacentally passed; they provide the developing infant with passive immunity. Passively acquired IgG has a half-life of approximately 20 days. All IgG subclasses cross the placenta. At birth, newborn IgG concentrations actually exceed maternal IgG levels, but they immediately begin to decrease until a nadir (400 mg/dL) is reached at 3 to 4 months of age in term infants. The IgG1 subclass represents approximately 70% of IgG and usually contains antiprotein antibody that also activates complement. The IgG2 subclass represents 20% of IgG and usually contains an antipolysaccharide antibody. The ability to produce antibodies to protein and carbohydrate antigens is different during the first 2 years of life. An infant's ability to produce IgG1 protein antibodies in the first few months of life is similar to that of adults. The infant's ability to produce IgG2 antibodies to polysaccharide antigens, however, is much lower, and effective antibody concentrations are not reached until after 2 years of age. Thus children younger than 2 years of age are predisposed to infections by polysaccharide-coated organisms (e.g., *H. influenzae*, pneumococcus, meningococcus). The IgG3 subclass represents 6% of IgG and usually contains antiviral protein antibody.[275,276]

Decreased passage of maternal antibody is suspected to be a major determinant in neonatal infections with GBS and *E. coli*, especially in premature infants. Before 32 weeks of gestation, the developing neonate's IgG concentrations are less than 50% of maternal values. Neonates with deficiency of type-specific antibody to the type III polysaccharide of GBS or the K-1 capsule of *E. coli*, whether because of inadequate maternal stores or decreased maternal antibody transport, are predisposed to GBS and *E. coli* infections.[277]

IgA antibodies are selectively transported across mucous membranes by secretory piece. These IgA antibodies prevent the attachment of microorganisms, absorb harmful antigens from mucous membranes, and are present in human breast milk. Secretory IgA is undetectable in the mucosal secretions at birth and first appears between 1 week and 2 months of age. It has been suggested that decreased levels of secretory IgA may predispose the developing neonate to colonization with pathogenic type III GBS.[278,279]

The IgE antibodies are found on mast cells and basophils and are primarily responsible for allergic reactions, protection against parasites, and anaphylaxis. The IgD antibodies serve as early antigen receptors on B cells; their role in host defense is currently under investigation.[246]

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László Maródi and Richard B. Johnston, Jr.

Host-Fungal Interactions Relevant to the Newborn Infant

Fungal infections are commonly classified as either endemic or opportunistic.[1] An endemic fungal infection may occur in any individual living in a geographic area that is the natural habitat of that fungus. Histoplasmosis, coccidioidomycosis, and blastomycosis, which are usually acquired by inhalation, are examples. Although exposure to endemic fungi might be possible in the newborn period, it is unlikely under normal circumstances. Therefore we do not discuss mechanisms of resisting these organisms but, rather, focus on resistance to opportunistic fungi.

Opportunistic fungal infections occur primarily in immunocompromised individuals.[2] Extensive investigation of host defense mechanisms in the newborn has indicated that multiple systems are partially compromised, or blunted, and it is probably this combination of partial deficiencies that places the newborn at risk.[3-5] Among the opportunistic fungi, *Candida* species stand out as particularly threatening to the newly born baby, especially the very-low-birth-weight and extremely low-birth-weight infant.[6-10] Although understanding of how the host resists fungal infection is incomplete, host-candida interactions have been relatively well studied. For these reasons, this chapter focuses primarily on candida, what is known about how humans resist its infection, and what mechanisms of host defense might be expected, on the basis of current knowledge, to be involved in defending the newborn against candidal infection.

Perineal and oral thrush caused by *Candida* species is common in otherwise normal neonates. In neonatal intensive care units, all newborns are at risk for more serious systemic candidal disease, even those with birth weights >2500 g.[9-12] *Candida albicans* remains the most common fungal species isolated in neonatal surveillance cultures[13] and the most common cause of neonatal candidiasis, but nonalbicans species such as *Candida parapsilosis* and *Candida glabrata* are also frequent causes of invasive neonatal infection, especially in low-birth-weight infants.[9,12,14-16] The frequency of serious candidal disease in newborns may be attributed to technical improvement of medical practices and intensive care measures on a backdrop of the newborn's blunted capacity to fight infection.[10,17,18] The prominence of neonatal candidal infections and the difficulties in diagnosis and treatment of invasive candidiasis emphasize the importance of acquiring a better understanding of the mechanisms by which this species causes infection in newborns.

We divide this chapter into discussion of antifungal defense mechanisms thought to be active at body surfaces and how these may be related to mucocutaneous candidiasis and a review of those expected to defend the newborn against invasive candidal infection. In discussing both forms of candidiasis, we use the experience of individuals with primary or acquired immunodeficiency diseases with fungal infections as a guide to understanding basic host-fungal interactions and how these might be expressed in newborn babies.

DEFENSE AGAINST MUCOCUTANEOUS CANDIDIASIS

Candida Virulence Factors and Surface Host Defense

Candida species cause a broad range of mucocutaneous infections involving the skin and mucous membranes.[19,20] *C. albicans* is part of the common commensal microbial flora of the oral cavity and the gastrointestinal and genitourinary tracts of healthy humans. Candidal vulvovaginitis occurs commonly in most women during pregnancy, and the body surfaces of newborns are colonized at birth. Overgrowth of these colonizing candida may lead to mucosal or cutaneous candidiasis characterized by local signs of infection and, in the mouth, visible white patches.

Transformation from the yeast phase to the filamentous form (pseudohyphae, hyphae, and germ tubes) appears to be essential for candidal pathogenicity.[21] Adhesins on blastospores and hyphae of *C. albicans*, like integrins on human leukocytes, can recognize arginine-glycine-aspartic acid (RGD) sequences on epithelial cells and several extracellular matrix proteins.[22,23] The candida gene *INT1* encodes an adhesin, INT1p, that recognizes the RGD-containing proteins and promotes the growth of filaments. This candidal adhesin is homologous to mammalian

integrins and itself contains an RGD site that may be recognized by integrins on human cells, facilitating further the adhesion of candida to the cell surface.[22] These findings provide at least a partial explanation for the symbiosis of candida with humans and the tropism of these fungi to intestinal and mucosal epithelium.

On the host side, secretory antibody, especially of the immunoglobulin (Ig) A class, the mucous layer, and cilia act to prevent adhesion to mucosal surfaces, and a variety of antimicrobial peptides protect both the skin and mucosae. These have the potential to prevent the penetration of commensal fungi such as candida. Demonstration that peptides with broad and potent antimicrobial properties—the magainins—exist in frog skin proved the principle.[24] These cationic peptides bind to anionic components of the microbial membrane and form pores that permeabilize the cell.Two major classes of cationic antimicrobial polypeptides have been described in human skin and epithelia, the β-defensins and the cathelicidins.[25] Both polypeptides can kill fungi. An additional peptide, dermcidin, which is secreted in sweat, can also kill fungi.[26] Nasal and lung secretions contain the antimicrobial polypeptide constituents lysozyme, lactoferrin, and secretory leukoprotease inhibitor, as well as neutrophil and epithelial defensins.[27] Absent function of these proteins across all epithelial surfaces has not been detected, and their precise role in defense against fungal (or any) infection is not yet fully defined. However, in the familial disorder Kostmann congenital neutropenia, patients' neutrophils are markedly deficient in antimicrobial peptides.[28] Normalizing neutrophil counts by treating with colony-stimulating factor prevents systemic infections, but destructive periodontitis continues until the patient's neutrophils are replaced with normal myeloid cells through bone marrow transplantation.[28]

Lessons From Primary and Acquired Immunodeficiency Disorders

Neonatal deficiency of T cell–mediated immunity may play a part in the frequent mucocutaneous candidal infections in newborns. Persistent mucosal candidiasis and oropharyngeal thrush are common complications of primary T-cell deficiencies such as DiGeorge syndrome and severe combined immunodeficiency disease.[5] Patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome, chronic mucocutaneous candidiasis, and hyper-IgE syndrome, all of which are characterized by subtle, nonlethal defects in T lymphocytes, have protracted candidal infections of the mucous membranes, nails, and skin.[5,19,29] These patients, however, are no more susceptible to life-threatening, generalized candidiasis than immunocompetent individuals.

Oral and gastrointestinal candidiasis is a common manifestation of human immunodeficiency virus infection, and esophageal candidiasis is one of the diagnostic criteria of the acquired immunodeficiency syndrome (AIDS).[30] Disseminated candidiasis is much less frequent in patients with AIDS than in immunosuppressed patients without AIDS and occurs only in end-stage disease when other immune functions are also severely impaired. Oral or esophageal candidiasis associated with AIDS is likely the consequence of a defect in T cell–mediated host defense.[30] An adequate number of functioning T cells are required for the development of a normal secretory immune response to candida, and T cells play an essential role in stimulating differentiation of IgA+ B lymphocytes into plasma cells that produce secretory IgA.[5]

Taken together, these observations support the concept that partial (selective) T-cell deficiency is responsible for the development of mucosal candidiasis in a variety of conditions, including chronic mucocutaneous candidiasis, secretory IgA deficiency, and AIDS, and, perhaps, in newborn infants. This concept is further supported by the observation in mice that recovery from oropharyngeal infection with *C. albicans* depends largely on $CD4^+$ T-cell augmentation of monocyte and neutrophil functions through activity of Th1-type cytokines such as interleukin (IL)-12, and interferon (IFN)-γ.[31]

Deficient T Cell–Mediated Immunity in Newborns

Neonatal T-cell responses are compromised at several steps including deficient production of cytokines by $CD4^+$ T cells. Deficiency in IFN-γ production by neonatal T cells has been well documented.[5,32] IFN-γ deficiency in neonates may be attributed to lymphocyte immaturity and also to decreased production of IL-12 by cord blood mixed mononuclear cells.[5,33-35] In addition, diminished production of IL-12 by cord-derived dendritic cells has been reported.[36] These studies suggest that neonatal T cells, mononuclear phagocytes, and dendritic cells do not mount a normally vigorous Th1 response. This apparent down-regulation of Th1 responses in human neonates[37] may play a part in the developmental immaturity of the immune system on mucosal surfaces and the susceptibility of newborns to oropharyngeal candidiasis.

DEFENSE AGAINST INVASIVE CANDIDIASIS

Invasive candidal disease may arise from translocation of the gut flora, and it may be propagated by the ability of candida to adhere to nonbiologic materials such as IV catheters.[20,38,39] Electron microscopy has shown that candida can burrow into the catheter and form a surrounding biofilm.[20] The use of heparin to prevent blood clot formation in newborns with intravascular catheters may accidentally promote invasive candidal disease. It has been proposed that heparin might facilitate the cleavage of the *C. albicans* surface adhesion protein INT1p, with release of the fungi from the catheter and generation of a cleavage product that stimulates release of inflammatory cytokines.[40]

Once in the bloodstream, candida are able to adhere to the vascular endothelium. Adherence of *C. albicans* exceeds that of less virulent species, and germinated *C. albicans* adheres better than do yeast-phase organisms.[39] INT1p appears to play an essential role in both adhesion and development of filamentous forms.[22] In mice, administration of broad-spectrum antibiotics and glucocorticoids, known risk factors for disseminated candidiasis in newborns,[10,16,18] accentuated the pathogenesis of *C. albicans* infection through an INT1p-influenced mechanism.[41]

Pulmonary surfactant protein-D (SP-D) has been implicated in host defense against *C. albicans* in alveolar compartments of the lung.[42] SP-D may facilitate mucociliary clearance by agglutinating *C. albicans* into large complexes and may decrease hyphal outgrowth. Decreased concentration of SP-D in neonates or alteration in levels of this protein because of lung disease and mechanical ventilation could disrupt local host defense and contribute to the development of pulmonary candidal infection.

Pseudomonas aeruginosa can form a dense biofilm on *C. albicans* filaments and kill the fungi.[43] This example of a bacterial antifungal suppressive mechanism emphasizes the risk for candida infection posed by giving an individual, particularly a newborn baby, broad-spectrum antibiotic therapy.[16,18]

Role of Phagocytes in Protection Against Invasive Candidiasis

Phagocytic cells are an essential defense against infections by candida and other fungi. Patients with chronic granulomatous disease, whose phagocytes cannot convert oxygen to toxic oxidants, and patients with primary or secondary neutropenia have increased susceptibility to infections by *C. albicans* and other fungi. This predisposition exists in the absence of antibiotic or corticosteroid therapy, depressed cell-mediated immunity, or IV lines.[44,45] A few individuals with complete deficiency of myeloperoxidase (MPO), which combines with hydrogen peroxide

to generate microbicidal oxidants, and diabetes mellitus have sustained systemic candidiasis,[46] and phagocytes from patients with MPO deficiency have an impaired ability to kill ingested *C. albicans*.[47] An important role for MPO-derived oxidants in the killing of candida is supported by the in vitro findings that MPO-rich human monocytes killed *C. albicans* significantly better than monocyte-derived macrophages, which have little MPO,[48] and that recombinant human MPO significantly enhanced the killing of *C. albicans* by macrophages in a concentration-dependent manner.[49] Relative resistance to MPO-derived oxidants appears to at least partially explain the greater pathogenicity of *C. albicans* compared with other *Candida* species.[48,50]

In vitro studies have suggested that antibody-mediated immunity can protect against invasive candidal disease.[51-53] We have found that optimal phagocytosis of *Candida* species by human mononuclear phagocytes requires opsonization by human serum, which supports this hypothesis.[50] However, no clinical observation suggests that patients with immunodeficiency disorders other than quantitative or functional phagocytic cell defects have increased susceptibility to invasive candidiasis. It is notable that patients with X-linked agammaglobulinemia or other severe hypogammaglobulinemia do not exhibit an increased susceptibility to either mucocutaneous or invasive candidal infections.

In patients with hypogammaglobulinemia, T cells, natural killer cells, and antimicrobial peptides may suppress candida at the surface, and macrophages and keratinocytes, which phagocytize and kill candidal yeasts through the mannose receptor,[50,54,55] might protect without the need for antibody opsonization. Mouse macrophages can mediate nonopsonic recognition of *C. albicans* and consequent release of cytokines through a complex of Toll-like receptor (TLR)-2 and dectin-1, the receptor for binding fungal-derived β-glucan, which comprises an inner skeletal layer of the candidal cell wall.[56] *C. albicans* cell-wall mannan can mediate binding to macrophages and release of cytokines through TLR-4 and its MyD88-dependent signaling pathway, as well as through the mannose receptor.[57] Neonatal monocytes and mononuclear leukocytes have been reported to have blunted TLR-4 responses to endotoxin and decreased MyD88 levels,[58,59] and cord blood mononuclear cells produced significantly less IFN-γ, IL-12, and IL-18 than adult cells on exposure to group B streptococci.[35] These abnormalities would be expected to predispose newborns to disseminated candidiasis.

Deficient Anticandidal Activities of Neonatal Monocytes and Macrophages

The extent of phagocytosis and killing of serum-opsonized candida by resident monocytes and monocyte-derived macrophages has been reported to be comparable in newborns and adults.[60] In the absence of serum, ingestion and killing by cord and adult cells were reduced by half but still equivalent. Mannan inhibited ingestion of unopsonized candida by macrophages in a concentration-dependent manner, indicating a role for the mannose receptor. However, exposure of cord and adult macrophages to IFN-γ gave quantitatively different results in candida killing, as well as in release of superoxide anion. Maximal increase in these functions with adult macrophages was achieved with 100 U/mL IFN-γ. No enhancement with cord macrophages could be detected after treatment with 100 U/mL, and at 500 U/mL killing and superoxide release by cord macrophages were still significantly lower compared with adult cells.[60] A similar blunted response of cord macrophages to IFN-γ–induced activation and increased killing was found by using group B streptococci as the target organism.[61] The number and binding capacity of IFN-γ receptors were greater on newborn than adult mononuclear phagocytes.[60] These data suggest that neonatal macrophages have a normal capacity to ingest and kill both opsonized and unopsonized candida but cannot be fully activated by IFN-γ, a finding that could not be attributed to lower expression of IFN-γ receptors on neonatal cells.[60]

Could the decreased activation of cord macrophages by IFN-γ be due to defective signal transduction? In response to IFN-γ, phosphorylation of signal transducer and activator of transcription (STAT)-1 was significantly decreased in neonatal monocytes and macrophages.[62] The STAT family are a group of key signaling molecules linking cytokine binding to the macrophage proinflammatory responses. These proteins are phosphorylated by Janus kinases that are activated by the binding of IFN-γ to its receptors. Expression of STAT-1 protein was equivalent in cord and adult monocytes and macrophages, but STAT-1 phosphorylation was deficient in cord cells.[62] Thus it is possible that the decreased capacity of cord macrophages to be activated is the result of defective signal transduction; this defect remains to be defined.

Intracellular parasites have evolved a variety of mechanisms to evade phagocytic killing by macrophages. Candida yeasts have a thick glycoprotein cell wall composed of mannoprotein and covalently linked chitin-glucan components that is highly resistant to lysosomal enzymes.[63] Phagocytosis of *C. albicans* by mouse macrophages reportedly induces rapid fusion of the phagolysosome membrane with late endosomes and lysosomes.[64] The ensuing acidification of the phagolysosome promotes germ tube formation, which can lead to distention of the phagocyte's membranes and eventual escape of the organism.

Ingestion of candida by macrophages can induce genes of the organism's glyoxylate cycle, a metabolic pathway by which two-carbon compounds are assimilated into the tricarboxylic acid cycle.[65] In *C. albicans* isolated from phagolysosomes of macrophages, the principal enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, were up-regulated. *C. albicans* mutants lacking isocitrate lyase were markedly less virulent in mice than wild-type *Candida*.[65] These findings demonstrate the significance of the glyoxylate cycle to candidal virulence and point to the complexity of the host-parasite interactions that have the potential to influence the outcome of invasive candidal disease.

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150 Host Defense Mechanisms Against Viruses

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OVERVIEW OF INNATE AND ADAPTIVE ANTIVIRAL IMMUNITY

Viral host defense mechanisms of vertebrates depend on a combination of innate and adaptive immune mechanisms that are tightly interlinked. Key innate immune mechanisms include antiviral and proinflammatory cytokines, such as type I interferon (IFN), IFN-γ, and tumor necrosis factor (TNF)-α, which have pleiotropic immunoregulatory effects and multiple potential cellular sources, including mononuclear phagocytes, dendritic cells, and natural killer (NK) cells. Dendritic cells are key for initiating the adaptive immune response and efficiently take up viral material in various forms, such as necrotic or apoptotic cellular debris. With some viral pathogens, such as human immunodeficiency virus (HIV), dendritic cells also may be directly infected. Dendritic cells process viral proteins and present these in the form of peptides bound to class I (human leukocyte antigen [HLA]-A, -B, and -C in humans) and class II (HLA-DR, -DP, and -DQ in humans) major histocompatibility complex (MHC) molecules for activation of $CD8^+$ and $CD4^+$ T cells, respectively.[1]

As a result of clonal expansion and differentiation, virus-specific $CD8^+$ and $CD4^+$ effector T cells are generated that carry out direct and indirect antiviral immune functions. $CD4^+$ T cells provide key help to B cells for the production of antiviral antibodies, and to $CD8^+$ T cells, which are a major source of cytokines with antiviral activity, and in some cases may also play a role in cell-mediated cytotoxicity. Effector $CD8^+$ T cells are the key cells involved with the clearance of virally infected cells from infected tissues by cell-mediated cytotoxicity, which is triggered when their T cell receptors (TCRs) recognize viral peptide–class I MHC complexes on the surfaces of target cells. B cells provide antiviral antibody that can neutralize viral attachment and entry into cells. Viruses, particularly herpesviruses, can block antigen presentation and may down-regulate the overall level of class I MHC expression on the cell, potentially thwarting $CD8^+$ T cell recognition. As a countermeasure, NK cells recognize and immediately kill cells with reduced class I MHC expression, providing innate and early protection against viral infection before the appearance of differentiated T cells and B cells. NK cell–mediated killing also is augmented by virus-specific antibody produced by B cells, a further example of the linkage of innate and adaptive immunity (Table 150-1). Unlike innate immune mechanisms, T cell– and B cell–specific viral immunity persists for years or for a lifetime.

TABLE 150-1

Innate and Adaptive Immunity

Property	Innate Immunity	Adaptive Immunity
Receptors	Fixed in genome (no rearrangement necessary)	Gene segments (rearrangement necessary)
Distribution	Nonclonal	Clonal
Recognition	Conserved motifs/ patterns	Specific structure
Self-nonself discrimination	Perfect	Imperfect
Timing	Immediate	Delayed
Response	Co-stimulatory molecules Cytokines (IL-1, IL-6) Chemokines (IL-8)	Clonal expansion or anergy IL-2 Effector cytokines (IL-4, IFN-γ)

The neonate is at risk for severe or rapidly progressive infection with certain viruses, most notably herpes simplex virus (HSV) type 1 and type 2 (HSV-1 and HSV-2), HIV, and enteroviruses. Another herpesvirus, human cytomegalovirus (HCMV), often causes severe disease in the premature neonate and is the major viral cause of congenital sensorineural deafness. Because viruses replicate intracellularly, mechanisms that control or block infection within cells, spread of virus from cell to cell, or both, are the most critical for effective host defense. Antibody and complement may also modify viral expression, especially by preventing spread of virus into the central nervous system (CNS), but cellular immune responses are the most central for control of viral replication and the elimination of virally infected cells.

This chapter presents a systematic review of the key components of human innate and adaptive antiviral immunity against viruses such as HSV, HCMV, HIV, respiratory syncytial virus (RSV), and enterovirus, including considerations specific to lack or failure of these components in the neonate. In addition to being "unprimed" as a consequence of lack of previous exposure to antigen, the neonatal immune system has functional deficiencies in both speed and magnitude of its innate and adaptive immunity.

HOST DEFENSE AGAINST HERPES SIMPLEX VIRUS AS A PROTOTYPE OF NEONATAL ANTIVIRAL MECHANISMS

HSV infection is severe in term infants if it is acquired at the time of parturition, as well as in the uncommon cases in which it is acquired *in utero*.[2] Similarly, enterovirus infections also are severe and may be fatal when acquired in the perinatal period. Both HSV and enteroviral infections have a similar propensity for dissemination and for causing CNS disease. Both infections, however, usually are effectively controlled when acquired after 4 weeks of age, suggesting that common developmental limitations in antiviral immunity may be responsible for neonatal vulnerability. The example of neonatal herpes infection illustrates well both the strengths and weaknesses of the neonatal immune system.

Herpesvirus Biology

HSV-1 and HSV-2 are two of eight herpesviruses that have been isolated to date from humans (the others include HCMV, varicella-zoster virus [VZV], Epstein-Barr virus [EBV], and human herpes viruses 6 to 8 [HHS-6 to HHV-8]).The HSV virion is composed of a 150–kilobase pair DNA genome, a capsid shell surrounded by a protein tegument and an outer lipid bilayer derived from the host embedded with a number of viral membrane proteins and glycoproteins.

Cellular targets of HSV include, but are not limited to, epithelial cells of skin and mucosa, neurons, and cells of the immune

system. The virus is capable of infecting other differentiated cell types, however, as is evident from disseminated disease that can occur in neonates and in immunocompromised individuals. Although humans are the only natural host for HSV-1 and HSV-2, a variety of laboratory animals and cultured cells from many vertebrate tissues also are susceptible.[3]

The initial interaction of HSV with the host cell surface usually is by binding of viral glycoprotein gB or gC to heparan sulfate chains on cell surface proteoglycans.[4] This is a reversible step and is not strictly required for viral entry. Entry depends on the binding of gD to one of its cell surface receptors.[5] These include HveA (herpesvirus entry protein A), also known as HVEM (herpesvirus entry mediator), which is a member of the TNF receptor family. Other receptors include nectin-1 (i.e., herpes immunoglobulin-like receptor [HIgR]) and nectin-2, cell adhesion molecules belonging to the immunoglobulin superfamily. These proteins also are known as herpesvirus entry (Hve) proteins C and B, respectively. Additionally, modified heparan sulfate (3-*O*-S HS) generated by cellular D-glucosaminyl 3-*O*-sulfotransferase can serve as the receptor for gD-mediated entry, in the absence of any known protein receptors.[4]

The final step in cell entry involves fusion with the host cell plasma membrane and involves the HSV glycoproteins gD, gB, and the gH-gL heterodimer. Although the key molecules involved in this process have been known for years, the exact mechanisms of membrane fusion are poorly understood. Analysis of amino acid residues 377 to 397 within the ectodomain of HSV-1 gH has identified a predicted membrane α-helix with attributes of a fusion peptide. Disruption of this α-helix abolishes HSV infectivity, and its replacement with other known fusion peptides restores fusion and infectivity.[6] Because the trio of gH, gL, and gB is conserved throughout the Herpesviridae family and is essential for infectivity in all examined herpesviruses, it is likely that gH serves as the principal fusion glycoprotein for the other herpesviruses as well and is part of a conserved mechanism of action.

Additionally, the protein encoded by a recently characterized human gene designated human fetal lung cDNA B5 (*hfl*-B5) has met criteria, when expressed in a model system, suggesting its function as a new class of HSV receptor or possibly a co-receptor.[7] With heptad repeat motifs and a proposed coiled-coil structure, the protein encoded by *hfl*-B5 is thought to function naturally in fusion as a cellular SNARE-like protein. Its HSV viral ligand currently remains unproved; however, because of its α-helix and peptide fusion motif, it is now postulated that gH is possibly the B5 receptor ligand as well.[6,8]

After entry, synthesis of the HSV DNA genome, as well as its assembly with capsid proteins, occurs in the nucleus. The production of HSV progeny results in the irreversible destruction of the host cell and subsequent viral release. Despite this lytic effect, a substantial proportion of both primary HSV-1 and HSV-2 infections are asymptomatic.[9] The hallmark of HSV gene expression is the regulated and coordinated expression of approximately 80 viral genes. This regulation occurs largely through modulation of RNA polymerase II transcription. Two viral proteins, VP16 and ICP4, function to activate transcription of the five immediate early genes and the rest of the HSV genome, respectively. Of the 80 or so genes within the HSV genome, fewer than one half are essential for replication. These essential genes encode functions related to entry of virus into cells, as noted previously, regulation of gene expression and replication, and packaging of viral DNA into virions. A large number of enzymes are involved in nucleic acid metabolism, some of which are targets for antiviral therapy (e.g., with thymidine kinase). The genes that are not essential for replication in culture play a key role in multiplication of the virus and its transfer from cell to cell. Additionally, they serve to complement cellular functions lost as a consequence of viral replication, fine-tuning viral gene expression, and overcoming the host's response to infection.

Upon entering the cell, HSV can enter either a lytic program in which ultimately the host cell is killed, or it can enter a latent state and reside silently for long periods, indeed a lifetime, in sensory neurons such as the trigeminal and sacral ganglia.[10] Mechanisms regulating these pathways are being elucidated and recently have been reviewed.[11] A key player in progression to productive infection is the HSV structural protein VP16, which is important in the initiation of the lytic program through the assembly of a multiprotein transcriptional regulatory complex involving two cellular proteins, octamer binding protein (Oct-1) and host cell factor-1 (HCF-1). This VP16-induced complex represents a regulatory switch. When "on," it promotes immediate early (IE) gene expression and lytic infection. When it is "off," IE-gene transcription is limited, favoring latent infection. Additional mechanisms involved in the establishment and maintainance of latency include maintainance of the viral genome itself, taking on a more stable circular DNA configuration, and posttranslational modifications of cellular histones. Here, for example, hyperacetylation of histones (enhanced by VP16) is associated with an "open chromatin" conformation and transcriptional activity,[12] but histone hypoacetylation is associated with condensed chromatin and gene silencing of IE genes and perhaps enrichment of the latency-associated promoter.[13]

The latency-reactivation cycle can be divided into three major steps: latency establishment, latency maintenance, and reactivation.[14] The latency period is characterized by prominent expression of a single viral gene, the latency-associated transcript (LAT). LAT appears to be important but not absolutely required for establishment of latency, as well as for maintaining the viability of HSV-infected neurons by inhibiting apoptosis[15,16] and by limiting entry into a lytic program. It remains controversial whether the regulatory effects of LAT on latency are mediated solely at the RNA level or require LAT messenger RNA (mRNA) translation into protein. No virally encoded functions are required for establishment of the latency, but a full complement of viral genes (including LAT) is essential for efficient reactivation of the virus from the latent state.

INNATE ANTIVIRAL IMMUNITY

The antiviral immune response generally can be divided into an early, nonspecific phase (typically the first 5 to 7 days in HSV infection) involving innate immune mechanisms, followed by a later, antigen-specific phase involving adaptive immunity by T and B cells.[17] The early phase is critical, because infection either may be successfully contained or, alternatively, may disseminate throughout the host. A closer look at the neonatal antiviral response is provided in the following discussion, which describes host recognition of viral infection, identifies the inflammatory mediators produced in response, and summarizesthe cells participating in these processes.

After recognition of pathogens (principally by dendritic cells), mediators such as interferons, cytokines, chemokines, and surfactant proteins are necessary in signaling the anti-viral state within the cell and activating and attracting other immune cells such as neutrophils, macrophages, and NK cells to orchestrate an effective antimicrobial response at the site of infection. The induction of the innate immune mechanisms is not pathogen specific but is dependent on interactions between pathogenic factors and host cell determinants. The aim of this early innate response is to either eliminate the pathogen or to avoid spread of the infection until elimination is achieved through the adaptive immune response. Additionally, the innate immune system serves to shape and regulate the developing immune response by promoting or inhibiting development of specific downstream effector mechanisms. This is exemplified by the ability of innate cytokines to improve the efficiency of antigen-presenting cells (APCs) and to direct the development of naive T cells into different subtypes expressing

distinct immune responses. Overall, regulation is important to a successful immune response, because many immune mechanisms can cause disease if they are left unregulated or are left in activated status inappropriately.

Pattern Recognition Receptors

Before mounting an antiviral response, the host must first recognize the infection. Unlike in adaptive immunity, which uses remarkably specific receptors and circulating factors to recognize highly variable antigens such as proteins, the receptors and factors of innate immunity react more broadly to less variable structures. The task of innate immune recognition is to identify key molecular signatures borne by pathogens, termed pathogen-associated molecular patterns (PAMPs),[18] by pathogen-recognition receptors (PRRs), leading to a cascade of intracellular signaling and ultimately the mobilization of soluble defense molecules, killing of infected cells, acquisition of specialized functions by sentinel cells, and priming of the adaptive immune response. This process starts with the identification of virions or viral products by extracellular and intracellular receptors. Several of the PRRs have been identified, including Toll-like receptors (TLRs), macrophage scavenger receptor (MSR), retinoic acid–inducible gene protein-1 (RIG-1), nucleotide-binding oligomerization domains (NODs), double-stranded RNA-activated protein kinase (PKR), and the 2′-5′oligoadenylate (2-5A) synthetase system (Table 150-2).

TLR3, TLR7, TLR8, TLR9, and to a lesser extent TLR2 and TLR4[19-21] have been associated with viral recognition by the innate immune system and subsequently acquired antiviral properties such as IFN production (Figure 150-1). Plasmacytoid dendritic cells (pDCs) recognize HSV, for example, by TLR9, expressed on endosomes to sense viral material. TLR9 identifies the HSV DNA because it contains abundant, unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs. These CpG motifs are underrepresented in mammalian DNA, where they generally are methylated. In response, pDCs secretes high levels of IFN-α and represent the so-called natural interferon–producing cells of the body.[22] Consistent with this recognition mechanism for HSV, human pDCs express very high levels of TLR9 compared with other dendritic cell populations, such as most myeloid dendritic cells.[23] The secretion of high levels of IFN-α by pDCs probably results in a systemic antiviral state, because receptors for type I interferons are essentially ubiquitous. Exposure of secondary lymphoid tissue to IFN-α also may enhance local adaptive immune responses.[24] TLR9 signaling of pDCs primarily involves the adapter protein myeloid differentiation primary response protein 88 (MyD88) and ultimately activates interferon regulatory factor-7 (IRF-7), which is constitutively expressed by pDCs and serves as a master regulator of IFN-α/β gene expression.[25] Although *in vitro* studies of pDCs in knock-out mice deficient in TLR9 and MyD88 demonstrate poor IFN response, no significant differences in morbidity or mortality were noted after HSV infection in these mice *in vivo*. This observation demonstrates that the presence of TLR9- and MyD88-independent pathways in cells other than pDCs can effectively compensate for defective interferon production by pDCs in response to HSV.[26] TLR8 in humans (TLR7 in mice) also is present on pDCs and serves as a PRR in recognition of single-stranded RNA (ssRNA) oligonucleotides rich in guanisine or uridine derived from ssRNA viruses such as HIV, influenza virus, and vesicular stomatitis virus. TLR7 signaling is also mediated through MyD88 pathways and, in pDCs, has high expression of IRF-7 with subsequent type I IFN production.

TLR3 also functions to recognize viral infection. TLR3, a known ligand for double-stranded RNA (dsRNA), also has been shown to initiate IFN production after viral infection in a MyD88-independent manner through an adapter protein named Toll/IL-1 receptor domain–containing adapter–inducing interferon (TRIF) by activation of IFN transcription regulatory factor-3 (IRF-3). IRF-3 is produced constituitively in most cell types and, in the absence of activation, is present in the cytoplasm in an inactive form. Activation occurs by means of two kinases: IκB kinase-ε (IKK-ε) and TANK-binding kinase-1 (TBK-1). Upon phosphorylation, IRF-3 dimerizes and translocates to the nucleus, where it interacts with coactivators CAAT-binding protein and p300. This complex serves as an enhanceosome complex that transactivates IFN-β gene expression in cooperation with activator protein-1 (AP-1) and NFκB. Infected cells secrete mainly IFN-β as an initial response to infection then switch to IFN-α during subsequent amplification of the IFN response.[27,28] It is not known if TLR3 binds only extracellularly derived dsRNA, for example, from lysed cells, or if dsRNA produced in the cell can be exported to the endosome and trigger TLR3.[29] In studies of mice deficient in TLR9 or TLR3 challenged with murine cytomegalovirus (MCMV), a significant abrogation in IFN production was demonstrated in both, and the mice were susceptible to MCMV infection.[30]

Besides TLRs, other extracellular receptors exist to identify pathogens within the innate immune system. Mannan-binding lectin (MBL) (which activates the complement system), C-reactive protein (CRP), serum amyloid protein (SAP), and the macrophage mannin receptor (MMR) are pattern recognition molecules that mediate phagocytosis of microorganisms, primarily bacteria and fungi. Another phagocytic pattern recognition receptor expressed on macrophages, macrophage scavenger receptor (MSR), has a broader role in host defense including recognition of viruses. Belonging to the scavenger receptor family (type A), it has specificity for a variety of polyanionic ligands, including dsRNA, lipopolysaccharide (LPS), lipotechoic acid (LTA), and acetylated low-density lipoproteins (LDLs). MSR-deficient mice have an increased susceptibility to HSV infection (as well as to other infections such as listeriosis and malaria).[18]

Extracellular receptors and TLRs are not the sole receptors controlling the innate immune response. Recognition of other families of molecules that play a key role in the sensing of infectious microorganisms are emerging. The existence of specific innate immunity receptors in the cytosol illustrates the topological problem

TABLE 150-2

Innate Recognition Receptors and Their Viral Ligands

PRR	Viral PAMP	Pathway/ Mechanism	Outcome
TLR-2	Envelope, lipopeptide	MyD88/Mal	INF-β, IL-6
TLR-3	dsRNA	TRIF/IRF-3	↑ type I IFN
TLR-4	Envelope	TRIF/IRF-3	↑ type I IFN
TLR-8	ssRNA	MyD88/IRF-7	↑ type I IFN
TLR-9	CpG	MyD88/IRF-7	↑ type I IFN
MSR	dsDNA	Phagosomal localization	Degradation/ apoptosis
RIG-1/MDA-5	dsRNA	NFκβ/IRF-3	↑TRAIL/apoptosis/↑ IFN
PKR	dsRNA	eIF-2α	↓ host translation/↑ IFN
OAS	dsRNA	2-5A–dependent RNase ligand	Cleaves viral RNA; ↓ transcription
Mx	ssRNA	GTPases	↓ viral transcription

dsRNA, Double-stranded RNA: *eIF-2α,* eukaryotic translation initiation factor 2 subunit α; *IL,* interleukin; *IFN,* interferon; *IRF,* interferon regulation factor; *Mal,* MyD88 adapter-like protein; *MDA-5,* melanoma-differentiation-associated gene 5; *MSR,* macrophage scavenger protein; *MyD88,* myeloid differentiation primary response protein-88; *NFκβ,* nuclear factor kappa-β; *OAS,* oligoadenylate synthetase; *PAMP,* pathogen-associated molecular pattern; *PKR,* dsRNA-dependent protein kinase; *PRR,* pattern recognition receptor; *RIG-1,* retinoic acid–inducible gene 1; *TLR,* Toll-like receptor; *TRAIL,* tumor necrosis factor–related apoptosis–inducing ligand; *TRIF,* Toll/IL-1 receptor domain–containing adapter–inducing interferon.

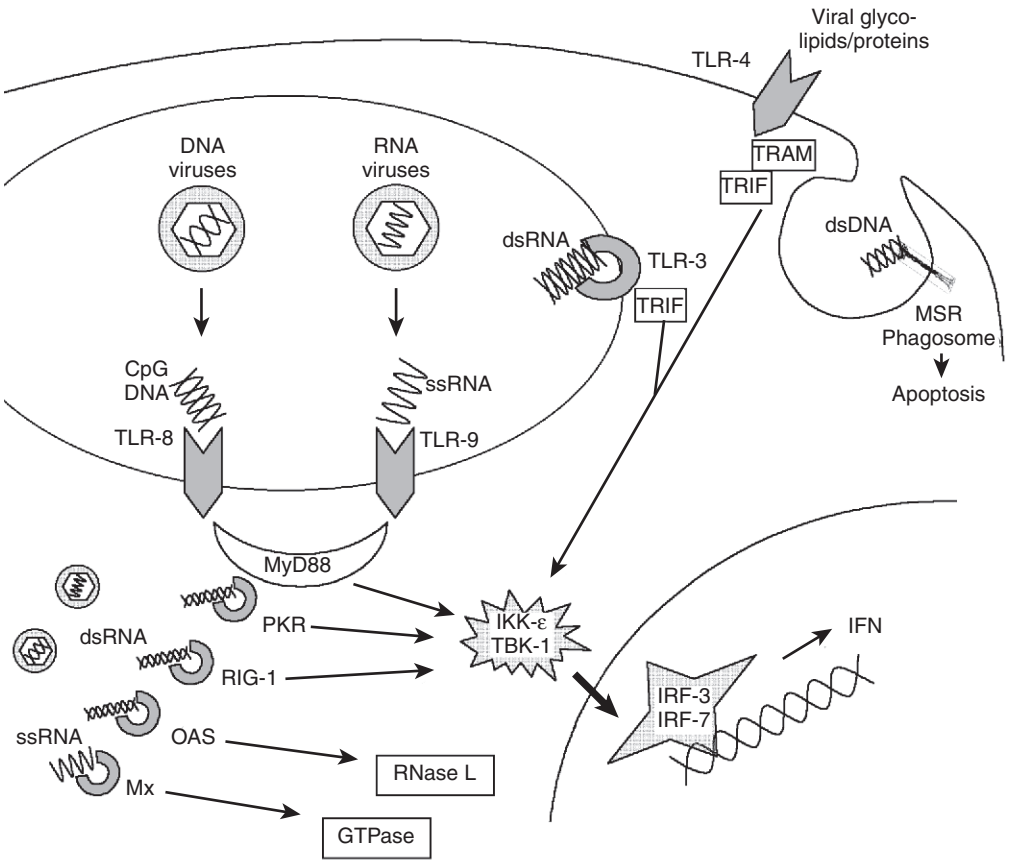


Figure 150-1 Examples of viral pattern recognition receptors and downstream signaling. *IFN*, Interferon; *IKK-ε*, IκB kinase-ε; *IRF,* interferon regulatory factor; *MyD88*, myeloid differentiation primary response protein 88; *OAS*, oligoadenylate synthetase; *PKR*, double-stranded RNA–dependent protein kinase; *RIG-1*, retinoic acid inducible gene-1; *TBK-1*, TANK-binding kinase; *TLR*, Toll-like receptor; *TRAM*, TRIF-related adapter molecule; *TRIF*, Toll/IL-1 receptor domain–containing adapter–inducing interferon.

posed by the cellular location of TLRs: These transmembrane receptors have access only to ligands that are either extracellular or are in the lumen of endosomes. TLRs are blind to microorganisms or their products that have penetrated the cytosol. Two such cytoplasmic receptors of dsRNA have been identified as members of a family of DExD/H box RNA helicases: RIG-1 and MDA5 (Helicard). The DExD/H box RNA helicases are defined by their ability to unwind dsRNA with intrinsic ATPase activity, and both RIG-I and MDA5 contain a caspase recruitment domain (CARD), which serves to activate NFκB and IRF-3, critical regulators of innate immune responses, in response to cytoplasmic dsRNA.

Other well-described cytosolic sensors of viruses include PKR, the 2-5A system, and the Mx proteins. PKR normally is inactive, but on binding dsRNA it undergoes autophosphorylation and subsequent dsRNA-dependent phosphorylation of substrates, including the α subunit of eukaryotic initiation factor 2 (eIF2-α), leading to rapid inhibition of translation.[31] The oligoadenylate synthetase (OAS) system is a multienzyme pathway in which IFN-inducible oligoadenylate synthetases are stimulated by viral dsRNAs to produce a series of short 2′,5′-oligoadenylates ("2-5A") that activate the 2-5A–dependent RNase ligand, which cleaves ssRNA halting viral and cellular processes. Similarly, the Mx proteins are IFN-inducible GTPases in the dynamin superfamily that interfere with viral transcription on encountering a wide range of ssRNA virus targets.

A large new family of cytosolic receptors is the nucleotide-binding oligomerization domain proteins (NODs), now designated nucleotide-binding domain, leucine-rich repeat–containing proteins (NLRs) (formerly called CATERPILLER).[32] They have been shown to be active in recognition of bacterial products such as peptidoglycans, modulated through a family of receptor-interacting (RIP) proteins.[33,34] Although preliminary data demonstrate possible activation of IFN-β through NOD recognition of *Listeria* and *Bacillus,* future studies will show whether NODs are able to recognize viruses and through which pathways they produce inflammatory mediators such as IFN.[35]

Interferons

The IFN system is indispensable for vertebrates to control viral infections. Knock-out mice with deletions in IFN receptors quickly succumb to viral infections despite having a normal adaptive immune system.[36] Likewise, children with genetic defects in the IFN-α/β system have been shown to die in infancy from viral infection, such as with HSV.[37] Animal studies modeling specific knock-out genes of effector molecules within this pathway also have revealed extreme susceptibility to various viral infections, demonstrating the significant power of this antiviral system.[36,38-43]

During viral infections, IFNs are some of the most prominent cytokines produced.[44] Named for their ability to interfere with viral replication,[45] IFNs are a large family of multifunctional secreted proteins involved in antiviral defense, cell growth regulation, and immune activation. They are classified into two types: *Type I* IFNs (IFN-α and IFN-β) are produced in direct response to viral infection and consist of the products of the IFN-α multigene family (14 genes), which are synthesized predominantly by leukocytes, and the product of the IFN-β gene, which is synthesized

by most cell types but especially fibroblasts. *Type II* IFN consists of the products of the IFN-γ gene and, rather than being induced directly by viral infection, is synthesized in response to the recognition of infected cells by activated T lymphocytes ($CD4^+$ T helper 1 cells and $CD8^+$ cytotoxic T cells) and NK cells.[46]

Both types of IFNs stimulate an antiviral state in target cells, whereby the replication of virus is blocked or impaired owing to IFN receptor signaling and the subsequent synthesis of a number of enzymes that interfere with cellular and viral processes. Additionally, IFNs make cells more susceptible to apoptosis, further limiting spread of virus. Finally, both types of IFN have profound immunomodulatory effects and stimulate the adaptive response. However, although both IFN-α/β and IFN-γ influence immune effector cells, they show significant differences, and it is these extended cytokine functions that probably account for the different spectrums of antiviral activities of the two types of IFN.

The signaling pathways for IFNs are well characterized, and the pathways involved in this interferon response circuit have been recently reviewed.[28,29,38,46,47] The biologic activities of IFNs are initiated by the binding of IFN-α/β and IFN-γ to their cognate receptors on the surface of cells, which results in the activation of distinct but related signaling pathways, known as the Janus kinase/signal transducer and transcriptional activator (Jak/STAT) pathways. The ultimate outcome of this signaling is the transcription of target genes that previously were quiescent or being expressed at low levels. The regulatory sequences of IFN-α/β genes contain a variation of the upstream consensus sequence called the Interferon-stimulated response element (IRSE), whereas IFN-γ–inducible genes contain the upstream IFN-γ activation sequence (GAS). The Stats are latent transcription factors that become tyrosine phosphorylated by the Jaks. After binding to their receptor (IFNAR), type I IFNs activate intracellular signaling including Jak1, Tyk2, STAT1, STAT2, and STAT3. The Jaks activate (phosphorylate) the STATs which dimerize, translocate to the nucleus, and induce IFN-simulated genes (ISGs). STAT1-STAT2 heterodimers complex with cofactor nuclear protein p48 forming a heterotrimeric complex known as ISGF3 and binds the ISRE sequence in the promotors of ISGs, influencing the transcription of a distinct group of genes.[48] These include the gene for IRF-7, which amplifies type I IFN production if viruses infect cells that previously have been exposed to type I IFN.[49] STAT1 homodimers and STAT1-STAT3 heterodimers activate GAS sequences.[50] Because STAT-1 but not other STATs is tyrosine-phosphorylated by IFN-γ binding to its specific receptor,[50] the potential exists for type I IFN to induce genes that are characteristic of IFN-γ responses, but not vice versa.

Patients who have genetic defects in STAT-1 that compromise the GAS pathway but not the interferon-stimulated response element (ISRE) pathway of gene regulation, are not prone to severe viral infections.[51] This point argues that the regulation of the GAS pathway by type I interferons, and the induction of certain gene products such as IRF-1,[52] are dispensable for antiviral immunity in humans. By contrast, complete lack of STAT-1 function and, as a result, loss of the ISRE-mediated pathway of signal transduction in humans resulted in recurrent HSV-1 encephalitis and death.[36] The critical importance of the ISRE pathway also is supported by the observation that mice genetically deficient in the type I interferon receptor are highly susceptible to HSV.[53]

IFNs can induce more than 300 cellular proteins.[54] However, the exact pattern of induction varies by cell type and type of IFN. The IFN-induced proteins include enzymes, signaling proteins, chemokines, antigen presentation proteins, transcription factors, heat shock proteins, and apoptotic proteins. Several of the cellular systems already discussed in the detection of viruses (PKR, Mx proteins, and the 2-5A system) also are IFN-driven, allowing for a positive reinforcement of these antiviral systems. Moreover, studies in mice have demonstrated that loss of any of these three systems as examples leads to increased susceptibility to viral infection.[55,56] Interestingly, despite their potency, knock-out mice lacking PKR, Mx, and OAS still exhibit a limited IFN-induced antiviral state, indicating additional pathways exist.[57]

Of note, ISG56 encodes P56, which is one of the most strongly induced genes among all of the IFN-stimulated genes. P56 binds to the large multimeric complex of eIF-3 through p48 and blocks its function in initiating protein synthesis. Of interest, ISG56 also is strongly induced by non-IFN pathways after viral infection (dsRNA).[58]

Interferons also have a profound effect on cells of the immune system that mediate innate and adaptive immunity. Members of the P200 family of structurally related proteins are strongly induced by type I IFNs and encoded by a gene cluster. The P202 protein impairs cell proliferation by inhibiting the functions of many cellular factors, including NFκB, E2F, TP53, c-Fos, c-Jun, MyoD, myogenin, c-Myc, and Rb.[59]

Interferon-γ is the major immunomodulator and activator of macrophages. Consequentally, mice defective in IFN-γ, IFN-γ receptor, or STAT1 genes are highly susceptible to infection with *Listeria, Toxoplasma, Leishmania,* and *Mycobacterium.*[60] IFN-γ has an important role in the development of $CD4^+$ helper T cells: It directs $CD4^+$ T cells toward the T_H1 direction and, by blocking IL-4 production, away from T_H2. This shift is facilitated by enhancing the synthesis of IL-12 in APCs, an effect that also helps develop T_H1 cells. IFN-γ also up-regulates $CD4^+$ T cell expression of IL-12 receptors.

Both type I and II IFNs promote the expression of MHC class I molecules and the development of $CD8^+$ T cell responses.[44] MHC class II induction occurs in mononuclear phagocytes, endothelial cells, epithelial cells, and T cells where it enhances $CD4^+$ responses. The positive role of IFNs in enhancing antigen presentation (besides up-regulating MHC molecules) is augmented by regulating the expression of many proteins responsible for generating antigenic peptides. This is accomplished through IFN-γ induction of three proteosome subunits—LMP2, LMP7, and MECL1. IFN-γ also induces the expression of PA28, the nonenzymatic subunit of the proteosome, and TAP1 and TAP2 (transporter associated with antigen-processing proteins), which transfer the proteosome-mediated peptides to the endoplasmic reticulum, where they bind nascent MHC class I proteins.[44]

In an early study, type I IFN production by neonatal lymphocytes and monocytes was equivalent to that in adult cells for a variety of inducers, including HSV and other viruses.[61] A more recent study,[217] however, found that type I IFN production by peripheral blood mononuclear cells and the frequency of IFN-α–producing cells (assayed by the ELISPOT technique) in response to fixed HSV were diminished in neonatal cells compared with adult cells, particularly for cells from prematurely born infants.

Interfering With Interferons

Shortly after the recognition that heat-inactivated influenza viruses would induce IFN, it was discovered that infection of cells with live virus inhibited the subsequent induction of IFN by an inactivated virus—"inverse interference."[45] The key role of both type I (α and β) and II (γ) IFNs as one of the first antiviral defense mechanisms is highlighted by the fact that anti-IFN strategies are present in most viruses.[46,62] Viruses have been reported to block nearly all aspects of the IFN regulatory pathway, the details of which are available in several reviews[46,63-65]; some of the medically relevant viral interactions with the neonatal host are summarized here. Viruses counteract the host IFN response in a number of ways including by inhibiting IFN production and signaling, and by circumventing IFN-induced antiviral effector molecules.

Substantial amounts of type I IFN are found in the tissues of HSV-infected animals and humans.[66,67] Although HSV efficiently induces the secretion of type I IFN, the virus is relatively resistant

to their antiviral effects. HSV attempts to block synthesis and function of type I IFN in infected cells by several mechanisms. In cells infected with HSV, viral DNA replication leads to the activation of another cytoplasmic receptor, PKR, that phosphorylates the α subunit of translation initiation factor 2 (eIF-2α) and thereby inhibits translation initiation.[31] As a countermeasure, the ICP34.5 protein is expressed by HSV to prevent the shutoff of protein synthesis. ICP34.5 interacts with cellular protein phosphatase 1 and redirects its activity to dephosphorylate eIF-2α.[68] Recent data demonstrate that in HSV-infected cells, eIF-2α dephosphorylation mediated by the ICP34.5 protein is tightly coupled with viral resistance to interferon.[69] As expected, ICP34.5-minus recombinant HSVs are attenuated in the host. Interestingly, the phenotype can be re-established by extragenic mutations in the herpes genome resulting in earlier expression of US11 protein. If the US11 protein binds PKR before its phosphorylation, US11 is able to fully compensate, demonstrating that HSV has utilized at least two ways to overcome PKR.[70]

ICP34.5 and US11 protein effectively negate a major effect of the type I IFNs. Second, HSV blocks the phosphorylation of Stat1, Stat2, and Janus kinases induced by interferons.[71] This may account for the limited ability of type I interferons to treat recurrences of established HSV infections, such as of the eye. A combination of IFN types I and II are effective in the control of HSV infection, at least in mice,[72] suggesting that cellular sources of IFN-γ *in vivo* (T_H1 $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells, NK cells, or NK T cells) play an important role in host defense against HSV by acting in concert with type I IFN.

The OAS (2-5A RNase L) pathway also is inhibited by HSV-infected cells by generation of substituting 2′,5′-oligoadenylate derivatives that inhibit RNase L.[73] HCMV, another herpesvirus, has been shown to have complex interactions with the IFN system of the host. HCMV binding of the cell by its gB glycoprotein activates type I IFN; at the same time, however, the virus disarms downstream effectors involved in the induction of the antiviral state. It does this by reducing the expression of Jak1 and IRF-9, thereby disrupting IFN signaling.[74]

Adenoviruses are another class of DNA viruses that are known to encode several mechanisms to antagonize IFNs. Adenovirus-infected cells express VAI viral RNA, which, like dsRNA, binds to PKR but, unlike dsRNA, does not activates PKR but in fact inhibits it. Additionally, adenovirus produces E1A protein, which inhibits ISGF3 complex by down-regulating the expression of STAT1 and IRF-9 and by directly binding to STAT1.[75] Influenza viruses are segmented negative-strand RNA viruses and counteract IFNs through production of a nonstructural protein, NS1. NS1 binds to dsRNA, prevents dsRNA-mediated activation of PKR and OAS, and prevents synthesis of IFN. The mechanisms by which NS1 achieves this inhibitory effect is through disruption of dsRNA-stimulated IFN gene transcription of IRF-3, IRF-7, and NFκB and activation of cellular protein (p58IPK), which inhibits PKR. NS1 also binds another IFN-induced protein, ISG15, and blocks its action.[76] The nonsegmented negative-strand RNA parainfluenza viruses encode proteins that inhibit IFN signaling by degradation of the Jak/STAT pathway.[77]

Hepatitis B capsid protein specifically inhibits MxA gene expression through an unknown mechanism.[46] Its open-reading frame C product and the viral terminal protein inhibit IFN production at the transcription level by sequestering dsRNA.[78] The sequestration of dsRNA by viral proteins probably has a wider role in protecting the virus from antiviral mechanisms, because dsRNA-activated PKR can activate NFκB and induce synthesis of immunomodulary genes, in addition to IFN.

HIV type I (HIV-1) RNA transcripts contain stem bulge loop structures, called transactivating response (TAR) elements, in their 5′-untranslated region, which are readily detected by PKR.[79] Its transcriptional transactivation protein (Tat) is able to compete with PKR for binding to eIF2-α, allowing protein synthesis and viral replication to proceed.[80] Of interest, the utilization of PKR-phosphorylated Tat by HIV-1 has been shown to increase the interation with its TAR-containing RNAs, thereby enhancing transcription.[81] HIV down-regulates RNase L (OAS) by inducing the expression of the RNase L inhibitor, which antagonizes 2-5A binding to RNase L, preventing its activation.[82]

Cytokines

Interleukin-1 and Interleukin-18

Interleukin (IL)-1 ligands (IL-1α and IL-1β, collectively referred to as IL-1) are pluripotent, proinflammatory cytokines that orchestrate inflammatory and host defense responses in the body. IL-1 augments T cell responses to mitogens (and indirectly activates B cells), increases expression of vascular adhesion molecules, and induces a number of other proinflammatory cytokines, chemokines, and inflammation-associated molecules that form an amplifying cascade to stimulate an immune response.[83] IL-1 affects nearly all cell types and often operates in concert with other cytokines and mediator molecules. IL-1 is a highly inflammatory cytokine, and the margin between benefit and unacceptable toxicity is quite narrow. Thus its gene expression, synthesis, secretion, and regulation are tightly controlled.[84] In fact, IL-1 actually has two receptors: one that transmits a signal (IL-1RI) and one that does not—a "decoy" receptor (IL-1RII) that serves as an IL-1 sink and is somewhat unique to cytokine biology.

Many viruses induce IL-1 production. IL-1 is induced at sites of HSV infection-induced inflammation, such as the cornea, and may contribute to pathogenic inflammation.[85] It is produced by many cell sources, including activated mononuclear phagocytes. Recombinant IL-1α administered to neonatal mice also can enhance survival,[86] but the role of endogenously produced IL-1 in the control of HSV infections is unclear. Some viruses have adapted ways to counteract IL-1, such as the generation of inhibitors to IL-1–converting enzyme (ICE), which blocks the post-translational activation of IL-1. Other viruses produce proteins that bind to and block the IL-1 receptor.[87,88]

The most salient property of IL-18 that separates it from IL-1 is its ability to induce IFN-γ in the presence of IL-12. Besides its IFN-γ–inducing activity, IL-18 enhances the Fas ligand and perforin-mediated T cell and NK cell cytotoxicity, stimulates T_H1 cell development, up-regulates granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2 receptor-α (IL-2α), decreases IL-10–expression in T cells, and modulates immunoglobulin secretion from B cells. The proinflammatory activity of IL-18 is mediated by the production of nitric oxide (NO), prostaglandins, and inflammatory cytokines such as TNF-α, IL-1β, and IL-13. The IL-18–induced chemokines IL-8, macrophage inflammatory proteins (MIP)-1α and MIP-1β, and monocyte chemotactic protein-1 (MCP-1) recruit monocytes and macrophages to the site of infection.[89,90]

IL-18 binds its receptor (IL18R) in a two-stage manner, first to its α-chain IL-18Rα; then, it forms a tricomplex with its IL-18Rα chain. Subsequently, MyD88 is recruited, followed by activation of IL-1 receptor–activating kinases (IRAKs), TNF receptor–associated factor-6 (TRAF6), and NFκB-inducing kinase (NIK). NIK phosphorylates the IκB kinase, which then degrades, liberating NFκB, which translocates to the nucleus to initiate gene transcription. Its functional regulation is principally by control over its post-translational processing. The ability of INF-γ to increase the synthesis of inducible nitric oxide is essential for killing intracellular organisms. In that regard, IL-18 as an IFN-κ–inducing factor plays a key role in controlling infections due to *Salmonella, Cryptococcus*, *Toxoplasma, Candida,* and *Mycobacterium.* Additionally, IL-18, through IFN-γ, helps to induce endothelial adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which facilitate the emigration of neutrophils and lymphocytes to the nidus of infection.[91]

It also functions in an antiviral capacity.[92] IL-18 administration to mice, including those lacking both T and B cell immunity, provides substantial protection against systemic HSV infection.[93] In wild-type mice, this protective effect depends largely on IFN-γ but not on NK cells.[93] Mice genetically deficient in IL-18 also die earlier and demonstrate an increased viral load compared with wild-type mice after a lethal intravaginal challenge with HSV.[94] The production of IL-18 during human HSV infection remains to be fully characterized.

Interleukin-12 and Interleukin-23

IL-12 is structurally related to several other cytokines, including IL-6, IL-23, and IL-27. IL-12 and IL-23 share a common p40 subunit and have distinct p35 and p19 subunits, respectively. The IL-12 receptor on cells consists of an IL-12Rβ1 and IL-12Rβ2 chain, whereas the IL-23 receptor consists of an IL-12Rβ1 and IL-23R subunit. IL-12 was first described for its ability to stimulate IFN-γ production by NK cells and enhance $CD8^+$ T cell cytotoxicity.[95] It also has been described as a key initiator of cell-mediated immune responses as well as playing a central role in promoting the differentiation of $CD4^+$ T cells into mature T_H1 effector cells. IL-12 does not induce proliferation of resting-blood T cells or NK cells, although it augments proliferation induced by mitogenic lectins, alloantigens, and CD3-specific antibodies, and it has a direct proliferative effect on preactivated T cells and NK cells. Stimulation through CD28 is synergistic with IL-12 for inducing proliferation of resting T cells and does not require the presence of specific antigens. Additionally, because the ligands for CD28 are surface molecules and IL-12 is a secreted product of APCs, their synergy also facilitates T cell proliferation and IFN-γ production after antigen presentation to T cells by APCs. Although IL-12 presumably may affect B cell proliferation, differentiation, and IFN-γ production directly, some of the reported effects of IL-12 on B cell activation and particular immunoglobulin isotypes are mediated indirectly by IL-12–induced T_H1 cells or their products (IFN-γ). IL-12 also induces T cells and NK cells to produce several other cytokines, including GM-CSF and TNF, but particularly IFN-γ.

Initially, this family of interleukins—IL-12, IL-23, and IL-27—was thought to be quite similar from a functional status as well as structural one. They respond to microbial and host immune stimuli such as TLRs and IFNs[96] and are tightly regulated, and each activates members of the Jak/STAT pathway. More recently, their unique functions have become more apparent. The finding that stimulation of activated and memory T cells in the presence of IL-23 (but not IL-12) led to the production of IL-17, but not IFN-γ or IL-4, provided the first evidence of unique role for IL-23 in the regulation of T cell effector function. IL-23 does not have the same ability as that of IL-12 to develop the T_H1 response. Instead, IL-23 induces a subset of T cells with a unique expression pattern—IL-6, IL-17A, IL-17F, and TNF. IL-17, for example, is implicated primarily in the regulation of local inflammation at multiple sites but predominantly in the intestines, lungs, and joints. IL-17 alone, or combined with TNF, can stimulate endothelial cells and macrophages to produce factors including chemokines, granulocyte colony-stimulating factor (G-CSF) and GM-CSF, IL-1, IL-6, and TNF.

The virus-targeted effects of IL-12 and IL-23 include their ability to promote $CD4^+$ and $CD8^+$ T cell proliferation and cytolytic function, and in augmenting NK cell activity. IL-12 is produced by myeloid dendritic cells and by plasmacytoid dendritic cells, after their exposure to HSV. Mononuclear phagocytes also produce IL-12 in response to HSV infection *in vitro*, and this appears to require intermediary synthesis of viral and host proteins and transcriptional activation by NFκB/Rel proteins.[97] HSV corneal infection also has been reported to induce IL-12 p40 mRNA by neutrophils,[98] but it is unclear whether these cells produce functional IL-12 or IL-23 protein. IL-23 transcripts have been detected in the ganglia of HSV-1 infected mice, but the cellular source has not been defined.[99]

IL-12 and IL-23 are important for directing the differentiation of naive $CD4^+$ T cells into T_H1 immune effectors that secrete high levels of IFN-γ in response to TCR stimulation.[100,101] They also can directly induce NK cells and effector T cells to produce IFN-γ. The production of IL-12 by plasmacytoid dendritic cells after their exposure to HSV may skew the immune response to HSV toward a T_H1 outcome. In addition, type I IFN also favors T_H1 differentiation, probably by increasing expression of the IL-12Rβ2 receptor on activated T cells.[102] Together, these mechanisms may account for the predominance of IFN-γ–producing effector and memory $CD4^+$ T cells after most viral infections, such as with herpesviruses.[103,104]

Nevertheless, the role of IL-12 and IL-23 in the control of HSV infection and other herpesviruses remains unclear. Mice lacking both IL-12 and IL-23 as a result of a gene disruption of the common p40 subunit die more rapidly than wild-type mice do after a lethal challenge with HSV.[94] Cellular, or T_H1, immunity has been suggested to play a vital role in the defense against intracellular pathogens such as viruses and a number of bacteria, parasites, and fungi. This view was supported largely by animal knock-out studies in mice lacking IL-12 or IL-12 receptor.[100] The description of human patients with inherited IL-12 or IL-12 receptor deficiency challenges the mouse model. IL-12 in humans was found to be redundant in defense against intracellular microorganisms other than *Mycobacteria* and *Salmonella.* In other studies, IL-12 also was found to be redundant even in defense against primary infection by *Mycobacteria* and *Salmonella.*[105] In fact, viruses almost ubiquitous in distribution—HSV, VSV, EBV, HCMV, influenzavirus and parainfluenza viruses, papillomavirus, molluscum contagiosum virus, rotavirus, enterovirus, parvovirus, adenovirus, RSV, calicivirus, and others—that are classic opportunistic organisms in immunocompromised patients do not cause severe disease in the IL-12 axis–deficient patients. It is therefore possible that human IL-12 alone is entirely redundant in protective immunity. Regardless of their role in primary control of HSV, IL-12 and IL-23 may contribute to inflammatory corneal scarring in HSV infection,[106] suggesting a deleterious impact in cases of established infection.

Interleukin-2 and Interleukin-15

IL-2 and IL-15 are structurally similar members of the short-chain four-helix bundle of cytokines and both utilize the β- and γ-chains of the IL-2 receptor for binding and signal transduction. IL-2 and IL-15 both are involved in adaptive immunity (especially in proliferation and maturation of both $CD4^+$ and $CD8^+$ T cells, the development of memoryphenotypic $CD8^-$ T cells, and the prevention of automimmunity).[107-109] However, these two cytokines, more than any others, are involved in the development, survival, proliferation, apoptosis, and effector functions of NK cells, including the stimulation of NK-derived cytokines such as TNF-α, IFN-γ, and GM-CSF. In contrast with IL-2, IL-15 is produced not by T cells but by other cell types, especially bone marrow stromal cells and activated mononuclear phagocytes. IL-15 also is a potent activator of NK cells for enhanced cytotoxicity against most herpesviruses, including HSV.[110] Exposure of human peripheral blood mononuclear cells to HSV also up-regulates NK cytotoxic activity by an IL-15–dependent mechanism.[111] Infection of mice results in a rapid and substantial increase in serum levels of IL-15, peaking at approximately 3 days after infection[112]—suggesting a role in the early innate immune response. IL-15 administration also is protective to wild-type mice given an otherwise lethal dose of HSV.[112]

IL-12 and IL-15 production by mononuclear cells (presumably mainly monocytes) from term neonates after stimulation with LPS was approximately 25% of that typical for adult cells.[113,114] Although this limited production could contribute to the observed reduced HSV-specific T_H1 response *in vivo* of neonates, such reduced responses are highly stimulus-dependent. For example, neonatal and adult blood mononuclear cells stimulated

with *Staphylococcus aureus* or meningococcal outer membrane proteins produce equivalent amounts of IL-12.[113-116] The production of cytokines that enhance defenses against intracellular pathogens by monocytes derived from newborn infants, such as TNF-α, also is modestly reduced.

Chemokines

Chemokines secreted by mononuclear phagocytes and dendritic cells, as well as nonhematopoietic cells, play an important role in the recruitment of NK cells and subsequently T cells to sites of infection and in maintaining inflammation. In the case of herpes keratitis, the production of the CC chemokine MIP-1α contributes to inflammatory disease but does not influence viral replication.[117] The CXC chemokine IL-8, which is a potent neutrophil chemoattractant, is produced by corneal keratocytes[52] and possibly by corneal epithelial cells,[118] and this property is likely to account for the prominence of neutrophils in HSV keratitis.

Elevated CSF levels of the CC chemokines, MCP-1, MIP-1α, and RANTES, and of IL-8 also have been documented in cases of human HSV encephalitis,[119] suggesting their role in CNS inflammation. Cerebrospinal fluid mononuclear phagocytes, such as macrophages or microglial cells, are likely sources of at least a portion of these chemokines—an idea supported by the ability of microglial cells or macrophages infected by HSV to produce chemokines such as RANTES.[120,121] In addition to their proinflammatory role, certain chemokines potentially may skew the outcome of the adaptive immune response to viral infection. For example, MCP-1 is produced in the cerebrospinal fluid of mice experimentally infected with HSV-2. This effect appears to result in decreased survival compared with that in mice in which this chemokine is neutralized.[122] MCP-1 appears to skew the $CD4^+$ T cell response toward a nonprotective T_H2 (IL-4, IL-5, and IL-13 predominant) response, rather than a protective T_H1 response. This observation is consistent with earlier studies in which administration of relatively small doses of IL-4 in an HSV-1 encephalitis mouse model was found to increase disease severity.[123]

Surfactant Proteins

Surfactant proteins SP-A and SP-D are members of the collectin family of lectins and are synthesized by type II alveolar epithelial cells, lung epithelial Clara cells, and certain extrapulmonary cell types.[124] SP-A and SP-D contribute significantly to pulmonary immunity by involvement in viral neutralization, clearance of bacteria, fungi, and apoptotic cells, and regulation of inflammation.[125] Their carboxyl-terminal (C-terminal) carbohydrate recognition domain can recognize carbohydrates or charge patterns on a wide variety of microbes, whereas the triple helical collagen region interacts with receptors on a variety of immune cells in order to initiate clearance mechanisms. Studies involving gene knock-out mice, murine models of lung hypersensitivity and infection, and functional characterization of cell surface receptors have revealed diverse roles for SP-A and SP-D[126]: They affect agglutination, opsonization, chemotaxis, and enhanced phagocytosis.[125] In mice lacking SP-A, viral load is increased after challenge with RSV, in association with increased neutrophil inflammation of the lung and production of proinflammatory cytokines.[127] SP-D has been shown to bind RSV proteins G and F and to reduce macrophage and neutrophil production of oxygen radicals.[128] SP-D binds *in vitro* to influenza A virus through its hemagglutinin molecule and can promote internalization of the virus by neutrophils. Mice lacking SP-D demonstrate reduced influenza A virus clearance, diminished myeloperoxidase activity, and excessive IFN-γ and IL-2 production in bronchial lavage fluid.[129] SP-A and SP-D both bind LPS from bacteria such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa,* although by different mechanisms. SP-A, for example, binds the lipid A region of LPS and CD14 and initiates TNF-α signaling. SP-A also has been shown to engage TLR4 and accessory protein MD-2 in an immunomodulatory role.[130] Many of the conditions that predispose to severe respiratory infection, including prematurity and bronchopulmonary dysplasia, are associated with decreased SP expression by the lung, and it is plausible that these decreases could contribute to disease severity. Of note, neither SP-A or SP-D is utilized in the artificial surfactant replacement therapies routinely given for infantile respiratory distress syndrome, although some experimental evidence supports the utility of SP-A and SP-D for antimicrobial therapy and to restore inflammation.[131]

Innate Cellular Mechanisms

Mononuclear Phagocytes and Inducible Nitric Oxide Synthase

Mononuclear phagocytes are widely distributed in the tissues as macrophages and are likely to play an important role in the local containment of viral infections such as HSV before activation of the adaptive immune response. In murine studies with intraperitoneal HSV or MCMV and HSV corneal infections, macrophages have been identified as the predominant cell type involved and demonstrate HSV-instigated production of TNF-α. TNF-α has direct anti-HSV effects, particularly in combination with IFN-γ,[132] and these two cytokines also may synergize to increase expression of leukocyte adhesion molecules and inducible nitric oxide synthase (iNOS) expression by mononuclear phagocytes. HSV infection of mononuclear phagocytes also synergizes with IFN-γ for promoting expression of iNOS,[133] which has anti-HSV effects *in vitro*,[134] and for production of TNF-α.[135] Systemic depletion of mononuclear phagocytes (by administration of liposomes containing dichloromethylene diphosphonate) or of γδ T cells (which produce IFN-γ), or inhibition of TNF-α, IFN-γ, or iNOS activity in the HSV corneal infection model, results in increased HSV-1 replication within the ganglion.[136,137] Specific inhibition of iNOS in mice challenged with intranasal aminoguanidine causes a dose-dependent increase in pneumonitis compared with that in control animals.[138] These complex interactions illustrate the importance of these cellular and cytokine components in local antiviral host defense. An antiviral role for iNOS also is suggested by the increased susceptibility of mice with complete genetic deficiency of iNOS to HSV infection from footpad injection. These mice also demonstrate exaggerated T_H1 responses, indicating that iNOS may have key antiviral effects, as well as inhibitory effects on T_H1 differentiation.[139]

However, innate antiviral immune mechanisms mediated by mononuclear phagocytes, such as iNOS induction, may not necessarily be beneficial in all contexts of HSV infection. In a murine model of HSV pneumonia, inhibition of iNOS activity markedly reduced pulmonary inflammation and improved survival, despite increases in the lung tissue HSV viral titer.[140] Inhibition of iNOS activity also was of clinical benefit in experimental HSV-1 encephalitis in rats and was not associated with increased viral load.[141]

Dendritic Cells

Dendritic cells form a network of immune sentinels throughout the body. Their function is to detect and capture foreign antigens, process them, and carry them to the lymph nodes, where the antigen can be presented to T cells in association with surface MHC molecules.[142] Immature dendritic cells residing in the peripheral tissues have relatively weak T cell stimulatory capacity and express low levels of MHC molecules and co-stimulatory molecules. Dendritic cells mature in response to a pathogen recognized by pattern recognition receptors or danger signals such as IL-1β or TNF-α secreted by surrounding cells. They migrate to a lymph node (in response to chemokine CCR7),[143] where they undergo a series of functional and phenotypic changes necessary for effective antigen loading and priming of naive T cells. Dendritic cell maturation is characterized by an increase in antigen

processing efficiency as well as increased surfaced expression of CD40, CD80, CD83, and CD86.

Most dendritic cells are found in one of three compartments. Epidermal Langerhans cells and the underlying dermal and interstitial dendritic cells constitute the first group and the predominant population within the skin and mucosa. Within peripheral blood, two additional populations of dendritic cells can be identified: myeloid and plasmacytoid.[142] Plasmacytoid dendritic cells ($CD11c^{-}CD123^{+}$) are involved in the production of high levels of type I IFNs (after TLR-9 signaling after endocytosis of antigen, for example) in response to invaders such as HSV, HIV, and influenzavirus. Myeloid dendritic cells ($CD11c^{+}CD123^{lo}$) in the blood are a circulating group of precursors of tissue-resident dendritic cells. Dendritic cells are able to capture antigen from infected cells or from direct contact with virions and present it for recognition by naive T cells. Dendritic cells play a pivotal role in the immune response to a variety of viral infections; thus successfully overcoming this obstacle by the attacking virus will allow persistence of viral infection within its host. Direct infection of dendritic cells is one such strategy and has been used by a number of viruses including influenzavirus, HIV, and HSV-1 and -2 to block the amplification of the immune response by dendritic cells. Indeed, myeloid dendritic cells generated from peripheral blood monocytes express HSV entry receptors Hve-A and Hve-B. Although attack of mature dendritic cells by HSV-1 generally results in a nonproductive or aborted infection, the mature cells generally are not found at the sites of HSV infection. Immature dendritic cells are, however, readily infected by HSV and subsequently down-regulate their co-stimulatory and adhesion molecules, but the expression of MHC molecules is largely unaffected.[144] The inability of HSV ICP47 to interfere with MHC class I expression probably is the consequence of innately high levels of the "transporter associated with antigen processing" (TAP) protein in dendritic cells. Down-regulation of other molecules does have an impact on its stimulatory ability; for example, down-regulation of CD40 expresison on HSV-infected dendritic cells may lead to decreased production of IL-12 on contact with T cells. Observed down-regulation of CD54 on the surface of immature dendritic cells may also effect generation of anti-HSV immunity. CD54/ICAM-1 is a surface glycoprotein that binds leukocyte function antigen-1 (LFA-1), facilitating a number of cellular events including antigen-specific T cell activation and leukocyte transendothelial migration. Binding of CD54 to LFA-1 appears to be of critical importance for the activation of naive T lymphocytes, especially at low antigen concentrations.[145] Infected dendritic cells subsequently are compromised in their ability to carry out their function in stimulating effector cells and may undergo rapid apoptosis. Although this destructive process removes a pool of cells that present antigen, thereby potentially giving a "head start" to viral infection, neighboring uninfected dendritic cells readily take up apoptotic cell debris for antigen presentation through MHC class I (an example of cross-presentation of antigen) and stimulate effector cells such as HSV-specific $CD8^{+}$ T cells. Transfer of viral peptide antigens from infected cells to neighboring bystanders through gap junctions also has been proposed.[146]

In neonatal herpes simplex, the diminished competence of dendritic cell function may explain in part why HSV infection is such a devastating disease in this population. Inherent defects in the ability of human and murine neonatal dendritic cells to mature, produce cytokines, and activate effector cells, in comparison with adult cells, are well recognized. Dendritic cells isolated from human newborn cord blood have reduced expression of accessory molecules and produce less IL-12 owing to reduced IL-12 p35 gene transcription, and less IFN-γ than those from adults. Growing evidence supports an association between HSV infection and suboptimal maturation and activation of the neonatal dendritic cell as well.[147] Treatment of neonatal mice with Flt-3 ligand and growth factors that increase the number and maturation of peripheral dendritic cells increases resistance of neonatal mice to HSV infection, however.

Studies using dendritic cell–enriched fractions that contain both myeloid and plasmacytoid dendritic cells suggest that neonatal dendritic cells overall are less able to activate $CD4^{+}$ T cells.[148,149] However, plasmacytoid dendritic cells make up a greater fraction of circulating dendritic cells in neonates than in adults, and plasmacytoid dendritic cells are relatively poor at activating T cells.[150] Therefore further investigation of myeloid dendritic cell function in neonates and in adults is needed for accurate assessment of developmental limitations in this cell population.

Myeloid dendritic cells derived from immature precursors in neonatal or adult blood can be primed for efficient antigen presentation by engagement of their CD40 molecule by CD154 expressed by activated T cells, or by their exposure to microbial products or inflammatory cytokines induced by these products. Experimental observations support the idea that overall myeloid dendritic cell maturity is increased after exposure to microbial products, such as from endogenous bacterial flora. Myeloid dendritic cells from adult mice lacking functional TLR4, which recognizes LPS and other bacterial products, have reduced levels of expression of co-stimulatory molecules and a decreased capacity to direct T cell activation.[151] Thus it is possible that the lack of previous exposure to microbes (which occurs in a cumulative manner after birth) could contribute to diminished dendritic cell function and the slow rate at which antigen-specific T cells develop.

Natural Killer Cells

NK cells are bone marrow–derived lymphocytes that lack the antigen-specific TCRs and immunoglobulins that are characteristic of T and B cells, respectively. They make up approximately 5% to 20% of lymphocytes in the spleen, liver, and peripheral blood and are present in lower frequencies in the thymus, lymph nodes, and bone marrow.[152] In humans, a minority population of $CD56^{hi}$ NK cells are primarily involved in producing large amounts of cytokines such as IFN-γ, GM-CSF, G-CSF, macrophage-CSF, TNF-α, IL-5, IL-10, and IL-13 and chemokines; $CD56^{low}$ NK cells express high levels of low-affinity Fc receptor for immunoglobulin G (IgG) (FcγRIII) (also known as CD16) that are powerfully cytotoxic, accounting for 90% of the NK population.[153] Activation of NK cells is mediated by cell surface interactions as well as cytokine signals, and in response to these stimuli, NK cells produce further cytokines and lyse target cells. NK function is tightly regulated by a balance of positive and negative signals. Unlike cytotoxic T lymphocytes (CTLs), NK cells do not require antigen-specific recognition to kill their targets.[154] They are activated by the differential engagement of cell surface receptors in combination with activities mediated by proinflammatory cytokines.

The two major groups of inhibitory NK receptors are the killer inhibitory receptors (KIRs) and CD94/NKG2A. The KIRs bind to portions of HLA-B and HLA-C molecules located outside the peptide-binding groove recognized by the TCR, whereas CD94/NKG2A binds to HLA-E, a nonclassic and nonvariable MHC molecule that requires hydrophobic leader peptides from HLA-A,-B, and -C for its surface expression. These NK cell receptors help counteract the ability of viruses to decrease surface expression of class I MHC molecules, thereby limiting $CD8^{+}$ T cell–mediated viral clearance. For example, the down-regulation of HLA-C by HSV results in killing by an NK cell clone by its release from inhibition of KIRs.[155]

NK cells also express positive activating receptors,[156] such as 2B4 (CD244), NKG2D, NKp30, NKp44, NKp46, and NKR-P1A (CD161), that are important for killing. NKG2D may be activated by binding to MHC class I chain A (MICA) or chain B (MICB) or UL-16–binding proteins (ULBPs) on target cells. These molecules are induced by cell stress. Direct activation by viral products

also is possible. For example, the Ly49H-activating receptor of murine NK cells specifically recognizes a MCMV-encoded protein, m157.[157] Viruses, including herpesviruses, use a wide variety of strategies to inhibit NK cell–mediated cytotoxicity (Figure 150-2), indicating the importance of this antiviral mechanism *in vivo*.[158,159] HCMVs interfere at multiple steps of cellular MHC-I production. For example, they cause sequestration of MHC-I before they reach the surface by interfering with peptide chaperones. Then they produce their own MHC-I viral homologues (HCMV UL18).

NK cells are a particularly important restraint on viral replication and dissemination prior to the appearance of adaptive immune responses mediated by T cells and B cells. This has been demonstrated most clearly in the severity of primary infections with herpesviruses, including HSV, HCMV, and VZV, in patients with a selective lack of NK cells.[160] Although the initial disease is severe, these patients are able eventually to clear virus, presumably by means of T cell–mediated immunity.[160,161] NK cells may contribute to the control of viral infection by the direct lysis of virally infected cells, as described earlier, or by lysis of virally infected cells through antibody-dependent cellular cytotoxicity (ADCC). In the case of ADCC, antibody that recognizes viral proteins on the surface of the infected cell can bind to the NK cell through surface Fc receptors and enhance NK cell recognition and lysis of infected target cells. NK cells also produce proinflammatory cytokines that increase intrinsic antiviral resistance (e.g., type I IFN) and promote the development of antigen-specific T_H1 cell immunity (both type I IFN and IFN-γ). Thus the NK cell may contribute both to the innate immunity phase and the late phase of control of herpesvirus infections and be of particular importance in settings in which class I MHC expression is down-regulated and $CD8^+$ T cell recognition of infected target cells is hampered.

Infection of mice with HSV also demonstrates the importance of NK cells in the early control of viral infection. If NK cells are eliminated by injection with specific antiserum concurrent with the administration of virus, the titers of HSV in internal organs and the mortality rate both are markedly increased, but this treatment has no significant effect if started 5 days after viral challenge.[162] Adoptive transfer studies in immunosuppressed mice demonstrate that significant protection against HSV is provided by NK cells.[163] A more detailed study found that after HSV inoculation into the anterior ocular chamber, NK cells accumulated within the eye by day 5 after inoculation and that depletion of these cells by specific antiserum resulted in spread of the infection to the retina.[164]

Studies with MCMV also suggest that NK cells are important in the early control of herpesvirus infection,[165] and that IFN-γ produced by NK cells is more important for this protection than is NK cell–mediated cytolytic activity.[166] The production of IFN-γ by NK cells *in vivo* during the early phase of MCMV infection uses IL-12 and is enhanced by TNF-α, but not type I IFN. By contrast, endogenously produced type I IFN, but not IL-12 or TNF-α, greatly enhances NK cell–mediated cytotoxicity and proliferation.[167] How IFN-γ is protective in early viral infection remains unclear, but it could act directly to limit productive viral infection in cells that are otherwise permissive for infection, such as hepatocytes. Some evidence points to a noncytolytic mechanism of protection of hepatocytes in hepatitis B and hepatitis C infections, particularly through effects mediated by IFN-γ or TNF-α .[168–170]

IFN-γ produced by NK cells also increases class II MHC expression on mononuclear phagocytes *in vivo* and probably other cell types in response to HSV or MCMV infection,[171] an effect that facilitates antigen presentation to $CD4^+$ T cells. Increased IFN-γ

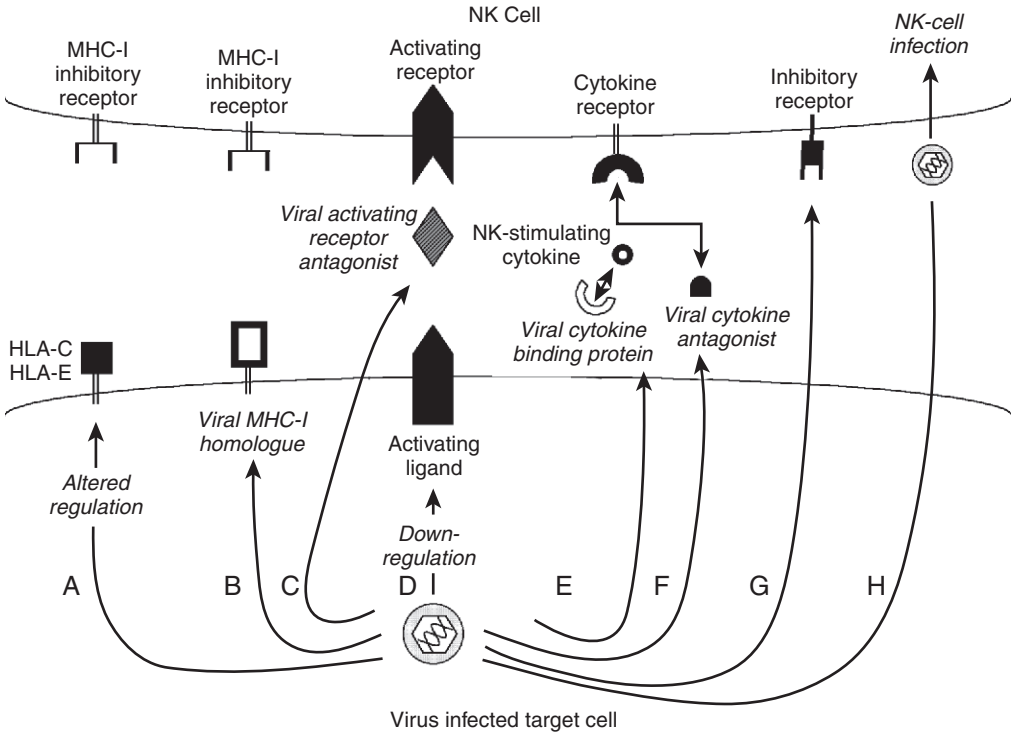


Figure 150-2 Viral mechanisms for evading a natural killer (NK) cell. NK function is tightly regulated by a balance of positive and negative signals. Viruses manipulate expression of native major histocompatibility complex (MHC)-I molecules **A,** express virally encoded MHC-I homologues **B** and generate activating receptor antagonists **C** to mimic "self." Additionally they down regulate target cell activating ligands **D** and block NK-activating cytokines with cytokine binding proteins **E** or NK cytokine receptor blockade **F.** Additionally viral proteins can act directly on NK inhibitory receptors **G,** or the virus can directly infect the NK cell **H.**

during the early phases of T cell activation and differentiation also may favor the development of T cells with an enriched capacity to secrete T_H1-type cytokines. This effect may be mediated by the ability of IFN-γ rapidly to increase expression by T cell T-bet, a key regulatory transcription factor that directs T_H1 development.[172]

More recently, NK cells have been observed to interact with dendritic cells in a number of experimental and *in vivo* settings including studies with herpesviruses and in fact have been shown to share some functional and phenotypic characteristics, such as NK-presenting antigens with MHC II and co-stimulatory molecules. Likewise, antigen-presenting dendritic cells with cytotoxic functions termed NK dendritic cells also have been described in mice.[173]

The cytolytic function of NK cells increases progressively during fetal life to reach values approximately 50% (range, 15% to 60% in various studies) of those for adult cells at term.[174-184] NK cells from the premature infant also have reduced cytotoxic function compared with those from the term neonate. An important point is that decreased cytotoxic activity by neonatal NK cells compared with adult cells also is consistently observed with HSV-infected[185-187] and HCMV-infected target cells,[188] but not with HIV-1 infected cells.[189,190]

Unlike adult NK cells, which are predominantly $CD56^{high}$, most neonatal NK cells are $CD56^{low}$. This $CD56^{low}$ subset, which is phenotypically immature, has relatively low NK cell activity compared with $CD56^{high}$ cells. However, the molecular basis for reduced activity of the $CD56^{low}$ subset remains poorly defined, as does the mechanism responsible for pathogen-specific differences in neonatal NK cell function.[1] It is clear that reduced neonatal NK cell activity is not determined at the level of the hematopoietic stem cell or later precursor cells of the NK cell lineage, because donor-derived NK cells appear early after cord blood transplantation and mediate robust cytotoxicity by way of the perforin/granzyme and Fas/Fas ligand cytotoxic pathways.[191]

As with their effects on adult NK cells, cytokines such as IL-2, IL-12, IL-15, type I IFN, and IFN-γ can augment the cytolytic activity of neonatal NK cells within a few hours[192-194]—an observation consistent with normal levels of expression of surface receptors for these cytokines.[195] However, neonatal NK cells are less responsive to activation by the combination of IL-12 and IL-15 than are adult NK cells, as reflected in the induction of CD69 surface expression.[196]

Reduced NK cell cytolytic activity and cytokine production may be an important contributor to the pathogenesis of neonatal HSV infection. In the murine neonatal model in which human cells are adoptively transferred, the age-related maturation of NK cell function parallels the development of resistance to HSV.[16,197] Neonatal mice, which like human neonates are more susceptible to HSV infection, can be protected by adoptive transfer of human blood mononuclear cells from adults but not those from neonates. Addition of IL-2 augments protection mediated by cells from adults but not cells from neonates, and protection depends on IFN-γ production by the transferred cells. The failure of human neonatal cells to transfer resistance in this model also can be corrected by the addition of IFN-γ, a cytokine produced poorly by neonatal T cells. This set of findings suggests that lack of IFN-γ production may be one important difference between adult and neonatal cells.

ADAPTIVE ANTIVIRAL IMMUNITY

Adaptive immune responses mediated by HSV antigen–specific T cells and B cells are first detected 5 to 7 days after the onset of primary HSV infection in adult humans, and the peak response is achieved approximately 2 to 3 weeks after infection.[198-200] In virtually all affected persons, antigen-specific immunity does not eradicate infection—that is, does not achieve sterile immunity—but rather terminates active viral replication and the acute infection. HSV is able to persist in the form of latent virus in neurons, leaving it potentially capable of causing recurrent localized disease. Recurrent viral replication may be frequent and, in many cases, asymptomatic.

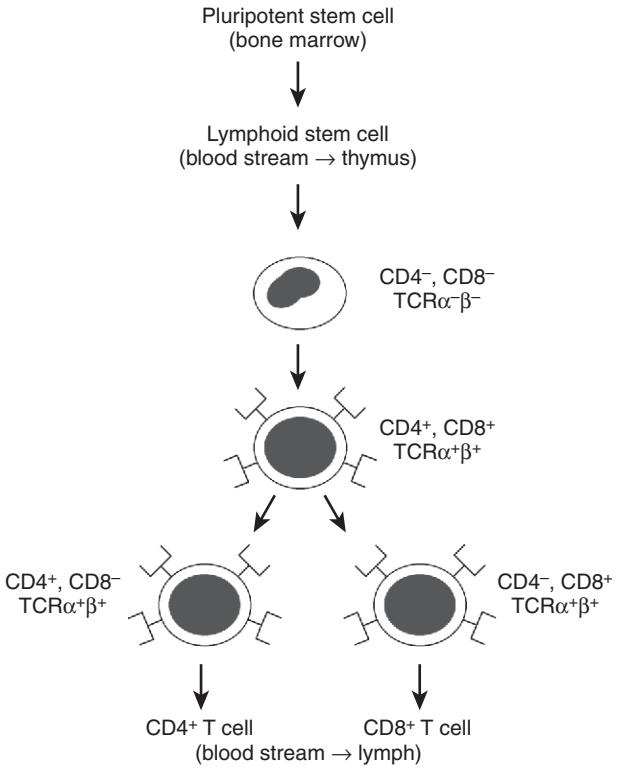


Figure 150-3 T cell maturation. T lymphocytes develop in the thymus where cells that recognize self-antigens are removed and TCRs that recognize foreign antigens in association with self–major histocompatibility complex (MHC) are selected. TCR expression commences in the double-positive $CD4^+CD8^+$ stage, beginning with low numbers of receptors on each cell and increasing as maturation proceeds. Single-positive, that is, $CD4^+$ or $CD8^+$ TCR-αβ–expressing, mature cells are selected from this population. A small population (approximately 5%) of double-negative T cells bearing TCR-γδ also leave the thymus[374] *(not shown).*

Antiviral T Cell Immunity: An Overview

The lymphoid stem cell derives from the pluripotent hematopoietic stem cell and differentiates into three types of cells—the T lymphocyte, the B lymphocyte, and the non-T, non-B NK cell progenitor, discussed previously. The lymphoid stem cells destined to be T lymphocytes (Figure 150-3) undergo positive and negative selection principally within the thymus and undergo development into descendant cells with rearranged α and β (or γ and δ) genes of its TCR. T cell surface expression of the TCR depends on coexpression with the CD3 molecule, which is a cluster of γ-, δ-, ε-, and ζ-chains. Together the TCR and CD3 molecules form the *TCR complex.* These early T cells are capable of distinguishing between self and nonself antigens in the context of self-MHC molecules and circulate within the bloodstream, the lymphatic system, and tissues. For the most part, three populations of T cells leave the thymus: TCR-αβ/$CD4^+$ T cells, TCR-αβ/$CD8^+$ T cells, and (as a minority) TCR-γδ T cells, which are predominantly

$CD4^-/CD8^-$. All bear the TCR complex; thus CD3 represents a universal T cell marker.

Although $CD4^+$ T cells are referred to as helper T cells and $CD8^+$ T cells as cytotoxic T cells or CTLs, they can have diverse functions and should be understood on the basis of their respective phenotypes and their response to MHC molecules. $CD8^+$ T cells recognize peptides in the context of class I MHC (displayed on virtually all nucleated cells), and $CD4^+$ T cells recognize antigens as presented in the context of class II MHC molecules, found on APCs such as monocytes, macrophages, B cells, and dendritic cells.

T cells play the critical role in resolution of active HSV infection and the maintenance of viral latency.[201,202] Adoptive transfer studies and T cell depletion studies using $CD4^+$ or $CD8^+$ monoclonal antibodies indicate that both $CD4^+$ and $CD8^+$ T cells contribute to clearance of HSV after acute infection in mice, acting, in large part, through the production of IFN-γ[203]; by contrast, B cells are not able to provide full protection in the absence of T cells.[200,204-206] The importance of T cells in the control of human HSV and other herpesvirus infections is indicated by the increased susceptibility of those with quantitative (purine nucleoside phosphorylase deficiency,[207] late HIV infection) or qualitative T cell and antigen presentation defects (Wiskott-Aldrich syndrome). Murine studies also suggest that $CD4^+$ T cells are more important than $CD8^+$ T cells in the control of HSV infections of the skin and peripheral nervous system,[204] suggesting that the relative importance of $CD4^+$ versus $CD8^+$ T cells in antiviral control is tissue-specific.

Comparison of newborn and adult T lymphocyte function explains some of the increased susceptibility to viral infections such as HSV infection in neonates. Although the mean T lymphocyte count is higher in neonates, neonatal T cells do not proliferate well in response to antigen, do not function efficiently as B cell helpers, and produce cytokines (such as IFN- and IL-4) in significantly lesser amounts.[208] Additionally, neonatal T cell cytotoxicity is limited in comparison with that of adult T cells. The specific role of T cell immaturity in severe clinical HSV infection in neonates is further suggested by the observation that neonates infected with HSV showed decreased antigen-specific cellular responses (including decreased proliferation and IFN-γ production) compared with adults who had primary HSV infection.[198]

$CD4^+$ T Cell–Mediated Antiviral Immunity

$CD4^+$ T cells appear to play a key role in protection from HSV and have been identified in the circulation[209] and from multiple sites of recurrent human HSV infection, such as the skin,[210,211] cervix,[212] cornea,[213] and retina.[214] Natural infection results in $CD4^+$ T cell recognition of peptide epitopes from a variety of HSV proteins, including those of the envelope, tegument, and capsid.[215,216] A number of mechanisms are likely to be important, reflecting the central and diverse roles of $CD4^+$ T cells in mediating and regulating antigen-specific immunity in HSV infection[1] (Box 150-1).

BOX 150-1

CD4 Anti–Herpes Simplex Virus (HSV) Mechanisms

- CD4 expression of IFN-γ, TNF-α, CD40 ligand[175,176,208]
- CD4 helper stimulation of anti-HSV effector $CD8^+$ CTLs[203,177,178]
- CD4 priming of dendritic cells (CD40 ligand)[179,180]
- CD4-expressed IL-2 expansion and differentiation of $CD8^+$ CTLs[202]
- CD4-expressed IFN-γ interference with ICP47-mediated MHC-I inhibition[202,181]
- CD4 cytolytic activity against cells expressing MHC-II–bound HSV[210,182,183]

CTLs, Cytotoxic T lymphocytes; *IFN-γ,* interferon-γ; *IL-2,* interleukin-2; *MHC-I, -II,* major histocompatibility complex class I, II; *TNF-α,* tumor necrosis factor-α.

T Cell Activation and T_H1/T_H2 Differentiation

In response to microbial pathogens, $CD4^+$ T cells differentiate into T_H1 or T_H2 phenotype. On the one hand, T_H1 cells principally produce IFN-γ and induce B cells to release IgG2 isotype antibodies, which are responsible for phagocyte activation of ADDC, and defense against intracellular pathogens. On the other hand, T_H2 cells produce IL-4, IL-5, and IL10 and induce immunoglobulin E (IgE) antibodies, which are responsible for immunity against parasitic infections.

As discussed previously, TLRs are important for innate immunity and activation of many cell types in response to recognition of pathogens through pattern recognition receptors. For example, the uptake of microorganisms by dendritic cells through TLRs induces the up-regulation of co-stimulatory and MHC molecules, the expression of chemokines, and migration of the dendritic cell to draining lymph node. There they prime and activate the adaptive immune response through interaction with T cells. The activation of T cells by APCs results from (1) T cell recognition of MHC class II–bound pathogen peptide, (2) activation of co-stimulatory molecules (such as CD28/CD80/86 or CD154/CD40), and (3) an initiation signal from specific cytokines, such IL-12 driving a T_H1-type response or IL-4/IL-10 driving a T_H2-type response.

After activation of T cells, a crucial step in tailoring adaptive immunity is determining T_H1 versus T_H2 phenotype.[217,218] Factors such as the density of peptides presented, types of co-stimulatory molecules expressed, and the state of APC activation influence whether the $CD4^+$ T cells differentiate into either T_H1 or T_H2 phenotype. However, the most important signal is the cytokine milieu present at the time of T cell stimulation, with either a T_H1-inducing profile represented by IL-12 family members and IFN-α or T_H2-inducing profile represented by IL-4 and IL-10.

Because presentation of antigen in the absence of co-stimulatory signals leads to anergy instead of activation, the stimulation of CD80 or CD86 by TLR-mediated signals is an important step in the activation of adaptive immunity.[219] In this way, TLRs participate in the translation of nonspecific information contained in conserved pathogen-associated molecular patterns into antigen-specific information and clonal expansion of T cells.

In vitro models of activation have shown that neonatal $CD4^+$ T cells differ from adult $CD4^+$ T cells in tending to become anergic (permanently nonresponsive to antigenic stimuli) unless antigen is presented by primed or mature dendritic cells expressing co-stimulatory molecules and cytokines.[220-223] This anergic tendency may apply to $CD4^+$ T cells that are expanded in instances in which neonates are infected with bacteria that express superantigen toxins, such as toxic shock syndrome toxin-1.[223]

Limitations in dendritic cell antigen presentation or co-stimulatory function could promote such T cell anergy or a failure of expanded $CD4^+$ T cells to acquire T_H1 effector activity. Of note, infants between 6 and 12 months of age exhibit lower $CD4^+$ T cell–derived IL-2 production in response to tetanus toxoid than that typical for older children and adults.[224] This observation supports the idea that either antigen-specific memory $CD4^+$ T cell generation or function is decreased not only for HSV but more generally during early infancy. *In vitro* studies suggest that reduced class II MHC antigen presentation could contribute to this outcome.[224]

T_H1 Cytokine Production

The HSV-specific $CD4^+$ T cell response of humans is characterized by a predominant T_H1 response.[184] The differentiation of naive $CD4^+$ T cells into T_H1 effector cells is favored by production of cytokines, such as IL-12, IL-23, and IL-27,[100] which are induced as part of HSV infection *in vivo*. These cytokines also can directly induce differentiated T_H1 cells to produce IFN-γ. HSV infection also may promote T_H1 immunity by inducing the production of osteopontin by T cells at an early stage of activation and differentiation. This cytokine may act on APCs by interacting with β3 integrins to maintain IL-12 production and with CD44 to inhibit IL-10 production, thereby favoring a T_H1 adaptive immune response.[225] Osteopontin is required for the development of delayed-type hypersensitivity responses (characteristic of T_H1 immunity) to HSV, and of HSV-induced corneal inflammation, which is T_H1-dependent.[225]

IFN-γ is a key immunoregulatory cytokine that is produced in substantial amounts by $CD4^+$ T cells of the T_H1 subset, $CD8^+$ T cells, γδ T cells, and NK cells. It is a key component in appropriately regulating adaptive and innate cellular mechanisms of antiviral control. The potential cellular sources of this cytokine and their temporal appearance (e.g., NK and γδ T cell–derived IFN-γ production preceding $CD4^+$ and $CD8^+$ T cell–mediated production), as well as their location, need to be considered when evaluating the importance of IFN-γ *in vivo*. IFN-γ acts to increase the antiviral state of many cell types, such as mononuclear phagocytes. In addition, this cytokine enhances the expression of the class I heavy chain, β_2-microglobulin, and TAP transporter. These effects help counteract the negative effects of HSV on class I MHC antigen presentation, and increase the ability of infected cells to be lysed.[181] CD40 ligand expression by $CD4^+$ T cells also is key for the antibody production by B cells and isotype switching.

Administration of neutralizing antibodies to IFN-γ increases the severity of HSV-1 infection in mice.[203] Likewise, knock-out mice lacking IFN-γ or its receptor are impaired in their ability to control HSV infection, as measured by higher mortality, prolonged infection, and more frequent reactivations. IFN-γ is a poor inhibitor of HSV replication *in vitro*; this is not a biologically relevant situation, however, because T_H1 cells do not secrete IFN-γ in environments devoid of other cytokines. *In vivo*, the secretion of other proinflammatory cytokines and chemokines occurs before and during the recruitment of T cells into sites of viral infection. In addition, IFN-γ has been found to act in concert with other cytokines such type I IFNs to effect potent inhibition of HSV *in vitro*.[72]

Virus-specific T_H1 cells also are major sources of TNF-α.[102] TNF-α has direct antiviral effects, particularly in conjunction with IFN-γ, and also proinflammatory effects (e.g., by increasing adhesion molecule expression by nonhematopoietic cells, such as endothelium, and production of chemokines). In human corneal infection with HSV, the combination of TNF-α and IFN-γ exerts a synergistic antiherpes effect within the corneal fibroblasts, chiefly attributed to the induction of IFN-β.[226] In the case of MCMV infection, some of these antiviral effects mediated by IFN-γ may be cell type–specific—for example, they may be operative for mononuclear phagocytes but not fibroblasts—and involve novel mechanisms that are shared with type I IFN.[227] Some of these T_H1 cells also are a rich source of IL-17, which is found at sites of HSV infection, such as the cornea. IL-17 may contribute to local inflammation by increasing the production of proinflammatory cytokines and neutrophil chemotactic proteins, such as chemokines.[228]

Some of the best-documented limitations in neonatal adaptive immunity come from two studies comparing T cell responses in neonates and in adults with primary HSV infection.[198,229] HSV-specific proliferation of peripheral blood mononuclear cells and production of both IFN-γ and TNF-α were diminished and delayed in the neonates compared with the adults. The neonates did not achieve adult levels of these responses for 3 to 6 weeks after clinical presentation, whereas the adults all exhibited robust responses by 2 weeks. These studies used ultraviolet light–irradiated viral preparations to stimulate peripheral blood mononuclear cells, in which viral proteins are processed mainly by the class II rather than the class I MHC antigen presentation pathway. Therefore they assayed mainly $CD4^+$ and not $CD8^+$ T cell function.

These results suggest a profound lag in the development of adaptive immunity to the HSV in normal neonates, during which viral replication and cell lysis could continue. Because, as discussed earlier, $CD4^+$ T cells provide multiple critical effector mechanisms that may be important for the resolution of HSV infection (including direct antiviral cytokine production and providing help for $CD8^+$ T cell and B cell responses), these findings suggest that a lag in T cell immunity may be a key contributor to the tendency of HSV infection to disseminate and to cause prolonged disease in neonates.

Studies of the responses of neonates and infants to viral vaccines also support the idea that $CD4^+$ T cell immunity to viruses may be blunted early in life. Peripheral blood mononuclear cells from neonates given oral poliovirus vaccine (OPV) at birth and at 1, 2, and 3 months of age exhibited decreased OPV-specific proliferation, IFN-γ production, and IFN-γ–positive cells in comparison with cells from immunized (but not reimmunized) adults.[230] By contrast, their antibody titers were higher than those for adults.

Antigenically naive $CD4^+$ T cells initially show a very limited capacity to express cytokines other than IL-2, whereas distinct subsets of memory $CD4^+$ T cells express particular cytokines. T_H1 and T_H2 cells produce IFN-γ and IL-4, respectively.[231] Thus the essential absence of memory $CD4^+$ T cells in the circulation of neonates accounts for the limited ability of neonatal $CD4^+$ T cells to produce IFN-γ compared with adult $CD4^+$ T cells.[232] If naive $CD4^+$ T cells are activated *in vitro*, they differentiate into effector-like cells that acquire the capacity to produce IFN-γ (T_H1 effectors) or IL-4 (T_H2 effectors), depending on the conditions used in terms of cytokine milieu and APC populations.[101,233-235] As mentioned earlier, however, studies suggest that neonatal naive $CD4^+$ T cells have a decreased capacity to become IFN-γ–producing cells compared with adult naive $CD4^+$ T cells in response to short-term (i.e., for 24 to 48 hours) stimulation by allogeneic dendritic cells.[1]

This decreased expression of IFN-γ by neonatal $CD4^+$ T cells is likely to be due to several factors. First, neonatal $CD4^+$ T cells are less effective than adult naive cells at inducing the co-cultured adult dendritic cells to produce IL-12, a key cytokine for promoting IFN-γ production.[1] Second, as discussed earlier, neonatal APCs, such as dendritic cells, may have a decreased capacity to produce IL-12 and related cytokines that are key for T_H1 differentiation.[236] It is interesting that this decreased capacity for IL-12 production by mononuclear cells may continue into early childhood, at least for certain stimuli, such as LPS.[237] Third, neonatal naive $CD4^+$ T cells exhibit a decreased expression of certain transcription factors that may play a role in the induction of IFN-γ gene expression, such as the NFATc2 protein.[238,239] Fourth, the greater methylation of DNA of the IFN-γ genetic locus in neonatal T cells also may contribute to a reduced and delayed acquisition of IFN-γ production after activation *in vitro*.[240] Some or all of these mechanisms are likely to contribute to the delay in the appearance of IFN-γ production by antigen-specific $CD4^+$ T cells after viral infections in the neonatal period.

CD154/CD40 Interactions

CD154 (CD40 ligand) is a member of the TNF ligand superfamily and is expressed abundantly on the surface of activated but not resting $CD4^+$ T cells (Figure 150-4). CD154 engages CD40, a molecule expressed by "professional" APCs,[241] including dendritic cells, mononuclear phagocytes, B cells, and possibly $CD8^+$

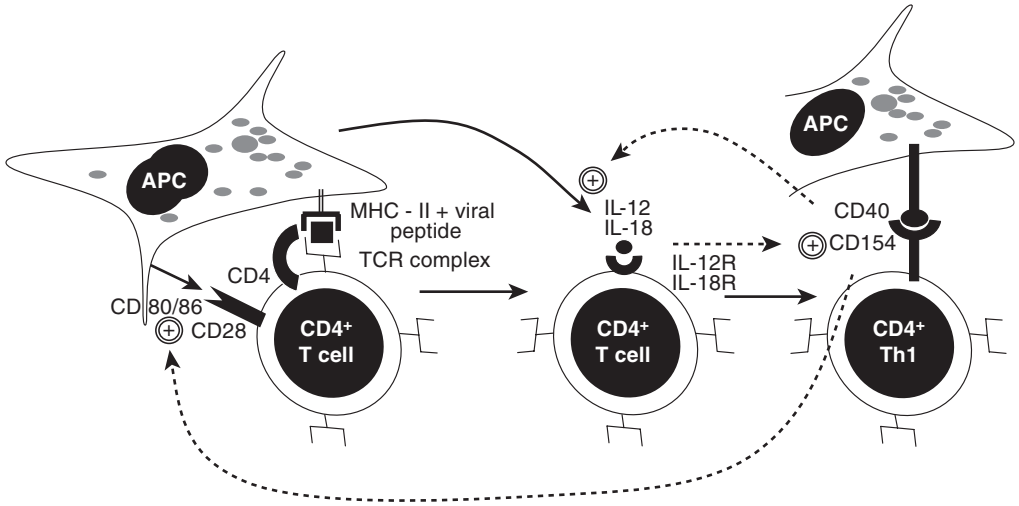


Figure 150-4 Helper T cell (T_H1) differentiation. The ligation of the T cell receptor on naive T cells by an immunogenic major histocompatibility complex (MHC)–peptide complex typically does not provide an adequate signal to induce their proliferation and differentiation. However, when antigen-presenting cells (APCs) such as dendritic cells (DCs) acquire antigens at sites of infection, they mature and express co-stimulatory molecules and cytokines that trigger the additional intracellular signaling required for $CD4^+$ T cell proliferation and differentiation. For instance, CD80 and CD86 expressed on a mature DC induce signaling through CD28 on naive $CD4^+$ T cells, and the interleukins IL-12 and IL-18 secreted by the mature DC induce signaling through IL-12 and IL-18 receptors. Such signaling transiently activates the maturing T cell, which then expresses a number of surface molecules including CD154 (a tumor necrosis factor [TNF] receptor family member) that binds CD40 (a TNF family member) on the DC, which leads to increased expression of CD80/CD86 and increased IL-12/IL-18. This reciprocal activation produces the robust T_H1 $CD4^+$ T cell response that is required to eradicate many viral infections. CD154 also appears to be important for the establishment of B cell and $CD8^+$ T cell memory.[245]

T cells.[242] Engagement of CD40 on dendritic cells is a potent maturation signal and an inducer of IL-12. This engagement also counteracts the inhibitory effect of other cytokines, such as IL-10.[243] The CD154/CD40 interaction is essential for many events in adaptive immunity, including the generation of memory $CD4^+$ T cells of the T_H1 phenotype and memory B cells, as well as most immunoglobulin isotope switching.[244]

The role of CD154 in viral host defense appears to vary with different pathogens but in general correlates with the particular requirement for $CD4^+$ T cells, indicating that $CD4^+$ T cells and CD154 are linked in mediating such help. CD154 deficiency in mice results in decreased $CD4^+$ and $CD8^+$ T cell responses to viral antigens and increased mortality after HSV-1 footpad infection. In studies of HSV corneal infection, the numbers of $CD4^+$ and $CD8^+$ T cells and B cells within draining lymph nodes dramatically expand within the first 7 days. However, no IFN-γ–secreting $CD4^+$ cells can be seen immediately. IFN-γ and a full T_H1 response do not appear until after 7 days after expression of CD154. These findings suggest that CD154 is not required for the initial expansion of HSV-1–specific $CD4^+$ T cells in the draining lymph node, but it is important for their differentiation into mature T_H1 effector cells. Exogenous IL-12 can at least partially relieve the requirement for CD154 in the T_H1 differentiation of HSV-1-specific T cells, consistent with the known capacity of CD40 signaling to induce IL-12 production in dendritic cells.[245]

Mice lacking CD154 as a result of selective gene disruption, or in which CD154/CD40 interactions were neutralized by monoclonal antibody, demonstrated an increased frequency of CNS infection and paralysis compared with wild-type mice after footpad inoculation with HSV.[228] These abnormalities correlated with reductions in $CD4^+$ but not $CD8^+$ T cell responses. The absence of CD154/CD40 interactions eliminates most antiviral antibodies and results in severe disease in viral infections in which antibody plays a critical role, such as in the dissemination of vesicular stomatitis virus to the CNS. In humans, CD154 also appears to play an important role in the expansion of $CD8^+$ T cell recognizing viral peptide antigens, such as from influenza A virus and HIV-1, based on an *ex vivo* assay using monocyte-derived dendritic cells as APCs.[246]

Freshly isolated monocytes from neonates or adults express low levels of CD40,[247] but whether they have similar capacity to produce cytokines in response to CD154 (CD40 ligand) engagement remains unclear. Using more physiologically relevant stimulation, neonatal $CD4^+$ T cells co-cultured with dendritic cells increase their expression of CD154[248] and induce IL-12 production by dendritic cells.[249] CD154 expression by purified neonatal naive $CD4^+$ T cells co-cultured with adult allogeneic dendritic cells was substantially less than by adult naive $CD4^+$ T cells after 24 to 48 hours of stimulation. This reduced CD154 production was accompanied by reduced IL-12 production (by monocyte-derived dendritic cells) and IFN-γ production (by naive $CD4^+$ T cells).[236] These observations suggest that CD154 surface expression is at least initially more limited for neonatal T cells, but that with continued priming *in vitro*, this limitation can be overcome. A likely consequence is that the differentiation of naive $CD4^+$ T cells into T_H1 effector cells by an IL-12–dependent and dendritic cell–dependent process may be delayed.

The generation of $CD4^+$ T effector cells is a complex process, involving antigen presentation by dendritic cells to naive $CD4^+$ T cells and multiple steps of T cell clonal expansion and differentiation; compromise of any of these steps could result in limited T_H1 immunity. Myeloid dendritic cells are essential to the differentiation of naive $CD4^+$ T cells to T_H1 effector and memory T cells. Key events promoting T_H1 differentiation include the elaboration by dendritic cells of cytokines, such as IL-12, IL-23, and IL-27, and their binding to specific receptors on $CD4^+$ T cells.[100] CD154 is expressed by naive $CD4^+$ T cells after the T cell recognizes peptide antigen/MHC complexes through the αβ-TCR and receives co-stimulation through CD28 binding to CD80 and CD86. The engagement of CD40 on the dendritic cell by CD154 on the T cell enhances the dendritic cell production of these T_H1-promoting cytokines and also increases the expression of CD80 and CD86, which helps sustain T cell activation. In persons who are

genetically deficient in CD40 ligand[250] or in the ability to respond to IL-12 and IL-23,[251] antigen-specific T_H1 responses are absent or severely depresssed, supporting the validity of this model.

The basis for the delayed development of HSV antigen-specific $CD4^+$ T cells in neonates is not known; it could reflect limitations at one or more of the steps just described. Neonates have been reported to have a lower frequency of precursor T cells capable of responding to HSV and HCMV.[252] This finding is unlikely to be due to a limitation in the diversity of the αβ-TCR repertoire[253-255] and is more likely to reflect intrinsic limitations in T cell function.

Cytolytic $CD4^+$ T Cell–Mediated Mechanisms

CTLs use two main pathways in executing cytotoxicity: (1) exocytosis of perforin and granzyme granules and (2) death receptor signaling through Fas/Fas ligand (CD95/CD95L) or TNF/TNF-R signaling. Both pathways trigger the apoptotic cascade and programmed cell death involving activation of caspase-3 and the cleavage of apoptotic substrates. Gene knock-out studies in mice defective in key elements of these pathways revealed that MHC-restricted cytolysis by murine $CD4^+$ T cells is mediated predominantly by the death receptor system, whereas MHC-I–restricted cytolysis by $CD8^+$ CTLs relies on perforins and granzymes. Human $CD4^+$ CTLs are able to utilize both pathways.[256]

$CD4^+$ CTLs make up a sizable minority of $CD4^+$ T cells in murine HSV infection (around 30%) and recognize HSV-specific antigens presented on MHC class II molecules.[257] In fact, early efforts to clone CTL specific for HSV resulted in $CD4^+$ instead of $CD8^+$ CTL clones.[258] Studies evaluating the $CD4^+$ CTL response suggest that activated $CD4^+$ T cells express perforin and granzyme A and B, in a fashion similar to that for $CD8^+$ cells; however, $CD4^+$ T cells express substantially lower amounts of perforin and granzymes in both cell numbers and amounts per cell, compared with $CD8^+$ T cells.[259] Human $CD4^+$ cytotoxic cells can recognize peptides derived from viral glycoproteins found in the HSV lipid envelope,[201,260,261] such as gB, gC, and gD,[152] and it is likely that these viral glycoproteins enter into the class II antigen-processing endocytic pathway by first fusing with the host cell membrane.

Although class II MHC normally is expressed by APCs, such as B cells, mononuclear phagocytes, and dendritic cells, a wide variety of cell types can express class II MHC and, in most cases, present antigen, after exposure to IFN-γ, GM-CSF, or TNF-α. These cell types, which include endothelial cells, enterocytes, renal epithelial cells, thyroid epithelial cells, microglial cells, epidermal keratinocytes, myoblasts, eosinophils, NK cells, and T cells themselves,[2] might then become potential targets for cytotoxic $CD4^+$ T cells. However, the importance of $CD4^+$ T cell–mediated cytotoxicity in the control of infection has been questioned, given that substantial $CD8^+$ T cell responses to HSV are generated in humans, and that HSV-specific $CD8^+$ T cells constitute a prominent part of the viral response in tissues such as the skin and cervix. Additionally, HSV-1 renders infected cells resistant to CTL-induced apoptosis[262] by mechanisms such as the down-regulation of MHC class II through the virion host shutoff protein (vhs) and the viral protein encoded by the $\gamma_1$34.5 gene.[263]

An expanded target cell range is possible, however, because class II MHC expression is up-regulated on many non-APC types in response to cytokines, such as IFN-γ. Thus it is plausible that $CD4^+$ cytolytic T cells play an important role in the resolution of HSV infection in humans. No studies have examined the degree of cytolytic activity mediated by neonatal $CD4^+$ T cells for HSV or other infections.

$CD8^+$ T Cell–Mediated Antiviral Immunity

$CD8^+$ CTLs are important in effective clearance of viral infections, including HSV infection,[210,264,265] and are found at sites of local recurrence of such infection such as the skin adjacent to the genital tract,[210,266] cervix,[212] and cornea.[267] $CD8^+$ T cells, when first activated by antigen, are not effective killers but subsequently, under the influence of cytokines such as IL-2 and IL-15, proliferate and differentiate into an effector cell lymphoblast population that efficiently kills. They kill target cells by exocytosing cytotoxic molecules such as perforin, granzymes, or granulysin.[268] Fas/Fas ligand and TRAIL/DR interactions also may play a part in this killing. This differentiation includes increased expression of molecules involved in cytotoxicity, such as perforin, granzymes, Fas ligand, and granulysin, as well as an increased capacity to produce cytokines, such as IFN-γ and TNF-α. The mature virus-specific $CD8^+$ CTL looks conspicuously different from the unprimed, naive $CD8^+$ T cell.

TABLE 150-3

Properties of $CD8^+$ T Cell Subsets

Expression Marker	Naive	Memory	Effector
CD45RA	+	−	+
CD45R0	−	+	−
CD27	+	+	−
CD28	+	+	−
CD57	−	−	++
CD95 (Fas)	−	++	+
Fas ligand	−	±	++
IL-2	+	++	−
IL-4	−	+	−
IFN-γ	−	++	++
TNF-α	−	++	++
Perforin/granzyme B	−	±	++

Compared with naive $CD8^+$ T cells, two discrete primed subpopulations can be found within the human $CD8^+$ lymphocyte compartment. First, the $CD45RA^{neg}$ $CD45R0^+$cells are similar to *memory-type* $CD8^+$ T cells in that they express CD95 (Fas) and integrin family members CD11a, CD18, CD29, CD49d, and CD49e and are able to secrete not only IL-2 but also IFN-γ, TNF-α, and IL-4. This subset does not exert cytolytic activity without previous stimulation but does contain virus-specific CTL precursors. A second primed population is characterized by $CD45RA^{pos}$ $CD27^{neg}$ $CD28^{neg}$ and contains T cells expressing CD11a, CD11b, CD18, and CD49d. These T cells do not excrete IL-2 or IL-15 but can produce IFN-γ and TNF-α. These *effector-type* $CD8^+$ T cells abundantly express Fas ligand and contain perforin and granzyme B and have high cytolytic activity as is.[375]

CTL, Cytotoxic T lymphocyte; *HSV,* herpes simplex virus; *IFN-γ,* interferon-γ; *IL,* interleukin; *TNF-α,* tumor necrosis factor-α.

$CD8^+$ T cell differentiation also is recognized by cell surface changes that reflect the stage of maturation and nomenclature of this population (Table 150-3). For example, in contrast with naive $CD8^+$ T cells that uniformly express CD45RA, HCMV-specific $CD8^+$ T cells early in infection exhibit CD45R0 surface expression. CD45 is a transmembrane phosphatase that regulates signaling through TCR CD3, and distinct isoforms of the molecule are generated through alternative splicing of amino-terminal (N-terminal) exons A, B, and C. The high-molecular-weight form (CD45RA) contains A, B, or C (or all three), whereas CD45R0 does not. Similarly, patterns of CCR7 expression change, indicating activated CTL homing signals. Chemokine receptor CCR7 binds CCL19 and CCL21 and directs the migration of lymphocytes to the lymph node. CD127 (IL7R-α) is expressed by naive T cells, disappears for the most part during active infection but reappears in a subset of virus-specific $CD8^+$ cells within the memory pool with the ability to respond to cytokine IL-7, enabling reactivation.

Studies using class I MHC/viral peptide tetramers, which allow direct detection of $CD8^+$ T cells based on their TCR specificity, have documented that virus-specific $CD8^+$ T cells undergo a dramatic expansion *in vivo* during viral infection. In some instances, such as with primary EBV infection, more than 40% of circulating $CD8^+$ T cells may be reactive with a single viral peptide epitope.[269] Most $CD8^+$ effector T cells have a relatively short life span and probably are eliminated by apoptosis after antigen clearance. $CD4^+$ T cells may aid directly in the generation of $CD8^+$

cytolytic T cells by the production of several cytokines, including IL-2 and IFN-γ. In addition, $CD4^+$ T cells may indirectly influence this process by enhancing dendritic cell function through a CD154/CD40 interaction; dendritic cells, in turn, may then help promote $CD8^+$ cytolytic T cell generation by secreting cytokines and engaging co-stimulatory molecules. In animal models, $CD8^+$ T cells also appear to be key in resolving HSV lytic infection of ganglion during primary infection,[270,251] and in preventing reactivation of virus from latency in sensory neurons[271]; this latter function may involve an IFN-γ–dependent mechanism.[272] Direct recognition of infected ganglion cells by $CD8^+$ T cells is likely, because HSV infection induces detectable class I MHC expression by sensory neurons.[273] Noncytolytic mechanisms,[274] such as the production of cytokines with antiviral activity (e.g., IFN-γ)[275] or the secretion of granzyme A,[276] may be key for $CD8^+$ T cells to maintain latency and to prevent viral spread. By contrast, cytolytic mechanisms, such as those mediated by perforin, may not be necessary for control of HSV infection at particular tissue sites, such as the cornea or brain, and may even contribute to inflammatory disease.[277,278]

Viral Inhibition of Antigen Presentation and Effect on the T Cell Immune Response

In view of the importance of class I MHC–restricted cytotoxicity in the control of viral infection, it is perhaps not surprising that a number of viruses, including HSV and HCMV,[278] have developed mechanisms to inhibit class I MHC antigen presentation. First, the virus can establish latency in sensory ganglia, an immunoprivileged site in which viral proteins are not expressed. During lytic replication, however, the HSV ICP47 protein (gene Us12) inhibits the MHC class I antigen presentation pathway by inhibiting the transporter associated with antigen presentation (i.e., TAP), which translocates peptides across the endoplasmic reticulum membrane.[226,279-282] The virion host shutoff protein, vhs, degrades host cell mRNA preventing synthesis of new HLA class I.[170] The combination of these two proteins is responsible for poor recognition of infected cells by $CD8^+$ CTLs; however, CTL toxicity is restored somewhat by exposure of those cells to IFN-γ, which up-regulates class I antigen processing and presentation. Certainly, the high levels of IFN-γ isolated from clinical lesions suggest that this effect occurs *in vivo* in addition to *in vitro*.[283]

CTL cytotoxicity also is inhibited by HSV products Us3 kinase, ICP22, ICP27, ICP4, gJ, and gD, which confer apoptosis resistance to infected cells.[262] When IFN-γ is present and the effects of ICP47 are not as evident, the second-line defense effects of Us3 (Us3 kinase) and Us5 (gJ) become more apparent. They both partially block CTL-induced chromium release. Us3 kinase blocks the cleavage of Bid by granzyme B. In addition, gJ prevents the activation of caspase-3 by granzyme B (Figure 150-5).

Despite these inhibitory mechanisms, class I MHC–restricted human $CD8^+$ T cells that can lyse HSV-infected targets can be isolated from HSV-2 lesions of patients with genital disease[132,202] or from the blood of HSV-immune donors.[284,285] The $CD8^+$ T cell response in humans appears to be dominated by recognition of HSV proteins that are internal structural components of the virion, such as those of the tegument and nucleocapsid, or those that are rapidly induced upon viral entry, such as certain immediate early genes.[286,287] These proteins apparently can be processed into peptides and presented on class I MHC molecules before the inhibitory effect of either the virion host shut-off or TAP inhibitor proteins. Similarly, immediate-early gene products and tegument and nucleocapsid proteins also may be important targets of the human $CD8^+$ T cell response against HCMV by being presented to $CD8^+$ T cells before the onset of multiple mechanisms that interfere with class I MHC antigen presentation. By contrast, viral glycoproteins that are found in the HSV lipid envelope do not appear to be important antigens for class I MHC–restricted responses, probably because they fuse with the host cell membrane and do not enter in the cytoplasm in substantial amounts.[226]

Inhibition of viral replication also can occur without killing of the infected cell. Studies using fibroblasts preinfected with HSV for 2 hours before exposure to CTLs, under conditions in which less than 10% lysis was observed, resulted in a 50% decrease in production of infectious virus.[288] Similarly, control of viral replication and reactivation from latency in infected neurons is controlled by nonlytic mechanisms.[289] Studies using Us12-deleted HSV ($ICP47^-$) led to a decrease in virus yield, especially when CTL attack occurred at early stages of viral replication. *In vivo*, the effect of ICP47 probably serves to open a short window of time in which infected cells are spared from recognition and attack by CTLs. ICP47 is not present in the virions but is one of the first proteins to be made in infected cells. Therefore, early on, before sufficient ICP47 is present, antigen is presented on the surface of the cells, permitting recognition by CTLs. At intermediate time points, the effect of ICP47 prevents recognition of infected cells, but once IFN-γ is up-regulated within lesions, recognition of infected cells is restored. At that time, the second lines of defense, such as inhibition of apoptosis, come to the fore.

HCMV also encodes proteins that inhibit class II MHC antigen presentation,[290] and the same has been found to be true of HSV, with vhs and $\gamma_1 34.5$.[263] Infection of mice with virulent HSV strains results in CNS lesions in which class II MHC expression remains intracellular, thereby avoiding any chance of detection by $CD4^+$ T cells.[291]

$CD8^+$ T cells can be sensitized *in vitro* for cytotoxicity using allogeneic (MHC other than self) stimulator cells, followed by testing for cytotoxic activity against allogeneic target cells. With this approach, most studies have found that neonatal T cells are moderately less effective than adult T cells as cytotoxic effector cells.[292-295] More substantial defects in T cell–mediated cytotoxicity by neonatal T cells after allogeneic priming are observed when no exogenous cytokines, such as IL-2, are added.[296,297] This finding suggests that such decreased cytolytic activity could be of physiologic significance *in vivo* if $CD4^+$ T cell help also was limited. The capacity of neonatal $CD8^+$ T cells to become cytolytic effector cells when primed using optimal APCs such as dendritic cells, remains to be determined.

The mechanism for reduced neonatal T cell–mediated cytotoxicity remains poorly understood. Two studies have found that only a low percentage of neonatal $CD8^+$ T cells constitutively express perforin, whereas approximately 30% of adult $CD8^+$ T cells contain this protein.[298,299] By contrast, another study found that approximately 30% of neonatal T cells expressed perforin—a frequency similar to that for adult T cells.[300] The capacity of neonatal $CD8^+$ T cells to express Fas ligand (CD95L) and to mediate Fas ligand–dependent cytotoxicity in response to priming by alloantigen also is not known.

Much of the studies of antigen-specific cytotoxicity to date have focused on congenital and perinatal HIV-1 infection. In congenital HIV-1 infection, an expansion of HIV-specific cytotoxic T cells was detected at birth, indicating that fetal T cells were activated by viral antigens.[301] In another case of *in utero* HIV infection, HIV-specific T cell–mediated cytotoxicity was detected at 4 months of age and persisted for several years despite a high HIV viral load.[302] Cytotoxic responses to HIV in perinatally infected infants suggest that, although $CD8^+$ T cells capable of mediating cytotoxicity have undergone clonal expansion *in vivo* as early as 4 months of age,[303] their cytotoxicity may be reduced and delayed in appearance compared with that of adults.[304] In many cases, HIV-1–specific cytotoxic lymphocyte responses were not detectable during the first few months of life.[301,304] When evaluated beyond infancy, cytolytic activity directed to HIV envelope proteins was commonly detected, but that directed against *gag* or *pol* proteins was rarely detected.[305] Also noted was decreased HIV-specific $CD8^+$ T cell production of IFN-γ by young infants

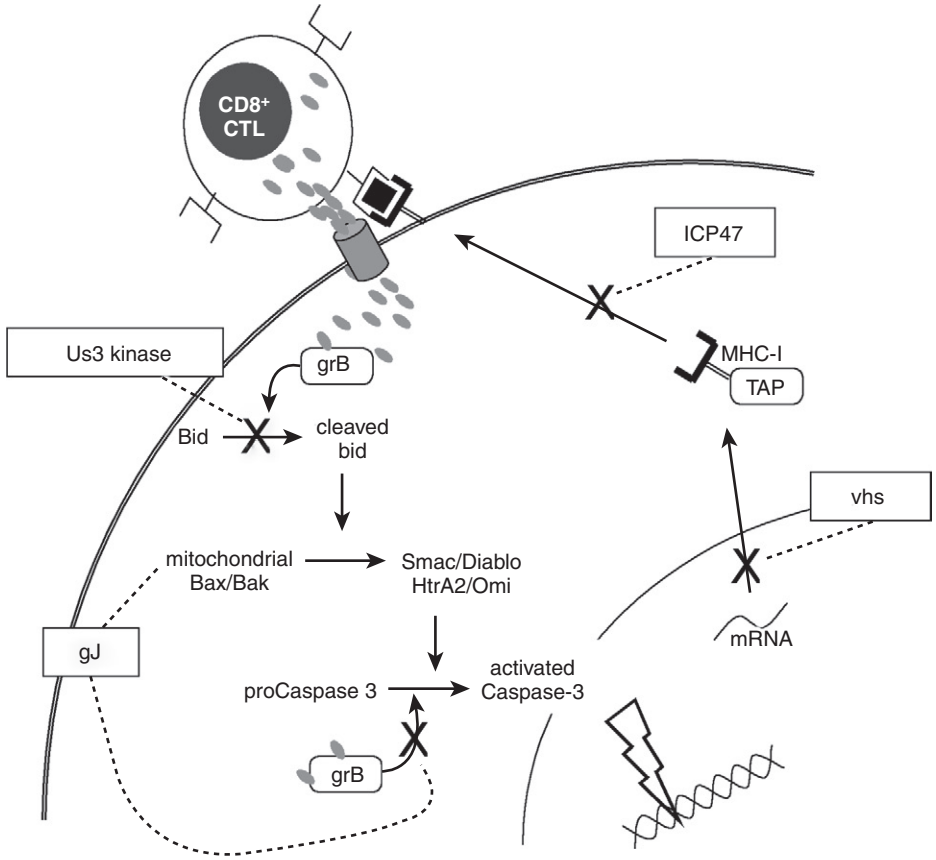


Figure 150-5 Herpes simplex virus (HSV) blocks cytotoxic lymphocyte (CTL)-driven apoptosis. On recognition of viral infection by major histocompatibility complex class I (MHC-I) bound viral antigen, $CD8^+$ CTL releases cytotoxic molecules including granzyme B (grB). grB cleaves proapoptotic molecule Bid, which then associates with proapoptotic protein Bax/Bak at the mitochondria to induce the release of Smac/Diablo, HtrA2/Omi, and cytochrome *c*. In turn, those molecules permit the activation of caspase 3. An additional cleavage of procaspase-3 occurs directly by grB but alone is insufficient for activation of caspase-3. Activated caspase-3 drives apoptosis by stopping translation and DNA repair and by damaging the genomic DNA through DNA fragmentation factor (DFF) and caspase-activated DNase (CAD). HSV proteins circumvent this process. ICP47 blocks TAP transport of MHC-I molecules to the cell surface. Virion host shutoff (vhs) protein cleaves host messenger RNA, blocking new protein synthesis and also effectively down-regulating MHC-I antigen presentation. Us3 kinase blocks grB cleavage of Bid, and gJ stabilizes the mitochondria and blocks grB activation of caspase-3.

with perinatal HIV infection,[306] along with an inability to generate HIV-specific cytotoxic T cells after antiretroviral therapy.[307] Together, these results point to the possibility of a delay in the development of $CD8^+$ T cell–mediated responses in comparison with that in adults and suggest that the nature of the target antigens recognized by neonatal $CD8^+$ T cells may be more limited. These developmental limitations in $CD8^+$ T cell function may not necessarily be limited to HIV-1, at least in early infancy, because one study of RSV-specific cytotoxicity found that it was more pronounced and frequent in infants 6 to 24 months of age than in younger infants.[308]

HIV-1 infection is characterized by a number of severely immunosuppressive features that may not apply to most other viral infections. These include impairment of antigen presentation,[309] decreasing thymic T cell output,[310] and promotion of T cell apoptosis.[311] Of note, these suppressive effects of HIV-1 on cytotoxic responses may be relatively specific for HIV-1, because HIV-infected infants who lack HIV-specific cytotoxic T cells may maintain $CD8^+$ T cell responses against EBV and CMV.[306,307] Whether a lag occurs in the acquisition of herpesvirus-specific $CD8^+$ T cell responses in neonates and infants (either HIV-infected or uninfected) compared with those in adults with primary infection remains unclear.

CD28/CD80–86 Co-Stimulation

In addition to engagement of the αβ-TCR by peptide/class II MHC antigen, full naive T cell activation is thought to require co-stimulation, such as by engagement of CD28 on the T cell by CD80 or CD86 on the APC (see Figure 150-4). Murine studies indicate that such co-stimulation is critical for the development of an effective adaptive immune response to HSV. CD28/CD80–86 interactions appear critical for paralysis-free survival as well as for the generation of both HSV-specific $CD4^+$ and $CD8^+$ T cell responses in the footpad injection model.[228] This was demonstrated using mice with selective genetic disruption of CD28, as well as in normal mice in which CD28/CD80–CD86 interactions were neutralized *in vivo* by administration of CTLA-4–Ig (cytotoxic T lymphocyte antigen-4–immunoglobulin),[228] a soluble fusion molecule that effectively competes with T cell–associated CD28 for CD80–CD86 binding. In an intravaginal HSV murine model, mice lacking both CD80 and CD86 were prone to severe local and disseminated disease and death[312] and, as in the other study, were unable to mount effective $CD4^+$ or $CD8^+$ T cell responses and exhibited more severe disease and increased mortality.[313]

Neonatal T cells express levels of CD28 similar to those in adult T cells and produce IL-2 and proliferate as well as adult T cells do in response to mouse APCs expressing human CD80

or CD86, and to anti-CD3 monoclonal antibody, indicating that CD28-mediated signaling is intact.[314] This finding also is supported by a study showing that anti-CD28 monoclonal antibody treatment of neonatal T cells markedly augments their ability to produce IL-2 and proliferate in response to anti-CD2 monoclonal antibody.[315] These results do not exclude the possibility that neonatal T cell co-stimulation by CD28 could be limited because of decreased expression of CD80 or CD86 by key APCs, such as neonatal myeloid dendritic cells.

Chemotactic and Homing Receptor Expression by Viral Antigen–Specific T Cells

The differential expression of chemokine and homing receptors by T cells is important in their selective trafficking, either to sites at which naive T cells may potentially encounter antigen for the first time, such as the spleen and lymph nodes, or to inflamed tissues for effector functions.[143] The homeostatic chemokines CCL19 and CCL21 have been shown to be essential in the transmigration of lymphocytes across high endothelial venules (HEVs) (highly specialized postcapillary venules within the lymph nodes and Peyer's patches), allowing the passage of lymphocytes bearing their shared receptor CCR7 from the blood compartment into secondary lymphatic organs. CCR7 is uniformly expressed on naive T cells as well as a subset of resting memory T cells known as central memory T cells. CCL21 is constitutively expressed within the lumen of HEVs, whereas CCL19 is produced by other cells within the lymph node, but becomes displayed on the HEV lumen after transcytosis across the endothelial barrier.

Naive T cells also express CCR9 and CXCR4. CCR9 ligand is CCL25 and may help traffic T cells into the gastrointestinal lymphatics. CXCR4's ligand is CXCL12 and is involved in transendothelial migration across HEVs. These are the principal chemokine receptors expressed on naive lymphocytes; this is contrast with the numerous chemokine receptors expressed on effector T cells.

Primed $CD4^+$ T cells briefly express CXCR5, which is a chemokine receptor otherwise expressed principally on B cells. Its ligand CXCL13 is produced within the B cell–rich follicular compartment of secondary lymphoid tissues but is absent from the adjacent T zone. The acquisition of CXCR5 by recently primed $CD4^+$ T cells temporarily relocates these cells to the outer follicle, where they can interact with B cells during antibody production. Accordingly, the $CXCR5^+$ $CD4^+$ T cells also are known as follicular B helper cells (T_{FH}). CXCR5 is rapidly lost during T cell proliferation, indicating that the homing program is an early and transient step in the T cell activation and differentiation process.[316]

Effector T cells are equipped with receptors for inflammatory cytokines and adhesion molecules that are greatly up-regulated during inflammation, so these cells efficiently home to sites of pathogen entry and disease. By contrast, memory T cells are believed to confer rapid and superior protection against pathogens that they have encountered during previous immune responses. They are divided into two subsets based on their different migratory and functional properties. The subset $CCR7^+CD62L^+$ memory T cells, termed central memory T (T_{CM}) cells, far outnumber the memory subset lacking these lymph node–homing receptors termed effector memory T (T_{EM}) cells. The utility of these T_{CM} cells in responding to recurrent herpes infection is unclear, because a recent study did not indicate accelerated proliferation kinetics or tissue infiltration of T_{CM} cells when compared with naive T cells. A more brisk effector (T_{EM}) response was demonstrated.[317]

In established HSV infection, most recurrences of viral replication occur in keratinocytes found at epithelial sites, such as the skin and genitourinary mucosa. Trafficking of T cells into this location is controlled by the expression of cutaneous lymphocyte-associated antigen (CLA), which is a fucose-containing carbohydrate that can decorate P-selectin glycoprotein ligand-1 on T cells. CLA is expressed from most T cells recovered from the skin and is on approximately 5% to 10% of circulating $CD8^+$ T cells. During symptomatic recurrences of virus at these cutaneous sites, virus-specific $CD4^+$ T cells and NK cells may infiltrate within 48 hours after the appearance of lesions. This is followed several days later by infiltration by virus-specific $CD8^+$ T cells, which are associated with viral clearance.[210] Consistent with the importance of $CD8^+$ T cell immunity at these local sites of replication, a majority of $CD8^+$ T cells that recognize a particular HSV peptide express CLA.[211]

It is likely that HSV-specific T cells that enter sites of viral replication, such as the CNS during HSV encephalitis, utilize a distinct combination of homing and chemotactic receptors to achieve selective trafficking. As indicated by studies in mice using other neutrotropic viruses, expression of CXCR3, CCR2 and CCR5 chemokine receptors may regulate $CD8^+$ T cells trafficking into the CNS.[318] Other chemokine and homing receptor combinations are likely to be important for trafficking of HSV-specific T cells to the liver and gastrointestinal tract in cases of disseminated disease.

As discussed earlier, down-regulation of CCR7 and up-regulation of other chemokine receptors by effector T cells probably are important for redirecting these cells from secondary lymphoid tissue to other sites of tissue inflammation, where they can carry out their effector functions. This appears to be true for herpesvirus-specific $CD4^+$ T cell responses, because CMV-specific human memory $CD4^+$ T cells, which are capable of producing antiviral T_H1 cytokines, such as IFN-γ, are essentially all $CCR7^{low}$ cells.[1] This restriction of CMV-specific T_H1 effector function to $CCR7^{low}$ cells has been observed after primary CMV infection in both adults and young children but has not been evaluated in cases of congenital or perinatal CMV infection or in cases of HSV infection.

Human neonatal naive T cells differ from those of adults in that they do not increase CXCR3 expression, which favors trafficking to inflamed tissues that have been exposed to IFN-γ, and they decrease CCR7 expression after activation by way of anti-CD3 and CD28 monoclonal antibodies.[319,320] The CCR7 expressed on neonatal T cells is functional and mediates chemotaxis of these cells in response to CCL19 and CCL21.[321] These findings suggest that activated neonatal T cells may be limited in their capacity to traffic to nonlymphoid tissue sites of inflammation and may recirculate between the blood and peripheral lymphoid organs. Neonatal T cells can increase the surface expression of CCR5 by treatment with either mitogen or IL-2[322] or after differentiation *in vitro* in a cytokine milieu that favors T_H1 development.[323-325] Whether CCR5 expression by T cells, which probably is important for their homing to the CNS, is up-regulated in cases of neonatal or postnatal HSV encephalitis is not known.

γδ T Cells

The γδ T lymphocytes are one of the first T cells to emigrate from the thymus and express a TCR heterodimer consisting of a γ-chain and a δ-chain, in association with CD3. A majority of these γδ T cells take residence in epithelial tissues including the skin, intestines, lung, and reproductive tracts. Their nomenclature is based on their TCR gene rearrangements; for example, the three γ genes are Vγ1 to Vγ3. Vγ3Vδ1 T cells are found principally in the skin and are known as dendritic epidermal T cells (DETCs) and are maintained by expression of zeta (ζ)-chain–associated protein 70 (ZAP-70) signaling molecule within the epidermis.[326] $V\gamma5^+$ γδ T cells, known as intestinal intraepithelial lymphocytes (IELs), are found predominantly in the intestines and are dependent on thymus IL-15 for development. The γδ T cells have multiple functions including tissue homeostasis and wound repair, facilitated by γδ expression of insulin-like growth factor-I (IGF-I) and keratinocyte growth factor-1 (KGF-1), respectively. Total parenteral nutrition

administration, which leads to villus atrophy in the small intestine, is associated with a down-regulation of IEL-derived KGF-1, whereas short bowel syndrome, a model of villus hypertrophy, is associated with up-regulation of IEL-derived KGF.[327] γδ T cells also regulate local inflammation. KGF-1 produced by DETCs, for example, induces expression of hyaluronan by keratinocytes which recruits inflammatory cells such as macrophages. γδ IELs also influence neighboring epithelial cells to produce proinflammatory factors such as chemokine IL-8 and IFN-γ–inducible protein-10.[328] γ δ T cells also are able to recognize and lyse (perforin-mediated) cancerous epithelial cells and lymphoma cells,[329] and they are able to recognize and fight infection. Although some γδ T cells respond to antigens (such as HCMV peptides in the context of MHC class I),[329] little is known about the antigens that stimulate a majority of γδ T cells. These antigens appear to be expressed on stress or transformation of tissues, leading to activation of γδ T cells and their subsequent expression of cytokines and growth factors that aid in tissue repair or lysis of an infected or transformed cell. Some γδ T cells, such as DETCs, have been shown to recognize antigens via TLRs.[330] In the case of herpes simplex infection, the absence of γδ T cells has been shown to lead to increased susceptibility to infection owing to a blunted $CD4^+$ T_H1 and IFN-γ response.[331,332]

Although human neonatal γδ T cells proliferate *in vitro* in response to mycobacterial lipid antigens,[333] they express lower levels of serine esterases than those typical for adult γ δ T cells, suggesting that they are less effective cytotoxic cells.[334] Clones of γδ T cells derived from cord blood also have a markedly reduced capacity to mediate cytotoxicity against tumor cells extracts.[335] Because these neonatal clones also have lower CD45R0 surface expression than the adult clones, their reduced activity may reflect their antigenic naive status.

Natural Killer T Cells

A small population of circulating human T cells express αβ TCRs, lack expression of both CD4 and CD8, and express NKR-P1A, the human orthologue of the mouse NK1.1 protein. These features, as well as others (such as CD56 and CD57 surface expression, along with a dependence on the cytokine IL-15 for their development[336]), are characteristic of NK cells. For this reason, these cells frequently are referred to as NK T cells, or natural T cells. Like murine T cells expressing NK1.1, human NK T cells have a restricted TCR repertoire and mainly recognize antigens presented by the nonclassic MHC molecule CD1d, rather than by class I or class II MHC molecules. These CD1d-restricted antigens that can be recognized by NK T cells include certain lipid molecules as well as hydrophobic peptides. NK T cells also have the ability to secrete high levels of IL-4 and IFN-γ and to express death-inducing ligands (Fas ligand and TNF-related apoptosis–inducing ligand [TRAIL]) on their cell surface on primary stimulation—a capacity not observed with most antigenically naive αβ T cells,[337,338] suggesting that they could play a role in the early stages of the immune response. Additionally, NK T cells have been shown to help B cells produce antibody. This interaction has been shown to require CD40-CD154 co-receptor interaction but not IL-4 or IFN-γ. This role does include augmenting $CD4^+$ T cell response (related to an increased availability of MHC-II–restricted T cell help to B cells), although in the absence of $CD4^+$ T cells, NK T cells have been shown to replace, at least in part, conventional $CD4^+$ T helper functions.[339] Murine NK T cells have been implicated in the control of zosteriform HSV infection,[194] but the antiviral mechanisms involved remain to be defined.[340]

Limitations in NK T cell–mediated immunity in neonates could contribute to the severity of HSV infection. At first, only a small number of NK T cells (accounting for less than 1% of circulating T cells) are present in the neonatal circulation, but this number subsequently increases with age. Neonatal NK T cells differ from adult NK T cells in producing only limited amounts of IFN-γ on primary stimulation, indicating functional immaturity.[341]

B Cells, Antibody, Antibody-Dependent Cellular Cytotoxicity, and Complement

In animal models, evidence points to a protective role of antibody against HSV infection and spread within the nervous system, acting by neutralization directed against glycoprotein B (gB), and gD or by ADCC (discussed further on) against gB, gD, and gC. ADCC in the presence of competent effector cells (NK cells) may be more effective against higher challenge doses of virus than neutralizing antibodies. In humans the role for antibody has been suggested by studies of vertical transmission resulting in neonatal herpes, where passive transmission of neutralizing antibody or antibody titers associated with ADCC have been reported to correlate with protection against disease.[342,343] The risk of neonatal herpes after primary infection is more than 10 times greater than that after recurrent infection, which may be related to the higher titers and longer duration of viral shedding within the genital tract associated with primary infection. Infants born to seronegative mothers with primary HSV infection are at much higher risk for the development of neonatal HSV compared with those born to mothers with recurrent disease (3% versus 30% to 50%).[344] This relatively low occurrence of neonatal HSV compared with those associated with primary maternal infection suggests that the transfer of preexisting HSV-specific antibody at our near the time of birth mediates protection.[345]

Children with agammaglobulinemia, however, do not exhibit an increased susceptibility to primary HSV infection.[346] In immunocompromised patients (such as transplant recipients or persons with acquired immunodeficiency syndrome [AIDS] or leukemia), chronic herpetic lesions are not associated with diminished specific antibodies. In fact, clinical recurrences of HSV often are associated with higher antibody titers in immunocompetent patients than in seropositive asymptomatic control subjects, and antibody titers do not change significantly after dermal recurrences. Nevertheless, it remains a matter of debate whether or not humoral immunity plays a significant part in the prevention of recurrent HSV infection.[347] Either way, antibody certainly may serve an auxiliary role in host defense against herpesvirus infection, and this capability may be especially important when cellular components of the response are deficient, such as in the human neonate. Antibody also has been shown to be important in the prevention of dissemination of viral infections to the CNS. This is quite evident in the case of enteroviral infections, in which echoviruses or coxsackieviruses have been associated with the development of paralytic poliomyelitis or severe chronic encephalitis in agammaglobulinemic or hypogammaglobulinemic patients.[348]

Murine models of HSV support the importance of humoral immunity in controlling HSV infection. Classic murine studies have convincingly demonstrated that both prenatal and postnatal transmission of HSV-specific antibody in neonatal mice will afford protection against severe HSV infection and death.[349] Prenatal transfer of HSV-specific antibody from repeatedly immunized females protects neonatal mice in a dose-dependent manner against HSV-associated mortality. Partial protection is afforded by both transplacental and transmammary transfer of HSV-specific IgG. Such antibody confers protection by both viral neutralization and ADCC.[350,351] In the cutaneous HSV model, mice lacking B cells as a result of selective gene targeting have been reported to be markedly more susceptible to infection and to exhibit impaired T_H1 responses.[352] The finding of decreased T_H1 responses is surprising in view of the normal T cell immunity observed in humans with X-linked agammaglobulinemia. B cell–deficient mice also exhibit more pronounced local inflammation and viremia than that seen in wild-type mice when challenged intravaginally with an attenuated strain.[353] It is interesting

that serum from nonimmune, wild-type mice transferred into B cell–deficient mice was found to decrease vaginal HSV shedding, suggesting that natural antibody may inhibit viral replication.[353] Whether such antibody activity is found in the human circulation is unknown.

Human HSV infection results in the induction of antibodies against a diverse set of proteins, including the surface glycoproteins involved in cell attachment and entry.[354] The production of HSV-specific antibodies by B cells is T cell–dependent and also requires the C3 and C4 complement components, as well as the CD21 complement receptor on B cells,[355] which acts as a co-stimulatory molecule. However, if antibody is passively acquired, as in the case of maternal-to-fetal transfer, humoral immunity may exist independently of T cell immunity. Although enveloped viruses, such as herpesviruses, can be lysed by antibody and complement *in vitro*, this is unlikely to be an important mechanism *in vivo* because of the high concentrations of antibody required.[356] Under relatively physiologic conditions, IgG and alternative pathway complement components can coat the surfaces of many viruses, including HSV, and effectively neutralize their infectivity, possibly by preventing their attachment or fusion with cell membranes.[356,357] Antibodies also can react with viral proteins found on the surface of infected cells. IgG and complement components can lyse human cells infected *in vitro* with a number of different viruses, including HSV and influenza, parainfluenza, measles, and mumps viruses.[358-360] The importance of complement in viral host defense is uncertain, however, because serious viral infection is not a feature of patients with inherited complement deficiencies[361] or of animals made acutely hypocomplementemic.[362]

A potentially more effective system for the elimination of virus-infected cells by specific antibody is by ADCC.[363] In this system, the killer cell is a nonspecific immune effector, such as a mononuclear phagocyte, an NK cell, or a polymorphonuclear neutrophil (PMN), which has surface receptors for IgG. NK cells probably are the most efficient effector cells for ADCC.[364] The specificity of cytotoxicity is due to the specific recognition by antibody of viral antigens present on the infected cell surface. *In vitro* ADCC requires relatively low concentrations of antibody[365] and occurs rapidly (within hours),[207] making it less likely that the virus will have had sufficient time to produce infectious particles. ADCC mediated by all types of effectors is up-regulated by cytokines, such as IFN-γ, type I IFN, IL-12, and IL-15.[366-368] Although *in vitro* ADCC activity correlates (in some cases) with protection against serious viral infections in humans and in animal models, the extent to which this occurs *in vivo* remains uncertain.

Paralleling the reduction in natural cytotoxic activity of neonatal cells, ADCC of neonatal mononuclear cells is approximately 50% of that by adult mononuclear cells, including against HSV-infected targets.[190] Decreased ADCC mediated by purified neonatal NK cells appears to be caused in part by a defect in adherence of NK cells to target cells.[369]

Part of the apparent limited impact of antibody and complement on HSV infection in humans may reflect viral immunoevasion mechanisms that have hampered antibody effector functions. For example, the HSV gC protein is an inhibitor of the complement cascade, especially by way of the alternative pathway,[370] and acts by directly binding C3.[371] The gE-gI heterodimer also binds the Fc portion of antibody molecules and ablates their neutralization activity.[372] It is possible that these or other HSV-encoded inhibitory mechanisms that block viral neutralization may not be as effective in mice as in humans, analogous with ICP47 inhibition of TAP. This might account for the striking differences in the effects of genetic ablation of B cells on HSV infection in humans versus mice.

The immunoglobulin M (IgM) response to most protein antigens in the neonate is intact; IgG responses to certain vaccines, such as hepatitis B surface antigen are only slightly limited. Nevertheless, clear differences are recognized between neonates and older infants in the magnitude of the antibody response to most protein neoantigens, but this response differential rapidly resolves after birth. Abnormalities may be more pronounced in those born prematurely, but, again, it resolves rapidly after the neonatal period. Thus chronologic (i.e., postnatal) age is more of a determinant of antibody responses to T cell–dependent antigens than is gestational age. Isotype expression by B cells after immunization with T cell–dependent antigens is limited by T cell function, such as reduced CD40 ligand production, and intrinsic limitations of B cell maturation and function.[1] These limitations are exaggerated in the fetus.

These limitations in response to T cell–dependent antigens also may apply to neonatal HSV infection. Proceeding from the assumption that neutralizing or ADCC HSV-specific antibody contributes to the control of primary HSV infection in humans, it is possible that limitations in isotype switching from IgM to IgG or a slower rate of somatic hypermutation of IgG responses could contribute to the severity of neonatal infection. The neonate also is unable to produce antibodies in response to T cell–independent type II antigens, such as the capsular polysaccharides of bacteria. This limitation, however, is unlikely to be an important factor in the severity of neonatal HSV infection, because no T cell–independent type II HSV antigen-specific responses by human B cells have been described.

The neonate also is partially protected from infection by passive maternal IgG antibody, transferred predominantly during the latter third of pregnancy. Fetal IgG concentrations are equal to or higher than maternal concentrations after 34 weeks gestation, reflecting active transport mechanisms. In newborn infants with gestational age less than 38 weeks, a greater fraction of this maternally derived HSV-specific IgG may enter into the CNS,[373] reflecting a generally less effective blood-brain barrier. Whether this IgG fraction enters in sufficient amounts to provide protection from CNS disease is unknown. Kohl[16] also has shown that among HSV-infected infants, those with greater concentrations of ADCC antibody had less severe disease. Of note, healthy adults and older children with primary HSV infection, who by definition lack antibody to HSV, do not develop severe disease, as do neonates with primary infection. This observation indicates that the deficits intrinsic to the neonate are the important factors predisposing the neonate to infection. Nevertheless, passively acquired antibody may play a role in decreasing transmission or ameliorating disease severity.

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151 T-Cell Development

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Hematopoetic stem cells (HSCs) give rise to committed progenitors of multiple lineages. Whereas maturation along myeloid, erythroid, and megakaryocytic cell lineages occurs within the bone marrow, differentiation into B lymphocytes initiates in the bone marrow and is completed in the periphery. By contrast, T lymphocyte development occurs within the thymus, where bone marow–derived common lymphoid progenitors migrate. In particular, interaction of lymphoid progenitors with thymic stromal cells permits differentiation of $CD4^-$ and $CD8^+$, double-negative (DN) cells into $CD4^-$ $CD8^+$, double-positive (DP) thymocytes within the thymus cortex. Subsequently, DP cells lose expression of either CD4 or CD8, becoming functionally active single-positive (SP) cells that populate the thymic medulla (Figure 151-1).

Although the thymus is particularly active early in life, it ensures lifelong generation of a pool of newly generated T cells. Of importance, the thymus also plays a critical role in purging self-reactive T lymphocytes. Therefore T cells produced in the thymus carry a seemingly unrestricted antigen receptor repertoire, whose reactivity is largely restricted to foreign antigens and is tolerant to self-antigens.[1]

THE THYMUS AND THE EMBRYOLOGY OF T CELLS

The human thymus originates as an epithelial outgrowth from the third and fourth pharyngeal pouches between the sixth and seventh weeks of gestation. It grows caudally into the mediastinum as two elongated structures that fuse at midlevel around week 8 of gestation.[2] In mice, the epithelial component of the thymus completes migration by day 10 of the 19- to 21-day gestation period, and lymphoid cells appear on day 11.[3] By days 12 to 13, these T lymphocyte precursors are proliferating, and by day 14, their surface phenotype shows that they have committed to the T-cell lineage. Further maturation of these precursors occurs over the following week, resulting in the development of distinct corticomedullary demarcation by the time of birth. The first wave of differentiation from precursors to mature T cells requires approximately 7 days in humans as well.

HSCs are present in the yolk sac during early embryonic life. They subsequently are found in the fetal liver and ultimately locate in the marrow, where they continue to seed the thymus. HSCs have high levels of CD34 on the cell surface, but they lack lineage-specific antigens such as the lymphocyte-specific CD52 molecule and the CD3, CD4, and CD8 molecules, which are restricted in expression primarily to thymocytes and mature T cells. Table 151-1 summarizes the relevant CD antigen designations. The least mature precursor cells in the marrow normally are resting and are triggered to divide by stem cell factor (SCF) and interleukin (IL)-3.[4] These and other cytokines important in T-cell development are listed in Table 151-2.

A family of Ikaros proteins, required for the differentiation of lymphoid precursors[5] in the bone marrow and thymus, regulates chromatin remodeling in preparation for commitment to lymphoid lineages. Subsequently, stromal cell–derived cytokines such as IL-7 and thymic stromal lymphopoietin (TSLP) signal through the IL-7 receptor complex to enhance proliferation of T-cell precursors and promote rearrangement of the T-cell receptor γ-chain locus (see later).[6] Another subpopulation of thymocytes, which does not rearrange T-cell receptor (TCR) genes, differentiates into natural killer cells after the binding of IL-15 to the combined IL-2–IL-15 receptor, signaling by way of the common γ-chain.[7,8] Many of these cytokine signals are transmitted through common pathways involving Janus kinases[9] (particularly JAK1 and JAK3) and their target signal transducers and activators of transcription (STAT3 and STAT5), suggesting that other growth and differentiation factors must modulate the responsiveness of developing precursors to these stereotypical signaling pathways.[10]

A functional thymic epithelium is required for the development of mature T cells.[11] Mice and humans with defects in the gene encoding the transcription factor FOXN1 (forkhead box protein N1), required for thymic epithelial development, have few T cells and an impaired ability to reject grafts.[12] In these circumstances, differentiation of hair follicles is also affected (hence the *Foxn1* mouse mutant also is known as *nude* mouse). Moreover, in humans, defects in thymic development (as observed in patients with DiGeorge syndrome) are associated with a variable degree of T-cell deficiency.[13] Actual cell-cell contact between lymphocytes and epithelial cells (and Notch-mediated signaling in particular) appears to be required for T lineage antigens to be expressed, because soluble factors derived from thymic epithelial cultures are unable to promote continued T-cell development. Functional T cells, however, also are required for inducing and maintaining the epithelial cells characteristic of the thymic cortex and medulla. Without continued contact between epithelium and developing T cells, the thymus may lose the ability to support full T-cell maturation with time.[14,15]

COMMITMENT TO THE T-CELL LINEAGE

Mouse T-cell precursors begin to express adhesion molecules common to hematopoietic lineages 2 to 3 days after their first appearance in the thymus. The adhesion molecule CD18/CD11a (also known as leukocyte function associated antigen-1 [LFA-1]) regulates the entry of mature leukocyte populations into sites of infiammation and enhances T-cell activation after antigen stimulation. LFA-1 is expressed on lymphoid precursors shortly after their entry into the thymus and is likely to mediate intercellular interactions that are required for subsequent steps in thymocyte development.[16] Receptors for growth and differentiation factors common to all hematopoietic precursors found in the bone marrow (e.g., c-Kit [CD117], which binds SCF) are still detectable at this stage as well. Commitment to the lymphoid lineage is demonstrated on a fraction of thymocytes by expression of CD25[17] as well as of the enzyme complex (recombinase) that generates functional lymphocyte antigen receptors (see later). Cells from this subset rapidly and selectively begin to activate and express the T cell–specific antigen receptors. They can efficiently repopulate a recipient thymus with developing T cells in cell transfer experiments. The interactions of IL-2, IL-7, and other growth factors with their receptors on thymocytes at this stage of development are likely to be important for the subsequent differentiation of

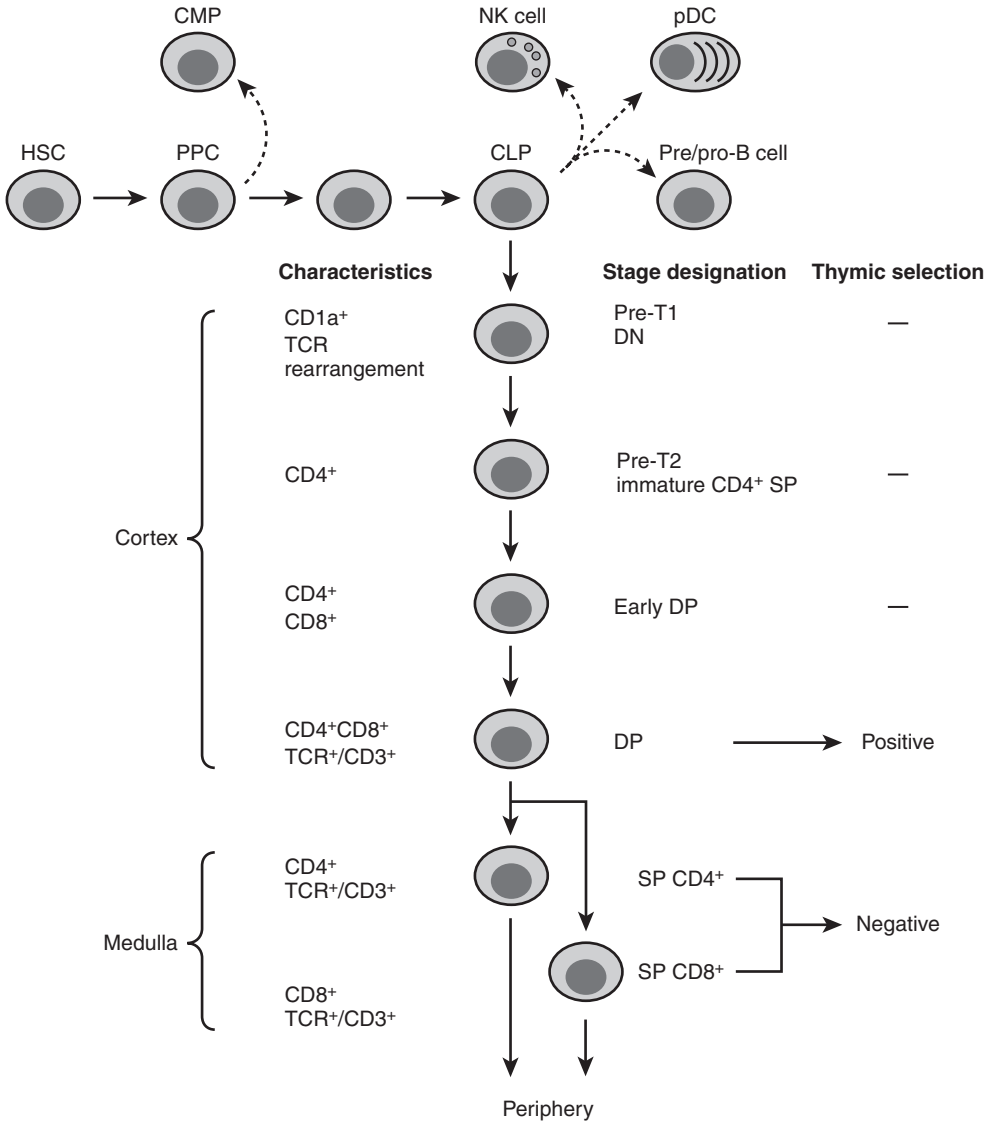


Figure 151-1 T-cell development and selection in the thymus. Hematopoietic stem cells *(HSCs)* with self-renewing capacity give rise to pluripotent progenitor cells *(PPCs)* that may differentiate to common myeloid progenitors *(CMPs)* or common lymphoid progenitors *(CLPs)*. CLPs in turn differentiate into T cell–restricted precursors (indicated in *green*), in addition to giving rise to natural killer *(NK)* cells, plasmacytoid dendritic cells *(pDCs),* and B cells. T cell lineage–restricted cells then pass through pre-T1, pre-T2, early double-positive *(DP),* DP, and single-positive *(SP)* stages in the thymic cortex and medulla. T cells at the pre-T1 differentiation stage express CD1a and initiate T-cell receptor *(TCR)* rearrangement. Next, they express CD4 but not CD8 and are referred to as $CD4^+$ immature cells. Further maturation gives rise to cells expressing both CD4 and CD8 markers, in addition to TCR/CD3. These cells are subjected to positive selection in the thymic cortex. DP thymocytes then differentiate into $CD4^+$ or $CD8^+$ SP cells. After post-selection maturation in the medulla, T cells are exported to the periphery.

mature T cells and may be the target of pharmacologic intervention (as shown by suppression of mature T-cell production by cyclosporine[18]).

Generation of Antigen Receptors

T cells are characterized by their specific cell–surface receptors for antigens (TCRs). Each antigen receptor is composed of two polypeptide chains with two internal disulfide-linked domains. The α-chains generally pair with β-chains, and δ-chains with γ-chains, defining two different populations of T cells in the mature animal.[19] Carboxyl-terminal domains include the peptides spanning the cell membrane. Each has 138 to 179 amino acids of relatively invariant sequence, which includes a hydrophobic transmembrane section and a short cytoplasmic tail. These are known as the constant (C) regions.

In a process called *rearrangement,* selected variable sequences are moved into close proximity to the C region–coding sequences by enzyme-catalyzed excision of the intervening loop of DNA, thereby creating a complete gene sequence for each polypeptide chain. This process is very similar to the rearrangement of immunoglobulin genes that occurs during B-cell development, and is accomplished by activation of a multicomponent recombinase complex that includes recombinase-activating genes 1 and 2 *(RAG1, RAG2)*.[20] The RAG-1 and RAG-2 proteins introduce DNA double-strand breaks that are eventually resolved by means of a

TABLE 151-1

CD Antigens and T-Cell Development

CD Antigen	Common Name	Present on	Function
CD1		Most nucleated hematopoietic cells	Antigen presentation
CD2			Adhesion, co-stimulation of TCR signals
CD3		T cells	TCR-mediated signal transducer
CD4		MHC class II–restricted T cells	Adhesion, co-stimulation of TCR signals
CD7		Most hematopoietic cells; high on immature cells	Co-stimulation of TCR signals
CD8		MHC class I–restricted T cells	Adhesion, co-stimulation of TCR signals
CD11a	LFA-1 α-chain	Most nucleated hematopoietic cells	Adhesion, co-stimulation of TCR signals
CD18	LFA-1 β-chain	Most nucleated hematopoietic cells	Adhesion, co-stimulation of TCR signals
CD25	High-affinity IL-2 receptor	Immature ($CD4^-CD8^-$) thymocytes, activated mature T cells	Supports proliferation, differentiation
CD28		Mature, activated T cells	Co-stimulation of TCR signals
CD34	Sialomucin	Multipotent bone marrow stem cells	Adhesion
CD40		B cells, monocytes, dendritic cells, endothelial cells	B-cell proliferation, Ig class switching
CD45	Leukocyte common antigen	Most hematopoietic cells	Co-stimulation of TCR signals
CD45RA		High levels of expression on naive T cells	Co-stimulation of TCR signals
CD45R0		High levels of expression on memory T cells	Co-stimulation of TCR signals
CD52		Most hematopoietic cells	Unknown
CD80	B7-1	Activated B cells, macrophages	CD28 ligand
CD86	B7-2	Activated B cells, macrophages	CD28 ligand
CD117	c-Kit	T-cell precursors	Proliferation of precursors
CD154	CD40 ligand (CD40L)	Activated T cells	Adhesion, transmission of signal to B cells

LFA-1, Lymphocyte function–associated antigen-1; *MHC,* major histocompatibility complex; *TCR,* T-cell receptor.

TABLE 151-2

Cytokines and T-Cell Development

Cytokine	Common Name	Produced by	Function
IL-1β	Interleukin-1β	Monocytes, macrophages	Enhanced inflammation; thymocyte proliferation
IL-2	Interleukin-2	Activated $CD4^+$ T cells	T-cell proliferation; thymocyte differentiation
IL-3	Interleukin-3	Stromal cells in bone marrow and thymus	TCR-mediated signal transducer
IL-4	Interleukin-4	Activated T_H2 cells	B-cell proliferation and antigen presentation; Ig production; switch to IgE
IL-7	Interleukin-7	Stromal cells: bone marrow, thymus; intestinal epithelium	Expansion of lymphoid precursors
IL-10	Interleukin-10	Activated T_H2 cells	Suppression of macrophage activation; B-cell proliferation
IL-12	Interleukin-12	Activated macrophages	IFN-γ production; macrophage activation
IL-13	Interleukin-13	Activated T_H2 cells, activated $CD8^+$ T cells	Similar to IL-4 with additional proinflammatory signals for fibroblasts and tissue granulocytes
IL-15	Interleukin-15	Many cell types	Differentiation and proliferation of NK cells, $CD8^+$ T cells
IFNγ	γ-Interferon, type II interferon	Activated T cells, NK cells	Enhanced antigen presentation, inflammation
SCF	Stem cell factor	Stromal cells: bone marrow, thymus; intestinal epithelium	Proliferation, differentiation of precursor cells
TGF-β	Transforming growth factor-β	Activated regulatory T cells	Suppression of mature T-cell activation; accelerated T-cell development
TSLP	Thymic stromal lymphopoietin	Stromal cells: thymus	Expansion of T-cell precursors; TCR γ-chain rearrangement

NK, Natural killer; *TCR,* T-cell receptor.

series of proteins belonging to the nonhomologous end-joining and DNA repair pathway. Mutations in the recombinase and DNA repair genes impair cell-mediated immunity by inhibiting productive rearrangements in lymphocyte receptor genes as well as repair of radiation-induced DNA strand breaks.[21]

Recombination of TCR genes, similar to that of immunoglobulin genes, follows a well-regulated sequence in the presence of a normal recombinase complex. In the generation of a functional β-chain, gene sequences coding for variable (V), diversity (D), and joining (J) segments, 18 to 20 amino acids long, are used. In this process, a D region segment is first brought into close proximity to the J region by looping out and deleting much of the intervening DNA. The excised DNA persists as a circle in the cell and is known as a *T-cell receptor excision circle* (TREC). The TREC does not replicate in parallel with nuclear DNA, so counting TRECs in blood T cells gives an indirect estimate of T-cell production from the thymus that also is affected by cell division.[22] Indeed, measurement of TRECs on dried blood spots at birth has been introduced in pilot programs of newborn screening for severe combined immunodeficiency in Wisconsin and

in Massachusetts. The rearrangement process that generates the TREC allows for transcriptional activity at the site as well as a commitment to express 1 of 12 potential J regions. A V segment is then selected and moved closer to the rearranged D-J segment by a similar process. δ-Chains resemble β-chains in having V, D, J, and C segments. α-Chains and γ-chains are somewhat simpler in structure and lack D regions. The rearranged V(D)J segments of TCR chains achieve additional diversity by addition or subtraction of nucleotides at the joints between the V, D, and J segments. This phenomenon appears to result from the properties of the recombinase enzymes acting in conjunction with an enzyme designated terminal deoxytransferase (Tdt) and is known as *N segment diversity.*

The thymocytes of fetal mice rearrange their β-, γ-, and δ-chain genes[18] at approximately 13 to 14 days gestation, and α-chain rearrangement follows 1 to 2 days later. Messenger RNA (mRNA) for α-chains is detectable on days 16 to 17 of gestation, and α-chains are present on the surface of fetal thymocytes on days 17 to 18, so that the appearance of complete γδ heterodimers precedes that of αβ heterodimers by 1 to 2 days. Expression of a rearranged β-chain suppresses further rearrangements of β-chain loci by means of signaling through a receptor-related tyrosine kinase, $p56^{lck}$, in a process known as *allelic exclusion.*[23] α-, γ-, and δ-chain gene rearrangements also are largely suppressed after the binding of mature TCR to MHC molecules on the surface of thymic macrophages or epithelial cells. However, a subset of $CD8^+$ cells appears to rearrange both α-chain loci, creating a small population of potentially bispecific cells.

When either pair of receptor genes (αβ or γδ) has been rearranged and transcribed, it is associated on the cell surface with the five polypeptides of the CD3 complex. The cytoplasmic tail of each TCR chain is small, and intracellular signaling leading to the activation of T-cell signaling cascades is actually mediated through the CD3 complex.[24]

Extrathymic T-Cell Development

Most lymphocytes carrying αβ or γδ antigen-specific receptors develop in the thymus and are exported to peripheral lymphoid organs. However, a small number of lymphocytes carrying T-cell surface markers may develop extrathymically. In particular, lymphoid cells may appear within the epithelium of the small and large intestine in the absence of a functioning thymus. Intraepithelial lymphocytes (IELs) in normal adult individuals are composed of both αβ and γδ $CD3^+$ cells. In contrast with their representation in the thymus, γδ cells are more frequent than αβ cells in the intestinal epithelium. Precursors for the γδ subset appear to begin differentiating within lymphoid aggregates associated with epithelial crypts in the small intestine. Cells in these *cryptopatches* express the recombinase-activating genes RAG-1 and RAG-2, as well as the c-Kit ligand and the IL-7 receptor.[25] Loss of these early-lineage determinants is coincident with expression of TCR and CD3 signaling molecules on lymphoid cells in these aggregates, suggesting that active T cell–like differentiation is occurring in situ. These cells can develop in athymic nude mice, albeit more slowly than in immunologically normal animals, suggesting that thymus-derived factors are not absolutely required for local development of T-like lymphocytes in the gut.

Intestinal immune responses are critical as a first line of defense against intestinal flora and have also been shown to be important in generating tolerance to ubiquitous environmental antigens. However, the importance of local extrathymic T-cell development in performing these functions is unknown. γδ Subsets found in other epithelial compartments (skin, alveolar and bronchial epithelium) seem to be dependent on thymic output, and evidence is lacking for significant extrathymic differentiation of the αβ subset.

CONTROL OF THE T-CELL REPERTOIRE FOR ANTIGEN RECOGNITION

Role of T Cell Receptor–Major Histocompatibility Complex Interactions in T-Cell Development

B and T cells, despite the similarities in the mechanisms of assembly of their antigen-specific receptors, interact with antigen in different ways. In generating a response from B cells, immunoglobulin carried on the B-cell surface binds directly to antigen, whether this is free in blood or tissues or expressed at the surface of a cell or microbe. By contrast, the TCR recognizes antigen in the groove of self major histocompatibility complex (MHC) molecules. The random selection of variable regions and the N region diversification described previously generate a diverse repertoire of antigen specificities. Estimates of the potential diversity of TCRs that can be achieved by the processes of V(D)J recombination and N region diversification exceed 10^{15} for both αβ and γδ heterodimers.[27] This repertoire is subjected to important selection steps in the thymus. Although the pool of T cells in an animal must have broad diversity to recognize a wide array of foreign antigens, for efficiency it must also be limited to those cells bearing TCR capable of binding to self-MHC.

Positive selection has been shown to occur through TCR interactions with MHC molecules expressed on thymic cortical epithelial cells during the $CD4^+CD8^+$ (DP) stage in thymocyte maturation; the large numbers of developing thymocytes that do not bind self-MHC die in the thymus. TCR-MHC interactions that exceed a threshold level, however, result in additional cell death. This negative selection step is the first level of protection against autoimmunity. Negative selection can be initiated by thymocyte contact with virtually any MHC^+ cell in the thymus, suggesting that positive and negative selection may be distinguished not only by the affinity of the TCR-MHC interaction but also by the requirement for epithelium-derived signals in positive selection.[28] The display of self-antigens by thymic epithelial cells is key to inducing tolerance of the T-cell compartment, a process enhanced by the autoimmune regulator (AIRE) gene, which is expressed by a subset of terminally differentiated medullary thymic epithelial cells (mTECs).[29] AIRE, as a transcriptional regulator of tolerance induction, promotes central tolerance by inducing the ectopic expression of a large number of self-antigens by mTECs. This mechanism permits that mTECs (which constitutively express MHC class II molecules) may present self-antigens to nascent thymocytes. Recognition of these antigens by self-reactive thymocytes leads to clonal deletion, thus representing a key mechanism of central tolerance to prevent autoimmunity.[30] Mutations of *AIRE* cause autoimmune polyendocrine syndrome type I, also known as autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED).

Expression of Other T Lineage–Specific Molecules

Although antigen specificity and activation signals are determined by the TCR complex, the CD4 and CD8 co-receptors on T cells play a significant role in thymocyte differentiation as well as in the postthymic activation of mature T cells. These molecules bind to MHC molecules at sites distant from binding sites for the T-cell antigen receptor. CD4 molecules bind to MHC class II molecules (HLA-DP, -DQ, and -DR in humans), which are expressed primarily on cells of hematopoietic origin and participate in generating an immune response. CD8 molecules bind to the MHC

class I molecules found on nearly every nucleated cell in the body (HLA-A, -B, and -C). The cells that first arrive in the thymus are negative for CD4 and CD8 cell-surface antigens. Expression of CD4 and CD8 molecules during thymocyte development is finely regulated. Maturation of early T-cell progenitors (which are CD4–CD8– [DN]) is marked by sequential expression of both CD4 and CD8, along with appearance of a functional TCR,[31] thereby enhancing the likelihood of TCR-MHC interactions during the critical steps of positive selection.

As thymocytes mature, expression of the CD4 molecules and that of CD8 molecules generally become mutually exclusive. CD4 and CD8 molecules potentiate TCR-mediated intracellular signaling because they are associated with the intracytoplasmic protein tyrosine kinase $p56^{lck}$. The switch from $CD4^-CD8^-$ cells to $CD4^+CD8^+$ thymocytes probably occurs by way of an immature thymocyte, which has low levels of surface CD3,[32] and a signaling molecule composed of the rearranged TCR β-chain and an invariant α-chain known as *pre-Tα,* which supports proliferation of T-cell precursors that have successfully rearranged a β-chain allele. In mice, this complex is first detectable at 16 days gestation. $CD4^+CD8^+$ cells first appear 1 day later, and in vivo labeling studies indicate that CD25 is lost immediately before CD4 and CD8 are expressed. Commitment to the CD8 pathway appears to require a specific signal, presumably mediated by binding to a class I MHC molecule. If this signal cannot be transduced, as, for example, in children who lack the Zap70 kinase, no CD8 cells appear in the blood.[33] $CD4^+CD8^+$ DP cells that survive positive and negative selection and that do not become $CD8^+$ cells appear to enter a default pathway, becoming $CD4^+$ SP cells in the blood.[34]

OBSERVATIONS IN HUMANS

T-Cell Phenotypes and Antigen Receptors

T-cell development in the human thymus starts at approximately 7 weeks of gestation[35] with the entry of precursors with the leukocyte antigens CD7 and CD45 on their surface. These cells mature to express first the CD2[36] co-stimulatory molecule and then, around 8.5 weeks gestation, CD3. By 9.5 weeks, 32% of the $CD3^+$ cells have the β-chain of the TCR in the cytoplasm, and at 10 weeks, many of the cells have α-chain in addition to β-chain in the cytoplasm. The proportion of cells expressing the δ-chain of the γδ TCR is highest at 9.5 weeks gestation (11%) and rapidly diminishes (to 4% at 10 weeks and 1% at 12 weeks). CD1a, CD4, and CD8 appear on lymphocytes in the thymus by 10 weeks gestation, and the high-density CD3 phenotype (associated with mature antigen receptor expression) appears on these cells at approximately 12 weeks. A population of immature $CD7^+$ cells with potential for multiple differentiation pathways is present in cord blood.[37]

The biochemistry of the human T cell–antigen receptor resembles that of mice. The αβ heterodimer has a molecular mass of approximately 90 kDa. The α- and δ-chain genes are on chromosome 14, and the β-chain genes are on chromosome 7. The complete αβ or γδ heterodimer is expressed on the T-cell surface in association with five invariant chains, which constitute the CD3 complex.

TABLE 151-3

Summary of T Lymphopoiesis in Human Fetuses

Age (weeks)	Developmental Step
7	Precursors in fetal liver
10	Thymus becomes lymphoid
12	Hassall's corpuscles in thymus
12+	T cells appear in blood

Some estimate of the time and rate of T-cell export from the human fetal thymus comes from measurement of T cells in the blood and spleen. Occasional $CD3^+$ cells are present in blood from the end of week 12 of gestation, their proportion rising from 20% to 30% at 14 weeks to 50% or more by 22 weeks (Table 151-3). Studies suggest that the range of VDJ recombinations that can be made before 14 weeks gestation is limited.[38,39] The mechanism for limited diversity during fetal life may relate to a limited use of N region diversification, secondary to low levels of the TdT enzyme in the thymus. It is interesting that the diversity of the fetal B-cell repertoire also limited, although a selective preference for certain VH genes appears to be the explanation in this case.[40] Because the T-cell diversity is limited, the fetus may have much less ability to respond to antigens (or to reject foreign grafts) than might be predicted from the presence of CD3 cells in the blood. Much remains to be learned about the range of TCR gene recombinations that can be made at different gestational ages.[41]

Of fetal $CD3^+$ cells in blood and spleen, 90% to 96% use αβ heterodimers, and the proportion of γδ receptor-positive cells is between 2% and 8% at 20 to 40 weeks of gestation; this subunit distribution is similar to that found in adults.[42] Fetal T cells resemble adult lymphocytes in expressing either CD4 or CD8 but not both. T cells also appear in the intestinal epithelium and lamina propria by 14 weeks gestation.[43] Cells carrying γδ TCRs populate the epithelium more slowly than do αβ TCR^+ cells and may arise in part through local differentiation, rather than exclusively from thymic sources. Gut-associated γδ lymphocytes increase rapidly in the fed neonate,[44] suggesting that local antigenic stimulation may be responsible for the expansion of this population.

By analogy with studies in animals, it seems likely that T cells accumulate in the blood and lymph nodes of fetuses during the second half of gestation, principally as a result of thymic emigration. This view is supported by studies of CD45 isoforms, which distinguish between naive and memory T cells. More than 99% of the lymphocytes in the cord blood of neonates express high-molecular-weight isoforms of CD45 ($CD45RA^+$ and $CD45RB^+$),[45] in association with the low levels of LFA-1 (CD11a/CD18) and other integrins; this expression pattern is characteristic of the naive T-cell phenotype (Table 151-4). When stimulated, naive cells of newborn infants switch to express the $CD45R0^-$ isoform of CD45. This isoform is present on the surface of adult circulating T cells that respond to recall antigens. The low number of $CD45R0^-$ cells in the blood of healthy term neonates suggests that little antigen-driven expansion of T cells occurs before birth. The minor population of memory phenotype cells that does appear during fetal life may be related to autoantigen stimuli (see later).

TABLE 151-4

Summary of Differences Between Naive and Memory T Cells

Characteristic	Naive T Cells	Memory T Cells
Definition	Cell that has not yet been triggered by antigen	Product of antigen-driven clonal expansion
CD45 phenotype	$CD45RA^+$, $CD45RB^+$, $CD45R0^-$	$CD45R0^+$
Other cell surface antigens	$LFA\text{-}1^{low}$	$CD2^{high}$, $LFA\text{-}1^{high}$, $LFA\text{-}3^{high}$
Cytokine response to stimulation	IL-2	IL-2, IL-4, IL-10, IL-12, γ-IFN
CD40L response to stimulation	Low	High

CD40L, CD40 ligand; *IFN,* interferon; *IL,* interleukin; *LFA,* leukocyte function–associated antigen.

Functional Responses of Fetal T Cells

In vitro tests indicate that several components of a T-cell response (proliferation to mitogens or to alloantigen in mixed lymphocyte culture, IL-2 production, and the expression of IL-2 receptors) exist from approximately 12 weeks gestation. In addition to these ligand-driven proliferative responses, T cells of neonates also can be maintained in culture using a combination of IL-4 and IL-2, or IL-4 and IL-12.[46] Evidence that fetal T cells can make antigen-specific responses comes from their proliferation in mixed lymphocyte culture, together with the generation of antigen-specific cytotoxic cells.[47] Other stimuli, such as *Staphylococcus* enterotoxins and anti-TCR antibodies, tend to elicit lesser responses by neonatal than by adult cells.[48] This difference is accounted for, at least in part, by the lower proportion of CD45R0 cells in neonates than in adults.[49]

The maturation of naive cells to the memory cell-phenotype most likely follows encounter with antigen presented on a specialized antigen-presenting cell, such as B lymphocytes or dendritic cells (DCs) in the lymph nodes. Several studies suggest that naive T cells that encounter antigen in the absence of co-stimulation enter a pathway that leads to unresponsiveness, also known as *anergy,* which may be temporary or permanent.[50] A polyclonal population of $CD4^+$ and $CD8^+$ T cells with a memory ($CD45R0^+$) phenotype makes up 25% of human fetal spleen cells; these T cells express high-affinity IL-2 receptors and proliferate in response to exogenous IL-2 but not to anti-CD2 or anti-CD3 antibodies.[51] Some of these cells may be autoreactive T cells destined to become unresponsive. Finally, umbilical cord blood contains a population of T cells with reduced in vivo responsiveness following ligation of cell surface CD47.[52]

The production of soluble mediators, or cytokines, by T cells that respond to antigen stimulation is essential for host defense.[53] Naive and memory T cells make similar amounts of IL-2, which is required for T-cell proliferation, whereas other cytokines, including IL-4, IL-10, IL-12 and γ-interferon (γ-IFN), are made largely by memory T cells. Consequently, T cells of newborns make little γ-IFN or IL-4 unless they first undergo several cycles of proliferation and maturation[54] or are cultured with stimulation that persists for several days.[55] These differences are likely to be important because T cells show functional specialization regarding their predominant cytokine production.[56] The cells making IL-4 and IL-10 are described as type 2 helper T (T_H2) cells. These cells have an essential role in promoting B-cell replication and increasing the production of immunoglobulin E (IgE) isotype antibodies. T_H1 cells make mainly γ-IFN, and they promote local inflammatory responses, as well as stimulating B cells to make IgG. A third class of T_H0 cells can make both γ-IFN and IL-4. The specialization of cells into a T_H1 or T_H2 phenotype from T_H0 cells is determined in part by the cytokine environment in which stimulation occurs. The presence of γ-IFN, for example, biases responses toward a T_H1 phenotype, whereas IL-4 tends to suppress γ-IFN production and to promote differentiation toward a T_H2 phenotype. Human newborn naive cells can mature into those making IL-4 apparently in the absence of preexisting IL-4.[57] IL-13, an important cytokine in the T_H2 pathway, is produced by newborns' $CD8^+$ T cells even when production of IL-4, IL-10, and γ-IFN remains low.[58] Different specificities of cytokine receptors on the surface of T_H1 and T_H2 cells cause them to have different homing patterns in intact animals. Responses to immunization can be markedly altered by deliberate manipulation of the cytokine milieu in which immunizing antigens are presented to the immune system. This strategy may be useful in the design of future vaccines.[59]

The extent of signaling through the TCR-CD3 complex can be modified also by simultaneous interactions of receptor-ligand pairs of *co-stimulatory molecules* on the surface of T cells and antigen-presenting cells. Interaction of CD28 molecules on the T-cell surface with its ligands B7-1 and B7-2 on B cells, macrophages, and dendritic cells markedly enhances the proliferation and cytokine production of "naive" T cells in response to specific antigen-MHC complexes. Other surface molecules with co-stimulatory function include LFA-1, the inducible costimulator ICOS (another CD28/B7 family member), and tumor necrosis factor (TNF) family members such as OX40. These membrane-bound receptors carry amino acid residues in their cytoplasmic tails that allow recruitment of activating kinases that can amplify intracellular signaling. On the other hand, other members of the CD28 family of molecules, such as CTLA-4, contain intracytoplasmic inhibitory sequences that can activate a series of intracellular phosphatases with opposite function. CTLA-4 also can bind B7-1 and B7-2 molecules on the surface of antigen-presenting cells, and its affinity for these molecules is higher than that of CD28; accordingly, interaction of CTLA with B7-1 (or with B7-2) actively suppresses intracellular TCR signaling. The balance of positive and negative *co-stimulation* shapes the final T-cell repertoire by regulating both clonal expansion and differential cytokine production of the responding T cells. Interruption of these co-stimulatory and regulatory interactions can markedly alter the development of antigen-specific T-cell responses. The effectiveness of blocking CD28 interactions with B7-1 and B7-2 in limiting or reversing rejection of allogeneic tissue grafts is currently being explored.[60]

Postnatal Development of Memory T Cells

Approximately 70% of naive T cells—the predominant population in human newborn infants—contain TRECs that identify recent thymic emigrants.[61] Recirculation of naive T cells through the specialized venules of lymph nodes and other lymphoid tissues allows them to migrate to the T cell–dependent areas of lymph nodes. T cells that do not encounter antigen leave the node in the efferent lymph and return to the bloodstream through the thoracic duct. By contrast, naive T cells, whose surface antigen receptors are cross-linked by antigen in the context of self-MHC molecules, enter a response cycle that is accompanied by expression of a surface receptor for IL-2. In the presence of a co-stimulator signal (such as binding of B7-1 or B7-2 to CD28), the T cells themselves release IL-2. This autocrine loop permits cell division of IL-2–dependent T cells for as long as the antigen stimulus persists. The development of a useful repertoire of T cells in the circulation is therefore best thought of as a consequence of antigenic experience stimulating T-cell proliferation. Specialized DCs play a key role in capturing antigens in tissues and at body surfaces and in transporting them through lymphatics to the draining lymph nodes. The migration of DCs is regulated by chemokine gradients (particularly that for CCR7), and their handling of antigen is affected by receptors for bacterial products such as lipopolysaccharides and certain unmethylated dinucleotides.[62] DCs are likely to contribute to the specialization of T cells along T_H1 or T_H2 pathways (described earlier). B lymphocytes also can present antigens, albeit at low concentrations, and hence may participate in T-cell activation.

The response of naive T cells to antigen stimulation depends largely on the co-stimulatory environment.[63] Stimulation results in an increase in the proportion of $CD45R0^+$ cells from less than 5% at birth to 35% to 45% by age 16 years. This increase occurs mainly during the first years of life,[49] and one attribute of memory cells, the production of γ-IFN, is mature around the age of 2 years. The expansion of populations with defined specificities has been documented for only a few antigens. For example, the frequency of lymphocytes in blood responding to varicella-zoster virus (VZV) antigen is less than 1 in 10^6 before immunization or infection.[64] After a varicella infection, VZV-responsive cells do not appear in the blood for approximately 10 days. At 1 month into convalescence, the responder-cell frequency is

approximately 1:20,000 cells, rising to 1:10,000 after 3 months.[65] VZV is latent and responder-cell frequencies in the range of 1:10,000 to 1:20,000 are maintained through the age of 50 years, when they start to decline. The frequency of T cells with specificity for a nonreplicating antigen, tetanus toxoid, fluctuates directly in relationship to immunization. Before infants receive tetanus toxoid, they have fewer than $1:10^5$ cells that respond to this antigen. Six weeks after the first immunization, the responder-cell frequency is still less than 1:40,000. With completion of the course of three injections by the age of 6 months, the frequency of responder cells increases to 1:10,000 cells, and this falls to less than 1:40,000 one year later.

Novel Subsets of CD4+ T Cells

A recently described effector population of CD4 cells is defined as T_H17 T cells, because they secrete several IL-17 cytokines. The generation of these T-cell subsets requires IL-6 and IL-1 in humans, and differentiation is amplified by IL-21 and IL-23.[66] Severe depletion of T_H17 cells was found in patients with STAT-3 deficiency and clinical manifestations of autosomal dominant and sporadic hyper-IgE syndrome.[67]

Negative selection in the thymic medulla delete many self-reactive T lymphocyte as potential sources of autoimmunity. However, self-reactive T cells slipping past negative selection require a different strategy to restore their naive status. This strategy involves $CD4^+CD25^+$ regulatory T cells (T_{reg} cells), which express the transcription factor Foxp3. Scurfy mice lack detectable T_{reg} function as a consequence of mutations of the *Foxp3* gene.[68] In humans, immune dysregulation–polyendocrinopathy–enteropathy X-linked (IPEX) syndrome is a primary immunodeficiency disease characterized by defective Treg function secondary to mutation in the *FOXP3* gene.[69]

IL-22–producing helper T cells (T_H22 cells) have been the latest addition to the $CD4^+$ T lymphocyte subsets.[70] T_H22 cells are characterized not only by producing IL-22 but also by their propensity to express skin-homing chemokine receptors.

HELP FOR B LYMPHOCYTES

B cells, which that bind antigen through their surface immunoglobulin receptors enter a pathway that leads either to self-destruction by apoptosis[71] or, if a T-cell signal is received, to proliferation and differentiation. The T-cell signal for B-cell proliferation is supplied by cytokines and is delivered mostly at the T cell–B cell interface in lymph nodes. T cells control the switch from IgM to IgG (isotype switching) as well as proliferation. The switch signal is supplied by binding a 39,000-kDa glycoprotein, termed CD40 ligand (CD40L), to the CD40 receptor, which is constitutively expressed on the B-cell surface. Resting memory T cells express CD40L within a few hours of a response to antigen, whereas naive T cells take longer to become CD40L-positive. In vitro experiments suggest that delay in CD40L expression on naive T cells is an important factor limiting the ability of human newborn infants to make antibody responses.[72] T_H1 cells help certain IgG responses, and IL-4 from T_H2-type cells promotes IgA and IgE responses. As noted previously, T_H2 cells are most efficiently generated in an environment already containing IL-4 and IL-13. IL-21 is a very important T cell–derived cytokine that is secreted by helper T cells in the lymph node follicles (the so-called follicular helper T cells) and promotes B-cell maturation and immunoglobulin secretion.

SUMMARY

T-cell development occurs in multiple discrete steps, beginning with lineage commitment by hematopoietic stem cells (HSCs) in the specialized environment of the thymus. Progression through the developmental pathway in the thymus is controlled by soluble cytokine growth factors and cellular interactions between the maturing thymocytes and the local stromal-cell compartment. The repertoire of antigen-specific TCRs expressed on mature thymocytes before export to the periphery results from both positive and negative selection of receptor specificities generated by random association of variable regions for TCR heterodimers and the addition of N region diversity through random nucleotide deletions or additions during the recombination events. After export from the thymus, the mature T-cell pool is further shaped by antigen exposure and the local production of cytokines during initial activation and clonal expansion in lymph nodes, spleen, and epithelial structures such as skin and intestine. Although mature T cells are present in significant numbers by midgestation in humans, a fully mature T-cell population develops slowly after birth in response to antigen exposure.

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152 B-Cell Development

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B-CELL DEVELOPMENT: OVERVIEW

Mammalian B-cell development represents a series of sequential developmental transitions that culminates in the establishment of an antibody repertoire essential for host immunity. Mutations in genes critical for the development and function of B cells can result in immune deficiency. This chapter focuses on the developmental biology of human B-lineage cells and perturbations in development that give rise to several antibody deficiency states.

SITES OF HUMAN B-CELL DEVELOPMENT

Like all blood cells that appear in the periphery, B cells (also called B lymphocytes) are derived from hematopoietic stem cells. The liver and omentum are the initial sites of B lymphopoiesis in the human fetus, where a detectable population of cytoplasmic μ heavy chain+ pre-B cells first appears at approximately 8 weeks gestation.[1,2] This population differentiates into immature surface immunoglobulin (Ig) M+ (IgM) cells by 8 to 10 weeks gestation, constituting the first wave of B-cell development.[1,3] Between 12 to 15 weeks gestation hematopoietic stem cells begin to home to the marrow, at which time multiple stages of B-lineage cells emerge.[4,5] Human B-cell development continues in the marrow from the midtrimester of fetal development through the eighth decade of life.[6,7]

STAGES OF MARROW B-CELL DEVELOPMENT

The stages of mammalian B-cell development have historically been defined by the rearrangement and expression of Ig gene segments. However, because detection of Ig rearrangements requires destruction of the cell, monoclonal antibodies recognizing cell surface antigens have been useful to define the developmental stages of intact B-lineage cells.[8]

Lymphoid cells committed to the B-lineage are derived from common lymphoid progenitors that develop from hematopoietic stem cells. Common lymphoid progenitors are a heterogeneous population with the potential to develop into B, T, natural killer, or lymphoid dendritic cells.[9-11] Initial rearrangements between diversity (D) and joining (J) gene segments occur in CD34+/CD10+/CD19− common lymphoid progenitors and CD34+/CD10+/CD22+/CD19− precursor-progenitor-B (pre-pro-B) cells.[12,13] Pre-pro-B cells with D to J rearrangements on both alleles then undergo further differentiation by completing variable (V) to DJ rearrangements and expressing the B lineage-specific cell surface antigen CD19. At this stage CD34 and CD10 expression is sustained and the resulting CD34+/CD10+/CD19+ surface phenotype defines progenitor-B (pro-B) cells. Pro-B cells that fail to produce functional VDJ rearrangements undergo apoptosis and are eliminated from further development. If the VDJ rearrangement on either allele is in-frame (i.e., encodes a μ heavy chain protein), pro-B cells then differentiate into large CD34−/CD10+/CD19+ precursor-B (pre-B) cells, defined by transient cell surface and constitutive cytoplasmic expression of the rearranged μ heavy chain. Cell surface μ heavy chain pairs with the surrogate light chain (a heterodimer of two proteins originally named VpreB and λ5 in the mouse), and the complex is referred to as the pre-B cell receptor (pre-BCR).[14] Pre-BCR expression is required for normal human B-cell development. Those pre-B cells that express cell surface pre-BCR undergo substantial proliferation (estimated to be four to six cell divisions), thereby expanding the number of cells with functional VDJ rearrangements.[15] Large pre-B cells eventually cease expression of the surrogate light chain and exit the cell cycle. At this stage they are designated small pre-B cells and initiate V to J rearrangement at the κ light chain (LC) locus. If the small pre-B cell makes a functional VJ κ rearrangement it expresses cell surface μ/κ Ig. If rearrangement of both κ alleles is out of frame, the pre-B cell can undergo rearrangement at the λ LC locus. Immature B cells harboring functional LC rearrangements express the κ or λ LC in complex with μ heavy chain on the cell surface as the B-cell receptor (BCR). Immature B cells express high levels of several B-lineage restricted cell surface markers, including CD20, CD21, CD22, and CD40.[8]

In addition to sequential rearrangement of Ig heavy chain and LC genes, an ordered expression of genes that mediate recombination and diversification of the Ig loci occurs. Recombination activating genes are required for initiation of Ig rearrangement, and both are involved in generation of DNA strand breaks at recognition signal sequences 5′ or 3′ of Ig gene segments.[15] RAG expression initially occurs in common lymphoid progenitor, is down-regulated in large pre-B cells following functional heavy chain rearrangements, and re-expressed at the small pre-B cell stage during LC rearrangement.[16] The expression of an enzyme that contributes to repertoire diversification, terminal deoxynucleotidyl transferase, is expressed in early B-cell development. Terminal deoxynucleotidyl transferase is a DNA polymerase that adds single nucleotides in a template independent manner to the 3′ overhangs within the CDR3 domain of functional VDJ rearrangements.[17]

A summary of the patterns of gene expression unique to individual stages of B-cell development is shown in Figure 152-1.

POSTMARROW B-CELL DEVELOPMENT

Coincident with or immediately following egress from the marrow, immature B cells co-express cell surface IgM and IgD. Cells at this stage are also designated transitional B cells to reflect the fact that they are transiting between the marrow and secondary lymphoid organs, such as spleen and lymph node. They can be distinguished from the bulk of naïve B cells in peripheral blood by differential expression of a variety of cell surface molecules.[18] *Naïve B cell* is the term applied to those cells that have not yet been activated following contact with foreign antigen. Naïve B cells circulate in peripheral blood or reside in primary follicles in lymph nodes to form the naïve follicular B-cell compartment.

Antigen induced activation of B-cell differentiation in the lymph node encompasses a series of developmental transitions collectively designated the germinal center (GC) reaction.[19,20] These transitions include clonal expansion, class switch recombination (CSR) at the IgH locus, somatic hypermutation of VH genes, and affinity maturation. The GC reaction is initiated when naïve B cells are triggered by antigen-driven BCR cross-linking in the presence of co-stimulatory signals from helper T cells. Naïve

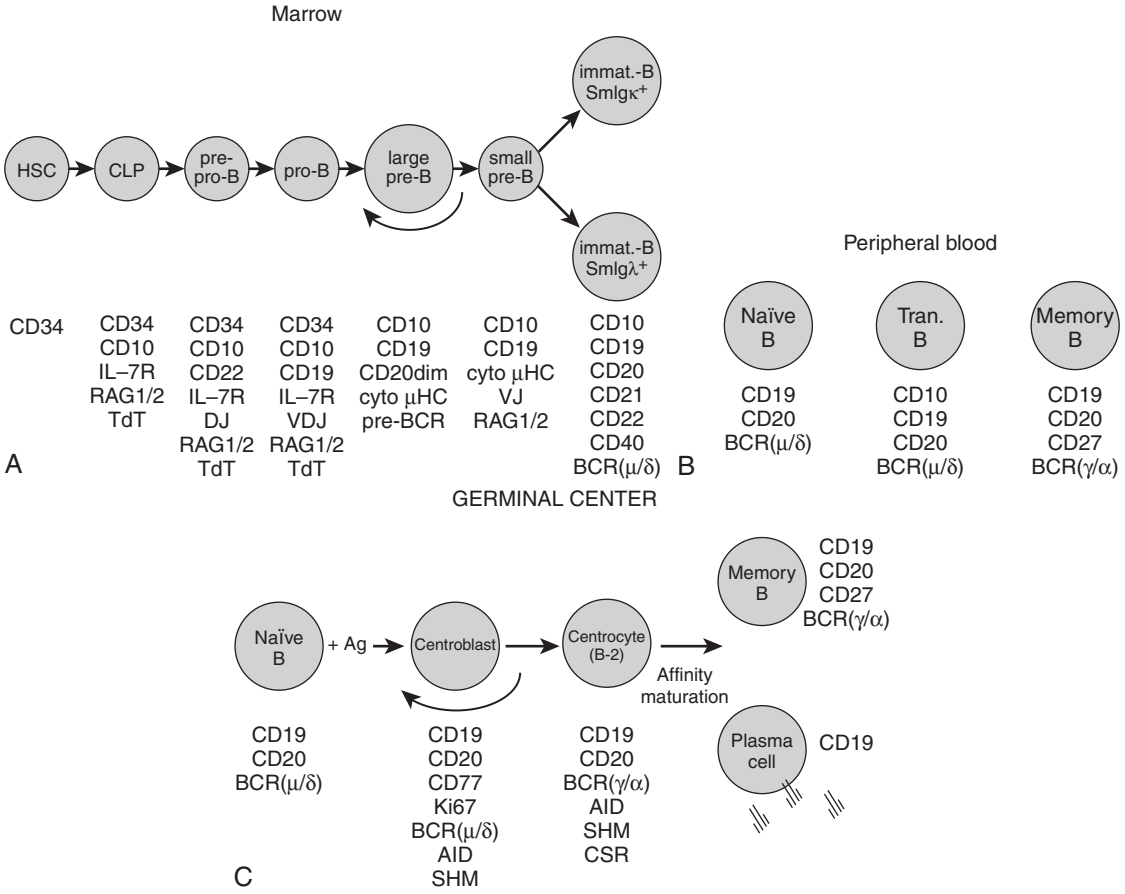


Figure 152-1 Developmental stages of human B cells. Expression of gene products that play a critical role in each B-lineage developmental stage, and/or are useful in characterizing each stage by flow cytometry are shown. **A, B-cell development in the marrow.** Commitment to the lymphoid lineage begins when hematopoietic stem cells *(HSC)* differentiate into the common lymphoid progenitor *(CLP)*. Commitment to the B-lineage is initiated when the IgH locus undergoes D to J *(pre-pro-B)* and then V to DJ *(pro-B)* rearrangement. Large pre-B cells that have successfully rearranged the IgH locus express the μ heavy chain protein paired with the surrogate light chain as the pre-B cell receptor *(pre-BCR)*. Large pre-B cells undergo several rounds of cell division *(curved arrow).* Rearrangement of the immunoglobulin light chain at either the κ or λ loci occurs in small pre-B cells, which then express successfully rearranged IgH and IgL genes as the BCR and become immature B cells. Most immature B cells express IgM and IgD. B-lineage cells that fail to make successful (i.e., functional) IgH and IgL rearrangements undergo apoptosis in the marrow. **B, Peripheral blood B cells.** After egressing from the marrow, IgM+/IgD+ cells circulate in the blood and/or migrate to secondary lymphoid organs such as spleen and lymph nodes. A small fraction of IgM+/IgD+ cells leaving the marrow have been characterized as the putative counterpart of mouse transitional B cells; a population of cells destined to migrate to the spleen and form the marginal zone B-cell compartment. These transitional B cells are rare (<2% of total peripheral blood B cells), and can be distinguished from the naïve and memory B-cell populations by expression of CD10. Memory B cells are identified by CD27 expression, frequently express IgG or IgA, and circulate in the peripheral blood following development in the germinal center reaction. **C, The germinal center reaction.** Naïve B cells encounter antigen in secondary lymphoid organs and, in cooperation with signals from CD4+ helper T cells, up-regulate markers of proliferation (e.g., Ki67) and the cellular machinery (e.g., activation induced cytidine deaminase) necessary for induction of somatic hypermutation. This highly proliferative *(curved arrow)* B-cell population is designated centroblasts. Centroblasts differentiate into centrocytes, which undergo class switch recombination and affinity maturation. During this transition B cells with low-affinity antigen receptors are deleted and those with high affinity receptors further differentiate into either CD27+ memory B cells or antibody-secreting plasma cells. The latter down-regulate all major surface markers with the exception of CD19 and home to the marrow.

B cells undergo robust proliferative expansion and at this stage are referred to as *centroblasts.* Populations of centroblasts can be morphologically identified as the so-called "dark zone" of the GC. Centroblasts are the stage of B-cell development that undergo somatic hypermutation in VH genes (see later discussion). After exiting the cell cycle, centroblasts differentiate into centrocytes, forming the so-called "light zone" of the GC. Multiparameter fluorescence activated cell sorting of tonsillar B-cell subpopulations and subsequent sequencing of VDJ rearrangements was used to demonstrate that somatic hypermutation is initiated in centroblasts.[21] Centroblasts can be distinguished from centrocytes by the expression of CD77 and Ki67 in the former but not the latter.[22] Centrocytes (also called B-2 cells) are highly enriched for mutated VH genes and undergo selection by virtue of binding to cognate antigen presented by follicular dendritic cells and T cells. The affinity of the interaction dictates whether centrocytes will undergo apoptosis (low affinity interaction) or be selected for further differentiation (high affinity interaction) into memory B cells or plasma cells. Some centrocytes also undergo CSR and shift from expression of cell surface IgM and IgD to IgG or IgA.

Multiparameter fluorescence activated cell sorting of peripheral blood B cells has also been used to identify distinct populations of naïve and memory B cells.[22] Expression of the tumor necrosis factor receptor family member CD27 correlates with the presence of somatically hypermutated VH genes. When coupled with analysis of Ig isotype expression, it is now possible to identify four separate CD27+ memory B-cell populations: IgM+/IgD+, IgM+/IgD−, IgM−/IgD+ and IgG+/IgA+. Likewise, CD27− naïve B cells comprise two separate populations: IgM+/IgD+/CD5+ B-1 cells (see below) and IgM+/IgD+/CD5− cells. The collective memory and naïve B-cell populations represent approximately 40% and 60% of peripheral blood B cells, respectively. However, not all memory B cells (i.e., cells with somatic hypermutation) express CD27.[23]

A distinct population of B cells with attributes of both naïve and memory cells are the splenic marginal zone B cells.[24,25] Marginal zone B cells are a first line of defense against encapsulated bacteria in rodents and primates, but there is uncertainty regarding the stage of development and function of marginal zone B cells in humans. This partially reflects the fact that the microscopic anatomy of the human splenic marginal zone differs substantially from rodents.[25] CDR3 spectratyping and gene expression analysis were used to demonstrate that the circulating IgM+/IgD+/CD27+ memory B-cell population is destined to migrate to the spleen to form marginal zone B cells in humans and may be the product of T cell–independent immune responses.[26]

Seminal studies demonstrated that CSR and somatic hypermutation are mediated by an enzyme designated activation-induced cytidine deaminase (AID).[27,28] As might be expected of an enzyme with this function, AID expression is induced in germinal center B cells where CSR and somatic hypermutation occur. There are two somewhat opposing theories on how AID functions to promote antibody diversification. One argument holds that AID subserves an "RNA editing" function; not being the source of hypermutator activity per se, but cooperating with another protein to mediate somatic hypermutation.[29] The prevailing view holds that AID acts directly as a DNA mutator to mutate Ig genes.[30] Interestingly, AID induction also occurs following infection by hepatitis C and the Epstein-Barr virus,[31,32] suggesting that AID plays a role in the innate immune response to viral infection.

As discussed elsewhere in this textbook, neonates are vulnerable to infection with selected pathogens (e.g., encapsulated bacteria) and respond poorly to immunization. In general the neonatal VH repertoire is more restricted than in adults,[33] but this appears to reflect environmental factors (i.e., antigen exposure, hormonal differences) instead of some inherent mechanistic difference in Ig gene rearrangement and VH diversification.

B-1 CELLS

B-1 cells represent a unique population of B cells, originally identified in humans[34] and mice[35] based on the expression of cell surface CD5, a molecule also expressed on most human T lymphocytes. B-1 cells have many developmental and functional attributes that distinguish them from the more conventional follicular-derived B-2 cells that undergo the GC reaction.[36] A wealth of published work in the mouse has revealed an association of B-1 cells with self-reactivity and autoimmunity.[36] Although originally defined by the expression of CD5, it is now known that there are two categories of murine B-1 cells: B-1a (CD5+) and B-1b (CD5−). The former secrete "natural antibodies" critical in the initial response to encapsulated bacteria, whereas the latter have been shown to be important in long-term protection against *Streptococcus pneumoniae.*[37] A long-standing controversy over whether murine B-1 cells originate in a progenitor distinct from B-2 cells may have been resolved by the identification of a B-1 restricted progenitor in murine fetal marrow.[38] Despite considerable knowledge on murine B-1a and B-1b cells, we know less about the identity and function of human B-1 cells. During human fetal development approximately 40% of B cells in the omentum and spleen are CD5+/IgM+, and splenic CD5+ cells reportedly secrete autoreactive IgM.[39] However, if human splenic CD5+ B cells are the counterpart of murine B-1 cells (which are known to play a critical role in immunity to encapsulated bacteria) then it is difficult to explain why infants are so vulnerable to pneumococcal infections.

PLASMA CELL DEVELOPMENT

Plasma cells are terminally differentiated B-lineage cells that secrete copious quantities of antibody central to the humoral immune response. After T cell–dependent or T cell–independent antigen stimulation, plasma cells can develop from all the major mature B-cell compartments, including marginal zone B cells, GC centroblasts, memory B cells, and B-1 cells.[40] Substantial progress has been made in elucidating the identity and function of multiple transcription factors that regulate the potential of an activated B cell to differentiate into a memory B cell or a plasma cell. The basic transcriptional regulatory mechanism comprises distinct genetic programs leading to the development of memory B cells or plasma cells. The transcription factors BCL-6 and PAX5 work in concert to suppress plasma cell development. A prominent target gene of BCL-6 is BLIMP1, a transcription factor the function of which is central to driving activated B cells into plasma cells.[41] PAX5 induces expression of AID[42] and other B-lineage specific genes important for maintenance of a B-cell identity (i.e., CD19 and BLNK).[43] Certain signaling events received at the surface of B-lineage cells may trigger a reduction in BCL-6 levels, which in turn may relieve suppression of BLIMP1. A negative feedback loop then ensues involving BLIMP1 suppression of BCL-6 that, when coupled with the action of two other transcription factors designated XBP1 and IRF4, promotes differentiation to the plasma cell stage.[40] Plasma cells generated in the spleen or lymph nodes during post-GC reactions migrate to the marrow, where they survive for 1 to 2 months. Various integrins and chemokine receptors (e.g., CXCR4) function to promote egress of preplasma cells from spleen/lymph node, followed by migration to and localization in the marrow.

CYTOKINES AND CHEMOKINES IN B-CELL DEVELOPMENT

B-cell development occurs in a complex microenvironment consisting of other lymphohematopoietic cells, stromal cells, and extracellular matrix components such as fibronectin.[44] B-lineage cells develop in the extravascular compartment and, as they differentiate to the immature B-cell stage, migrate to medullary vascular sinuses as a prelude to entering the bloodstream. The term stromal cell is a generic term encompassing multiple cell types, including adipocytes, endothelial cells, osteoblasts, and fibroblastic reticular cells. Marrow stromal cells secrete cytokines and chemokines such as IL-7 and CXCL12 (see later discussion) that mediate development of B-lineage cells. In the mouse, the specific stromal cells that secrete these two molecules may be distinct from one another.[45] In vitro studies using human marrow-derived fibroblastic reticular cells indicated that vascular cell adhesion molecule-1 VCAM-1 plays an important role in the adherence of normal human B-lineage cells.[46,47] A single study to date that examined the physical disposition of human B-lineage cells and marrow stromal cells in situ indicated that B-lineage cells were prominently juxtaposed to CD10+ stromal cells, and that B-cell differentiation was spatially oriented with the most mature cells progressing toward marrow sinusoids.[48] Using gene targeting of cytokines and their receptors, a large number of studies in the mouse have implicated the IL-7 and FMS-related

tyrosine kinase ligand (FLT3-L) as well as the chemokine CXCL12 in the development of murine B-lineage cells.[49] Cytokines such as B cell–activating factor (BAFF), a-proliferation-inducing-ligand (APRIL), and CD40-ligand (CD40L) are expressed by various cell types in the spleen and lymph node and play prominent roles in B-cell survival in the periphery. This section briefly summarizes the effect of these cytokines and chemokines on human B-cell development.

Interleukin-7

The role of interleukin (IL)-7, and its receptor subunits the IL-7Rα chain and the γc chain, have been extensively studied in murine and human lymphopoiesis.[50,51] After its discovery as a B-lineage stimulatory molecule,[52] the physiologic importance of IL-7 was clarified by the demonstration that targeted disruption of the IL-7[53] or IL-7Rα chain[54] genes leads to profound disruption of B-cell development in adult mice. Targeted disruption of the genes encoding the γ_c subunit of the receptors for IL-2, 4, 7, 9, 15, and 21,[55,56] and the JAK3 tyrosine kinase[57,58] also leads to severe disruptions in B-cell development. This is consistent with the now well known structure of the IL-7 receptor and the activation of JAK3 following IL-7 stimulation.[51] Recent studies have clarified the contribution of IL-7 in murine B-cell development. IL-7 deficient mice undergo normal fetal and perinatal B-cell development, culminating in B-1 cell development and a normal-sized marginal zone B-cell compartment.[59] Therefore, B-cell development in fetal and neonatal mice has an element of IL-7 independence. In contrast, mice doubly deficient for FLT3 and the IL-7Rα chain fail to develop fetal and neonatal B-lineage cells.[60] Thus IL-7 and FLT3-L are cytokines that collectively orchestrate fetal and adult murine B-cell development.

Insight into the role of IL-7 in human B-cell development has been gleaned from analysis of patients with congenital and acquired immune deficiency. Patients with severe combined immune deficiency (SCID) with mutations in genes encoding components of the IL-7R signaling pathway often have been cited as evidence that human B-cell development does not have an absolute dependence on IL-7. Similarly, patients with X-linked SCID with γ_c subunit mutations have severe defects in T- and NK-cell development, but have normal or even elevated numbers of peripheral blood B cells.[61] Moreover, patients with autosomal recessive mutations in the JAK3 tyrosine kinase exhibit a lymphocyte developmental phenotype indistinguishable from X-linked SCID,[62,63] including normal numbers of circulating B cells. Further studies have indicated that mutations in the IL-7Rα chain are now the third most common cause of SCID.[64,65] An increase in percentage or absolute number of peripheral blood B cells occurred in essentially all these patients. Thus no doubt exists that circulating B cells in these collective patients with SCID do not have an absolute requirement on IL-7 signaling for their development. However, a normal peripheral blood B cell count does not necessarily equate with normal B-cell development in these patients with SCID. No data are published on the characteristics of marrow B-cell development or the origin and functional capability of circulating B cells in patients with SCID with IL-7R mutations. Furthermore, the requirement for IL-7 signaling could vary in humans as a function of ontogeny, with fetal/neonatal B-cell development being relatively IL-7 independent, as it is in the mouse.

Studies of patients with non-SCID immune deficiency provide a separate perspective on the possible role of IL-7 in human B-cell development. In a clinical study evaluating if in vivo IL-7 administration could restore T-cell numbers in patients with lymphopenic cancer, marrow biopsies performed before and after IL-7 administration revealed a three- to eight-fold increase in the percentage of CD19+/CD10+ B-lineage cells in four of six patients after IL-7 administration.[66] Administration of IL-7 may therefore have induced the expansion of B-cell precursors in the marrow of some patients. Studies in HIV patients[67] and patients with idiopathic CD4+ T lymphocytopenia,[68] the latter being an HIV-independent syndrome, revealed a positive correlation between serum levels of IL-7 and the existence of circulating CD10+/CD27− immature/transitional B cells. In vitro studies have shown that IL-7 transduces a replicative signal to normal human B-lineage cells that is complemented by additional stromal cell–derived signals that cooperate with IL-7 to promote normal human B-cell development.[69] In summary, the precise role of IL-7 in fetal, neonatal, and adult human B-cell development, and the identity of the chemokines that cooperate with and/or compensate for IL-7, remain to be clarified.

FLT3-L

The role of FLT3-L during B-cell development has been facilitated using mouse models, and this signaling pathway may exert an effect at the common lymphoid progenitor stage of development.[70] Similarly, repression of FLT3 expression may be a necessary event for commitment to the B-lineage.[71] Some evidence suggests that FLT3-L can synergize with IL-7 to enhance the proliferation of human pro-B cells isolated from human fetal marrow,[72] and enhance differentiation of cord blood–derived stem cells into B lymphocytes.[73] However, the degree to which FLT3-L plays a role in human B-cell development and whether it can subserve a function distinct from IL-7 signaling is unknown.

CXCL12 (SDF-1)

The chemokine CXCL12 was originally identified as a stromal cell–derived factor that promoted pre-B cell growth in the absence of IL-7.[74] Subsequent studies revealed that CXCR4, one of the receptors used by HIV to gain entry into T lymphocytes, is the receptor mediating CXCL12 effects. CXCL12 has been identified as a chemokine that plays an important role in retention of murine B-cell precursors in the marrow.[75] Studies with human B-lineage cells have indicated that CXCL12 signaling regulates adhesion[76] and migration[77] of early B-lineage cells to stromal cells but plays no role in promoting proliferation.

CD40 ligand (CD154)

The CD40 ligand (CD154) is expressed by T cells and platelets, but not by B cells or monocytes. CD40, a member of the tumor necrosis factor receptor superfamily, is the glycoprotein receptor for CD154.[78,79] CD40 is initially expressed at low level on pro-B cells, is subsequently expressed at high levels on immature and mature B cells, and undergoes down-regulation on plasma cells. B-cell functions that depend on T-cell help often require activation of the CD40/CD154 signaling axis. Engagement of CD40 by CD154 in the presence of cytokines such as IL-4 and IL-13 results in a multitude of functional outcomes specific to the maturational stage of the B cell.[78,79] These range from up-regulation of antiapoptotic genes that promote cell survival, cell cycle entry and proliferation, and CSR and somatic hypermutation during the GC reaction. Thus the differentiation of mature B cells into memory B cells and plasma cells relies heavily on appropriate signals transduced by this axis.[78,79]

BAFF/APRIL

B cell–activation factor of the TNF family (BAFF) and a proliferation inducing ligand (APRIL) are members of the TNF ligand superfamily. Receptors for these molecules include BAFF-R, TACI and BCMA and are differentially expressed initially in transitional B cells. BAFF is secreted by gamma interferon or IL-10 activated macrophages, dendritic cells, and granulocyte colony-stimulating factor–stimulated neutrophils. BAFF/APRIL stimulation leads to

up-regulation of antiapoptotic factors and prolongation of B-cell survival, even in the context of autoreactivity-induced deletion stimuli. Proapoptotic molecules are down-regulated in response to BAFF signaling, further protecting the cell from apoptosis. BAFF signals are also sufficient to induce CSR, although an additional stimulus such as cross-linking of the BCR is needed to induce secretion of class switched immunoglobulin.[80,81]

B-CELL IMMUNODEFICIENCIES

Given the unique and critical role of gene products expressed in early B-cell development, it is no surprise that mutations in some of these genes can have dire consequences on the development and function of human B cells.[82] If a genetic disease affecting B cells deserves the title of a "classic," it would be X-linked agammaglobulinemia. The pediatrician O. C. Bruton[83] originally described an 8-year-old boy with recurrent bacterial infection and no detectable serum gamma globulins. Subsequent studies showed that the disease was inherited in an X-linked pattern[84] and was characterized by an absence of circulating B cells,[85] thus the designation X-linked agammaglobulinemia. Following the characterization of cell surface antigenic maps that define specific stages of B-cell development, it became clear that B-cell development was "arrested" between the pro-B and pre-B stages.[86] Two landmark reports[87,88] then identified the gene mutated in X-linked agammaglobulinemia, and it was christened Bruton tyrosine kinase (Btk). Btk plays a critical role in a variety of biochemical signaling pathways.[89] Notably, after pre-BCR and BCR cross-linking Btk is activated and phosphorylates phospholipase C $\gamma 2$, ultimately culminating in a sustained calcium flux central to proliferation.[89] Hundreds of distinct mutations in Btk have been identified in X-linked agammaglobulinemia, but a relationship between a specific mutation and severity of the disease does not exist. Gammaglobulin is used to treat patients and must be administered throughout life. Rare cases of autosomal recessive agammaglobulinemia have also been described. This heterogeneous cohort includes patients with mutations in the μ heavy chain, the $\lambda 5$ subunit of the surrogate light chain, the pre-BCR/BCR associated signaling molecule Igα, and the adapter signaling molecule BLNK.[82] These patients have similar clinical characteristics to patients with X-linked agammaglobulinemia.

Patients with hyper-IgM syndrome have recurrent infections, elevated serum IgM, a relative paucity of other serum Ig isotypes, and a general failure of B cells to undergo CSR and somatic hypermutation.[90] Mutations in five distinct genes have been found to give rise to this congenital immunodeficiency. The most common is a mutation in CD154 (CD40 ligand) expressed on activated T cells that is inherited as an X-linked mutation. Patients with rare mutations in CD40, IKK-gamma/NEMO, and uracil-DNA glycosylase have also been reported.[90] The critical function of AID in development of a functional Ig repertoire is underscored by the fact that patients with an autosomal recessive form of the hyper-IgM syndrome harbor a deficiency of this enzyme.[91]

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Kurt R. Schibler

Mononuclear Phagocyte System

ORIGIN AND DEVELOPMENT OF MONONUCLEAR PHAGOCYTES

Tissue phagocytes have been recognized as fundamental components of host immune defense. The term *mononuclear phagocyte system* (MPS) identifies a specific lineage of phagocytic hematopoietic cells that share certain morphologic, cytochemical, biochemical, genetic, and functional characteristics.[1,2] In this system, mononuclear phagocytes comprise a cell lineage in which tissue macrophages are derived from circulating monocytes and immature bone marrow precursors. Mononuclear phagocytes share common functional characteristics including: (1) phagocytosis and digestion of invading microorganisms and tissue debris, (2) biosynthesis of numerous secretory products, (3) antigen processing and interaction with lymphocytes in development of the primary immune response, (4) generation of cytoxic response to tumor cells, and (5) performance of specialized tissue functions in concert with functions of other tissue cell types to maintain a homeostatic extracellular environment.[2,3]

Although dendritic cells differ in several morphologic, cytochemical, and functional features from classical cells of the MPS, evidence indicates that at least one population of dendritic cells, the myeloid dendritic cells, are derived from the same bone marrow hematopoietic progenitors as circulating monocytes and macrophages.[4] Because dendritic cells and mononuclear phagocytes share a common progenitor pathway, consideration must be given to including dendritic cells as members of the MPS.[5] Dendritic cells exhibit most characteristics just enumerated. First, dendritic cells are phagocytic cells in which captured material enters endosomes for degradation and antigen processing. Second, they synthesize cytokines that direct actions of innate and adaptive immune cells. Third, dendritic cells are highly effective antigen-presenting cells. Fourth, dendritic cells provide signals for macrophage and T-cell activation. Finally, dendritic cells exhibit specialized functions to maintain homeostasis of the extracellular environment. In this chapter, myeloid dendritic cells are discussed as components of the MPS.

Overview

The mononuclear phagocyte cell lineage originates from a committed progenitor cell present in the bone marrow (Fig. 153-1). Pluripotent stem cells give rise to progenitor cells committed to a program of proliferation and differentiation. Progenitor cells committed to the myeloid lineage, called *colony forming unit–granulocyte macrophages* (CFU-GMs), are supported along various myeloid pathways by actions of glycoproteins called *colony-stimulating factors* (CSFs). CSFs involved in mononuclear phagocyte differentiation include interleukin-3 (IL-3), granulocyte macrophage colony–stimulating factor (GM-CSF), and monocyte colony–stimulating factor (M-CSF or CSF-1). These factors synergize to support differentiation of CFU-GM into macrophages in vitro.[6-8] Addition of M-CSF preferentially induces macrophage differentiation. Inclusion of GM-CSF in combination with IL-4 and tumor necrosis factor–α (TNF-α) generate dendritic cells in vitro.[9-12]

Experiments in which the genes encoding the CSFs have been inactivated in vivo through homologous recombination in mice have increased our understanding of the in vivo role of the CSFs in regulating MPS cell production. Homologous inactivation of the GM-CSF gene did not appear to have a significant effect on fetal, neonatal, or adult growth and did not alter many aspects of granulocyte and macrophage physiology with one notable exception. Affected mice succumbed to alveolar proteinosis as a result of defective alveolar macrophage function.[13,14] Similar results were observed in affected mice when the common β-subunit for the receptors for GM-CSF, IL-3, and IL-5 was inactivated.[15] Shibata and colleagues[16] identified multiple defects in alveolar macrophage function in GM-CSF–deficient mice, including adhesion, phagocytosis, microbicidal activity, surfactant metabolism, and expression of Toll-like receptors (TLRs). Mice suffering from an inherited form of osteopetrosis (op/op) have been shown to have a mutation resulting in M-CSF gene inactivation.[17] Homozygous mutants have a defect in some but not all macrophage populations. The most profound macrophage derangement is in osteoclast numbers leading to severe bone and tooth abnormalities.[18] As op/op mice age, IL-3 expression increases and any of the deficits in the alveolar macrophage population resolve.[19] These studies confirm the importance of CSF function in vivo in MPS development, and, in addition, demonstrate the redundancy in these factors.

The identification and characterization of numerous lineage-restricted transcription factors that regulate the pattern of gene expression in hematopoietic cells suggest that transcription factors may play a key role in regulating hematopoietic progenitor cell proliferation and differentiation.[20] One such transcription factor that plays a significant role in MPS development is designated PU.1 (Fig. 153–2). The importance of this factor is underscored by its developmental expression in alveolar macrophages of GM-CSF knockout mice. Defective PU.1 expression in the alveolar macrophages of GM-CSF–deficient mice leads to impaired host defense functions and surfactant homeostatic mechanisms. These functions are restored using retroviral transduction of the PU.1 gene into alveolar macrophages. Homozygous PU.1-deficient mice also exhibit lack of myeloid dendritic cell development.[21]

Kinetics of Peripheral Blood Monocytes

The normal circulating monocyte count in the healthy human adult is estimated to be between 300 and 450 monocytes/mm^3. Peripheral blood monocyte counts have been observed to vary in a cyclic fashion over a 3- to 6-day period.[22,23]

The production and kinetics of circulating human adult mononuclear phagocytes have been studied by using in vivo pulse labeling of mononuclear phagocytes with tritiated thymidine followed by autotransfusion of radiolabeled leukocytes. The number of promonocytes present in bone marrow has been estimated to be between 3% and 5%, with approximately 40% of the promonocytes actively proliferating.[24] The promonocyte cell cycle rate varies between 30 and 48 hours. These rapidly dividing cells achieve a production rate between 7×10^5 and 7×10^6 monocytes/kg of body weight per hour. After 60 hours of maturation in the bone marrow, mature monocytes move into the intravascular space where they

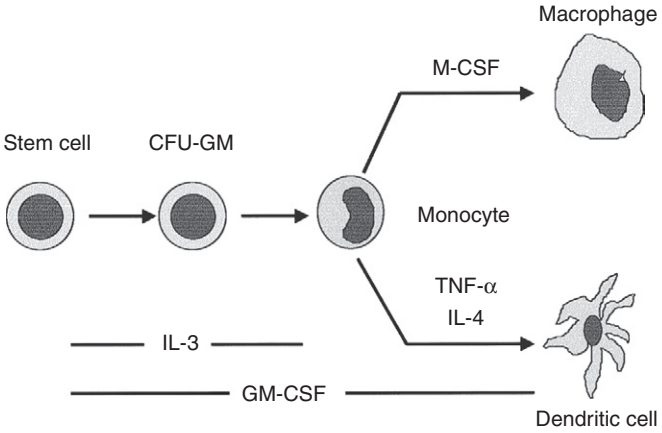


Figure 153-1 Mononuclear phagocyte cell lineage. *CFU-GM,* Colony-forming units granulocyte macrophage; *IL-3*, interleukin-3; *GM-CSF*, granulocyte macrophage colony-stimulating factor; *M-CSF*, macrophage colony–stimulating factor; *TNF-α*, tumor necrosis factor–alpha; *IL-4*, interleukin-4.

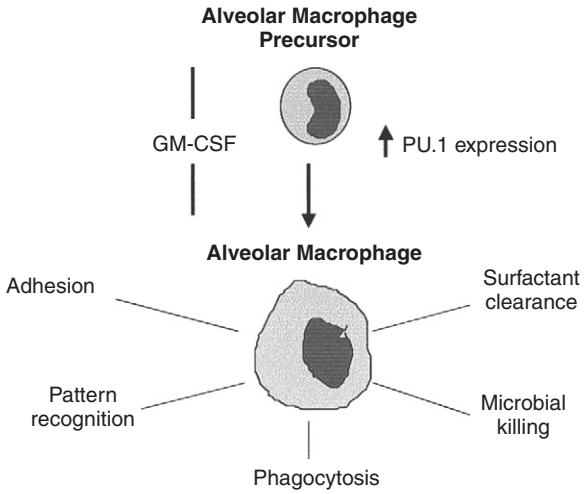


Figure 153-2 Role of granulocyte macrophage colony–stimulating factors (GM-CSF) and PU.1 in regulation of differentiation and function of the alveolar macrophage. GM-CSF induces the transcription factor, PU.1, in macrophage precursors in the lung. PU.1 is required for terminal maturation of alveolar macrophages, and it enables these cells to play critical roles in maintenance of lung homeostasis and innate immune responses.

circulate for approximately 3 days.[24] Van Furth and co-workers[2] calculated that 1.66×10^{10} circulating monocytes leave the intravascular space/hour. Therefore these investigators estimated the turnover of monocytes to be 0.4×10^{9} cells/day. Whitelaw[25] has suggested that the total intravascular monocyte pool is composed of a marginated pool and a circulating pool. He calculated that the marginated pool was 3.3 times greater than the circulating pool. Other investigators have failed to confirm a marginated pool of monocytes in human subjects.[2] Experiments in mice and rats provide evidence for marginated monocyte pools.[26,27]

Kinetics of Macrophages

Analysis of tissue macrophage kinetics is difficult given that the ultimate fate of tissue macrophages is not precisely known. Alveolar macrophages purportedly move from the respiratory tract to the alimentary tract, where they are eventually cleared.[28] The fate of other tissue macrophages has not been determined.

Tissue macrophages are derived from bone marrow precursors. The first evidence for the bone marrow origin of macrophages was obtained in mouse chimeras and rat parabiosis experiments.[29,30,31] Studies in human subjects have confirmed the data observed in animal experiments. In several patients undergoing bone marrow transplantation, isolation and characterization of lung and liver macrophages provided evidence that these cells contained the karyotype of the bone marrow donor.[32,33] Thus terminally differentiated macrophages must have been derived from transplanted bone marrow. Furthermore, after disappearance of the Y-body–containing macrophages from male patients who received female donor marrow, the half-life of the alveolar macrophage was an estimated 81 days.

The size of the tissue macrophage compartment is considerable. By some estimates, the tissue macrophage compartment is 500 to 1000 times larger than the bone marrow compartment. Some organs, such as the lung and liver, may have particularly large macrophage populations that may account for between 20% and 30% of the total cell number. These cells are long lived, and in a number of tissues have been estimated to survive for several months.[28,32,33]

Fetal Period

Embryonic macrophages can be identified early in hematopoietic tissues of the yolk sac. Tissue macrophages present at 3 to 4 weeks gestation do not appear to be associated with mononuclear phagocyte precursors (promonocytes and monocytes). Determination of the origin of these cells challenges the mononuclear phagocyte concept, in which macrophages are believed to be derived from immature precursors. The true origin of these macrophages remains to be clarified, although some investigators believe that these cells differentiate directly from hemocytoblasts.[34] Cline and Moore[35] have demonstrated that murine macrophages develop in vitro from primitive yolk sac precursors. They propose that the macrophage develops from a progenitor in the yolk sac that is proximal to promonocyte and monocyte stages of maturation. Later in gestation when the yolk sac is open to the fetal circulation, promonocytes, monocytes, and macrophages are all readily identifiable.

The liver becomes the predominant hematopoietic organ in the second month of gestation. At 4.5 weeks gestation, approximately 70% of blood cells present in the liver are morphologically identifiable as macrophages.[36] Over the next 6 weeks, macrophages decrease to approximately 1% to 2% of all differentiated blood cells in the liver, with erythroblasts becoming the most numerous blood-cell type in that organ. Primitive hemocytoblasts never account for more than 5% of all hematopoietic cells in the liver.

Although monoblasts and promonocytes may be identified during the period of liver hematopoiesis, true mature monocytes do not appear in the intravascular system of the fetus until the fifth month of gestation.[37] Circulating monocyte numbers remain low until after the sixth month of gestation, at which time bone marrow hematopoiesis predominates. During the first trimester of gestation, monocytes account for 2% to 4% of all hematopoietic cells. At approximately 30 weeks gestation, monocytes constitute 3% to 7% of all hematopoietic cells.[38]

Analysis of mononuclear phagocyte kinetics has not been possible in the fetal tissue examined. Mitotic figures have been seen in 0.1% to 1.2% of all hematopoietic cells in tissues. However, because of the difficulty of adequate preparation of tissues between delivery and the time of analysis, the number of cells in mitosis might actually be higher.[34] Therefore the kinetics of mononuclear phagocyte production and distribution to the tissues during the fetal period remain to be determined.

Neonatal Period

Analysis of the origin and development of mononuclear phagocytes in the neonatal period has been limited by the minimal number of approaches available to sample tissue macrophage populations in these patients. The total cellularity of the bone marrow averages 1.36×10^{11} cells/L on the first day of life but declines to 3.5×10^{10} cells/μL by the ninth day of life.[39] Analysis of bone marrow preparations from premature and term infants suggests that there are no significant differences in the differential counts of marrow elements for these populations of newborn infants.

There is a relative monocytosis present throughout the neonatal period. Peripheral blood monocyte counts are highest during the first 24 hours of life and decline over the remainder of the first month of life. Xanthou[40] reported that the peripheral monocyte counts of newborn infants in the first week of life range from 300 to 2100 cells/mm^3 with a mean of 1000 cells/mm^3. Weinberg and colleagues[41] analyzed the peripheral blood monocyte count in newborn infants and found a range between 0 and 1912, with a median of 600 monocytes/mm^3 in the first 60 hours of life. They also observed a decline in the upper limit of normal during the first month of life, but the median value of 600 cells/mm^3 remained constant. One problem encountered by all of these investigators, which may account for discrepancies in absolute numbers detected, is that mononuclear phagocytes in the newborn period are morphologically heterogeneous. Some monocytes are difficult to differentiate from myelocytes or metamyelocytes belonging to the neutrophil series, whereas other monocytes appear to be morphologically similar to large immature granular lymphocytes.[42]

Analysis of tissue macrophage kinetics in newborn infants has been limited to the examination of pathologic specimens or those gathered at autopsy of cases of stillborn or neonatal death. Alenghat and Esterly,[43] who performed histologic examination on the lungs of 200 consecutive infants who were stillborn or who died during the neonatal period, reported that the lungs of stillborn infants rarely contained alveolar macrophages. The presence of alveolar macrophages was confirmed in only 4 of 100 stillborn infants, and evidence of congenital pneumonia was present in several of these cases. In those babies who survived fewer than 48 hours, alveolar macrophages were observed in 33 of 46 patients. Infants who survived for longer than 48 hours uniformly had alveolar macrophages present in their lungs; however, in most of these cases, pneumonic infections were identified. Alveolar macrophages were detected in infants of all gestations (that is, longer than 30 weeks) who survived longer than 48 hours after birth. These investigators concluded that few alveolar macrophages were present in fetal lungs before birth, but that postnatally, a rapid influx is highly probable.

Experiments in a variety of animal models have confirmed the observation that fetal lung tissue contains few alveolar macrophages.[44-47] Soon after birth in most animal species studied, there are significant increases in the number of alveolar macrophages. Analysis of macrophage kinetics in these studies suggests that macrophage populations in the lungs are replenished both by a continuous monocyte influx and by local proliferation of macrophages.

CHARACTERIZATION OF MONONUCLEAR PHAGOCYTES

Morphology

The morphology of human monocytes and macrophages has been extensively studied at light and electron microscopic levels.[48] The ultrastructure of monocytes is notable for a well-developed Golgi apparatus with a small endoplasmic reticulum containing ribosomes and polysomes. Mitochondria are abundant. Numerous granules containing various lysosomal enzymes are distributed throughout the cytoplasm. The surface membrane of the monocyte includes an extensive system of microvilli. Macrophages are larger than monocytes. The Golgi apparatus, lysosomal granules, and mitochondria are larger than in monocytes. A prominent feature of macrophages is the presence of electron-dense lysosomes along the cell surface. Granules and vacuoles are seen along the periphery of the cell, indicating enhanced pinocytosis. Phagosomes containing ingested material are present at various stages of degradation.

Dendritic cells received their name from their distinctive morphology. They are notable for their irregular shape and numerous membrane processes including spiny dendrites, bulbous pseudopods, and lamellipodia or veils.[49] Distinctive electron microscopic features include prominent mitochondria, endosomes, and lysosomes in the cytoplasm.

Histochemistry

Monocytes and macrophages can be delineated from other leukocytes by cytochemical techniques that detect cellular constituents and biochemical processes. One of the most reliable of these markers is nonspecific esterase, which can be demonstrated using α-naphthyl butyrate as a substrate.[1] Many cell types contain nonspecific esterase, but the isozyme present in monocytes can be identified by inhibition with sodium fluoride.[50] Nonspecific esterase is expressed on the surface of peripheral blood monocytes. As monocytes differentiate into macrophages, additional isozymes of nonspecific esterase are expressed, leading to intense cytoplasmic staining.[51] Cord blood monocytes stain positive for nonspecific esterase, however, the proportion of esterase-positive cells is lower than in adult peripheral blood.[52] Peroxidase activity is also prominent in monocytes and diminishes as they mature into macrophages.[53] No differences are observed in peroxidase activity between monocytes from neonates and adults.

Dendritic cells lack many of the enzymes associated with other members of the MPS. Cytochemical reactions show that dendritic cells lack myeloperoxidase and have low levels of 5-nucleotidase, dipeptidylpeptidase, and cathepsin B activity.[54-57] Other intracellular enzymes, such as nonspecific esterase, acid phosphatase, and lysosomal CD68 may be present in dendritic cells, but their intensity is less than in monocytes or macrophages.[58] Nonspecific esterase decreases with activation of dendritic cells.[59]

Cell Surface Receptors

Numerous cell surface receptors have been identified on human mononuclear phagocytes. Ligands recognized by mononuclear phagocyte receptors include bioactive lipids, carbohydrates, complement components, cytokines, extracellular matrix, growth hormones, pathogen-associated pattern recognition, polypeptide hormones, and lipoproteins.

Fc receptors (FcRs) for immunoglobulin G (IgG) were identified nearly four decades ago with the observation that IgG antibodies could be directly cytophilic for macrophages when opsonized to erythrocytes.[60] Subsequent in vitro studies established the role of these receptors in triggering leukocyte effector functions such as macrophage phagocytosis and neutrophil activation by IgG immune complexes.[61,62] Our current understanding of IgG FcRs has been greatly enhanced by molecular cloning of the murine genes and their human counterparts and by the recently described crystal structures for both receptors.[63-68] Two general classes of FcRs are now recognized: activation receptors characterized by the presence of a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) sequence; and an inhibitory receptor, characterized by the presence of an immunoreceptor tyrosine inhibition motif (ITIM) sequence.[69,70] Immunoreceptor activation and inhibitory receptors bind IgG with comparable affinity and specificity; therefore coengagement of both signaling pathways sets the threshold and magnitude for the

effector cell response.[71] The balanced function of these receptors has been comprehended through analysis of mice deficient in either the receptor or signaling pathway component.

Activation FcRs include an ITAM motif either intrinsic to the receptor, as in the case of FcγIIA, or as part of an associated subunit, as in FcγI and FcγRIIIA. Cross linking of the ligand-binding extracellular domain results in tyrosine phosphorylation of the ITAM by members of the src family kinases. This leads to recruitment of SH2-containing signaling molecules that engage phosphorylated ITAM. Depending on the particular cell type activated by FcR, different kinases are involved in these signaling pathways. Early signaling events include activation of phosphatidylinositol-3 kinase (PI3 kinase), the enzymatic activity that leads to production of phosphatidyl-inositol-3,4,5-trisphosphate (PIP3) and recruitment of binding proteins of the pleckstrin homology (PH) domain class such as phosholipase Cγ (PLCγ).[72-74] Ultimately, activation of PLCγ leads to sustained calcium mobilization. The significance of this activity for FcR function has been determined through analysis of PLCγ2-deficient mice.[75] Cellular processes associated with FcγR activation receptors include degranulation, phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), transcription of cytokine genes, and release of inflammatory mediators.[61,62]

Both activation and inhibitory receptors are present on cells of the MPS, notably monocytes, macrophages, and dendritic cells. In general, activation and inhibitory FcγRs are coexpressed on the same cell, a physiologically important means of establishing a threshold for activating stimuli, because the IgG ligands engage both receptors. The ratio of these two opposing signaling systems determines the cellular response. Importantly, these receptors are modulated in their expression during development and differentiation of effector cells by the cytokine activation of these cells.

Microbes display molecular arrays or patterns that are recognized by pattern-recognition molecules. These patterns are shared among groups of pathogens. Representative examples are the lipopolysaccharide (LPS) of gram-negative bacteria, the glycolipids of mycobacteria, peptidoglycans and lipoteichoic acid of gram-positive bacteria, the mannans of yeast, and the double stranded RNA of viruses.[76-78] Important features of these microbial patterns are that they: (1) represent invariant structures shared by large groups of microorganisms, (2) are absolutely essential for the microbe's survival, and (3) are not produced by the host organism.[79] To limit infection the mammalian host uses an array of pattern-recognition molecules. These include complement, the collectins, and a battery of antimicrobial peptides that act with effector cells to combat infectious challenge.

The immune system detects and eliminates invading pathogenic microorganisms by discriminating between self and nonself. In mammals the immune system can be divided into branches: innate immunity and adaptive immunity. Adaptive immunity detects nonself through recognition of peptide antigens using antigen receptors on the surface of B and T lymphocytes. In order to respond to a wide range of potential antigens, B and T lymphocytes rearrange their immunoglobulin and T-cell receptor genes to generate more than 10^{11} different species of antigen receptors. Engagement of antigen receptors by cognate antigens triggers clonal expansion of lymphocytes and further production of antigen-specific antibodies. This sophisticated system is operative only in vertebrates. In contrast, the innate immune system is phylogenetically conserved and is present in almost all multicellular organisms.[80] The mechanisms by which the innate immune system recognizes nonself remained unknown until quite recently. The identification of Toll-like receptors in mammals—and the recognition of their role in detection of specific patterns of microbial components and in regulation of both innate and adaptive immune functions—represents a major advance in our understanding of immunity.

The involvement of Toll receptors in innate immunity was first described in *Drosophila*. The signaling pathway of *Drosophila* Toll shows remarkable similarity to the mammalian IL-1 pathway, which leads to activation of nuclear factor κB (NF-κB), a transcription factor responsible for many aspects of inflammatory and immune responses. The cytoplasmic *Drosophila* Toll and the mammalian IL-1 receptor are highly conserved and are referred to as the *Toll/IL-1 receptor* (TIR) domain. Base on this similarity it was suggested that the Toll pathway might mediate immune responses.[81] This was clearly demonstrated in a study of mutant *Drosophilia* lacking certain components of the Toll pathway.[82] Each mutant was highly susceptible to fungal infection caused by its inability to express the antifungal peptide drosomycin. Subsequent studies indicate that the Toll pathway is required for resistance to gram-negative and gram-positive bacterial infections in addition to fungal infections.

A year after the discovery of the role of *Drosophila* Toll in the host defense against fungal infections, a mammalian homologue of the *Drosophila* Toll was identified.[83] Subsequently, a family of proteins structurally related to *Drosophila* Toll was identified. This family of proteins is collectively referred to as the Toll-like receptors (TLRs), Currently, the TLR family consists of 10 members (TLR1-TLR10) in humans.[83-88] Ectopic expression of TLR4, the first mammalian TLR identified, was shown to induce genes for several inflammatory cytokines.[83] Therefore it was anticipated that the TLRs might be instrumental in immune responses, in particular in the activation of innate immunity. In 1998 it was determined that TLR4 was involved in the recognition of LPS, a major component of gram-negative bacteria. Thereafter, other members of the TLR family were shown to be essential for the recognition of a range of microbial components (Table 153-1). The structural similarity of TLRs seems to reflect their common function in recognition of microbial components.

TABLE 153-1

Toll-Like Receptors and Their Ligands

TLR Family	Ligands (Origin)
TLR1	Triacyl lipopeptides (bacteria, mycobacteria)
	Soluble factors *(Neisseria meningitidis)*
TLR2	Lipoproteins/lipopeptides (variety of pathogens)
	Peptidoglycan (gram-positive bacteria)
	Lipoteichoic acid (gram-positive bacteria)
	Lipoarabinomannan (mycobacteria)
	Porins *(Neisseria)*
	Zymosan (fungi)
	Atypical LPS *(Porphyromonas gingivalis)*
	HSP70 (host)
TLR3	Double-stranded RNA (virus)
TLR4	LPS (gram-negative bacteria)
	Taxol (plant)
	Fusion protein (RSV)
	Envelope protein (MMTV)
	HSP60 *(Chlamydia pneumoniae)*
	HSP60 (host)
	HSP70 (host)
	Type III repeat extra domain A of fibronectin (host)
	Oligosaccharides of hyaluronic acid (host)
	Polysaccharides of heparin sulfate (host)
	Fibrinogen (host)
TLR5	Flagellin (bacteria)
TLR6	Diacyl lipopeptides (mycoplasma)
TLR7	Imidazoquinolone (synthetic compound)
	Loxoribine (synthetic compound)
	Bropirimine (synthetic compound)
TLR8	Unknown
TLR9	CpG DNA (bacteria)
TLR10	Unknown

Recognition of LPS is an important function of innate immunity that can have profound consequences for the host. Failure to contain the infection can result in gram-negative sepsis, shock, and death as a result of LPS release. Two LPS-binding proteins have been characterized in mammals; these are the bactericidal permeability–increasing protein (BPI) and LPS-binding protein (LBP).[89,90,91] Binding of LPS by these molecules results in markedly different functional consequences.[92] BPI is a neutrophil granular pattern–recognition molecule that exhibits selective toxicity against gram-negative bacteria. BPI has two distinct functional domains, one that binds LPS and is antimicrobial and the other that is opsonic.[93] BPI is most effective at the site of infection and acts in synergy with other antimicrobial peptides such as defensins and the membrane attack complex of complement. In contrast, LBP enhances the sensitivity to LPS by binding to the lipid A moiety of LPS thus allowing triggering of circulating effector at subpicomolar concentrations of LPS. LBP plays a significant role in clearance of bacteria from the circulation. This clearance is mediated by activation of NF-κB through the CD14/TLR4 signal transduction pathway. LBP enhances the efficiency of this pathway by binding LPS and shuttling it to the CD14/TLR complex.

Two mouse strains, C3H/HeJ and C57BL10/ScCr, have long been known to be hyporesponsive to LPS. The C3H/HeJ mouse strain was found to have a point mutation in the intracellular region of the *Tlr4* gene leading to replacement of a highly conserved proline with histidine. This mutation results in generation of a dominant negative allele leading to defects in TLR4-mediated signaling and suppression of the response to LPS.[94,95] The C57BL10/ScCr mouse strain was found to have a null mutation in the *Tlr4* gene.[96]

The LPS-LBP complex is subsequently recognized by CD14, a molecule preferentially expressed on monocytes, macrophages, and dendritic cells. LPS stimulation is followed by close physical proximity of CD14 and TLR4. MD-2 was identified as a molecule associated with the extracellular portion of TLR4 whose presence enhances LPS responsiveness.[97,98] Indeed, generation of MD-2–deficient mice demonstrates the essential role of MD-2 in the response to LPS. Macrophages and dendritic cells from MD-2–deficient mice display severely impaired responses to LPS.[99]

The engagement of TLRs by LPS and other TLRs by microbial components triggers the activation of signaling cascades (Fig. 153-3). After binding, TLRs dimerize and undergo conformational changes that enable recruitment of TIR domain–containing adaptor molecules to the TIR domain of the TLR. There are four adaptor molecules: (1) MyD88, (2) TIR-associated protein (TIRAP/MyD88 adaptor-like, or Mal), (3) TIR domain-containing adaptor protein–inducing inerferon (IFN-β) (TRIF)/TIR domain-containing molecule 1 (TICAM1), and (4) TRIF-related adaptor molecule (TRAM).[100-102] The differential responses mediated by distinct TLRs can be explained by selective usage of adaptor molecules. MyD88 and TRIF activate signaling pathways leading to production of proinflammatory cytokines and type I IFNs, respectively.

MyD88 is a critical component for signaling of all TLRs except TLR3. Upon stimulation, MyD88 associates with the cytoplasmic portion of TLRs and then recruits IL-1R–associated kinase 4 (IRAK-4) and IRAK-1 through a homophilic interaction. In TLR2 and TLR4 another adaptor molecule, TIRAP/Mal, is required for MyD88 engagement with the receptor.[103-105] After IRAK-1 associates with MyD88 it is phosphorylated by the activated IRKA-4 and subsequently associates with TNFR-associated factor (TRAF) 6, which acts as a ubiquitin protein ligase (E3).[106] TRAF6, in combination with a ubiquitination E2 enzyme complex consisting of UBC13 and UEV1A, catalyzes the formation of a K63-linked polyubiquitin chain on TRAF6 and on IKK-γ/NF-κB essential modulator (NEMO).[107] A complex including TGF-β-activated kinase 1 (TAK1) and the TAK1 binding proteins TAB1, TAB2, and TAB3 is also recruited to TRAF6.[108] TAK1 phosphorylates IKK-β and mitogen-activated protein (MAP) kinase kinase 6 (MKK6), which in turn modulates the activation of NF-κB and MAP kinases, resulting in induction of genes involved in the inflammatory response. In addition to NF-κB, the transcription interferon regulatory factor (IRF)5 is involved in regulation of cytokine genes.[109] Upon stimulation of the TLR, IFR-5 translocates to the nucleus and binds IFN-stimulated response elements (ISRE) motifs present on the promoter regions of cytokine genes.

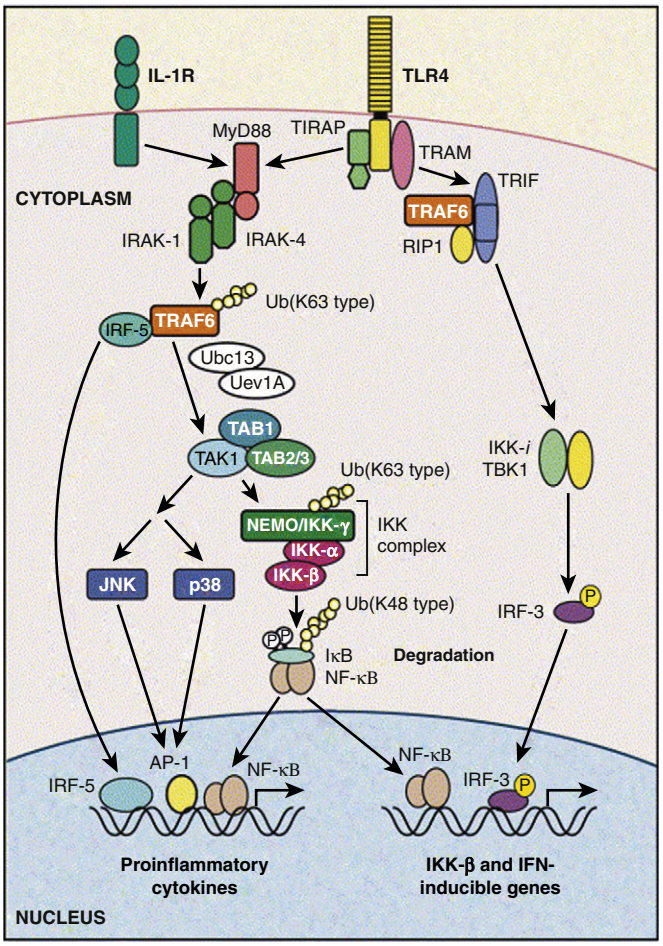


Figure 153-3 The TLR signaling pathway. TLRs and IL-1R share common signaling pathways in general. Stimulation with their ligands recruits TIR domain–containing adaptors including MyD88 and TIRAP to the receptor and the subsequent formation of a complex of IRAKs. TRAF6, and IRF-5 is induced. TRAF6 acts as an E3 ubiquitin ligase and catalyzes the K63-linked polyubiquitin chain on TRAF6 itself and NEMO with E2 ubiquitin ligase complex of UBC13 and EUEV1A. This ubiquitination activates the TAK1 complex, resulting in the phosphorylation of NEMO and activation of IKK complex. Phosphorylated of IκB undergoes K-48-linked ubiquitination and degradation by the proteosome. Freed NF-kB translocates to the nucleus and initiates the expression of proinflammatory cytokine genes. Simultaneously, TAK1 activates the MAP kinase cascades, leading to the activation of AP-1, which is also critical for the induction of cytokine genes. TLR4 triggers the MyD88-independent, TRIF-dependent signaling pathway via TRAM to induce type I Fins. TRIF activates NF-κB and IRF-3, resulting in the induction of proinflammatory cytokine genes and type I IFNs. TRAF6 and RIP1 induce NF-κB activation and TBK1/IKK-I phosphorylates IRK-3, which induces translocation of IRF-3. *(From Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity,* Cell *124:783-801.)*

Stimulation with TLR3, TLR4, TLR7, and TLR9 ligands induces type I IFN production in addition to proinflammatory cytokine signals. TLR3 and TLR4 have the ability to induce IFN-inducible genes in the absence of MyD88 signaling. The activity of these pathways leads to dendritic cell (DC) maturation, costimulatory molecules, and secretion of IFNα/β.[110] This MyD88 independent pathway is initiated by TIR domain-containing adaptor, TRIF.[111,112] TRAM, another TIR domain-containing adaptor, is specifically associated with TLR4 activation of TRIF. TRIF interacts with receptor-interacting protein 1 (RIP1), which is responsible for the activation of NF-κB.[113] In addition TRIF activates TRAF family member–associated NF-κB activator (TANK) binding kinase 1 (TBK1). TBK1 comprises a family with inducible IκB kinase (IKK-i), and these kinases directly phosphorylate IRF-3 and IRF-7.[114] Phosphorylated IRS-3 and IRF-7 form homodimers, translocate to the nucleus, and bind to the ISREs resulting in expression of a set of IFN inducible genes.

Förster-Waldl and co-workers[115] found reduced TLR4 surface protein and TLR4-specific mRNA in monocytes derived from preterm infants compared with those of mature infants and adults. They found that reduced TLR4 expression paralleled diminished in vitro LPS-stimulated IL-1β, IL-6, and TNF-α secretion. Levy and colleagues[116] reported a striking decrease in sensitivity of monocytes in human neonatal cord blood, compared with monocytes in adult peripheral blood, to the TNF-α–inducing effect of multiple TLR ligands, including bacterial lipopeptides (BLPs), LPS, and the imidazoquinoline compound, imiquimod. In marked contrast, they found that TNF-α release in response to R-848, a TLR ligand that is a congener of imiquimod, was equivalent in newborn and adult blood. Differences in ligand-induced TNF-α release correlated with divergent ligand-induced changes in monocyte TNF-α mRNA levels. Newborn and adult monocytes did not differ in basal mRNA or protein expression of TLRs or mRNA expression of functionally related molecules. However, newborn monocytes demonstrated diminished LPS-induced, but equivalent R-848–induced, phosphorylation of p38 mitogen-activated protein kinase and altered BLP- and LPS-induced acute modulation of cognate receptors, suggesting that the mechanism accounting for the observed differences may be localized proximal to ligand recognition by surface TLRs. Interestingly, newborn plasma substantially reduced BLP-, LPS-, and imiquimod-induced TNF-α release in adult monocytes without any effect on R-848–induced TNF-α release, reflecting differences in a plasma factor(s) distinct from soluble CD14. Impaired response to multiple TLR ligands may significantly contribute to immature neonatal immunity.

Cell Surface Antigens

Morphologic, cytochemical, and enzymatic characteristics have been traditionally used for the identification of mononuclear phagocytes. Monoclonal antibody techniques have provided a more precise description of the mononuclear phagocyte lineage relationships and differentiation sequence.[48] As a result of the large number of monoclonal antibodies generated against cell surface antigens of leukocytes, an international workshop was convened to classify and characterize monoclonal antibodies and their leukocyte cell surface antigens. The Human Leukocyte Differentiation Antigen Workshop has evaluated thousands of antibodies to develop common nomenclature.[117] Monoclonal antibodies having similar reactivity patterns to cellular antigens are given a common cluster of differentiation (CD) designation. Table 153-2 summarizes several CD groupings for the MPS discussed in this chapter. Well-described class I and class II major histocompatibility antigens are not included in the table.

During ontogeny, macrophages in the fetal liver express CD11b as early as 12 weeks gestation.[118] Another antigenic determinant, CD14, appears to be a more mature antigen. Its expression does not appear until 15 to 21 weeks gestation. CD14 expression in cord blood and adult blood monocytes is equivalent.[119] In contrast, CD11a, CD11b, and CD11c are expressed at lower density on cord blood monocytes than on cells from adults. Several investigators have reported that human leukocyte antigen (HLA)-DR, HLA-DP, and HLA-DQ are expressed at lower density on neonatal monocytes than on adult monocytes.[52,120-122] Furthermore, HLA-DR expression is decreased in monocytes from preterm neonates when compared with term neonates.[123,124] Class II histocompatibility antigen density on macrophages has been correlated to antigen presentation capacity in vitro.[125] It remains to be determined whether developmental differences in these antigens account for deficient antibody responses to certain foreign antigens in the newborn period.

Dendritic cells lack expression of surface antigens specific for other leukocytes including T cells (CD3), monocytes (CD14), neutrophils (CD16), B cells (CD19), and natural killer (NK) cells (CD56).[126] Myeloid dendritic cells differentiate directly from CD34+ precursors or indirectly from CD14+ monocytes.[127,128] They express surface markers associated with myeloid cells, such as CD11b and CD11c.

TABLE 153-2

CD Antigens on Mononuclear Phagocyte Cells

CD	Ligand, Receptor, Associated Molecules	Cell Type
CD4	MHC Class II, Gp120, IL-16	DC, Mono
CD11a	ICAM 1,2,3	Mono, Mac, Myeloid
CD11b	iC3b	DC, Mono, Mac, Myeloid
CD11c	iC3b	DC, Mono, Mac
CD14	LPS	Mono, Mac
CD16	Fc	DC, Mac
CD18	CD11a,b,c	Mono, Mac
CD40	CD40L	DC, Mono, Mac
CD80	CD28, CD152 (CTLA-4)	DC, Mono, Mac
CD123	IL-3	DC, Mono, Mac

BIOCHEMISTRY AND METABOLISM OF MONONUCLEAR PHAGOCYTES

Monocyte Metabolism

Metabolic activities of the cells of the MPS vary considerably based on their stage of development, site of origin, and state of activation. Human adult peripheral blood monocytes require an intact glycolytic pathway that is not adversely affected by inhibition of oxidative phosphorylation.[129] Peripheral blood monocytes placed in tissue culture environment demonstrate increased glycolysis with concomitant time-dependent increase in lactate accumulation in the culture media.[130]

Prindull and Prindull[131] compared the glycogen content of mononuclear phagocytes from premature and term newborn infants with that of adult subjects. The glycogen reserves under resting conditions were similar; however, during experimental inflammatory conditions mononuclear phagocytes from adult subjects increased their glycogen content, presumably through pinocytosis of neutrophil glycogen. In contrast, the glycogen content of mononuclear phagocytes from newborn infants decreased. In other studies, the glycolytic activity of cord-blood mononuclear cells (preparations included monocytes and lymphocytes) from preterm and term newborn infants was compared with mononuclear cells from adult peripheral blood.[132] Mononuclear cells from term and premature cord blood cells demonstrated decreased glycolysis and little metabolic activity in response to phytohemagglutinin (PHA) stimulation, whereas cells from adult blood completely converted glucose to lactate and demonstrated a significant increase in glycolysis in response to stimulation with

PHA. In addition, cord blood monocyte pyruvate kinase activity and adenosine triphosphate concentrations were significantly diminished compared with measurements obtained from adult peripheral blood monocytes. These investigators proposed that diminished energy metabolism might contribute to the dysfunction in host defense exhibited by newborn infants.

Macrophage Metabolism

Human adult macrophages are capable of using both glycolytic and oxidative phosphorylase pathways to derive their energy requirements. The operative pathway depends on the site of tissue origin. The pulmonary alveolar macrophage exhibits a high degree of oxygen consumption, whereas peritoneal macrophages depend predominantly on anaerobic glycolysis to provide their energy needs.[133] Analysis of the glycolytic enzymes lactate dehydrogenase and pyruvate kinase and the mitochondrial enzyme glutamate dehydrogenase in macrophages suggests that the metabolic activity of tissue macrophages may be determined by the oxygen tension in the environment. This hypothesis was substantiated by Bar-Eli and colleagues[134] who reported that macrophages derived from a single progenitor cell can develop different metabolic features in response to the oxygen tension in the environment in which they mature. The metabolic state of fetal and neonatal human tissue macrophages has not been determined.

Biosynthesis of Reactive Oxygen Intermediates

Mononuclear phagocytes are capable of either reducing or exciting oxygen molecules to produce highly reactive intermediates, such as superoxide anion, hydrogen peroxide, and the hydroxyl radical. Shortly after the mononuclear phagocyte interacts with stimuli, oxygen consumption increases dramatically. This respiratory burst is associated with enhanced microbicidal activity, particularly in circulating blood monocytes. The respiratory burst and production of reactive oxygen intermediates are discussed in detail in separate studies by Babior[135] and by Nathan and Tsunawaki.[136]

The nitroblue tetrazolium (NBT) dye test is a screening device used to determine deficiencies in oxidative metabolism. The NBT assay is based on the ability of reactive oxygen intermediates to reduce the soluble yellow NBT dye to a blue-black (formazan) insoluble material that precipitates in the cytoplasm and is readily identified by light microscopy.[137] Cells derived from patients with inherited phagocyte deficiencies of oxidative metabolism fail to reduce NBT.[138] Light emission (chemiluminescence) can also be used to detect the respiratory burst when mononuclear phagocytes generate reactive oxygen intermediates in response to external stimuli. This property provides a semiquantitative assay for the formation of oxygen radicals.

Investigators comparing the respiratory burst generated by cord and adult blood monocytes using the quantitative spectrophotometric NBT assay found no differences in the ability of these cells to reduce NBT in vitro.[139] Direct quantitative measurement of oxygen consumption, superoxide anion levels, and hydrogen peroxide production have been performed in parallel studies of cord blood and adult peripheral blood monocytes. These studies suggest that the cord blood monocyte has a respiratory burst that is similar in magnitude to that of adult peripheral blood monocytes.[140]

Macrophages derived from cord blood and adult peripheral blood were found to be equivalent in their capability to generate superoxide in response to soluble stimuli.[140] Treatment of cord blood and adult blood monocyte–derived macrophages with LPS or muramyl dipeptide enhanced production of superoxide anion in response to a soluble stimulus.[141] The superoxide generation by muramyl dipeptide-primed cord blood monocyte–derived macrophages was significantly less than that generated by primed cells from adult blood.[140] These investigators postulated that diminished superoxide generation in response to a priming stimulus indicates that cord blood monocytes may have a diminished capacity to differentiate into activated macrophages.

Biosynthesis of Complement Proteins

The complement (C) system is composed of at least 20 circulating plasma proteins, five membrane-bound proteins that regulate the binding or degradation of complement components, and seven membrane-bound proteins that bind complement components or cleave fragments of components. The complement cascade may be activated through either a classical or an alternative pathway to generate numerous biologically active products. Activation of this cascade through either pathway produces low-molecular-weight inflammatory peptides (termed *anaphylatoxins*) and a final large complex of proteins (termed *the membrane attack complex*) that insert into the target cell membrane, resulting in cell lysis.[142] The anaphylatoxins bind cell surface receptors on many target tissues, which results in release of vasoactive substances, cytokines, arachidonic acid metabolites, and reactive oxygen intermediates. Anaphylatoxins, including opsonic C3b, and chemotactic C5a, stimulate multiple leukocyte functions involved in killing microbial pathogens.[143-145] The significance of the complement system in amplifying the host immune system is demonstrated in animal models and in patients with inherited deficiencies in complement components.[146,147]

The appearance of serum complement components, complement-mediated hemolytic activity, and complement regulatory proteins occurs between 8 and 14 weeks in the human fetus.[148] These components are products of fetal synthesis rather than maternal components acquired transplacentally.[149,150] In the adult human, complement component synthesis (with the exception of C7) occurs predominantly in the liver.[151] In the embryo and fetus, the major sites of synthesis have not been clearly defined, but extrahepatic sites are thought to predominate. The MPS is likely an important source for complement proteins.[152]

In various animal models, serum complement concentrations and complement-mediated hemolytic activity gradually increase over the course of gestation; however, adult serum concentrations are not achieved until several weeks after delivery.[153,154] In newborn infants, serum complement concentrations are diminished compared with findings in adults. Deficiencies in complement components are more pronounced in infants delivered prematurely.[155,156] Serum complement components generally achieve adult levels by 6 to 18 months of age.

Biosynthesis of other Secreted Products

Characterization of mononuclear phagocyte secretory products is a major focus of research. Diverse products synthesized by mononuclear phagocytes include polypeptide hormones, complement components, coagulation factors, enzymes, cytokines, extracellular matrix proteins, other binding proteins, bioactive oligopeptides, bioactive lipids, sterol hormones, purine and pyrimidine products, reactive oxygen intermediates, and reactive nitrogen intermediates.

Production of secretory products by mononuclear phagocytes during fetal and neonatal life is limited. Synthesis of the polypeptide hormones interferon (IFN)-α, IL-1, and TNF by term cord blood monocytes appears to be quantitatively similar to those of adult peripheral blood monocytes.[157-159] However, TNF production by freshly isolated monocytes derived from the cord blood of premature infants is lower than that of term cord blood and adult peripheral blood monocytes.[159] Other investigators described diminished IL-1 and TNF-α synthesis by preterm and term cord blood monocytes stimulated with LPS.[160] Kaufman

and co-workers[161] described a variety of synthetic deficits in LPS-stimulated adherent monocytes from preterm compared with monocytes from full-term infants. These included diminished generation of superoxide, TNF-α, and CD11b/CD18 receptors. They observed no differences in IL-1β or IL-6 production. These investigators postulated that adherence functions such as CD11b/CD18 ligation might be important in modulating subsequent cellular activation and cytokine secretion activities. Synthesis and secretion of several cytokines in vitro including G-CSF, GM-CSF, IL-6, IL-8, IL-10, and IL-12 by stimulated monocytes isolated from preterm and term cord blood monocytes are significantly lower at the protein and mRNA level than those measured from stimulated adult blood monocytes.[162-167] Diminished production of GM-CSF by PHA and phorbol myristate acetate (PMA)-stimulated newborn mononuclear cells has been determined to be secondary to increased levels of AUF1 isoform (RNA binding proteins) that affect mRNA stability.[162,168] Regulation of IL-10 also appears to be developmentally regulated at the post-transcriptional level.[166]

Goriely and colleagues[169] hypothesize that deficits in neonatal dendritic cell function are responsible in part for impaired T-helper 1 (Th1) function in human newborns. Their investigations of neonatal monocyte-derived dendritic cells revealed diminished expression of HLA-DR and costimulatory molecules CD40 and CD80. In addition they observed a selective defect in IL-12 synthesis at the protein and messenger RNA level in response to LPS, CD40 ligation, or poly-deoxy-inosinic acid:poly-deoxy-inosinic-deoxy-cytidylic acid (poly[d(I-C)]). Moreover, they found that neonatal dendritic cells were less effective than adult dendritic cells in promoting IFN-γ production by allogeneic adult T cells. This deficit was corrected with addition of recombinant IL-12.

FUNCTIONAL ASPECTS OF MONONUCLEAR PHAGOCYTES

Morphogenesis

Macrophages are critical during morphogenesis of the fetus. In addition to their role in immunity, macrophages participate in modeling organs, and they engulf debris and apoptotic cells during embryogenesis.[170] For example, they are present in the urogenital ridge, where they clear degenerated cells of the Müllerian duct in the male and the Wolffian duct in the female.[171] They appear to play a similar role in the retina and brain.[172,173] Macrophages have also been observed in close proximity to areas of apoptosis in the developing fetal mouse footplate.[174]

Movement

Mononuclear phagocytes exhibit both random and directed movement.[175] Random or nondirected movement occurs in the absence of attracting substances. Directed movement along a concentration gradient of chemical attractants is essential for the effective localization of mononuclear phagocytes to sites of infection and inflammation. This directional movement, called *chemotaxis*, is governed by chemoattractant molecules that bind to receptors on the cell surface. Chemotactic substances are produced through activation of the complement, fibrinolytic, and kinin systems or are directly produced by microorganisms. These chemoattractants may also accentuate random migration of mononuclear phagocytes in the absence of a concentration gradient. An entire family of proinflammatory molecules called *chemokines* has been identified and many of its members have been characterized.[176] Several members of this family serve as potent chemoattractants for mononuclear phagocytes. Monocyte chemotactic protein 1; macrophage inflammatory protein 1; and regulated upon activation, normal T-cell expressed and secreted (RANTES) are key regulators of monocyte migration. Dendritic cells migrate to lymphoid organs in response to secondary lymphoid chemokine.[177,178]

Mononuclear phagocyte migration in cells derived from cord blood and neonatal peripheral blood has been compared with migration in adult peripheral blood monocytes. In most reports, random movement by cord blood monocytes appears to be equivalent to that of adult peripheral blood monocytes.[179-181] On the contrary, several investigators have reported diminished directed movement by cord blood and neonatal peripheral blood compared with that of adult peripheral blood monocytes. Yegin[182] reported that monocyte chemotaxis increased gradually during childhood and was equivalent to adult chemotactic activity after 5 to 6 years of age. These results are consistent with those of other investigators who reported similar chemotactic activity by cord blood and adult peripheral blood monocytes, but dramatically decreased chemotaxis by neonatal peripheral blood monocytes in the first few days of life.[183] Monocyte chemotaxis gradually increased but remained lower than adult peripheral blood monocyte activity over the first 6 months of life. Other investigators report a slightly increased chemotaxis of cord blood monocytes in response to endotoxin-activated adult serum or activated adult lymphocytes.[184] Differences in methods of measurements applied, patient populations examined, and monocyte isolation used, likely account for the contradictory results reported by these investigators.

Adherence

Adhesion is a requisite function of mononuclear phagocytes for egress from the circulation, movement through interstitial tissues, and interaction with a wide variety of cell and tissue types. In the systemic circulation, movement of monocytes into tissues occurs in three independent steps. First, monocytes rolling along the vascular endothelium have transient low affinity focal interactions with endothelial cells through adhesion molecules called *selectins*.[185] L-selectin is constitutively expressed on the cell surface of blood monocytes and interacts with a variety of endothelial cell surface glycoproteins to promote transient monocyte-endothelium contact.[186] E-selectin and P-selectin are adhesion molecules expressed by vascular endothelium after activation.[187,188] These molecules recognize glycoprotein ligands on the cell surface of monocytes to facilitate monocyte-endothelium adhesion. The second step of monocyte egress from the vascular space involves halting the rolling, shedding of monocyte surface selectins, and firm adhesion of the monocyte to the endothelial cell surface. This firm adhesion is generated by activation of members of the integrin family on the monocyte cell surface. These integrins include CD11a-c and CD18 on monocytes and intercellular adhesion molecule (ICAM-1) or ICAM-2 on the endothelial cell surface.[189] An additional adhesion interaction pathway used by monocytes includes monocyte integrin α4β1 and endothelial vascular cell adhesion molecule-1 (VCAM-1). Both pathways promote firm adhesion of monocytes to endothelium and enable the final step of extravasation to occur. Monocytes migrate through the endothelium in response to a concentration of chemotactic signals to arrive at a focus of infection or inflammation. People who exhibit moderate to severe deficiency of cell surface integrins or selectins are susceptible to recurrent bacterial infections as a result of their inability to recruit neutrophils and monocytes to the site of infection. These defects can be visualized using a Rebuck skin window test.[190-192]

Dendritic cells are involved in a number of adhesive interactions that facilitate their migration and interaction with T cells. The initial tethering and rolling may prove to be mediated by selectins. More firm adherence and migration through the endothelium is likely to involve integrins and ICAMs or other immunoglobulin superfamily members. Integrins and CD44 or syndecans may mediate interstitial tissue interactions. Dendritic cell interactions with epithelium probably involve cadherins.

Adhesive properties of fetal and newborn monocytes have been examined to a limited degree. Boner and colleagues[193] reported equivalent adherenceto glass coverslips in both cord and adult blood neutrophils and monocytes. In others studies, Speer and co-workers[194] isolated mononuclear cells from cord and adult subjects and subjected these cells to a nylon-wool packed column. More than 85% of esterase positive monocytes from cord and adult blood adhered to the column. Other investigators evaluated the capacity of cord blood monocytes to adhere to denatured collagen and fibronectin-coated culture plates.[195] These studies also demonstrated comparable adherence of cord and adult blood monocytes to these substrates. In summary, monocyte adhesiveness appears to be unimpaired in neonatal blood compared with adult blood.

Endocytosis

The ability of mononuclear phagocytes to ingest a variety of materials is crucial to their immunologic function. Endocytosis is important in antimicrobial resistance, removal of old cells, antigen processing, and multiple other functions. Endocytosis is mediated through pinocytosis and phagocytosis. These two processes are believed to use similar mechanisms; however, distinctions have been drawn between them based on size and composition of the particles being ingested. Pinocytosis refers to ingestion of microscopic fluid droplets. Phagocytosis describes ingestion of large particulate matter.[196] Both processes involve internalization of extracellular materials. Phagocytosis is enhanced by opsonization of particulate materials with immunoglobulins or complement components that bind to Fc and C3 receptors on the cell surface of mononuclear phagocytes.

Historically, macrophages and dendritic cells were recognized as separate populations of adherent cells based, in part, on differences in endocytic capabilities. A model of antibody response by mouse spleen cells developed by Mishel and Dutton[197] required cooperation of two populations of cells distinguished by buoyant density and the capacity to adhere to glass or plastic. The high density, nonadherent cells were predominantly lymphocytes, whereas the low density, adherent cells consisted of a macrophage and a nonmacrophage (dendritic cell) population.[49,198] Macrophages had numerous lysosomes and actively internalized any small tracer molecules including microorganisms, antibody-coated particles, soluble horseradish peroxidase, and colloidal carbon. The other adherent cell population called *dendritic cells* based on their morphologic appearance had distinct features, including a paucity of lysosomes, numerous multivesicular vacuoles, and an inability to internalize particulate and fluid phase molecules.[49,198-200] The multivesicular vacuoles were later recognized to be major histocompatibility complex (MHC) class II–rich vesicles. A lack of endocytic activity was reported in many of the initial descriptions of dendritic cells.[200-203] After methods were developed to separate dendritic cells from macrophages, several laboratories found that dendritic cells exhibited two features that had not been previously encountered. They expressed high levels of MHC class II products and exhibited remarkable stimulatory capacity for T-cell–dependent responses (reviewed in Steinman's[126] study). The paucity of lysosomes and weak endocytic activity of dendritic cells, coupled with strong antigen- presenting functions were enigmatic to many investigators. The ability to isolate and culture dendritic cells has allowed resolution of certain aspects of this dichotomy.

Macrophages have the ability to internalize a broad spectrum of substrates in digestive lysosomes. Scavenging is a high capacity form of endocytosis that culminates in complete digestion of substrates. In studies applying fluid phase solutes to macrophages, these cells accumulated 10^5 soluble protein molecules per hour (on average) and substrates were digested to the level of amino acids.[204] Adsorptive uptake using Fc receptors enhances clearance properties of macrophages approximately 1000-fold.[205] In contrast, antigen presentation by dendritic cells requires a relatively small number of MHC-peptide complexes. For example, MHC II-restricted T-T hybrids can respond to presentation of a few hundred specific MHC-peptide complexes.[206,207] Similarly, naive T-cell stimulation can be achieved by presentation of only a few hundred complexes of MHC and superantigen by dendritic cells.[208]

Dendritic cells, like other antigen-presenting cells, have receptors that mediate adsorptive uptake and delivery of peptides to MHC products rather than lysosomes. Dendritic cells can present immune complexes and self-antigens by Fcγ receptors.[209,210] Furthermore, epidermal Langerhans cells (a specialized tissue macrophage) contain Birbeck granules that can be coated and mediate adsorptive uptake.[211,212] Sallusto and colleagues[213] demonstrated that the mannosyl-fucosyl C-type lectin receptor mediates adsorptive uptake of horseradish peroxidase and FITC-labeled dextran. Other C-type lectin domain–containing receptors have been identified and linked to antigen uptake and presentation by dendritic cells.[214,215]

Recycling of internalized membrane is a general feature of endocytosis. Levine and Chain[216] described significant trafficking of endocytic vesicles through endosomes but not lysosomes in dendritic cells. They examined endocytic activity using fluorescent solutes and cytofluorography and concluded that a significant portion of the internalized solute was discharged through the recycling process. On the contrary, Sallusto and coworkers[213] showed accumulation and retention of fluorescent solutes for longer periods of time. Their cell population contained more abundant MHC class II compartment (MIIC) vesicles compared with the cells used by Levine and Chain; thus MIICs might retain internalized substances longer for the purpose of antigen presentation.

Endocytosis of substrates is limited to discrete stages of the dendritic cell life cycle in response to certain signals. Romani and co-workers[217] and Streilein and Grammer[218] reported that dendritic cells isolated from skin efficiently presented protein antigens; however, after 12 hours in culture this capacity is lost. Splenic dendritic cells also capture antigens on isolation, but lose endocytic activity after overnight culture.[213,219] The proposal that macropinocytosis is increased in dendritic cells by growth factors is consistent with cytokine-induced macropinocytosis in other cell types.[220,221]

During the fetal period, macrophages are capable of immune protection of the fetus. In animal models, primitive macrophages are highly mobile and are capable of ingesting latex particles injected into the yolk sac and carrying them to the mesenchymal stroma and loose connective tissue.[222] Fetal Kupffer cells may detoxify circulating endotoxin. Endotoxin that reaches the amniotic cavity is swallowed by the fetus and gains access to the liver through the portal circulation.[223] Macrophages internalize endotoxin through the LPS receptor. This receptor is present on human fetal Kupffer cells and can be up-regulated as early as 8 weeks postconception by IL-3.[224] Later, macrophages contribute to the adaptive immune response as antigen-presenting cells and as producers of a variety of cytokines including TNF-α, IL-1, and IL-6.

The kinetics of phagocytosis by monocytes from cord blood and adult peripheral blood have been studied by Schuit and Powell.[225] These investigators found that monocytes isolated from adult peripheral blood ingested all polystyrene particles within 50 minutes. In comparison only 38% of cord blood monocytes had initiated phagocytosis in the same time period. The cord blood monocytes were able to ingest the particles by 100 minutes. These studies were not influenced by the presence of serum or heat-inactivated serum. Other investigators have reported that opsonized IgG sheep erythrocytes, *Staphylococcus aureus, Escherichia coli, Streptococcus pyogenes, Toxoplasma gondii,* and type II herpes simplex are ingested by cord blood monocytes as effectively as monocytes derived from adult peripheral blood.[194]

Defective phagocytosis of *Streptococcus agalactiae* by cord blood monocytes has been reported by Marodi and colleagues.[226] Monocytes derived from term cord blood ingested fewer group B Streptococci than monocytes isolated from adult blood. Other studies suggested that the phagocytic defect of group B *Streptococcus* by cord blood monocytes could be overcome by inclusion of adhesive glycoprotein fibronectin in the culture.[44] Although fibronectin alone did not enhance phagocytosis, preopsonizing group B *Streptococcus* with IgG preparation in combination with fibronectin enhanced phagocytosis by cord blood monocytes.

The phagocytic activity of cord blood monocyte–derived macrophages has been compared with that of macrophages derived from adult blood monocytes. After a 10-day culture period, Speer and co-workers[227] determined that cord blood–derived macrophages ingested complement opsonized *S. aureus* to the same extent as macrophages derived from adult blood. Other investigations compared the phagocytic activity of alveolar macrophages from intubated newborns with alveolar macrophages from bronchoalveolar lavage of adult subjects. Phagocytosis of *Candida albicans* by newborn and adult alveolar macrophages was determined to be equivalent with respect to rate and number of organisms ingested.[228]

Dendritic cells derived from cord blood monocytes by culture with IL-4 and GM-CSF exhibited decreased endocytic function compared with adult monocyte–derived dendritic cells.[229] Liu and colleagues[229] suggested that decreased mannose receptor expression might contribute to this developmental deficit. Zheng and co-workers[230] reported that after the addition of TNF-α to cultures, monocyte-derived cord blood dendritic cells exhibited equivalent endocytic capacity to adult monocyte–derived dendritic cells. The results of these studies suggest that mononuclear phagocytes in the newborn period have a well-developed endocytic pathway for most microorganisms. Phagocytic activity of mononuclear phagocytes from the fetus and from prematurely born infants has not been reported.

Microbicidal Activity

Several different antimicrobial mechanisms have been identified in mononuclear phagocytes. These are generally divided into oxygen-dependent and oxygen-independent mechanisms. The microbicidal mechanism used by mononuclear phagocytes varies depending on the state of maturation, differentiation, activation, and tissue of origin. The oxygen-dependent mechanism, termed the *respiratory burst*, is initiated with activation of the transmembrane electron transport system involving a reduced nicotinamide adenine dinucleotide, extracellular or phagosomal oxygen, and additional plasma membrane enzymes and cofactors.[135] Superoxide anions are the initial product of the respiratory burst. This molecule is directly toxic to certain proteins and membrane constituents; however, it is often converted to a more powerful antimicrobial agent, hydrogen peroxide, through a reaction catalyzed by superoxide dismutase. Hydrogen peroxide is highly toxic to a number of microorganisms. Hydrogen peroxide toxicity can be further enhanced when it is used as substrate for the oxidation of halides (for example, iodide, bromide, and chloride) to hypohalous acids and other reactive intermediates.[136] This reaction is catalyzed by the phagocytic enzyme myeloperoxidase. Halide oxidation products are themselves powerful oxidants and probably attack bacterial cell wall components leading to microbial killing. Proposed chemical groups involved in these interactions include amino acids, which are decarboxylated and deaminated; lipid membranes, in which polyunsaturated fatty acids are oxidized; and sulfhydryl groups, which are readily oxidized.[231]

Oxygen-independent antimicrobial mechanisms used by mononuclear phagocytes include acidification of the phagosome and the synthesis and secretion of cationic proteins called *defensins, lipid hydrolases*, *proteases*, and *nucleases.*[232] The extent to which these mechanisms contribute to antimicrobial activity in vivo is uncertain. However, in patients with chronic granulomatous disease, despite a deficient oxygen-dependent antimicrobial system, mononuclear phagocytes are capable of killing microbial pathogens.[233]

Nitric oxide (NO) is an additional molecule that plays an important role in oxygen-independent macrophage antimicrobial activity.[234] Initially, NO was identified as an antimicrobial and tumoricidal product of activated macrophages generated from the amino acid L-arginine by the enzymatic activity of inducible nitric oxide synthase (iNOS). NO plays many roles in the immune system as well as in other organ systems. In addition to macrophages, several other immune cells (dendritic cells; NK cells; mast cells; and phagocytic cells, including monocytes, microglia, Kupffer cells, eosinophils, and neutrophils) produce and respond to NO. Three isoforms of NOs are known: iNOS and two constitutive forms collectively called *constitutive NOS* (cNOS). Although the three isoforms catalyze the same reaction, they differ in their regulation, amplitude, and production of NO, and in cellular and tissue distribution.[235] NOS activity is determined by several mechanisms, many influenced by immunologic stimuli. The activity of NO is not restricted to the site of its production. It is a highly diffusible gas and can act locally or can be distributed widely in the body through NO vehicles such as *S*-nitrosothiols, *S*-nitrosylated proteins, and nitrosyl-metal complexes. Unlike cytokines, the interaction of NO is not restricted to a defined receptor. Rather, it can interact with other inorganic molecules (for example, oxygen or superoxide); structures in DNA (for example, pyrimidine bases); prosthetic groups (for example, heme); or proteins leading to *S*-nitrosylation of thiol groups, nitration of tyrosine residues, or disruption of metal-sulfide clusters (for example, zinc-finger domains or iron-sulfide complexes). Considering that many targets of NO are regulatory molecules it is evident that NO exerts diverse effects.

NO has a diverse spectrum of activities against infectious pathogens, including antiviral, antimicrobial, immunostimulatory (proinflammatory), immunosuppressive (antiinflammatory), cytotoxic (cell-damaging), and cytoprotective (tissue-preserving). The analysis of iNOS–/– mice confirms that most of these effects are mediated by iNOS-derived NO.[236-238] Under different circumstances, depending on the species, strain, infective dose, or route of pathogen entry, iNOS was indispensable or helpful in controlling the infection, had no effect, or worsened the disease.[235] The antimicrobial activity of NO results from a variety of mechanisms including mutation of DNA; inhibition of DNA repair or synthesis; inhibition of protein synthesis; alterations of proteins by S-nitrosylation, adenosine diphosphate (ADP)-ribosylation or tyrosine nitration; inactivation of enzymes by disruption of Fe-S clusters, zinc-fingers or heme groups; or peroxidation of lipid membranes.[237,239] These mechanisms likely account for the most actions against infectious agents. Peroxynitrite ($ONOO^-$), a reaction product of NO and O_2^-, is a potent antibacterial effector molecule. The nitrating efficiency and production capacity of peroxynitrite by macrophages have been controversial; however, recent investigations have demonstrated that $ONOO^-$ might be formed within the microbe by host-derived NO and microbe derived O_2^-. The significance of $ONOO^-$ is underscored by the fact that pathogens such as *Mycobacterium tuberculosis* and *Salmonella typhimurium* have developed mechanisms to detoxify $ONOO^-$ to nitrite.[240]

The iNOS-dependent killing of parasites by macrophages can result from depletion of arginine. For certain strains of *Leishmania* it has been shown that L-hydroxyarginine can inhibit arginase activity in the macrophage or parasite promoting parasite killing. Also, arginine is required for polyamine and DNA synthesis in *Leishmania* and African trypanosomes by the ornithine decarboxylase pathway and by *Trypanosoma cruzi* through the arginine decarboxylase pathway.[241]

Cytotoxic Activity

Under some circumstances mononuclear phagocytes are capable of killing other formed elements of the blood, normal cells, and tumor cells. Mononuclear phagocyte cellular toxicity may occur through means of specific antibodies, called antibody-dependent cellular cytotoxicity (ADCC), or it may occur in the absence of specific antibodies to the target cell.[242] Mononuclear phagocyte–mediated ADCC has been studied extensively. Because antibody specificity is the primary determinant of ADCC reactions, this means of cellular killing is used for disposal of both malignant tissue and normal cells. In ADCC, the immunoglobulin-bound target interacts with the Fc receptor on the mononuclear phagocyte. This initial binding is necessary but not sufficient to kill the target. Reactive oxygen intermediates, in particular hydrogen peroxide, are the major secretory products of the mononuclear phagocyte that lyse the target cell.[243] Serine proteases and tumor necrosis factor might also be involved in this lytic mechanism.

Antibody-independent cellular toxicity is highly selective for killing various types of malignant cells of syngeneic, allogeneic, or xenogeneic origin without damaging tissue-matched nonmalignant cells. This cytotoxicity is mediated through cell-cell contact and is usually highly selective and nonphagocytic in nature. Mononuclear phagocytes may have plasma membrane receptors for certain target cells.[242] TNF, reactive oxygen, NO intermediates, and the putative 40-kDa serine protease have been implicated as major secretory products involved in antibody-independent cellular cytotoxicity.[242]

Cord blood monocytes have been reported to exhibit normal ADCC activity to herpes simplex–infected hepatocytes and to antibody-coated human erythrocytes.[244,245] Antibody-independent cytotoxicity in term cord blood monocytes has been reported to be diminished by some investigators but to be normal by others.[244,246] The ontogeny of mononuclear phagocyte cytotoxic functions during fetal development has not been examined.

Antigen Processing and Presentation

The MPS is integral in the establishment of an effective immune system. In addition to the innate immune functions aimed at eradicating invading microorganisms, mononuclear phagocytes are involved in initiating and propagating the adaptive immune response. A complex relationship exists between antigen-presenting cells and antigen-specific T cells. The mechanism by which antigen-presenting mononuclear phagocytes interact with T cells requires internalization, degradation, and display of antigenic fragments on the cells' surface in association with MHC class II molecules. Macrophages and dendritic cells both participate in this function, although their respective roles likely differ.

Mechanisms of antigen uptake might differ between various cell types in the MPS. Macrophages may be more effective than dendritic cells as scavengers as a result of their extensive repertoire of scavenger receptors.[247] Macrophages also appear to be more effective than dendritic cells at ingestion of large antigenic materials and whole cells.[198] For example, colloidal carbon administered intravenously is predominantly ingested by macrophages. Several investigators have observed limited phagocytosis of large particles such as intact cells, bacteria, and synthetic beads in bone marrow–derived murine dendritic cells.[248-250] Macrophages may contribute to antigen uptake by lysing larger particles such as bacteria, viruses, or tumor cells before transferring the degradation products to dendritic cells.[251,252]

Dendritic cells are highly active in endocytosis and pinocytosis. This is the process by which small fluid phase particles, usually smaller than 0.5 μm, are internalized.[253] Mouse and human dendritic cells bind antigen at the cell surface and internalize these along with small portions of their plasma membrane.[254] This activated fluid phase endocytosis explains the low density characteristics acquired by dendritic cells in vitro. Monocyte-derived dendritic cells generated from peripheral blood mononuclear cells treated with GM-CSF and IL-4 take up extracellular fluid, actively processing approximately 100 times their volume per hour.[220]

Nonspecific inflammatory signals may be required to initiate phagocytosis, endocytosis, and expression of antigen capture receptors. Bacteria express complex carbohydrate molecules on their surface, which may bind to pattern recognition receptors, many of which have lectinlike specificity.[255] Mannose-containing residues bind mannose receptors thus facilitating phagocytosis by macrophages. Antigen presentation by dendritic cells has been associated with expression of DEC-205 and BDCA-2. DEC-205 is a membrane protein homologous to the macrophage mannose receptor, and BDCA-2 is a type II C-type lectin.[214,215] Down-regulation of these receptors may reduce the risk of extraneous antigen presentation on egress of antigen-presenting cells from the tissue and reprogramming of dendritic cell machinery toward antigen presentation to T cells.

The MHC gene codes for two families of cell surface glycoproteins (classes I and II). Class I MHC molecules include the classical transplantation antigens and are displayed on all cells in the body. Class II MHC molecules are heterodimeric glycoproteins found on the surface of antigen-presenting cells. The MHC gene is polymorphic, therefore a wide variety of class II molecules can be expressed on the surface of antigen-presenting cells.[256]

Endogenous antigen is degraded by intracellular proteases and peptidases to provide peptides in the cytosol. These peptides bind to transporter (TAP) gene products and are delivered to the class I MHC compartment where they are incorporated into the peptide-binding groove generated by folding of the class I molecule before moving to the surface. This pathway requires intact proteosome and Golgi transport function. In dendritic cells, additional mechanisms are involved in class I antigen processing. First, dendritic cells internalize antigens through macropinocytosis and then release antigens into the cytosol for classical TAP-dependent MHC class I presentation.[257,258] Second, peptides produced in high concentrations may be exchanged with peptides on mature MHC class I molecules. Third, carrier molecules or chaperones capture peptides and deliver them through the dendritic cell surface to the endoplasmic reticulum where they are released, processed, and incorporated into MHC class I molecules.

Exogenous antigens including intact organisms, particulates, and soluble antigens are internalized by the phagocytic or endocytic pathway and must undergo degradation and proteolysis before interacting with the MHC class II compartment where peptide loading occurs. Class II α and β chains are synthesized, combined, and transported in association with HLA-DM to the class II compartment. In this compartment HLA-DM dissociates; the class II molecules are membrane bound with their peptide-binding site protected by class II-associated invariant chain peptide (CLIP). Maturation of these vesicles occurs after fusion with the endocytic lysosome pathway.[259] The invariant chains dissociate allowing the appropriate peptides to bind with MHC.[260,261]

Although expression of class II molecules on antigen-presenting cells is assumed to be a constitutive process in macrophages, studies of antigen processing and presentation in dendritic cells demonstrate the complexity of these mechanisms in this antigen-presenting cell subtype. During the immature stages of dendritic cell development, cell surface expression of MHC class II and costimulatory molecules is low and endocytic activity is high.[262,263] On encountering inflammatory stimuli or microbial products, these cells undergo a maturational process in which class II and costimulatory molecules are up-regulated and endocytosis is reduced. After this programmatic shift, dendritic cells are capable of effective activation of naive T cells.[264]

Increased expression of MHC class II during dendritic cell maturation is believed to be primarily regulated by the rate of invariant chain proteolysis by cathepsin S and the subsequent

binding of antigenic peptides to the newly formed class II αβ dimer in lysosomes.[265,266] In immature dendritic cells, cathepsin S activity is inhibited by cystatin C, preventing invariant chain (Ii) proteolysis. This leads to sequestration of nonfunctional class II αβI complexes in the lysosomes. Activation of dendritic cells promotes down-regulation of cystatin C, thus liberating cathepsin S activity. This permits Ii proteolysis and enhanced trafficking of functional class II MHC molecules to the cell surface.

Although Ii proteolysis is assumed to be the primary regulator of class II trafficking during dendritic cell differentiation, additional levels of regulation have recently been identified. Different rates of macropinocytosis and MHC class II recycling have been observed in immature and mature dendritic cells, suggesting that these factors, rather than Ii proteolysis might control class II MHC surface expression.[267] Macropinocytosis in dendritic cells is controlled by small guanosine triphosphatases such as Rac1 and Cdc42, suggesting molecular mechanisms for regulation of class II transport during dendritic cell maturation.[268] Furthermore, cytokines can modulate class II trafficking in monocytes and can regulate cathepsin activity in dendritic cells.[269,270] It is apparent that antigen-presenting cell function is tightly regulated at the level of MHC class II transport.

The CD4 subset of T cells recognizes antigen presentation by cells expressing the class II MHC antigens. The density of MHC class II molecules appears to correlate with their ability to present antigens to the CD4-positive T cell. Lymphokines such as IFN-γ increase the expression of MHC class II antigens and enhance antigen presentation. In contrast, prostaglandins of the E class and glucocorticoids inhibit MHC class II antigen expression and antigen presentation.

Fetal and Newborn Antigen-Presenting Cell Capabilities

Investigations of antigen presentation and processing by mononuclear phagocytes from fetal blood have not been reported. Antigen-processing capabilities of monocytes derived from term cord blood have been evaluated by Hoffman and co-workers.[271] In their experiments, maternal and neonatal blood samples were paired so that single-haplotype sharing between mother and child was sufficient to allow for T-cell recognition of cord blood mononuclear phagocytes.[271] When cord blood–adherent mononuclear phagocytes were exposed to tetanus toxoid antigen and cultured with tetanus-toxoid–specific T cells, a T-cell proliferative response was observed. This response was equivalent to the response seen when maternal blood adherent mononuclear cells were cocultured with antigen and T cells. These investigators concluded that newborn mononuclear phagocytes were competent in presenting tetanus toxoid antigen, but they cautioned that additional studies would be required to determine whether antigen processing of other microbial products was as efficient.

Zlabinger and colleagues[272] examined the capacity of cord blood mononuclear phagocytes to present *E. coli* or tetanus toxoid antigen to cord or paternal T cells. In these studies, T cells were cocultured with either antigen-pulsed or control-adherent mononuclear phagocytes. When cord blood T cells were cocultured with pulsed-adherent mononuclear phagocytes from cord blood, the proliferative response was significantly less than was seen when paternal T cells were cocultured with adherent mononuclear phagocytes. However, in experiments in which cord blood–adherent mononuclear phagocytes pulsed with *E. coli* were cocultured with paternal T cells, enhanced T-cell proliferation was of similar magnitude as that seen with adult autologous antigen-presenting mononuclear phagocytes. These investigators also reconfirmed the observations by Hoffman and colleagues[271] that cord blood mononuclear phagocytes were fully capable of processing and presenting tetanus toxoid antigen to antigen-specific T cells.

Few studies of dendritic cells have been done in human neonates, and those that exist were done with dendritic cells differentiated in vitro from circulating monocytes with a cocktail of cytokines (that is, IL-4 in combination with GM-CSF). These cord blood monocyte–derived dendritic cells have limited antigen-presenting cell function and defective IL-12 production.[169,229,273] However, it is always difficult to generalize data obtained from these in vitro differentiated cells, because they are derived in a static environment containing high concentrations of cytokines and reduced cellular interactions, which might not mimic the physiologic milieu.

Intrinsic functional defects have been identified in immature dendritic cells derived from cord blood monocytes. Hunt and co-workers[274] described low expression of MHC class I and II molecules and adhesion molecules on cord blood–derived dendritic cells. They postulated that this diminished expression contributed to diminished allostimulatory capacity of these dendritic cells compared with adult monocyte–derived dendritic cells. Investigations by Liu and colleagues[229] concurred with findings that cord blood monocyte–derived dendritic cells exhibited reduced CD1a and MHC II expression. They also demonstrated diminished endocytosis related to low mannose receptor expression and reduced allostimulatory activity by cord blood compared with adult dendritic cells.[229]

Defective antigen-presenting cell function has been hypothesized to contribute to increased infection susceptibility in newborns. Drohan and colleagues[275] characterized antigen-presenting cell subsets in adult peripheral blood and cord blood. Adult peripheral blood had a higher proportion of CD11c+ dendritic cells, whereas cord blood mainly contained CD123+ dendritic cells. Adult blood was enriched in CD16+CD11c+ dendritic cell subset, whereas CD34+CD11c-CD123lo cells were prominent in cord blood. LPS-induced TNF-α production was dampened in myeloid dendritic cells and monocytes from cord blood, whereas IL-1α production was not different. These investigators reasoned that reduction in TNF-α response did not result from reduced surface detection of LPS, because CD14, Toll-like receptor (TLR)-4 and TLR-2 levels were not reduced in cord blood compared with adult antigen-presenting cells. Also, no correlation was observed between TLR-2 or TLR-4 levels and TNF-α production in myeloid dendritic cells and monocytes. Cord blood monocytes had lower surface HLA-DR immediately ex vivo. Both adult blood and cord blood monocytes up-regulated HLA-DR after incubation, but an additional LPS-induced increase in HLA-DR was suggested only in adult blood monocytes.

Mononuclear Phagocyte Activation

The enhanced activity of mononuclear phagocytes against microorganisms and tumor cells requires interaction with sensitized lymphocytes.[276] T lymphocytes stimulated by previous exposure to specific antigens release soluble factors that induce mononuclear phagocytes to enhance microbicidal and tumoricidal activity.[277] Considerable evidence now exists that IFN-γ is the factor produced by T lymphocytes that induces macrophage activation.[276]

IFN-γ is a highly basic heterogeneous glycoprotein with a molecular weight of 40,000 to 70,000 daltons.[277] The biologically active form of the natural protein consists of dimeric aggregates. Recombinant IFN-γ is not glycosylated and has a molecular weight of 17,000 daltons. The effects of IFN-γ on mononuclear phagocytes are initiated by engagement of IFN-γ with specific receptors on the cell surface.[278] Adams and Hamilton[279] have reviewed the signal transduction mechanism through which IFN-γ binding produces immediate and sustained functional responses. These investigators have proposed that mononuclear phagocyte activation should not refer solely to the effects of a stimulus such as IFN-γ thus leading to a proscribed change in morphology or function, but rather activation may be used to describe a composite of multiple specific capacities that enable the mononuclear phagocyte to perform a complex function.[279]

Mononuclear phagocytes play a key role in the interface between innate and adaptive immunity. Both tissue macrophages and dendritic cells recognize pathogens through a variety of receptors, induce potent stimulatory molecules, and initiate microbicidal pathways. Brightbill and colleagues[280] observed production of IL-12 and NO synthase by macrophages in response to microbial lipoproteins recognized through TLRs. Dendritic cells also produce IL-12, which is capable of inducing T lymphocyte activation and subsequent IFN-γ production.[281,282]

Marodi and co-workers[283] examined the mechanisms used by cord blood and adult blood monocyte–derived macrophages to ingest and kill *C. albicans.* They determined phagocytosis and killing of *Candida* sp. to be equivalent between cord blood and adult blood monocyte–derived macrophages both in the presence and the absence of serum. Exposure of cord and adult macrophages to IFN-γ resulted in quantitatively different results. Candidacidal activity and superoxide anion generation was maximal for adult macrophages at 100 units/mL interferon-γ, whereas the magnitude of Candidacidal activity and superoxide production by cord blood–derived macrophages remained low at a dose up to 500 units/mL of interferon-γ. The mechanism accounting for this disparity in macrophage activation was not caused by differences in interferon-γ receptor density. Recent work by Marodi and co-workers[284] analyzed interferon-γ signaling through STAT-1 in cord blood and adult blood monocytes and monocyte-derived macrophages using flow cytometry. They found comparable expression of STAT-1 between cord and adult cells; however, phosphorylation of STAT-1 in response to interferon-γ was significantly decreased in neonatal monocytes and monocyte-derived macrophages compared with findings in adult cells. These data suggest that defective interferon-γ signaling through STAT-1 in neonatal mononuclear phagocytes might contribute to the unique susceptibility of neonates to intracellular pathogens.

Neonatal peritoneal and blood macrophages are known to be ineffective in antigen-presentation functions, and this manifests as inefficient MHC class II expression in response to IFN-γ. The underlying mechanisms responsible for this maturational deficiency have not been completely elucidated. Lee and co-workers[285] reported that MHC class II expression in alveolar macrophages from neonates are refractory to IFN-γ stimulation, and by examining the intracellular pathway leading to MHC class II expression they demonstrated that the site of the impairment is at the level of transcription. Thus expression of mRNA encoding the class II transactivator (CIITA), MHC class II, and Ii was low or undetectable in neonatal alveolar macrophages stimulated with concentrations of IFN-γ that induced adult AM to up-regulate MHC class II expression. The failure of alveolar macrophage from young animals to express MHC class II was not simply the result of deficient IFN-γ receptor function because IFN-γ-responsive genes such as IRF-1, IRF-2, and IP-10 were up-regulated in a dose-dependent manner from animals of all ages investigated. The responsiveness of neonatal alveolar macrophages to IFN-γ, as determined by MHC class II expression, could be modulated to adult levels when pre-cultured in vitro. This suggests that microenvironmental factors operative in vivo may play a role in suppressing the expression of MHC class II in alveolar macrophages from young animals. Type I interferons were not found to be responsible for the inability of alveolar macrophages from juvenile animals to induce MHC class II in response to IFN-γ.[285]

Monocyte Function in the Fetus and Preterm Neonate

The innate immune system of the preterm infant is considered to be immature because multiple host defense proteins are low, immune cell numbers are low, and immune cell functions are low compared with cells from older children or adults.[286] Very little information is available about innate immune responses and their modulation in the fetus, although such responses might be central to a number of injuries associated with preterm birth. Fetal exposure to inflammation might either increase or suppress subsequent immune actions.[286] Immune responsiveness could be decreased by exposure to inflammatory products resulting in the fetal equivalent of endotoxin tolerance. For example, plasma interleukin IL-6 concentrations are strikingly decreased in fetal sheep after repetitive administration of low dose *E. coli* endotoxin.[287] Alternatively, increased responsiveness is characterized by increased production of hydrogen peroxide and augmented phagocytosis by blood monocytes in fetal sheep seven days after maternal glucocorticoid exposure.[288]

The fetus is generally recognized as immunologically naive, but chronic indolent chorioamnionitis caused by commensal or low pathogenic organisms occurs in the majority of pregnancies complicated by preterm labor and delivery before 30 weeks gestation.[289-291] Fetal exposure to infection or a fetal systemic inflammatory response increases neonatal morbidity and mortality.[292-295] Approximately 30% of very-low-birth-weight preterm infants also develop nosocomial infections that have no identifiable source.[296] The role of in utero infection, inflammation, and medication exposure on the developing immune system is complex.

Temporary suppression of innate immune cell response has been referred to as endotoxin tolerance.[297,298] The concepts of endotoxin tolerance have been developed primarily in mice and in adult human. In adult mice, endotoxin exposure decreases TNF–α and IL-1 secretion by monocytes.[297] Mechanisms proposed for endotoxin-induced tolerance include down-regulation of endotoxin receptors, LPS-binding protein, or CD14 and the alteration of signaling pathways to decrease NF-κB nuclear translocation. Alternatively, changes in cytokine expression profiles or in inhibitory pathways in the cells might be operative.

Endotoxin tolerance has been used to describe in vivo models in which normally lethal doses of endotoxin can be tolerated by animals that were previously exposed to sublethal doses of endotoxin.[297] Chronically catheterized preterm sheep respond to intravascular endotoxin with a tolerance response.[287] Preterm infants are extremely susceptible to nosocomial infections several weeks after preterm birth, at a time when they are clinically stable.[299] This susceptibility to infection is consistent with immune tolerance.

The responses of monocytes to endotoxin are complex. In fetal sheep models of intraamniotic endotoxin exposure, suppressed production of hydrogen peroxide and IL-6, and decreased expression of CD14 and MHC II receptors have been observed.[288] These responses are typical of endotoxin tolerance. In contrast, increased hydrogen peroxide and IL-6 production and increased TLR4 expression two weeks after in utero endotoxin exposure indicate an augmented innate immune response. The enhanced response 14 days after endotoxin exposure might represent recovery from endotoxin tolerance or maturation of monocyte function. Unique aspects of studies in which intraamniotic endotoxin was administered include the induction of lung maturation and impaired lung development.[300,301] Despite the use of higher endotoxin doses that would have been lethal if given intravascularly, no impairment of fetal well being or growth was observed. The lung inflammation required direct contact of endotoxin with the fetal lung, presumably by mixing of fetal lung fluid with amniotic fluid as a result of fetal breathing. Provision of intraamniotic endotoxin more than 30 days before preterm delivery resulted in increased monocytes and lymphocytes in the alveolar fluid of preterm lambs. It is noteworthy that intraamniotic endotoxin also modified the systemic monocytes to an immune tolerance phenotype. In a similar manner, repeated exposure to endotoxin at 7-day intervals resulted in profound suppression of hydrogen peroxide production by systemic monocytes from preterm sheep.[288] Clinically, chorioamnionitis is often indolent and persists for weeks or months.[290] Chronic fetal exposure to inflammation could cause a profound and persistent immune tolerance and thus put these infants at risk for nosocomial infection.[302]

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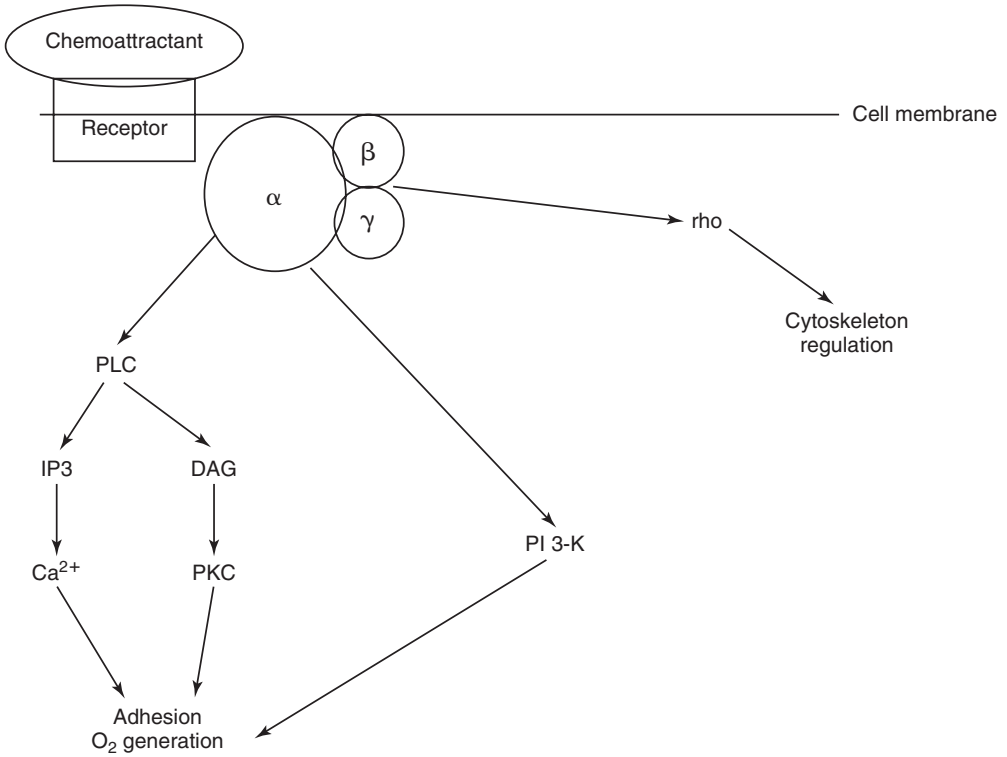


Figure 154-1 Neutrophil activation signaling pathways. *Ca^{2+},* calcium ions; *DAG,* diacylglycerol; *IP3,* inositol triphosphate; *PI 3-K,* phosphatidylinositol 3-kinase; *PKC,* protein kinase C; *PLC,* phospholipase C; *rho,* Rho subfamily protein. *(Modified from Burg ND, Pillinger MH: The neutrophil: function and regulation in innate and humoral immunity,* Clin Immunol *99:7-17, 2001.)*

have been demonstrated to correlate with the inability of neonatal PMNs to achieve a fully bipolar shape change on stimulation.[15]

Measurement of light-scattering response to FMLP has been used to assess whether defective neonatal neutrophil tubular assembly is a result of impaired signal perception or immature responsiveness of the cytoskeleton. Light-scattering effects are the result of an extremely synchronous and massive rearrangement of organelles in the responding neutrophils. Wolach and colleagues[16] compared the response of neonatal and adult neutrophils with FMLP stimulation. Cell polarization of adult neutrophils and that of neonatal neutrophils were similar when these cells were stimulated with low FMLP concentrations, but with increasing FMLP concentrations, neonatal PMN polarization was reduced compared with adult PMNs. These observations suggest that neonatal neutrophils have a fully functional signal perception, but that their cytoskeleton framework fails to comply with the demands of a strong signal.

Taniuchi and associates[17] confirmed these findings by demonstrating differential actin polymerization in adult and neonatal PMNs after stimulation with FMLP. No difference was found in binding capacity of FMLP receptors between adult and cord neutrophils, but actin polymerization was significantly decreased in cord blood neutrophils compared with adult PMNs.

Merry and associates[18] investigated the role of actin polymerization and its regulation by protein kinase C and by phosphatases in the defective neutrophil chemotaxis of the human newborn. Neutrophils isolated from adult volunteers and healthy term newborns were studied for assessment of chemotaxis by flow cytometry. The neutrophils from neonates demonstrated reduced actin polymerization in response to chemotactic agents that act through cell surface receptors, but not with those that act directly on cytoplasm. This finding suggests that a defect in cell signal transduction may be one of the factors implicated in defective chemotaxis. Phosphatase 1 and 2A inhibitors inhibit chemotaxis but not actin polymerization. Therefore, phosphatase 1 and 2A can be considered chemotaxis regulators, but not through actin polymerization.

ADHERENCE

The events of phagocytosis begin within minutes of tissue damage or pathogenic invasion with the accumulation and adherence of neutrophils to endothelial surfaces at the site of infection. Recruitment of neutrophils to the site of infection involves a coordinated series of events mediated by three families of adhesion receptors: selectins, integrins, and the immunoglobulin G (IgG) gene superfamily. The selectins promote the initial rolling or tethering of the neutrophils to the endothelium under the shear force of blood flow, whereas the integrins induce firm adhesion.[19] This initial process is mediated by three selectins, which have been identified as P- and E-selectins (CD62P, CD62E), induced by inflammatory cytokines on endothelial cells, and L-selectin (CD62L), constitutively expressed on circulating neutrophils. On the neutrophil surface, L-selectin (CD62L) interacts with specific oligosaccharide moieties on endothelial cell surface glycoproteins, whereas on the endothelium, E-selectin (CD62E) and P-selectin (CD62P) similarly recognize specific neutrophil carbohydrate moieties.

Selectins are critical to neutrophil transmigration by virtue of their ability to tether leukocytes in a reversible fashion under conditions of shear flow, a process that mediates their rolling on the endothelium of inflamed vessels. Within minutes of activation with certain agonists (phorbol myristate acetate, histamine, thrombin, C5a, or oxidants) or inflammatory mediators (interleukin-8 or platelet-activating factor), selectins are translocated to the plasma membrane, following which neutrophil attachment is facilitated by β_2-integrins, leukocyte factor antigen-1 (i.e., CD11a/CD18), and macrophage-1 antigen (Mac-1) (i.e., CD11b/CD18).

Thus contact with selectins begins the process of neutrophil adhesion by slowing the velocity of neutrophil flow and increasing the duration of intercellular and membrane contact with the endothelium. Next, firmer attachment is accomplished through the action of the β_2-integrins and intercellular adhesion molecules ICAM-1 and ICAM-2, which mediate neutrophil arrest and transmigration. ICAM-1 (CD54) and ICAM-2 are members of the IgG gene superfamily and are expressed constitutively on endothelial cells, increasing when the endothelium is inflamed. ICAM-1 is the ligand for leukocyte factor antigen-1 and Mac-1 and is required for neutrophil arrest and transmigration.[20] Neutrophil-neutrophil adhesion (aggregation) also involves initial tethering through L-selectin followed by β_2-integrin–dependent firm adhesion.[21]

Neutrophils use various signaling pathways to link receptors for adhesion with effector molecules in the cytoplasm and nucleus. The Rho family of GTPases, members of the Ras superfamily, plays a key role in regulating these responses. The role of the Rho GTPases in phagocyte cell function has been studied. Williams and associates,[22] using a murine model genetically deficient in hematopoietic-specific Rho GTPase, Rac-2, demonstrated that Rac-2 plays an essential and unique role in neutrophil rolling by L-selectin, chemotaxis, and phagocytosis.

Several investigators have reported deficient adhesion of neonatal neutrophils to endothelial cells as a result of decreased expression of integrins, diminished expression of L-selectin, and a rigid cytoskeleton that prevents redistribution of adhesion sites. Lorant and associates[23] demonstrated decreased P-selectin expression in endothelial cells lining mesenteric venules and umbilical veins of premature infants compared with term infants. Reports differ regarding the resting and stimulated expression of Mac-1 on neonatal neutrophils. Several investigative groups have demonstrated that the expression of Mac-1 in neonates is equal to that in adults; however, the total cell content of Mac-1 is decreased.[24] Rebuk and co-workers,[25] however, reported a decrease in baseline expression of neonatal neutrophil Mac-1 and a stimulated expression equal to that in adults. Additional studies have shown that neonatal neutrophils fail to up-regulate Mac-1 surface expression to the same extent as for adult neutrophils in response to chemotactic stimulation. Mariscalco and colleagues[24] demonstrated no significant difference in the stimulated expression of Mac-1 in neonatal and adult neutrophils but showed a significant decrease in the expression of L-selectin in neonatal over than in adult neutrophils and a significant decrease in the interaction of neonatal neutrophils with human umbilical vein endothelial cells compared with those from adult subjects.

HEMATOPOIETIC GROWTH FACTORS IN NEONATAL SEPSIS

Sepsis continues to be an important cause of morbidity and mortality among both full-term and preterm infants, secondary to an immaturity in neonatal host defenses. The dysregulation of the expression and production of hematopoietic cytokines in the neonate contributes to the quantitative and qualitative deficiencies in neonatal myeloid progenitor activity and decreased availability and function of mature effector neutrophils. Two myeloid-stimulating cytokines, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), have been shown to augment the number of circulating neutrophils, promote their maturation and activation, and extend neutrophil lifespan.

Granulocyte Colony-Stimulating Factor

G-CSF is a lineage-specific hematopoietic growth factor that stimulates the proliferation and differentiation of myeloid progenitor cells in the bone marrow, induces the egress of mature cells from the neutrophil storage pool, and enhances mature neutrophil effector function.[26] G-CSF improves neutrophil function by increasing adhesion and by stimulating chemotaxis and phagocytosis, and it could improve neutrophil functions that are deficient in the neonate. Significant differences between adults and newborns, especially preterm infants, have been reported in GCSF serum levels, mRNA expression, and activity. Laver and colleagues have studied the plasma levels of G-CSF and GM-CSF in the umbilical cord blood of normal term newborns and have reported higher levels than those seen in adults.[27] Ohls and co-workers[28] reported that in term and preterm neonatal neutrophils, G-CSF significantly increased expression of β_2-integrin to a greater extent than levels observed in adult neutrophils. However, L-selectin expression in term and preterm neonatal neutrophils decreased after G-CSF incubation and decreased further after stimulation.[28] Cairo and associates have shown that G-CSF production from stimulated adult mononuclear cells was higher than that from cord blood mononuclear cells ($P < .007$).[29] Additionally, significantly more G-CSF mRNA transcripts were produced from adult than from cord mononuclear cells. No differences were found in affinity, binding, and number of G-CSF receptors between cord and adult peripheral effector cells.[29]

Granulocyte-Monocyte Colony-Stimulating Factor

Bailie and co-workers[30] have reported an inverse linear relationship between maternal GM-CSF levels and gestational age. In neonates, a quadratic (nonlinear) association was found between GM-CSF and gestational age. Furthermore, levels were not related to neutrophil count, perinatal complications, or the presence of infection.

Cairo and associates[31] previously reported the effects of GM-CSF on neonatal neutrophils. In a study of the effect of GM-CSF on neonatal neutrophil function, GM-CSF directly up-regulated the expression of the C3bi (complement receptor) in neonatal neutrophils, which resulted in increased aggregation and adherence. Results of a phase I/II trial using GM-CSF in very low-birthweight neonates demonstrated that a 7-day regimen of GM-CSF in this patient population appears to be safe and well tolerated. Additionally, the use of GM-CSF induced a significant increase in the absolute neutrophil count, absolute monocyte count, platelet count, neutrophil C3bi receptor expression, and bone marrow neutrophil storage pool.[32]

A study by Molloy and colleagues[33] evaluated the effect of G-CSF and GM-CSF on neonatal neutrophil function in both normal mothers and neonates and those at high risk for sepsis. Results indicated that G-CSF enhances neonatal neutrophil survival with no significant impact on neutrophil function (CD11b, reactive oxygen intermedicates (ROI). By contrast, after LPS priming, GM-CSF significantly increased neutrophil respiratory burst and CD11b expression. In summary, Molloy's group showed that GCSF has a greater postsurvival effect in neonatal neutrophils than GM-CSF, even after LPS priming. However, GM-CSF augments neonatal neutrophil function by up-regulating both CD11b expression and ROI activity, either alone or in response to LPS. A recent randomized control trial comparing G-CSF and GM-CSF in neonates found that the absolute neutrophil count was significantly increased in the G-CSF group compared with the placebo or GM-CSF therapy group.[34] G-CSF has the greatest antiapoptotic effect on neonatal neutrophils, and GM-CSF has the greatest effect on function.

DEFORMABILITY

The movement of adherent neutrophils through vessel walls requires the expansion of pseudopods between endothelial cells, opening a passageway for the remaining portion of the leukocyte. The neutrophil subsequently moves freely in the intercellular

spaces under the influence of various chemotactic agents. This movement and diapedesis depend on energy, provided by ATP, and on the neutrophil locomotor apparatus.[11]

Neutrophils from newborn infants, compared with PMNs from adults, have been demonstrated to have significantly decreased deformability,[35,36] especially in immature forms.[37] Immature neutrophils from neonates and adults are both less deformable and larger than with mature PMNs. During stressful conditions, such as neonatal sepsis, up to 100% of the circulating neutrophils may be immature. In addition, evidence suggests that neutrophils activated in vivo or in vitro with chemoattractants are much stiffer than unactivated (basal) neutrophils. In particular, pseudopods, which are formed as a result of polymerization and alignment of actin molecules near the surface membrane, are extremely rigid.[38]

Studies of neutrophil transmigration through endothelial monolayers have shown that term neonatal PMNs display approximately 50% transmigration relative to that in adult PMNs, and antibody-blocking studies have demonstrated that these neonatal PMN abnormalities may be explained, in part, by reduced Mac-1 expression.[39]

Lorant and associates[23] demonstrated that delayed transmigration of neutrophils in the neonatal rat correlates with a defect in the expression of P-selectin on the surface of the endothelial cells. In an animal model of inflammation, radiolabeled neutrophils from adult rats transmigrated into the peritoneum of adult rats five times more efficiently than they did through the peritoneum of neonatal rats. The decreased P-selectin expression was associated with decreased numbers of P-selectin storage granules and decreased P-selectin transcription.

CHEMOTAXIS

The most consistently observed functional abnormality of neonate neutrophils is reduced chemotaxis. *Chemotaxis* is the directed migration of a cell toward a chemoattractant along a concentration gradient. In most assays, neutrophils from newborn infants migrate at approximately half the speed observed for adult cells.[40-42] The ability of neutrophils to undergo chemotaxis implies the possession of a sensory mechanism that must be linked to the locomotor apparatus of the cell. After chemotactic factor-receptor interaction, a complex sequence of events occurs: activation of phospholipases, breakdown of phosphoinositol, activation of protein kinase C, an increase in intracellular calcium, release of eicosanoids, and activation of kinases and phosphatases.[7,43] This sequence of events leads to neutrophil-directed migration. The cells migrate from an area of low concentration (i.e., blood vessel walls) to an area of high concentration (site of infection or inflammation), whereupon the chemotactic factors become potent activators of neutrophils.[44]

Since the 1980s, several investigators have studied neonatal neutrophil responses to chemoattractant agents and have determined that neonatal PMNs have normal random migration, but a decreased ability to move toward defined chemoattractant stimuli in comparison with adult PMNs.[42,45,46]

The neutrophils of clinically stable preterm neonates as immature as 24 weeks gestation appear to be functionally very similar to those of term infants.[47] Expression of both L-selectin and Mac-1, after stimulation, is similar to that in term PMNs.[25,47] However, in a study of preterm neonates, PMN Mac-1 content (both in the cytoplasm and on the cell surface) ranged from 10% of adult levels at 27 weeks gestation to 48% at 36 weeks gestation to 57% in term neonates. This study showed a high correlation between PMN Mac-1 content and gestational age.[48]

Sacchi and colleagues[49] demonstrated that chemotactic responses of PMNs in term neonates mature to those of normal adult PMNs by 2 weeks of postnatal age, and those in preterm neonates (born at 34 to 36 weeks) reach adult values by 40 to 42 weeks of postnatal age. Eisenfeld and co-workers[50] similarly demonstrated improvement in term neonatal PMN chemotaxis in term neonates from 40% of adult values at birth to normal adult values between 10 and 32 days of postnatal age. Carr and associates[47] studied preterm infants (24 to 32 weeks gestational age) over a 2-month period and found that chemotaxis improved during the second month of life but remained abnormal in comparison with that in adults.

Impaired expression and up-regulation of receptors may additionally contribute to defective chemotaxis. Falconer and associates[51] demonstrated that neutrophils from preterm and stressed neonates have significantly fewer receptors for complement factors C3b and iC3b than those from term neonates and adults.

Reddy and co-workers[52] studied CR3 expression in neonatal and adult PMNs. Neonatal neutrophils, on average, expressed less CR3 in comparison with adult PMNs. However, because of the higher concentration of neutrophils in umbilical cord blood versus the adult, the absolute number of neutrophils in cord blood expressing high amounts of CR3 was equivalent to that in adult blood. High proportions of immature PMNs in cord blood may explain the previous findings of deficient CR3 expression. Therefore the typical neutrophilia of cord blood can be considered a compensatory event in providing the adult concentration of mature neutrophils.

Human complement component C5a is an important mediator of the inflammatory response, neutrophil chemotaxis, and activation. Nybo and associates[53] demonstrated that C5a uptake was significantly lower in neonatal neutrophils than in adult PMNs. Using FMLP and C5a as stimuli, less migration was observed in neonatal neutrophils than in adult neutrophils.

Various abnormalities of Mac-1 receptors correlate closely with the reduced chemotaxis observed in neonatal neutrophils. In resting neutrophils, Mac-1 receptors are distributed evenly on the cell surface, but during cell locomotion, these receptors are translocated to the tail of the cell, whereas new receptors are translocated from stores in cytoplasmic granules to the leading edge of the migrating cell. This process in neonatal neutrophils is not up-regulated to the same extent as in adult PMNs. The defect is thought to be caused by both a reduced total PMN content of preformed Mac-1, which in term neonates is approximately 60% of adult PMN Mac-1 levels, and a reduced translocation of preformed new receptors from cytoplasmic storage to the PMN surface. The secondary translocation of these receptors to the uropod is also impaired in the neonatal PMN.[54]

Abnormalities in events that follow initial chemotactic factor-receptor interaction have been suggested to contribute to defective PMN chemotaxis. Changes in membrane potential, in the generation of inositol, and in release of calcium ions from intracellular stores have been reported to be impaired in neonatal neutrophils.[55-57] Specific cytoplasmic granules constitute essential supply for the new membrane receptors required for PMN movement. The number of lactoferrin-containing granules appears to be decreased in neonatal neutrophils.[58] Chemoattractant-induced hexose uptake by neonatal neutrophils[59] and release of leukotriene B_4[60] also are decreased when compared with adult PMNs.

Chemotaxis of neonatal and adult neutrophils was compared after stimulation with two chemoattractants: FMLP and DL1.2, a chemotactic monoclonal antibody that binds to a specific neutrophil antigen.[61] Stimulated neonatal neutrophils demonstrated reduced chemotaxis in comparison with adult PMNs, suggesting an aberration in chemoattractant-induced signaling.[62] FMLP induced a rapid increase in intracellular calcium in both adult and neonatal PMNs, but this response was of lesser magnitude in neonatal PMNs. Moreover, a subpopulation of unresponsive neonatal neutrophils was identified.

Impaired polarization and chemotaxis in neonatal neutrophils may result from insufficient production of hematopoietic growth

factors, such as G-CSF and GM-CSF. The in vitro effects of G-CSF and GM-CSF on neutrophil chemotaxis and polarization were studied in isolated PMNs of neonates compared with adults. Both G-CSF and GM-CSF significantly enhanced the chemotaxis of neonatal neutrophils, thereby normalizing their chemotactic defect. This effect was more pronounced when chemotaxis was severely impaired. The reduced polarization, observed in neonatal neutrophils, was corrected after in vitro incubation with both G-CSF and GM-CSF.[16]

PHAGOCYTOSIS

Neutrophils are the predominant mobile phagocytes of the circulating blood. They move from the circulation by adherence to vascular endothelium and migrate through the walls of small blood vessels to the site of infection, where they kill and ingest foreign organisms. Neutrophils possess a selective ability to recognize foreign antigens and have cell surface receptors for the Fc portion of the IgG molecule as well as the C3b complement component. Humoral factors known as *opsonins* coat the foreign antigen and make it more susceptible to phagocytosis after the specific adherence between the opsonized antigen and the neutrophil cell surface receptors. The best-described opsonins are heat-stabile antibodies, immunoglobulins, and complement, particularly the heat-labile component C3b.[61] Preterm and term neonates have reduced production of immunoglobulins. Because most of the IgG is transferred during the last weeks of gestation, premature infants have decreased levels of circulating IgG. Preterm neonates have significantly lower concentrations of complement components and total hemolytic complement activity compared with term neonates. Thus decreased IgG and complement components in the neonate may result in deficient opsonization.[63]

Both the Fab and Fc portions of the IgG immunoglobulin molecule enhance phagocytosis. The Fab portion adheres to the bacteria, whereas the Fc portion adheres to receptors on the neutrophil. Neutrophils express three different types of receptors for the Fc component of IgG: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). FcγRII and FcγRIII are low-affinity receptors normally expressed on neutrophils, and they bind to polyvalent IgG complexes. FcγRI is a high-affinity receptor for IgG and is absent from resting neutrophils; it is considered to be a marker of activated neutrophils. The interaction of these Fc receptors and immunoglobulin initiates the process of phagocytosis, degranulation, and antibody-dependent cellular cytotoxicity (ADCC). Neutrophils from preterm infants express FcγRII and FcγRIII to a significantly lesser extent than neutrophils from term infants and adults.[64,65] Fjaertoft and associates[66] reported that the expression of FcγRI on neutrophils from preterm infants was similar to that in term infants, older infants, children, and adults. Most neutrophils from patients with sepsis express CD64 (FcγRI), and up-regulation of CD64 on neonatal neutrophils is regarded as an indicator of sepsis.[67] This correlation also has been observed in newborn infants: The expression of FcγRI was significantly higher in neutrophils from neonates with bacterial infection than in both noninfected preterm and term newborn infants.[65] Other investigators demonstrated that FcγRI (CD64) expression also is a very sensitive marker for diagnosing late-onset nosocomial infection in very low-birth-weight infants.[68]

The functional response of the stimulated neutrophil, including the cross-linking of Fcγ receptors, involves a complex cascade of biochemical events. The regulation of phagocytosis has been associated with the cross-linking of Fcγ receptor by the induction of tyrosine phosphorylation of FcγRII itself. After ligand binding, there is a rapid induction of protein phosphorylation of the several nonreceptor proteins by the activation of multiple kinases, including tyrosine kinase, protein kinase C, and mitogen-activated protein (MAP) kinase. Protein kinase C and Raf-1 have been shown to translocate to the plasma membrane during the first minutes of neutrophil phagocytosis, in which Raf-1 initiates the MAP kinase cascade, activating extracellular signal-regulated protein kinases 1 and 2 (ERK1 and ERK2), which are required for the ingestion of IgG-opsonized particles.[69] Ydrenius and associates[70] reported that cyclic adenosine monophosphate (cAMP) and cAMP-dependent protein kinase (cAPK) regulate F-actin reorganization during IgG-FcR receptor–mediated neutrophil phagocytosis.

The γ subunit of the Fc receptor contains a cell activation motif referred to as an immunoreceptor tyrosine-based activation motif (ITAM). ITAM tyrosines are phosphorylated after Fcγ clustering, and the phosphorylated ITAMs create sites for the assembly of Src homology 2 (SH2) domains including the Syk tyrosine kinase.[71] Syk plays a critical role in the phagocytic pathway mediated by the FcγRs. Matsuda and associates,[72] using antisense oligonucleotides, reported that ablation of Syk expression significantly reduced FcγR-mediated phagocytosis in human monocytes. Furthermore, transfection of Syk into COS1 cells has been shown to enhance FcγR-mediated phagocytosis, and Syk-deficient macrophages do not signal for FcγR-mediated phagocytosis.[73] Hunter and co-workers[74] identified the regions of Syk that are critical for interaction with the phagocytic pathway and demonstrated that an intact kinase domain is required for facilitating phagocytic signaling by FcγRs.

Activation of the complement system, resulting in the formation of C3b, can be initiated either by the bacteria itself through the alternate complement pathway or by the interaction of specific antibody with microbial antigen and activation of the classical pathway. The complement receptor 3 (CR3, Mac-1, CD11b/CD18) functions as a nonopsonic receptor and is the specific receptor for the complement fragment C3bi. CR3 (a β_2-integrin) is also a receptor for extracellular proteins and, as a lectin, recognizes many different sugars. CR3 is a heterodimer consisting of CD18, the protein common to the β_2-integrins, and CD11b, which contains the C3bi binding site and recognition domain of matrix proteins. An internal peptide sequence in the fibrinogen γ chain has been shown to function as the high-affinity binding site for C3R on neutrophils. Soluble human fibrinogen has been demonstrated to activate neutrophils through a CD11b-dependent mechanism that results in an up-regulation of CD11b, enhancement of phagocytosis, and increased degranulation.[75]

C3R also is involved in the selective internalization of pathogenic mycobacteria. The phagocytosis of mycobacteria by neutrophils under nonopsonic conditions elicits the production of superoxide (O_2^-) and the release of specific granule proteins. The mechanism of the nonopsonic phagocytosis is reported to involve CR3, which associates with a glycosylphosphatidylinositol-anchored protein (GPI) and relocates to cholesterol-rich domains in the cell membrane where the mycobacteria are internalized. When CR3 is not associated with a GPI, it remains outside these domains and mediates phagocytosis of opsonized particles.[76]

Once the opsonized microbe and neutrophil establish contact, a cohesive force prevents their separation. The microbe binds to specific receptors on the surface of the neutrophil through microbe-bound opsonins (IgG, C3b). The sequential interaction between opsonins and membrane receptors leads to submembranous activation of neutrophil contractile microfilaments, resulting in the flow of pseudopods completely surrounding the microbe. The opposed membranes of the pseudopods fuse and then invaginate the captured microbe, enclosing it in an internalized vacuole called a *phagosome.* Ingestion of the foreign particle is an active process requiring ATP glycolysis, glycogenolysis, and oxidative phosphorylation. After the phagosome is formed, degranulation begins, and cytoplasmic azurophilic and specific granules fuse with the phagosome. The granules rupture and discharge toxic chemicals into the phagosome.

Colony-stimulating factors and cytokines have been reported to enhance neutrophil function. Interferon-γ treatment is associated with a significant increase in the expression of the high-affinity Fcγ receptor (peaking 48 hours after exposure to the cytokine), as well as a significant increase in neutrophil phagocytosis of opsonized *Staphylococcus aureus*. This increased phagocytosis after interferon-γ treatment correlates with FcγRI expression by the neutrophils.[77] Recombinant GM-CSF and G-CSF have been shown to enhance phagocytic activity of mature neutrophils.[78] In vitro studies have demonstrated enhanced neutrophil C3bi expression and bacterial killing in neonatal neutrophils incubated with GM-CSF.[31] In addition, Campbell and associates[79] reported an enhancement of phagocytosis of opsonized type III group B streptococci (III GBS) by neonatal neutrophils after pretreatment with G-CSF and GM-CSF but not tumor necrosis factor-α. These workers also demonstrated the abrogation of the heightened killing of III GBS by blocking FcγRIII receptors and that phagocytosis was further enhanced by the addition of purified IgG containing III GBS–specific antibody to the GM-CSF–treated neutrophils.[79]

DEGRANULATION

Bacterial killing occurs within a matter of minutes after phagocytosis. The neutrophil makes tight contact with its target, and the plasma membrane flows around the surface until the bacterium is completely enclosed, thus forming a phagosome. The formation of a phagosome limits the amount of extracellular fluid, and the exclusion of external medium allows a new environment to be established that will provide favorable conditions for the biochemistry of oxidant production and bacterial killing. Killing is initiated within seconds of ingestion by two interrelated processes: *degranulation,* the release of granule contents into the phagosome, and the *respiratory burst,* which consists of a series of oxidation-reduction reactions. Degranulation begins with the fusion of cytoplasmic azurophilic (or primary) and specific (or secondary and tertiary) granules with the phagosome. The granules then rupture and discharge their contents into the phagosome[80] Figure 154-2). Thus the contents of the cytoplasmic azurophilic and specific granules constitute a significant proportion of the phagosomal volume. These granule contents generally are membrane-active cationic proteins and peptides[79] whose affinity for the negatively charged microbial surface depends not only on electrostatic interactions but also on their tertiary structure[81,82] (Table 154-1).

Azurophil (primary) granules can be distinguished from specific granules by their affinity for the basic dye, azure A, owing to a high content of acid mucopolysaccharide and myeloperoxidase, which are present only in azurophil granules. Azurophil granules are further characterized by their content of hydrolytic and bactericidal proteins such as elastase, bactericidal permeability-increasing protein (BPI), defensins, and myeloperoxidase. The contents of the azurophil granules are released into the phagosome, thereby exposing ingested microorganisms to high concentrations of hydrolytic enzymes, antimicrobial proteins and peptides, and reactive oxygen intermediates.

Myeloperoxidase, a major constituent of the azurophilic granules, is critical for oxidative killing. It is a classic heme peroxidase that uses hydrogen peroxide (H_2O_2) to oxidize a variety of aromatic compounds and is unique in readily oxidizing chloride ions to the strong nonradical oxidant HOCl. HOCl is the most potent bactericidal oxidant known to be produced by the neutrophil, and many species of bacteria are killed by the myeloperoxidase-H_2O_2-chloride process.[82] Bacterial targets include iron-sulfur proteins, membrane transport proteins, ATP-generating systems, and the origin of replication sites for DNA synthesis. Chloramines are generated indirectly through the reaction of HOCl with amines, and these also are bactericidal. Myeloperoxidase can also generate peroxides and hydroxylated derivatives of phenolics, such as salicylate in the oxidative reactions.[82]

BPI is a 55-kDa cationic protein produced only by precursors of neutrophils and stored in the azurophilic granules. BPI plays a dominant role in the bactericidal activity against gram-negative

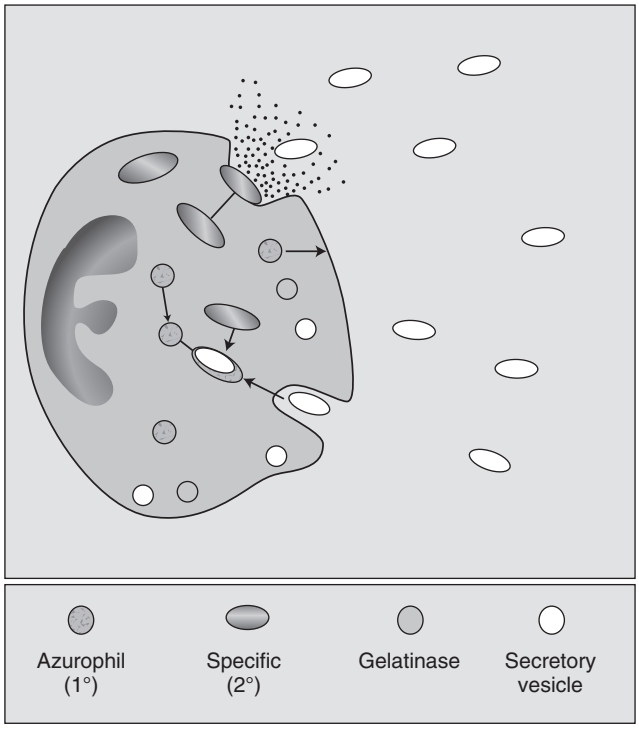


Figure 154-2 Neutrophil degranulation of antibiotic proteins and peptides. An activated neutrophil in the process of phagocytosis of gram-negative bacteria *(open ovals)* is demonstrated. As shown, specific (secondary) granules are more prone to discharge their contents (including lactoferrin and cathelicidins) into the extracellular space. By contrast, with azurophil (primary) granules, containing bactericidal permeability-increasing protein and defensins, degranulation occurs predominantly into the phagolysosome. To a lesser extent, specific granules also release their contents into the phagolysosome, and primary granules into the extracellular space *(arrows).* Neutrophil granule populations, including gelatinase granules and secretory vesicles, are depicted at the *bottom* of the figure. *(From Levy O: Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents,* Blood *96:2664-2672, 2000.)*

TABLE 154-1

Antimicrobial Proteins and Peptides of Neutrophils

Protein	Size (kDa)	Neutrophil Granule	Mechanism
Lactoferrin	80	Secondary	Iron-binding/ membrane-active
BPI	55	Azurophil	Binds lipid A of lipopolysaccharide
Serprocidins	~30	Azurophil	Proteolysis/membrane-active
Cathelicidins	10-20	Secondary	Release of membrane-active peptides
Lysozyme	14.5	Azurophil and secondary	Lysis of peptidoglycan/ membrane-active
Defensins	4	Azurophil	Formation of voltage-gated multimeric pores

BPI, Bactericidal permeability-increasing protein.

bacteria and is bactericidal in nanomolar concentrations. The biologic effects of BPI are linked to its high-affinity binding to bacterial lipopolysaccharides, which causes immediate growth arrest; actual killing is accomplished by its interaction with lipid phosphate groups and acyl chains of the membrane lipids perturbing the structures, thereby leading to outer and inner membrane lysis. The antibacterial and antiendotoxin activities of BPI are fully expressed by the amino-terminal (N-terminal) half of the molecule.[80,83] Furthermore, activated neutrophils release BPI into inflammatory fluids, where it is potently bactericidal. Levy and colleagues[84] reported that neutrophils of newborns are selectively deficient in BPI. These investigators demonstrated that newborn neutrophils from cord blood contain at least three to four times less BPI per cell than adult neutrophils, and this deficiency correlated with decreased antibacterial activity of newborn neutrophil extracts against serum-resistant *Escherichia coli.*[84] Further studies reported that supplementing newborn cord blood with recombinant BPI potently inhibited the tumor necrosis factor–inducing activity of gram-negative bacteria and in some cases enhanced bactericidal activity. These results suggest that the administration of recombinant BPI may be of clinical benefit to neonates suffering from gram-negative bacterial infection or endotoxemia.[80]

Serprocidins, 25- to 37-kDa serine proteases localized in the azurophil granules, include elastase, cathepsin G, proteinase 3, and azurocidin/CAP37 (heparin-binding protein) and are related to the granzymes of cytotoxic T cells. The serprocidins exert broad cytotoxic activity against gram-negative and gram-positive bacteria through proteolytic action and direct membrane perturbation.[80] Elastase participates in generating antimicrobial activity during inflammatory responses by cleaving cathelicidin proforms to generate active antimicrobial peptides.[85] Proteinase 3 has the ability to degrade extracellular matrix proteins and also plays an important role in myeloid differentiation. Fractionation studies of neutrophils have shown that proteinase 3 could be detected not only in azurophil granules, but also in specific granules and in the plasma membrane–enriched fraction containing secretory vesicles.[86]

Defensins, a family of 4-kDa peptides with broad cytotoxic activity against bacteria, fungi, parasites, and viruses, are found in the azurophil granules. The activity of defensins depends on their cationicity and three-dimensional structure. Defensins form multimeric voltage-dependent pores that permeabilize cellular membranes. α-Defensins are expressed in human neutrophils and are characterized by six invariant cysteine residues forming three disulfide bonds. The activity of defensins is limited by monovalent and divalent cations, a finding suggesting that the action of these peptides is limited to prevent indiscriminate cytotoxicity.[80]

Peroxidase-negative specific granules contain distinct antimicrobial proteins and peptides that are deployed toward the leading edge of the "chemotaxing" neutrophil, thus accomplishing extracellular degranulation.[81] The peroxidase-negative specific granules can be subdivided into secondary granules, defined by the high content of lactoferrin, and tertiary granules, defined by a high concentration of gelatinase. These granules play different functional roles in the process of degranulation. Three novel proteins of specific granules—neutrophil gelatinase-associated lipocalin (NGAL), human cathelicidin (i.e., human cationic antimicrobial protein [hCAP-18]), and SGP28—have been confirmed to contribute significantly to the function of neutrophils. NGAL is a 25-kDa FMLP-binding protein that may play a role in regulating the inflammatory response by binding small lipophilic mediators such as FMLP, platelet-activating factor, leukotriene B_4, and lipopolysaccharide. Synthesis of NGAL has been shown to be induced in peripheral blood neutrophils treated with GM-CSF.[81,87] The only human member of the cathelicidin family of bactericidal peptides, hCAP-18, is a major component of specific granules, in which it is co-localized and co-mobilized with lactoferrin.[88,89] The antibacterial and endotoxin-binding domain resides in the carboxyl terminus (C terminus) of hCAP-18 and is thought to be unleashed by the proteases from the azurophil granules. The 28-kDa glycoprotein SGP is a member of a family of cysteine-rich secretory proteins (CRIPs) and shows high amino acid sequence homology to pathogenesis-related proteins, which are thought to be important for resistance to viral, bacterial, and fungal infections.[81]

Lactoferrin, an 80-kDa member of the transferrin family also localized to the secondary granules, exerts a direct microbicidal effect by membrane disruption. It also has been shown to bind to the lipid A moiety of gram-negative bacterial lipopolysaccharide, thereby neutralizing its endotoxic activity. Lysozyme is a 14-kDa enzyme that degrades bacterial peptidoglycans by cleaving the glycosidic bond of *N*-acetyl glucosamine. This enzyme is stored in both the primary and secondary neutrophil granules. The activity of lysozyme is synergistic with other granular proteins including defensins and lactoferrin.[80]

Stimulation of the bacterial tripeptide receptor FMLP induces the exocytosis of primary and secondary granules and the process of degranulation. FMLP stimulation has been shown to induce a rapid induction of tyrosine phosphorylation of several intracellular proteins including tyrosine kinases and MAP kinases. Studies of adhesion-dependent degranulation demonstrate that the release of lactoferrin is blocked by a selective inhibitor for the Src family kinases. Additional studies with mice deficient in two Src family kinases, Frg and Hck, have demonstrated the essential role these kinases play in degranulation.[90] Mócsai and co-workers,[91] using broad-specificity tyrosine kinase inhibitors, found that FMLP-induced degranulation of primary and secondary granules of neutrophils is mediated by p38 MAP kinase activated by the Src family of tyrosine kinases. Degranulation also involves the rapid activation of p38 MAP kinase and phospholipase D after ligand binding to FMLP receptors. Phospholipase D, found primarily in secretory vesicles, is mobilized to the plasma membrane on stimulation and is activated by small GTPases of the ARF and Rho families. Mansfield and associates[91] reported that neutrophil degranulation is regulated by ceramide, an inhibitor of kinase phosphorylation, through the inhibition of phospholipase D. These investigators demonstrated that phospholipase D activity is significantly increased in neutrophils primed with G-CSF and is stimulated with FMLP; however, the activity was completely inhibited by ceramide.[92]

MICROBICIDAL ACTIVITY

Bacterial killing is initiated by two interrelated processes: oxidative and nonoxidative microbicidal activity. The oxygen-dependent mechanism uses a multicomponent enzyme that is dependent on reduced nicotinamide adenine dinucleotide phosphate (NADPH) to convert molecular oxygen into reactive oxygen-derived species, including (O_2^-) and H_2O_2. These molecules in turn act in concert with PMN-derived granule components such as myeloperoxidase to exert antimicrobial activity.

Oxidative Microbicidal Activity

The *respiratory burst* is characterized by a several-fold increase in oxygen consumption and a 10-fold increase in glucose utilization by activation of the hexose monophosphate shunt. The respiratory burst results in the production of and release of large quantities of O_2^- and H_2O_2 by the phagocytes into their surroundings. The respiratory burst consists of a series of reactions, all of which depend on the activity of the phagocyte oxidase (phox). Current evidence suggests that the phox is a complex multicomponent system that catalyzes the one-electron reduction of oxygen to O_2^- at the expense of NADPH. In the resting phagocyte, the phox system includes a 91-kDa flavoprotein and a

22-kDa heme protein, usually referred to as flavocytochrome b_{558}. Both are plasma membrane–associated, and they act with at least three soluble cytosolic components—$p47^{phox}$, $p67^{phox}$, and rac2.

Defective or absent oxidase activity, manifested clinically as chronic granulomatous disease, can be explained on the molecular level as a disorder of flavocytochrome b_{558}, $p47^{phox}$, or $p67^{phox}$, and full activity can be demonstrated using recombinant forms of these factors with added rac2.[93,94] In unstimulated PMNs, each molecule of flavocytochrome b_{558}, with its two heme groups and flavin adenine dinucleotide and NADPH-binding sites, is located primarily intracellularly, in the membrane of secretory vesicles and specific granules, with a lesser amount in plasma membrane.[81] The segregation of functionally competent components of the oxidase in intracellular compartments allows resting PMNs, cells with limited synthetic activity, to maintain the response in an inactive but ready-to-be-recruited state.[95] The activation of PMNs triggers degranulation and results in increased surface expression of flavocytochrome b_{558}. Biosynthesis and assembly of mature flavocytochrome require coordinated expression of genes encoding each subunit, because they are located in different chromosomes. For example, analysis of the X-linked chronic granulomatous disease PMNs demonstrated a membrane defect consistent with the localization of $gp91^{phox}$ on the X chromosome, whereas $p47^{phox}$ is localized to chromosomal region 7q11.23 and $p67^{phox}$ to 1q25.[96]

The biochemical events of the respiratory burst are summarized as follows:

$$2O_2 + NADPH \xrightarrow[phox]{} 2O_2^- + NADP^+ + H^+$$

Most of the O_2^- is rapidly converted to H_2O_2 by dismutation either occurring spontaneously or mediated enzymatically by superoxide dismutase (SOD):

$$2O_2^- + 2H^+ \xrightarrow[SOD]{} H_2O_2 + O_2$$

NADPH consumption rises because of the O_2^--forming reaction as well as the detoxification of some of the H_2O_2 by NADPH in a glutathione-dependent process:

$$H_2O_2 + NADPH + 2GSH \xrightarrow[glutathione\ peroxidase]{} H_2O_2 + GSSG + NADP^+ + H^+$$

The oxidation of glucose by the hexose monophosphate shunt, involving glucose-6-phosphate dehydrogenase (G6PD), is stimulated by NADPH consumption and leads to the replenishment of NADPH:

$$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow[G6PD]{} \text{5-carbon-P} + CO_2 + NADPH$$

The respiratory burst produces microbicidal agents by the partial reduction of oxygen. However, the most potent of these agents are products from further reactions involving O_2^- and H_2O_2. O_2^- and H_2O_2 react in the presence of iron to form the hydroxyl radical and possibly singlet oxygen.

$$H_2O_2 + O_2^- \xrightarrow[Fe^{3+}]{} HOX + OH^- + O_2$$

In the best-defined microbicidal system, myeloperoxidase derived from the azurophilic granules catalyzes H_2O_2 in combination with chloride, bromide, or other halide to form HOCl or HOBr:

$$H_2O_2 + 2Cl^- \xrightarrow[MPO]{} 2HOCl$$

These reactive species halogenate bacterial cell walls, which lose integrity, resulting in cell death. Oxidizing radicals such as O_2^- also contribute significantly to bacterial killing. Microorganisms vary relatively little in their susceptibility to these oxygen-dependent antimicrobial systems.

Several investigators have explored PMN respiratory burst activity in the newborn. The activities of glutathione peroxidase and catalase, the major enzymes responsible for detoxifying H_2O_2 within the cell, were decreased in neonatal neutrophils in comparison with adult PMNs.[97] Neonatal PMN SOD activity, however, was normal[97] or slightly decreased[98] compared with that in adult PMNs.

Adult neutrophils exposed to low concentrations of gram-negative lipopolysaccharides become primed and exhibit an increased oxidative response to a second stimulus.[99] Bortolussi and co-workers[100] demonstrated lipopolysaccharide-deficient priming activity of neonatal PMNs, and Qing and colleagues[101] identified an association of this reduced priming activity with decreased expression of CD14 on neonatal neutrophils. Subsequently, Bonner and associates[102] investigated the role of ERK1 and ERK2 and found that lipopolysaccharide activated ERK1 and ERK2 in PMNs of adults and newborns, suggesting that the ERK pathway was not responsible for the lack of lipopolysaccharide priming.

In 1986, Allen and associates[103] discovered that activated neutrophils could generate light—the phenomenon of chemiluminescence. Sources of this light emission include singlet oxygen, which releases some of its energy as a photon as it decays to the ground state, and carbonyl compounds in excited states. As a sensitive, noninvasive technique, chemiluminescence has been used extensively to measure respiratory burst activity by neutrophils.

Kallman and colleagues[104] examined the respiratory burst activity of adult and neonatal term and preterm PMNs after activation and opsonization. Term neonate neutrophils exhibited a chemiluminescence response identical to that in adult neutrophils, but preterm (28 to 34 weeks) neutrophils responded with a very much smaller chemiluminescence peak. The normal chemiluminescence response in neutrophils of full-term neonates and the reduced respiratory burst activity in those of preterm infants have been confirmed by other investigators.[105-107]

In contrast with that of adult PMNs,[108] the respiratory burst of term neonatal neutrophils becomes less active when it is challenged by sepsis or other clinical stress.[109] At the time of diagnosis of sepsis, increased levels of cytokines and intensity of oxidative metabolism were found in serum of newborns by Sikora and associates.[110] Over the course of time, however, these findings were further accompanied by prolonged neutrophil stimulation and, eventually, by functional exhaustion and diminished oxidative metabolism in PMNs.

Term neonates with GBS sepsis demonstrated neutrophil chemiluminescence responses of only 50% to 75% those measured in uninfected infants, and this was associated with a 10-fold reduction in ability to kill opsonized GBS in vitro.[111] A similar, although less marked, reduction in chemiluminescence was found in uninfected infants with respiratory distress.[112]

Postnatal maturation of the respiratory burst was studied by Driscoll and co-workers[113] in 57 premature infants over a 2-month period. During the first postnatal week, most preterm infants exhibited a lower chemiluminescence peak. During subsequent maturation, mean PMN chemiluminescence continued to be lower than in adult control subjects. The suppression of the respiratory burst seemed to be more closely associated with clinical stress and prolonged intensive care than with low gestational age. Usmani and associates[107] likewise demonstrated suppression of PMN chemiluminescence at 21 days after preterm birth.

An intriguing aspect of the neonatal respiratory burst is that although the generation of hydroxyl radicals is reduced, the initial phase of the respiratory burst, O_2^- generation, is increased in neonatal neutrophils compared with that in adult PMNs.[114] Measurement of light scattering in neutrophils from 90 healthy neonates and 96 healthy adults demonstrated that O_2^- generation in response to FMLP of neonates exceeds that of adult PMNs.[16] Ambruso and colleagues[115] demonstrated that the

kinetics of the neonatal NADPH oxidase system, which generates O_2^-, was different from that in adult PMNs, and an increase was observed in O_2^- production by cord blood neutrophils collected after labor, in contrast with that observed after cesarean section.[116] The paradoxical findings of reduced bactericidal oxidants and increased generation of O_2^- may be explained by inadequate stores of lactoferrin and myeloperoxidase to catalyze the later stages of the respiratory burst. Lactoferrin, if saturated with iron, enhances the production of hydroxide ions.[117] Neonatal neutrophils contain only half the amount of lactoferrin found in adult cells, and lactoferrin levels in premature neonatal PMNs are even lower.[118] Myeloperoxidase, which is involved in generation of hypochlorous acid from H_2O_2, has been found in normal concentrations in the azurophil granules of term neonatal PMNs,[114] but it is reduced in PMNs from preterm infants.[119]

Reddy and co-workers[52] studied CR3-dependent respiratory burst activity in neonatal and adult leukocytes. Neonatal neutrophils, on average, expressed less CR3 in comparison with that in adult PMNs. Because of the higher neutrophil concentration in cord blood versus in adult blood, the calculated number of neutrophils in cord blood expressing high amounts of CR3 was equivalent to that in adult blood.The size of CR3-dependent respiratory burst correlated with CR3 expression; therefore respiratory burst activities were equivalent in newborn and adult cells. High proportions of immature cells in cord blood may explain in part the deficiency in CR3 expression and neutrophil-dependent killing activities.

Nonoxidative Microbicidal Activity

Neutrophil cytoplasmic granules contain antimicrobial agents that are released into phagolysosomes and do not require the production of oxidants for activity. The granules are extremely heterogeneous in terms of shape, size, and content.

The first granule type produced during neutrophil maturation is the azurophilic granule, which contains myeloperoxidase, defensins, cathepsin G, BPI, hydrolases, elastase, and collagenase.[120] Defensins possess antimicrobial activity through their ability to bind to the negatively charged cell surface of the organism and thereby interfere with growth. Cathepsin G microbicidal activity does not rely on proteolysis but depends on inhibition of macromolecular synthesis (proteins, RNA, and DNA) of the invading bacteria.[121] BPI contributes to the ability of neutrophils to kill gram-negative bacteria.[82,122,123] During microbial ingestion, acid hydrolases have a digestive role against proteins, complex lipids, polysaccharides, and nucleic acids.

PMN secondary and tertiary granules are produced later in development, and they are twice as abundant as azurophilic granules. They include lysozyme, lactoferrin, gelatinase, and β-glucuronidase. Lysozyme can kill some saprophytic bacteria within the phagocytic vacuole by hydrolyzing the mucopolysaccharides of the bacterial cell walls, as well as by digesting the glycopeptide cell wall debris of dead bacteria. Lactoferrin exerts bacteriostatic activity by competitively binding the iron required as an essential nutrient for microbial growth.[124] Lactoferrin also makes the bacteria more accessible to lysozyme, leading to alterations in the bacterial cell wall.[125]

To investigate specific deficits in neutrophil microbicidal activity as a risk for neonatal sepsis, Levy and co-workers[83] compared the relative contents of BPI in the neutrophils of neonates and adults. Newborn and adult PMNs contain nearly identical amounts of myeloperoxidase and defensin peptides in their azurophilic granules, but neonatal neutrophils contain three to four times less BPI than that in adult PMNs.These data suggest a selective deficit of BPI in neonatal neutrophils, which may partly explain the increased incidence of gram-negative sepsis in the neonate.

Studies of bactericidal activity by neonatal neutrophils indicate that phagocytic killing of different microorganisms is normal in most healthy neonates but is reduced in preterm infants.[126,127] The relevance of reduced microbicidal activity to increased sepsis in preterm infants is supported by in vitro studies of bacterial killing. Intracellular killing of *S. aureus* or *E. coli* by neutrophils of term neonates has consistently been demonstrated to be normal,[128-130] whereas killing of staphylococci was impaired in preterm neonates with birth weights less than 2000 g.[57] Various conditions, such as respiratory distress and sepsis, have been associated with decreased bacterial killing.[131] A depression of microbicidal capacity also was demonstrated when neonatal neutrophils were challenged with a large number of *E. coli* organisms.[132] *Candida*-cidal activity has been shown to be normal in neonatal PMNs.[133] In one study, however, neonatal PMNs were unable to kill *Candida albicans* as efficiently as adult PMNs.[134]

ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

Neutrophils are the predominant mobile phagocytes of the circulating blood and constitute the first line of defense against infection. They also are active in immunosurveillance against tumor cells.[135-137] The lytic disruption of target cells, cytolysis, is a fundamental immunologic effector function mediated by neutrophils as well as by cytotoxic T cells, natural killer cells, and macrophages. One mechanism of cytolysis is ADCC. Neutrophils exhibit fast recruitment activity in vivo, and rapid, potent neutrophil cytotoxicity toward various tumor targets has been documented.[138,139]

ADCC requires opsonization of a target cell with immunoglobulin and the engagement of effector cell surface receptors for the Fc domains of immunoglobulin molecules. Neutrophils can trigger ADCC by engagement of FcγRI (CD64), FcγRIα (CD32A), and FcαRI (CD89). FcαRI has been identified as the most effective FcR for neutrophil-mediated tumor cell killing. FcαRI is constitutively expressed on neutrophils and has a medium affinity for both IgA1 and IgA2 that is increased on exposure to G-CSF or GM-CSF.[140,141] Neutrophil-mediated GM-CSF/Lym-1 cytotoxicity has been reported to require the intervention of FcγRII and CD11b-CD18 integrins.[142] Furthermore, studies with transgenic mice also have demonstrated the requirement of CD11b/CD18 (CR3) for FcαRI-mediated ADCC.[143] The role of CR3 in neutrophil Fc-mediated lysis of tumor cells was further characterized in the CR3-deficient mouse. ADCC was abrogated in $CR3^{-/-}$ neutrophils and in human neutrophils blocked with anti-CR3 monoclonal antibodies.[138]

Nagarajan and associates[144] reported that affinity modulation of FcγRIIa also is one of the mechanisms by which neutrophils regulate their FcγR-dependent functions. These studies demonstrate that resting neutrophils express FcγRIIa in a low-affinity state, but on activation, FcγRIIa is converted to a high-affinity state that leads to FcγRIIa-dependent ligand binding and signaling for tumor cell cytotoxicity.[144] Membrane-associated CD45, a protein tyrosine phosphatase essential for antigen receptor kinase–mediated signaling in lymphocytes, also has been shown to regulate and enhance the stimulation and function of neutrophils mediated through Fcγ receptors. Gao and associates[145] demonstrated that neutrophils pretreated with anti-CD45 have a reduced ability to perform ADCC compared with untreated neutrophils.

Antibodies against HLA class II or related epitopes are highly effective in recruiting neutrophils. Whole blood from patients during G-CSF therapy exhibits significantly enhanced lysis of breast cancer target cells in the presence of Her-2/neu antibody, and the extent of tumor killing correlated positively to neutrophil blood counts.[146] Furthermore, separation of whole blood into plasma, mononuclear cells, and neutrophils demonstrated that significant cytotoxicity was found in the neutrophil fraction.[146] Bispecific antibodies contain one specificity against a

tumor target and another specificity against selected epitopes of an activating Fc receptor on neutrophils. Preclinical studies of bispecific antibodies and tumor targets have demonstrated that neutrophils are the most active effector cell population in tumor cytolysis. During G-CSF therapy, bispecific antibodies (FcαRI × Her-2/neu) are reported to induce enhanced killing of Her-2/neu$^+$ breast cancer cells.[140] This enhanced cytotoxicity is attributed to the increased neutrophil counts that resulted in higher effector-to-target cell ratios in G-CSF–primed blood.[140] Neutrophils also were found to effectively lyse malignant B cells with an FcαRI × CD20 bispecific antibody.[147]

The particular role of extracellular and intracellular domains of CD19 and Her-2/neu for ADCC by neutrophils was examined by creating Her-2/CD19 and CD19/Her-2 chimeric target molecules. Their ability to trigger ADCC was compared with that for wild-type Her-2/neu and CD19 targets.[148] Neutrophils predictably killed wild-type Her-2/neu but not CD19 targets; however, neutrophils also were effective against chimeric CD19/Her-2–transfected but not Her-2/CD19$^-$ transfected target cells. These results suggest that intracellular domains of target antigens may contribute to effective neutrophil ADCC.[148]

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Melvin Berger

The Complement System of the Fetus and Newborn

Complement is a system of more than 30 soluble proteins, cell surface regulatory factors, and receptors that work together to accomplish a wide variety of functions.[1-4] These are important not only in host defense and inflammatory responses but also in normal physiologic homeostasis. By effecting proper disposal of apoptotic cells and antigens from infectious agents, complement plays an important role in preventing autoimmune and immune complex diseases under normal conditions. In infections, complement fragments rapidly activate and attract leukocytes and macrophages, and complement-derived opsonins facilitate clearance and killing of infectious organisms. Complement also provides two-way bridges between the innate and adaptive immune systems. Besides its traditionally understood roles in amplifying the effector functions of antibody, it also has important influences on antigen presentation and B cell activation, giving it a key role the afferent limb of antibody responses. As expected with a system that ties together so many different physiologic activities, disorders of complement can have wide-ranging effects.

From a biochemical standpoint, it is fascinating to study the processes by which circulating proteins that seem inert when considered individually can transform themselves into complex multisubunit enzymes,[4] form multichain integral membrane proteins that insert into cells from the outside, provide address tags that direct trafficking of a wide variety of cellular and foreign materials, and stimulate vigorous inflammatory responses. These transformations are achieved by two main mechanisms: limited proteolytic cleavage and conformational changes. At each stage of activation, newly revealed conformations are accompanied by new properties, such as enzyme activity and markedly increased binding activity for other components or cell surface receptors. In the first several steps of activation, newly formed enzymes can cleave many molecules of the next component in the sequence, further amplifying the signal. For this reason, the activation pathways are considered "cascades," in analogy with the cascades of the clotting or contact activation (kinin) systems. Understanding the way the complement proteins are activated is the first step toward understanding the functions of this system.

ACTIVATION PATHWAYS

There are three major pathways by which complement activation can be initiated. Because complement was originally discovered as a set of heat labile serum proteins that could cause lysis of erythrocytes sensitized with antibody, the antibody-dependent or "classical" pathway has been best studied. Although it is phylogenetically the newest pathway, its activation is easiest to describe, so it will be presented first (Fig. 155-1, *A*.)

The Classical Pathway

C1, its first component, is composed of 22 peptide chains arranged into three subcomponents termed C1q, r, and s.[5] C1q itself contains six sets of trimers, each of which has a collagen-like stalk and a globular head. Electron micrographs show the six trimers arranged with their stalks together and the globular heads all at the same end, giving the appearance of a bunch of tulips. Around the stalk are wrapped two molecules each of C1r and C1s, which are latent proteases. Classical pathway activation begins when two or more of the six globular immunoglobulin-binding domains of C1q become attached to the Fc regions of antigen-bound immunoglobulin M (IgM) or IgG molecules. This causes internal rearrangements in the complex, leading to activation of latent proteolytic activity of C1r, which then cleaves and activates the C1s molecules.[5,6] Because of the requirement for two or more of the globular heads of C1q to be engaged for activation to begin, IgM, which has five Fc regions attached together, is a very efficient activator of the classical pathway. In contrast, many molecules of IgG must be bound to the surface of a target cell or bacterium in order for two of the IgG molecules to find themselves close enough together to bridge a single C1q. The other classes of immunoglobulins, IgD, IgA, and IgE, do not bind to C1q and hence do not activate the classical pathway. C1s, when activated, also has proteolytic activity. This enzyme can cleave synthetic esters in vitro, in addition to its substrates in the complement system, and it is hence often referred to as *C1 esterase*. Its physiologic role is to carry out limited proteolytic cleavage of the next two components in the reaction sequence: C4 and C2.[5,6] The components were numbered in the order in which they were discovered rather than the order in which they act, hence C4 comes before C2. C1s cleaves the largest of the three chains of C4 at a single site, liberating a 9-kDa peptide (C4a) and leaving the larger fragment (C4b) with a transient ability to form covalent bonds to the target.[7] The C4b fragment also takes on a new conformation in which it can bind a molecule of C2 so that the C2 be cleaved by C1s as well. Again, a small fragment is released and diffuses away, and the conformation and activity of the larger fragment are changed. In this case, the larger fragment is C2a. The newly formed C2a has its own proteolytic active site, and together with C4b, which can also hold onto a C3 molecule, forms an enzyme that can cleave and activate C3. This complex is therefore called *C3 convertase*.[1-4] This classical pathway C3 convertase, composed of one molecule of C4b covalently attached to the target and one molecule of C2a loosely bound to that, is capable of cleaving many molecules of C3. C3 is homologous with C4. On cleavage, the larger fragment also transiently has the ability to bind covalently to the surface or target on which activation is occurring.[7-9] The heavy chain of immunoglobulin molecules provides particularly good "acceptors" for covalent attachment of C3b.[10] Many molecules of C3 can be cleaved by a single convertase, but the convertase eventually loses activity when the C2a subunit diffuses or is pushed away. Some of the newly formed C3b molecules will diffuse away before they can bind to the surface. Many others will bind to the surface at short distances around the convertase; these may serve to "opsonize" (facilitate phagocytosis of) the target. Some of the C3b molecules may deposit in very close proximity to the C4b2a complex and actually join with it. These C3b

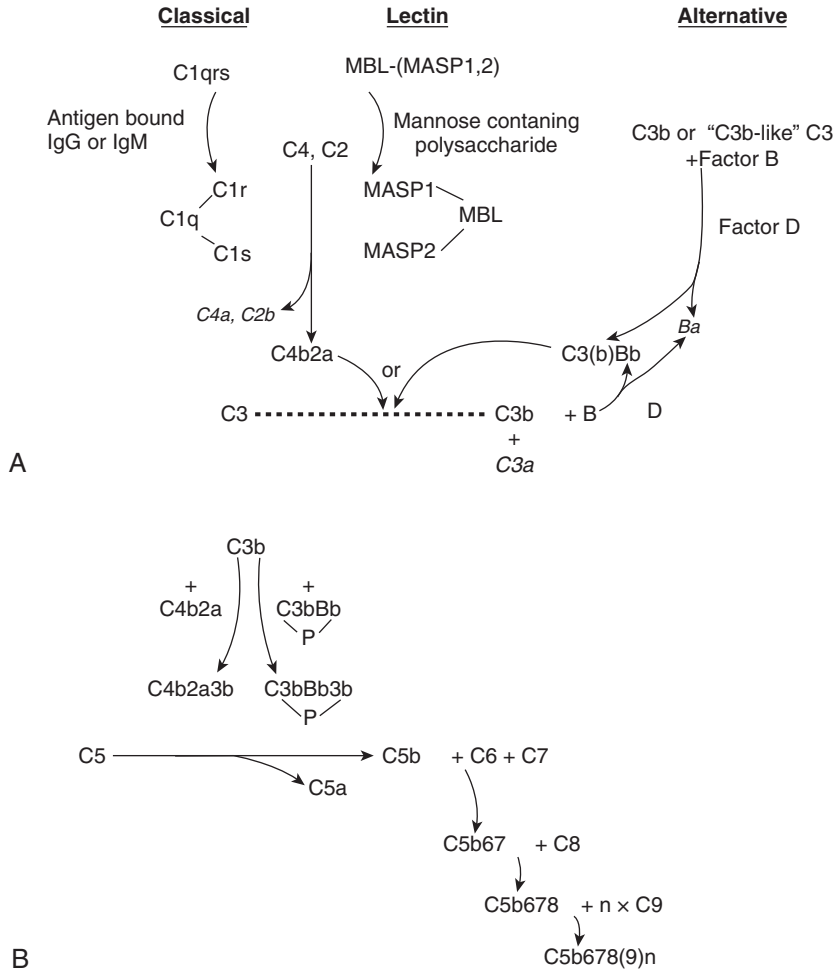


Figure 155-1 A, The early steps of complement activation drawn to emphasize homology between different pathways. The classical pathway is activated when C1q in the C1 complex binds to antigen-bound immunoglobulin Gs (IgGs) or IgM. *C1r* then activates C1s. The lectin pathway is activated when the MBL-MASP complex binds to mannose residues of a polysaccharide. *MASP 1* activates MASP 2. *C1s* and *MASP 2* cleave C4 and C2, which form a C3 convertase, *C4b2a*. The inactive *C4a* and *C2b* fragments *(italics)* diffuse away. The convertase cleaves C3 into *C3a*, an anaphylotoxin that diffuses away, and the major fragment, C3b. C3b can bind a molecule of B, which makes the latter susceptible to cleavage by *D*. *Ba* diffuses away, but Bb binds to C3b and forms the alternative pathway C3 convertase, which is homologous with *C4b2a*. **B,** Activation of C5 and formation of the membrane attack complex. Addition of another molecule of C3b provides a binding site for C5 and changes the C3 convertase into a C5 convertase. C5 can be cleaved by the classical/lectin pathway convertase, *C4b2a3b,* or the alternative pathway convertase, *C3bBb3b* (here shown in the form stabilized by properdin, P). The smaller fragment, *C5a,* is an anaphylotoxin and chemoattractant that diffuses away. The larger fragment binds to C6, forming a lipophilic complex that may insert into cell membranes. C5b6 binds C7 and then C8 without proteolytic cleavage, and this complex activates and binds multiple molecules of C9, forming the complete membrane attack complex.

molecules can provide binding sites for C5 molecules, which will be cleaved by the proteolytic active site on the C2a. Thus the complex C4b2a3b serves as the classical pathway C5 convertase.[11] Like C4 and C3, the cleavage of C5 liberates a small fragment (C5a), but unlike C4 and C3, C5 cannot form a covalent bond with the surface. The larger fragment, C5b, however, undergoes a conformational change that allows it to bind C6. This leads toward formation of the lipophilic membrane attack complex (MAC), discussed later.

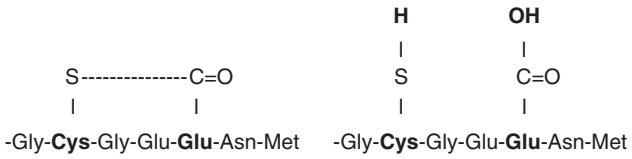
The Lectin Pathway

Activation by this route begins with the protein mannan binding lectin (MBL), Like C1q, this multichain molecule has collagen-like stalks composed of three protein chains that form a globular binding domain at one end.[12] In the case of MBL, however, the binding sites recognize polysaccharides containing mannose, hence its name. Because MBL, C1q, and other similar molecules have collagen-like regions and binding sites that often have lectin-like activity in recognizing polysaccharides, they are often considered to be members of the same family of collagen-like lectins, or "collectins."[13] Lung surfactant proteins A and D are also members of this collectin family. Different isoforms of MBL may contain between two and eight of the basic trimer subunits. Because many bacteria and fungi are coated with mannose-containing polysaccharides, the MBL pathway of complement activation is considered part of the innate immune system, as is the alternative pathway, which is discussed later. Phylogenetically, the alternative and lectin pathways likely originated earlier than the classical pathway. In a sense, C1q may be

seen as an adaptor protein that allowed this important part of the innate immune system to be used to enhance the activity of antibody produced by the adaptive immune system. The ability of the lectin and alternative pathways to activate complement in the absence of antibody may be particularly important in the neonate, while the adaptive immune system is immature. Similarly, early in the course of infections, before antibody production and other effector mechanisms of the specific immune system are brought to bear, MBL and the lectin and alternative pathways provide an important innate system for distinguishing self from non-self. Like the binding of C1q to the Fc regions of IgG or IgM, the binding of MBL to mannose-containing polysaccharides leads to activation of latent proteases. In this pathway, the proteases that are analogous to C1r and C1s are termed *MBL-associated serine proteases* (MASPs).[14] Two major MASPs have been identified and may circulate in the plasma as a large complex together with MBL and another protein component whose function has not yet been determined. When MBL is engaged with polysaccharides, MASP 1 becomes activated and cleaves MASP 2. Both MASP 1 and MASP 2 have been shown to be capable of cleaving C3,[15] but some studies suggest that they actually act more like C1r and C1s in cleaving C4 and C2. Just as in the classical pathway, the larger fragments of those components go on to form a C3 convertase, and the addition of a molecule of C3b confers C5 convertase activity on the complex. The physiologic importance of the lectin pathway is illustrated by the occurrence of an increased susceptibility to infections and also to autoimmune diseases like lupus in patients with MBL deficiency.[16,17]

The Alternative Pathway

The alternative pathway, also known as the *properdin pathway*, shares with the lectin pathway the ability to activate complement without antibody. Alternative pathway activation is initiated by C3b, which may be formed by one of the other pathways, by nonspecific proteolytic cleavage of C3, or after the spontaneous activation of C3 by water molecules[18] (see later). Most investigators visualize native C3 in the circulation as a kind of coiled spring, which holds a unique structure called a *thioester* in an internal hydrophobic pocket.[7,8] This highly unusual chemical structure is formed by the binding of a thiol on the side chain of one of the protein's cysteine residues with the carboxyl on the side chain of a glutamate three residues away in the polypeptide.[7,8]



C4 and α_2-macroglobulin also contain this internal thioester. Although the cleavage site on the protein chain containing the thioester is distant from that structure, cleavage by a convertase or nonspecific protease causes conformational changes in which the coiled spring is released, the thioester is transiently exposed, and the carbon on the glutamate can transfer one of its bonds from the sulfur of the cysteine to another acceptor.[8,9] It is the ability of these molecules to transfer a carbon bond to hydroxyl or amino groups on other proteins or to sugar molecules that allows C3b and C4b to bind covalently during complement activation. If the transfer to another protein or sugar does not occur within milliseconds, the bond will transfer to the hydroxyl group of a water molecule, whose other hydrogen atom will bind to the sulfur of the cysteine to create a free thiol (−SH). This process of transfer or hydrolysis of the internal thioester is greatly accelerated by the conformational changes that follow cleavage of the protein chain. However, water molecules may occasionally penetrate into the hydrophobic pocket and hydrolyze this bond even in intact molecules of C3. Once that occurs, similar conformational changes in the protein chains will follow, even though no cleavage fragment has been removed. That process goes on constantly at a slow rate, hence there is always some turnover of C3. These C3 molecules with hydrolyzed thioester bonds assume a conformation similar to that of C3b and are thus called "C3b-like" C3 molecules.[18] This constant low-grade turnover of C3 may be thought of as the idling of a car engine: the system is always turned on and ready to accelerate at any time. This allows the alternative pathway of complement to play an important role in recognition of foreign invaders as part of the innate immune system and also allows it to play a role in eliminating dead or damaged body cells. However, it also means that the system must be carefully regulated, as discussed later in the section, Control of Complement Activation.

C3 and C4 are highly homologous proteins, and the alternative pathway C3 convertase is highly homologous to the classical pathway convertase and is formed in an analogous way.[1-4,18] This is initiated when a molecule of C3b-like C3, or C3b itself, which acts like C4b in the classical pathway, binds with a molecule called *factor B*, which is homologous with C2. The B is then cleaved by factor D, a proteolytic enzyme that circulates in its active form all the time but can only act on factor B when it is held in the proper configuration by C3b. Cleavage of factor B is analogous to cleavage of C2, but the large fragment is named Bb. Like C2, factor B has latent protease activity. The Bb fragment that results from cleavage by factor D has its proteolytic active site exposed, and this enzyme can cleave C3 and C5. The C3b or C3b-like C3 molecule that initiated the pathway also serves the same role as the C4b in the classical pathway C3 convertase: holding additional molecules of C3 substrate in place, which are then cleaved by Bb. Just as in the classical pathway, some of the newly cleaved C3b molecules will deposit sufficiently close to the C3bBb enzyme to join with it. This new molecule of C3b can provide a binding site for C5, which will be cleaved by Bb. Thus, the addition of this new C3b molecule changes the C3 convertase *C3bBb* into the alternative pathway C5 convertase, *C3bBb3b*.[11,18]

The alternative pathway contains an additional molecule not found in the classical or lectin pathways, which is called *properdin*, or *factor P*. The discovery of this molecule led to the elucidation of the alternative pathway, and thus it is sometimes referred to as the *properdin pathway*.[19] P is capable of stabilizing the alternative pathway convertases, which might otherwise lose activity when the Bb, which contains the proteolytic active site, diffuses away.[18] This may also occur pathologically in the presence of *C3 nephritic factor*, an autoantibody with properdin-like properties found in some patients with glomerulonephritis.[20]

Activation of C5 and Formation of the Membrane Attack Complex (MAC)

One of the most dramatic features of complement activation is the conversion a series of separate, water-soluble, circulating plasma proteins into a large multisubunit, lipophilic membrane channel that can insert itself into plasma membranes, spanning both leaflets. Insertion of just one of these channels into an erythrocyte allows enough water to rush into the cell to cause explosive lysis. This ability of a heat labile fraction of plasma proteins to lyse erythrocytes sensitized by heat stabile antibodies was recognized more than 100 years ago and led to the elucidation of the complement reaction sequence because the liberated hemoglobin provided a convenient end point for assays. It is arguable, however, that complement-mediated lysis is not the most important function of the complement system in vivo, as discussed in subsequent sections. The transmembrane water channel formed

by the assembly of C5 through C9 is the MAC, and its formation begins with activation of C5 by a proteolytic cleavage.

After C5 has been activated, regardless of which pathway was responsible, the remaining steps of activation of C6 through C9 follow the same sequence. Unlike the early activation pathways, however, activation beyond C5 and formation of the MAC do not involve proteolytic cleavages or other enzymatic activity. C5 does not contain the same internal thioester as its homologues C3 and C4, and C5b cannot form covalent bonds with the target. However, just after cleavage, C5b gains the ability to interact with C6. Interaction with C6 increases the lipophilicity of C5b, and the C5b6 complex can insert into lipid membranes at some distance from the C5 convertase, even on adjacent cells.[21] This phenomenon can lead to "bystander lysis" of normal cells in the vicinity of soluble immune complexes or bacteria that are being attacked. The C5b6 complex can then attract and bind a molecule of C7. The complex of C5b67 is highly lipophilic and, if formed in the fluid phase, will rapidly insert into plasma membranes, where it will serve as a binding site for C8. Binding of C8 to C5b67 creates an active complex, which can disrupt the phospholipids of target cell membranes and can cause lysis of erythrocytes, even without the addition of C9.[22,23] The C5b-8 complex can induce conformational changes in soluble C9 molecules.[24,25] These conformational changes include elongation and unfolding of the C9 molecules, exposing previously protected hydrophobic regions as well as potential sites for disulfide bonding between chains.[25] These changes, in turn, give the C9 molecules the ability to polymerize and cause their addition to the complex. Isolated C9 can also be induced to undergo these conformational changes and polymerize in vitro.[23-25] These polymers may contain up to 12 molecules of C9 linked in dimers by disulfide bonds. Electron micrographs of these C9 polymers look very similar to MACs isolated from cells or artificial membranes that have been attacked by complement. They appear like grommets or donuts, with a top rim 15 to 20 nm in diameter sitting above a slightly narrower cylinder, 15 to 16 nm in length, which spans the plasma membrane.[26] The conformational changes that accompany C9 polymerization are recognized by monoclonal antibodies that do not recognize native, circulating C9 molecules.[27] These antibodies to the C9 "neoantigen" that is formed on activation may be used to identify sites of MAC deposition in tissue sections[27] and are now widely used in enzyme-linked immunosorbent assays of complement activity.

CONTROL OF COMPLEMENT ACTIVATION

Besides the ability to lyse cells, complement activation liberates C3a and C5a, which are potent mediators of inflammation. The deposition of large amounts of C3b can opsonize, or target, our own cells for destruction by macrophages in the reticuloendothelial system, just as it can opsonize invading microorganisms. The apparent dichotomy between complement's role as a part of the innate immune system that is always turned on to detect foreign invaders and its potential for tissue damage and destruction requires that a delicate balance must be maintained at all times. This balance depends on a set of control mechanisms no less intricate than the activation pathways discussed previously.

Control of the Alternative Pathway

The need to control alternative pathway activation becomes apparent when one considers the fact that the alternative pathway can be initiated wherever C3b has been deposited, whether by any one of the activation pathways, by the action of a nonspecific serine protease, or even spontaneously after lysis of the thioester by water.[18] The alternative pathway is often visualized as an amplification loop that can feed forward whenever C3b is formed (Fig. 155-1, *A*). The danger of uncontrolled alternative pathway activation is illustrated by the case of a patient with congenital deficiency of the complement regulatory protein, factor H (see later).[28] This patient lacked detectable C3 activity in his serum, giving him an increased susceptibility to infection; but he did have C3b deposited on his red blood cells, causing mild but chronic hemolytic anemia.[28] In this patient, most of the C3 was being consumed continuously, by nonspecific activation of the alternative pathway, as fast as it was synthesized, and some of the C3b was deposited nonspecifically on his own red blood cells.[28]

First of all, the activity of complement is controlled by maintaining low circulating concentrations of the complement proteins. C3, C4, and other components are "acute phase reactants," whose synthesis by hepatocytes and other types of cells can be increased rapidly in response to cytokines, hormones, and other signals.[29] Thus, local synthesis and changes in vascular permeability are important determinants of the amounts of the components available at any site or time. Activation of the cascades of the early activation pathways discussed previously is also inherently limited by the instability of the multisubunit convertase enzymes. In these bimolecular and trimolecular complexes, the proteolytic active sites are on the C2a and Bb fragments, which are not covalently bound to the target. These proteolytically active subunits have relatively weak, noncovalent interactions with the C4b, C3b, or both subunits, which bind the next component, the substrates C3 or C5. The mechanism by which properdin facilitates activation of the alternative pathway is by holding together the two subunits of the noncovalent *C3bBb* enzyme so that it will maintain activity.[18,19] In contrast, dissociation of the active subunit (C2a or Bb, respectively, in the classical or alternative pathways) causes loss, or "decay," of the convertase activity. This decay may occur spontaneously, or it may be accelerated by proteins that bind to the covalently attached member of the convertase and "push away" the proteolytically active subunit, thereby "accelerating decay" of the complex.[30] Several proteins have this function; some are soluble and some are membrane-bound (Table 155-1). Many of these proteins share homology with each other. This is not unexpected because they all bind to C3, C4, or both, which are themselves homologous. These proteins belong to the regulators of complement activation (RCA) family and are encoded by genes in the RCA gene cluster on chromosome 1.[31,32] Members of this family are all composed of variable numbers of short homologous repeats, each of which has 60 to 65 amino acids and two internal disulfide bonds, which give it a double-looped structure. These are further grouped into long homologous repeats, which form the binding sites for C4b and C3b.[32] A prototypic member of this family is called *decay accelerating factor* (DAF) (CD55).[30,33] DAF is bound to the plasma membrane by a glycolipid anchor, which is believed to give it increased lateral mobility in the membrane, to prevent attack of complement on our own cells.[33] The major function of DAF is to bind to C3b and C4b molecules and push off Bb or C2a. Besides the ability to bind to and push off (or block binding of) active subunits of convertases, some of the regulatory proteins can also facilitate degradation of the C4b or C3b to which they bind. A circulating protease, called *C3b/C4b inactivator* (factor I), can cleave C3b and C4b into forms that will no longer bind other components of the convertases. This factor I can only cleave its substrates when an additional cofactor is present. Soluble cofactors include C4 binding protein (C4bp) and factor H (formerly termed *β-1-H globulin*). Several integral membrane proteins also have this kind of cofactor activity, including complement receptor type 1 (CR1, CD35),[34] commonly considered the C3b receptor, and a separate membrane cofactor protein (MCP) (CD46).[35] DAF and MCP only bind to C3b and C4b that have been deposited on same cell on which they reside, so they are considered important in protecting our own cells from becoming activators or targets of complement. CR1 can also bind C3b on other cells or circulating

TABLE 155-1

Regulators of Complement Activation (RCA) Proteins

Protein*	Abbreviation	Molecular Weight	No. of Short Consensus Repeats (SCRs)	DISSOCIATION OF C3 AND C5 CONVERTASES		FACTOR 1 COFACTOR ACTIVITY ON:	
				Alternative	Classical	C3b	C4b
Factor H, β_{1H}	H	150,000	20	+	−	+	−
C4 binding protein	C4bp	570,000 (Octamer)	8	−	+	−	+
Decay accelerating factor (CD55)	DAF	70,000-80,000	4	+	+	−	−
Membrane cofactor protein (CD46)	MCP	45,000-70,000	4	−	−	+	+
Complement receptor type 1 C3b/C4b receptor (CD35)	CR1	205,000-250,000	22-30	+	+	+	+
Complement receptor type 2 C3d receptor (CD21)	CR2	145,000	15/16	−	−	−	−

Modified from Ahearn JM, Fearon DT: Structure and function of the complement receptors, CR1 (CD35) and CR2 (CD21), *Adv Immunol* 46:183-219, 1989; and Weisman HF, Bartow T, Leppo MK, et al: Soluble human complement receptor type 1: In vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science* 249:146-151, 1990.

*Factor H and C4bp are soluble. All others are membrane proteins indicated by CD number in parentheses.

complexes, so it is important in processing these complexes and in promoting phagocytosis of particles opsonized with C3b or C4b. The enzymatically inactive fragments are termed *iC4b* and *iC3b*, respectively. The latter still serves as an important opsonin, however, by binding to its own receptor, termed *CR3*.

From the viewpoint of a C3b molecule, regulation of the alternative pathway might be seen as a competition between binding of factor B, which would lead to additional activation, and factor H, which would lead to inactivation.[36] The enzymes that execute those actions, factor D and factor I, respectively, are both present in their active forms in the circulation at all times. An important determinant of the fate of any given C3b molecule is the chemical nature of the surface on which it is bound because the surface has an important role in influencing the binding of B compared with H. Surfaces that are rich in sialic acid, like our own cells, favor the binding of H and thus promote the action of I.[36,37] These surfaces are thus poor activators of the alternative pathway. In contrast, many bacteria and cells from some other species of mammals lack sialic acid. On these surfaces, binding of B is favored. Those cells are good activators of the alternative pathway. Thus, the alternative pathway can be considered an important part of the "innate" immune system because it can distinguish self from non-self, even in the absence of antibody or a rearranged T cell receptor. It is interesting in this context that bacteria, such as K12 *Escherichia coli*[38] and type III group B *Streptococcus,*[39] which have terminal sialic acid residues on their surface carbohydrates or capsules, are dangerous pathogens, particularly for newborns who lack specific antibody. Related bacteria, without the sialic acid, are much less virulent. Antibody molecules provide good acceptor sites for C3b deposition, on which B is favored over H and the C3b is "protected" against inactivation.[10] This allows the alternative pathway to amplify initial signals generated by antibody and the classical pathway. Besides their coating with sialic acid, most of our own cells are protected from amplification of the alternative pathway by the presence of DAF and MCP. Some cell types, including erythrocytes, are also similarly protected by CR1.

Control of Early Steps of the Classical and Lectin Pathways

Besides these critical mechanisms of regulation of the activation of the alternative pathway, there is a specific plasma protein, called *C1 inhibitor* (C1 INH), or *C1 esterase inhibitor*, that can complex with C1r and C1s and terminate their activity.[40] This protein has also been shown to inhibit the MASPs.[41] All these enzymes contain serine in their active sites, and C1 INH is a member of the serine protease inhibitor or serpin family of proteins. C1 INH also can also inhibit serine proteases in the clotting and kallikrein-kinin systems. Congenital deficiency of this protein results in the autosomal dominant condition, hereditary angioedema (HAE), which is manifest clinically in repeated episodes of swelling, including attacks of laryngeal edema that can be fatal.[40]

Control of Membrane Attack Complex Formation

Activation of the complement system beyond C5 involves conformational changes in individual soluble protein molecules that expose hydrophobic domains and lead to assembly of a multimeric lipophilic complex. Several circulating molecules are capable of interacting with the newly developing lipophilic sites on C5b-6 and C5-7 and preventing attachment of these intermediate complexes to plasma membranes. Some of these proteins may also interfere with binding of C8 or C9 and may bind to nascent MACs forming on cells, preventing their completion. Proteins with these properties include some of the lipoproteins, a protein called *clusterin* (apolipoprotein J)[42]; a specialized plasma protein called *S-protein* or *vitronectin*,[43,44] which also inhibits the blood clotting system; and others. Besides these plasma proteins that inhibit the formation of MACs before they insert into cells, there are membrane proteins that protect the cells on which they reside.[45-48] These include homologous restriction factor (HRF), also known as *protectin* (CD59), which inhibits polymerization of C9 and its incorporation into complexes containing C8.[47] This blocks formation of the full MAC. Like DAF (CD55), CD59 is not a true transmembrane protein but rather is linked to the cell membrane by phosphatidyl inositol glycolipid anchors.[48] This linkage may give them high mobility in the plasma membrane, facilitating their ability to move to sites of complement activation. Mutations in the enzymes that produce this type of anchor lead to the disease paroxysmal nocturnal hemoglobinuria, in which there can be spontaneous lysis of the unprotected cells.[48]

PHYSIOLOGIC EFFECTS OF COMPLEMENT ACTIVATION AND DEPOSITION

C3a and C5a

C3a is a 9.6-kDa fragment released from the N-terminal of the α chain of C3 when it is cleaved by convertases or other serine proteases. C5a is a similarly released on cleavage of C5, but C5a is heavily glycosylated and has a molecular weight of about 11 kDa.

TABLE 155-2

Properties of Complement Components and Inhibitors

Component	Molecular Weight	Serum Concentration (μg/mL)	Chain Structure
Classical Pathway			
C1q	390,000	190	6 each α, β, γ
C1r	95,000	100	s*
C1s	85,000	80	s
C4	209,000	430	α, β, γ
C2	117,000	30	s
C3	190,000	1400	α, β
C5	206,000	75	α, β
C6	95,000	60	s
C7	120,000	55	s
C8	163,000	80	α, β, γ
C9	79,000	160	s
Alternate Pathway			
P (properdin)	223,000	25	4 identical chains
D	25,000	1-5	s
B	100,000	200	s
Mannan Binding Lectin Pathway			
Mannan Binding Lectin	32,000	0.5 ng-5 μg/mL	3 identical chains per unit circulates as assembly of 2-8 units
MASP 1	90,000	1.6-7.5 μg/mL	s
MASP 2	74,000	0.4 μg/mL	s
Control Proteins			
Soluble Control Proteins			
C1 esterase inhibitor (C1EI, C1 INH)	105,000	180	s
C4 binding protein	570,000	150	6-7 α chains, 1 β chain
I (C3b/C4b inactivator, C3 INA)	100,000	50	α, β
H (β_{1H} globulin)	150,000	520	s
Clusterin (apolipoprotein J)	80,000	50-100	2
S protein (vitronectin)	80,000	600	s
Membrane Bound Control Proteins		**CD Designation**	
Decay accelerating factor (DAF)	70,000-80,000	CD 55	s
Membrane cofactor protein (MCP)	48,000-68,000	CD46	s
Complement receptor type 1 (CR1)	190-220,000	CD35	s
Protectin, homologous restriction factor (HRF)	20,000	CD59	s

CD, Cluster of differentiation; *MASP,* mannose binding lectin-associated serine proteins.
*Single chain.

Both of these peptides can bind to specific receptors on several types of cells, including mast cells, causing non–IgE-mediated degranulation, with release of histamine and other mediators. Thus, the actions of C3a and C5a mimic anaphylaxis, and they are known as *anaphylotoxins*.[49] C4a has only weak anaphylotoxin activity. Expression of the receptors[50] for C3a and C5a on epithelial, gland, and smooth muscle cells in the lung likely contributes to non-histamine-mediated mucus secretion and bronchoconstriction, and there are reports that these anaphylotoxins can induce interleukin-6 (IL-6) production,[51] providing a mechanism by which local complement activation can contribute to the systemic response to infection. All three of these peptides end with a C-terminal arginine. Removal of this residue by the enzyme carboxypeptidase N (an "anaphylotoxin inactivator") markedly decreases the ability of the "des-Arg" forms of the anaphylotoxins to activate their receptors.[49-53]

The carbohydrate moiety is important in the binding of C5a to its receptor (CD88) on myeloid cells, making this peptide one of the most potent known chemoattractants and activators of neutrophils, monocytes, and macrophages.[53] The des-Arg form of C5a has reduced, but still very potent, chemoattractant and proinflammatory activity.[53] Exposure of neutrophils to C5a or C5a des-Arg causes rapid up-regulation and activation of CD11/CD18 on the surface of polymorphonuclear leukocytes (PMNs), which allows the neutrophils to adhere to surfaces or aggregate with each other, or both.[54] In some cases, neutrophil aggregates formed in response to C5a or C5a des-Arg released by intravascular complement activation may plug pulmonary capillaries, which can cause transient pulmonary dysfunction or acute respiratory distress syndrome.[54] This may occur in sepsis as well as in burns and ischemia-reperfusion injury, in which damaged tissues can activate the alternative and lectin pathways. This pathophysiology also occurs when blood comes into contact with artificial membranes in oxygenators and during hemodialysis.[54] It has been hypothesized that part of the pulmonary dysfunction that accompanies amniotic fluid embolism may actually be caused by PMN aggregates induced by C5a produced intravascularly when complement is activated by fetal antigens and intracellular cellular debris from the placenta entering the maternal circulation.[55]

Bound C3 Fragments and Their Receptors

C3 is present in serum at a higher concentration than any other complement protein (approximately 100 mg/dL in normal adults) (Table 155-2). On activation, C3b can become covalently bound to its target, whether that is a bacterium, human cell, or soluble antigen. Subsequent metabolism of bound C3b involves

an orderly sequence of limited proteolytic cleavages, resulting in fragments that bind to distinct receptors on different types of cells.[56,57] The stage of degradation of C3 fragments on any target of complement activation plays a major role in determining the fate of that target and the host's response to it.[58]

When the α chain of C3 is cleaved or when the internal thioester is opened, the resulting C3b or C3b-like C3 molecule undergoes conformational changes that markedly increase its affinity for CR1 (CD35), the C3b receptor.[59,60] Uncleaved, native C3 does not bind to this receptor,[59] but C4b can also bind, so it is often termed the *C3b/C4b receptor*. This receptor is a member of the RCA family, but its cytoplasmic domain is short and cannot activate cells. Hence, its main function is to hold onto C3b-bearing immune complexes and C3b-opsonized bacteria and cells. By itself, it does not actually induce the internalization phase of phagocytosis.[61] That usually requires a "second" signal, such as that generated by binding of IgG to certain Fcγ receptors.[61] The conformational change that accompanies conversion of native C3 to C3b is followed by changes of similar magnitudes if and when the bound C3b is converted to iC3b.[60] That form of the molecule has a greatly reduced affinity for CR1 (CD35) but increased affinity for CR3 (CD11b/CD18). CD11b/CD18 is a multifunctional molecule that is a β2 integrin and serves as the neutrophil's major adhesion protein. Mutations in the CD18 subunit interfere with expression of this class of molecules, resulting in the condition known as leukocyte adherence deficiency (LAD) type 1.[62] Reduced intracellular pools of this molecule likely contribute to decreased chemotactic and phagocytic responses of neonates' neutrophils.[63] Because there are fewer CR3 molecules on neonates' neutrophils, more iC3b must be on the opsonized target to achieve the same number of adherence interactions.[64] Further cleavage of iC3b results in the release of a large soluble fragment termed *C3c*, which contains fragments of the cleaved α chain as well as the entire β chain.[56,57] The smaller fragment of the α chain remains covalently bound to the target. This is termed *C3dg* or *C3d*, depending on whether additional cleavages occur. This fragment binds to another distinct receptor, CR2 (CD21), which is also a member of the RCA family.[57,58] CD21 is most notably expressed on B lymphocytes and follicular dendritic cells. Simultaneous engagement of CR2 by C3d on an antigen or immune complex and the receptor (surface immunoglobulin) for that antigen on B cells markedly enhances the B cells' response to that antigen.[65] Persistence of C3d-bearing antigen complexes on the surface of dendritic cells and costimulation of B cells by additional bound C3d fragments is probably the major mechanism by which complement contributes to the afferent arm of "adaptive" immune responses (see later).[65,66]

C3 and Disposal of Immune Complexes and Apoptotic and Necrotic Cells

Although the proinflammatory activities of complement usually receive the greatest emphasis, it is important to realize that a major physiologic function of the complement system actually limits excessive inflammatory reactions by targeting the stimulus for elimination. Similarly, a major function of complement is to prevent tissue pathology by promoting solubilization and appropriate disposal of antigen-antibody complexes.[67] Activation of the complement cascades can deposit many C4b and C3b molecules onto antigen-antibody precipitates. The covalent binding of these water-soluble polypeptides disrupts the lattice-like structure of the antigen-antibody precipitate, forming smaller, soluble C3b-bearing complexes. The C3b targets these smaller complexes for phagocytosis and digestion by cells in the reticuloendothelial system which are specialized to perform those functions. Thus, C3b deposition can solubilize large antigen-antibody aggregates that might otherwise be trapped in tissues by ionic interactions with basement membranes (e.g., in the kidney). Interestingly, in the circulation, C3b-bearing complexes adhere to CR1 on the surface of erythrocytes (in primates only), and the erythrocytes thus carry the complexes on their surfaces into the narrow sinusoids of the liver and spleen.[68] This "immune adherence" of the C3b-bearing complexes to CR1 on the nonphagocytic erythrocytes slows down transit of the complexes and increases the ability of the macrophages to phagocytose the complexes, by stripping them off the erythrocyte's surface. Studies in patients with deficiencies of C3 or other early complement components demonstrate the importance of these mechanisms in normal immunologic homeostasis. Thus, for example, patients with congenital C3 deficiency have an increased incidence of glomerulonephritis, presumably owing to the failure of their reticuloendothelial macrophages to remove antigen-antibody complexes that result from common infections.[69] The lack of complement to solubilize and opsonize antigen-antibody complexes allows their accumulation in the kidneys, where they induce pathology by other pathways.[69]

Patients with deficiencies of the early components of the classical pathway or MBL have an increased incidence of autoimmune diseases like SLE.[10,69-71] C1q can bind to proteins that are usually intracellular but become exposed when cells undergo apoptosis or necrosis.[72] Such cells can also bind "natural antibodies" and MBL, and they can activate complement by the classical or lectin pathway. C3b deposited on apoptotic cells or their fragments can enhance their phagocytosis in situ by tissue macrophages, thus facilitating "disposal" of this cellular "waste."[3,72,73] It is hypothesized that the failure to efficiently clear cellular debris and proteins that would normally not be seen by the immune system allows potentially antigenic material to accumulate.[3] Epitopes not normally exposed could then stimulate autoimmune responses. Viewed in this way, a properly functioning complement system actually plays an important role in preventing inappropriate inflammatory and immune responses.

Complement in Host Defense Against Infectious Agents

Viruses

Because viruses employ specific proteins to adhere to and enter host cells, binding of antibody or complement directly to these proteins or to nearby sites on the virus surface can block this interaction, neutralizing the infectivity of the virus.[74] With enveloped viruses, insertion of the MAC can disrupt or lyse the envelope.[75] Some viruses can activate the alternative pathway at sites where the complement causes little damage to the virus; in those cases, antibody may play an important role by redirecting the complement to more critical sites. Retroviruses may activate the classical pathway directly by interacting with C1 without requiring antibody.[76] However, this does not usually lead to viral neutralization or lysis of the envelope. C3b fragments deposited by this route may actually facilitate entry of the virus into host macrophages that carry CR1. Other pathogens may also employ similar strategies of subverting host defenses to increase their virulence (see later).

Bacteria

The most important functions of complement in host defense are its contributions to control of bacteria, as evidenced by the increase in bacterial infections in patients with complement deficiencies.[77,78] Gram-positive bacteria have thick cell walls and must be phagocytosed and killed intracellularly to be eradicated.[79] The MAC cannot penetrate the cell wall to reach the cell membrane. Although the cell walls themselves often activate the alternative pathway, with many organisms, a polysaccharide capsule surrounds the cell wall. The capsule is permeable to proteins but holds off other cells at a distance. Thus, with encapsulated gram-positive organisms, C3 fragments can become bound to the

cell wall, but phagocyte receptors cannot reach them.[78-80] Organisms with capsules that lack sialic acid will be good activators of the alternative pathway, and complement will easily limit their pathogenicity. On the other hand, organisms that have terminal sialic acid on their capsular polysaccharides will not activate the alternative pathway. With these types of organisms, which include group B streptococcus, K12 *E. coli*, and others, anticapsular antibody plays a critical role by binding to the capsule, activating the classical pathway, and providing good acceptor sites for C3b.[79] Phagocytes, attracted and activated by the C5a generated during complement activation, will then rapidly phagocytose the C3b-opsonized organisms, especially if the anticapsular antibody is IgG. The roles of antibody and complement in clearance of circulating bacteria from the bloodstream are similar to those in phagocytosis of the bacteria at peripheral sites of tissue invasion.[81] Efficient killing of gram-negative bacteria requires insertion of the C5 through C9 MAC into their cell membranes, which are more accessible than the membranes of gram-positive organisms.[78,79] Patients deficient in components C5 to C9 thus have an increased susceptibility to serious infection with *Neisseria* species,[77,82] but these often do not present until the teenage years.

Protozoans

The cell membranes of protozoans are generally susceptible to attack by the C5 to C9 components of complement, which can lyse the organisms.[83,84] Thus many can be killed by antibody and the classical or the alternative pathways. However, several protozoans have adapted elements of the complement system to their own advantage. For example, *Plasmodium falciparum* has a surface protein that binds to CR1and other mammalian cell surface proteins, allowing it to adhere to erythrocytes and endothelial cells in the microvasculature and avoid destruction in the liver or spleen.[85] There is some evidence suggesting that expression of different allotypes of CR1 correspond with differences in susceptibility to malaria.[85] Some types of leishmanial promastigotes may also be killed by complement, but deposition of C3 fragments on their surfaces allows some of them to be phagocytosed through interactions with complement receptors on macrophages before they are killed.[83] These organisms can then survive intracellularly by altering trafficking of lysosomes and inhibiting microbicidal mechanisms. Other protozoans, including *Trypanosoma cruzi,* can produce complement regulatory proteins that function like DAF.[84,86-88] Many protozoans produce extracellular proteases that may degrade complement proteins, protecting the organisms. In addition,[88] some protozoans and bacteria produce enzymes that can transfer sialic acid onto their surfaces in mammalian hosts.

Molecular Mimicry and Complement Evasion Strategies of Pathogens

Many organisms that are significant pathogens have either adapted elements of the complement system to facilitate invasion of the host or have developed adaptations to evade complement attack to increase their pathogenicity.[86-88] For example, adenovirus, human herpesvirus type 6, measles virus, and other pathogens use CD46 (membrane cofactor protein) and other cell surface proteins as attachment sites to facilitate their entry into a variety of host cell types.[89] The surface protein GP350/220 of Epstein-Barr virus (EBV) contains a nine-residue sequence that is similar to the sequence on C3d that interacts with CR2.[90] This allows EBV to bind to and activate B lymphocytes and is believed to account for the tropism of that virus for those cells and the induction of polyclonal B cell activation during EBV infection.[90] C3d on the surface of HIV serves to hold the virus on CR2 on the surface of follicular dendritic cells in lymph nodes, accounting for a major reservoir of the virus in most infected individuals.[91] Mycobacteria may become coated with complement without being damaged. The mycobacteria then use the complement fragments to induce phagocytosis through the complement receptors on macrophages,[92] in which these intracellular pathogens survive and replicate.

Besides putting sialic acid on their capsules to inhibit alternative pathway activation, many other adaptations help pathogenic bacteria evade complement.[78,88] The side chains of some gram-negative bacterial lipopolysaccharides are able to hold active complement components at a distance from the cell membrane or cause shedding of the nascent MAC as it is being formed. Many gram-positive bacteria have surface proteins that mimic or interact with complement control factors. For example, streptococcal M proteins and other somewhat similar proteins on pneumococci can bind C4 binding protein and factor H from human serum, then these proteins inhibit complement activation on the bacteria.[93] A similar strategy has been developed by *Borrelia burgdorferi,*[94] the spirochete that causes Lyme disease, and is also used by the blood forms of protozoans and parasitic worms. In addition, some bacteria secrete enzymes that can degrade the complement components in their milieu.[95] Viruses also have specific proteins that allow them to evade complement: the surface glycoprotein gC on herpes simplex–type 1 virus binds C3,[96] and orthopoxviruses, including both variola and vaccinia, code for proteins that are homologous with RCA family members and that protect virus-infected cells from attack.[97] In yet another variation, cytomegalovirus infection up-regulates synthesis of CD46 (MCP), protecting infected cells from complement attack and increasing pathogenicity of the virus.[98] The blood phases of treponema, other protozoans, and schistosomes and other parasites also often bind factor H or other complement regulatory proteins, thereby protecting themselves.

Role of Complement in Antibody Responses

Complement plays a major role in localization and persistence of antigen on follicular dendritic cells in germinal centers, most importantly by the interaction of C3d with CR2.[99] Complement-bearing antigens are much more efficient in activating B cells than are non-complement-bearing antigens, and coengagement of CR2 and antigen receptor surface immunoglobulin increases processing and expression of major histocompatibility complex (MHC) class II antigen peptide on the B cells, which probably contributes to T cell activation in vivo.[65,66] Some T cells themselves have complement receptors, and recent studies show that complement may provide costimulation for T cells.[100] These effects, particularly those involving antigen persistence and localization, likely explain the in vivo observations that depletion of complement in mice decreases secondary antibody responses, particularly to T cell–dependent antigens.[101] Similar results are found in humans and guinea pigs congenitally deficient in C4.[102] Primary IgM responses are not affected much, but on reimmunization, the secondary IgG response is markedly diminished, and IgM production resembles the response to the primary immunization.[102] Thus, by directing antigen localization and by increasing B cell activation, bound C3 fragments play a major role in immunologic memory and immunoglobulin class switching.

COMPLEMENT GENES AND THEIR ORGANIZATION

The genes for each of the complement proteins and major receptors and control proteins have all been identified and sequenced.[103] As expected, because many of the proteins are homologous with each other (i.e., C1r with C1s and the MASPs; C2 with factor B; C3, C4, and C5 with each other), studies of the structures and locations of various genes suggest that these homologies have arisen by gene duplication then divergence.The three different polypeptides that make up the trimeric subunits of C1q are all encoded by different genes that are clustered together on the short arm of chromosome 1 and organized similarly.

The homologous genes for MBL, surfactant proteins A and D, and other members of the collectin family are on chromosome 10.[104] C1r and C1s are encoded by separate but adjacent genes on chromosome 12, which share about 40% homology.[105] The homologous MASP genes are on chromosome 3 (*MASP1*) and chromosome 1 (*MASP2*). The genes for C2 and its homologue, factor B, and the gene for C4 are on chromosome 6 and are considered part of the MHC complex,[106] termed MHC class III genes. Although less diverse than MHC class I and II proteins, these and many other complement proteins also have different allotypes, which also correlate with susceptibility to different autoimmune diseases, although the reasons for this have not been clearly elucidated. There are two separate loci for C4, with multiple allotypes at each.[107] The proteins encoded by the different genes have different tendencies to form amide versus ester bonds when they transfer their internal thioesters, and they may have different activities in hemolytic compared with other types of assays. It is possible to have homozygous null mutations at one locus but still produce the other type of C4. The two slightly different gene products are distinguished as the Chido and Rogers blood group antigens when their fragments are displayed on red cells. Like its homologues C3 and C5, C4 is synthesized as a single chain precursor pro-C4, which is cleaved to form the predominant form in plasma, but as much as 5% of the circulating C4 may be the single chain pro-C4 form.[108] Unlike the other multichain components, the three chains of C8 are the products of two separate genes. Most of the genes for the homologous proteins that regulate complement activity by binding to C3 or C4 are found in the RCA gene cluster on chromosome 1(q32).[31] CR1, the C4b/C3b receptor, has structural allotypes as well as genetically determined quantitative differences in expression.[109] Allotypic differences in specific components' functions, as well as in receptor expression and function, may be related to differences in the efficiency of clearance of immune complexes and therefore to the susceptibility to autoimmune and inflammatory diseases.

COMPLEMENT SYNTHESIS

Complement synthesis by fetal tissues has been observed as early as the sixth week of gestation,[110,111] and most of the components are detectable in fetal serum by the 10th to 12th weeks.[110,111] Studies of maternal-fetal pairs that express different allotypes of C3[112] and other components and studies in babies of complement-deficient mothers[113] indicate that complement proteins do not cross the placenta and that fetal synthesis accounts for the complement components easily measurable in cord blood. Complement synthesis has been documented in kidney and other fetal tissues[114-116] in addition to the liver. The liver is the major site of production of most of the complement proteins in adult's plasma.[117] For example, the colon may be the major site of production of C1 in fetal life[118] and continues to be an important site of synthesis in the adult. Various components can also be synthesized by myeloid and other types of cells, and local synthesis may play important roles in inflammatory reactions (i.e., in inflamed joints, lung, and sites of infection). The levels of most components increase with gestational age and reach 50% to 75% of the levels in maternal serum at term.[119-127] However, the levels of most of the complement proteins in maternal serum also increase during pregnancy, so the levels in the term newborn correspond more closely to about 80% of normal adult levels.[120-122] The levels of C8 and C9 in term babies are lower than that, at only about 10% of the level in maternal serum.[122] The addition of normal human C9 in vitro increases the hemolytic activity and the ability of neonatal sera to kill gram-negative organisms.[128] In contrast to the active components, C1 INH reaches the same concentration as in adult serum by the 28th week of gestation.[110]

As expected, because the concentrations of most components increase progressively during gestation, preterm babies have lower levels of complement activity than full-term babies. The concentrations usually climb to within the normal adult range by 3 months of postnatal life. The difference between the actual functional amount of complement at a site of infection in a baby compared with an adult, however, may be greater than the difference in the serum levels because synthesis in the baby may not increase in response to infectious stimuli in the same way as the adult.[129] However, it is not clear whether this difference resides in the regulation of the complement proteins themselves compared with differences in other proteins, such as interferon-γ, which are involved in the response to lipopolysaccharides and other stimuli.[29] The relatively lower levels of C3 and factor B in neonates' sera, together with the decreased levels of CR3 in neonate's neutrophils, likely both contribute to compromise phagocytic defenses,[130] even in term newborns.

COMPLEMENT IN MATERNAL-FETAL MEDICINE

Recent studies suggest that there is ongoing low-level complement activation during pregnancy, as evidenced by increased concentrations of C3a and other split fragments in maternal circulation.[131] Both fetal and maternal complement components are present in the placenta, and alloantibodies in the mother have the potential for activating complement in the placenta. Expression of the complement regulatory proteins DAF (CD55), MCP (CD46), and protectin (CD59) on trophoblasts has been documented[132] and may play an important role in maintaining the fetus. Homozygous deletion in mice of the major complement receptor and control protein, Crry, which is a homologue of CR1 and CR2, causes embryonic lethality,[133] suggesting the importance of restricting complement activation in the placenta. Elegant studies in mice show that passive transfer of antiphospholipid antibodies purified from women with recurrent pregnancy losses or other signs of antiphospholipid antibody syndrome cause fetal loss.[134] Studies of this model in the presence of complement inhibitors and in complement knockout mice strongly suggest that complement can play an important role in damaging the placenta.[134] Even in the absence of antibody, complement activation and generation of C5a can lead to inflammatory infiltrates in the placenta, intrauterine growth retardation, and loss of fetuses.[135] Furthermore, it has been reported that there may be complement-fixing antitrophoblast or antiendothelial cell antibodies in the serum of women with recurrent fetal losses who do not have antiphospholipid antibodies.[136] Complement does not play a major role in Rh(D) hemolytic disease of the newborn because the density of D antigens on erythrocytes are too few and far apart for the IgG antibodies to attach to and activate C1 molecules. Destruction of the fetal red cells in this case is carried out by FcγR-mediated macrophage phagocytosis.

A growing body of evidence suggests that maternal neutrophil activation occurs in, and probably contributes to, the pathophysiology of preeclampsia and eclampsia.[137,138] Some reports suggest that these conditions are associated with activation of complement and liberation of C5a, which activates the neutrophils and may induce formation of aggregates that occlude capillaries. In addition, local production of complement fragments and release of mediators like tumor necrosis factor from complement-activated leukocytes could certainly induce prothrombotic changes in endothelial cells, promoting microthromboses.[139] The resulting restriction of blood flow could contribute to hypertension in the mother and intrauterine growth restriction in the fetus.[138] This process could certainly be initiated by binding of maternal alloantibodies to trophoblasts or by formation of immune complexes in the placental circulation. One report suggests that the risk for preeclampsia is higher in women with the low expressing allele of CR1, whose erythrocytes have less capacity to bind and carry away immune complexes than the erythrocytes of women with medium- or high-level expression of CR1.[140] Meconium has also

recently been shown to be a potent activator of the alternative pathway,[141] and the severity of lung damage or inflammation in an animal model of meconium aspiration correlates with the degree of complement activation induced by instillation of meconium into the lungs.[142]

THERAPEUTIC MANIPULATION OF COMPLEMENT ACTIVITY

In many diseases that involve autoantibodies or alloantibodies formed by the mother against antigens expressed by the fetus but not herself, complement may be physiologically activated after antibody binding. However, because in these situations, the antigen is a normal body constituent, the complement activation causes pathology. This may occur if the mother produces IgG antibodies against fetal antigens because that is the only class that crosses the placenta. Complement activation by the alternative pathway may also occur after ischemia-reperfusion injury.[34] In all those situations, production of C5a may cause local neutrophil activation and aggregate formation, which can plug capillaries; deposition of C3 fragments may lead to attack on cells and interstitial structures, and deposition of MACs may cause endothelial or parenchymal cell damage. It may be thus be desirable to inhibit complement activation as part of the therapy. It is possible that augmentation of normal C1 esterase inhibitor activity may be beneficial because of the broad range of activity of this inhibitor.[40,143] Small molecule inhibitors of the complement convertases are under development, but they have not yet come into use clinically. As noted earlier, by binding to C3b, CR1 can inhibit both the classical and alternative pathways. A recombinant form of soluble CR1 has been prepared, which inhibits both the classical and alternative activation pathways.[34] This molecule impressively limits the extent of myocardial infarction in an animal model of transient ischemia,[34] and some clinical trials have been started. A monoclonal antibody against C5 has been developed, which has shown very promising results in inhibiting lysis of paroxysmal nocturnal hemoglobinuria erythrocytes[144] and may prove useful in other disorders in which MAC formation participates.

As mentioned earlier, IgG antibody molecules provide very good acceptors for the nascent covalent binding site that is exposed during C3 activation.[10] Activating complement in the presence of increased concentrations of IgG results in distraction of some of the C3b molecules from the target to some of the extra IgG molecules in solution.[145] In this way, excess IgG can decrease C3b deposition and can prevent hemolysis of sensitized erythrocytes and other forms of tissue damage.[145-147] Therapeutically, administration of high doses of intravenous immune globulin (IVIG) (1 to 2 g per kg) may have this effect in vivo, and inhibition of complement deposition and reticuloendothelial clearance may be major mechanisms by which IVIG treatment acts in autoantibody- or alloantibody-mediated diseases, such as thrombocytopenia due to maternal alloantibodies. IVIG also exerts additional antiinflammatory effects whose mechanisms are not well defined, and this is the mainstay of treatment for Kawasaki syndrome. Whether IVIG is really effective in preventing fetal loss due to antiphospholipid antibodies or other alloantibodies remains to be proved, and the exact circumstances in which it is most likely to be effective must be defined by additional studies.

CONCLUSION

Complement is an important part of the innate immune system, which may be particularly important in neonates, who have low IgM concentrations and do not produce much of their own IgG. When antibody is present, complement can serve as a major amplification and effector pathway for the adaptive immune system. As with other aspects of complement's activities, its ability to attract and activate leukocytes is a double-edged sword that is necessary for normal host defense but that can cause serious pathology when activated inappropriately. Complement plays important roles in limiting inflammatory responses by removing infectious agents and noxious stimuli and supports normal homeostasis by directing trafficking and disposal of apoptotic cells and immune complexes. However, complement can contribute to local pathology when these mechanisms are overwhelmed or function imperfectly. Fortunately, an elaborate set of soluble and cell surface regulatory molecules keeps this system in delicate balance.

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Laurie E. Kilpatrick and Mary Catherine Harris

Cytokines and Inflammatory Response in the Fetus and Neonate

CYTOKINE STRUCTURE AND FUNCTION

Inflammation is a protective response to infection or injury. The several different components of this response act to mobilize host defense systems.

The inflammatory response is controlled primarily by cytokines, which are endogenous mediators of the immune system. An understanding of how the inflammatory response is regulated at both local and systemic levels requires an appreciation of the wide range of biologic activities of this set of multifunctional proteins. To provide this background, an overview of the production and physiologic functions of cytokines is presented next.

General Description of Cytokines

Cytokines are produced by various different cell types in response to multiple types of stimuli and have overlapping biologic activity.[1-5] Cytokines are potent endogenous mediators whose synthesis and secretion are under tight regulatory control. These endogenous mediators are not stored as preformed molecules in producer cells; rather, new synthesis is required for secretion.

Once these mediators are released, their half-life is relatively short, further limiting their biologic activity. Cytokines act in minute quantities (10^{-9} to 10^{-12} mole) by binding to specific cellular receptors that are members of distinct structural families. Most cellular responses to cytokines result in an altered pattern of gene expression that leads to alterations in cellular physiology.

An important function of cytokines is cell-to-cell communication. Cytokines are unusual in that they can communicate in autocrine, paracrine, or endocrine mode.[1,2,5] Most cytokine activity occurs on the local level, where these proteins act in paracrine fashion to exert their effects by simple diffusion or by cell-to-cell contact. Some cytokine producer cells also express cytokine receptors, and secreted cytokines can bind to their producer cells, whereby they can modulate cell function. Cytokines also can leave the local environment, enter the circulation, interact with different organ systems, and alter host physiology.

A group of cytokines is produced specifically in response to inflammatory stimuli. The function of these *proinflammatory* cytokines is to communicate to surrounding tissue the presence of infection or injury.[3-5] Principal proinflammatory cytokines are tumor necrosis factor-α (TNF-α); the interleukins IL-1, IL-6, IL-8, IL-12, IL-18; and interferon-γ (IFN-γ). TNF and IL-1 are the principal mediators of the inflammatory response and are thought to have a critical role in orchestrating the local response through cell activation and triggering a cytokine cascade.[3,6] Proinflammatory cytokines also can enter the systemic circulation and can produce immune cell activation and significant alterations in host physiology, such as fever, and the acute phase reaction.

Inflammatory stimuli also trigger the synthesis of *antiinflammatory* cytokines and specific cytokine inhibitors that serve to modify the host inflammatory response.[6-8] Antiinflammatory cytokines limit the inflammatory response by inhibiting proinflammatory cytokine synthesis; key antiinflammatory cytokines include IL-10, IL-13, IL-4, and IL-11.

Naturally occurring proinflammatory cytokine inhibitors neutralize proinflammatory cytokine bioactivity by blocking cytokine: cytokine receptor interactions, by binding to either the proinflammatory cytokine itself or to its specific receptor. These naturally occurring inhibitors include soluble proinflammatory cytokine receptors, decoy receptors, receptor antagonists, and cytokine binding proteins. The interplay among these proinflammatory cytokines, antiinflammatory cytokines, and naturally occurring cytokine inhibitors determines the inflammatory response and its effectiveness.

Proinflammatory Cytokines

Tumor Necrosis Factor

TNF-α is one of the principal mediators of the inflammatory response and has a critical role in orchestrating the local inflammatory response through cell activation and initiation of a cytokine cascade. TNF is synthesized as a 26-kDa transmembrane protein, which is processed to the mature 17-kDa form after cleavage of the residue signal peptide by a matrix metalloproteinase disintegrin.[9,10] TNF can exert activity as a transmembrane cell-associated species as well as in a secreted form.

TNF synthesis is triggered by endotoxin, enterotoxin, toxic shock syndrome toxin-1, viruses, mycobacterial cord factor, C5a, fungal and parasitic antigens, IL-1, and TNF itself.[6,11-13] The primary producer cells of TNF are monocytes and tissue macrophages. TNF also is produced by many other cell types, which include lymphocytes, natural killer (NK) cells, neutrophils, mast cells, endothelial cells, keratinocytes, smooth muscle cells, astrocytes, and microglial cells.

Tumor Necrosis Factor Receptors

The tight regulation of TNF signaling is important in the control of TNF-induced cellular alterations. Most cell types possess two TNF receptors: a 55- to 60-kDa receptor, TNFR-1 (i.e., CD120a), and a 75- to 80-kDa receptor, TNFR-2 (i.e., CD120b).[14,15] These two receptors are structurally related and are members of the tumor necrosis factor (TNF)/tumor necrosis factor receptor (TNFR) family. The extracellular domains of the TNFR-1 and TNFR-2 share 28% homology, but no homology is observed in the cytoplasmic domain.[16] The lack of homology of the two receptors suggests that they mediate discrete signaling pathways.[16]

TNFR-1 is largely responsible for proinflammatory cellular responses.[11,12,17-19] TNFR-1 also is unique in that it regulates signaling pathways linked to both proapoptotic and antiapoptotic responses (as reviewed by Aggarwal,[11] MacEwan,[12] and Wallach[19]). The cellular response to TNF is dependent on numerous environmental factors, including input from other signaling pathways.

TNFR-1 (Figure 156-1) contains a cytoplasmic sequence of approximately 80 amino acids termed the *death domain.* This region regulates apoptosis through its association with effector proteins that activate programmed cell death, but the death domain also is the region in this receptor that mediates the cell's survival or antiapoptotic pathways.[20]

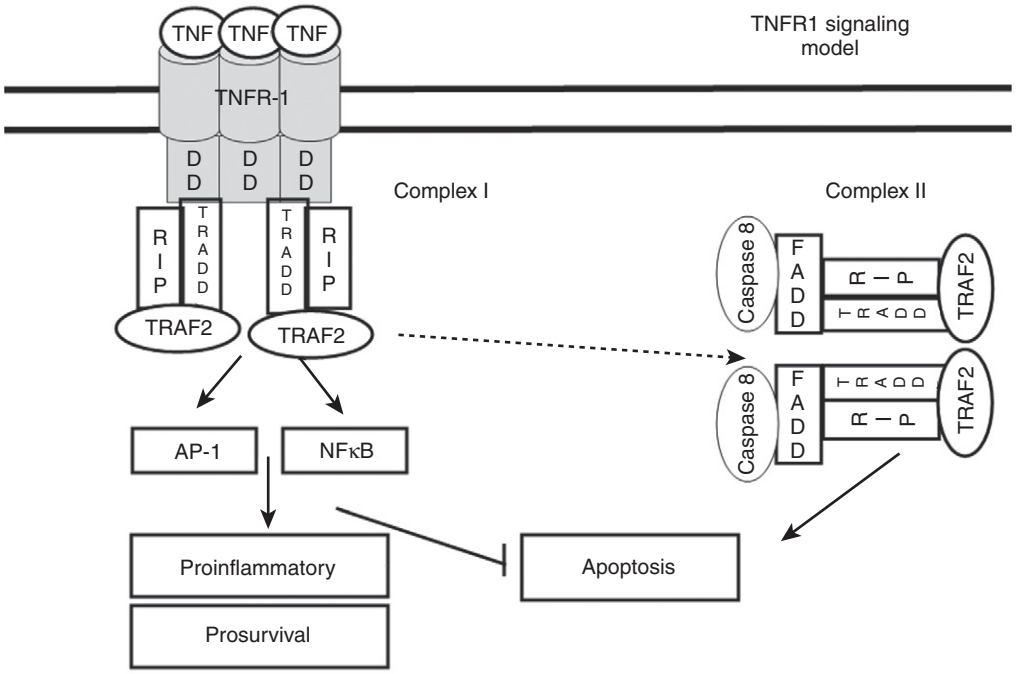


Figure 156-1 The tumor necrosis factor receptor-1 (TNFR-1) signaling complex. Binding of tumor necrosis factor (TNF) to TNFR-1 results in the recruitment and formation of a TNFR-1 signaling complex. TRADD (TNFR-associated death domain) associates with TNFR-1 through death domain (DD) interactions and serves as a platform for the recruitment of other adaptor proteins including TRAF-2 (TNFR-associated factor-2) and RIP (receptor-interacting protein) to form complex I. These adapter proteins recruit additional components necessary for the activation of proinflammatory and antiapoptotic pathways through the activation of transcription factors, such as nuclear factor κ-B (NFκB). Complex II is formed when TRADD:RIP:TRAF2 disassociate from TNFR-1. Subsequent recruitment of FADD (Fas-associated death domain) and caspase-8 to this complex initiate TNF-mediated apoptosis. *(Adapted from Micheau O, Tschopp J: Induction of TNF receptor 1-mediated apoptosis via two sequential signaling complexes,* Cell *114:181, 2003.)*

Additional studies have provided insight into the assembly of the TNFR-1 signaling complex and the regulation of proapoptotic and antiapoptotic signaling.[21] Activation of the TNFR-1 by TNF results in the formation of a receptor trimer through self-association with the death domains. Receptor trimerization leads to recruitment of effector proteins to form a signaling complex at the plasma membrane.[17] The TNFR-1–associated death domain protein (TRADD) associates with TNFR-1 and acts as a scaffold for recruitment of the effector proteins RIP (receptor-interacting protein) and TRAF2 (TNFR-associated factor 2) to form the complex TNFR-1:TRADD:TRAF2:RIP. This complex, termed complex I, controls both antiapoptotic and proinflammatory pathways primarily through activation of mitogen-activated protein (MAP) kinases and transcription factors such as nuclear factor-κB (NFκB) and activator protein-1 (AP-1), leading to the coordinated expression of antiapoptotic proteins, proinflammatory cytokines, chemokines, enzymes, and adhesion molecules.[22-24] Several studies suggest that the complex that signals apoptosis is distinct from complex I.[18,21,25]

A second complex termed complex II can be assembled when TRADD-TRAF2-RIP complex disassociate from TNFR-1 to form a second cytosolic complex with FADD (fas-associated death domain protein). FADD is essential for apoptosis through its association with and activation of caspase-8.[17] Apoptosis is activated by complex II under conditions in which the signal from complex I fails to activate NFκB.

Interleukin-1

IL-1α (or IL-1F1), IL-1β (or IL-1F2), and IL-1 receptor antagonist (IL-1ra)(or IL-1F3) (see the section on proinflammatory inhibitors) are members of the IL-1 ligand superfamily.[26-28] IL-1α and IL-1β are agonists, regulated independently, that are products of distinct genes located on chromosome 2.[27,29] The two molecules share little sequence homology (22% to 26%) but have similar tertiary structures, bind to the same receptors, and share biologic activities.[30]

Both IL-1α and IL-1β are synthesized as large 31-kDa leaderless precursor proteins (proIL-1α and proIL-1β).[27,31] Most of IL-1α remains in the cell cytosol in the pro form, where it can function in an autocrine fashion or be transported to the cell surface and participate in cell-to-cell communication.[27,32] IL-1α also is biologically active when it is cleaved to the 17.5-kDa mature protein by membrane-associated cysteine protease calpains and released from the cell. IL-1α is rarely observed in the circulation and appears systemically only during severe disease.[33] By contrast, IL-1β is active only in its cleaved mature form, and after secretion, it is the principal mediator of the systemic effects of IL-1. Activation and release of the mature biologically active 17-kDa form require the cysteine protease, caspase-1 (also called IL-1β–converting enzyme [ICE]).[34] IL-1β processing and secretion constitute a highly regulated event that involves multiple steps.[35] Initially, the IL-1β precursor and inactive procaspase-1 co-localize in the specialized secretory lysosomes. A molecular scaffold termed the IL-1β inflammasome activates the inactive procaspase-1 through proteolytic cleavage to caspase-1.[36] Caspase-1 in turn cleaves proIL-1β to its active form IL-1β. IL-1β is then secreted via a mechanism that may be linked to the nucleotide P2X7 receptor and activation of phospholipases.[28,31]

IL-1 synthesis is triggered by many of the same stimuli that activate TNF production (i.e., microbial products of inflammation). IL-1 is synthesized by many different cell types, including monocytes, macrophages, neutrophils, epithelial and endothelial cells, fibroblasts, B and T lymphocytes, smooth muscle cells, microglial cells, and astrocytes.[33]

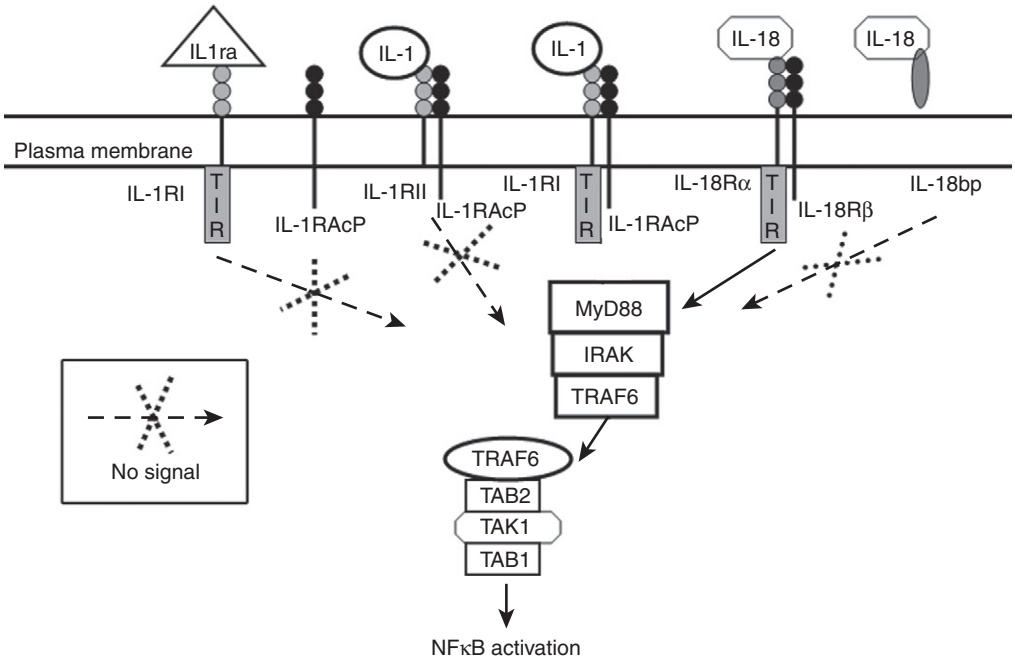


Figure 156-2 Interkeulin-1 (IL-1) and IL-18 receptor family. The IL-1 and IL-18 receptors are members of the interleukin-1/Toll-like receptor family. IL-1 can bind to either the type I IL-1 receptor (IL-1RI) or the type II IL-1R (IL-1RII). IL-1 binding to IL-1RI results in the association of the IL-1R accessory protein (IL-1RAcP) to form a complex that recruits the MyD88 (myeloid differentiation protein 88) to the TIR (Toll/interleukin-1 receptor) domain, activation of MYD88 signaling cascade, and subsequent activation of NFκB and proinflammatory gene transcription. Members of the IL-1RI signaling complex include MyD88, IRAK (IL-1R–associated kinase), TRAF6 (TNFR-associated factor 6), TAK-1 (transforming growth factor-β–activated kinase), and TAB-1 and TAB-2 (TAK-1 binding protein-1 and -2). IL-1 receptor antagonist (IL-1ra) also binds to the IL-1RI but does not trigger the association of IL-1RAcP and activation of the MyD88 signaling cascade. IL-1RII can bind IL-1, but binding does not result in the formation of a signaling complex. IL-1RII lacks the TIR domain and may act as a decoy receptor. IL-18 is also a member of the IL-1/Toll-like receptor family. IL-18 binding to the IL-18Rα triggers the association of IL-18Rβ and activation of the MyD88 signaling cascade. IL-18 also can bind to IL-18bp (IL-18 binding protein), a soluble inhibitor that prevents IL-18 interaction with the IL-18 receptor. *(Adapted from Mantovani A, Locati M, Vecchi A: Decoy receptors: a strategy to regulate inflammatory cytokines and chemokines,* Trends Immunol *22:328, 2001.)*

Interleukin-1 Receptors

Two different receptors bind IL-1: the type I IL-1 receptor (IL-1RI) and the type II IL-1R (IL-1RII). Both receptors are members of the IL-1 receptor/Toll-like receptor (IL-1R/TLR) superfamily[30,37-39] (Figure 156-2). This receptor family also includes receptors for the proinflammatory cytokine IL-18 and receptors for both gram-negative and gram-positive bacterial products. Most members of this family of receptors contain a conserved cytosolic region termed the Toll–IL-1 receptor (TIR) domain.[30,39] The IL-1RI and IL-1RII have similar extracellular characteristics, with three immunoglobulin domains and a high degree of sequence homology.[27,39] IL-1β binding to the IL-1RII, however, does not trigger signal transduction, because it contains a cytoplasmic region of only 29 amino acids that does not contain a TIR domain.[37,39] This receptor may serve as a decoy receptor and may decrease the availability of IL-1β to bind to the functionally active IL-1RI (see the section on cytokine inhibitors). IL-1RI is found on most cell types and is important in transducing the action of IL-1. IL-1 binds to IL-1RI with low affinity and requires the recruitment of the IL-1 receptor accessory protein (IL-1RAcP) to the receptor to form a high-affinity receptor complex, a requirement for optimal signal transduction.[37,39]

IL-1β, similar to TNF, activates the nuclear transcription factors NFκB and AP-1, which trigger the synthesis of numerous proinflammatory mediators[39-41] (see Figure 156-2). IL-1 binding to IL-1RI elicits the formation of a receptor complex with IL-1RAcP. This trimeric complex then recruits the cytosolic protein MyD88 (myeloid differentiation protein 88).[42] MyD88 acts as an adapter protein and mediates the recruitment of members of the IRAK family (serine/threonine IL-1 receptor–associated kinases) with the receptor complex.[43] IRAK-4 is activated and phosphorylates IRAK-1.[44] Phosphorylation of IRAK-1 triggers dissociation from MyD88 and subsequent association with TRAF6 (TNF receptor–associated factor 6). TAB-1 and TAB-2 (transforming growth factor-β–activated kinase-1 (TAK-1)-binding protein-1 and -2) are recruited to the activated TRAF6 complex that, in turn, phosphorylates TAK-1 (transforming growth factor-β–activated kinase 1).[45] TAK-1 initiates signaling, leading to the activation of NFκB and the MAP kinases p38 MAPK and JNK. Although IL-1 and TNF share many of the same postreceptor signaling pathways, they are not entirely redundant cytokines. IL-1, unlike TNF, cannot activate cellular programmed cell death pathways.

Interleukin-6

IL-6 is a pleiotropic cytokine with a wide range of biologic functions and is an important regulator of inflammation, immune responses, oncogenesis, and hematopoiesis.[46-48] IL-6 is a potent inducer of the acute phase response, as well as specific cellular and humoral immune responses including B cell differentiation and T cell activation.[48] IL-6 also is an important regulator of the transition from acute to chronic inflammation and of the shift from neutrophil to mononuclear cell infiltration.[49] IL-6 is not expressed constitutively, and IL-6 production is triggered in response to viral and bacterial infections and other cytokines such as TNF, IL-1β, and IFN-γ. IL-6 is a single-chain 21- to 28-kDa

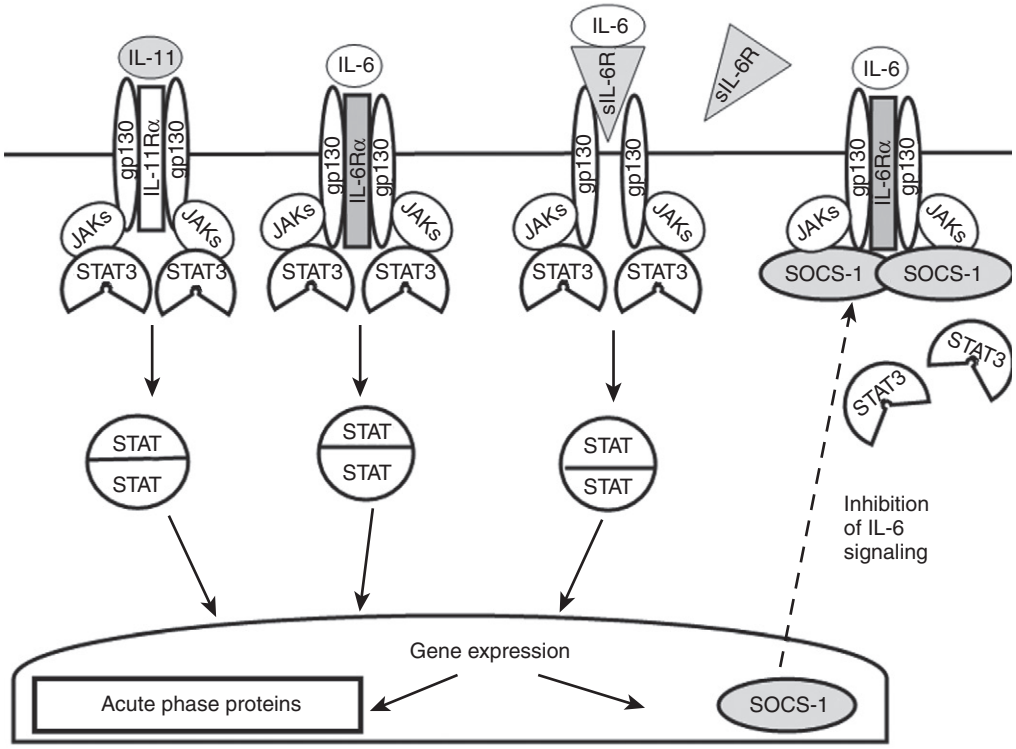


Figure 156-3 Interleukin-6 (IL-6) and IL-11 receptors are members of the IL-6 receptor family and utilize a common signal transducing subunit gp130. IL-6 and IL-11 bind to unique receptor α-chains IL-6Rα and IL-11Rα, respectively. A soluble form of IL-6R (sIL-6R) can also bind IL-6 and this complex can then associate with gp130 and initiate IL-6 signaling through a process termed trans-signaling. Receptor binding triggers association of gp130, activation of JAKs (Janus kinases) and STAT3 (signal transducer and activator of transcription), and the subsequent activation of gene transcription. STAT3 induces transcription of multiple genes including acute phase proteins and SOCS-1 (suppressor of cytokine signaling-1). SOCS-1 acts as a negative feedback regulator of IL-6 signaling by preventing JAK/STAT signaling.

protein produced by a variety of cells including B and T cells, monocytes, fibroblasts, endothelial cells, and some tumor cells.[47,50,51]

Interleukin-6 Receptors

The IL-6 receptor system consists of two components.The ligand-binding molecule, or IL-6R, is an 80-kDa glycoprotein[52] (Figure 156-3). Mutation studies have demonstrated that the cytoplasmic domain of IL-6R is not essential for signal transduction. The second component of the IL-6 receptor system is gp130, a non–ligand binding signal transducer.[53] IL-6 binding to the IL-6R leads to the association of gp130 with the receptor complex. Both IL-6R and gp130 are members of the cytokine receptor family, which is characterized by four conserved cysteine residues and a tryptophan-serine-X-tryptophan-serine motif above the transmembrane domain.[54] The gp130 also is the common signal-transducing subunit for other members of the cytokine receptor family including IL-11, oncostatin-M, ciliary neurotrophic factor, cardiotrophin-1, leukemia inhibitory factor, and the newly discovered interleukins IL-27 and IL-31, leading to redundancy in cytokine activity.[55,56]

Although the membrane IL-6R is expressed on hepatocytes, monocytes, macrophages, neutrophils, and some types of lymphocytes, a naturally occurring soluble form of the IL-6R (sIL-6R) also is present in most body fluids.[56] The sIL-6R is produced by proteolytic cleavage or through translation from alternatively spliced messenger RNA (mRNA). IL-6 binding to sIL-6R triggers the association with the signal-transducing gp130 component in cells that do not express the membrane-bound form of IL-6R.[56,57] Thus, unlike most other soluble cytokine receptors, sIL-6R can act as an agonist and through trans-signaling can activate cell types which do not express the IL-6R. In contrast with sIL-6R, soluble gp130 (sgp130) inhibits IL-6 activity by binding to IL-6/sIL-6R complexes and interfering with IL-6 trans-signaling.[56]

Formation of the IL-6 receptor complex leads to activation of gp130 associated tyrosine kinases of the Janus family (JAK) resulting in tyrosine phosphorylation of the cytoplasmic domain of gp130.[53,58,59] These phosphorylated sites serve as docking sites for the transcription factors STAT 1 (signal transducer and activator of transcription-1) and STAT 3. STAT1 and STAT3 also are kinase substrates, and after phosphorylation, they disassociate from the receptor complex and translocate to the cell nucleus, where they initiate expression of target genes. JAK kinases also activate other tyrosine kinases, which in turn activate MAP kinases and the transcription factors AP-1 and nuclear factor IL-6 (NF-IL6).[53] IL-6 signaling is regulated by feedback inhibition of the JAK-STAT pathway. Activation of STAT3 also induces transcription of SOCS-1 (suppressor of cytokine signaling-1), which binds to JAK and acts as a negative regulator of gp130 signaling.[46,47]

Interleukin-8 and Chemokines

IL-8 is the prototype of a family of cytokines that are chemotactic for leukocytes. Chemotactic cytokines or chemokines are proinflammatory mediators that orchestrate the recruitment of leukocytes to sites of inflammation. IL-8 is synthesized as a precursor form of 99 amino acids.After cleavage of a signal sequence, IL-8 is secreted in the mature form, approximately 8.4 kDa in size.[60] IL-8 is produced by monocytes, macrophages, fibroblasts, endothelial

cells, epithelial cells, hepatocytes, keratinocytes, synovial cells, chondrocytes, and some tumor cells.[60] Endotoxin, TNF, and IL-1, but not IL-6, trigger production of IL-8. IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), lectins, phorbol esters, immune complexes, and phagocytosis also stimulate IL-8 production.

IL-8 (also termed CXCL8) is a member of the chemokine supergene family which is composed of close to 50 members.[61-64] Little sequence homology exists between chemokines, but they are remarkably similar in their three-dimensional structure.[63,64] The chemokine family is divided into four structural groups characterized by the number and spacing of conserved cysteine residues: CXC, CC, C, and CX_3C.The CXC chemokine group contains one amino acid between the first two cysteine residues.The CXC chemokine subgroup is further divided into two groups based on the presence or absence of a Glu-Leu-Arg (ELR) motif located before the first cysteine residue and termed ELR^+CXC and ELR^-CXC chemokines.[65] ELR^+CXC chemokines act primarily on neutrophils, whereas ELR^-CXC chemokines interact with mononuclear leukocytes. The ELR^+CXC family includes several chemokines with biologic activity similar to that of CXCL8 (IL-8), such as CXCL1 (GRO-α), CXCL2 (GRO-β), CXCL3 (GRO-γ), CXCL7 (neutrophil-activating peptide-2 [NAP-2]), CXCL5 (epithelial cell–derived neutrophil activating protein-78 [ENA-78]), and CXCL6 (granulocyte chemotactic protein-2 [GCP-2]).[61]

CC chemokines—the second structural group—contain adjacent cysteine residues. CC chemokines act principally on monocytes, basophils, eosinophils, and some lymphocyte subpopulations.[61,66] The best-characterized CC chemokine is CCL2 (monocyte chemotactic protein 1 [MCP-1]). Other members of the CC chemokine family include CCL8 (MCP-2), CCL7 (MCP-3), CCL5 (RANTES), CCL3 (macrophage inflammatory protein [MIP-1α]), and MIP-1β (CCL4).

The C chemokine structural group has a single amino-terminal (N-terminal) cysteine and contains two highly related chemokines, XCL1 (lymphotactin) and XCL2 (SCM-1β).[62] The last subgroup, CX_3C, contains a single member, CX3CL1 (fractalkine), which is characterized by separation of the first two cysteine residues by three amino acids. CX3CL1, as well as CXCL16, is present in two forms: a membrane-bound form, which acts as a leukocyte adhesion receptor, and a cleaved form, which is secreted and acts as a soluble chemoattractant.[61,64] Chemokines also have been classified as constitutive and inducible to differentiate other chemokines involved in homeostatic trafficking and those involved in inflammation.[62,64] However, a number of chemokines have overlapping characteristics, depending on environmental factors.

Chemokine Receptors

Chemokine receptors are a family of G-protein–coupled receptors (GPCRs) composed of at least 10 CC chemokine receptors, 6 CXC chemokine receptors, 1 CX_3C chemokine receptor, and 1 C chemokine receptor[61,67] (Table 156-1).An unusual feature of this receptor family is their ability to bind more than one chemokine within their particular subclass. An exception is the DARC receptor (Duffy antigen receptor for chemokines) located on erythrocytes and endothelial cells, which will bind both CC and CXC chemokines. Engagement of this receptor does not appear to activate signal transduction, and DARC may limit leukocyte activation by serving as a chemokine sink.

Two distinct CXCL8 (IL-8) receptors—CXCR1 and CXCR2—have been cloned and demonstrate 77% sequence homology.[68,69] CXCR1 binds CXCL8 (IL-8) with high affinity and CXCL1 (GRO-α) and CXCL7 (NAP-2) with low affinity, whereas CXCR2 binds most ELR^+CXC chemokines.[67] In neutrophils, CXCL8 (IL-8) binding to CXCR1 or CXCR2 leads to the activation of G-protein–sensitive phospholipase C, which, in turn, results in the formation of inositol triphosphate and elevation of cytosolic calcium.[62,66,67] These receptors also activate other signaling pathways coupled to activation of phosphoinositide 3-kinase, tyrosine kinases, MAP kinase, and low-molecular-weight proteins Rho and Ras. CXCL8 (IL-8) binding also triggers receptor phosphorylation and desensitization, a process thought to be important for continued cellular ability to detect chemotactic gradients.[70]

Interleukin-12

IL-12 is a potent proinflammatory cytokine that plays a major role in linking innate and adaptive immune responses.[71,72] IL-12 is a member of the IL-12 cytokine family comprised of structurally related cytokines including IL-12, IL-23, and IL-27.[73-75] IL-12 is a heterodimer composed of two subunits, an α-chain (p35 subunit) and a β-chain (p40 subunit), which are encoded by separate genes and covalently linked by disulfide bonds.[72,76,77] The p40 subunit of IL-12 contains the receptor binding site,

TABLE 156-1

Chemokine Receptors and Their Ligands

Receptor	Ligand(s)*
CXC Chemokine Receptors	
CXCR1	CXCL6 (GCP-2), CXCL7 (NAP-2), CXCL8 (IL-8)
CXCR2	CXCL1 (GROα), CXCL2 (GROβ), CXCL3 (GROγ), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL7 (NAP-2), CXCL8 (IL-8)
CXCR3	CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC)
CXCR4	CXCL12 (SDF-1)
CXCR5	CXCL13 (BCA-1)
CXCR6	CXCL16
CC Chemokine Receptors	
CCR1	CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL13 (MCP-4), CCL14 (HCC-1), CCL 15 (HCC-2), CCL16 (HCC-4), CCL23 (MPIF-1)
CCR2	CCL2 (MCP-1), CCL7 (MCP-3), CCL8 (MCP-2), CCL13 (MCP-4), CCL16 (HCC-4)
CCR3	CCL5 (RANTES), CCL7 (MCP-3), CCL11 (eotaxin), CCL 15 (HCC-2), CCL16 (HCC-4), CCL24 (eotaxin-2), CCL26 (eotaxin-3)
CCR4	CCL17 (TARC), CCL22 (MDC)
CCR5	CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL8 (MCP-2)
CCR6	CCL20 (MIP-β)
CCR7	CCL19 (ELC), CCL21 (SLC)
CCR8	CCL1 (I-309)
CCR9	CCL25 (TECK)
CCR10	CCL27 (CTACK), CCL28 (MEC)
C Chemokine Receptor	
XCR1	XCL1 (lymphotactin), XCL2 (SMC-1β)
CX3C Chemokine Receptor	
CX3CR1	CX3CL1 (fractalkine)

*Ligands are listed under the new nomenclature, with the old name in parentheses.

BCA-1, B cell–attracting chemokine-1; *CTACK,* cutaneous T cell–attracting chemokine; *ELC,* Epstein-Barr virus–induced receptor ligand chemokine; *ENA-78,* epithelial neutrophil-activating protein-78; *GCP-2,* granulocyte chemoattractant protein; *GRO,* growth-related oncogene; *HCC,* hemofiltrate CC chemokine; *IL-8,* interleukin-8; *IP-10,* interferon-γ–inducible protein; *I-TAC,* interferon-inducible T cell α-chemoattractant; *MCP,* monocyte chemotactic protein; *MDC,* macrophage-derived chemoattractant; *MEC,* mammary-enriched chemokine; *MIG,* monokine induced by interferon-γ; *MIP-1,* macrophage inflammatory protein-1; *MPIF,* myeloid progenitor inhibitory factor; *NAP-2,* neutrophil-activating protein-2; *RANTES,* regulated on activation normal T cell expressed and secreted; *SCM,* single C motif; *SDF-1,* stromal cell–derived factor-1; *SLC,* secondary lymphoid tissue chemokine; *TARC,* thymus- and activation-regulated chemokine; *TECK,* thymus-expressed chemokine.

whereas the p30 subunit is required for signal transduction. The two subunits demonstrate little sequence homology, but the p35 subunit shows sequence homology with IL-6 and G-CSF, whereas the p40 shows homology with the extracellular domain of the IL-6 receptor.[77] The p40 chain of IL-12 can also associate with another molecule termed p19 to form a distinctive heterodimeric cytokine, IL-23.[78] A homologue of IL-12p40, Epstein-Barr virus–induced gene 3 (*EBI3*), forms a noncovalent association with another homologue of IL-12p35 (p28) to produce IL-27.[79]

IL-12 is produced principally by phagocytic cells, such as monocytes, macrophages, and neutrophils, and by microglial and dendritic cells, in response to bacteria, bacterial products, intracellular pathogens, and viruses.[72] Similar to IL-12, IL-23 and IL-27 are produced predominantly by macrophages and dendritic cells in response to inflammatory stimuli.[71,75] IL-12 induces production of interferon-γ (IFN-γ) and regulates T helper (T_H1)-type inflammatory responses. IL-27 also promotes T_H1 cell differentiation and IFN-γ production, but preferentially acts on naive $CD4^+$ T cells.[73,74] By contrast, IL-23 acts on several T cell types and promotes synthesis of IL-17, a proinflammatory cytokine involved in neutrophil-mediated inflammation.[75] Both IL-12 and IL-27 act to suppress the development of IL-17–secreting T cells and limiting IL-17–mediated proinflammatory actions.[74,75]

Interleukin-12 Receptors

IL-12 receptors are expressed primarily on NK and T cells. The receptor is composed of two subunits—IL-12Rβ1 and IL-12Rβ2—and is a member of the gp130 cytokine receptor superfamily.[80] IL-12 signaling is through the JAK/STAT family signal transduction pathway.[81] IL-12 binding to the IL-12 receptor leads to tyrosine phosphorylation of JAK2 and TYK2; subsequent phosphorylation of STAT1, STAT3, STAT4, and STAT5; and synthesis of IFN-γ.[72,75] IL-23 binds to a receptor composed of the IL-12Rβ1 subunit and an IL-23R subunit and activates a similar pattern of JAKs and STATS.[72] The IL-27 receptor also is principally located on NK and T cells but is composed of an orphan receptor subunit WSX1/TCCR linked to a gp130 subunit. IL-12 and IL-27 signaling is mediated principally though STAT4, whereas IL-23 signaling is mediated through STAT3.[72,74,75]

Interferon-γ

IFN-γ is a pleiotropic cytokine produced by activated T cells and NK cells in response to IL-12, IL-27, and IL-18.[77,82,83] IFN-γ is a critical mediator of T cell recruitment and phagocyte-mediated clearance of pathogens.[4] In macrophages, IFN-γ triggers cytokine (including TNF) and chemokine synthesis, enhanced oxygen radical generation and nitric oxide (NO) production, and induction of major histocompatibility complex (MHC) class II expression.[4,83] The cellular targets of IFN-γ are numerous because IFN-γ receptors are present on virtually every cell type, and most of the biologic effects mediated by IFN-γ are through transcriptional regulation of approximately 500 genes.[82,83]

Interferon-γ Receptors

Similar to other cytokine receptor systems, the receptor is composed of two subunits, IFN-γR1, which is necessary for ligand binding, and IFN-γR2, which is involved in signal transduction.[77,82,83] IFN-γ binding to the IFN-γR1 subunit leads to receptor dimerization and the association of two accessory chains—the IFN-γR2 subunits. Both IFN-γR1 and IFN-γR2 have specific JAK kinases constitutively associated (JAK1 and JAK2, respectively).[84] The formation of the receptor complex results in transphosphorylation of the JAK kinases and subsequent phosphorylation of the IFN-γR1. Tyrosine phosphorylation of the receptor provides a docking site for the cytosolic factor STAT1, which, after receptor phosphorylation, is also phosphorylated. STAT1 phosphorylation results in the formation of STAT complexes, translocation of these complexes to the nucleus, and the regulation of immunological relevant genes.

Interleukin-18

IL-18 originally was designated the IFN-γ–inducing factor, a factor that stimulated the synthesis of IFN-γ by $CD4^+$ T lymphocytes and had biologic activity similar to that of IL-12.[85] Later studies demonstrated that the IL-18 receptor is a member of the IL-1/Toll-like receptor superfamily, and that IL-18 has structural homology with IL-β[86] Similar to IL-1β, IL-18 is synthesized in a proform with an unusual signal peptide that lacks the amino acid sequence necessary for secretion.[87,88] Unlike that of IL-1β, the precursor form of IL-18 has no biologic activity. ProIL-18 also is cleaved by caspase-1 to an 18-kDa nonglycosylated protein. IL-18 is produced by a wide range of cell types including Kupffer cells, macrophages, monocytes, T and B cells, dendritic cells, epithelial cells, and osteoblasts in response to inflammatory stimuli, such as lipopolysaccharide (LPS), exotoxins from gram-positive bacteria, and a variety of other microbial products.[89,90]

Interleukin-18 Receptors

The IL-18 receptor system is similar to that of the multicomponent IL-1β receptor system and expressed in a variety of cells including macrophages, neutrophils, NK cells, smooth muscle cells, and dendritic cells (see Figure 156-2).[30,91] The IL-18 receptor is composed of two subunits, an IL-18–binding component (IL-18Rα) and a signal-transducing component (IL-18Rβ) that is a homologue of AcP. IL-18 binds to IL-18Rα with low affinity that is significantly enhanced with the recruitment of IL-18Rβ. Similar to IL-1β receptor system, IL-18Rβ does not bind to IL-18; rather, it binds to the IL-18–IL-18Rα complex. IL-18 binding to the IL-18 receptor complex recruits the same adapter proteins as for IL-1β, including MyD88, IRAK, and TRAF-6. These adapter proteins activate the transcription factor NFκB and p38 MAPK. The p38 MAPK in turn activates another transcription factor, C/EBPβ. These transcription factors stimulate the synthesis of several proinflammatory genes including other cytokines and cytokine receptors, chemokine and chemokine receptors, NO synthase, adhesion molecules, and the Fas ligand.[30,90] Although several similarities between the biologic properties of IL-1 and IL-18 have been identified, significant differences are recognized. Unlike IL-1, IL-18 is not able to induce fever but can stimulate IFN-γ synthesis in T cells and NK cells.[90-92]

Antiinflammatory Cytokines

Interleukin-10

IL-10 was first described as a cytokine synthesis-inhibiting factor and a potent inhibitor of IFN-γ.[93] It is now recognized that IL-10 possesses both potent antiinflammatory and immunosuppressive properties. IL-10 inhibits mononuclear cell synthesis of a wide range of proinflammatory cytokines including TNF, IL-1β, IL-1α, IL-6, IFN-γ, IL-12, IL-18, IL-8, and both CXC and CC chemokines.[7,94-97] In addition to inhibiting the production of proinflammatory cytokines, IL-10 also stimulates the production of natural proinflammatory cytokine inhibitors such as IL-1ra and sTNFRs (see the section on inhibitors of proinflammatory cytokines).[7,94,96] IL-10 further modulates the proinflammatory response by down-regulating MHC class II expression, oxygen radical production, NO synthesis, and intercellular adhesion molecule-1 (ICAM-1) expression. These properties make IL-10 a critical factor in the regulation of the magnitude of the inflammatory response and in the prevention of an overwhelming response to inflammation.[94-96,98] IL-10 is synthesized by numerous cell types including monocyte-macrophages, B and T cells, dendritic cells, and epithelial cells in response to bacteria, bacterial products, proinflammatory cytokines, parasites, fungi, and viruses.[94,98]

IL-10 is a 37-kDa homodimer whose tertiary structure resembles IFN-γ, a cytokine whose actions are antagonized by IL-10.[94,96,97,99]

Interleukin-10 Receptors

IL-10 receptors are members of the class II cytokine receptor family and are expressed predominantly on immune cells.[94,100] IL-10R is composed of two subunits, IL-10RI, a ligand-binding subunit, and IL-10RII, an accessory subunit. The accessory subunit associates with the receptor after IL-10 binding and is required for signal transduction. Similar to other members of this cytokine receptor family, the receptor complex interacts with members of the JAK family, specifically JAK1 and TYK2. IL-10 binding triggers tyrosine phosphorylation and activation of the transcription factors STAT3, STAT1, and STAT5.[95,97] Critical to its potent antiinflammatory properties, IL-10 inhibits nuclear translocation of the transcriptional factor NFκB and subsequent synthesis of proinflammatory cytokines, as well as promoting degradation of mRNA of proinflammatory cytokines.[95,96,99] The activation of STAT3 by IL-10 also triggers the synthesis of SOCS-1 and SOCS-2, effector proteins that can interfere directly with proinflammatory signaling.[97]

Interleukin-4 and Interleukin-13

IL-13 and IL-4 are members of the T_H2-type cytokine family and are the products of $CD4^+$ T cells, $CD8^+$ T cells, mast cells, basophils, eosinophils, and NK cells.[101-104] IL-4 is a 20-kDa glycosylated protein, and IL-13 is a 10-kDa nonglycosylated protein.

Although IL-4 and IL-13 have only 20% to 25% amino acid sequence homology, their tertiary structures are highly homologous, and they possess numerous overlapping biologic properties.[7,102,105,106] Both IL-4 and IL-13 are potent antiinflammatory cytokines that are capable of inhibiting monocyte-macrophage production of proinflammatory cytokines and chemokines. Moreover, IL-4 and IL-13 up-regulate the synthesis of antiinflammatory IL-1ra and promote adherence by increasing expression of adherence molecules. The overlapping biologic properties of these two cytokines are the result of a common component in their respective receptor complexes.[107] However, the two cytokines do not share all biologic properties; most notable is that IL-4, but not IL-13, can promote T_H2 lymphocyte differentiation.[106] The functional differences between these two cytokines are the result of cell-dependent differential receptor expression.

Interleukin-4 and Interleukin-13 Receptors

IL-4 can bind to two receptor complexes, type I and type II[106-109] (Figure 156-4). The type I IL-4 receptor consists of a ligand-binding chain (IL-4Rα) and a common γ-signaling chain (γc), which also is an essential component of IL-2, IL-7, IL-9, and IL-15 receptors. The type II IL-4R receptor is composed of IL-4Rα subunit and IL-13Rα1 subunit. Unlike the type I IL-4R, both IL-4 and IL-13 can bind to the type II IL-4R. A second IL-13 binding component has been identified, IL-13Rα2 receptor subunit, which binds IL-13 with high affinity but does not initiate cell signaling.[110] IL-13Rα2 serves as a decoy receptor to down-regulate IL-13 activity. Signaling of both IL-4 and IL-13 is mediated principally through the IL-4Rα chain and involves activation of the transcription factor STAT6.[107-109,111,112] IL-4 binding to type I IL-4R induces the phosphorylation of the JAK1 associated with the IL-4Rα chain and JAK3 associated with the γc chain. These kinases, in turn, phosphorylate the IL-4Rα chain itself, as well as STAT6, resulting in STAT6 translocation to the nucleus and transcription of IL-4 responsive genes. Phosphorylation of IL-4Rα also stimulates recruitment of IRS-2, a member of the insulin substrate family, which activates the phosphoinositide 3-kinase pathway—a pathway critical in IL-4/IL-13 induced cell survival and protection from apoptosis.[107,108] IL-13 also activates similar signaling pathways through interaction with the type II IL-4R.[109] IL-13 binding to the IL-13Rα chain induces heterodimerization with IL-4Rα and stimulates phosphorylation of JAK1 associated with the IL-4Rα chain and another member of the JAK family, TYK2, associated with the IL-13Rα1 chain. The IL-13Rα1 can both amplify IL-4Rα

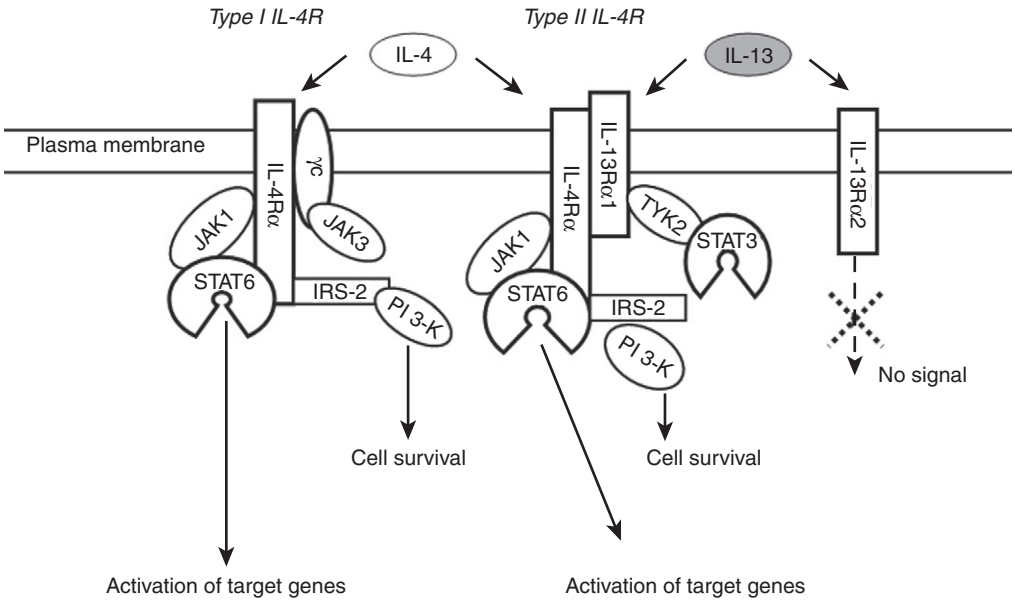


Figure 156-4 Interleukin-4 (IL-4) and IL-13 receptor complexes. IL-4 can bind to two different receptors: type I IL-4R and type II IL-4R. Type I IL-4R is composed of IL-4Rα and the common γ subunit (γc), whereas the type II IL-4R contains both IL-4Rα and IL-13Rα subunits. IL-4 binds to both type I IL-4R and type II IL-4R by way of the common IL-4Rα-chain. By contrast, IL-13 binds only to the type II IL-4R by way of the IL-13Rα1 subunit. Both receptors activate STAT6 and IRS-2 (insulin receptor substrate 2) signaling pathways resulting in the induction of gene expression and cell survival pathways. IL-13 also binds to IL-13Rα2 with high affinity. This receptor does not initiate signaling, however, and may serve as a decoy receptor to suppress IL-13 signaling.

signaling through activation of STAT6 and signal independently through the activation of other STAT proteins such as STAT1 and STAT3.[107]

Interleukin-11

IL-11 is a 23-kDa nonglycosylated protein produced by fibroblasts, epithelial cells, and endothelial cells in response to various stimuli, including IL-1, TGF-β, TNF, parathyroid hormone, respiratory syncytial virus, rhinovirus, and parainfluenza virus.[113-116] IL-11 is a member of the IL-6 family, proteins that share a four-helix bundle fold motif.[114] This pleiotropic antiinflammatory cytokine can stimulate hematopoiesis, regulate T cell function, activate macrophage proliferation and differentiation, and confer mucosa-protective effects.[114,116] Similar to IL-6, IL-11 inhibits production of proinflammatory cytokines and NO, and it is able to induce the acute phase response.[117]

Interleukin-11 Receptor

The IL-11 receptor is similar to other members of the IL-6 family of receptors and is composed of an IL-11–specific α-chain that binds IL-11 and the gp130 subunit, which is the common receptor β-chain subunit and functions as the signal transduction component of this receptor family (see Figure 156-3).[114,118] The binding of IL-11 to the IL-11α receptor triggers homodimerization of the gp130 subunit and formation of an IL-11 receptor complex. Signaling through the gp130 receptor occurs by way of JAK/STAT pathways.[114,115] Activation of this signaling pathway by IL-11 is mediated predominantly by JAK1 and STAT3.[119] Other signaling pathways activated by IL-11 include the Ras/MAP kinase pathway, protein kinase C, and Src families.[115]

Inhibitors of Proinflammatory Cytokines

Soluble Tumor Necrosis Factor Receptors

Soluble cytokine receptors are highly selective inhibitors of proinflammatory cytokines and represent another level of regulation of proinflammatory signaling. Both the TNFR-1 and TNFR-2 receptors are expressed on the surface of most cell types.

In response to various proinflammatory stimuli such as IL-6, IL-1β, IL-2, IFN-γ, and TNF itself, TNFR-1 and TNFR-2 can be proteolytically cleaved and the extracellular cytokine binding domains released from the cell surface.[120,121] TNF-α–converting enzyme (TACE), a member of the ADAM (a disintegrin and metalloprotease) family, has been implicated as the protease involved TNFR cleavage.[122]

A second pathway for the release of soluble TNFRs has been described in which full-length TNFR-1 can be released extracellularly in exosome-like vesicles.[123,124] Both forms of the soluble TNFRs (sTNFRs) retain their ability to bind TNF and compete with membrane-bound TNF receptors, leading to decreased availability of circulating TNF.[2,123,125] The shedding of these receptors from the cell surface also serves to decrease cellular responsiveness to TNF by decreasing the receptor number. The sTNFRs are thought to be a part of a negative feedback loop to regulate TNF bioactivity, to control inflammation, and to restore homeostasis. However, sTNFRs can act as agonists as well as antagonists of TNF bioactivity.[120,122,126] TNF binding to sTNFRs forms a stable complex and enhances the half-life of circulating TNF.[120] The TNF-sTNFR complex may act as a reservoir and may prolong TNF bioactivity. Soluble TNFRs have been detected in the plasma and urine of patients with sepsis, and increased TNF-sTNFR ratios are correlated with increased mortality.[127,128]

Interleukin-1ra and Interleukin-1RII

IL-1ra and IL-1RII are two natural regulators of IL-1 biologic activity. IL-1ra is a member of the IL-1 gene family.[7,32,129,130] Synthesis of IL-1ra is triggered by the same stimuli that trigger IL-1 production, and it is produced by the same cell types. IL-1ra is structurally similar to IL-1α and IL-1β and has a molecular weight similar to that of the mature form of IL-1β. Sequencing studies have shown 26% homology with IL-1β and 19% homology with IL-1α.

IL-1ra is a competitive inhibitor and binds to the IL-1RI with similar affinity as IL-1α and IL-1β, but it has no agonist activity (see Figure 156-2). IL-1ra binding to the IL-1RI does not trigger the association of IL-1RAcP and the formation of a heterodimer.[131] During the inflammatory response, IL-1ra levels tend to increase later than IL-1, suggesting that IL-1ra functions to block further IL-1 activity and have a role in the termination of the inflammatory response.[132] Administration of IL-1ra to animals reduced inflammation in various different diseases.[7,129,130]

IL-1RII is present as both a membrane-bound receptor and, in a truncated form, as a soluble receptor.[37,133] The receptor acts as a decoy receptor for IL-1 and is the principal IL-1 receptor on neutrophils, monocytes, macrophages, and B cells.[134] The extracellular domain of the IL-1RII is similar to that of IL-1RI and has high affinity for IL-1β but much lower binding affinity for IL-1α and IL-1ra. After IL-1 binding, IL-1RII associates with the IL-1AcP. However, IL-1RII lacks the TIR (Toll/IL-1R) region, and without this domain, the receptor cannot interact with MyD88 and initiate signal transduction (see Figure 156-2). Thus the membrane-bound IL-1RII may act as ligand sink by competing for IL-1 binding with the active IL-1RI and by reducing the concentration of available AcP.[32,37,134] IL-1RII also exists in a soluble truncated form, and circulating levels are increased during inflammation.[135,136] The extracellular domain of the IL-1RII is released from the cell surface by proteolytic cleavage by matrix metalloproteases to form a soluble IL-1RII (sIL-1RII).[137] Shedding of the IL-1RII is regulated by two major mechanisms.[39,133] Antiinflammatory agents, such as IL-4, IL-13, and glucocorticoids, increase gene expression of IL-1RII, enhanced cell surface expression, and subsequent extracellular shedding. Alternatively, rapid shedding of the IL-1RII is triggered by reactive oxygen intermediates, LPS, TNF, and chemoattractants. sIL-1RII binds IL-1β but not IL-1α or IL-1ra and may serve to inhibit the systemic effects of IL-1.

In summary, IL-1 activity is modulated by two different modes of regulation. IL-1ra competitively blocks IL-1 binding to the signaling IL-1RI. A second mechanism of regulation is IL-1RII or decoy receptor, which can bind both IL-1 and IL-1AcP and can prevent their interaction with an active receptor (i.e., IL-1RI). Furthermore, cleavage and release of sIL-1RII into the circulation further serve to modulate systemic IL-1 activity.

Interleukin-18–Binding Protein

Another member of the Toll/IL-1R family is IL-18. IL-18, similar to IL-1 and TNF, has a naturally occurring circulating inhibitor.[138] However, unlike sTNFR or IL-1RII, IL-18–binding protein (IL-18bp) is not a truncated form of the extracellular domain of the IL-18 receptor. Rather, IL-18bp is a naturally occurring antagonist of IL-18 that binds to IL-18 with high affinity and prevents binding to the IL-18 receptor (see Figure 156-2).[139,140] IL-18bp is composed of a single immunoglobulin domain and lacks a transmembrane and cytoplasmic domain. IL-18bp is not membrane-associated and is secreted rather than cleaved from the cell surface.[138,140] Circulating IL-18bp is present in healthy individuals and is significantly elevated in sepsis.[139] Additional studies have shown a beneficial protective effect of exogenously administered IL-18bp in sepsis; however the therapeutic benefit correlates with the inflammatory status.[89]

Inflammation

Inflammation is a protective mechanism in response to invasion by exogenous agents (microbial, chemical, or physical) or endogenous factors (immunologic or neurologic).[141] The host

inflammatory response is composed of innate and acquired immunity and cytokines play an important role in both components of the immune system. The inflammatory response is characterized by the accumulation and subsequent activation of leukocytes in affected tissue. The inflammatory response is initiated when immune cells, such as macrophages, dendritic cells, neutrophils, and NK cells, are activated after detection of microbial molecules through specific pattern recognition receptors (PPRs), which recognize specific pathogen-associated molecular patterns (PAMPs).[142-145] Activation of PRRs, such as Toll-like receptors (TLRs), which can recognize specific PAMPs from bacteria, viruses, fungi, and protozoa, leads to the activation of the transcription factor NFκB and the rapid production of early proinflammatory cytokines such as TNF, IL-1, and IL-6. TNF and IL-1 amplify the immune response through activation of the cytokine cascade and the production of other proinflammatory cytokines and chemokines. In addition to cytokines, other proinflammatory mediators such as NO, platelet-activating factor (PAF), prostaglandins, and leukotrienes also are produced.[2,146,147] These cascades are responsible for activating complement, coagulation, and kinin cascades as well. As inflammation progresses, antiinflammatory cytokines and cytokine inhibitors are produced, which serve to contain the inflammatory response, bring about resolution, and limit host tissue damage.

The accumulation of specific subpopulations of leukocytes at the site of inflammation is the result of a series of events: (1) endothelial cell activation and the expression of adhesion molecules; (2) leukocyte expression of adhesion molecules and leukocyte-endothelial cell adhesion; (3) leukocyte transendothelial migration; (4) leukocyte migration along a chemotactic gradient; and (5) the release of reactive oxidants, proteinases, and antimicrobial polypeptides at the site of inflammation.[146] Cytokines are involved at each step of this process and act both locally and systemically to initiate, maintain, and finally resolve the inflammatory response.[4]

Local Effects

At the site of inflammation, the local release of TNF and IL-1 and other cytokines including IL-8, IL-4, IL-13, IL-18, IL-6, and IFN-γ leads to the activation of endothelial cells and the expression or up-regulation of adhesion molecules such as selectins, ICAMs, platelet-endothelial cell adhesion molecule-1 (PECAM-1), and vascular cell adhesion molecule-1 (VCAM-1).[148,149] This event initiates leukocyte endothelial interaction. Increased endothelial cell surface expression of the adhesion molecules E-selectins and P-selectins (in conjunction with the constitutively expressed L-selectin on leukocytes) mediates the initial adhesive interaction resulting in the leukocyte's rolling along the endothelium.[150]

The endothelium also is a source of cytokines such as TNF, IL-1, IL-4, IL-11, IL-8, and IL-18.[151] These cytokines, as well as IFN-γ, promote leukocyte activation, surface expression of the adhesion molecules β-integrins, and the shedding of L-selectins.[67,149,150] The interactions between leukocyte β_2 integrins and endothelial counterligands such as ICAM-1, ICAM-2, and ICAM-3 mediate the firm attachment of the leukocytes to the endothelium and subsequent migration. Activated endothelium also produces CC and CXC chemokines, which are released or localized on the luminal surface of the endothelium to further enhance leukocyte adhesion.[149-151] Transmigration occurs by migration through endothelial junctions (paracellular transmigration) or through the endothelial cell itself (transcellular migration).[150,152-154] Paracellular migration involves the release of endothelium–expressed VE-cadherin (vascular endothelial cadherin) and involves PECAM-1, ICAM-1, ICAM-2, CD99, ESAM (endothelial cell–selective adhesion molecule), and JAMs (junctional adhesion molecules). The specific adhesion molecules involved in transmigration are both stimulus- and cell-dependent.

Movement of leukocytes to the inflammatory site is directed along a chemotactic gradient, where the strongest concentration of chemoattractants is at the site of inflammation. Local production of chemokines by both immune and nonimmune cells serves as a source for long-lasting chemoattractants. The movement of specific populations of leukocytes into the inflammatory site depends on the specific stimuli and the resultant type of chemokines synthesized (i.e., CXC chemokines versus CC chemokines).[150] Thus the movement of leukocytes is a cytokine-mediated process and the result of coordination of endothelial expression of adhesion molecules, leukocyte adherence, and specific chemotactic gradients.[155]

Cytokines also are directly involved in the activation of cells at the inflammatory site. After recruitment of inflammatory cells to the site of inflammation, cytokines can further amplify the inflammatory response through the activation of transcription factors, such as NFκB, which regulate proinflammatory gene expression. Both TNF and IL-1 increase the expression of genes for proinflammatory cytokines, chemokines, adhesion molecules, inducible NO synthase, matrix metalloproteinases, and cyclooxygenase-2 (COX-2).[156] Cytokines and chemokines also can effect the activation of resident and recruited phagocytic cells. Both CXC and CC chemokines are capable of triggering, in their specific target cells, the generation of oxygen radicals. Chemokines also trigger exocytosis of secretory vesicles and specific granules, resulting in release of enzymes and other soluble proteins.[67,157,158] In adherent neutrophils, TNF triggers the release of oxygen radicals and degradative granule enzymes such as elastase and promotes cytoskeleton reorganization.[159,160] IFN-γ also acts directly on phagocytic cells and enhances bactericidal activity, phagocytosis, NO synthesis, and oxygen radical production.[83] Proinflammatory cytokines such as TNF, IL-1, and IFN-γ also stimulate the release of potent lipid mediators such as PAF, leukotrienes, thromboxane, and prostaglandins.[161] IL-18 has a direct role in neutrophil activation and can induce cytokine and chemokine release, increased β_2 integrin expression, and degranulation.[162] Antiinflammatory cytokines also modulate local activation of phagocytic cells. IL-10, IL-11, IL-4, and IL-13 can inhibit proinflammatory cytokine synthesis, suppress oxygen radical production, and down-regulate NO synthesis, thereby further limiting the local inflammatory response, 163.[95-98,105,106,114,116,117,163]

Mature neutrophils are end-stage cells and have a relatively short life span. Once released into the circulation, neutrophils undergo constitutive apoptosis, or programmed cell death. Enhanced neutrophil survival at the site of inflammation promotes increased bactericidal activity but also may play a role in acute inflammatory damage through excessive release of oxygen radicals, proteases, lipid mediators, and cytokines. TNF and other proinflammatory cytokines are important regulators of neutrophil function during the inflammatory response through activation of proinflammatory signaling and suppression of neutrophil apoptosis.[164-167] Thus cytokines are involved both in trafficking specific populations of leukocytes to the site of inflammation and in the activation process once the leukocytes arrive at their destination.

Coagulation

The coagulation system is activated during the inflammatory response and is an essential component of the host response. Coagulation is initiated by the expression of tissue factor (TF) on the surface of endothelium and monocytes.[168] The proinflammatory cytokines TNF, IL-1, and IL-6 promote the expression of TF and activation of the extrinsic pathway of coagulation resulting in thrombin production and ultimately fibrin deposition.[169-171] Studies also have implicated IL-12, IL-8, and the chemokine CCL2 as procoagulation mediators.[8,169,172] TNF and IL-1 further enhance the procoagulation state by also inhibiting key anticoagulation pathways. TNF and IL-1 inhibit the synthesis and release of thrombomodulin, resulting in the impairment of

thrombin-mediated protein C activation and the subsequent inhibition of that anticoagulation pathway.[146,173]

Fibrinolysis is suppressed by TNF through the inhibition of tissue-type plasminogen activator release.[170] Tissue-type plasminogen activator is essential for the conversion of plasminogen to the active protease plasmin. Plasmin can dissolve the fibrin network in thrombi. Fibrinolysis also is limited by TNF and IL-1–mediated release of plasminogen-activator inhibitors (e.g., PAI-1), which further inhibit the conversion of plasminogen to plasmin.[146,174] The stimulation of the extrinsic coagulation pathway, coupled with the inhibition of fibrinolysis, is thought to promote containment and localization of the inflammatory site. However, the activation of the coagulation pathway can further up-regulate the inflammatory response through cross-talk between the two pathways. For example, thrombin binding to its receptor triggers the activation of NFκB, the synthesis of proinflammatory cytokines, and the release of NO.[168,170]

Antiinflammatory cytokines also regulate coagulation. In vivo administration of IL-10 reduced LPS-activation of the coagulation pathway as well as modulated the fibrinolytic system.[170,175] Although the coagulation system is critical for maintaining homeostasis and the containment of inflammatory stimuli, if it is not adequately regulated, it can lead to the development of disseminated intravascular coagulation and multiple organ failure.[171]

Systemic Effects

Proinflammatory cytokines, either acting alone or synergistically, can produce systemic alterations in host physiology. These systemic alterations include induction of fever, stimulation of hepatic acute phase response, and nonspecific activation of host immune systems.

Fever

IL-1 (previously known as endogenous pyrogen), TNF, and IL-6 are able to raise the temperature set point of an organism and cause fever.[176,177] These cytokines stimulate production of inducible cyclooxygenase (i.e., COX-2), which induces the hypothalamic production of prostaglandins, particularly PGE_2. PGE_2 stimulates the release neurotransmitters such as cyclic adenosine monophosphate (cAMP) and increases body temperature.[28,177] Other proinflammatory cytokines, such as IFN-γ, may indirectly cause fever through induction of IL-1 and TNF synthesis. Cytokines that are members of the same receptor families as those of pyrogenic proinflammatory cytokines do not necessarily trigger fever. The IL-18 receptor is a member of the IL-1/Toll-like receptor superfamily, but unlike IL-1 and LPS, IL-18 does not trigger PGE_2 synthesis in vitro or cause fever when it is administered in vivo.[92,177] Similarly, IL-11 and IL-6 both are members of the gp130 cytokine family, and both activate the acute phase response.[48,117] In contrast with IL-6, IL-11 does not produce fever. IL-10 is an endogenous antipyretic cytokine and probably modulates fever through inhibition of proinflammatory cytokine synthesis.[178]

Acute Phase Reaction

The synthesis and release of hepatic acute phase proteins constitute an important mechanism in modulating the inflammatory response and the restoration of homeostasis. Acute phase proteins have diverse biologic activities and include antiproteases, antioxidants, activators of the complement system, blood-clotting agents, and immune response modulators.[48,179]

The hepatic acute phase response is regulated principally by IL-6, but other members of the IL-6 family, including IL-11, also can induce the acute phase response.[48,55] TNF, IL-1, IFN-γ, and possibly IL-8 also induce synthesis of various hepatic acute phase proteins.[48,179-181] The acute phase reaction is terminated indirectly through the inhibition of proinflammatory cytokine synthesis by IL-4, IL-13, and IL-10.[182] Natural inhibitors of TNF, IL-1, and IL-6, including soluble receptors and receptor antagonists, also can remove excess IL-6, IL-1, and TNF. IL-4, in contrast with other antiinflammatory cytokines, can inhibit synthesis of select hepatic acute phase proteins.[183] Acute phase proteins themselves also are capable of modulating the proinflammatory response through the induction of cytokine antagonists such as IL-1ra.[184] Thus cross-talk between cytokines and the hepatic acute phase response contributes to the resolution of the inflammatory response.

Inflammatory Diseases

Proinflammatory cytokines play a central role in inflammatory diseases of infectious or noninfectious origin. TNF triggers a cytokine cascade that initially is composed of the proinflammatory cytokines (IL-1, IL-6, IL-8, IL-12, IFN-γ, IL-18, and TNF itself).[2,6,8,185,186] These cytokines serve to contain and resolve the inflammatory foci through activation of local and systemic inflammatory responses. TNF also triggers a cytokine cascade of the antiinflammatory cytokines that block proinflammatory cytokine synthesis, as well as cytokine inhibitors that block proinflammatory cytokine actions.

In most cases, the inflammatory response is successfully resolved. Overzealous production of cytokines or the inability to shut down proinflammatory cytokine production, however, can lead to increasing concentrations of cytokines in the systemic circulation ("cytokine storm"). This continued cytokine production can have a deleterious effect on the host, with the development of hypotension, intravascular thrombosis, pulmonary edema, and hemorrhage; if this process is left unchecked, it can lead to multiple organ failure and death. This condition often is referred to as the *systemic inflammatory response syndrome* (SIRS).[187] This term describes the clinical manifestations of widespread endothelial inflammation that leads to increased vascular permeability.[170,188-190] This condition is the initiating pathologic process in a group of diverse disorders, such as bacterial sepsis, ischemia, burn injury, trauma and tissue injury, and hemorrhagic shock.

It has become apparent that interactions between proinflammatory and antiinflammatory mediators regulate the inflammatory response.[5,7,8,125] Antiinflammatory cytokines, in particular IL-10, inhibit proinflammatory cytokine synthesis and adhesion molecule expression while increasing the levels of specific cytokine inhibitors. Excess production of antiinflammatory cytokines, however, can compromise the host's ability to clear microorganisms through suppression of immune cell function. If a balance is not maintained, the result is either an excessive proinflammatory response or, alternatively, immunosuppression and increased susceptibility to secondary infection. Thus, although the cytokine cascade triggered by TNF is often beneficial to the host by initiating the inflammatory response, overproduction or underproduction of proinflammatory or antiinflammatory endogenous mediators may actually be deleterious to the host. Studies suggest a genetic predisposition that determines the balance of proinflammatory and antiinflammatory cytokines and, hence, susceptibility to disease.[3,7,191-193] Various polymorphisms have been identified within cytokine and cytokine receptor genes that alter their expression. These cytokine and cytokine receptor polymorphisms may determine the balance of proinflammatory and antiinflammatory cytokines in the inflammatory response.

To date, therapeutic strategies targeting proinflammatory cytokines such as TNF and IL-1β have proved ineffective in the treatment of SIRS.[1,125,194] These cytokines are critical to the initiation of the inflammatory response and may have peaked before the clinical signs and symptoms of SIRS become apparent. Current investigational approaches are now focused on mediators that appear later in disease progression. Two important late

proinflammatory mediators are high-mobility group box protein type 1 (HMGB1) and macrophage inhibitory factor (MIF). HMGB1, originally identified as a DNA-binding protein, is now recognized as a late mediator of sepsis and SIRS.[195] HMGB1 is actively released extracellularly by macrophages and endothelial cells during the inflammatory response as well as passively from necrotic cells. HMGB1 mediates numerous proinflammatory actions both locally and systemically.[196] Administration of recombinant HMGB1 to mice produces the clinical features of SIRS and multiple organ failure. Furthermore, antibodies or antagonists directed against HMGB1 are protective in animal models of sepsis and SIRS.[195,197]

Although the proinflammatory mediator MIF originally was identified as a modulator of macrophage migration, it is now recognized to be a critical regulator of the inflammatory response.[198] Unlike other proinflammatory cytokines, MIF can be induced by glucocorticoids.[143] MIF is released in the periphery by several types of immune cells in response to infection. MIF also can be released centrally from the anterior pituitary gland, and this central release contributes to sustained plasma elevations of MIF in response to inflammation. Furthermore, in animal models of infection and sepsis, anti-MIF therapy significantly improved survival.[143,198,199] Thus these "late" proinflammatory mediators may provide novel therapeutic targets for the treatment of SIRS.[143,186,199,200]

CLINICAL APPLICATION OF CYTOKINE PHYSIOLOGY IN NEWBORN INFANTS

Cytokine Physiology in Newborn Infants

The newborn period represents a time of increased risk for the development of bacterial infection and increased mortality from sepsis.[201,202] Among other factors known to increase this risk is a functional immaturity of newborn immune mechanisms. Both immunoglobulin and complement levels are low, and leukocyte functions, including the secretion of inflammatory mediators, may be deficient.[203-205] Only recently has attention been directed to developmental aspects of the immune response.

The fetus and newborn infant are unique from the standpoint of immunity and infection. The fetus lives in a sterile, immunologically protected environment. However, it is during this time that the development of the immune system is initiated—a highly complex process mediated, at least in part, by the expression of cytokines.[206,207] Fetal cytokines are known to play a role in the regulation of hematopoiesis and to protect the fetus against rejection. Placental and fetal cytokines also protect against infection.[206,207] Evidence suggests that cytokines in the fetus and neonate play a role in the pathophysiology of severe neonatal diseases including sepsis, bronchopulmonary dysplasia (BPD), and necrotizing enterocolitis (NEC).[208-211]

In the past, therapeutic strategies were aimed exclusively at killing the bacteria that caused neonatal infection. It has become evident, however, that many infants die despite the sterilization of blood cultures with antimicrobial agents.[212,213] It is now appreciated that the physiologic derangements that occur during sepsis are secondary to the host response induced by pathogenic microorganisms. During overwhelming sepsis, the host produces proinflammatory cytokines that initiate a cascade of events resulting in tissue injury at distant sites and generalized multiorgan system failure.[213,214] The balance of proinflammatory and antiinflammatory cytokines may ultimately determine the outcome with sepsis in newborn infants. This inflammatory response may have its origins during the fetal period.[206,207] Thus the heightened morbidity and mortality in neonatal sepsis result from physiologic deficiencies in immune function, as well as the pathophysiologic alterations produced by bacterial byproducts.[212,213] This section discusses the development of the inflammatory response in the fetus and neonate, the production of inflammatory mediators, and their role in the inflammatory response to specific disease processes in newborn infants.

Cytokine Production in Newborn Infants

In comparison with adult data, considerably less is known regarding the production and release of cytokines in the fetus and newborn infant. Studies indicate that both proinflammatory and antiinflammatory cytokines mediate many of the pathophysiologic events during bacterial sepsis. Moreover, growing evidence suggests that the fetal inflammatory response plays a major role in the induction of several neonatal diseases. Thus attention has been focused on determining the capability of monocytes from newborn infants to produce these cytokines and the balance of proinflammatory and antiinflammatory mediators in the fetus and neonate.

Tumor Necrosis Factor

TNF, an early proximal mediator in the cytokine cascade,[215,216] is produced by human fetal Kupffer cells as well as placental mononuclear cells in response to stimulation by LPS.[217,218] TNF can be found in amniotic fluid during the second and third trimesters, and TNF levels increase with premature rupture of membranes.[219]

Several (but not all) investigators have demonstrated significantly decreased TNF production by cord blood monocytes.[220-224] In addition, preterm infants born before 30 weeks gestation demonstrated significantly diminished LPS-stimulated TNF secretion when compared with neonates of later gestational ages or adults.[222] Levy and co-workers[225] further demonstrated a specific inhibitory effect of adenosine on TNF production during the perinatal period. When analyzed in culture, monocyte-macrophages derived from newborn infants also secreted diminished amounts of TNF when compared with adult cells.[224,226] Kwak and colleagues[227] examined TNF production by cord blood mononuclear cells in response to several stimuli. In contrast with the immediate response to LPS (in which TNF, IL-1β, IL-6, and IL-8 appeared almost simultaneously), stimulation by group B streptococci resulted in increased TNF production but a delayed appearance of the other cytokines. Thus, abnormalities of TNF production and release, particularly in preterm infants, may increase susceptibility to bacterial infection in this age group.

TNF receptor expression also may be diminished. Using flow cytometry and a human recombinant TNF that binds to the 75- and 55-kDa receptors, Chheda and co-workers[226] demonstrated reduced expression of TNF receptors on cord blood monocytes when compared with that on adult cells. Moreover, these findings suggest a dissociation between the production and release of TNF and other cytokines, such as IL-1 and IL-6.

Interleukin-1

IL-1 also is an integral mediator in the cytokine cascade that is produced by fetal Kupffer cells in response to LPS.[217] IL-1 is present in amniotic fluid and increases with premature rupture of membranes.[219] Production of IL-1 by cord blood monocytes is comparable with that in adult control subjects, even in monocytes obtained from preterm infants.[220,221,228-231] However, in preterm infants with bacterial sepsis, monocyte IL-1 secretion was lower during the acute infectious phase, but improved significantly during the convalescence period.[232] Bry and co-workers[233] found gender differences in the antiinflammatory IL-1ra responses in newborn infants (female > male). Moreover, increased cord IL-1ra levels were associated with neonatal morbidity and adverse outcome in preterm infants.[234]

Although monocytes, endothelial cells, and fibroblasts are thought to be the primary sources of cytokine production, neutrophils also possess that capability, albeit in lesser amounts. After stimulation with LPS or TNF, Contrino and associates[235]

found significantly increased expression of IL-1 by cord blood neutrophils compared with that in cells from adults. Although neutrophils function primarily as rapid effector cells with little immunoregulatory function, IL-1 secretion by neutrophils may be important in the amplification of the early inflammatory response.

Interleukin-6

IL-6 is a pleiotropic cytokine that induces the acute phase response with additional important antiinflammatory properties.[223] After stimulation with LPS, IL-6 is produced by human fetal Kupffer cells as early as 13 weeks after conception.[218] This cytokine also has been detected in the amniotic fluid during infection.[236]

Several studies have evaluated IL-6 production by monocytes from newborn infants.[222,223,228,236-238] Liechty and colleagues[236] demonstrated equivalent IL-6 production by fetal and maternal mononuclear cells after stimulation with IL-1. Angelone and coworkers[223] demonstrated that LPS-stimulated cord blood IL-6 production and IL-6/TNF ratios were higher than in adult peripheral blood. This robust IL-6 production was linked to both cellular (monocyte) and humoral (serum) factors. However, IL-6 production was reduced in cells from preterm neonates, perhaps contributing to their enhanced susceptibility to overwhelming bacterial infection.[236,239] Using flow cytometry and LPS stimulation, Schultz and associates[208] demonstrated that both term and preterm monocytes displayed a higher percentage of IL-6–positive cells than in those from adults, suggesting an enhanced inflammatory response in these infants.

Cord blood lymphocytes express both the gp80 and the gp130 IL-6 receptor, although generally at lower levels than in adult cells.[240] The immunofluorescence staining patterns for IL-6 receptors on monocytes were similar for cord and adult cells; however, the available data are limited.

Interleukin-8

IL-8 is a low-molecular-weight cytokine produced by mononuclear and endothelial cells that attracts and activates neutrophils.[241] Little is known about the expression and function of IL-8 in the fetus. Using cord blood mononuclear cells stimulated with endotoxin, Taniguchi and co-workers[242] found that IL-8 production was significantly greater in neonates born to mothers with chorioamnionitis than in those born to healthy women. Although early studies suggested deficiencies in IL-8 production by neonatal mononuclear cells, more recent literature indicates an intact proinflammatory cytokine pathway.[213,214,243,244] After LPS stimulation of whole blood cultures, IL-8 production by neonates exceeded adult levels.[245] Moreover, IL-8 production was independent of gestational age.[239] Using flow cytometry to analyze cytokine production at the single cell level, Schultz and colleagues[208] also found enhanced IL-8 production by neonatal monocytes after LPS stimulation. In this study, cells from both term and preterm infants displayed a higher percentage of IL-8–positive cells than in adult cells, a finding challenging the existing view of an immature neonatal inflammatory response.[208] IL-8 levels in whole cord blood specimens increased in response to the stress of labor and delivery.[246] Furthermore, elevated IL-8 levels have been found in neonates with diseases including cerebral white matter damage, BPD, and NEC.[208]

Interleukin-10

IL-10 is an antiinflammatory cytokine that decreases the expression and function of proinflammatory cytokines, including TNF, IL-1, and IL-8.[100,102] In addition, IL-10 limits the participation of helper T cells in delayed hypersensitivity and protects against inflammation. During the fetal period, placental production of IL-10 may prevent immune rejection.[207,247] Neonates exhibit decreased IL-10 production.[209,248,249] Both term and preterm infants demonstrate significantly diminished IL-10 mRNA expression and IL-10 production after stimulation.[250] Although IL-10 is less well explored than other cytokines in this respect, diminished production of IL-10 and immaturity of the antiinflammatory response may contribute to the enhanced proinflammatory state in newborn infants.

Interleukin-18

Il-18, a more recently described member of the IL-1 family, is an important regulator of both innate and acquired immune responses. Overall, data for newborn infants are lacking, but a single study found diminished IL-18 production in response to stimulation with group B streptococci.[251] This deficiency may contribute to the notable susceptibility of the neonate to group B streptococci and other pathogens.

Fetal and Neonatal Cytokines in the Inflammatory Response

The fetus and newborn infant are uniquely susceptible to the development of bacterial infection as well as heightened mortality from infectious disease. In adults, cytokines have been implicated as endogenous mediators elaborated during the pathogenesis of sepsis and shock. Work in developmental immunobiology also has suggested that cytokines contribute to the inflammatory response in the fetus and newborn infant with chorioamnionitis, sepsis, NEC, and BPD.

Chorioamnionitis and Brain Injury

Evidence suggests that intrauterine infection is a cause of cerebral palsy in both term and preterm infants. Both clinical and subclinical chorioamnionitis may trigger an inflammatory response in the mother and fetus associated with the elaboration of proinflammatory cytokines. This fetal inflammatory response is thought to contribute to the development of neonatal brain injury and subsequent cerebral palsy.[252-254]

The cytokines TNF, IL-1β, and IL-6 normally are present in amniotic fluid, with levels increasing until term gestation.[252] During the course of intrauterine infection, however, levels of proinflammatory cytokines are significantly increased and have been correlated with the initiation of preterm labor.[255] Although chorioamnionitis traditionally was considered a maternal infection, more recent evidence suggests that the systemic fetal inflammatory response is more important in contributing to neonatal brain injury.[256] Observations by Dammann and Leviton[257] support the hypothesis of a fetal vasculitis as part of the response to perinatal infection.

Exposure to LPS during pregnancy is associated with elevated concentrations of the proinflammatory cytokines TNF, IL-1, IL-6, and IL-8 in both amniotic fluid and neonatal plasma, suggesting both maternal and fetal involvement.[258-260] In turn, elevations of these cytokines are associated with increased risk for neonatal brain injury and cerebral palsy.[255,257,261] Yoon and associates[255,258,259] demonstrated higher levels of TNF, IL-1β, IL-6, and IL-8 in amniotic fluid in infants who subsequently developed periventricular leukomalacia. Other investigators have reported higher umbilical cord plasma IL-6 levels in neonates with white matter damage.[261-263] Shimoya and colleagues[264] found elevated IL-8 levels in cord sera from preterm fetuses with chorioamnionitis, which increased proportionately with the severity of disease as graded histologically. These workers suggested that the measurement of IL-8 may be more sensitive (97%) and specific (89%) for the diagnosis of chorioamnionitis than conventional markers, such as leukocytes, C-reactive protein, and immunoglobulin M. Pacora and associates reported an association between elevations of IL-18 in amniotic fluid and intrauterine infection.[265] Minagawa[266] further suggested a possible correlation between elevations of cord blood IL-18 levels in preterm infants and the

development of periventricular leukomalacia and cerebral palsy. A recent study has also noted the relationship of higher levels of IL-6 and IL-10 in cerebrospinal fluid and magnetic resonance imaging (MRI)-defined cerebral white matter injury.[267] Furthermore, TNF and IL-6 have been demonstrated directly on histopathologic examination of white matter lesions in newborn infants.[255] These findings lend further direct support to the association of an inflammatory process in the central nervous system with neurologic injury in vulnerable infants.

Several mechanisms have been suggested to explain the link between infection/inflammation in pregnancy and cerebral white matter damage.[255,268-270] Intrauterine infection stimulates the fetal production of proinflammatory cytokines, which increase the permeability of the blood-brain barrier, thereby allowing inflammatory products into vulnerable areas of the developing brain.[268,271] Dammann and Leviton[268] proposed that proinflammatory cytokines cross the blood-brain barrier and then cause white matter damage directly, or induce further cytokine production by microglial cells and astrocytes. The brain-derived cytokines exert a direct neurotoxic effect on oligodendrocyte precursors and inhibit their subsequent differentiation into mature myelinating oligodendrocytes.[271,272] Inflammatory cytokines also activate astrogliosis and NO release, producing mitochondrial damage and cellular energy failure.[271,272] The final result is the accumulation of reactive oxygen species and apoptosis of oligodendrocytes.[271] Furthermore, elaboration of inflammatory mediators may increase the susceptibility of the cerebral white matter to other insults, such as hypoxia or ischemia.[256]

Epidemiologic data suggest that preterm infants exposed to an intrauterine infection are more likely to develop cerebral palsy. Positive associations have been reported between cystic periventricular leukomalacia and intraamniotic, brain, and umbilical cord cytokine levels.[252-254,259,270] In a meta-analysis of data for women with histologic and clinical chorioamnionitis, a relative risk of 1.8 (confidence interval, 1.5 to 2.3) was found for cerebral palsy in association with chorioamionitis.[254] Although these data do not establish causality between cytokines and perinatal brain injury, they do provide evidence of the association of cerebral palsy with an inflammatory environment at birth.[254]

Genetic polymorphisms in cytokine expression may influence the host response to chorioamnionitis and the development of cerebral palsy.[252,259] These polymorphisms may, in turn, explain differences in outcome after intrauterine infection and predict which infants will incur brain damage.[252,254,257,273] Using multivariate analysis, Annells and colleagues found an association between polymorphisms in the immunoregulatory genes for TNF and IL-10 and the presence of histologic chorioamnionitis.[274] Gibson and co-workers[275] further demonstrated that carriage of polymorphisms in TNF genes was associated with an increased risk for cerebral palsy, either directly through cytokine-mediated brain damage or indirectly in association with an altered immune response to infection. These associations warrant further investigation.

Bacterial Sepsis

Sepsis and septic shock are important determinants of morbidity and mortality in the neonatal intensive care unit (NICU). Cytokines play a key role in this response in newborn infants. Although the immune system produces cytokines to protect the host when threatened by microbial invasion, overproduction or an imbalance of proinflammatory versus antiinflammatory mediators may sometimes occur, with potentially deleterious effects.[212,214]

Several groups of investigators have examined levels of the proinflammatory cytokines TNF, IL-1, IL-6, and IL-8 during the evolution of bacterial sepsis in newborn infants.[276-280] In an early report, Girardin and associates[277] demonstrated significantly higher TNF levels in infants with systemic infection than in infants in other clinical groups; the highest levels were found in the neonates with circulatory failure, particularly those who died. TNF levels were unrelated to gestational age or to the Gram stain characteristics of the infecting organism. Similarly, Roman and co-workers[281] correlated the degree of TNF elevation with the severity of sepsis, with the infants who died having the highest levels of TNF.

Miller and colleagues[282] prospectively measured levels of TNF, IL-1, and IL-6 in cord blood samples from neonates born after complicated perinatal courses. In this study, IL-1β levels were significantly elevated in the presence of severe perinatal complications, whereas TNF and IL-6 levels did not reflect the type of delivery or severity of the perinatal course. In infants with strictly defined infectious complications, however, cord blood IL-6 levels were markedly elevated.[282] In neonates with herpes simplex virus infection, Kawada and co-workers[283] demonstrated significantly higher IL-6 and sTNF-R1 in disseminated disease than in infection confined to the central nervous system or skin, eyes, and mouth. Furthermore, concentrations of inflammatory cytokines were correlated with viral load.[283]

Numerous studies have demonstrated elevations of IL-6 and IL-8 in infected neonates, although the methodology used to measure cytokine levels has varied, and a group of neonates with "probable" infection sometimes is included.[284-286] Santana and associates found elevated cord blood levels of IL-6 in newborn infants with various infectious and noninfectious conditions, whereas IL-8 was the best predictor of early-onset bacterial infection.[286] Using a chemiluminescence immunoassay that allowed results within 1 to 2 hours, Krueger and co-workers[287] found elevations of IL-6 and IL-8 in preterm infants with culture-proven sepsis. These investigators suggested that this method may allow an immediate diagnosis of infection in preterm infants. Using reverse transcriptase–polymerase chain reaction analysis, Berner and colleagues[288] also found elevations of IL-8 mRNA to be highly sensitive (86%) and specific (96%) for the detection of neonatal infection.

Lehrnbecher and colleagues[289] extended these findings by measuring both proinflammatory mediators (IL-6, IL-8) and sIL-6 receptors in cord blood from infected neonates. Although IL-6 and IL-8 levels were significantly elevated, as predicted from previous studies, sIL-6R levels were significantly reduced in sepsis.[289] This phenomenon has also been described in adults with sepsis, and it is speculated that the reduction of sIL-6R during sepsis may alter the biologic activity of IL-6. Døllner and co-workers[290] found elevated TNF receptors (p55 and p75) as well as IL-1ra in infected neonates. In an experimental mouse model of group B streptococcal sepsis, Cusmano and associates detected significant elevations of IL-18 in infected animals; however, survival was improved after the administration of recombinant IL-18.[291]

A recent study by Harris and colleagues[284] measured levels of IL-8, IL-10 and IL-18 in critically ill infants with bacterial sepsis versus sepsis syndrome. Infants with bacterial sepsis had significantly higher plasma levels of IL-8 and IL-10, but not IL-18, suggesting differential regulation of this cytokine.[284] Plasma IL-8, IL-10 and IL-18 levels were correlated with mortality.[284] Romagnoli and co-workers[292] also demonstrated that persistently high IL-10 levels correlated with death from sepsis. In preterm infants weighing less than 1500 g at birth, Ng and colleagues[279] found elevations of both proinflammatory (TNF, IL-6) and antiinflammatory (IL-10) cytokines in infected infants versus noninfected control infants. Measurements of IL-6 and IL-10 at clinical presentation predicted the development of disseminated intravascular coagulation in infected neonates, with the sickest infants demonstrating higher concentrations of inflammatory cytokines than in those with milder infections.[293] These studies suggest that interactions of both proinflammatory and antiinflammatory cytokines play a critical role in the clinical manifestations of and outcomes with neonatal sepsis.

Several studies also have monitored cytokine levels as a guide to the duration and efficacy of antimicrobial therapy.[294-296] DeBont and associates[294] measured TNF, IL-1, and IL-6 in newborn infants hospitalized for clinically suspected sepsis. Plasma TNF and IL-6 levels were significantly increased in the infants with sepsis, although IL-1 levels were only slightly elevated. After the start of antimicrobial therapy and concomitant clinical improvement, TNF, IL-1, and IL-6 levels decreased and normalized within 2 days. Sikora and colleagues[295] found elevations of both proinflammatory (IL-1, IL-6, IL-8) and antiinflammatory (IL-10, IL-13) cytokines early in the course of sepsis, which decreased after antimicrobial therapy. Levels of cytokine inhibitors (IL-6sR, sTNFR II) also decreased after treatment.[295] Franz and co-workers[297] suggested use of a diagnostic algorithm measuring IL-8 and C-reactive protein to reduce unnecessary antibiotic exposure in newborn infants evaluated for early-onset sepsis. However, infants who were rapidly deteriorating or in septic shock were excluded from this trial.[297] Cytokine elevations also have been demonstrated in newborn infants with nosocomial sepsis. Groll and associates[296] reported elevations of IL-6 in 8 of 10 infants at the onset of proven nosocomial infection; in 7 infants, these values returned to normal on completion of antibiotic therapy.

In view of the excessive morbidity and mortality from bacterial sepsis in newborn infants, several investigators also have addressed the predictive value of cytokine measurements in the early diagnosis of bacterial infection.[290,298-300] In one report, Mehr and Doyle[301] reviewed results of studies published between 1996 and 1999 using TNF, IL-6, and IL-8 as markers of bacterial sepsis in newborn infants. Although the patient populations studied and the cytokines measured varied, several conclusions can be drawn from these data. Of all the cytokines measured, the most consistent results were found using IL-6.[301] Less consistent results were obtained with TNF; however, TNF is an evanescent cytokine whose levels peak early and decline rapidly during sepsis. IL-1 was an unreliable indicator of bacterial infection. Finally, IL-8 may prove most useful for the diagnosis of bacterial infection. More recently, Verboon-Maciolek and colleagues[302] compared levels of IL-6 and IL-8 in bacterial versus viral sepsis in the NICU. Although elevated levels of both cytokines were found in bacterial sepsis, normal values were noted in infants with enterovial disease, suggesting that cytokine measurements may aid in the differentiation of bacterial versus viral infection.[302]

Genetic polymorphisms in cytokine expression may influence neonatal susceptibility to infectious disease as well as host response to infection.[303] The most consistent results to date have been obtained for TNF. TNF-308 is the best-studied polymorphism and has been associated with increased mortality from sepsis in ventilated preterm infants.[304] Other polymorphisms related to IL-6 and IL-10 have been reported to increase the risk of sepsis in adult patients; however, studies in neonates have thus far produced conflicting results.[303,304]

Necrotizing Enterocolitis

NEC is a disease frequently encountered in premature infants in the NICU. Although the manifestations of the illness are quite variable, the development of fulminant sepsis and shock is not uncommon in affected infants.[305] As with the involvement of cytokines in bacterial sepsis, the generation of cytokines in NEC occurs in response to circulating bacteria and their byproducts as well as local mediator release associated with tissue inflammation.[306]

Data from both animal and human studies support the role of PAF and TNF in the pathophysiology of NEC.[307,308] Caplan and associates[308] demonstrated higher circulating levels of PAF and TNF in infants with NEC than in age-matched control patients. Feedings alone increased circulating PAF levels, which preceded the development of NEC. Plasma TNF levels also were elevated in affected infants, thereby augmenting the inflammatory response.[308] Harris and colleagues[309] measured TNF and IL-6 levels in infants with suspected sepsis or NEC at clinical presentation and at 12, 24, and 48 hours thereafter. IL-6 levels were highest (10-fold greater) in infants with bacterial sepsis plus NEC, intermediate in infants with bacterial sepsis alone, and lowest in control infants with negative findings on evaluation for infection. Elevated IL-6 levels also were associated with increased mortality. By contrast, plasma TNF levels did not reliably identify infants with sepsis or NEC.[309] These data suggest that increased plasma IL-6 levels in NEC may reflect the combined effects of bacterial invasion of the bloodstream and the systemic response to severe bowel injury. Morecroft and co-workers[310] also demonstrated elevated TNF levels in a majority of samples (71%) from infants with NEC but found no correlation with severity of disease. By contrast, IL-6 levels were significantly increased in infants with definite (stage II) and advanced (stage III) NEC. These investigators concluded that although TNF may be an early marker of disease, IL-6 correlates best with disease severity. Furthermore, therapy with anti-TNF antibodies in animals reduced the incidence and severity of disease.[311]

Expression of both proinflammatory and antiinflammatory cytokines in NEC has been studied.[211,284] Edelson and colleagues[211] found circulatory levels of both proinflammatory (IL-1, IL-8) and antiinflammatory (IL-1ra, IL-10) cytokines to be elevated at the onset of NEC in infants with severe disease. Moreover, an elevated IL-1ra concentration had a high sensitivity (100%) and specificity (92%) for the presence of severe disease. In a more recent study, Harris and colleagues[284] reported elevations of IL-8 and IL-10 in infants with NEC versus sepsis syndrome controls, reflecting a response to circulating bacteria and their byproducts as well as local mediator release associated with tissue inflammation. Elevated IL-10 levels in preterm infants with NEC versus control infants were reported previously;similar to the findings by Harris' group,[284] these levels were lower than in infants with bacterial sepsis.[292]

More recent evidence suggests that inflammatory mediators produced locally in the intestine may contribute to the pathogenesis of NEC. Viscardi and associates[312] measured TNF, IL-1, IL-6, and IL-8 mRNA in surgical specimens of NEC and normal intestine. Although TNF and IL-1 mRNA levels were higher in intestinal specimens from infants with severe disease, IL-6 and IL-8 mRNA levels did not distinguish between infants with NEC and control infants. By contrast, Nadler and colleagues[313] found up-regulation of intestinal IL-8 mRNA in acute NEC; however, elevations of IL-8 did not correlate with mortality. IL-11 mRNA levels also were increased; this increase may represent a protective response to limit the extent of intestinal damage. Levels of IL-8 and IL-11 returned to baseline at the time of stomal closure. Elevations of ileal IL-18 also have been demonstrated in NEC[313]; however, Harris and co-workers[284] found no significant elevation in plasma IL-18 levels in critically ill infants with NEC versus sepsis syndrome control infants.

A more recent study of HMGB1, a late but potent mediator of intestinal inflammation associated with NEC, found elevated levels in ileal samples from infants undergoing intestinal resection for NEC.[314] Thus investigations of plasma and intestinal mRNA levels suggest elevations of both proinflammatory and counterinflammatory cytokines in NEC.

Bronchopulmonary Dysplasia

Since the mid-1990s, improvement in neonatal respiratory care has enhanced the survival rate of premature infants who develop chronic lung disease. In these infants, the pathogenesis of BPD is complex and probably multifactorial, but evidence suggests the development of an early and persistent inflammatory response in the lung.[209,315,316] This inflammatory response may have its origins during the fetal period after intrauterine infection.[317] As hypothesized separately by Speer[210] and Jobe,[318] antenatal

inflammation may prime the lung so that minimal postnatal injury can produce an excessive inflammatory response.

Yoon and colleagues[319,320] reported elevated levels of TNF, IL-1, IL-6, and IL-8 in amniotic fluid from mothers of infants who subsequently developed BPD. In addition, elevated cord blood IL-6 levels, suggesting a fetal inflammatory response, were shown to be an independent risk factor for the development of BPD.[320] An and co-investigators[321] found elevated IL-6, IL-8, and sTNFR-1 levels in cord blood from infants with subsequent chronic lung disease, whereas IL-1, IL-2, IL-4, IL-10, and IFN-γ were undetectable. IL-6 levels also were higher in the infants who required prolonged oxygen therapy.

The presence of cytokines in bronchoalveolar lavage specimens may also serve as markers of inflammation. Cayabyab and co-workers[322] detected IL-1 in bronchoalveolar lavage (BAL) specimens from preterm infants within the first hour of life, which correlated with the presence of maternal chorioamnionitis, the presence of other proinflammatory cytokines (TNF, IL-6, IL-8), and increased risk for the development of chronic lung disease. In a more recent study of preterm infants in whom BPD developed subsequently, Kakkera and colleagues[323] found higher levels of IL-1ra as well as IL-1 in tracheal aspirates on day 1 of life. By days 5 to 7, increasing IL-1 expression (relative to IL-1ra) favored a proinflammatory response and the development of BPD. Other investigators have reported elevated IL-6 levels in lung lavage samples from infants in whom chronic lung disease developed later on.[324,325]

Jones and associates[209] measured IL-8 levels in BAL supernatants from term and preterm infants. Whereas IL-8 levels declined in term infants, elevations persisted in preterm infants over a 96-hour observation period. In infants with hyaline membrane disease, Kotecha[325] correlated the development of chronic lung disease with increased BAL IL-8 levels. Huang and co-workers[326] measured IL-8, IL-10, and TNF levels on the first day of life in BAL samples from preterm infants intubated for respiratory disease. Levels of IL-8, but not TNF or IL-10, were correlated with the development of BPD. Similarly, De Dooy and associates[327] found high levels of IL-8 (but not TNF, IL-1, IL-6, or IL-10) to be associated with adverse respiratory outcome in infants younger than 28 weeks gestation by multivariate analysis. These results are in accord with those reported by Kwong and colleagues[328] who found the highest IL-8 levels on day 12 of life in preterm infants with diffuse alveolar disease, which then declined to low levels by 28 days of age. Furthermore, tracheal aspirate concentrations of monocyte chemoattractant protein-1, -2, and -3 and macrophage inflammatory protein-1α and -1β were significantly increased in oxygen-dependent preterm infants at 28 days of age.[329]

By contrast, Jones and associates[209] reported undetectable levels of IL-10 mRNA in BAL samples from preterm infants with respiratory distress syndrome but measurable levels in term infants with meconium aspiration syndrome. These investigators speculated that the susceptibility of the preterm infant to chronic lung disease may in part reflect an inability to generate the antiinflammatory cytokine IL-10.[209,210] In a more recent study, Blahnik and colleagues[330] found that whereas TNF expression was nearly identical in term and in preterm neonates, a trend toward diminished levels of IL-10 was evident in the preterm group. In preterm infants born before 27 weeks gestation, a strong inverse relationship was found between constitutive IL-10 production by lung cell cultures and the development of BPD.[331] However, IL-10 production induced by LPS was correlated to gestational age. In contrast with the foregoing studies, McColm and co-workers[315] found that IL-10 was readily detectable in BAL samples from ventilated preterm infants with respiratory failure, most of whom developed chronic lung disease. Overall, however, data are consistent with a proinflammatory-antiinflammatory imbalance that favors the development of chronic inflammation and contributes to the pathogenesis of BPD.[319,331]

Investigations also have focused on genetic polymorphisms that may increase the risk and severity of chronic lung disease. A TNF polymorphism (TNF-α-238) in which lower TNF levels are produced after stimulation has been associated with diminished severity of BPD.[332] However, the IL-10-1082 G/A polymorphism did not influence the development of BPD in ventilated infants weighing less than 1500 g at birth.[333] Future multicenter studies in large populations are needed to address the potential associations of genetic susceptibility to inflammatory states.

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M. Michele Mariscalco

Integrins and Cell Adhesion Molecules

Adhesion is of fundamental importance to cell functioning. Besides providing "anchorage," it also provides cues for migration, signals for growth, differentiation, and cell activation. There are two principal types of cell adhesion: cell to extracellular matrix and cell to cell. Embryonic development, maintenance of tissue architecture, wound healing, hemostasis, inflammation, and the immune response all involve the interaction of cells with extracellular matrices or adjacent cells. The seminal role of adhesion molecules in embryogenesis and tissue morphogenesis cannot be overemphasized. Indeed much of the understanding of cell adhesion derives from this prolific area of research.[1] The focus of this chapter will be on the role of cell adhesion necessary for leukocyte and platelet localization in inflammation and the immune process, emphasizing what is known regarding the changes in these mechanisms in the immature host.

With the use of intravital microscopy, several investigators in the 19th century established white cells adhering to the blood vessel lining as the hallmark of the acute inflammatory response. In the absence of inflammatory stimuli and under conditions of flow, leukocyte contact with the endothelium is random and circulating leukocytes do not adhere to the vascular endothelium. With inflammation, leukocytes marginate within the venules where they are only loosely tethered to the endothelial wall and roll along the surface. After rolling, leukocytes become firmly adherent to the endothelium and become "activated," changing shape from a spherical to a flattened configuration. The leukocytes finally crawl along through the endothelium lining of the blood vessel (This process also is known as transendothelial migration or diapedesis). Though the initial observations of this phenomenon were made more than 150 years ago, only in the past several decades with the identification of specific cell adhesion and chemotactic-activator molecules have the molecular mechanisms of what has been termed the *leukocyte-endothelial cascade* been elucidated.[2] It is important to emphasize that this cascade does not necessarily occur in all areas of the body, and that other mechanisms appear to be operative in tissues such as the liver, lung, and kidney.

The susceptibility of human neonates to localized soft tissue infections, as well as systemic infections caused by bacterial or fungal agents, has prompted extensive investigations of neonatal host defense mechanisms. Among the most consistently observed functional abnormalities are those related to leukocyte migration. Compared to adult cells, neonatal neutrophils and monocytes have diminished random and directed migration (chemokinesis and chemotaxis, respectively) and decreased adhesion to substrates.[3-6] Motile function is affected by gestational age in that more severe abnormalities have been recognized in premature infants than in term neonates.[7-9]

In vivo studies employing Rebuck "skin windows" in human neonates provide limited data suggesting that inflammatory

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In vivo studies employing Rebuck "skin windows" in human neonates provide limited data suggesting that inflammatory

responses as reflected by leukocyte exudation may differ from those in older children and adults.[10] Neonatal animal studies have provided insight into these differences, revealing diminished localization in response to inflammatory agents to the peritoneum and lung and other organs compared to adult animals. The basis for this defect in neonatal leukocyte motility is likely multifactorial and possibly includes diminished decrease in f-actin polymerization and abnormalities of microtubule assembly, as well as diminished adhesion receptor number and function.[5,11,12,13]

CELL ADHESION MOLECULES

Three families of cell adhesion molecules have been found to play a central role in leukocyte-endothelial interactions: the integrins, the immunoglobulin (Ig) gene superfamily, and the selectins.[2,14] The ligands for selectins constitute a fourth family of adhesion molecules.[15] Newer adhesion molecules identified in the last decade add increasing complexity to this area. These include new members of the Ig gene superfamily, junctional adhesion molecule (JAM) -1, -2, -3, and CD99.[16] Other molecules, such as platelet endothelial cell adhesion molecule 1 (PECAM1), found on the cell surface modulate transendothelial migration. PECAM-1, with a structure that originally placed it in the Ig gene superfamily, appears to have a signaling-inhibitory function as well as an adhesive function.[16] It is beyond the scope of this chapter to cover each of these molecules in detail, but the following will serve as an introduction to the basic elements so that "differences" in neonate, compared to "mature host," adhesive molecule function can be explored.

Integrins

Overview

The integrins are transmembrane cell surface proteins. Unlike the other families of cell adhesion molecules, the integrins are heterodimers, consisting of noncovalently associated α and β subunits of 150 and 100-kDa, respectively. Each subunit crosses the membrane once, with most of each polypeptide in the extracellular space (≥1600 amino acids) and a short cytoplasmic tail (30-50 amino acids). The term *integrin* was originally suggested to describe membrane receptors that "integrate" the extracellular environment (matrix or other cells) with the intracellular cytoskeleton. At present, 18 α- and 8 β-chains have been identified with the formation of 24 distinct integrins. The adhesion molecules also may have a cluster designation (CD) classification (Table 157-1). As seen in Figure 157-1, β subunits define the subfamily of integrin receptors and can associate with more than one α subunit. Several α subunits may associate with more than one β. Phylogenetically ancient integrins recognize the tripeptide sequence arginine (R)-glycine (G)-aspartic acid (D) in fibronectin, fibrinogen, and vitronectin whereas other integrins mediate the adhesion to the basement membrane laminin (Figure 157-1). Collagen receptors have inserted (I) domains, which is discussed in more detail in subsequent paragraphs. A pair of related integrins (α4β1, α9β1) recognizes both extracellular matrix proteins such as fibronectin and an Ig gene superfamily cell surface counterreceptor, vascular cell adhesion molecule (VCAM-1). The β2 and β7 integrins (Figure 157-1) are restricted to leukocytes; their counterreceptors are members of the Ig gene superfamily; they mediate heterotypic cell-cell interactions, and several mediate adhesion to extracellular matrix proteins. Ligand specificity relies on both subunits of the αβ heterodimer pair. Each of the integrins has a specific function that is mostly nonredundant. The phenotypes of genetically altered mice in which the specific integrin is "knocked out" of the genome details the ligand specificity of these molecules and their various roles.[17] The phenotypes range from a complete block in preimplantation development (β1) to defects in leukocyte (αL, αM, αE, β2, β7) or platelet function (αIIbβ3).

Although integrins appear to have a nonredundant function in regard to their roles in adhesion to extracellular matrix ligands or counterreceptors on adjacent cells, they nonetheless share a number of characteristics. On the cytoplasmic side, integrins coordinate the assembly of cytoskeletal proteins and signaling complexes. On the extracellular side, integrins engage either extracellular matrix macromolecules or counterreceptors on adjacent cell surfaces. The adhesiveness of the integrins is regulated through a process known as *inside-out* signaling. In contrast, ligation of the integrin transduces signals from the extracellular integrin domains to downstream cytoplasmic pathways. This is known as *outside-in* signaling.[2] Thus the integrins link signaling and extracellular matrix assembly spatially and bidirectionally thereby "integrating" cells with their microenvironment.

Integrin Structure

Integrins are not constitutively "active" and usually exist in an "off" state. As such, integrins do not bind ligand, and they do not signal. One of the most important mechanisms of integrin function is the rapid transition from a nonadhesive, low-affinity state to an intermediate state which can transition to a very transient high-affinity state and then return to a low affinity state (Figure 157-2). This change in affinity is caused by intracellular signaling, or inside-out signaling. Members of the β2 integrin subfamily, which are expressed on leukocytes, are inactive when the cells are quiescent. However β2 integrins become rapidly activated in response to cell activation with low dose chemokines, cytokines, and with shear stress.[18] The activated β2 integrins may then bind to their ligands on the endothelial surface, other cells, subendothelial matrix, or complement-coated bacteria. Activation of the integrins is quite transient, with rapid return to an inactive state.[19] Clearly this function of integrins is necessary for maintenance of a "nonthrombotic," "noninflamed" state in the host.

At least two mechanisms are proposed to explain how integrin activation increases integrin-ligand interactions.[19,20] These mechanisms include conformational changes in integrins (regulating the affinity of the integrin for the ligand) and clustering of integrins and association with the cytoskeleton (regulating the avidity of integrin-ligand binding). Using crystal structure analysis, electron microscopic images, and electronic magnetic resonance, extensive progress has been made in elucidating conformational regulation of ligand binding by integrins.[19,20] The N-terminal portions of α and β subunit form a globular head containing the ligand-binding region. It is connected to the cell membrane by a stalk approximately 170 Å in length containing C-terminal segments from the both α and β subunits. As seen in Figure 157-2, in the α subunits, there are 7 segments of about 60 amino acids each that have weak homology to each other, but which are predicted to fold into a seven-bladed β propeller domain with Ca^{2+} binding motifs in 4-7. As outlined earlier and demonstrated in Figure 157-1, nine of the integrin α subunits contain an I domain also called a von Willebrand factor (vWF) A domain (A), as it resembles this structure. The I domain is the exclusive ligand binding site in those integrins. Divalent cations are necessary for ligand binding, and the residues coordinating metal binding are referred to as the metal ion-dependant adhesion site (MIDAS). The remaining α subunits are non-αI, and ligand binding involves specific regions of the N-terminal portions of both the α and β subunit. The remainder of the extracellular α subunit is predicted to be three β sandwich domains designated the thigh, calf-1, and calf-2 domains (see Figure 157-2).

The N-terminal cysteine-region of the β subunit is termed the PSI domain—for plexins, semaphorins, and integrins—because it shares sequence homology with these proteins. The first cysteine of the PSI domain forms a long-range disulfide bond to the C-terminal cysteine-rich region further down the β-subunit (see Figure 157-2). Although the β subunit lacks a true I domain, it

TABLE 157-1

Adhesion Molecules in Inflammation and Hemostasis

Name	CD Classification	Primary Cell Expression	Ligand
Integrin Family			
β1 Integrins	CD49a/CD29	T and B cell subsets, Mono	COL I, COL IV, LN
α1β1 (VLA-1)	CD49b/CD29	T cell subsets, Mono, PLT, PMN	COL I, COL IV, LN
α2β1 (VLA-2)	CD49c/CD29	T cell subsets, Mono	FN, COL I, LN
α3β1 (VLA-3)	CD49d/CD29	T cell, B cell, Eos, Mono, PMN, Baso	FN, VCAM, Tsp, JAM2
α4β1 (VLA-4)	CD49e/CD29	T cells, PMN, PLT	FN, Tsp
α5β1 (VLA-5)	CD49f/CD29	T cells, PMN, PLT, EC	LN
α6β1 (VLA-6)		PMN, Mono	VCAM-1, OSP, tenascin
α9β1			
β2 Integrins	CD11a/CD18	PMNS, T & B cell, Eos, Mono, NK, Macro	ICAM-1, ICAM-2, ICAM-3, JAM-A
αLβ2 (LFA-1)	CD11b/CD18	PMNS, Mono, Eos, NK, Macro, Lymph subset	ICAM-1, iC_3b, Fg, FN, Factor X, JAM-C,
αMβ2 (Mac-1) , CR3	CD11c/CD18	PMN, Mono, Eos, Lymph subset, dendritic cells	and many others
αXβ2 (p150,95) ,CR4	CD11d/CD18	Lymph, Mono, Macro, PMN	iC_3b, ICAM-2, VCAM-1, Fg
αDβ2			ICAM-3, VCAM-1
β3 Integrins	CD41/CD61	PLTS	VWf, FN, Fg, VN, Tsp, COL
αIIbβ3 (GPIIbIIIa)	CD51/CD61	LAK, Macro, Mono, T cell, PLT, EC, PMN	VWf, VN, FN, Fg, PECAM-1, Tsp, LN, OSP,
αVβ3 (vitronectin receptor)			tenascin, COL
β7 Integrins	CD49d/β7	Gut-associated lymphocyte	VCAM, MAdCAM-1, FN
α4β7	CD103/β7	Gut-associated lymphocytes	E-CAD
αEβ7			
Immunoglobulin Superfamily	CD54	T+B cell, Mono, EC, pneumocyte, hepatocytes,	LFA-1, Mac-1
(IGSF)	CD102	Epithelial cells, fibroblasts	LFA-1
ICAM-1	CD50	EC	LFA-1, αDβ2
ICAM-2	CD106	Lymph, PMN, Mono	α4β1, α4β7; α9β1, αXβ2, αDβ2
ICAM-3	CD31	EC, Epithelial cells, fibroblasts, smooth muscle	LFA-1, JAM-A
VCAM-1		cells	JAM-C, JAM-B, α4β1
JAM-A (JAM-1, F11R)		EC, Epithelial cells, PMNs, Mono, Lymph, PLTS	Mac-1, JAMC
JAM-B (JAM2)		HEV, EC	α4β7, L-selectin
JAM-C (JAM3)		EC	
MAdCAM-1		Peyer's patch HEV, mesenteric LN,	
PECAM-1*			
Selectins	CD62-L	Lymph, Mono, Eos, Baso, PMN	PSGL-1, PNAd CD34, GlyCAM-1,
L-selectin	CD62-P	EC, PLTS	MAdCAM-1, sLe^x-bearing ligands
P-selectin	D62-E	EC	PSGL-1, E-selectin, GPIb- IX-V
E-selectin			PSGL-1, sLe^X-bearing ligand(s), CLA
			CD44, PSGL-1, sLe^X-bearing ligand(s)
Selectin Ligands	CD162	PMN, Eos, Mono, Lymph	P-selectin, L-selection
PSGL-1	CD34	Lymph node + lung EC	E-selectin
GlyCAM-1	CD44	Peripheral LN HEV, leukocyte precursors	L-selectin
CD34		Leukocytes	L-selectin
CD44			E-selectin
"Other"			
CD99	CD99	T cells, endothelial cells, Mono	CD99
PECAM-1*	CD31	EC, PMN, PLT, Mono, lymphocyte subsets	PECAM-1, αVβ3

Baso, Basophil; *CLA,* cutaneous lymphocyte-associated antigen; *COL I,* collagen type I; *COL IV,* collagen type IV; *EC,* endothelial cell; *E-CAD,* E-cadherin; *Eos,* eosinophils; *Fg,* fibrinogen; *FN,* fibronectin; *GlyCAM-1,* glycosylation-dependent cell adhesion molecule-1; *GP1b-IX-V,* glycoprotein complex present on the surface of platelets; *iC3b,* inactivated form of complement component C3b; *ICAM,* intercellular adhesion molecule; *JAM,* junctional adhesion molecule; *LAK,* lymphokine-activated killer cells; *LN,* laminin; *Lymph,* lymphocytes; *Macro,* macrophages; *MAdCAM-1,* mucosal addressin cell adhesion molecule-1; *Mono,* monocytes; *NK,* natural killer cells; *OSP,* osteopontin; *PECAM-1,* platelet endothelial adhesion molecule–1 (*Though PECAM-1 is now considered a member of the ITIM family, by convention it is still listed in the IGSF and "other"); *PLT,* platelet; *PMN,* polymorphonuclear leukocyte; *PNAD,* peripheral node addressin; *PSGL-1,* P-selectin glycoprotein ligand-1; *sLe^x,* sialyl 6-sulfo Lewis-X antigen; *Tsp,* thrombospondin; *VCAM,* vascular cell adhesion molecule; *VLA,* very late antigen; *Vn,* vitronectin; *vWf,* von Willebrand factor.

does have a region of about 240 residues, which is highly conserved among species. It has been termed the *I-like* domain because it has a metal binding motif similar to that of MIDAS region in the I domain, a weak amino acid sequence homology with the I domain, and similar secondary structure. The I-like domain appears to directly bind ligands in integrins that lack I domains and to indirectly regulate ligand binding by integrins that contain I domains.[19] Mutations in this region result in lack of association—or loss of function—of the integrin β2 subunit with the α, resulting in leukocyte adhesion deficiency (LAD).[21] As seen in Figure 157-2, the hybrid domain is folded with the I-like domain in the middle, with two covalent connections between the I-like and hybrid domains. There is a cysteine rich region with four repeats with structural similarity to epidermal growth factor (integrin-EGF or I-EGF). The cysteine-rich repeats have unique structural properties that make this region rigid and suited for transmission of structural motion in signaling. Many antibodies, which "activate" the integrins or only bind when the integrin is in an activated state, bind to this region.[20]

The most widely accepted model of integrin activation is that of a global change in conformation. Integrins can exist in a number of activation states. The integrin in the "inactive" conformation is "bent over" (see Figure 157-2, *B*). The ligand-binding headpiece is folded back on the tailpiece and thus is unavailable for binding to ligand. This occurs in the presence of Ca^{2+}. With activation, and/or in the presence of Mn^{2+} the integrin straightens like a "switchknife," and the I domain orients away from the membrane for optimal ligand binding. This is an "intermediate" activation state and is described as the extended confirmation. With the straightening and separation of the "legs" of the integrin, there are epitopes in the I-EGF and the PSI regions of the β subunit that are then "unveiled" and are now available to bind to

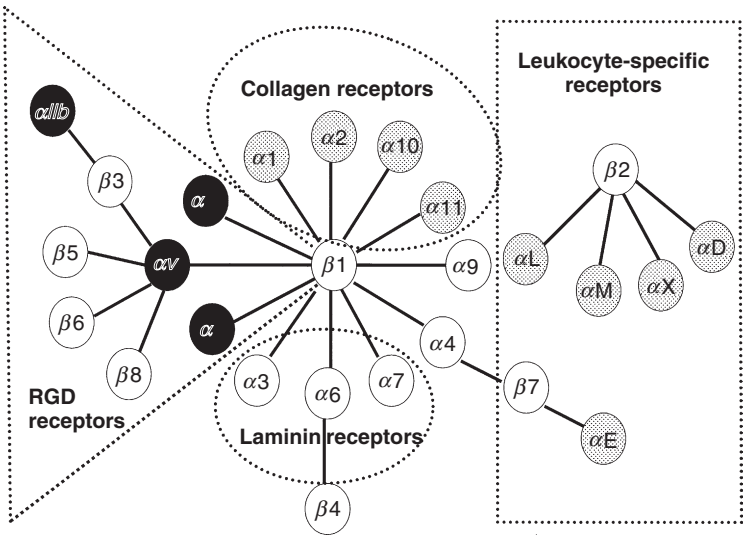


Figure 157-1 The integrin family. Integrins are αβ heterodimers. The figure depicts the mammalian subunits and their αβ associations; eight β subunits can associate with 18 α subunits to form 24 distinct integrins. α Subunits with *gray stippling* have inserted I domains (see text). α Subunits with I domains are found only in chordates, as are α4 and α9 and subunits β2-β8. In contrast, α subunits specific for laminin or RGD (*black spheres*) are found throughout the metazoa. Note that β2 and β7 integrins are restricted to leukocytes only. *(From Hynes RO: Integrins: bidirectional, allosteric signaling machines.* Cell *110:673-687, 2002.)*

antibodies, which bind only when the integrin is activated.[20] In the extended confirmation with high affinity, local conformational changes within the α and β I and I-like domains result in the hybrid domain swinging out and the subunit separating at the genu. This remodeling of the I-domain ligand-binding causes increased affinity for ligand (see Figure 157-2, *C*).

Much of our understanding of integrin physiology has been based on work examining the leukocyte integrins: lymphocyte function-associated antigen-1 (LFA-1 [CD11a/CD18, αLβ2]); Mac-1 (CD11b/CD18, αMβ2); α4β1 (very late antigen [VLA-4])]; and the platelet integrin (GPIIbIIIa [CD41/CD61, αIIbβ3]) individually.[19] Chemokine-receptor signaling leads to extension of the bent "low affinity" form of CD11a/CD18 (LFA-1), and this happens in less than 0.4 seconds. The extended form of LFA-1 is proposed to be in an intermediate state of affinity simply as a result of the greater accessibility of the ligand-binding I domain compared with the bent form. It has been proposed that the interaction of intermediate-affinity LFA-1 with its ligand intercellular adhesion molecule 1 (ICAM-1) leads to the high-affinity conformation that stabilizes adhesion.[20] However, there now is increasing evidence that mechanical forces can result in allosteric alterations of integrin conformation. For leukocytes, the interaction with the endothelial lining of the blood vessel under flowing conditions would be the major supplier of this force.[15]

Bidirectional Signaling and Integrins

Integrins are activated by inside-out signaling. The key target of this pathway is the GTPase Rap1. Agonist activation (such as chemokine stimulation) leads to relocalization of Rap1 to the membrane through several steps. The localization of Rap1 to the plasma membrane results in recruitment of the cytoskeletal protein talin to the β subunit of the integrin cytoplasmic domain. The recruitment is modulated by the cytoskeletal protein Kindeln3. It is the recruitment of talin to the cytoplasmic tail of β subunit which brings about the conversion of the integrin conformation to one of higher affinity.[20,22] Rap1 can be activated by the second messenger Ca^{2+} and diacylglycerol (DAG) and protein kinase C. At least in the case of lymphocytes it appears that there may be several mechanisms to activate Rap1. A fast route occurs with chemokine stimulation, in that Rap1 is activated within seconds and lasts a few minutes. A more sustained activation of Rap1 occurs through the T cell receptor.[20]

Ligand binding by the integrins then results in outside-in signaling (see Figure 157-2, *C*). It appears that high-affinity LFA-1-talin connection favors the formation of stable LFA-1 clusters. Macroclustering of LFA-1 strengthens adhesion to the LFA-1 ligand ICAM-1 (see Table 157-1).[19,20] It is accepted that the successful functioning of all leukocytes depends on cytoskeletal remodeling. Outside-in signaling of integrins causes cell spreading and migration. This implies that cytoskeletal reorganization occurs downstream of integrin-signaling. It is clear that β2 integrins mediate adhesion, spreading, chemotaxis, and production of reactive oxygen species and the release of cytokines from neutrophils and macrophages. At least part of this signaling is through Src kinase, which is constitutively associated with the β2 and β3 cytoplasmic tails.[20] Integrin activation results in Src kinase activation. It in turn phosphorylates adaptor molecules, which ultimately recruit Syk kinase to the integrin β tail.[23] Syk kinase activation results in downstream activation of several key proteins critical for spatio-temporal control of actin polymerization. These proteins include Vav—a guanine exchange factor for the Rho family GTPases—Rac, and Cdc42, all of which are major regulators of the organization of the actin cytoskeleton during leukocyte polarization and migration.[24]

INTEGRIN FUNCTION: ADDITIONAL LEVELS OF CONTROL

Integrin receptor expression can also be modulated at the transcriptional and translational level. During leukocyte differentiation, the levels of β2 integrins are differentially regulated. αLβ2 appears in early progenitor cells, the expression of αMβ2 (Mac-1), and αXβ2 (p150,95) occurs in later maturational stages. Numerous stimuli increase the expression level α4β1on lymphocytes and macrophages through transcriptional control. In contrast, rapid alterations in levels of integrins expressed on the surface of neutrophils and platelets can be achieved by mobilization of intracellular pools of integrins in response to extracellular stimuli and chemotactic factors (Table 157-2). Stimulation of neutrophils or monocytes can increase the αMβ2 and αXβ2surface expression tenfold, as these integrins are stored in secretory and tertiary

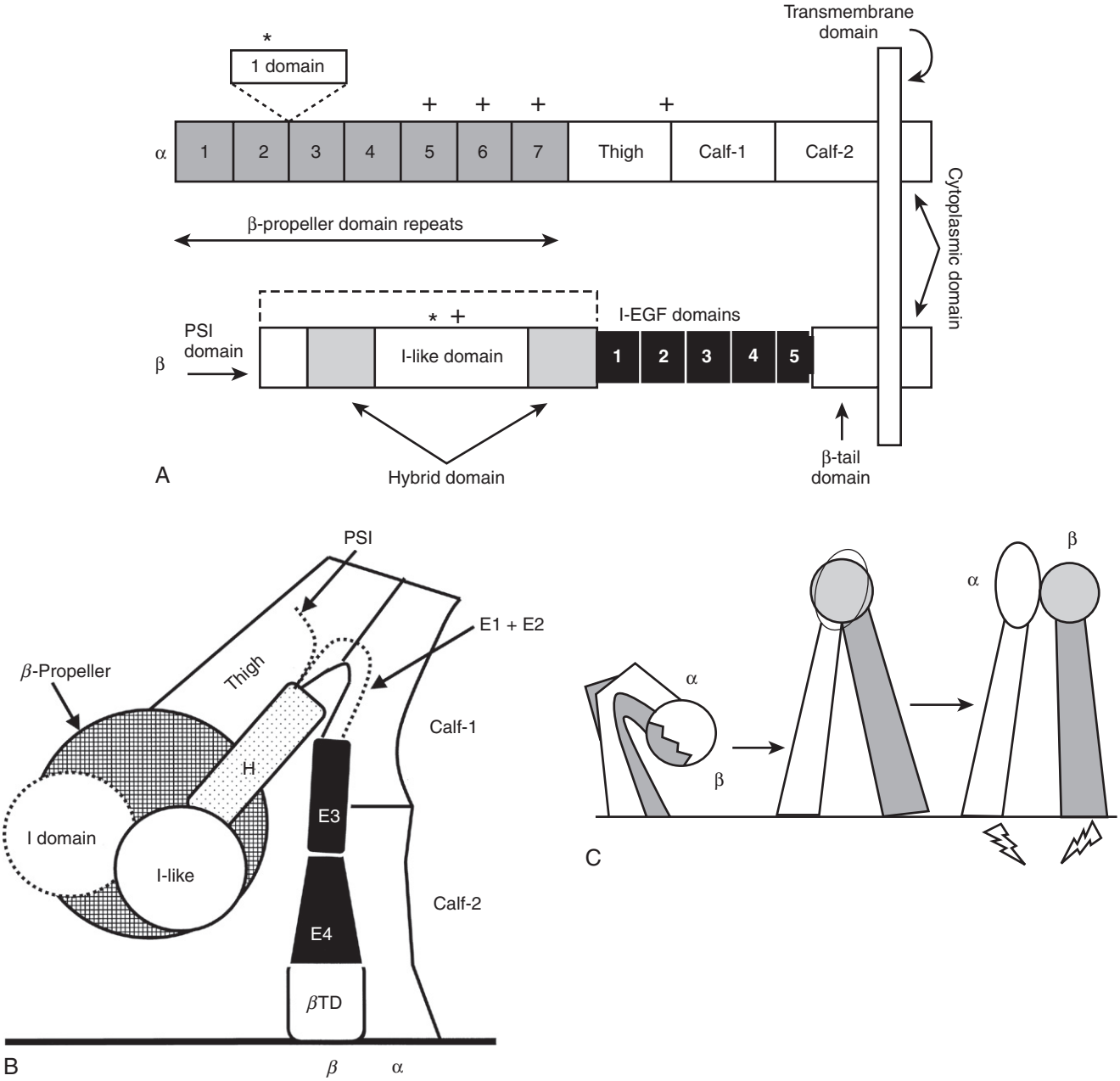


Figure 157-2 Integrin structure. A, General structure of the α and β subunits. Depending on the α subunit, there may also be an inserted I domain as outlined in the text. Mg^{2+} and Ca^{2+} binding sites are shown as asterisks *(*)* and plus signs *(+)* respectively. Only the disulfide bonds within the β chain between the PSI domain and the first I-EGF domain are shown (*dotted line*). Both chains have numerous disulfide bonds that are not included, but which account for the complex tertiary structure. **B,** Arrangement of the domains based upon the three-dimensional structure of αvβ3 with an added I domain. Domains refer to those listed in A. PSI and I-EGF domains 1 & 2 are referenced by a *dotted line* as they are "out of the plane" of the molecule. The transmembrane and cytoplasmic domains are not included. **C,** Hypothetical model for integrin activation. In the inactive state, the integrin is "bent over" with low affinity for ligand *(figure on left)*. In the *central figure*, there is an extended conformation with intermediate ligand affinity and closed headpiece. In the *figure on right*, there is a high ligand affinity conformation with an extended conformation that has open headpiece when bound to ligand, here RGD for αvβ3. The transient high affinity conformation results in "outside-in-signaling," demonstrated in this figure by the *lightning bolts*. *I-EGF,* Integrin-epidermal growth factor-like; *PSI,* plexin, semiphorins, integrins. *(From Shimaoka M, Springer TA: Therapeutic antagonists and the conformational regulation of the β2 integrins.* Curr Top Med Chem *4:1485, 2004.)*

granules.[25] Although not present or present at very low levels under resting conditions, neutrophils that have undergone migration can express large amounts of α2β1, α4β1, α5β1, α6β1, and α9β1, which assist the neutrophil in migrating through extracellular matrix.[26] It is unclear whether all these integrins are present in granules or are synthesized de novo. Platelets contain reserves of αIIbβ3 (GPIIb/IIIa, the receptor for fibrinogen, vWF, Table 157-1) in the α granules. With platelet activation, fusion of α granules with the plasma membrane occurs, effectively doubling the number of αIIbβ3 complexes.

TABLE 157-2

Activating Factors for Leukocyte-Platelet Endothelial Adhesion

ACTIVATING AGENT	TARGET CELL				
	Endothelial Cell	Neutrophil	Monocyte	Lymphocyte	Platelets
Cytokines					
TNFα	+	+			
IL-1	+	+			
IFNγ	+	+			
IL-4	+				
Lipids					
Bacterial endotoxin (LPS)	+	+	+	+	+
PAF	+	+	+		+
LTB_4		+	+		+
LTC4					
Thromboxane A2					
Peptide Chemoattractants					
C5a		+	+		
N-formyl peptides (fMLP)		+	+		
C-X-C Chemokines					
CXCL8(IL-8)	+	+	+	+	+
CXCL10 (IP10)	+	+	+	+	
CXCL5 (ENA 78)	+	+	+	+	
CXCL1 (GRO-α, MGSA-α)	+	+	+	+	
CXCL7 (NAPII)	+			+	
CXCL12 (SDF)				+	
C-C Chemokines					
CCL3 (MIP -1α)		+	+	+	+
CCL4 (MIP -1β)		+	+	+	+
CCL5 (RANTES)			+	+	+
CCL2 (MCP-1)			+	+	+
CCL8 (MCP-2)			+	+	
CCL7 (MCP3)			+	+	
Others					
Histamine	+	+			+
Thrombin	+				+
Hydrogen peroxide	+				+
Collagen					
Adenosine diphosphate					

C5a, Protein produced from cleavage of complement protein C5; *ENA 78,* epithelium-derived neutrophil attractant 78; *fMLP,* n-formyl-methionine-leucine-phenylalanine; *GRO-α,* growth regulating protein α; *IFN-γ,* interferon-γ; *IL-1,* interleukin-1; *IL-4,* interferon-4; *IL-8,* interleukin-8; *IP10,* γ-interferon-induced peptide; *LPS,* lipopolysaccharide; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; *MCP-1,-2,-3,* monocyte chemotactic peptide -1,-2,-3. Names in parenthesis indicate the other names of chemokines; *MGSA,* melanocyte growth-stimulating activity; *MIP-2α,* macrophage inflammatory peptide α; *NAP-2,* neutrophil activating peptide-2; *NAP-2,* neutrophil activating peptide-1; *PAF,* platelet activating factor; *RANTES,* regulated on activation, normal T cell expressed and secreted; *TNFα,* tumor necrosis factor α.

Integrin Ligands

The integrin ligand binding characteristics have been extensively studied, and clusters of integrin-ligand combinations reflecting a structural basis for molecular interactions are being characterized. All five αV integrins, two β1 integrins (α5, α8) and αIIbβ3 share the ability to recognize ligands containing an Arg-Gly-Asp (RGD) tripeptide active site. The Leu-Asp-Val (LDV)-binding integrins bind ligands with an acidic motif similar to LDV. Although fibronectin contains the prototype LDV ligand, other ligands such as VCAM-1 and MAdCAM-1 use similar motifs for binding. LDV-binding integrins include α4β1, α4β7, α9β, the β2 integrins, and α4β7. As seen in Table 157-1, there are extracellular matrix proteins that are dominant ligands for the β1 integrins. These include fibronectin, fibrinogen, laminin, and thrombospondin. Cell surface ligands for integrins include members of the Ig gene superfamily, which are discussed in following sections (see Table 157-1).[2,27]

β2 Integrins

It is the β2 subgroup (CD18), found exclusively on leukocytes, that is the major contributor to leukocyte motility and function. All members share the common β2 (CD18) subunit (Figure 157-1 and Table 157-1), but have unique α (CD11) subunits that primarily confer ligand specificity. αLβ2 (LFA-1,CD11a/CD18) is located on all leukocytes. αMβ2 (Mac-1, CD11b/CD18), and αxβ2 (p150,95, CD11c/CD18) are located on neutrophils, monocytes, macrophages, natural killer (NK) cells, and some lymphocytes. LFA-1 and Mac-1 appear to interact equally with their endothelial ligand, ICAM-1. LFA-1 also binds to both ICAM-2, -3, and JAM-A. Lymphocyte adhesion to the vessel wall involves the interaction of LFA-1 with its endothelial ligands, ICAM-1 and -2. However lymphocyte LFA-1 participates in many other cell-cell interactions such as T cell activation by antigen-presenting cells or the killing of virus-infected cells by cytotoxic T lymphocytes.

Mac-1 and p150,95 are capable of binding to a wide range of ligands including fibrinogen, factor X, denatured albumin, and the complement fragment iC3b. This rather "promiscuous" attachment to a number of native and denatured proteins is of importance during leukocyte migration over a variety of substrata, as well as adhesion to and phagocytosis of foreign particles. Neutrophil and monocyte adhesion to endothelium relies mainly on LFA-1 and Mac-1 with only a minor role for p150,95.

β1 and β7 Integrins

Most integrins are members of the β1 (CD29) or VLA subfamily (see Figure 157-1 and Table 157-1). VLA derives from *very late antigen* because the first ones to be identified (VLA-1 and VLA-2) were expressed only at a late stage after T cell activation. They comprise a series of cellular receptors for extracellular matrix proteins including fibronectin, collagen, and laminin. α4β1 (VLA-4, CD49d/CD29) is expressed at substantial levels on most mononuclear leukocytes including lymphocytes, monocytes, eosinophils, basophils, and NK cells, whether the cells are in circulation, within lymphoid organs, or resident in other tissues. α4β1 is also expressed on transmigrated neutrophils.[28] α4β1 binds to vascular cell adhesion molecules-1 (VCAM-1), mucosal addressin cell adhesion molecule-1 (MAdCAM-1), and to the alternatively spliced connecting segment (CS-1) of fibronectin. The site of α4β1 binding to VCAM-1 is distinct from that involved in fibronectin binding. α4β1 is of critical importance for monocyte and eosinophil trafficking to inflamed tissues and to lymphocyte migration and differentiation. Unlike LFA-1 and Mac-1, α4β1 can function both as a selectin, that is, mediate the initial tether of the leukocyte from the free stream to VCAM-1 and MAdCAM-1, as well as transition to a more stable adhesion.[29]

α1ββ1, α2β1, α3β1, α5β1, α6β1 (see Table 157-1) are expressed on T cell subsets or resting T lymphocytes and generally are of importance to lymphocyte-extracellular matrix interactions. Their ligands include the extracellular matrix proteins collagen, laminin, and fibronectin. Neutrophils also express α2β1, α5αβ1, α6β1 as they infiltrate lungs, joints, peritoneum, and spleen, and adhere to and migrate through interstitial matrix via pathways that are β2 integrin independent.[26,28] α9β1 is abundantly expressed on neutrophils, but not on lymphocytes and modestly on monocytes. Its ligand is VCAM-1. As with α4β1, α9β1 mediates neutrophil transendothelial migration.[26,28]

The α4β7 (CD49d/β7) and αEβ7are expressed primarily on gut-associated T lymphocytes. α4β7 is required for lymphocyte recirculation to specific areas of the gut containing MAdCAM-1, which is expressed on Peyer's patch high endothelial venules and postcapillary venules in the lamina propria. αEβ7 is expressed on T lymphocytes in the intestinal mucosa, genitourinary tract, lymphocytes derived from the lung, and on intraepidermal T cells in some skin disorders. It is also present on 90% of $CD8^+$ T cells. Its primary ligand is epithelial (E) -cadherin.[30]

β3 Integrins

Like Mac-1, αvβ3 (CD51/CD61), also known as the vitronectin receptor, and αIIbβ3 (gpIIb/IIIa) are promiscuous receptors and have overlapping ligands including fibrinogen, vWF, fibronectin, collagen, and vitronectin, though each may bind to different regions of the same molecule. αvβ3 is present on a large number of cell types and has been shown to play a role in tumor invasion, proliferation and metastasis, bone resorption, angiogenesis, differentiation, development, and the immune response. It serves as an accessory molecule for T cell activation and is of importance for complement deposition on platelets and cells. Additionally αvβ3 is expressed on macrophages in a maturationally related process and mediates recognition of apoptotic cells (both neutrophils and lymphocytes). Thus αvβ3 represents a mechanism that limits tissue injury through macrophage clearance of leukocytes at an inflamed site.

Many integrins are found on platelets including α2β1, α5β1, α6β1, and αvβ3and are of importance to either stimulatory or adhesive-cohesive phases of platelet function. αIIbβ3 (gpIIb/IIIa) is found exclusively on platelets and it is largely responsible for the final cohesive phase of platelet activation in vivo, that is, platelet aggregation supported by the binding of adhesive proteins like fibrinogen or vWF.[31] Coincident with platelet activation, surface expression of αIIbβ3 increases through release from α granules. Additionally, αIIbβ3 undergoes profound conformational changes with resultant binding to fibrinogen; resting platelets do not bind fibrinogen. With binding of αIIbβ3 through outside-in signaling, the platelet is stimulated further and can potentiate clot retraction.[31]

Immunoglobulin Gene Superfamily

The Ig gene superfamily encompasses a large group of molecules with multiple Iglike domains in the extracellular portion of the molecule resembling the second domain of the constant region of Ig (Figure 157-3). At least seven members of this family are involved in leukocyte-leukocyte or leukocyte-endothelial interactions. ICAM-1, CD54; ICAM-2 CD102; VCAM-1, CD106; PECAM-1, CD31; MAdCAM-1; and JAM all serve as endothelial ligands for leukocytes. ICAM-3, CD50 is expressed on leukocytes and is of importance for leukocyte-leukocyte adherence and cell signaling.[2,16,32]

ICAM-1, ICAM-2, and ICAM-3 were all initially identified by their ability to interact with LFA-1, and each is the product of distinct though homologous genes (see Figure 157-3). ICAM-1 contains five Ig domains, but ICAM-2 contains two; both domains are highly homologous to the two NH_2-terminal domains of ICAM-1. ICAM-3 also contains five Ig domains that are highly homologous to ICAM-1 and -2. The binding site for LFA-1 has been mapped to the first Ig domain for each of the molecules. In contrast, ICAM-1 has been found to bind to Mac-1 through a distinct site in its third Ig domain. ICAM-1 is constitutively present on endothelial cells as well as on other cell types including lymphocytes, dendritic cells, fibroblasts, hepatocytes, and epithelial cells such as type I pneumocytes. ICAM-1 is expressed on resting endothelium, but its expression increases dramatically after stimulation with a number of cytokines including tumor necrosis-α (TNF-α), interleukin-1(IL-1), and interferon-γ (IFN-γ) (Figure 157-4).

VCAM-1 exists predominantly as a 7 domain transmembrane protein in humans, though an alternatively spliced minor form exists (see Figure 157-3). VCAM-1 binds to αβ41 at its first and fourth domains. It also binds to α4β7 and α9β1 (see Table 157-1). VCAM-1 is expressed constitutively on some nonvascular cells including dendritic cells in lymphoid tissues and skin, epithelial cells, some monocyte-derived cells, and bone marrow stromal cells. VCAM-1 is absent on resting endothelial cells, but is induced in response to IL-1 and TNF with maximal expression reached by 6 to 12 hours (see Figure 157-4). VCAM-1 is required for lymphocyte, eosinophil, and monocyte adherence and emigration and appears critical in the initiation of atherosclerosis. Unlike ICAM, it is able to participate in the tethering and rolling of leukocytes.[29]

PECAM-1, CD31 is a six Ig domain molecule which mediates both leukocyte- and platelet-endothelial adhesion and leukocyte transendothelial migration. PECAM-1 is present on platelets, bone marrow precursor cells, neutrophils, monocytes, and certain subsets of lymphocytes. It is highly expressed on endothelial cells, but much less so on neutrophils. With its Iglike extracellular domains, it was classified initially as a cell adhesion molecule. However recent work has demonstrated that its cytoplasmic domain may be the "work" end of the molecule. PECAM-1 is a member of the Ig immunoreceptor tryosine-based inhibitory motif (Ig-ITIM) family. When members of this family are engaged by their extracellular ligand, their cytoplasmic ITIM domain becomes phosphorylated, which serves as a docking site for intracellular lipid and protein phosphatases. Once these catalytic enzymes become localized to

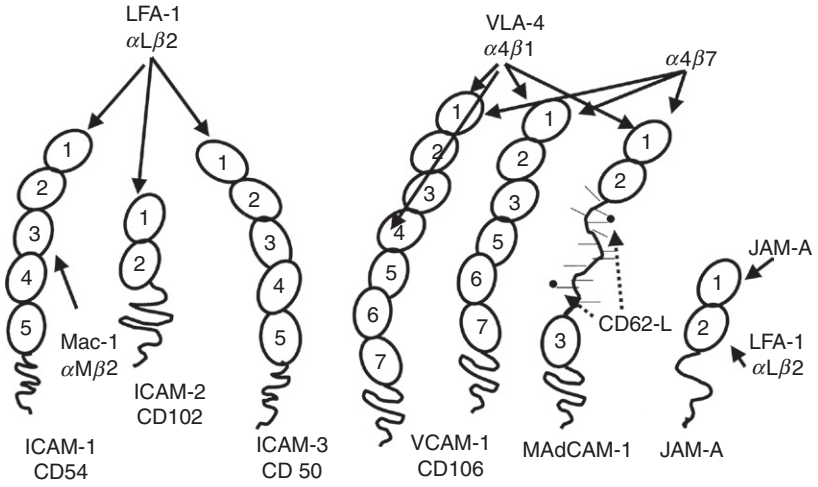


Figure 157-3 Immunoglobulin gene superfamily. The immunoglobulin gene superfamily (IGSF) comprises a large group of molecules, each of which contains multiple extracellular domains resembling the second domain of immunoglobulin *(oval)*, a transmembrane domain, and cytoplasmic tail. Note that there is an alternatively spliced form of VCAM-1 which does not contain domain 4. The *sticks* and *lollipops* on MAdCAM-1 refer to the N-linked and O-linked glycosylation sites, respectively. Included also on the structure diagrams are the domains to which specific adhesion molecules bind. *CD62L,* L-selectin; *ICAM,* intercellular adhesion molecule; *JAM,* junctional adhesion molecule; *MAdCAM,* mucosal addressin cell adhesion molecule; *VCAM,* vascular cell adhesion molecule.

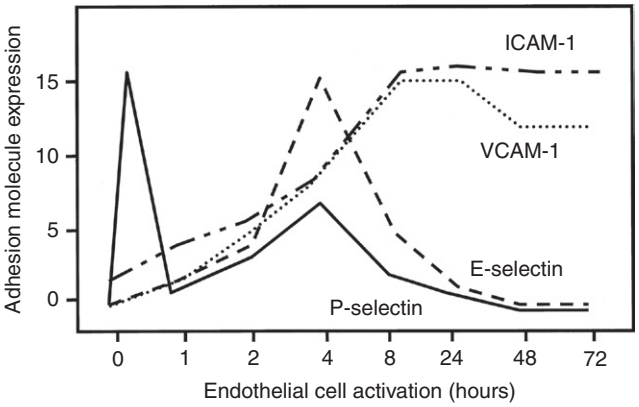


Figure 157-4 Temporal expression of adhesion molecules induced by cytokines. Note that P-selectin has two peaks at 0.5 hour and 4 to 8 hours. The first peak is caused by release of P-selectin from Weibel-Palade bodies; the second is caused by protein synthesis. There is constitutive expression of ICAM-1 (intercellular adhesion molecule). *VCAM,* Vascular cell adhesion molecule. *(From Imhof BA, Dunon D: Leukocyte migration and adhesion.* Adv Immunol *58:345-416, 1995.)*

their cytoplasmic anchors and activated, they affect a wide range of cellular functions including events which inhibit signaling, proliferation, and cellular activation. In particular, PECAM-1 appears to modulate activation of β1, β2, and β3 integrin function on platelets, lymphocytes, and neutrophils. PECAM-1 participates in homophilic (i.e., binds to another PECAM-1 molecule on adjacent cells) and heterophilic (binds to other ligands such as αvβ3) interactions. PECAM-1 is critical for leukocyte transendothelial migration.[16]

An adhesion molecule for lymphocyte recirculation to mucosa is expressed on Peyer's patch high endothelial venules, as well as other venules. Known as MAdCAM-1, this adhesion molecule contains three Iglike domains and a mucinlike region between domain 2 and 3 (see Figure 157-3). Mucins are serine- and threonine-rich proteins that are heavily glycosylated through an O linkage. They are rigid and have an extended structure (see following discussion on selectin-ligands.) MAdCAM-1 binds both α4β7 and α4β1. MAdCAM-1 is unique in that with the presence of a mucinlike domain it is also able to bind L-selectin and mediate lymphocyte rolling.[33]

JAM-A, -B, -C are some of the new members of the Ig family (see Table 157-1).[16] The JAMs have two extracellular Ig domains and are present on endothelial and epithelial cells at the tight junctions. Thus they are critical to maintaining barrier function. With stimulation by proinflammatory cytokines JAMs move away from the borders and appear to mediate permeability.[16] JAMs are also present on neutrophils, lymphocytes, and platelets. Recent studies demonstrate that these molecules may play unique roles in leukocyte transendothelial migration as they are ligands for LFA-1 and α4β1.[16]

CD99 does not belong to the Ig superfamily. It is a heavily glycosylated transmembrane protein present on many hematopoetic cells and has been shown to increase the presence and activity of LFA-1 and α4β1. It has also been found on endothelial cells and is concentrated at the borders between confluent cells. CD99 on monocytes and neutrophils appears to mediate homophilic binding to endothelial CD99 and mediate transendothelial migration.[34,35]

Selectins

There are three members of the selectin (CD62) family of adhesion molecules: E- (CD62-E), P- (CD62-P), and L-selectin (CD62-L). This family mediates the earliest events in leukocyte-endothelial adhesion in that they "capture" leukocytes from the free-flowing blood stream and allow rolling to occur along the blood vessel lining. The name incorporates the term *lectin*, which refers to a wide range of carbohydrate-binding proteins, reflecting the fact that this family of adhesion molecules recognizes carbohydrate ligands. Each is an integral membrane protein with an N-terminal, Ca^{2+}-type lectin domain followed by an EGF-like module, multiple copies of the consensus repeat units characteristic of complement-binding proteins, a transmembrane segment, and a short cytoplasmic domain. The amino acid sequences of the lectin and EGF domain of all three selectins are highly conserved, suggesting that both domains participate in ligand recognition. The lectin domain of P- and E-selectin has a single Ca^{2+}-binding site located on the face of the molecule opposite where the EGF domain is attached. Although there is strong homology between different selectins in the extracellular domains, there is none in

the cytoplasmic tails, suggesting selectin-specific functions for these domains (see Table 157-1).[18,36]

The characteristic feature of selectins is their ability to recognize and bind to specific carbohydrate determinants on selectin ligands in a calcium-dependent manner.[36] Binding takes place under dynamic conditions where continuous shear forces, exerted by the flowing blood, act on leukocytes rolling along the endothelium at rolling velocities ≈100- to 1000-fold slower than the mean blood flow velocity. To achieve controlled and stable leukocyte rolling under these conditions, selectin binding to selectin ligands needs to comply with the following three requirements: (1) rapid bond formation at the leading edge, (2) high tensile strength during binding, and (3) fast dissociation rates. Selectins can adjust to increasing flow rates by locking their bonds with their ligands more tightly. This enables leukocytes to roll even at high shear rates. However, there is a shear rate limitation, and once this is reached the bond properties change toward a slip bond behavior, which eventually leads to breakage of the bond. Thus these critical properties create a breaking system that recruits free flowing leukocytes to the endothelial wall and prepares them (during rolling) for subsequent adhesion and transmigration.

L-selectin functions as the peripheral lymph-node homing receptor on lymphocytes and binds through its lectin domain to carbohydrate-containing ligands on high endothelial venules in peripheral lymph nodes (see Table 157-1). It also serves as an important adhesion molecule for monocyte, neutrophil, and eosinophil trafficking. The cell surface expression of L-selectin is down-regulated after activation of both lymphocytes and myeloid cells. The process involves the shedding of L-selectin rather than the internalization or a conformational change and arises through proteolytic cleavage of the molecule at the extracellular part close to the transmembrane region. L-selectin has been demonstrated to be located at the tips of the surface folds of unstimulated neutrophils. In this position, L-selectin would be in a particularly advantageous position to contact the endothelium in the initial margination of neutrophils. Inhibition of L-selectin shedding in mice results in decreased leukocyte rolling velocity and increased number of arrested and transmigrated leukocytes.[37]

P-selectin is constititutively synthesized by megakaryocytes and endothelial cells, but is sorted into secretory granules (α granules in platelets and Weibel-Palade bodies in endothelial cells). Upon stimulation by thrombin, histamine, hydrogen peroxide, and other secretagogues, P-selectin is rapidly transported to the cell surface by fusion of granule membranes with the plasma membrane (see Table 157-2). Once mobilized, P-selectin usually remains on the cell surface for less than an hour. Lipopolysaccharide (LPS) and other inflammatory cytokines induce P-selectin expression in a manner similar to that of E-selectin. The kinetics for cytokine-induced P-selectin are similar to those occurring after cytokine induced E-selectin expression (see Figure 157-4). P-selectin mediates adhesion between leukocytes and platelets as well as leukocyte-endothelial adhesion (see Table 157-1).

The expression of E-selectin on endothelial cells requires the *de novo* synthesis of both mRNA and protein. Many stimuli—including the cytokines IL-1, TNF-α and lymphotoxin TNF-β, and LPS (see Table 157-2)—are capable of inducing the expression of E-selectin. It is also induced on dermal endothelial cells in delayed type hypersensitivity reactions and in chronically inflamed skin. Maximal expression of E-selectin peaks at 4 to 6 hours and then declines to near basal levels within 24 hours (see Figure 157-4).

Selectin Ligands

All three selectins bind sialylated and fucosylated oligosaccharides, of which sialyl Lewis^X (sLe^X) is the prototype (see Table 157-1).[2] Most but not all of the selectin ligand glycoproteins are mucinlike. Mucins are serine- and threonine-rich proteins, they are heavily O-glycosylated, extended, and rigid. The mucin P-selectin glycoprotein ligand-1 (PSGL-1) is expressed constitutively by most leukocytes (see Table 157-1). It is a disulfide-bonded homodimer with two 120-kd subunits containing functional sialylated O-glycans on its serine and threonine residues. It is modified with α2,3 linked sialic acid and α1,3-linked fucose. It is sulfated on 1 or more of 3 tyrosine residues on the N terminal region.[18,36] PSGL-1 recognizes P-selectin, E-selectin, and L-selectin.[18,36] L-selectin and P-selectin recognize the region containing sulfated tyrosines, and E-selectin recognizes the sialylated and fucosylated core-2 O-glycans. As is the case with L-selectin, PSGL-1 is localized to the tips of the surface folds of unstimulated neutrophils, providing a topographical advantage in the initial interaction of leukocytes with endothelial cells. As with integrin ligands, there are many different selectin ligands. CD44 is present on leukocytes and can function as an E-selectin ligand in vivo.

Lymphocyte L-selectin recognizes mucins synthesized by the high endothelial veins (HEV) of lymph nodes and Peyer's patches: glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), CD34, and MAdCAM-1 (see Table 157-1). GlyCAM-1 is expressed by the high endothelial venules of peripheral lymph nodes and mesenteric lymph nodes and is secreted. CD34 has a much broader tissue distribution with constitutive expression on endothelial cells in a diversity of blood vessels. GlyCAM-1 and CD34 along with podocalyxin and endomucin are known as peripheral node addressins (PNAds). No one molecule is absolutely required for L-selectin–dependent lymphocyte recruitment, thus there is probably overlapping L-selectin ligand function by all members of the PNAd group.[18,36]

LEUKOCYTE, PLATELET, AND ENDOTHELIAL ACTIVATION

Mobilization of leukocytes to sites of acute inflammation is a critical factor in successful host defense. Because activated leukocytes can damage host tissue, their localization and activation is tightly controlled. A number of factors have been identified that activate leukocytes or the cells to which they bind, resulting in increased cell adhesion molecule expression and/or chemotactic migratory response (see Table 157-2). Chemoattractants are important in integrin activation and in directing the migration of leukocytes. In chemotaxis, cells move in the direction of an increasing concentration gradient. For example, bacterial peptides N-formyl-methionyl-leucyl-phenylalanine (fMLP), anaphylatoxins produced by generation of complement (C5a), lipids such as LTB_4 and platelet activating factor (PAF) all elicit varying leukocyte populations. Leukocytes can sense a chemoattractant concentration difference of 1% across their diameter and therefore migrate in the direction of the attractant. However, the generation of these chemoattractants at sites of inflammation can be rapidly diluted and swept downstream by blood flow unless they are retained at the site of production. PAF is produced by activated endothelial cells when stimulated by histamine, thrombin, and leukotriene C_4. Although other cell types release PAF, endothelial cells express and retain PAF on their surface under the same conditions that P-selectin occurs.[38]

Chemokines are a group of small (8-14 kDa) molecules that regulate cell trafficking of all types of leukocytes. Chemokines interact with a subset of seven transmembrane spanning G-protein coupled receptors (GPCR) on the leukocyte surface. The first chemokines identified were those produced by many cell sources such as IL-8. The more recently identified are much more tissue and cell specific. Table 157-2 includes some of these chemokines. Chemokines are subdivided into two major subfamilies based upon the arrangement of the two N-terminal cysteine residues, CXC and CC, and depending on whether the first two cysteine residues have an amino acid between them (CXC) or are adjacent (CC). Two other groups—the CXC3 (fractalkine) and the XCL (lymphotactin)—exist. *L* in the name refers to the ligand

(i.e., chemokine), and the number is the gene that encodes it. IL-8, one of the first chemokines identified, is CXCL8, as it is in the CXC subfamily and is a ligand (L) encoded on gene 8. The receptors for chemokines are classified by *R*. There may be more than one receptor for a chemokine, for example for IL-8 (CXCL8) the receptors are CXCR1 and CXCR2. Chemokines can also share receptors, that is, CXCR1 also bind CXCL1 (Groα/MGSA-α), CXCL6 (GCP-2), and CXCL7 (NAP-2).[39,40]

Chemokines have a wide range of effects on cell types beyond the immune system, for example in the central nervous system and on the endothelial cells where they result in either angiogenic or angiostatic effects. Their function in the immune system is important in antigen presentation and development of the immune response. Chemokines are also critical for leukocyte activation and localization. They can induce changes in cell shape, release of intracellular enzymes, formation of bioactive lipids, the respiratory burst, and the activation of integrins and chemotactic migration.[14] Chemokines presented on the surface of endothelial cells can participate in the rapid activation of the leukocyte integrin assisting in the "breaking" mechanism of the rolling leukocyte.[15] Chemokines can be "presented" on the surface of endothelial cells by glycosaminoglycans and the Duffy antigen/receptor for chemokines (DARC).[41] In such a manner, chemokine-induced activation of leukocytes can occur.

As previously discussed, cell adhesion molecules may themselves generate biochemical signals within the cells in a process known as outside-in signaling. The cytoplasmic tails of most of the cell adhesion molecules are relatively short and do not appear to have any intrinsic enzymatic activity generally. Any signals that are generated therefore must occur through coupling of the cytoplasmic tail with cytoplasmic proteins that nucleate the formation of large protein complexes containing both cytoskeletal and catalytic signaling proteins (see Figure 157-2, *C*). For neutrophils, ligation of either Mac-1 or LFA-1 results in potentiation of the respiratory burst. TNF-induced degranulation and release of proteases is dependent on Mac-1. On lymphocytes, LFA-1 can cosignal along with antigen to yield an augmented T cell response in vitro, with increased calcium flux, increased cytokine production, and increased proliferation. One of the most exciting areas of integrin adhesion biology has been the observation that adhesion and transmigration of leukocytes in an integrin-dependent manner result in the expression of a large number of genes associated with the inflammatory response termed *immediate-early* (IE) response genes. Many of these genes code for cytokines such as IL-1, TNF, and IL-8.[42] Thus the integrins can not only modulate the response on the adherent cell, but also influence the inflammatory milieu by resulting in production of chemokines. Selectins can also activate leukocyte integrin function and production of oxidative radicals, as well as production of cytokines and chemokines.[18,36]

LEUKOCYTE-PLATELET-VESSEL WALL INTERACTIONS

Leukocyte Localization to Inflammatory Sites

Direct observations of microvascular beds have revealed a consistent pattern of leukocyte endothelial interactions. Leukocytes are seen rolling along the walls of small venules but not arteries.[43] Following introduction of inflammatory stimuli, the number of rolling leukocytes increases, and many leukocytes become firmly adherent to the endothelium and change shape from a spherical to a flattened configuration. Leukocyte-leukocyte and leukocyte-platelet aggregates are often seen. Adherent leukocytes then migrate through the endothelium and into the region between the endothelium and it basement membrane. After pausing briefly in this location, the leukocytes migrate into the surrounding tissue (Figure 157-5).[2,18]

Each member of the selectin family can support leukocyte rolling under conditions of flow. In inflammatory states, endothelial L-selectin ligands E- and P-selectin are up-regulated. Leukocyte L-selectin and PSGL-1 "tether" to their endothelial ligands, which helps capture the leukocyte from the free flowing stream and "slow it down," even under high shear. This occurs through the specific selectin ligand bond characteristics (described previously) (see Table 157-2).[36] Cells then "roll" at a constant shear rate. α4β1, constitutively present on all mononuclear leukocytes (also all mouse neutrophils and some human neutrophils under specific conditions) can also participate in "tethering" to VCAM, itself up-regulated on the inflamed endothelial cell surface.

Selectins are able to slow, but are insufficient to arrest rolling leukocytes. Leukocyte arrest requires the activation of the β2 integrins and α4β1, which then bind to their respective endothelial ligands (see Table 157-1). It requires several signals for the β2 integrins and α4β1 to transition from a low affinity to the transient high affinity state; at least one of these signals is that provided by selectin-selectin ligand interactions. It has been estimated that contact duration of more than 25 msec is needed for β2 integrin bonds to form, and this is provided by the selectin-selectin ligand interaction. Other activating signals, PAF and chemokines, derived in part from the endothelial surface itself, are required. Constitutive endothelial expression of ICAM-1 and ICAM-2 can function as ligands for LFA-1 and Mac-1. Even though high levels are found on resting endothelium, inducible expression of ICAM-1 remains crucial for leukocyte-endothelial interactions. VCAM-1 is present only on inflamed endothelia (see Figure 157-5).

After arrest, leukocytes have been observed in animal models to crawl along the luminal surface to arrive at a point of transendothelial migration. This crawling is dependent on both leukocyte LFA-1 and Mac-1 and the downstream signaling molecule Vav-1. In vivo, leukocytes using "mechanosensing" crawl perpendicular to the flow of blood to the point of transmigration.[44] Passage through the endothelium requires active participation of the leukocyte and the endothelium. The route of transendothelial migration can be between the endothelial cells by penetrating junctions that lie within the intercellular cleft (paracellular route) or across endothelial cell (transcellular route).[45] Although it is not clear what determines the route, it is clear that integrins, particularly β2 and α4 integrins depending on the leukocyte subset, are required. Adhesion molecules toward the apical surface, including E-selectin, VCAM-1, ICAM-1, and PECAM-1 (CD31) are required. CD99, JAM-A, and ICAM-2 located at the interendothelial junctions are also critical (see Figure 157-5).

Leukocytes must then migrate through the extracellular matrix for localization to inflammatory sites.[46] As outlined in Table 157-1, extracellular matrix ligands for integrins include fibrinonectin, laminin, collagen, entactin, tenascin, thrombospondin, and vitronectin. Although Mac-1 and αXβ2 are known to bind fibrinogen after activation, they are not critical for adhesion to and emigration through other extracellular matrix proteins. β2 Integrin-deficient– activated neutrophils adhere to fibronectin, vitronectin, and laminin through α5β1, αvβ3, and α6β1, respectively.[47] Migratory cells use adhesion receptors and proteolytic enzymes, such as the urokinase-urokinase receptor/plasmin system, to regulate the function of each for the other and migrate through the extracellular matrix.[46] Using peptides, which mimic extracellular proteins, Gonzalez and colleagues[48] demonstrated that "naive neutrophils" (i.e. those freshly isolated from whole blood) utilize Mac-1 and α4β1 for migration. In contrast, neutrophils that had migrated across IL-1β–stimulated endothelial cells utilized αvβ3, α5β1, α4β1, and α6β1, while Mac-1 contributed to the speed of migration. Thus transmigration results in a change in the neutrophil adhesion molecules which enhances migration on proteins found in the extracellular matrix.

Leukocyte Localization in Adaptive Immunity

With inflammation, lymphocytes, like neutrophils and monocytes, extravasate to the affected site. However, for the immune response the mechanism is different in lymphoid organs.

Lymphocytes exit the blood stream in the postcapillary venules at specialized postcapillary vascular sites called high endothelial venules (HEV). This is the major route for constitutive extravasation, and most recirculating lymphocytes selectively bind to the endothelial cells of the HEV and do not interact with quiescent vascular endothelium. HEV is found in all secondary lymphoid organs (except the spleen). In chronically inflamed nonlymphoid tissue, HEV-like vessels are observed and support lymphocyte recruitment. HEVs are found in peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN), Peyer's patches, and tonsils.

It is the controlled expression of adhesion molecules on the lymphocyte and the counter ligands on the specialized endothelial

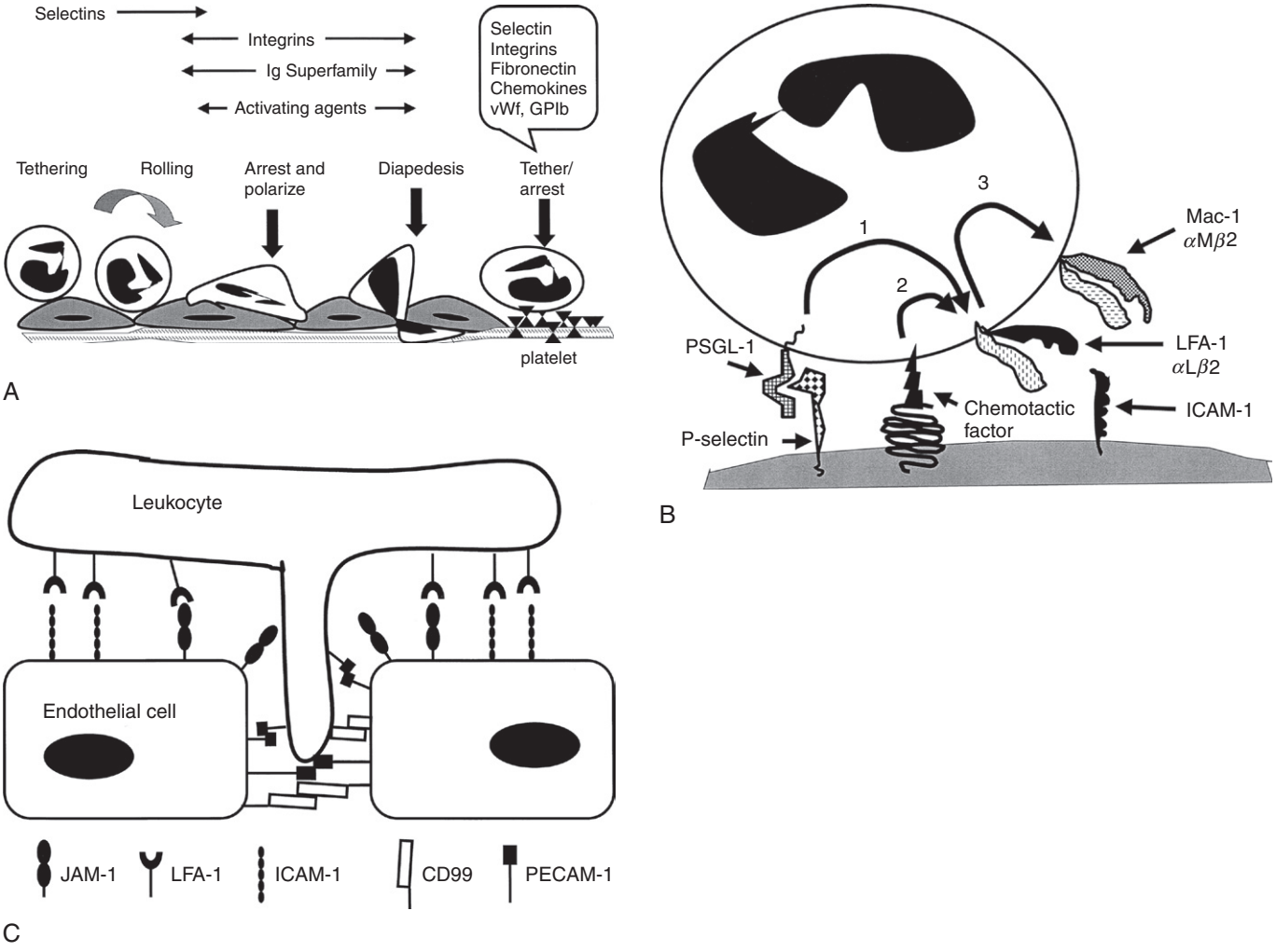


Figure 157-5 Leukocyte localization. A, Leukocytes are captured from the free-flowing stream (tether) and roll on the endothelial lining of the blood vessel. This interaction is mediated by all three members of the selectin family. The leukocyte slows, arrests, and changes shape (polarizes). Activating agents presented on the endothelial cells (or by activated platelets) are necessary for the transition from rolling to arrest. Integrins and their ligands, the Ig superfamily (IGSF), mediate these steps. The cells then "crawl" or diapedese over the surface of the endothelial surface until they migrate through the endothelium. The integrins, members of the IGSF, and CD99 have a role at this point. A leukocyte may also be tethered by adherent platelets *(as shown here)* or adherent leukocytes. Platelets through β3 integrins and GPIb- IX-V can bind directly to collagen or fibronectin on exposed basement membrane, or alternatively to von Willebrand factor bound to basement membrane. They can release chemokines, which can directly activate leukocytes once tethered. Leukocytes can bind to platelets directly through integrins or to fibrinogen which is bound to platelets. **B,** Model of leukocyte activation leading to arrest. Leukocytes tether to endothelial cells expressing P-selectin via P-selectin-glycoprotein ligand 1 (PSGL1). In the presence of shear, P-selectin–PSGL-1 interactions can result in leukocyte (and LFA-1) activation, *(arrow 1).* Chemotactic factors expressed at the endothelial surface can also result in activation of both Mac-1 and LFA-1. Finally LFA-1-ICAM-1 (intercellular adhesion molecule) interactions can augment the adhesivity of Mac-1. **C,** Proposed mechanisms for leukocyte transendothelial migration. With stimulation of the endothelial cell, ICAM-1 is up-regulated; JAM-1, which is localized at the interendothelial cleft, appears to be mobilized away from the cleft. With activation of the leukocytes (see Figure 157-5B) migration across the vascular endothelium can then occur via LFA-1, PECAM-1, JAM-1, and CD99. Leukocyte LFA-1 can bind to JAM-1 and ICAM-1 on the endothelial cell. Leukocytes may then traverse the interendothelial cleft through sequential transhomophilic interactions of PECAM-1 and CD99. Although CD99 is abundant on lymphocytes and monocytes, it does not appear to be so on neutrophils. *Ig,* Immunoglobulin; *JAM,* junctional adhesion molecule; *PECAM,* platelet endothelial adhesion molecule; *vWf,* von Willebrand factor. *(Part **B** from Smith CW: Possible steps involved in the transition to stationary adhesion of rolling neutrophils: a brief review.* Microcirculation *7:385-394, 2000. Part **C** from Aurrand-Lions M, Johnson-Leger C, Imhof BA: The last molecular fortress in leukocyte trans-endothelial migration,* Nature Immunology *3:116-118, 2002.)*

cells of the HEV of PLNs (draining the skin) and the lymphoid tissues of the mucosa (Peyer's patches, tonsil, and appendix) that ensures this selectivity of lymphocyte homing. Chemokines specific for lymphoid organs are required to activate the lymphocyte integrins for the transition from a rolling to adherent cell capable of transmigration. Although many chemokines specific for lymphocyte integrin activation are produced in inflammatory states, many serve a "housekeeping" function and are constitutively expressed at high levels in the thymus, lymph nodes, and certain lymphoid tissues.[30] L-selectin is present on virtually all lymphocytes and is required for homing to lymph nodes. Naive lymphocytes have abundant L-selectin, while specific activated or memory helper T cells lack L-selectin expression, but regain it when they transform into fully mature memory cells. L-selectin binds to ligands on PLN HEV via PNAd, as discussed above.

Cell-Blood Vessel Interactions in the Absence of the Cascade

There are many instances in which leukocyte and platelet recruitment to the vascular endothelium does not occur in the sequential manner described above. Neutrophils already localized to inflamed endothelium can recruit other neutrophils. In vascular beds where blood flow is diminished either by geometric constraints or by vascular occlusion/vasoconstriction, activation of the β2 integrins alone in the presence of a chemotactic gradient may be sufficient for neutrophil transmigration.[49] In the lung, leukocyte sequestration and transmigration appear to occur primarily in the capillaries. Leukocytes appear to "hop" through the capillaries rather than roll.[50] Neutrophils may spend considerable periods of time in stationary contact with capillary endothelium, and this predisposes to adhesive interactions between the neutrophil and the subjacent endothelium. In inflammatory states such as sepsis, the neutrophils become increasingly less deformable, resulting in increased retention. Though Mac-1 and LFA-1 are not required for the sequestration of neutrophils in the vascular bed of the lungs, they do mediate neutrophil migration. However nonβ2 integrin–dependent mechanisms are also present. The type of stimulus, the site of administration (lung versus peritoneum) or route (intravenous administration versus intratracheal) will affect the mechanisms required for lung recruitment. Leukocyte localization in the liver and kidney likewise is affected by geometric constraints and specialized endothelia.[51,52]

Activated platelets can adhere to endothelial cells, denuded vessel walls, and leukocytes. In response to vessel injury, vWF is exposed. Platelets, which are recruited from the free flowing blood, attach to vWF via the glycoprotein platelet receptor complex GPIbαβ-IX-V primarily through GPIbα. Platelets are recruited into the growing thrombi by release of activating substances from the platelets themselves. However release of P-selectin from the platelet α granules allows tethering under shear conditions to PSGL-1 on leukocytes. Leukocyte activation might then follow as a result of signaling through PSGL-1 or platelet-derived chemokines.[53] Firm adhesion of the leukocyte to the platelet involves leukocyte integrins, such as Mac-1, binding to platelet glycoprotein Ibα. Leukocyte-platelet aggregates are readily seen in human subjects in many inflammatory states. Selective platelet depletion in animal models affects leukocyte accumulation in various organs.[53] Leukocytes are critical to the development and maintenance of the hemostatic plug and thrombus as demonstrated by models in which inhibition of P-selectin function accelerates thrombolysis and prevents reocclusion.[54]

ADHESION MOLECULES AND THE DEVELOPING HOST

As outlined in the beginning of this chapter, several lines of investigation suggest that functional deficits in neonatal leukocyte adhesion increase the infectious susceptibility of the neonate. However, these functional deficits may play a physiologic role in limiting inflammatory responses that are possibly deleterious in immature hosts such as bronchopulmonary dysplasia (BPD). The remainder of this chapter will be devoted to the genetic defects of adhesion molecules, LAD I, LAD II, LAD III and Glanzmann's thrombasthenia (GT), which may present in the neonatal period, as well as our understanding to date of the neonatal developmental deficits of adhesion molecule expression and function.

TABLE 157-3

Leukocyte Adhesion Deficiencies

	LAD I*	LAD II	LAD III
Clinical Manifestation			
Recurrent severe infections	+++	+	+++
Neutrophilia			
Basal	+	+++	NL
With infection	+++	+++	+++
Gingivitis	++	++	?
Skin infections	++	+	+++
Delayed separation of the umbilical cord	+++	–	+
Developmental abnormalities	–	+++	–
Laboratory Findings			
β2integrins	↓, ↓↓, ↓↓	NL	NL
sLe^X expression	NL	Absent	NL
Neutrophil rolling	NL	↓↓↓	NL
Neutrophil adherence	↓↓↓	↓	↓↓,↓↓↓
Neutrophil emigration	↓↓↓	↓	↓↓↓
Opsonophagocytic activity	↓	NL	↓↓
Oxidative burst	↓↓↓	NL	↓
T + B cell function	NL	NL	↓↓↓
Platelet aggregation	NL	NL	↓↓↓
Gene defect	ITGB2	FUC1	FERMT3

*For LAD I, minimal (+), moderate (++), and severe (+++) decreases in β2-integrin expression have been described, leading to at least three phenotypes of the disease. *LAD,* Leukocyte adhesion deficiency; *NL,* normal. (See text for details.)

Genetic Deficiencies of Adhesion Molecules

Leukocyte Adhesion Deficiency I

Leukocyte adhesion deficiency is an autosomal recessive trait of humans, dogs, and cows characterized by recurrent, necrotic bacterial infections, impaired pus formation and wound healing, and abnormalities of many adhesion-dependent functions of granulocytes, monocytes, and lymphoid cells (Table 157-3).[55,56] With the targeted mutation of each of the β2 integrins in mice, we have found both similarities to and disparities with the syndrome as occurs in humans and other animals.[17,57] The affected gene, ITGB2, is located on 21q22.3, which encodes the common β-chain of the β2 integrin family. LAD I results from a decrease or absence of the cell surface expression or function of the entire β2 integrin family, which is, in turn, a result of heterogeneous mutations of the CD18 gene.[58] The α and β subunits are initially assembled in the cytoplasm of the cell, and only then is the complete β2 integrin heterodimer expressed at the membrane. Both subunits are required for expression. Given the diverse functional characteristics of β2 integrins it is not unexpected that patients with LAD I have profound deficits of acute inflammatory function and specific immunologic defects. Clinical and histopathologic features of LAD I are remarkably similar among human, canine, and bovine subjects. Recurrent necrotic and indolent infections of soft tissues primarily involving the skin, mucous membranes, and intestinal tract are the clinical hallmarks of this disease.[59,60] Superficial infections on body surfaces may invade locally or systemically. Typical small, erythematous nonpustular skin lesions often progress to large, well-demarcated ulcerative craters that

heal slowly or with a dysplastic scar. Septicemia progressing from omphalitis associated with delayed umbilical cord severance has been observed in infants from several affected families.[56,61] Perirectal abscess or cellulitis leading to peritonitis and/or septicemia has been reported in multiple patients, and facial or deep neck cellulitis has been observed to progress from ulcerative mucous membrane lesions of the oral cavity. Recurrent invasive candidal esophagitis, erosive gastritis, acute appendicitis, and necrotizing enterocolitis have been reported. Common respiratory infections include severe bacterial laryngotracheitis, recurrent pneumonitis, and sinusitis. Severe gingivitis and/or periodontitis are major features among all patients who survive infancy.

The recurrent infections observed in affected patients reflect the profound impairment of leukocyte mobilization into extravascular inflammatory sites. Skin windows and biopsies of infected tissues demonstrate inflammatory infiltrates devoid of neutrophils. This histopathologic feature is particularly striking considering that marked peripheral granulocytosis (fivefold to twentyfold higher than normal) during episodes of infection is a common finding. Two LAD I phenotypes were originally described. Patients with severe deficiency had essentially undetectable expression of all three complexes on their neutrophils. These patients either died in infancy or demonstrated a susceptibility to severe, life-threatening systemic infections. In contrast, among the six patients with moderate deficiency, life-threatening infections have been infrequently observed. Patients with moderate deficiency expressed 2.5% to 6% of all three β2 complexes. Patients with moderate disease may die in their second to fourth decade of life. In some moderately affected patients skin lesions may disappear after the first few years of life, recurring only with occasional infections.[56,59] Severe gingivitis is always observed in these patients and may be the presenting symptom.[62]

Abnormalities of neutrophil or monocyte antibody-dependent cellular cytotoxicity have also been observed in individuals with LAD I.[63] Most patients have normal and self-limiting courses of varicella and other viral infections. The predominance of recurrent bacterial infections in patients with LAD I implies that the functions of neutrophils and monocytes are more profoundly affected than those of lymphocytes. However T lymphocyte–mediated killing, proliferative responses, natural killing, and antibody-dependent killing by patient's lymphocytes are deficient compared to adult controls. Nonetheless delayed cutaneous hypersensitivity reactions are normal in most patients tested, and most individuals demonstrate normal specific antibody synthesis.[56,59]

The diagnosis of severe LAD I is suspected in the newborn or within the first few months of life when the infant displays extreme prolonged neutrophilia associated with recurrent severe infections (Table 157-3). Delayed separation of the umbilical cord occurs more frequently in patients with the severe phenotype, but is not universally found.[61] Delay in cord separation alone does not indicate a child has LAD I. The mean age of umbilical cord separation in normal neonates is reported to be 15.0 ± 7.2 days, standard deviation, with the range of 3 to 67 days.[64] In patients with the severe form of LAD I, prolonged cord retention was associated with omphalitis and extreme neutrophilia. The diagnosis of LAD I is made by immunophenotyping leukocytes using flow cytometry techniques. With immunophenotyping in classic LAD I there will be a few or no β2 integrins on the neutrophil or lymphocyte surface. The patients who were described as having "moderate" LAD I by clinical course had 2.5% to 6% of Mac-1, LFA-1, and p150,95 compared to normal adults.[64] Infants with this immunophenotype can still have clinically severe disease and succumb to overwhelming infections within the first year of life.[60] Treatment at present for patients with LAD I is limited to surveillance for recurrent infections, prompt institution of antibiotic therapy, and, when indicated, granulocyte transfusions.[65] Bone marrow transplantation has been advocated for these patients. The experience of the European Group for Bone Marrow Transplantation is that LAD I patients have good outcomes after bone marrow transplantation though graft versus host disease is a frequent complication.[66] The introduction of a normal β2 subunit gene (ITGB2) into hematopoietic stem cells has the potential to cure children with LAD I. Successful gene therapy has been reported in canine model of LAD I, implying that this procedure may be beneficial also in humans in the future.[67]

Glanzmann's Thrombasthenia

Glanzmann's thrombasthenia is a relatively rare autosomal recessive disease that is characterized clinically by purpura, epistaxis, gingival hemorrhage, and bleeding resulting from qualitative or quantitative defects of platelet αIIbβ3.[68] The diagnosis is virtually assured when there is absence of platelet aggregation in response to all agonists. Platelet αIIbβ3 binds fibrinogen, and, under shear, vWF. In GT, platelets attach to exposed subendothelium but do not spread, a process dependent on αIIbβ3. Formation of platelet-fibrinogen and platelet-vWF bridges do not occur, and clot retraction is often defective. In addition, prothombin, which can bind directly to platelets, is unable to do so in patients with GT and significantly less thrombin is generated in response to tissue factor. As with LAD I, large series of mutations have been described. However, unlike LAD I in which mutations occur primarily in the β subunit, in GT, mutations have been described in genes encoding either the αIIb or β3 gene. Classic GT occurs when there is insufficient or absent expression of αIIbβ3 on the cell surface. Variant GT occurs when there is quantitatively normal expression of αIIbβ3, but there are functional defects present. As such, patient presentation is heterogeneous. Bleeding difficulties range from severe to mild. Patients with either type of GT may be relatively asymptomatic, though they may have profuse bleeding after minor operative or dental procedures. Alternatively they may present in the newborn period and require frequent blood transfusions.

As many of the integrins share common pathways for activation, it is not surprising that individuals are now being described that have defects in β1, β2, and β3. These individuals have normal or near normal expression of cell surface molecules, but have features of both GT and LAD I (see following discussion regarding LAD III).[69]

Leukocyte Adhesion Deficiency II

An immune deficiency resembling LAD I was first described in 1992. In this syndrome there is a defect in the gene Slc35c1, which leads to the congenital disorder of glycosylation IIc (CDG2c), or CDG IIc. It is characterized by recurrent infections, failure to form pus, gingivitis, and pronounced neutrophilia (see Table 157-3).[70,71] Although the severity of the infectious complications resembles that of the moderate type of LAD I, the patients express normal levels of the β2 integrins and L-selectin. Additionally, these patients exhibit mental and growth retardation and the rare Bombay blood phenotype. LAD II is caused by a congenital defect of endogenous fucose metabolism. These patients demonstrate markedly decreased levels of sLe^x on leukocytes and other fucose-containing structures. Neutrophils from these patients bind minimally or not at all to E-selectin and P-selectin though β2 integrin function is normal. In vivo they have a marked decrease in migration into skin chambers or skin windows.[72] Using fluorescein-labeled cells from LAD I and LAD II patients in intravital microscopy (mouse mesenteric preparation after stimulation with TNF) the following observations were made: (1) LAD I neutrophils demonstrated normal rolling, but were unable to stick and emigrate with chemotactic factor stimulation; and (2) neutrophils from LAD II patients rolled very poorly and failed to stick and emigrate under the shear forces provided by flow. However, once flow was reduced the cells adhered and emigrated in response to LTB_4.[72] Similar to the studies of mice that are deficient in P-selectin and E-selectin, these studies clearly demonstrate the significance in humans of selectin-carbohydrate

ligand interactions in initiating low affinity adhesion manifested by rolling under conditions of flow.[73] When these interactions are not possible, leukocyte emigration cannot occur. Initial studies in infants suggest that high doses of oral fucose supplementation may mitigate some of the clinical effects.[74]

Leukocyte Adhesion Deficiency III

There are an increasing number of patients with an immune deficiency resembling LAD I with abnormal clotting, that is, like GT. These individuals have "normal" expression of Mac-1, LFA-1, α4β1, and αIIbβ3 integrins on the cell surface, but these integrins do not function properly. Although leukocytes from patients with LAD III rolling on endothelial surfaces were normal, these cells failed to arrest on endothelial integrin ligands in response to endothelial displayed chemokines. Their platelets also failed to aggregate, thus these individuals have a defect in β1, β2, and β3 function.[75-78] Studies confirm that this defect arises from an inability of the cell to "signal" integrin activation by chemokine activation, though other functions of the chemokine G-coupled protein receptor (GCPR) appeared to be normal.[79] Although initial work suggested that downstream mediators of GCPR, such as RAP-1, may be affected, recent work supports that Kindlin-3 is defective in most if not all of the kindreds.[80] Kindlin-3 binds the cytoplasmic tails of β1, β2, and β3 integrins at the same protein motif (NPXY) in all three. The evidence suggests that the binding of Kindlin-3 enhances talin binding at the membrane-proximal NPXY site, which causes an increase in integrin affinity. It appears that Kindlin-3 is essential for the generation and/or maintenance of integrin activity on human leukocytes and platelets and that this crucial step is faulty in individuals with LAD III.

Neonatal Integrin Expression and Function

β2 Integrin Function

It has been known for several decades that neutrophils from cord blood do not adhere to matrix proteins and migrate to the same extent as adult neutrophils in response to a chemotactic stimulus.[6,81-83] With the identification of the function of the β2 integrins and description of LAD I, a molecular basis for diminished neonatal neutrophil adhesion and motility was first detailed.[56,59,84] As we have outlined in previous sections, low levels of Mac-1 are present on the adult neutrophil cell surface. During cell activation, outside-in signaling activates Mac-1. This activated Mac-1 engages its ligand(s) and then translocates to the tail (uropod) of the cell. New Mac-1 is released from secretory and secondary granules, which may then be activated, and locomotion continues.[85] Several investigators have demonstrated that neonatal neutrophils when stimulated in vitro do not upregulate Mac-1 to the same extent as adult cells.[7,8,25,84,86] This is likely caused by diminished total cell content of preformed Mac-1, which is approximately 60% of that found in adult cells.[12] In addition, it appears that translocation of receptors to the uropod is also diminished.[82] Delays in chemotaxis appear to reverse by 1 month of age.[87] Stimulated Mac-1 expression remains depressed until about 11 to 12 months of age.[88] That Mac-1 expression on stimulated neonatal neutrophils is diminished compared to adult cells is controversial as others have not found this to be so.[89,90] It is unclear as to why these discrepancies exist though possibilities include large variance in expression for the neonatal population, preparation methods, and potentially larger proportion of "immature cells." In premature infants, Mac-1 expression is also diminished compared to adults, which also appears to correlate with their ability to undergo adhesion and chemotaxis.[8] It appears that the more "premature" the infant, the lower the levels of Mac-1.[91,92] This may be the result of even more marked decreases in the premature total cell content of Mac-1 compared with that in term neonates (30% and 60% of adult values respectively).[93] Using assays of neutrophil adhesion and transmigration through endothelial monolayers, term neonatal cells display about 50% transmigration compared to adults, and this appears to be completely caused by LFA-1. The diminished transmigration can almost entirely be explained by reduced Mac-1 expression.[84] In the preterm neonate, LFA-1 expression (and total cell content) remains less than adult values before 35 weeks gestation.[93]

Complementing the in vitro studies using neonatal or infant leukocytes, delays in leukocyte localization have also been demonstrated consistently in neonatal animals.[94-98] Peritoneal leukocyte accumulation is delayed in neonatal animals in response to a variety of chemotactic agents and bacteria. Leukocyte extravasation into the lung is also delayed in young animals in response to various bacteria and their products and in response to mechanical injury or hyperoxia. Using a rabbit model, we demonstrated that many of the changes in leukocyte integrins observed in the developing rabbit appear to generally model what has been described in neonatal human studies.[99] One-day-old rabbit neutrophils have decreased surface expression of the β2 subunit compared to adult and juvenile rabbits (14 days old), and, with chemotactic factor stimulation, failed to up-regulate Mac-1 (CD11b subunit) to the same extent as adult neutrophils. In addition, neonatal rabbits have diminished neutrophil accumulation in the sterilely inflamed peritoneum compared to adult or juveniles, and their extravasated neutrophils have diminished Mac-1 expression. β2 Integrin–dependent neutrophil accumulation in this model was caused by LFA-1, as an antiMac-1 monoclonal antibody had no effect on blocking leukocyte emigration.[100]

At least in adult humans and in mice, there are distinct roles for Mac-1 and LFA-1 for leukocyte localization in the host response.[101,102] In humans neutrophils, under conditions in which neutrophils can aggregate with cells expressing ICAM-1 under shear conditions, LFA-1 but not Mac-1, mediated the initial interaction as a result of shear alone. Chemotactic factors boosted LFA-1–dependent aggregation fourfold. Under the same conditions, Mac-1–dependent aggregation was only one third as efficient. However, Mac-1 was necessary for aggregate stability particularly at increased shear rates.[102] In assays in which human neutrophils transition from rolling to arrested cells, Mac-1 and LFA each may support the transition independently.[101] Mice deficient in LFA-1 or Mac-1 can use the alternative mechanism to "slow down" the rolling leukocytes, although LFA-1 appears to have a greater role in the transition to firm arrest.[103] Using intravital microscopy, we directly examined leukocyte recruitment in rabbit pup mesenteric vessels 6 hours after intraperitoneal injection of IL-1β.[104] Neonatal animals (≤1 week old) have diminished leukocyte arrest and emigration compared to adult animals.[104] Four-week old pups demonstrated diminished arrest of leukocytes compared to adult animals, though leukocyte emigration was not different. Using antiLFA-1 and antiMac-1 antibodies we demonstrated that in rabbits: (1) LFA-1 in all age animals was primarily responsible for leukocyte arrest and subsequent emigration; (2) Mac-1 dependent arrest and emigration was not present in neonatal animals, and accounted for about 30% of leukocyte arrest and adhesion in adult animals; and (3) leukocyte arrest in 4-week-old animals was not dependent on Mac-1 though Mac-1 accounted for 30% of transmigration.[105] Thus mechanisms for "slowing," "arrest," and subsequent emigration may change with maturation of the host.

The adhesion molecules involved with leukocyte mobility are dependent upon the organ and inflammatory stimulus used in adult animals. β2 Integrins do not appear to be necessary for leukocyte accumulation in the lungs of mice and rabbits infected with streptococcal pneumoniae.[106] This appears to be true also for neonatal animals. Term and preterm neonatal rabbits had increased alveolar neutrophils after infection with group B *Streptococcus*.[107] Anti-β2 monoclonal antibodies (mAbs) failed to inhibit neutrophil influx, though they did inhibit killing of the *Streptococcus*.[107] The age-dependent differences in neutrophil migration appear to be species dependent; neonatal bovine

neutrophils had levels of Mac-1, LFA-1, and p150,95 equal to that of adult bovine neutrophils. There were no delays in adhesion to or migration through stimulated bovine endothelial monolayers.[108]

The β2 integrins modulate a number of other events including phagocytosis of iC3b-coated particles, neutrophil homotypic aggregation, and adhesion-dependent oxidative burst. Phagocytosis of iC3b-coated particles was not different between term or premature neutrophils compared to adults, and the degree of phagocytosis could not be correlated with Mac-1 levels.[109] Neonatal monocyte phagocytosis of iC3b-opsonized *Candida* or unopsonized *Candida* (in which ingestion occurs via Mac-1) was equal to that of adult monocytes.[110] Thus it appears that the neonatal neutrophils utilize LFA-1 for adhesion and emigration. Decreased Mac-1 function and/or amount appears to contribute to the deficits in neonatal neutrophil motility. However, other Mac-1 functions may be intact.

β1 Integrin Function

As described above, neutrophil β1 and β3 integrin expression and function are affected by transendothelial migration. Neutrophil migration in the extracellular matrix is dependent on both β1 and β3 integrin function. Neonatal neutrophils at baseline and after transendothelial migration have equivalent amounts of α4β1, α5β1, α6β1, and β3 compared to their adult counterparts.[111] Using peptide mimetics, in which we could dissect the contribution of each integrin to neutrophil motility, we demonstrated that neonatal neutrophil β1 and β3 was equivalent to that of adult neutrophils, though β2 function remained diminished.[48,111] Thus it is possible that once neonatal neutrophils traverse the endothelial barrier, their motile function is equal to that of adults.

Using flow cytometry, it has been shown that in healthy 1- to 3-day-old infants there are elevated proportions of total T cells (CD3+), helper T cells (CD3/CD4+) with decreased proportions of CD3/CD8+, and NK cells in the blood compared to adults.[112] The newborns have a higher proportion of immature B cells and CD8+ T cells that express the costimulatory molecules CD28 or CD38. Compared to adult blood cells, the neonatal blood contained a lower proportion of activated T cells.[112] Such studies and others support that neonatal lymphocytes are "naive" and prepared to "home" to secondary lymph organs for priming for an immune response, but do not respond to sites of infection. Those CD4+ cells that are CD45RO+ (memory) have concomitantly increased expression of β1, α5, LFA-1, and CD2, but not necessarily α2, α4, and CD44 (another adhesion molecule).[113] Locomotion of T lymphocytes in extracellular matrices are caused both by the expression and function of cell adhesion markers, such as α4β1 and α2β1, and loss of L-selectin.[114] Although T cells from children have decreased β1 and α5 compared to adults, they have comparable levels of α4, (probably as α4β7).[115] In addition, child CD45RA+ lymphocytes are β1 integrin "high," while in adults β1high T cells are CD45R0+.[115] In contrast, lymphocytes from cord blood are uniform for β1 expression; the single peak is between the two peaks usually seen in adult cells.[115] Thus, for cord blood lymphocytes and those from very young infants, α4β1 integrin expression is present at moderate levels and can function as a ligand for VCAM-1.

As with neutrophils, lymphocyte chemotactic responses depend upon chemokine receptor expression. Cord blood lymphocytes in general have diminished chemotactic responses to CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CXCL9 (MIG), and CXCL10 (IP-10) compared to adult peripheral blood lymphocytes because they lack the respective chemokine receptor.[116] However, cord blood lymphocytes express both CCR7 and CXCR4, and as such they respond robustly to CCL19 (MIP-3β) and CXCL12 (SDF), respectively, in assays of directed migration. We have shown that cord blood lymphocytes can interact (tether and roll) on cytokine-stimulated endothelial cells to the same extent as adult peripheral blood cells, and this is dependent on α4β1.[117] In the presence of shear stress, cord blood and adult lymphocytes roll, arrest, and migrate across stimulated endothelial cells when the vascular endothelium bears apical CXCL12 stromal-derived factor (SDF).[118,119] Thus neonatal cord blood lymphocytes, though naive, maintain the capability to localize to an inflammatory response if the "signals" for localization are present in the microenvironment (i.e. VCAM-1 and chemotactic chemokines for which neonatal cells have receptors).

Neonatal Selectin Function

In assays where L-selectin is known to be operative for the initial tether of flowing neutrophils, neonatal cells are less able to interact with stimulated endothelial cells compared to adult cells.[120] Neonatal cells have diminished surface expression of L-selectin, and antiL-selectin monoclonal antibodies have minimal effect on neonatal cell rolling.[120] In contrast, in the presence of antiL-selectin antibodies, adult cell rolling is decreased to levels comparable to neonatal cells.[120] Thus neonatal cells have a relative L-selectin deficiency. Diminished neutrophil L-selectin has been confirmed in other studies of neutrophils and eosinophils from term and preterm infants.[86,121] In contrast, neonatal monocytes and fetal neutrophils and eosinophils demonstrate "adult" levels of L-selectin.[86,122] As L-selectin can be shed from the cell surface, it remains unclear whether the decrease in neutrophil surface L-selectin in the term and preterm neonate is the result of activation in utero or whether this was caused by maturational changes in neutrophil content of L-selectin. Though diminished total cellular L-selectin is found in cord neutrophils, soluble L-selectin (that which is cleaved from the cell surface) is also decreased in the serum compared to adult.[123] Additionally cord neutrophils demonstrated diminished down-regulation of L-selectin compared to adult neutrophils with chemotactic peptides and granulocyte-macrophage colony stimulating factor (GM-CSF), though the response to IL-8 was equal to that of adult neutrophils.[124]

Neutrophils from 1-day-old rabbit pups have approximately 60% of adult rabbit levels of L-selectin, and, similar to human neonatal neutrophils, failed to show a significant decrease after chemotactic factor stimulation.[99] Neutrophil L-selectin expression remained depressed until about 14 days of age when it was equal to that of adult rabbits. Systemic administration of an antiL-selectin mAb to rabbits with sterile peritonitis resulted in a significant decrease in peritoneal neutrophil accumulation both in adult and 14-day-old animals. In contrast there was no effect in the 1-day-old animals.[99] We examined L-selectin–dependent leukocyte localization in the mesenteric vessels of rabbits in response to IL-1β using intravital microscopy.[104] Inhibition of L-selectin had greater effects on arrest and emigration of leukocytes in adult animals than neonatal animals especially at those stages in which L-selectin serves as a signaling molecule for integrin activation. Inhibition of L-selectin also had less of an effect on leukocyte rolling in neonatal than in adult rabbits.

Human neonatal neutrophils appear to have diminished amounts and function of PSGL-1. Neonatal neutrophils demonstrate decreased rolling and arrest compared to adult neutrophils on either endothelial cells or transfected cell lines expressing E&P selection.[125-128] Neonatal neutrophils also have decreased interaction with platelet monolayers, and this appears to be caused by diminished tether duration of neonatal cell PSGL-1 to platelet P-selectin. As discussed, selectin-ligand interactions have a rapid "on" rate and much longer "off" rate, allowing cells to attach quickly and then remain attached for longer periods thus facilitating the transition to β2 integrin mediated arrest. At least for P-selectin, the "off" rate is much shorter for neonatal cells, thereby requiring these cells to arrest in a shorter period of time than that required by adult cells. As β2 integrin function is depressed in neonatal cells, this puts the neonatal cells at an even greater disadvantage to transition to arrest.

At present there is little understanding of the developmental differences in adhesion molecule expression at the level of the endothelium and other nonleukocytic cells. Human neonatal platelets have decreased up-regulation of P-selectin.[129] However, neonates have increased amounts of larger multimerized vWF, and as such cord blood platelet deposition on subendothelium may be equal to or exceed that of adult blood.[130] In the context of the developmental biology of endothelial cell adhesion molecules it is critical to highlight that much of the in vitro work has been performed on human umbilical vein endothelial cells (HUVEC). However, endothelial cells from umbilical veins in culture may not reflect what occurs in vivo in either the neonate or mature host.The use of cultured human foreskin provides some insight as to the relevance of these molecules in the human neonate, but there is no comparative data with adult tissue samples. Cultured foreskin postcapillary venular endothelium up-regulated ICAM-1 and E-selectin in response to cytokines and growth factors, and the effect was greater than predicted with isolated HUVEC assays.[131] Animal models have also been useful, but again there is uncertainty in extrapolating animal (particularly rodent) findings to humans. In neonatal rats subjected to sterile peritonitis, leukocyte accumulation is diminished compared to adult animals.[13] However this did not appear to be caused by decreased leukocyte responsiveness, as adult cells injected into neonatal animals also demonstrated decreased localization. Furthermore there was diminished P-selectin expression on neonatal mesenteric vessels compared to adult animals.[13] Inflamed endothelial cells cultured from midgestation fetal pigs had diminished P-selectin expression and decreased leukocyte localization compared to cells obtained from adult pigs.[132] Fetal mice that were deficient in P-selectin had diminished leukocyte influx in excisional wounds compared to their wild type controls.[133] That such differences in P-selectin expression in human neonates may also be operative are based upon the following observations: (1) P-selectin expression is diminished on the surface of endothelial cells from human newborn mesentery and expression increases with age. PECAM-1 and ICAM-1 staining was not affected by age; (2) endothelial cell stores of P-selectin and vWF increase with gestational age in humans; and (3) there is diminished mRNA in endothelial cells from premature neonates, and decreased P-selectin in endothelial cells from premature neonates is associated with fewer Weibel-Palade bodies.[13] An examination of healthy term and preterm infants demonstrated no polymorphisms in E- or P-selectin that had an effect on sepsis, development of BPD, or early birth.[134] Carriers of the 213Ser L-selectin allele were found to be more prevalent in low-birth-weight infants, particularly in those with BPD.There was no association between the L-selectin polymorphism and early postnatal sepsis.

Adhesion Molecules in Diagnosis and Therapy

Adhesion Molecules as Markers of Inflammation

In 1985 Arnaout[135] described eight patients who developed neutropenia while undergoing dialysis. The neutropenia was associated with increased surface expression of Mac-1 on circulating neutrophils. Because this first description there has been an explosion of studies quantifying the expression of adhesion molecules either on leukocytes or circulating in the sera as indication of leukocyte activation. An increase in neutrophil Mac-1 has been documented in patients undergoing cardiopulmonary bypass, with sepsis, and at early time points in the adult respiratory distress syndrome (ARDS).

Term infants with early-onset sepsis or suspected sepsis have increased Mac-1 expression on circulating neutrophils compared to those who do not.[92,136] One study did not demonstrate an increased surface expression of Mac-1 in septic premature neonates though other markers of leukocyte activation (CD33 and CD66b) were present.[137] However when premature infants were followed serially, and Mac-1 was determined daily for 3 to 4 weeks, those infants who subsequently developed sepsis had an increase in leukocyte Mac-1 compared with those who did not.[137] It also appears that for preterm infants with respiratory distress syndrome, neutrophil Mac-1 was elevated compared to cord blood from term infants and from preterm infants not requiring mechanical ventilation.[92,138] As has occurred in adult patients, the onset of ventilation in premature infants induced Mac-1 expression on circulating leukocytes.[139] Thus it appears that leukocytes from term and preterm infants with sepsis and those with respiratory distress syndrome have evidence of neutrophil activation, at least as demonstrated by Mac-1 expression. Furthermore the "act" of mechanical ventilation appears to activate neutrophils in premature infants, as demonstrated by increased Mac-1.

L-selectin is shed from the leukocyte cell surface with cell activation and is thought to occur as the leukocyte transitions from rolling to arrest. Neutrophils deficient in L-selectin can not localize to an inflammatory site.[140] L-selectin shedding may be a mechanism to release a slowly rolling, activated cell and inhibit it from interacting at a site distant from the original focus. L-selectin expression has been shown to be decreased in neutrophils and monocytes from the cord blood of infants with acute bacterial infection and in adult patients with surgical sepsis.[141,142] However L-selectin expression decreases in the neutrophils as they are released from the bone marrow and as they age.[143] In addition, inflammatory mediators such as glucocorticoids, IL-6, and granulocyte colony-stimulating factor (G-CSF) all result in decreased L-selectin on circulating cells, likely as a result of shedding of L-selectin during marrow release.[144] As outlined earlier, L-selectin is depressed on circulating newborn neutrophils and does not demonstrate further decrement with cell activation. Although soluble L-selectin is found in the plasma, its significance is unclear though it is present at levels in humans and rodents that can potentially inhibit L-selectin-dependent leukocyte function.[145]

With the observation that there are soluble forms of ICAM-1, VCAM-1, L-, E-, and P-selectin in normal human sera and/or in sera from patients with inflammation, much investigation has explored whether soluble adhesion molecules could be useful monitors of disease activity.What is unclear is whether determining the absolute level of soluble circulating adhesion molecules will have the necessary specificity and sensitivity required to predict disease severity or outcome. In term, infection-free newborns, plasma sL-selectin levels are diminished while sE-selectin levels are increased on the second and fifth day of life compared to adult values.[146] From day 2 to day 5, there is a decrease in plasma sE-selectin.The baseline level of sL-selectin, which is low compared to normal adults, remains unchanged. In a separate large cohort of infants, those neonates with definitive infection and those with possible infection had increased plasma levels of C-reactive protein (CRP), IL-6, sICAM-1, and sE-selectin compared to those neonates without infection.[147] However, using multiple logistic regression modeling, IL-6 in addition to CRP predicted sepsis, and sICAM-1 and sE-selectin did not contribute significantly to the prediction model. In a group of 25 neonates with clinical sepsis followed prospectively, only s-ICAM-1 appeared to correlate with severity of infection.[148] Neither sE-selectin, s-VCAM-1, nor sL-selectin changed during the infection, nor were they significantly different than that found at baseline in healthy newborns.[148]

In premature infants, plasma sL-selectin was not different between those with respiratory distress and those without respiratory distress.[138] However, bronchoalveolar fluid sL-selectin is increased in premature infants at risk for chronic lung disease compared to more mature infants, and those infants who developed chronic lung disease had persistently elevated bronchoalveolar lavage (BAL) sL-selectin.[149] Premature infants had increased plasma sP-selectin in cord blood on the first day of life, regardless of whether they developed chronic lung disease (CLD).[150] In contrast, plasma sE-selectin was increased in cord blood and in serum

samples on day of life 1 and 3 in those infants that developed CLD compared to those who did not.[150,151] Plasma sICAM-1 levels were also increased later in the first week and during the second week of life in those who developed CLD compared to those who do not.[150] Dexamethasone treatment increases the concentration of plasma sL-selectin and decreases sE-selectin.[144,152] Thus it appears that premature infants who develop CLD have evidence of increased inflammation in their early perinatal and neonatal course, as measured by sE-selectin, sL-selectin, and sICAM-1. It also appears that dexamethasone modulates the inflammatory response as measured by circulating adhesion molecules and those present on circulating cells. Platelet P-selectin and activated αIIbβ3, at least early in life, are elevated, most likely secondary to platelet activation at the time of birth.[153,154]

Modulation of Adhesion Molecules as Adjunctive Therapy

A new and exciting era began with the application of antiadhesive strategies to ameliorate tissue injury. Perhaps the most far-reaching has been the use of antiαIIbα3 (platelet glycoprotein IIb/IIIa) "chimeric" antibody in patients who have undergone coronary angioplasty for acute ischemic heart disease. Platelets play a large role in ischemic complications of coronary angioplasty, such as abrupt closure of the coronary vessel during or soon after the procedure. At 30 days and 6 months after coronary angioplasty, patients who received the antibody had markedly reduced rates of reinfarction or repeat urgent interventions compared to patients who did not receive the antibody.[155] The blockade of cell adhesion molecule function with specific murine antibodies is limited in the treatment of human disease because of generation of antimurine antibodies in the recipient. However the development of small molecular peptides that inhibit adhesion molecule function has revolutionized their use.[156] Antiadhesive therapy is used for Crohn's disease, psoriasis, multiple sclerosis, and other chronic inflammatory disorders. A large number of preclinical studies supported the concept that blocking endothelial-leukocyte interactions would be beneficial in models of ischemia/reperfusion injury such as stroke or myocardial infarction, traumatic shock, cardiopulmonary bypass, sepsis, and acute lung injury. However, longer clinical studies did not support these immunologic approaches.[157] Occasionally drugs are developed that appear to have an unexpected antiadhesive effect. Such has occurred with HMG-CoA reductase inhibitors like lovastatin. Some of these drugs "lock" LFA-1 into an inactive conformation close to the I domain, thereby inhibiting its ability to adhere to ICAM-1.[158] Thus, for these patients, inhibition of the inflammatory response in atherosclerosis may have beneficial effects in addition to the treatment of hypercholesterolemia.

The experiments in nature, the genetic deficiencies resulting in LAD I, LAD II, LAD III, and GT, should be a warning to the clinician scientist attempting to manipulate adhesion molecule function without discrimination. For those caring for the premature and term newborn, understanding adhesion molecule biology will be crucial in supporting immune function as well as attempting to modulate the effects of inflammatory-mediated injury such as BPD.

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Immunology of Human Milk and Host Immunity

In 1891, it was discovered that immunity could be transmitted through breast-feeding in experimental animals.[1] In the 1920s,[2-4] the incidence and severity of diarrheal diseases were found to be much lower in breast-fed than cow's milk–fed infants. Those clinical observations were confirmed repeatedly,[5-8] and it was ascertained that breast-feeding protected against many bacterial and viral enteric pathogens.[7-13] Three explanations for the protection were advanced. The first explanation: Because human milk is less contaminated with enteropathogens than formula feedings, breast-fed infants are exposed to fewer infectious agents. The second: Increased birth-spacing as a result of the contraceptive effects of lactation decreases the number of children who transmit common contagious agents to susceptible siblings.[14] The third: Breast-fed infants are rarely in group-care facilities and are thus less exposed to communicable infections.

These propositions did not, however, entirely explain the protection provided by breast-feeding, for breast-fed infants are asymptomatic even when they are exposed to bacterial enteropathogens such as *Shigella* that contaminate the mother's nipples, colostrum, and milk.[5] Further, breast-fed infants are more resistant to common respiratory infections.[15-18] Much of the protection is provided by a complex immunologic system in human milk. Furthermore, antimicrobial agents, which were the first parts of the immunological system to be recognized,[19-21] have certain shared features (Box 158-1). The inverse relationship between the quantities of many agents in human milk and the production of these agents by the infant suggested a relationship between the development of the infant's immune system and the ability of the lactating mammary gland to produce the immune factors.[22-24]

After the concept of an immune system in human milk was formed,[19] antiinflammatory[21,25,26] and immunomodulating agents[21,26] were discovered to be part of that system. Thereafter the evolutionary relationships between the immune system in human milk and the development of the immune system in the infant were appreciated.[22-24]

1. Certain postnatal developmental delays in the infant's immune system are compensated by the transmission of the same agents in human milk.
2. Other postnatal delays in components of the immune system in the infant are compensated by dissimilar agents in human milk.
3. Some agents in human milk initiate or augment functions poorly expressed in the infant.
4. Many antimicrobial agents in human milk act synergistically.
5. Some agents in human milk alter the physiological state of the alimentary tract from one suited for fetal life to one that is appropriate for extrauterine life.
6. Antibodies in human milk are produced by plasma cells that transformed from B cells that originate in the maternal intestines and bronchi.
7. Specialized living leukocytes are found in human milk.
8. Defense agents in human milk protect against microbial pathogens without provoking inflammation in the infant.
9. Some agents in human milk inhibit inflammation.
10. Some agents in human milk are immunoregulators.
11. Some agents in human milk are antineoplastic.
12. Defense agents in human milk resist enzymatic digestion and thus function in the recipient's GI tract.
13. Certain defense agents are created in the infant's GI tract by partial digestion of substrates in milk.
14. When defense agents in human milk interact with some pathogens, the infant develops specific adaptive immune responses but no symptomatic infections. Such a sheltered immunization is similar to immunizing with an attenuated microbial pathogen.
15. Agents in human milk augment the growth of commensal enteric bacteria adapted to infants that produce compounds that protect against bacterial pathogens and convey other immunologic benefits.[27,28]
16. There is often a reciprocal relationship between the defense agents that are transmitted in milk and those transmitted during fetal life via the placenta.

BOX 158-1

Features of Antimicrobial Agents in Human Milk

- Heterogeneous array of biochemical agents and live leukocytes
- Agents not well represented in other mammalian milks used to feed human infants
- Common to mucosal sites
- Adapted to persist in the gastrointestinal tract
- Often inhibit or kill microbial pathogens synergistically
- Often multifunctional
- Do not trigger inflammation
- Production often inversely related to the production in the infant

ANTIMICROBIAL FACTORS

The physical features, functions, and quantities of antimicrobial agents in human milk are summarized in Table 158-1. The proteins will first be considered.

Antibodies

IgM, IgG, IgD, and IgA are in human milk. IgM concentrations are much lower in human milk than in serum.[29] IgM molecules in blood and milk are pentamers. However, unlike serum IgM, human milk IgM is bound to secretory component.

The concentrations of IgG in human milk are lower than IgM in human milk and are much less than IgG in human serum.[29] All IgG subclasses are in human milk,[30] but the relative proportion of IgG4 is higher in human milk than serum.[30] Very little IgD is present in human milk.[31] IgE, the immunoglobulin responsible for immediate hypersensitivity, is essentially absent.[32] In contrast, IgA in the form of secretory IgA comprises more than 95% of human milk immunoglobulins.[29] Although some trimers and tetramers of IgA are in human milk, most secretory IgA consists of two identical IgA monomers united by a 15-kD polypeptide called the joining chain and complexed to a 75-kD glycoprotein, the secretory component.[33-35] Secretory IgA is assembled when dimeric IgA produced by plasma cells in the stroma of the mammary gland binds to the first domain of polymeric immunoglobulin

TABLE 158-1

Principal Antimicrobial Agents in Human Milk

Agents	Main Functions
Proteins and peptides	Microbiostatic and microbiocidal
Lactoferrin	Lyses siderophilic pathogens by chelating Fe^{+++}
Lysozyme	Lyses certain bacteria by degrading exposed cell wall peptidoglycans
SIgA	Binds adherence sites, toxins, virulence factors on intestinal and respiratory pathogens
α-Lactalbumin	Kills *Streptococcus pneumoniae*
CCL28	Kills *Candida albicans* and many gram-positive and gram-negative bacteria
MUC1	Blocks binding of S-fimbriated *Escherichia coli* to epithelium
Lactadherin	Blocks attachment of rotavirus to mucosa
C3 and fibronectin	Augment phagocytosis of pathogens
β-Defensin-1 and α-Defensin-1,2, and 3	Lyses bacteria and inhibits HIV-1, respectively
Oligosaccharides	Receptor analogues inhibit binding to epithelium
Glycoconjugates	
GM_1 gangliosides	*Vibrio cholerae* and *E. coli*
Globotriaosylceramide Gb3	Shigatoxin β subunits
Fucosyl oligosaccharides	*E. coli* stable toxin, *Campylobacter jejuni*
G1cNAc(β1-3) Gal-disaccharide	*Streptococcus pneumoniae*
Sulfated glycolipids	HIV-1
Glycoaminoglycans	HIV-1
Lewis X component	HIV-1
Monoglycerides and fatty acids from digested milk lipids	Inactivate enveloped viruses, certain bacteria, *Giardia lamblia* and *Entameoba histolytica*

TABLE 158-2

Antigen-binding Repertoire of SIgA Antibodies in Human Milk

Bacteria and Their Products	Viruses	Fungi	Parasites	Autoantigens
Escherichia coli	Adenoviruses	*Candida* sp	*Giardia lamblia*	DNA
Helicobacter pylori	Cytomegalovirus			RNA
Clostridium botulinum	Polioviruses and other enteroviruses			CCR5
Klebsiella pneumoniae	Rotaviruses			
Campylobacter sp	RSV			
Shigella sp				
Salmonella sp				
Vibrio cholerae				
Streptococcus pneumoniae				
Group B *Streptococcus*				
Haemophilus influenzae				

receptors on the basolateral surface of epithelial cells.[35] The complex is internalized, the original cytoplasmic part of the receptor is cleaved off, and the remaining assembled protein is transported across the cell into milk.

Secretory IgA antibodies in human milk are directed principally against enteric and respiratory pathogens (Table 158-2). The precursors of the cells that generate those antibodies originated at those mucosal sites. In fact, those precursors are released from the maternal mucosal sites because of immunogen-triggered events.[36] Hormonal stimulation during lactation causes antigen-stimulated B cells from Peyer's patches of the lower small intestinal tract to switch from the IgM to the IgA isotype and migrate to the mammary gland.[37,38] A similar B-cell pathway links bronchial lymphoid tissues to the mammary gland.[39] The details are as follows.

After antigenic stimulation, cytokines from mononuclear leukocytes in Peyer's patches induce local B cells to switch from IgM to IgA.[40,41] The IgA^+ B cells then migrate sequentially into local intestinal lymphatic channels and lymph nodes, the cisterna chyli, the thoracic duct, and the vascular circulation. During lactation, they home to the stroma of the mammary gland. Experimental evidence suggests that the chemokine CCL28 and its receptor CCR10 are crucial to this process: (1) CCL28 is up-regulated in the murine mammary gland epithelium during lactation;[42] (2) most IgA^+ B cells in the enteromammary gland pathway display CCR10;[43] and (3) IgA^+ B cells that are $CCR10^+$ move toward CCL28, which is expressed by mammary gland epithelium during lactation.[43]

IgA^+ B cells in the mammary gland differentiate into IgA-producing plasma cells that remain in the lamina propria of the organ. IgA dimers produced by mammary gland plasma cells principally contain λ-light chains, whereas κ-light chains predominate in serum immunoglobulins.[44] IgA dimers released from those plasma cells bind to polymeric immunoglobulin receptors on the basolateral external membranes of mammary gland epithelial cells.[33,34,45,46] The resultant receptor-dimeric IgA complex is transported to the apical side of the cell where the intracytoplasmic portion of the receptor is removed. The remaining molecule, secretory IgA, is secreted into milk. Thus enteromammary and bronchomammary pathways protect the immunologically immature infant against pathogens that the mother encounters (see Table 158-2). This is important since secretory IgA antibodies and the antigen-binding repertoire of immunoglobulins are developmentally delayed during early infancy.[47] Also, some secretory IgA molecules in human milk are antiidiotypic antibodies that function as immunogens.[48]

Certain secretory IgA antibodies in human milk are directed against the autoantigen CCR5, which is not only a chemokine receptor but is also the co-receptor for R5-tropic strains of HIV-1 that permits macrophages and immature dendritic cells to become infected with HIV-1.[49] The antibodies from uninfected as well as HIV-1 infected women bind to the second extracellular loop of CCR5 and thus block infection of macrophages and dendritic cells by HIV-1. In addition, secretory IgA antibodies to DNA enzymatically cleave DNA.[50,51] Therefore the DNA is recognized as an autoantigen and a substrate by the antibody enzyme and may hydrolyze free nucleic acids in the recipient's intestinal and respiratory tracts.

Secretory IgA in human milk also facilitates mucosal immunization against enteric microorganisms.[52] Secretory IgA adheres selectively to cells in Peyer's patches. Subsequently, the antibodies are transported to the underlying lymphoid tissue. There they bind to and are internalized by dendritic cells in the subepithelial dome region of Peyer's patches. If foreign enteric protein antigens are coupled to these antibodies, they will be processed by dendritic cells and then presented to T cells. Once the processed antigens are presented to TcRs on helper T cells, the T cells are activated. The activated T cells secrete cytokines that activate local cytotoxic T cells or stimulate local IgM^+ B cells to proliferate and switch their isotype to IgA. That eventually leads to the production of secretory IgA at mucosal sites.

The quantity of secretory IgA in human milk declines as lactation proceeds, but considerable secretory IgA is transmitted to the infant throughout breast-feeding.[54-57] Concentrations of secretory IgA in human milk are highest in colostrum[50] and gradually plateau later in lactation to about 1 mg/mL.[56] The approximate mean daily intake of secretory IgA in healthy full-term

breast-fed infants is 125 mg/kg/day at 1 month and 75 mg/kg/day by 4 months.[57]

The pattern of immunoglobulins in human milk differs from the pattern in other mammals except closely related primates.[25] For example, the dominant immunoglobulin in bovine colostrum, IgG, is absorbed into the calf's blood. IgG production is developmentally delayed in those newborns. Without colostrum, they remain IgG-deficient and very susceptible to intestinal infections.

Secretory IgA resists intestinal proteases such as pancreatic trypsin.[58] Bacterial proteases attack the hinge region of IgA1,[59] but the second subclass, IgA2, is resistant to those proteases and is disproportionally increased in human milk.[30] Furthermore, secretory IgA antibodies against bacterial IgA proteases are present in human milk.[59] In that respect, the amount of secretory IgA excreted in stools of low birth weight infants fed human milk is about 30 times that in infants fed a cow's milk formula.[60] In addition, urinary excretion of secretory IgA rises in infants fed human milk.[61,62] It is unlikely that the antibodies are from human milk because there is no mechanism for the their transport from the gastrointestinal tract to the blood. Thus human milk may stimulate the infant to produce the antibodies and transport them into the urinary tract.

Free Secretory Component

Free secretory component is also secreted into human milk, and this peptide inhibits the adherence of certain enterobacteria to epithelial cells.[63,64]

Lactoferrin

Lactoferrin is a single-chain glycoprotein with two globular lobes, each of which displays a binding site for ferric iron.[65] In 90% of lactoferrin in human milk,[66] iron-binding sites are available to compete with siderophilic bacteria and fungal enterochelin for ferric iron.[67-69] The iron chelation disrupts the proliferation of those pathogens. Lactoferrin also kills by damaging outer membranes of many gram-positive and gram-negative bacteria.[70-72] This process is conducted by a peptide, lactoferricin, which is usually comprises 18 amino acid residues from the N-terminal region of lactoferrin that are formed by gastric pepsin digestion.[71,72] Furthermore, lactoferrin inhibits certain viruses by a chelation-independent mechanism[73-76] and interferes with the adhesion of *Escherichia coli* to epithelial cells.[68]

The mean concentration of lactoferrin in human colostrum is between 5 and 6 mg/mL.[54] As the volume of milk production increases, the concentration falls to about 1 mg/mL at 2 to 3 months of lactation.[55] The mean intake of milk lactoferrin in healthy breast-fed full-term infants is about 260 mg/kg/day at 1 month and 125 mg/kg/day by 4 months.[57] Because human lactoferrin resists proteolysis[77] and the concentration of lactoferrin is much greater in human than bovine milk,[25] the excretion of lactoferrin in the stools is higher in infants fed human milk than in those fed a cow's milk formula.[60,78] The quantity of lactoferrin excreted in stools of low-birth-weight infants fed human milk is approximately 185 times that excreted by infants fed a cow's milk formula.[60] That estimate, however, may be too high because of immunoreactive fragments of lactoferrin in the stools of human milk-fed infants.[79] There is also a significant increment in urinary excretion of intact and fragmented lactoferrin as a result of human milk feedings.[62,79] Although the transport mechanism is unknown, the increase is a result of absorbed human milk lactoferrin and it fragments.[80]

Lysozyme

Lysozyme, a 15-kD single chain protein, lyses susceptible bacteria by hydrolyzing β-1,4 linkages between N-acetylmuramic acid and 2-acetylamino-2-deoxy-D-glucose residues in cell walls.[81] High concentrations of lysozyme are in human milk throughout lactation.[54-56] Longitudinal changes in lysozyme during lactation are unlike most other immune factors in human milk. The mean concentration of lysozyme is about 70 μg/mL in colostrum,[54] 20 μg/mL at 1 month and 250 μg/mL by 6 months of lactation.[55] The high content of lysozyme in human milk and its resistance to proteolysis lead to an eightfold increase in lysozyme in the stools of low-birth-weight infants fed human milk.[60] The urinary excretion of this protein is not increased in infants fed human milk.[62]

α-Lactalbumin

α-Lactalbumin is expressed only in the lactating mammary gland. A folding variant of the protein kills *Streptococcus pneumoniae* in vitro.[82] Furthermore, a molecular complex consisting of partially unfolded α-lactalbumin and oleic acid kills many types of tumor cells in vitro by several mechanisms including apoptosis and macroautophagy.[83,84] The protein invades tumor cells, depolarizes mitochondrial membranes, releases cytochrome c, exposes phosphatidyl serine, reduces caspase responses, and activates 20S proteasomes. α-Lactalbumin also translocates to tumor cell nuclei to cause chromatin disruption, loss of transcription, and nuclear condensation. The protein also kills certain skin papillomas and bladder cancers in situ. However, the in vivo antineoplastic action of the protein in human milk upon the recipient infant is undetermined.

CCL28

CCL28 is not only a chemokine; it also kills *Candida albicans* and many gram-positive and gram-negative bacteria. The killing is mediated by the 28 amino acid C-terminus of the molecule.[85]

MACROPHAGE MIGRATION INHIBITORY FACTOR

Macrophage inhibitory factor (MIF), a constituent of human milk,[86] is a proinflammatory cytokine that also up-regulates TLR-4[87] and aids in killing *Mycobacterium tuberculosis* in human macrophages.[88]

Fibronectin

Fibronectin facilitates the uptake of many particulates by mononuclear phagocytes. The in vivo effects of this opsonin in human milk[89] are unknown.

Complement

All components of the classical and alternative pathways of complement are in human milk, but their concentrations are much lower than those in serum.[90-92] Based upon in vitro experiments,[93] degradation products of C3 (C3b or C3bi) in human milk may augment phagocytosis of microbial pathogens in the infant's gastrointestinal or respiratory tracts.

Human Milk Mucin

Milk mucins are high-molecular-weight, highly glycosylated proteins.[94] Two thirds of mucin in human milk is bound to milk fat globule membranes. The concentration of mucin in human milk ranges between 50 and 90 mg/mL. Human milk fat globules and mucin from their membranes inhibit the binding of S-fimbriated *E. coli* to human epithelial cells.[94]

The most prominent mucin, MUC1, resists intragastric digestion in preterm infants.[95] Major fragments of MUC1 are in feces of breast-fed infants,[96] and mucins from such feces inhibit the adhesion of S-fimbriated *E. coli* to epithelial cells.[97]

Lactadherin

Human milk mucin was thought to defend against rotavirus in experimental mice,[98] but the protection turned out to be lactadherin,[99] a 49-kDa glycoprotein on milk fat globules that resists intragastric digestion.[95]

A protein analogous to human lactadherin, MFG-E8, is found on murine milk fat globules.[100] MFG-E8 on murine macrophages binds to phosphatidyl serine exposed on the outer membranes of apoptotic cells.[101,102] It also binds to α1β3 and α1β5 integrins on macrophages in the spleen and lymph nodes and elicited peritoneal macrophages via a tripeptide (RDG) motif within the second of its two EGF repeats. Because of the double linkage, apoptotic cells are phagocytized without inducing inflammation. Human lactadherin may have similar effects.

Low-Molecular-Weight Antimicrobial Peptides

In addition to antimicrobial peptides generated by partial digestion of lactoferrin, cysteine-rich, cationic low–molecular-weight peptides are in human milk including β-defensin-1,[103] which disrupts *E. coli,* and the α-defensins -1,-2, and -3 (HNP -1, -2, and -3)[104] and α-defensins, which inhibit HIV-1 replication and may interfere with postpartum transmission of HIV-1.[104]

Oligosaccharides and Glycoconjugates

Oligosaccharides in human milk are produced by mammary gland glycosyltransferases. Their concentrations in colostrum and mature milk are about 20 mg/dL and 12 mg/dL, respectively.[105] Many oligosaccharides are in human milk,[106] and they differ from those found in cow's milk. Although the quantities of total gangliosides in human and bovine milk are similar, the frequencies of each type of ganglioside in the milk of the two species are different. For example, greater amounts of monosialoganglioside 3 and GM_1 are found in human than in bovine milk.[106,107]

Because these agents resist enzymatic digestion, it was predicted that they would have nonnutritional functions. Indeed, some are receptor analogues that inhibit the in vitro binding of certain enteric or respiratory bacterial pathogens and their toxins to epithelial cells.[106,108,109] The chemistry of these compounds dictates the specificity of their binding. For example, GM_1 gangliosides are receptor analogues for *Vibrio cholerae* and *E. coli* toxins,[109] whereas the globotriaosylceramide Gb3 binds to b subunits of shigatoxin.[110] A fucosyloligosaccharide inhibits the stable toxin of *E. coli*,[111] whereas a different one inhibits *Campylobacter jejuni*.[112] Human milk oligosaccharides interfere with the attachment of *Haemophilus influenzae* and *S. pneumoniae* to respiratory epithelium,[113] and G1cNAc(β1-3) Gal-disaccharide subunits block attachment of *S. pneumoniae* to respiratory epithelium.[113]

The severity of *Campylobacter* or calcivirus enteritis in breast-fed infants is inversely proportional to concentrations of oligosaccharides in maternal milk that consist mainly of α-(1→2) oligosaccharides.[114] It is unclear whether the risk to other enteropathogens is related to quantitative variations in other oligosaccharides in human milk.

In addition, sulfated glycolipids, glycosaminoglycans,[115] and Lewis X component[116] in human milk inhibit in vitro infection by HIV-1. Polymers of Lewis X component interact with a dendritic cell–specific ICAM3-grabbing nonintegrin that facilitates the transfer of HIV-1 from dendritic cells to $CD4^+$ T cells. Consequently, gp120 on the envelope of HIV-1 is unable to bind to those T cells.

Animal experiments suggest that oligosaccharides and glycoconjugates in human milk protect against certain enteric bacterial infections.[117] In that regard, certain human milk oligosaccharides survive passage through the alimentary tract[118] and some are absorbed and then excreted into the urinary tract.[119] This may account for some protection by human milk against urinary tract infections.[120] Sugars in the glycoconjugates mucins, lactadherin, and secretory IgA also interfere with binding of bacterial pathogens to epithelial cells.[93,94,121]

In addition to the direct antibacterial effects of the carbohydrates in human milk, nitrogen-containing oligosaccharides, glycoproteins, and glycopeptides in human milk are growth promoters for lactobacilli and bifidobacilli.[122,123] For example, the growth-promoter activity associated with caseins may reside in the oligosaccharide moiety of those complex molecules.[123] These factors are responsible for the predominance of lactobacilli and bifidobacilli in the bacterial flora of the large intestine of breast-fed infants. The commensal bacteria produce large amounts of acetic acid, which suppress the multiplication of enteropathogens. The *Lactobacillus* strain GG may also aid in the recovery from acute rotavirus infections[124] and may enhance the formation of specific circulating antibodies during enteric infections.[29] In addition, enteric commensal bacteria may stimulate the production of IL-12[125] and low-molecular-weight antibacterial peptides such as defensins.[126] These latter defense mechanisms may contribute to the comparative paucity in stools of breast-fed infants of bacterial pathogens most often found in urinary tract infections such as P-fimbriated *E. coli*.[127]

Lipids

Fatty acids and monoglycerides generated by the enzymatic digestion of lipid substrates in human milk disrupt enveloped viruses.[128,129] These antiviral lipids may aid to prevent coronavirus infections of the intestinal tract[130] and defend against intestinal parasites such as *Giardia lamblia* and *Entameoba histolytica*.[131,132] Monoglycerides from milk lipid hydrolysis also inactivate certain gram-positive and gram-negative bacteria.[133] These lipids may act synergistically with one another and with antibacterial peptides.[134] Hydrolysis of milk lipids occurs in infants because of lingual lipase and the activation of human milk bile-salt stimulated lipase in the duodenum. Thus the products of lipid digestion may help defend breast-fed infants against enteric infections in the proximal gastrointestinal tract.

LEUKOCYTES IN HUMAN MILK

Living leukocytes are found in human milk,[135] and virtually all of them are activated.[136,137] In contrast to B cells that transform into plasma cells that remain sessile in the mammary gland, other leukocytes attracted to the site traverse the mammary epithelium and become part of milk secretions. The highest concentrations of leukocytes in human milk occur in the first few days of lactation ($1–3 \times 10^6$/mL). The several types of leukocytes and their major features are as follows.

Lymphocytes

The relative frequencies of T cells and B cells among lymphocytes in early human milk secretions are 83% and 6%, respectively.[136] The small number of natural killer cells in human milk[136] is in keeping with the low cytotoxic activity of human milk leukocytes.[138] The numbers of B cells in human milk are small because most B cells that enter the lamina propria of the mammary gland transform into sessile plasma cells.

Both $CD4^+$ (helper) and $CD8^+$ (cytotoxic/suppressor) T-cell subpopulations are present in human milk.[136] But compared with human blood T cells, the proportion of $CD8^+$ T cells in human milk is greater.[136] $CD4^+$ and $CD8^+$ T cells in human milk bear markers of cellular activation including CD45RO and HLA-DR.[136] Moreover, a greater percentage of human milk $CD8^+$ T cells express the intestinal homing receptor, CD103, and the mucosal homing receptor, CCR9, than those found in blood.[139]

T cells in human milk produce certain cytokines such as interferon-γ.[140] Additional cytokines are produced by human milk leukocytes,[141] but the extent of their production and secretion is undetermined.

Neutrophils and Macrophages

Neutrophils and macrophages are the dominant leukocytes in human milk. Both types of cells are laden with milk fat globules and other membranes that have been phagocytized. Because of

these intracytoplasmic bodies, they are difficult to recognize by common staining methods. They can be identified however by the presence of myeloperoxidase in neutrophils),[141,142] nonspecific esterase in macrophages,[141,142] or CD14 or MHC class II molecules in macrophages.[141] Both types of cells in human milk are phagocytic. A respiratory burst occurs in milk macrophages after stimulation.[142,143] Superoxide anion generation by those cells is more marked after exposure to mannose-receptor ligands.[143] The macrophages also process and present antigens to T cells.[144]

In contrast to blood neutrophils, human milk neutrophils do not increase adherence, polarity, directed migration,[145] or deformability after exposure to chemoattractants.[146] These alterations may be due to agents in human milk. For example, the decreased calcium influx found in human milk neutrophils is duplicated by incubating blood neutrophils in human milk.[147]

Unlike human milk neutrophils, the motility of macrophages in human milk is increased compared with blood monocytes.[148] The features of these cells in human milk are likely due to cellular activation, because they display phenotypic features of activation, including an increased expression of CD11b/CD18 and a decreased expression of CD62L.[137] The activation may be due in part to ingestion of milk fat globules or other membranous materials in human milk.[137]

Potential In Vivo Effects

The in vivo fate and role of human milk leukocytes in the infant are poorly understood. Only a small numbers of memory T cells are detected in infancy[149]; thus maternal memory T cells in milk may compensate for that developmental delay in the infant. There is evidence from experimental animal studies that milk lymphocytes enter tissues of the neonate,[141] but that has not been demonstrated in humans. In addition, cellular immunity may be transferred by breast-feeding.[150]

ANTI-INFLAMMATORY PROPERTIES

Inflammatory agents and systems that give rise to them are poorly represented in human milk.[25,26] These include (1) the coagulation system; (2) the kallikrein-kininogen system; (3) most complement components; (4) IgE; (5) basophils, mast cells, eosinophils; and (6) cytotoxic lymphocytes. Certain proinflammatory cytokines (see subsequent discussion) are found in human milk, but there is no clinical evidence that they generate inflammation in the recipient.

Human milk also contains many antiinflammatory agents[25,26] including (1) factors that promote epithelial growth and thus strengthen mucosal barriers; (2) antioxidants; (3) agents such as lactoferrin that interfere with some complement components[26,151]; (4) enzymes that degrade mediators of inflammation; (5) protease inhibitors[152]; (6) agents that bind to substrates such as lysozyme to elastin[153] and lactoferrin to the toxic moiety of lipopolysaccharide, lipid A[154]; (7) cytoprotective agents such as prostaglandins E_1, E_2, and $F_2\alpha$,[155,156]; and (8) agents that inhibit the functions of inflammatory leukocytes[26] such as binding of LPS to CD14 by lactoferrin[154] and the down-regulation by lactoferrin of LPS-induced cytokine production by mononuclear phagocytes via NFκB[157] (Table 158-3). Furthermore, many antiinflammatory agents in human milk are adapted to survive in the alimentary tract.

The main antioxidants in human milk include an ascorbate-like compound,[158] uric acid,[158] α-tocopherol,[159,160] and β-carotene.[159,160] Blood levels of α-tocopherol and β-carotene are higher in breast-fed than formula-fed infants not supplemented with those agents.[160] The in vivo action of those agents in human milk is unknown.

Mucosal growth factors in human milk include epithelial growth factor,[161] lactoferrin,[162] cortisol,[163] polyamines,[164,165] and peptides produced from α-lactalbumin and lysozyme.[166] Other hormones and growth factors in human milk[167] also affect the growth and differentiation of epithelium and thus limit the penetration of antigens and pathogens into the intestines. In that respect, the biophysical and biochemical organization and functions of mucosal barriers in adults and neonates are different.[168,169] Furthermore, their maturation may be accelerated by human milk.[170,171]

TABLE 158-3

Antiinflammatory Agents in Human Milk

Categories	Examples
Cytoprotectives	Prostaglandins E2, F2α
Epithelial growth factors	EGF, lactoferrin, polyamines
Maturational factors	Cortisol
Enzymes that degrade inflammatory mediators	PAF-acetylhydrolase
Binders of enzymes	α1-Antichymotrypsin
Binders of substrates of enzymes	Lysozyme to elastin
Binders of toxins	Lactoferrin to lipid A of LPS
Modulators of inflammatory leukocytes	IL-10, TGF-β1
Antioxidants	Uric acid, β-carotene, ascorbate

Enzymes in human milk degrade inflammatory mediators. Platelet-activating factor (PAF) plays a role in an intestinal injury in rats induced by endotoxin and hypoxia.[172] Human milk, however, contains an acetylhydrolase that degrades PAF,[173] whereas the production of human PAF-acetylhydrolase is developmentally delayed.[174] As a result of these agents in human milk, intestinal permeability is lessened in breast-fed infants.[175-177]

IMMUNOMODULATING AGENTS

Observations suggest that immunomodulating agents in human milk are important:

1. Epidemiologic investigations suggest that older children who are breast-fed during infancy may be at less risk for developing certain chronic diseases mediated by immunologic, inflammatory, or oncogenic mechanisms. The diseases are type 1 diabetes mellitus,[178-180] type 2 diabetes mellitus,[181] lymphomas,[182,183] acute lymphocytic leukemia,[183,184] and Crohn's disease.[185,186]
2. Increased levels of certain immune factors in breast-fed infants cannot be accounted for by passive transfer of those substances from human milk. Breast-feeding primes the recipient to produce higher blood levels of interferon-α in response to respiratory syncytial virus (RSV) infections.[187] In addition, increments in blood levels of fibronectin achieved by breast-feeding cannot be accounted for by the amounts of that protein in human milk. Moreover, breast-feeding leads to a more rapid development of systemic[188] and secretory[188,189] antibody responses and of secretory IgA in external secretions[60-62] including urine,[61,62] which are far removed from the route of ingestion. There is no evidence that those increments are caused by absorption of those same factors from human milk.
3. Thymic growth,[190] T-cell emigration from the thymus possibly caused by increased IL-7 in human milk,[191] T-cell maturation, and IL-10 production[192] are increased in breast-fed infants compared with infants who are not breast-fed.
4. All leukocytes in human milk are activated.

Cytokines

After it was ascertained that human milk leukocytes were activated, activating agents in human milk were sought. It was found that human milk enhances the movement of blood monocytes in vitro and that much of that motility was abrogated by antibodies to tumor necrosis factor-α (TNF-α).[193] Subsequently, TNF-α

TABLE 158-4

Cytokines in Human Milk

Types	Examples
T-cell production augmentation	IL-7
Cellular immunity enhancement	Interferon-γ, TNF-α, IL-12, and IL-18
Humoral immunity enhancement	TGF-β2, IL-4, IL-10
Macrophage stimulation	IL-1β, IL-6, IL-6, MIF
Chemokine activities	IL-8, RANTES, MIP-1, CCL28
Interferon-inducible proteins	IP-10 and MIG
Antiinflammatory actions	TGF-β1, IL-10
Growth stimulation	EGF, M-CSF, G-CSF, erythropoietin

in human milk was detected immunochemically.[194] Since then, many cytokines have since been found in human milk (Table 158-4). They include the following:

1. IL-7, which promotes intrathymic development of T cells and maintenance of mature T cells in the peripheral lymphoid system.[192]
2. IL-2 with IL-7, which promotes the proliferation of recent thymic immigrants.[195]
3. Th1 (interferon-γ,[196] IL-12,[197] and IL-18[198]) and Th2 (IL-10[199,200] and IL-4[201]) cytokines.
4. Macrophage-stimulating cytokines including IL-1β,[202] IL-6,[203] and MIF.[86]
5. Chemotaxins including IL-8,[204] RANTES,[201] CCL28,[85] and eotaxin.[201]
6. Interferon-inducible proteins IP-10 and MIG.[205]
7. Antiinflammatory agents such as Il-4,[201] TGF-β1,[206] TGF -β2[207] and IL-10[199,200]
8. Growth factors EGF,[161,167] granulocyte colony-stimulating factor,[208] macrophage-CSF,[209] hepatic growth factor,[210] Il-4[196] and erythropoietin.[211]

It should be pointed out that the in vivo fate, action, and interactions of these cytokines in human milk are complex and largely unexplored. For example, IL-2 greatly decreases the expression of IL-7 receptor α-chains (IL-7Rα).[212] Because IL-7Rα is a component of receptors for IL-7 and thymic stromal lymphopoietin, IL-2 may negatively regulate signals by each of these cytokines. It is unclear whether the in vitro actions pertain to the in vivo effects upon the infant.

Other Modulators

Other immunomodulating agents in human milk include β-casomorphins,[213] prolactin,[167,214] antiidiotypic antibodies,[48] α-tocopherol,[159,160] nucleotides that enhance NK-cell, macrophage, and Th1 activities,[215] cell adhesion molecules ICAM-1, VCAM-1, E- and L-selectin,[216] mannan-binding lectin—which activates complement by the lectin pathway after recognizing surface saccharide motifs on microorganisms[217] and soluble CD14, a B-cell mitogen.[218]

Toll-like receptors (TLR) in human milk are being investigated. Soluble TLR-2 is present in human milk.[219] Furthermore, a protein of approximately 80 kD in human milk enhances the response of TLR4 and TLR5 receptors on umbilical cord blood mononuclear leukocytes.[220]

In addition to the antimicrobial and antiinflammatory functions of lactoferrin, this single-chain glycoprotein also promotes the differentiation of dendritic cells from monocytes.[221] It will be important to establish whether lactoferrin in human milk has a similar in vivo effect in the recipient infant.

In addition to their antimicrobial properties, some oligosaccharides are immunomodulatory. Lacto-N-fucopentaose III and lacto-N-neotetraose increase the production of murine IL-10.[222] Furthermore, human milk acidic oligosaccharides increase the number of interferon-producing $CD4^+$ and $CD8^+$ T cells and IL-13 production by $CD8^+$ T cells.[223] In addition, more CD25 is expressed on $CD4^+$ T cells after exposure to those oligosaccharides.[223]

TABLE 158-5

Immune Factors in Human Milk Whose Production Is Delayed in the Recipient

Immune Functions	Representative Agents
Antimicrobial	SIgA
	Lactoferrin
	Lysozyme
Antiinflammatory	IL-10
	PAF-acetylhydrolase
	Lactoferrin
	Lysozyme
Immunomodulatory	Memory T cells
	IL-4
	IL-10
	IL-12
	G-CSF
	TNF-α
	Interferon-γ
	RANTES

IMMUNE SYSTEM IN HUMAN MILK AND THE RECIPIENT INFANT

Several evolutionary outcomes concerning the relationships between the immune status of infants and defense agents in human milk have been recognized. In respect to one of the main evolutionary outcomes, many aspects of the human immune system are incompletely developed at birth, and the immaturity is most marked in very-low-birth-weight infants. They include (1) the mobilization and function of neutrophils,[224-226] (2) the maturation of dendritic cells,[227,228] (3) the recognition by monocytes and macrophages to bacterial agents by toll-like receptors TLR2 and TLR4,[229] (4) the production of lysozyme[230] and secretory IgA,[231] (5) memory T cells that bear CD45RO,[149] (6) the full expression of the antibody repertoire,[232] and (7) the production of certain cytokines including TNF-α,[233-235] IL-4,[236,237] interferon-γ,[237-239] IL-6,[234,240] IL-10,[233,241] IL-12,[242,243] IL-18,[243] G-CSF,[244] GM-CSF,[245] IL-3,[244] and RANTES.[246]

Many developmentally delayed defense agents are present in human milk (Table 158-5). For instance, secretory IgA antibodies in milk compensate for low production of secretory IgA during early infancy.[248] Human milk antibodies are polyclonal and directed against protein and polysaccharide antigens. This is important because infants display a more restricted antibody clonality[248] and do not produce IgG antibodies to polysaccharides.[249] Conjugate vaccines have been introduced, but antibody responses to them are higher in breast-fed than cow's milk-fed infants.[250]

An additional example is the interrelationship between the production of lysozyme by the infant and the mammary gland. High lysozyme levels in human milk[54-56] are coupled to low production of the protein by tracheo-bronchial mucosal cells during infancy.[230] Indeed, normal intraluminal concentrations of lysozyme in infancy may depend on breast-feeding. This is in keeping with higher lysozyme activities in stools of breast-fed infants than in infants who are not breast-fed.[60]

The functions of immune factors in human milk in the recipient infant depend on their survival in the infant. The following are germane: (1) Proteins may affect the epithelium, leukocytes, or other cells of the proximal GI or respiratory tracts where proteolytic enzymes are not produced. (2) Some proteins are inherently resistant to proteolysis. (3) Ingested proteins may escape

digestion because of developmental delays in production of gastric HCl and pancreatic proteases.[251]

This resistance to digestion may be augmented by (1) the buffering capacity of human milk that shields some acid-labile components of milk, (2) antiproteases in human milk,[152] (3) the inherent resistance of many defense agents in human milk to digestive processes, and (4) the compartmentalization of some defense agents in human milk.[86,94,95] In that respect, much of the TNF-α in human milk is bound to soluble receptors.[252]

PROTECTION OF PREMATURE INFANTS BY HUMAN MILK

Maturational delays of the immune system are generally more profound in premature infants. Furthermore, the problem is compounded by the shortened duration of placental transfer of IgG to the fetus,[253] medical problems during the newborn period,[254] nutritional imbalances, and invasive clinical procedures that increase the risks to infections.

Milk from women who deliver prematurely contains many of the same antimicrobial factors that are found in milk from women who have delivered after a full-term pregnancy. These include secretory Ig, lactoferrin, and lysozyme.[255] The concentrations of some defense agents are higher in preterm than term milk. Those higher concentrations may be in large part due to a lower production of milk by women who deliver prematurely. However, that may not be the total explanation for the higher concentrations in that patterns of the concentrations of some antimicrobial agents in preterm and term milk are not the same.[255]

Bacterial Sepsis

In addition to the protection against enteric infections and respiratory infections, there are several indications that human milk feedings protect premature infants against systemic infections that are prone to occur in such infants.[256-258]

Necrotizing Enterocolitis

Human milk protects against necrotizing enterocolitis (NEC).[259] Human and experimental animal studies suggest that IgA,[260] erythropoietin,[261,262] PAF-acetylhydrolase,[172-174] and IL-10[199,200,263,264] protect against NEC. These possibilities are in keeping with two important findings: 1) each of these defense agents is well represented in human milk and not in artificial feedings, and 2) the production of each agent in human infants is developmentally delayed.

One animal model suggests that IL-10 in human milk may prevent intestinal inflammation. Mice homozygous for IL-10 null genes develop a fatal enterocolitis that begins soon after weaning and is dependent on an enteric bacterial flora.[263,264] The enterocolitis had some features of Crohn's disease and NEC. Much of the enterocolitis in those animals is prevented by intraperitoneal injections of IL-10 given at the start of weaning.[263]

One study suggests that variations in the concentrations of IL-10 in human milk may be responsible for some of the risk of NEC in premature infants.[200] Two distinct populations of women were found in respect to the concentrations of IL-10 in their milk—72% were high producers, and 28% were very low producers.[200] In women whose infants developed NEC while receiving their own mother's milk, IL-10 was barely detected or undetected in milk from more than 90% of them.[200] The study awaits verification.

Lung Disease

Although it is unknown whether human milk protects against pulmonary and vascular effects of hyperoxia, α_1-antitrypsin prevents many of the features observed in hyperoxic neonatal rats including elevated pulmonary elastolytic activity.[265] Furthermore, a murine model suggests that TGF-β1 in human milk protects against certain pulmonary inflammatory diseases. Mice homozygous for the TGF-β1 null gene display infiltrations of macrophages and T cells in many organ sites, particularly the lungs, heart, and salivary glands.[266-268] Further, the effects of TGF-β1 deficiency are mitigated by ingestion of TGF-β1 in murine milk.[268] Furthermore, one study suggests that greater exposure to human milk TGF-β1 lessens the risk of asthma in the first year of life.[206]

ATOPIC DISEASE

Some studies suggest that human milk protects against atopic dermatitis[269] and asthma[206,270] and that some of the protection against asthma is mediated by TGF-β1[206] and soluble CD14 in human milk.[270] However, there is no consensus whether as to whether breast-feeding protects against atopic diseases.[271] Much of the disagreement may be due to confounding variables including variations in genetic predisposition to atopic disorders, the sufficiency of breast-feeding, unappreciated dietary exposures, and exposures to inhaled allergens or irritants. Further, increased exposures to infectious diseases facilitate Th1 responses that lead to cellular immunity, whereas lower exposures engender Th2 responses that lead to antibody formation and hence to possible IgE-mediated hypersensitivity. Thus, the effect of breast-feeding on atopic diseases may depend on factors that are not equally represented in all investigated populations.

Moreover, the question is complicated by foreign food antigens in human milk[272] and the triggering of allergic reactions by those antigens in some infants.[273] To test whether a breast-fed infant reacts to a foreign food antigen in human milk, dietary elimination and oral challenge with the food in question in the breast-feeding mother are needed.[273] If the infant reacts to a foreign food antigen in human milk, then the food should be eliminated from the maternal diet. If the allergen is a basic food, the elimination diet should contain the correct types and quantities of nutrients to meet the needs of the mother.[20] If dietary elimination is impractical, breast-feeding may be stopped and a hypoallergenic formula instituted. In addition, allergic disease in breast-fed infants may be due to alterations in fatty acids in human milk.[273-275]

IMMUNOLOGIC TOLERANCE

Evidence for induction of immunologic tolerance by breast-feeding comes from studies of alloreactivity. Maternal renal allografts are better tolerated in children from women who breast-feed transplant recipients than those who do not.[276,277] The difference in alloreactivity is also shown with blood lymphocytes from mothers and their children. Less alloreactivity occurs when lymphocytes from the mother (stimulators) are co-cultured with her breast-fed child's cells (reactors).[278] The tolerance may be induced by HLA-DR antigens on fat globules[279,280] and macrophages[137,141,144] in human milk.

CONCLUSION

Although much has been learned, there is much to be discovered. This includes: 1) other defense agents present in human milk, 2) regulation of production of the agents during lactation, 3) the precise molecular form of each defense agent in human milk, 4) where compartmentalized and soluble-receptor bound agents in human milk are released, 5) whether other defense agents are created in the infant's gastrointestinal tract by partial digestion of substrates in human milk, 6) mechanisms responsible for activating leukocytes in human milk, 7) in vivo fate and action of the

defense agents in human milk, 8) effects of commensal bacterial flora induced by breast-feeding upon the immune system of the recipient, 9) tolerogenic effects of human milk, and 10) long-term effects of antiinflammatory, immunomodulating, and antineoplastic agents in human milk.

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Neonatal Pulmonary Host Defense

Like the skin and gastrointestinal (GI) tract, the lungs are a mucosal organ with a large surface area exposed to the external environment. Unlike the skin and GI tract, the lung is considered to be largely sterile below the glottis whereas the skin and GI tract are colonized with bacteria termed "commensal flora." Despite the lower airway being sterile, the upper airway becomes rapidly colonized with bacteria that can be aspirated into the lower airway, thus the lung has evolved an array of host defense mechanisms to prevent development of infection in the air space. This robust development of pulmonary host defense mechanisms was an essential step in the evolution of air-breathing animals. The major physiologic aspect of the lung is to perform gas exchange, namely the exchange of oxygen and carbon dioxide across the alveolar capillary membrane. To maintain this function, the lungs must have buffering capacity in the airway and alveolar space to neutralize potentially injurious agents including pathogens. In a 3.5-kg neonate with a typical minute ventilation ranging from 100 to 150 mL/(kg•min), the lungs are required to filter approximately 30 L of inhaled air hourly. This is a problematic task in that the alveolar surface area requiring protection is 20 times the average neonatal body surface area.[1] In addition to normal tidal breathing or gas exchange the lung must be able to handle larger insults because of what may occur upon aspiration of oropharyngeal or gastric contents.

Available pulmonary host defenses can be broadly categorized as either structural or immunologic. Examples of structural defenses include the larynx and epiglottis (which are

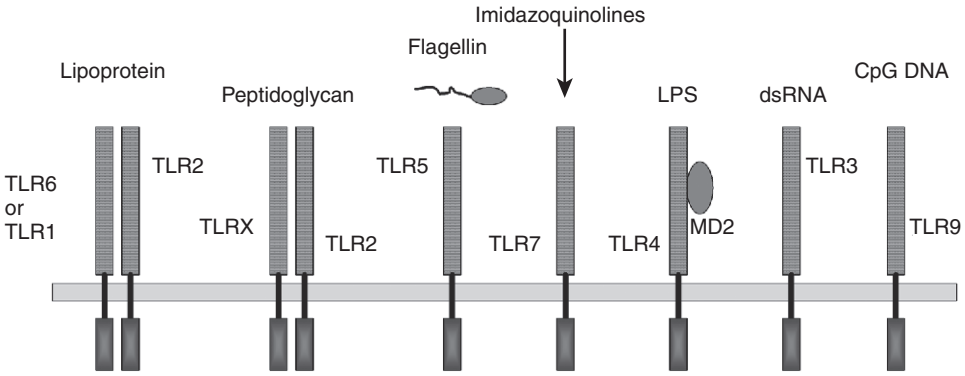


Figure 159-1 Toll-like receptors and their ligands. Toll-like receptors function as molecular recognition receptors with specific ligand recognition motifs encoded by their leucine-rich repeat domains *(horizontal lines)*. The TLR2/6 heterodimer recognizes bacterial lipoproteins, and TLR2 also recognizes peptidoglycans. TLR5 recognizes flagellin whereas TLR7 recognizes molecules such as Imiquimod. TLR4 in conjunction with MD2 recognizes LPS, but TLR4 is critical for signaling induced by LPS stimulation. TLR3 recognizes double stranded RNA, and TLR9 recognizes hypomethylated CpG DNA.

anatomically situated to minimize aspiration of oropharyngeal material), airway angulation, mucus secretion, and mucociliary clearance mechanisms including the cough reflex. These mechanisms result in progressive filtering of about 99% of inhaled particles as they pass through the conducting airways so that overall level of antigen exposure at a given site is inversely related to its depth within the respiratory tree.

Immunologic mechanisms can be divided into innate immune response and adaptive immune response. Innate immunity is highly conserved from lower order species such as worms *(Caenorhabditis elegans)* and fruit flies *(Drosophila melanogaster)*. Innate immunity is rapid (within minutes to hours) and generally lacks specificity toward the pathogen. Adaptive immunity takes longer to develop (days as opposed to minutes to hours), is more pathogen specific, and is characterized by recombination of cell surface receptors to achieve pathogen specificity in both B-lymphocytes and T-lymphocytes. Adaptive immunity is present in higher order eukaryotes such as fish and mammals. Although innate immune responses are thought to be nonspecific, they often rely on host recognition of pathogen-associated molecular patterns or specific molecules such as peptidoglycans, lipopetides, lipopolysaccharides (LPS), flagellin, fungal glucans, or specific nucleic acid structures such as single stranded RNA or unmethylated CpG DNA (Figure 159-1).[2] A major class of molecules that recognizes these specific molecules are the Toll-like receptors (TLR) (see Figure 159-1). These receptors are expressed on both blood-derived cells such as lung dendritic cells and alveolar macrophages and on nonmyeloid cells such as the lung epithelial cell.[3,4] Ligation of TLRs can initiate early alarm cytokines such as TNFα and IL-1β in the lung, and these can, in turn, stimulate lung endothelial expression of adhesion molecules, which augments binding of inflammatory cells to endothelium.[4a] Additionally, TLR signaling and the alarm cytokines can amplify the production of specific chemokines such as C3a, CXCL1, CXCL2, and CXCL8, which can augment neutrophil recruitment into the air space. Mice deficient in CXCR2, which is the receptor for CXCL1, CXCL2, and CXCL5 in the mouse, have significantly attenuated neutrophil recruitment in response to bacteria and hence have markedly reduced survival as a result of an inability to control bacterial growth in the lung.[5]

The lung is unique among mucosal organs in that it expresses surfactant proteins. Although surfactant was originally described as the critical phospholipid component of the alveolar lining fluid that reduces surface tension and allows successful transition from liquid breathing in the fetus to air breathing in the neonate, surfactant also plays critical roles in innate immunity.[6] Specifically, the surfactant proteins (SP) A and D have collagen stalks with lectin-binding domains termed "collections." SP-A and SP-D can act as opsonins and have been shown to be critical for host defense against bacterial, fungal, and viral pathogens.[7,8] These proteins can interact with carbohydrates on pathogens to directly neutralize the pathogen or augment macrophage-mediated uptake of these pathogens. Macrophages express a variety of phagocytic receptors to ingest and kill pathogens including Dectin-1, macrophage mannose receptors, and scavenger receptors.[9] Immature dendritic cells (iDCs) are also capable of ingesting pathogens-antigens, and when activated, iDCs can become mature DCs that express high levels of class II major histocompatibility complex for antigen presentation and costimulatory molecules such as CD80 and CD86. In addition, maturation leads to up-regulation of CCR7, a G-coupled chemokine receptor that is critical for migration of DCs to lymphoid tissue for antigen presentation to CD4+ T cells. Additionally it has recently been shown that cytokines, specifically IL-12p40, are required for DC migration to lymph nodes[10] possibly through the regulation of CCR7.[10] It is partly through these mechanisms that innate immunity can markedly drive adaptive immunity. For example, antigen presentation to CD4+ T cells in the absence of costimulation can lead to anergic T-cell responses that may be a key mechanism of achieving antigen tolerance in the lung. This is important because the delicate lining of the lung cannot afford to have strong immune reactions to all inhaled particles (such as ubiquitous mold spores) because the inflammation could adversely affect gas exchange. Thus the lung comprises macrophages and dendritic cells, which likely respond to specific thresholds of stimulus that are important in regulating decision points of antigen-specific tolerance versus antigen-specific immunity.

In addition to macrophages, dendritic cells, and epithelial cells, the lung also contains cells that are capable of "bridging" immunity. These cells include γδ T cells and natural killer (NK) T cells that often respond to canonical antigens (and cytokines). Thus these cells can secrete cytokines that control mucosal immunity but undergo limited T-cell receptor rearrangement compared with classic αβ T cells, which orchestrate more specific adaptive immunity. The recruitment of αβ T cells and B cells heralds the onset of the adaptive immune response. These cells express T-cell and B-cell receptors that undergo genetic reengagement so that the receptor has high affinity for peptides that are antigen specific. These cells consist of an effector population and a memory population. The latter population is capable of responding much more rapidly than naive cells if the antigen is reencountered by the host. Adaptive immunity thus initiates a targeted response aimed at

containment and clearance of a specific antigen, allowing titration of nonspecific—and potentially host injurious—alveolar inflammation. Although it is convenient to think of innate and adaptive immunity as innate immunity occurring proximal to adaptive immunity, complex pulmonary immune responses, as seen in pneumonia, require interplay between innate and adaptive elements.

STRUCTURAL MECHANISMS OF HOST DEFENSE

Structural defense exists throughout the respiratory tract. During air entry, the nose performs humidification of entrained air and also serves as an important filter. Particle filtration occurs through nasal hairs and through nasal turbinates. The turbulent airflow that exists in the upper airway facilitates particle deposition in the upper airway. These structures can filter particles as small as 10 μm. The next barrier to protect the lower airway is the larynx, which consists of the epiglottis, aryepiglottic folds, and glottis. Nervous innervation into these structures provides a gag and cough reflux which minimizes oropharyngeal aspiration. Particles smaller than 10 μm can be inhaled into the lower respiratory tract, and particle deposition is greatest at areas of airway bifurcation. An important clearance mechanism in the lower airways is cough and mucociliary cleanse, which is coordinated by specialized epithelia cells.

Epithelial Cells

Airway epithelial cells consist of both ciliated and nonciliated cells. The cells in the large airways exist as a pseudostratified epithelium with each cell having contact with the basement membrane. These cells express apical-tight junctions that restrict passive diffusion or pathogen movement across the lateral intracellular space, augmenting local mechanical barriers.[11] The cilia extend apically from the epithelium into the airway surface liquid (ASL) which has been estimated to be 7 to 8 μm in height.[12] On top of this liquid layer there is a mucous layer which is the site of particle deposition. The height of the ASL can markedly affect mucociliary clearance and is regulated by both sodium and chloride transport across the epithelium. Excluding regions of the larynx and pharynx where squamous epithelium predominates, ciliated epithelium is present from the upper respiratory tract to the level of the respiratory bronchioles. In addition to the ciliated epithelium, there are a variety of different nonciliated cell types including mucus-producing goblet cells, serous cells, Clara cells, neuroendocrine cells, and basal cells.[13,14] Ciliated cells are in physical contact at tight junction desmosomes and in physiologic communication via gap junctions.[15] Columnar ciliated cells may carry up to 200 cilia, ranging from 4 μm in the distal airways to 6 μm centrally. Ciliogenesis commences in the upper trachea early in gestation (week 7) and proceeds distally; by week 24 of gestation, the fetal airway epithelium resembles that of the mature trachea.[16] Although fetal cilia are motile, the function of ciliary activity in utero is unknown.

Epithelial cell types responsible for mucus production include the goblet cells, serous cells, and Clara cells. Goblet cells are found predominantly in large airways and are the principal source of mucus. Clara cells are found only in small airways and produce watery secretions; serous cells have similar location and function, but have been identified only in fetal tissues. Clara cells also serve as a likely stem cell for regeneration of ciliated epithelium.[17] Submucosal glands located in the cartilaginous portion of the airways also contribute to overall mucus secretion.

There is hierarchy of cell populations as one travels from the large airways to the alveolar ducts. In the trachea and large airways, ciliated columnar epithelium forms an essentially continuous epithelium, with a ratio of ciliated cells to goblet cells of 5:1.[18] In the lower airways, ciliated and goblet cell numbers are reduced, a ciliated cuboidal epithelium predominates, and Clara cells become the only secretory cells present.[19]

As alluded to above, particle deposition occurs in the mucus layer and the apical situated glycocalyx layer, which consists of mucins tethered to the epithelial cell surface.[20] The epithelium expresses both tethered mucins and nontethered mucins.[20] MUC1 and MUC4 are genes that encode tethered mucins, which are highly glycosylated and extend up to 1500 nm into the airway lumen. These mucins and other glycoproteins in the glycocalyx can bind particles and bacteria, facilitate clearance, and form an anatomic barrier between the particle and the epithelial cell. The glycocalyx has also been shown to be a critical barrier to prevent infection of the epithelium by various respiratory viruses.[21]

Ciliary Function

Human cilia are composed of nine doublet microtubules (subfibers) surrounding two microtubules. The major microtubular axonemal proteins are dynein, an adenosine triphosphatase (ATPase), and tubulin.[22] At the tip of the cilium, the nine subfibers simplify into nine single fibers that insert into a common cytoplasmic extension (Figure 159-2) through which they mechanically engage the overlying mucus sheet during the effective ciliary stroke.[23-25] At the base of the cilium, the nine subfibers end in the basal body, which is anchored to the cytoskeleton.[25] Because all basal bodies of a cell are oriented in approximately the same direction, the effective strokes of all cilia of a given cell have a similar orientation; however, neither the orientation of these structures nor the orientation of the ciliary beats is necessarily identical in adjacent cells.[26,27] During ciliary motion, dynein projections from one subfiber transiently interact with the nondynein-containing subfiber of an adjacent microtubule doublet and, using energy from dynein-mediated adenosine triphosphate (ATP) hydrolysis, induce a conformational change resulting in subfiber movement. Repetition of this process in adjacent doublets, in a unidirectional front circumferentially, causes the sequential movement of fibers that effect cilia motion.[28] From a resting state, a cilium in its recovery stroke swings close to the cell 180 degrees backward, then fully extends and moves through its effective stroke, an arc of approximately 110 degrees in a plane perpendicular to the cell surface.[29] During the effective stroke, the ciliary tip engages the overlying mucus, advancing it in the same direction. Following this, the cilium rests then repeats this sequence. As a cilium swings backward into its recovery stroke, it engages other resting cilia, stimulating them to begin a recovery stroke; this mechanical recruitment is pivotal for the coordinated beating of airway cilia.[30]

Cilia of human nasal, tracheal, and bronchial mucosa beat at 11 to 15 Hz at body temperature, with progressively slower frequencies noted in proximal bronchi and bronchioles.[25] Because the combined surface areas of the distal airways are exponentially greater than those of the central airways, this differential ciliary beat frequency allows efficient handling of the relatively large mucus loads eventually delivered to the trachea. Endogenous mechanisms that allow such local ciliary beat modulation have been described. Local physiologic loads to airway epithelium, such as increased quantities of mucus, can mechanically stimulate increased ciliary beating in vivo; this effect appears to be mediated by increased cytosolic calcium and can be reproduced pharmacologically by agents that modulate intracellular calcium levels.[31,32] Ciliary beat frequency is also exquisitely sensitive to alterations in temperature; cilia of the small airways beat optimally near body temperature, decreasing and increasing their beat frequencies in response to decreased and increased body temperatures, respectively.[32] Increased ciliary beat frequency in response to increased ambient air concentrations of nitric oxide (NO) has been described, suggesting that local cellular production of this bioactive substance may modulate ciliary function.[32] These

observations are consistent with in vitro data demonstrating that ciliated epithelium produces NO, which regulates ciliary beat frequency in an autocrine manner via a cyclic guanosine monophosphate (GMP) signaling pathway.[33] In addition to the dynein ATPase, a cyclic adenosine monophosphate (cAMP)-dependent kinase also facilitates control of ciliary beating.[32] Whether intrinsic neural control of ciliary beat frequency exists is unclear. Although acetylcholine increases ciliary beat frequency in vitro, suggesting a potential role for cholinergic regulation of ciliary "tone," there is no cholinergic efferent innervation of the superficial airway epithelium, making it unlikely that neural mechanisms regulate lung mucus transport.[34] Finally, recent interest has focused on autocrine and paracrine airway epithelial signals generated by local purinergic pathways.[32] In this model, luminal ATP and uridine triphosphate (UTP) induce increased ciliary beat frequency by binding to G protein–coupled purinoreceptors on the epithelial apical membrane. Nucleotide-hydrolyzing enzymes of the airway surface liberate adenosine, which acts through a separate receptor to sustain the ciliostimulatory effects of ATP, while cilia-derived phosphatases hydrolyze ATP and UTP, down-regulating ciliary beat frequency. Nucleotide release by airway epithelia is induced by shear stress and possibly other stimuli so that local mechanical or metabolic perturbations may modulate ciliary activity.[32]

Ciliary function may also be affected by a variety of exogenous substances introduced into the airway. Inhaled β-adrenergic agonists may increase ciliary beat frequency by increasing cellular cAMP.[35] Inhaled NO, via metabolism to S-nitrosothiols, may increase ciliary motility.[36] Conversely, ciliary activity may be diminished by a number of anesthetic gases or by exposure to high concentrations of inspired oxygen.[32] Finally, ciliary function may be impaired by infection. Specific pathogens, including *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*, produce soluble products inhibiting ciliary function.[32] These products may induce ciliary beat slowing, ciliary beat disorientation, ciliostasis, or frank ciliary lysis. Some of these substances are directly ciliotoxic, whereas others act by inducing local macrophages to generate hydrogen peroxide, which has a local cilioinhibitory effect.[32] Lipid-derived inflammatory mediators, such as platelet-activating factor (PAF) induce dose-dependent slowing of ciliary beat, whereas proteins released into the airway lumen during inflammation, such as leukocyte elastase and neutral protease, are also ciliotoxic.[32] Although other inflammatory mediators stimulate ciliary beating in vitro, overall mucociliary function is typically diminished during inflammation in vivo.[32]

Airway Surface Liquid

Airway surface liquid (ASL) is a thin, nonviscous secretion lying just between the epithelial cell and overlying mucus, bathing the basal portion of the cilia and providing an environment of low resistance for the cilia to move during the backstroke portion of the movement cycle. The level of the ASL is critical for effective mucociliary function.[12] If the fluid level is too high, the ciliary tips will not be able to engage the overlying mucus and effect its movement. If the fluid level is too low as occurs in cystic fibrosis (CF), the cilia will not be able to disengage from the mucus layer during the recovery stroke and ciliary beating will be impeded.[12]

As mentioned above, ASL is regulated by active ion transport across the epithelial cells with concomitant fluid movement that is either passive or aquaporin regulated.[12] The bulk of fluid transport is regulated by both sodium and chloride transport. The predominant apical sodium channel is the epithelial sodium channel (ENaC), and chloride conductance is regulated by the cystic fibrosis transmembrane conductance regulator (CFTR) and

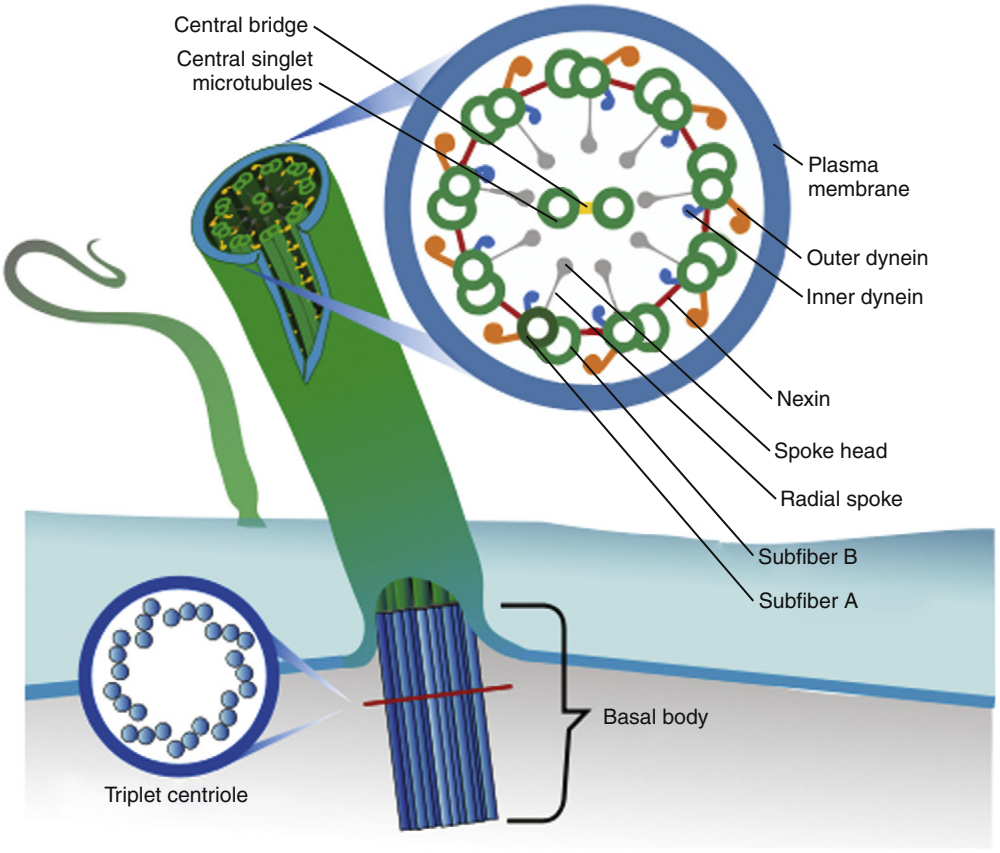


Figure 159-2 Schematic diagram of a cilium. Note the relationship of dynein projections from one subfiber with the adjacent subfiber *(inset)*. ATP hydrolysis results in conformational changes of the dynein arm, inducing subfiber movement and, ultimately, ciliary movement.

calcium-activated chloride channels (Figure 159-3). These channels are subject to precise regulation by environmental stress such as particle deposition or infection. ENaC is regulated by proteases as proteolytic cleavage of ENaC subunits can markedly augment channel activity.[37] CFTR is regulated by cAMP, and thus this channel is subject to influences such as osmotic stress or cytokine stimulation that influence cAMP levels.[38] In addition to these sodium and chloride channels, there are also bicarbonate exchangers that regulate ASL pH.[39] Passive efflux of water across the apical epithelial membrane results in cell shrinkage, triggering release of epithelial-derived mediators, including NO; these mediators increase blood flow and water content of the submucosa. Movement of water from the submucosa across the basolateral epithelial membrane, possibly regulated by aquaporin 4, restores epithelial cell volume.[40] In contrast, fetal lung fluid results from active secretion of Cl^- by all respiratory tract epithelium, with passive cation and fluid transit into the airway lumen.[41] In the distal airways these mechanisms persist, providing a source of periciliary fluid and mucus hydration for the rest of lung. Conversely, tracheobronchial epithelium gradually transitions postnatally from Cl^- secretion to Na^+ absorption, and Na^+ absorption eventually predominates in the larger airways under basal conditions.[12] At the same time, production of mucus components transiently increases. Mucociliary clearance has been shown to be reduced in neonates, presumably the result of increased ASL secretion and impaired ciliary tip engagement of mucus.

Mucins and Mucus

Mucins are high molecular weight, heavily glycosylated proteins that contribute to the gel-like qualities of mucus and are critical for mucosal host defense. Multiple mucin genes are expressed in the airways, but the primary gene products in mucus are MUC5AC and MUC5B.[42] Mucins are formed in the Golgi apparatus, concentrated in vesicles, and released by exocytosis from goblet cells and mucus cells of submucosal glands. Once secreted, condensed mucin polymers are hydrated, increasing their volume exponentially.[43,43a] Mucin proteins typically exhibit several hundred sugar side chains that contribute to their structural and barrier functions. As a result of their extensive glycosylation and their long carbohydrate side chains, mucins are capable of binding virtually any inhaled particle they encounter, facilitating particle entrapment within the mucus layer. Both MUC5AC and MUC5B show extensive regulation by both Th2 cytokines, including IL-9 and IL-13, and Th17 cytokines, namely by IL-17.[44,45] Additionally proinflammatory molecules such as histamine, prostaglandins, leukotrienes (LT), PAF, and tumor necrosis factor-α (TNF-α) can regulate mucin expression.

Mucus also contains proteoglycans, other proteins, lipids, water, and DNA. DNA is found in significant concentrations only during infection or inflammation of the airways originating from dead leukocytes or denuded epithelium; large amounts of DNA markedly increase mucus viscosity.[46,47] DNA is an important component of neutrophil extracellular traps (NETs), which consist of products of degranulated neutrophils such as granular proteases and antimicrobial peptides. NETs have been shown to serve as traps for bacteria and possibly a way to concentrate antimicrobial peptides at the host pathogen interface.[48] Other antimicrobial proteins including lysozyme, lactoferrin, human beta defensin-2 (HB-2), and immunoglobulin A (IgA) are secreted into the ASL-mucus layer.[49] Mucus rheology is determined by mucus hydration and by polyionic interactions between mucin molecules. These factors, in turn, depend on the pH and salt content of the ASL, and this may explain the thick, relatively immobile mucus seen with the impaired transepithelial Cl^- secretion and accelerated Na^+ absorption characteristic of cystic fibrosis.[50]

Mucociliary Function

Optimal mucociliary function depends on complex interactions among cilia, the mucus layer, and the intervening ASL. Both the mucus layer and the ASL are moved unidirectionally along

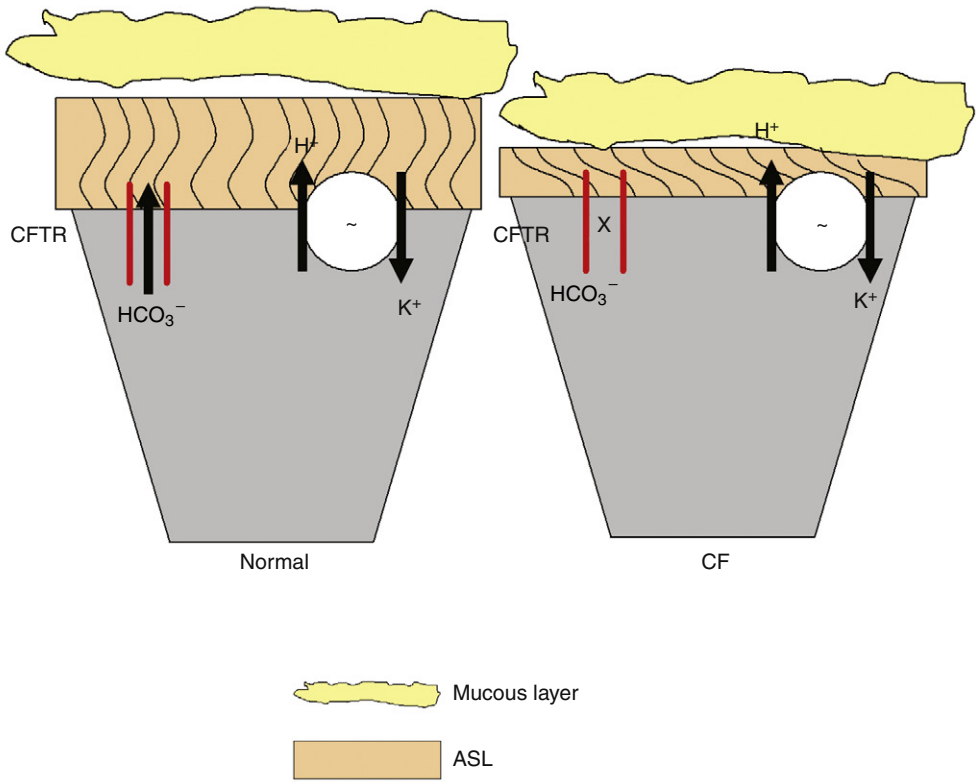


Figure 159-3 Regulation of the ASL by CFTR. The ASL is regulated by active ion transport across the epithelial cells with concomitant fluid movement that is either passive or aquaporin regulated. CFTR regulates both Cl^- and HCO_3^- transport, and, along with the epithelial sodium channel (ENaC), regulates the height of the ASL. Disease-causing mutations of the CFTR result in defective Cl^- and HCO_3^- conductance and reduction of the ASL. This leads to ciliary dysfunction and mucous impaction.

airway surfaces by ciliary action; cilia effect movement of the mucus layer through direct contact while frictional interaction with the mucus layer "drags" periciliary fluid. Beyond their propulsive function, cilia also impart vertical movements within the mucus layer; inhaled particles deposited within the airways are effectively "churned" into the mucus layer, facilitating binding by mucins and retention within the mucus layer until forced removal by mucociliary transport occurs.[51] Using aerosols of technetium-labeled albumin one can measure mucociliary clearance in humans. Mucociliary transport has been estimated to be 4 to 5 mm/min in the trachea, decreasing in the smaller airways to less than 0.4 mm/min in the bronchioles. Overall mucociliary clearance of the lung is achieved in two phases: (1) an initial rapid phase (half-life of about 4 hours) representing mucociliary clearance of the tracheobronchial tree and (2) a simultaneous slow phase lasting weeks to months, which represents alveolar clearance by nonmucociliary transport mechanisms.[52] Humans exhibit a wide range of mucociliary clearance rates, implying some endogenous control of this process. As noted earlier, purinergic regulatory mechanisms have been postulated, given the demonstrated effect of secreted nucleotides on ciliary beat frequency, goblet cell degranulation, and periciliary fluid secretion.[53] Mucociliary function continues to develop postnatally, gradually becoming adultlike over several weeks.

Mucociliary function can be perturbed by alterations in ciliary activity, changes in ASL composition, mucus secretion, or mucociliary interactions. Relevant environmental influences include temperature and humidity. Ciliary beat frequency and mucus transport rates are diminished at conditions below body temperature. Environmental tobacco smoke also reduces mucociliary clearance.[54] Furthermore, high inspired concentrations of oxygen can also damage the respiratory epithelium and impair mucociliary clearance.

Bacterial exotoxins can also perturb mucociliary clearance. Cholinergic agonists increase mucus clearance by increasing rates of mucus secretion and ciliary beat frequency, whereas atropine decreases mucociliary clearance and transport rates.[55] β-Adrenergic agonists increase intracellular cAMP levels, which increase ciliary beating ion and fluid secretion and mucus elaboration, resulting in the expected increase in mucociliary transport rates.[32,55] Methylated xanthines also enhance mucociliary clearance, presumably through similar actions on epithelial cell cAMP levels.

The critical role of cilia in mucosal host defense in humans has been clearly demonstrated by disorders that disrupt ciliogenesis or result in impaired ciliary function. These patients often lose sterility of the lower respiratory tract and suffer from sinusitis, bronchitis, and ultimately bronchiectasis. Primary ciliary dyskinesia (PCD) is a group of genetic disorders that result in congenital ciliary dysmotility state. Some mutations result in an absence of an intermediate chain dynein important in assembly and coordination of heavy chain dyneins of the ciliary shaft.[56] A subgroup of PCD is manifested by the triad sinusitis, bronchiectasis, and situs inversus termed "Kartagener's syndrome."[57] Other structural defects such as abnormal cilia length and orientation have been described in association with bronchiectasis and recurrent pulmonary infection. There is an ongoing effort to establish a better phenotype of these patients to allow better gene association studies to advance the gene-based diagnosis of these disorders. In addition to congenital disorders, many respiratory infections including both viral and bacterial infections can impair ciliary function. Adenovirus infection, particularly serotype 7, has been shown to cause marked damage to lung epithelium resulting in impaired ciliary function and the development of bronchiectasis.[58] As a result of marked decrease in blood flow to the damaged lung, the patient may develop a hyperlucent lung as described by Swyer and James.[59]

Another important cause of acquired ciliary dysfunction is the use of positive pressure ventilation in the neonate. Ventilator-associated lung injury and bronchopulmonary dysplasia (BPD) can impair mucociliary clearance. The airway epithelium is exquisitely sensitive to mechanical injury, and manipulations such as endotracheal intubation and/or the use of suction catheters have been shown to cause flattening or denuding of the epithelium in concert with a local inflammatory response. Prolonged mechanical ventilation has been shown to result in squamous metaplasia of the respiratory epithelium. Additionally, BPD is associated with bronchomalacia, which may further impair regional mucociliary clearance.[60]

AIRWAY REFLEXES

Integration of Breathing and Swallowing

Shared upper pathways for breathing and swallowing require that these functions be closely coordinated to prevent pulmonary contamination with oropharyngeal contents. In the neonate, multiple protective processes functionally separate the respiratory and alimentary tracts during swallowing.[61] The more cranial position of the larynx provides closer approximation of the epiglottis and soft palate, better isolating the oral cavity from the rest of the upper airway. Because newborns have lower resistance across the nasal passage than across the oral passage, owing to deficient control of oropharyngeal musculature, nasal breathing is favored, especially by preterm newborns; this may also facilitate functional separation between the upper tracts. Finally, it is well documented that neonatal breathing is interrupted by airway closure during nutritive sucking.[62]

Swallowing occurs early in fetal life, and three distinct stages have been identified: oral, pharyngeal, and esophageal. In neonates, the oral stage is coincident with nutritive sucking. During the pharyngeal stage, the bolus moves through the pharynx into the esophagus; the larynx is elevated and pulled forward; the epiglottis covers the laryngeal opening, which is further sealed by contraction of the laryngeal adductors; and inhibition of breathing occurs. At the end of the swallow, the airway reopens, and the esophageal phase follows.[63] This transient apneic response is also seen in nonfeeding swallows, which allow the infant to clear the airway of oral secretions or regurgitated materials before resuming breathing.[64,65] Premature infants typically exhibit incomplete integration of swallowing and breathing functions, such that oral feeding may result in pronounced apneic pauses.

When studied, intact cough reflexes were found in only 50% of term infants and only 25% of preterm infants; therefore, when challenged by a swallowing misadventure, neonates are more likely to defend the airway by sustained laryngeal closure than by coughing. Reflex laryngeal closure is mediated by stimulation of receptors in the laryngeal mucosa innervated by branches of the superior laryngeal nerves.[66] Although potentially pathologic, this response is also adaptive because attempts to breathe against an obstructed airway are reduced. Conversely, failure of laryngeal closure and premature termination of the apnea before airway clearance has occurred results in aspiration. As maturation occurs, coughing replaces apnea as the primary response to stimulation of these receptors.[67]

Cough and Forced Exhalation

In addition to mucociliary transport, cough is an important mechanism to accelerate the transport of inhaled or aspirated particulates out of the lung. The accessory muscles of expiration are used for coughing and sneezing. Contraction of the internal intercostal muscles decreases the antero-posterior diameter of the thorax, aiding in expiration. The abdominal muscles aid in forceful expiration (such as coughing) by increasing intraabdominal pressure, which is transmitted through the diaphragm to the pleural space. To move the mucus in the direction of the large airways, the shear force generated by the airflow must overcome

mucus viscosity and gravitational forces. At high airflow velocities (2500 cm/second), mucus is rendered off the surface as droplets, producing the mist flow characteristic of sputum expectoration from the trachea; such airflow velocities can be reached in the small airways during cough or forced exhalation.[67]

For a cough maneuver, there is rapid inhalation of a supranormal tidal volume followed by glottis closure, an intrathoracic pressure increase (50 to 100 mm Hg), and glottis opening with rapid exhalation. Although the cough reflex is quite effective in older children and adults, the cough is not as effective in neonates. This is the result of the markedly enhanced compliance of the chest wall and cartilaginous airways that impairs the ability to transfer pleural pressure to transpulmonary pressure. In addition, as the airways are more compliant, the neonate is subject to more airway collapse during the cough reflex. Coughing is also less effective in settings of respiratory muscle weakness or inability to close the glottis as when endotracheally intubated. A coordinated cough maneuver relies on local chemoreceptors and mechanoreceptors, vagal afferents, signal integration in the dorsal medulla, and efferent innervation to relevant muscles of inspiration and expiration. The receptors inducing cough are poorly characterized, but may be activated by local mediators including histamine, tachykinins, and substance P.[68] Cough can also be induced by stimulation of other vagally innervated sites such as the external auditory canal or esophagus. The afferent limb involves the vagus nerve and vagally innervated structures such as the larynx and conducting airways. Of these, the larynx and upper airways seem more sensitive to mechanical stimulation, whereas the lower airways are more chemosensitive.[68] Vagal afferents from these sites synapse in the nucleus tractus solitarius of the doral medulla, the putative "cough center." Efferent pathways include the recurrent laryngeal nerve, which stimulates glottic closure, and spinal nerves from C3 to S2, which innervate intercostal, abdominal, and pelvic muscles required to achieve sufficient tidal volume and expiratory pressure. As noted earlier, the cough reflex of premature neonates tends to be immature or absent, which is attributed to incomplete myelination of vagal afferents.[43a]

In the absence of effective cough, additional airway protective reflexes exist. Mechanical irritation of the epipharynx via the glossopharyngeal nerve elicits the sniff-like aspiration reflex by which foreign particles can be removed from the back of the nose to be either swallowed or expelled by mucociliary clearance. Mechanical stimulation of the vocal folds by means of the superior laryngeal nerve elicits a brief expiratory effort without preceding inspiration (the expiration reflex).

INNATE IMMUNE MECHANISMS OF HOST DEFENSE

Despite barriers to microbial colonization and invasion discussed earlier, particles of 2 to 10 μm in diameter may still infiltrate the lower airways and alveoli. Furthermore, anatomic barriers may be inadequate in situations of overwhelming pathogen inoculum via the air stream or ineffective in the situation of lung microbial invasion through hematogenous routes. Immune protective mechanisms are therefore frequently employed to contain penetrant microorganisms. Such mechanisms may be broadly characterized as either innate or adaptive. Adaptive immunity relies on specialized antigen-presenting cells to activate T cells and B cells, resulting in clonal expansion of lymphocyte pools unique for a specific antigen. Although advantageous for their specificity and capacity for long-term "memory," such responses are also delayed, owing to the multiple cellular interactions required. This is especially true in the setting of an "immunologically naive" host such as the newborn. Because delay in this setting may be deleterious to survival, capacity for more immediate immune response must also exist. The soluble and cellular components of this immediate host response constitute innate immunity.

Innate immunity is phylogenetically primitive, nonspecific, immediate, and the primary antimicrobial defense of the naive host. Innate immune responses recognize and target structurally conserved molecular sequences shared among groups of pathogens; these include LPS of gram-negative bacteria, lipotechoic acids of gram-positive bacteria, bacterial lipoproteins or peptidoglycans, mycobacterial glycolipids, fungal mannans or beta-glucans, unmethylated bacterial DNA sequences, and double-stranded viral RNAs. These are referred to collectively as "pathogen-associated molecular patterns" (PAMPs) and are recognized and ligated by pattern recognition receptors (PRRs) of the innate immune system. PRRs are nonclonal and do not depend on immunologic memory because they are germline encoded. These receptors are expressed on barrier and effector cells—such as epithelial cells, endothelium, and phagocytes—of innate immunity where binding and activation induce either direct phagocytosis or cellular signals culminating in leukocyte recruitment and nonspecific inflammation.[69] Alternatively, PRRs are also components of soluble or secreted humoral proteins. When present in the airway lining fluid, they constitute an additional "chemical barrier" and complement mucociliary clearance.[70,71]

Soluble Elements of Innate Immunity

As noted earlier, bacterial clearance from peripheral airways by mucus transport may require up to 6 hours; because bacteria can exhibit doubling times of fewer than 20 minutes, forced mucociliary clearance alone is potentially inadequate to maintain alveolar and airway sterility. Therefore concurrent mechanisms to contain bacterial growth must be present. Products of the neonatal lung that may confer innate, or nonspecific, antimicrobial protection include complement, other opsonins such as fibronectin, antimicrobial peptides (defensins, cathelicidins), lysozyme, collectins, and NO.

Complement

The complement system is a sentinel limb of innate immunity because it can be directly activated by component recognition of PAMPs. As such, it is a potentially important neonatal host defense providing a mechanism for direct bacterial lysis in the absence of optimal cellular immune responses. Specific complement components also function as microbial opsonins to facilitate phagocytosis and as chemotaxins to facilitate granulocyte recruitment.[72-75] The complement system consists of more than 20 proteins synthesized by cells of the liver or the reticuloendothelial system. Once activated, most components exhibit a proteolytic function responsible for activation of subsequent components in orderly series. Although normally quiescent, circulating and local complement components may be activated by any one of three major pathways.

The classical complement pathway is initiated by the binding of C1q to the Fc portion of either IgG or immunoglobulin M (IgM) complexed to antigen. This results in an amplified cascade leading sequentially to activation of C4b2a ("C3 convertase"), generation of C3a and C3b, and formation of the C4b2a3b complex that cleaves C5 into its split components C5a and C5b. The MB lectin pathway is mediated by mannan-binding lectin (MBL), an acute phase protein of the collectin family structurally similar to C1q.[76-79] MBL possesses domains that recognize and bind membrane glycoproteins of bacteria, yeast, mycobacteria, and certain viruses.[80,81] Pathogen binding induces an MBL conformational change, exposing two MBL serine proteases that activate C4, ultimately generating classical C3 convertase.[82-85] The alternative pathway is initiated by a number of substances, including endotoxin, complex polysaccharides, immune complexes, and surface components of intact cells such as certain bacteria and fungi.[86-91] Binding of nascent C3b fragments to these substances

protects C3b from inactivation by serum factors H and I. Subsequent cleavage and binding of factor B and the stabilizing protein properdin yield C3bBb, alternative pathway "C3/C5" convertase, which enzymatically cleaves C5 into the active components C5a and C5b. Each pathway ultimately activates terminal components of the complement system to form C5b-9, which inserts into lipid bilayers to form a transmembrane pore, permitting bidirectional solute flow and ultimately cell wall lysis.

Although there is considerable variability in data regarding neonatal complement levels and function, some generalizations can be made. Although many neonates have complement levels within the adult range, the level of components and activity of the alternative pathway are consistently diminished relative to those in the classical pathway. Marked deficiencies of C8 and C9 are seen and appear associated with poor killing of gram-negative bacteria in vitro.[92,93] Poor cross linking of C3 may contribute to decreased lytic and opsonic activity.[94,95] Preterm neonates have more consistent impairment of both pathways.[96,97] Complement levels increase steadily postnatally and reach adult levels by 12 to 18 months of age.[98,99] Finally, whereas MBL serum levels of term neonates approach those of adults, values in preterm neonates are roughly 60% of levels at term; the absence of increased morbidity or infection suggests only an adjunctive role for the MBL pathway in this population.[100,101]

Complement components are found throughout the lower respiratory tract and are produced locally by alveolar macrophages, pulmonary fibroblasts, and type II epithelial cells (Figure 159-4).[102-104] In this milieu, complement activation can be initiated by PAMPs or in the immune host by IgM or IgG microbial binding. Each pathway results in binding of component C3b, which is recognized by specific receptors on neutrophils and macrophages, facilitating phagocytosis. C3a and C5a are potent neutrophil chemoattractants, whereas C5b initiates the membrane attack complex effecting direct, non leukocyte–mediated bacterial killing. Evidence that complement is critical in defending against some pulmonary infections is provided by murine models of acquired and inherited hypocomplementemia, in which pulmonary clearance of *S. pneumoniae* and *P. aeruginosa* is impaired.[105-107]

Fibronectin

Fibronectins are extracellular matrix glycoproteins capable of interacting with a number of macromolecules and cells bearing specific fibronectin receptors.[108] The ability of specific mesenchymal cells such as fibroblasts to elaborate fibronectin during embryogenesis is critical in thoracic development. After birth, basal expression of fibronectin is limited primarily to hepatocytes, which produce circulatory fibronectin, and to the respiratory tract where fibronectin is present in saliva produced by bronchoepithelial cells and constitutively secreted by alveolar macrophages.[109] Serum concentrations of fibronectin are diminished in neonates (particularly in preterm infants), whereas bronchoalveolar lavage (BAL) levels appear inducible in settings of lung injury.[110-112] Enhanced fibronectin production by pulmonary fibroblasts, alveolar macrophages, and epithelial cells is induced by local production of macrophage-derived cytokines associated with acute inflammation, TNF-α, IL-1β, transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF).[113] In acute lung injury, fibronectin stimulates fibroblast and epithelial cell recruitment and endothelial cell proliferation to facilitate tissue reparative processes. However, the ability of fibronectin to bind certain bacteria and to augment leukocyte adherence and migration suggests an additional role for this molecule in lung antimicrobial defense.

Fibronectin is bound by a number of pathogenic bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, and *P. aeruginosa*) as well as *Mycobacterium* species, *Pneumocystis,* and fungi.[114-119] Because fibronectin binds multiple microorganisms it was originally thought to promote phagocytosis and destruction of the invasive organism before the onset of a specific immune response; studies demonstrating increased neutrophil and macrophage binding of *S. aureus* supported this theory.[120-122] Opposing studies suggested that fibronectin by itself may possess only weak opsonic activity.[123,124] More recent data indicate that fibronectin-binding proteins (FnBPs) present on the surface of *S. aureus* and streptococcal organisms promote adhesion of the bacteria to host tissue leading to increased

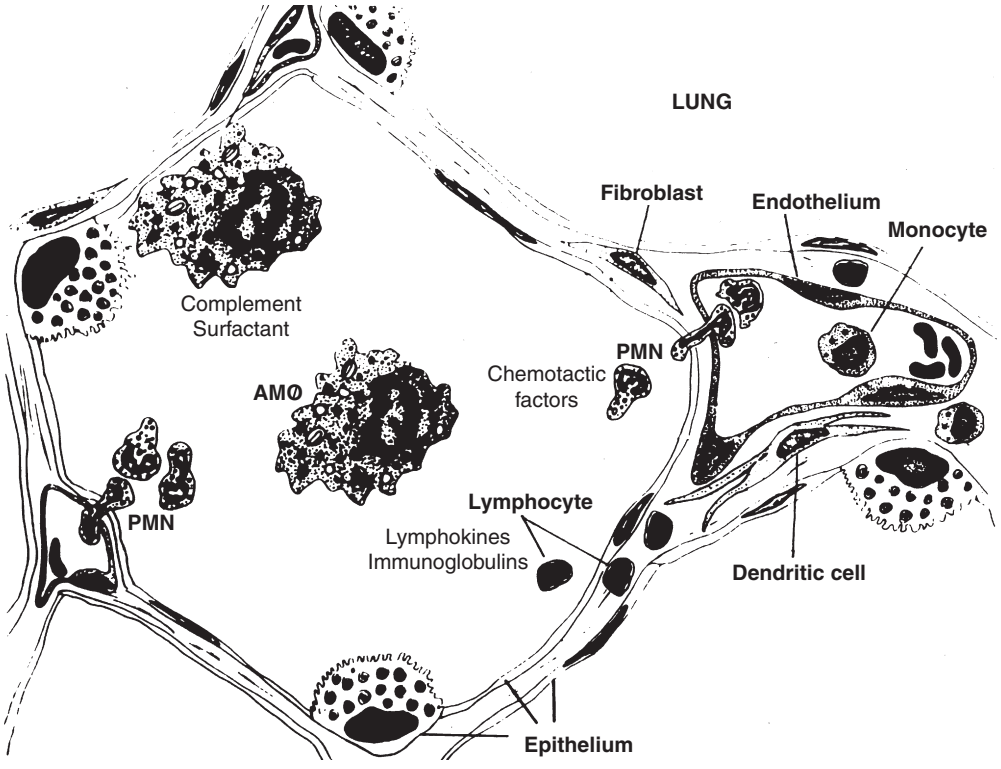


Figure 159-4 Normal alveolar architecture. Note specifically the relative location of structural cells (fibroblasts, epithelial cells, endothelial cells), which may be stimulated to participate in modulating local immune-inflammatory events. As noted in the text, vascular endothelial cells are strategically located to directly facilitate immune cell recruitment to the alveolar space. *(From Toews GB: In Shelhamer JH [moderator]:* Ann Intern Med *117:415, 1992.)*

colonization and infection.[125-127] This would be particularly problematic in cases of impaired mucociliary clearance or epithelial cell damage, yielding a partial explanation of pulmonary microbial tropism in these settings. In vitro studies have demonstrated that *S. aureus* can be internalized by a variety of nonphagocytic cells including epithelial and endothelial cells, fibroblasts, osteoblasts, keratinocytes, and kidney cells.[128-132] Furthermore, recent studies have shed light on a newly discovered mode of internalization through fibronectin-binding proteins, which may avoid fusion with the degrading lysosomes of phagocytic cells.[133-136] In the normal lung, the most important host defense role of fibronectin may be to facilitate binding of, and local colonization by, nonpathogenic organisms.

Iron-Binding Proteins

The availability of iron in the host environment and its effect on bacterial growth is one of the best studied aspects in pathogenicity.[137,138] Iron is an essential element for bacterial growth and survival, thus pathogens have evolved various ways to compete for the host's iron. Some of the strategies developed during the coevolution of host and pathogen include the production of low-molecular-mass iron–chleating compounds (siderophores); expression of transferrin and lactoferrin receptors; proteolytic cleavage of nonbinding glycoprotiens; disruption of iron-binding sites; and reduction of ferric to ferrous complex.[97,139] Host defenses that have evolved to restrict bacterial colonization or invasion include mechanisms to sequester elemental iron either in cells or complexed to transport proteins.[140] There are currently five known iron-binding proteins that have been described in human innate immunity: lipocalin, hepcidin, Nramp, the transferring family of proteins (lactoferrin and transferrin), and ferritin (cytsolic and plasma).[141-155] The primary iron-binding protein in serum is transferrin, whereas lactoferrin predominates in mucosal secretions. Because each of these proteins is present in airway mucosal secretions and airway lining fluid, they may be important local protective factors in containing microbial growth.Transferrin is found in BAL fluid in amounts between 1 and 2 times greater than matched serum; human lactoferrin is stored in specific granules of polymorphonuclear neutrophils (PMNs) from which it is released following activation.[140] It binds with high affinity to lipid A and may play an antibiotic role by depriving invading microorganisms of iron.[146,156] In contrast, lactoferrin is the predominant iron-binding protein of airway secretions and is rarely present in deep alveolar lavage unless inflammation is actively occurring; it is also stored in neutrophil granules in addition to its secretion on mucosal surfaces.

Iron-binding proteins effectively complex free iron available in mucosal secretions and alveolar lining fluid, thereby limiting the growth of most iron-dependent pathogens; notable exceptions include *Moraxella* and *Neisseria* species, which are able to obtain iron from these proteins.[157] In addition to iron binding, human lactoferrin has other antimicrobial activity against specific bacteria, including *E. coli*, *S.pneumoniae*, and *Legionella pneumophilia.*[83,158] Lactoferrin binds to LPS, disrupting gram-negative bacterial membranes, and this effect may be synergistic with the effects of other soluble components of the mucociliary layer such as lysozyme.[159] Lactoferrin also demonstrates antiviral activity, binding to HIV and cytomegalovirus (CMV), preventing their uptake by host cells.[160] Cleavage of lactoferrin by either proteases or pepsin yields a small cationic peptide, lactoferricin, with structure and antimicrobial activity similar to those of the defensins.[161] Finally, recent work suggests a unique role for lactoferrin in prevention of bacterial biofilms. Biofilm formation is a growth mode specialized for long-term bacterial colonization of surfaces, as is seen in chronic *Pseudomonas* infection; once established, organisms within biofilms are notoriously resistant to host eradication.[162] By chelating iron, lactoferrin stimulates "twitching," a pilimediated bacterial motility that deters biofilm formation. Once a biofilm has been established, however, sensitivity to this lactoferrin effect is lost.[147]

Beyond these antimicrobial effects, lactoferrin may also play an important role in modulation of local immune response. As a component of neutrophil secondary granules, lactoferrin is delivered to sites of inflammation. Here, lactoferrin binds LPS, effectively competing with LPS-binding protein and minimizing endotoxin presentation to immune cells.[163] Similarly, lactoferrin also recognizes and binds recurrent "motifs" of bacterial DNA, potentially dampening the broad proinflammatory immune responses that would ordinarily be triggered.[164] Teleologically, this may allow lactoferrin to limit excess immunostimulatory activity at mucosal surfaces with microbial exposure.

In addition to the primary iron-binding proteins, lactoferrin and transferrin, recent studies have provided insight into the antimicrobial roles of lipocalin, hepcidin, and natural resistance-associated macrophage protein (Nramp). Using a mouse model, host lipocalin has been shown to bind bacterial siderophores, thereby preventing iron acquisition by invading pathogens.[141,165] The human analogue of lipocalins was recently renamed siderocalin after demonstrating its dramatic up-regulation following bacterial colonization with *S. pneumoniae* to potentially bacteriostatic levels, stongly indicating its role as a host defense mechanism in the upper respiratory tract.[142]

Hepcidin, first discovered by Park and colleagues,[144] possesses antimicrobial activities. Originally, only hepatocytes were thought to produce this iron regulatory protein. Recent evidence, however, has demonstrated that alveolar macrophages also produce hepcidin in addition to expressing the iron importer divalent metal transport (DMT1) and iron exporter ferroportin1 (FPN1) proteins.[166] Further studies suggest that upon exposure to LPS, iron mobilization by alveolar macrophages is reduced and is mediated by hepcidin-induced degredation of the FPN1; as such, less iron is available to the pathogen.[166]

Nramp was discovered more than 30 years ago to possess antimicrobial properties. It is now widely accepted that the expression of Nramp1 (also called Slc11a1) confers innate immune defense against certain bacterial infections. It is thought to work by sequestering iron from bacteria within the phagosome or through a reaction in which iron catalyzes the production of toxic hydroxyl radicals.[167] Nramp1 has further been shown to play a role in early macrophage activation, and its expression is up-regulated by interferon γ (IFNγ), LPS, and granulocyte–colony stimulating factor (G-CSF).[87,168-171]

Antimicrobial Peptides (Defensins, Cathelicidins, Lysozyme)

One form of host defense, found in both plants and animals and therefore predating the separation of the plant and animal lineages, is the production of antimicrobial peptides. These endogenous peptides complement the activity of the larger opsonizing or nutrient-binding proteins in maintaining airway sterility (Table 159-1). They act by disrupting cell membranes of a wide range of pathogens including bacteria, viruses, and fungi; each peptide manifests a broad but fixed spectrum of activity, underscoring the need for multiple peptide classes within the airway lining fluid.[172-174] Within the respiratory tree they are either produced by epithelial cells, where their expression is both constitutive and inducible, or delivered to vulnerable loci by circulating leukocytes. Apart from their direct antimicrobial activity, which is nearly immediate in onset, these peptides can also activate cellular immunity, amplifying host response as necessary. The major human antimicrobial peptides are lysozyme, cathelicidins, and the defensins.

Defensins are small (3 to 6 kDa, 29-40 amino acids) cationic peptides containing six conserved cysteine residues. They are divided into α and β subclasses on the basis of their secondary

TABLE 159-1

Antimicrobial Products in the Mucosa (Partial Listing)

Product	Relative Concentration	Source
SLPI	μg/mL	Epithelia, Paneth cells, macrophages
Lactoferrin	μg/mL	Epithelia, Paneth cells, neutrophils
SP-A, SP-D	ng-μg/mL	Epithelia, Type II pneumocyte
α-Defensins	ng-mg/mL	Paneth cells, neutrophils
β-Defensins	ng-mg/mL	Epithelia, neutrophils, glands
Reg3γ/Reg3β	ng-mg/mL	Paneth cells
Lipocalin-2	ng/mL	Epithelia, neutrophils
CCL20	ng/mL	Epithelia, neutrophils, macrophages
PGLYRP-1	μg-mg/mL	Neutrophils, M cells
PGLYRP-3, PGLYRP-4	ug/mL	Epithelia, glands
Cathelicidins (LL37)	ug/mL	Epithelia, neutrophils

structure, but their gene locations imply a common evolutionary origin.[175] Structurally they share a β-sheet conformation that spatially segregates their cationic and hydrophobic amino acid clusters into an amphipathic motif.[176] When secreted, defensins are driven into anionic phospholipid bilayer membranes by electromotive forces where they multimerize into channels, disrupt normal membrane function, and induce cell lysis.[176,177] Although host cells are potential "bystander" targets, their lower anionic lipid content and the presence of cholesterol as a membrane stabilizer imbue significant protection.[178,179] Apart from their structural differences, the α and β defensins differ in their sites of expression and roles in airway defense.

The human α-defensins constitute a subclass of six members: four "human neutrophil peptides" (HNP-1 through HNP-4) and two "human defensins" (HD-5 and HD-6). HD-5 and HD-6 are secreted by Paneth cells of the small intestine and epithelia of the female urogenital tract and are presumed to attenuate local commensal burden; respiratory epithelia is not a source of α-defensins.[180,181] HNPs 1 through 4 are abundant (up to 50% of total protein) in primary granules of neutrophils, where they assist in nonoxidative killing, and are also found in NK cells, monocytes, and lymphocytes.[182,183] Individual α-defensins have unique spectra of antibacterial activity against gram-positive and gram-negative species, as well as activity against enveloped viruses (including *herpes* species) and activity against *Candida.*[173,175,184] The α-defensins also exhibit both proinflammatory and antiinflammatory activities. As discussed later, they are chemotactic and induce chemotaxins for a variety of immune cells. They also bind to serine protease inhibitors, amplifying PMN-derived elastase activity.[185] Conversely, they can inhibit complement activation by binding to C1q and participate in local wound repair by stimulating epithelial cell proliferation.[186,187] Extracellular α-defensins are scavenged by α_2-macroglobulin, which minimizes cytotoxicity to host cells and down-regulates defensin concentration at inflammatory sites.[188] Immunohistochemical analysis has demonstrated HNPs on bronchial epithelial surfaces and in mucinous exudate in the air spaces, presumably derived from PMN granule release within the airway.[189] In adults, α-defensins have been found in BAL fluid of patients with pneumonitis, correlating with chemotaxin concentration and PMN counts.[190]

Unlike the α-defensins, the β-defensins are produced by airway epithelium. This subclass contains four identified human β-defensins (HBD-1 through HBD-4), although more than 20 genes are postulated to exist.[191] As a group, these peptides demonstrate antimicrobial activity against several gram-positive and gram-negative organisms, as well as *Candida* and *Aspergillus* species; however, differences in antimicrobial spectra exist between individual peptides.[181,192] Histologically, β-defensins are expressed in the epithelia of airways and lung and in the serous cells of submucosal glands.[192,193] HBD-1 is found in the BAL specimens of healthy adults; its expression in the airways appears to be constitutive and noninducible.[194] In contrast, the remaining β-defensins are all inducible, with HBD-2 the best characterized. Endotoxin, *Pseudomonas,* and the cytokines TNF-α and IL-1β each induce HBD-2 expression, and this induction may be amplified further by concurrent alveolar macrophage stimulation. Apart from this interaction with alveolar macrophages, HBDs further interface with cellular immunity by functioning as chemoattractants, or "microchemokines." HBD-1 and HBD-2 are each chemotactic for immature dendritic cells (DCs), as well as memory T cells by means of ligation of the chemokine receptor CCR6. Sustained β-defensin induction by noncontained microbial stimuli may thus invoke cellular and adaptive immune responses. In vivo, tracheal aspirates of term and preterm newborns demonstrate similar levels of HBD-2 with increased levels seen in local and systemic infections. These data imply that β-defensin responses are intact even in preterm infants and that neonates may up-regulate some facets of pulmonary innate immunity in the context of a systemic inflammatory response.[195] More recent information confirms that HBD-2 is the predominat β-defensin in human neonatal lung; however, its abundance in neonatal tracheal apsirates increases as a function of gestational age. Compared to HBD-2, HBD-1 had a lower level of expression, and HBD-3 expression was absent in neonatal airway epithelial cultures.[196] Although no direct correlations have been made, it is likely that the lower levels of HBD-2 in infants may contribute to the increased susceptibility of premature infants to pulmonary infections.

Cathelicidins are a diverse family of vertebrate antimicrobial proteins found in leukocytes and on epithelial surfaces where they function like defensins. They are produced as preproproteins, stored as inactive proforms, and require enzymatic cleavage for bioactivity. The sole known human cathelicidin is hCAP-18, which requires proteolytic activation by proteinase 3 to liberate the antibacterial peptide LL-37—a 37 amino acid molecule whose amphiphilic α-helical structure facilitates affinity to and disruption of bacterial membranes. Human LL-37 displays a range of antimicrobial activities. It is a broad-spectrum antimicrobial with effects against *Actinobacillus*, *E. coli*, *P. aeruginosa*, *Enterococcus faecalis*, and *S. aureus,* and it demonstrates synergy with the defensins. LL-37 also directly binds endotoxin and is chemotactic for eosinophils, neutrophils, monocytes, and T lymphocytes.[197] LL-37 is found in primary granules of neutrophils and has also been shown in NK cells, lymphocytes, and monocytes. Expression of LL-37 in the lung is seen in cells of the submucosal glands and surface epithelia of the proximal airway, where its elaboration can be induced by up to fiftyfold in states of inflammation. Interferon-γ (IFN-γ) has been shown to induce LL-37 release, but it concurrently down-regulates gene transcription, perhaps balancing antimicrobial effects against host cytotoxicity.[192] At high concentrations, LL-37 can manifest cytotoxicity toward eukaryotic cells, and hosts have scavenging mechanisms as exist for the defensins. In vivo, comparable levels of LL-37 are found in the tracheal aspirates of term and preterm newborns, with increased levels seen in local and systemic infections. A recent study demonstrated that LL-37 is present in the airway secretions of newborns, but there was no association between concentration and gestational age.

Lysozyme is a 14-kDa cationic enzyme that hydrolyzes glycosidic bonds of bacterial cell wall peptidoglycan. This peptide is found in the granules of neutrophils and mononuclear phagocytes and is also secreted by airway epithelial cells of the pulmonary tract where it confers nonspecific antimicrobial protection. Lysozyme is highly active against many streptococci, but resistance to its enzymatic activity is common among other gram-positive organisms and nearly universal among gram-negative organisms. This is likely caused by variable accessibility of

vulnerable glycosidic bonds within the cell wall matrix with the outer membrane of gram-negative bacteria providing an additional barrier to the penetration of lysozyme. However, in the presence of other membrane-targeting substances such as complement or hydrogen peroxide, lysozyme enhances the destruction of *E. coli* and other gram-negative bacteria. Lysozyme is also capable of direct antimicrobial activity toward *Streptococcus sanguinis* and *Steptococcus faecalis* species by virtue of its cationic properties and possesses fungicidal activity against *Candida albicans* by targeting the glycosidic bonds of fungal chitin. Although lysozyme may appear redundant in the presence of other antimicrobial peptides, emerging data suggest otherwise. In vivo, a transgenic murine model of lysozyme overexpression imbued increased resistance to pulmonary infection from either *P. aeruginosa* or group B *Streptococcus*. Furthermore, lysozyme exhibits important antimicrobial synergy with HBD-2, with LL-37, and with lactoferrin. More recently, immunodepletion studies have suggested that lysozyme is a major antibacterial component secreted by submucosal glands within the tracheobronchial airways.[198] Together, these findings suggest a critical contribution by lysozyme to airway defense.

Lipopolysaccharide-Binding Protein

LPS-binding protein (LBP) is a soluble 60-kDa glycoprotein that recognizes and binds the lipid A moiety of LPS, enhancing host immune response to endotoxin. LBP is homologous to other phospholipid transport proteins and functions as a transport protein that disaggregates soluble LPS and presents it to targets on cellular membranes. Membrane bound CD14 is a PRR for LPS; however, it does not contain a cytoplasmic domain and cannot transduce activating signals across the cell membrane.[199] Instead CD14 complexes with Toll-like receptor 4 (TLR4) to form the cellular pattern recognition receptor for LPS, with TLR4 acting as the transmembrane signal-transducing portion of the receptor. LPS-induced signaling through the CD14/TLR4 complex, expressed on inflammatory cells, is distinctly enhanced by the presense of LBP, resulting in the production of "early response" cytokines TNF-α and IL-1β.[200,201] LBP is produced primarily in the liver as an acute phase protein, and its plasma concentration increases exponentially during acute inflammatory responses. LBP is also a normal constituent of lung fluid, with alveolar concentrations estimated at 1 μg/mL; these concentrations also increase exponentially with pulmonary inflammation, likely owing to capillary leak of plasma LBP and enhanced local generation. The relevance of LBP to pulmonary host defense is suggested by murine transgenic models of LBP deficiency; such mice exhibit blunted alveolar bacterial clearance with increased bacteremia and lethality in response to pneumonia. Despite these findings, pulmonary sources of LBP remain a focus of inquiry. Human alveolar type II epithelial cells demonstrate capability ex vivo to up-regulate LPB production in response to mediators (TNF-α, IL-1β, IL-6) that similarly induce hepatic acute phase production of LBP. Additionally, animal models suggest that in the neonate, alveolar macrophages may be a concurrent source of LBP in the lung. Although elevated serum levels of LBP have been reported in neonatal sepsis, pulmonary LBP expression in response to respiratory infection remains unquantified in this population. The most recent studies of infant LBP suggest its use as a viable diagnostic tool for neonatal bacterial infections.[202]

Pulmonary Collectins

Pulmonary surfactant is a bioactive material that bathes the alveolar surfaces and keeps alveoli from collapsing during expiration by reducing surface tension at the air-liquid interface.[203] It is a mixture of lipids and proteins, whereby dipalmitoylphosphatidylcholine (DPCC) functions as the primary lipid for lowering surface tension. The four known surfactant protein are SP-A, SP-B, SP-C, and SP-D. Together, they have several important functions including regulation of surfactant lipid metabolism, lipid membrane organization, and pulmonary host defense.[194] As with the lipid components of pulmonary surfactant, SP-B and SP-C are extremely hydrophobic and function primarily to reduce surface tension and maintain pulmonary homeostasis after birth.[204] The role of pulmonary host defense rests with SP-A and SP-D, which along with mannan-binding lectin make up a family of carbohydrate-binding proteins known as collagenous C-type lectins or collectins. These proteins are characterized by a discrete, four-domain primary structure consisting of a cysteine-containing N-terminus, a subsequent collagen-like region, a coiled neck region, and a C-terminal carbohydrate recognition domain (CRD).[205] Collectins function as soluble scavenger receptors, interacting through their lectin CRDs with microbial carbohydrate and glycolipid PAMPs to enhance phagocytosis and pathogen clearance; through this mechanism, collectins exhibit activity against a broad range of bacterial, viral, and fungal pathogens. Beyond these opsonizing qualities, SP-A and SP-D each exerts specific immunomodulatory effects that titrate the magnitude of inflammation and influence pulmonary immune responses.[93,206]

SP-A and SP-D share many characteristics related to their synthesis and bioactivity. Both collectins are produced by type II alveolar epithelium and Clara cells and are secreted into the alveoli and distal airway. Production and secretion of both proteins increase dramatically during the third trimester of fetal lung development and appear to be further inducible in utero in response to dexamethasone; both proteins are also up-regulated in response to acute lung injury or epithelial activation by microbial products such as LPS. The pulmonary collectins directly interact with a variety of microorganisms.[207-209] Both SP-A and SP-D bind with broad specificity to bacteria including *Klebsiella pneumoniae, P. aeruginosa, H. influenza, S. pneumoniae, S. aureus, E. coli, Mycobacterium tuberculosis,* and *Mycobacterium avium.* Pathogen encounter with lung collectins results in agglutination and/or opsonization. Agglutination impedes microbial invasion and colonization and facilitates clearance by the mucociliary escalator, whereas agglutination of viruses enhances their internalization by neutrophils. Alternatively, SP-A or SP-D may act as opsonins by bridging between PAMPs on the microbial surface and collectin receptors on phagocytes. The pulmonary collectins also enhance specific leukocyte functions. Both SP-A and SP-D are chemotactic for neutrophils and macrophages, although SP-D is more potent in this regard. SP-A and SP-D both enhance alveolar macrophage phagocytic function and oxyradical production. Induction of this latter effect requires the collectin CRD(s) to concurrently engage ligand; this presumably minimizes spurious up-regulation of potentially injurious mediators. Finally, although earlier studies suggested a direct inhibition of T-cell proliferation by SP-A and SP-D, recent evidence demonstrates that this inhibition may be a result of the presence of TGF-β in surfactant protein preparations.[210] Although the relationship between SP-A and TGF-β has not been fully described, it is postulated that SP-A acts as a storage site for latent TGF-β in the lung, thereby indirectly participating in the control of inflammation.[210] Despite their many shared functions, SP-A and SP-D exhibit several biochemical differences that result in distinctive, unshared activities in vivo. Relevant differences include solubility, CRD specificity, length of collagen domain, and affinity for available collectin receptors.

About 30% of the pulmonary collectin pool is made up of SP-D, the product of a single gene. SP-D exists primarily in a cruciform structure: four homotrimeric subunits radiating from a disulfide-linked hub. Unlike SP-A, it is relatively impervious to proteolytic or elastase degradation. Its up-regulation secondary to LPS-induced cytokines confirms its role in innate host defense. SP-D binds specifically to carbohydrates containing glucopyranosides, resulting

in affinity for the core oligosaccharides of LPS, the mannose-rich oligosaccharides of influenza A hemagglutinin, and fungal cell wall glycoconjugates of *Candida* and *Pneumocystis carinii*. SP-D binds to the putative collectin opsonin receptor, gp-340, a macrophage scavenger receptor, and CD14 via interactions between the CRD and the N-linked oligosaccharides on CD14; SP-D inhibits the interactions between CD14 and LPS.[211,212] Attempts to further clarify the role of SP-D in host defense have led to the development of transgenic murine models of SP-D deficiency. Such mice exhibit a phenotype of pulmonary alveolar proteinosis, with foamy activated macrophages, hypertrophy of alveolar type II cells, and increased inducible inflammatory responses.[213] SP-D-deficient mice show impaired functions of host defense against *H. influenza,* group B *Streptococcus* (GBS), influenza type A virus, and respiratory syncytial virus (RSV), but lack of SP-D does not affect newborn survival from infection.[214-216]

In contrast, about 70% of the pulmonary collectin pool is made up of SP-A, the product of two genes, SP-A1 and SP-A2, each producing different chain types. The SP-A subunit can therefore exist as a homotrimer or heterotrimer, introducing heterogeneity to this protein. Differential tissue expression has been reported, with SP-A1 expressed in the lower respiratory tract and SP-A2 expressed in the tracheal and bronchial epithelium and submucosal glands. Its production is up-regulated by interferon-γ (IFN-γ) and other LPS-induced cytokines. Its short collagen domain and preferred hexameric structure result in a "flower bouquet" pattern, and this clustering of CRDs influences ligand selectivity. SP-A binds specifically to carbohydrates including fungal wall glycoconjugates and some capsular polysaccharides. It also binds a variety of lipids including the lipid A moiety of LPS. Leukocyte receptors for SP-A include the C1q receptor, SPR210, the 210-kDa receptor specific for SP-A, the gp-340 receptor, and CD14. Multiple studies suggest that SP-A influences LPS-CD14 binding; however, reports disagree on whether SP-A promotes or inhibits this interaction.[212,217,218] SP-A–deficient mice exhibit increased susceptibility to many pathogens including GBS, *H. influenza,* RSV, *P. aeruginosa,* and *Pneumocystis jirovecii.*[214, 219-221]

SP-A exhibits a variety of immunoregulatory activities within the alveolus. It suppresses IFN-γ, an activator of leukocyte and macrophage inflammatory activity and an essential mediator for transition to a lymphocyte-mediated adaptive immune response. SP-A may also dampen LPS-dependent cellular activation by competing with LBP. Taken together with the reported SP-A/TGF-β inhibition of T-cell proliferation, these functions suggest that SP-A acts, at least in part, by tempering inflammation that might induce alveolar injury. Such a host-protective role would be consistent with transgenic models of SP-A deficiency, where microbial challenge results in delayed pathogen clearance, increased pathogen dissemination, and enhanced inflammation with increased production of TNF-α, IL-1β, and IL-6. This paradigm, however, is complicated by contradictory data regarding SP-A induction of proinflammatory cytokines and up-regulation of inducible NO synthase (iNOS). These disparate findings are reconciled by recent observations that the response to SP-A is determined by the state of cell activation and concurrent stimuli. Thus SP-A up-regulates iNOS in alveolar macrophages primed by IFN-α and stimulated by LPS, but conversely inhibits LPS-induced iNOS if IFN-γ priming has not occurred. SP-A, therefore, augments an inflammatory response already under way but dampens similar responses evolving de novo. In this context, SP-A induction of iNOS also exerts host protective effects. In a murine model of tuberculosis, exogenous SP-A enhances pathogen entry into alveolar macrophages by almost fivefold. This enhanced entry resulted from SP-A ligation of the SPR210 receptor with increased endocytosis of the SP-A:BCG complex; SP-A–mediated up-regulation of NO; and TNF-α production, achieving increased mycobacterial killing. SP-A similarly enhances *Mycoplasma* killing through analogous NO-dependent mechanisms.

SP-A and SP-D exhibit only partial redundancy of their bacterial specificities, owing to their distinct structures and their differing affinities for specific carbohydrates and lipids. These differences may result in complementary functions that enhance the antimicrobial activity of surfactant in toto. One example of such synergy is illustrated by collectin interactions with *Klebsiella,* a pulmonary pathogen that can reversibly switch between encapsulated and unencapsulated phenotypes. Unencapsulated forms of this organism allow optimal adhesion to the epithelial surface, facilitating colonization; these forms predominate early in infection. As noted earlier, SP-A binds to lipid A of LPS whereas SP-D preferentially binds to the LPS core sugars. Because lipid A is embedded within the bacterial cell wall and inaccessible to SP-A, SP-D is the primary collectin ligand for LPS expressed on the surface of unencapsulated forms. Encapsulation limits interaction of SP-D with the underlying LPS but invokes significant binding by SP-A, which recognizes the capsular polysaccharides expressed by this pathogen. In this scenario, SP-A and SP-D thus fulfill distinct but complementary opsonizing roles in the innate host response, as depicted in Figure 159-5. Similar complementary function is illustrated by collectin interaction with influenza A virus (IAV). SP-D binds viral envelope glycoproteins (hemagglutinin and neuraminidase) through its CRD and induces massive agglutination of IAV particles; this agglutination is facilitated by higher-order oligomerization, most achievable by SP-D by virtue of its longer collagen domain. Such agglutination generates particle size sufficient for mucociliary clearance and also directly enhances neutrophil uptake and respiratory burst. Alternatively, SP-A activity results from IAV recognition and binding of sialic acid residues on this collectin. SP-A has much less agglutinating capacity, but interacts with a broader range of IAV strains including those that are highly resistant to inhibition by SP-D or serum collectins. SP-A forms much smaller aggregates, resulting in less augmented neutrophil uptake but enhanced uptake by alveolar macrophages. As before, the differing collectin structures and binding affinities result in a broader range of antimicrobial activity and immune cell activation, which ultimately prove to be beneficial to the host.

Immunomodulating Role of Surfactant

Apart from the specific actions of its collectins, surfactant also exerts a host of immunomodulatory effects within the alveolus. Surfactant enhances phagocytosis by means of a mechanism separate from the actions of SP-A. Interestingly, this prophagocytic effect is more pronounced for resident alveolar macrophages than for recruited peripheral blood monocytes. Whole surfactant reportedly suppresses mononuclear cell oxyradical production by inhibiting the intracellular assembly of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, impairing hydrogen peroxide generation. However, specific lipid components of surfactant may exert different modulatory effects. Therefore, although macrophage oxidative burst is blunted by phosphatidylglycerol moieties, it is enhanced by phosphatidylcholine components. Enhanced microbicidal function of phagocytes and down-regulation of Fc receptors have each been reported and attributed to the lysophospholipid and free fatty acid components of surfactant. Whole surfactant also modulates phagocyte chemotaxis. Macrophage movement into the alveolus postnatally coincides with increased surfactant synthesis, suggesting a chemotactic effect, possibly SP-A mediated. Conversely, DPCC treatment of alveolar macrophages in vitro decreases their migration in response to serum chemotaxins; this is consistent with a teleologically preferred "default" attenuation of inflammatory

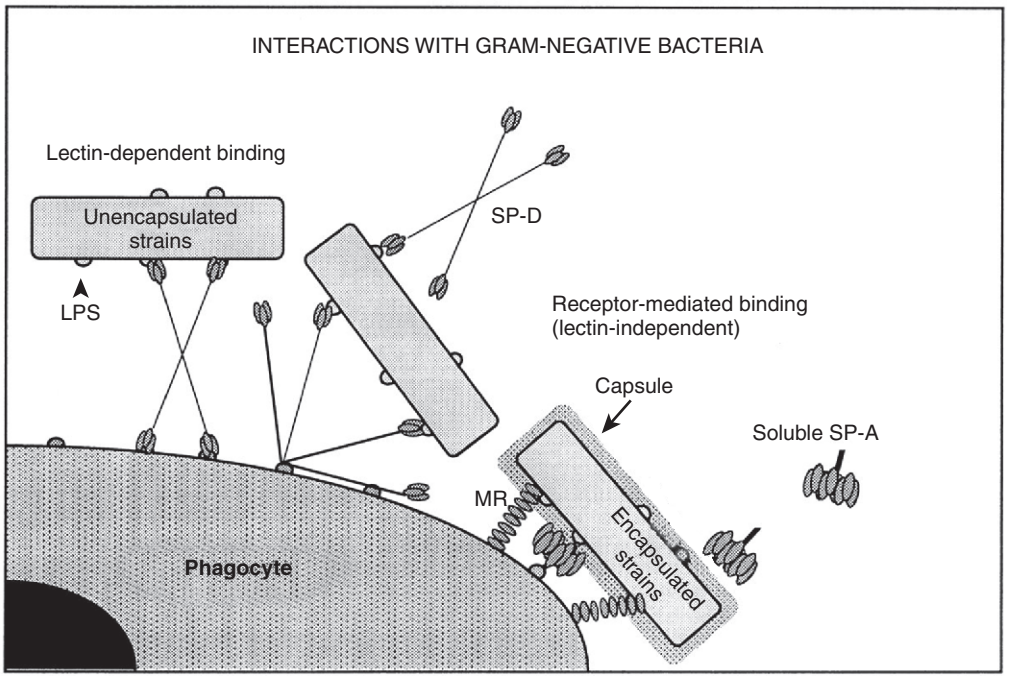


Figure 159-5 SP-A and SP-D are complementary opsonins for gram-negative bacteria. SP-D binds to LPS core sugars exposed by nonencapsulated variants, the phenotype required for colonization and invasion. Encapsulation increases pathogenicity and limits SP-D binding, but allows SP-A recognition and binding of capsular polysaccharides. Subsequent phagocytosis may occur through SP binding to phagocyte surface carbohydrates (lectin-dependent) or may be receptor mediated (lectin-independent), as depicted by SP-A engagement of mannose receptors. *(From Crouch EC:* Am J Respir Cell Mol Biol *19:177–201, 1997.)*

response designed to preserve alveolar function. Lastly, surfactant reportedly modulates lymphocyte function as well. Lymphocytes exposed in vitro to surfactant are less responsive to mitogens and exhibit depressed function; under similar conditions, cytotoxic T cells, B cells, and NK cells are all inhibited to various degrees. As discussed earlier, however, some lipid components (DPCC, phosphatidylglycerol) are more suppressive, whereas less abundant components (cholesterol, sphingomyelin) stimulate lymphocyte proliferation; this suggests that conditions that alter these phospholipids ratios may alter adaptive immune responses. Additionally, the differing effects of SP-A and surfactant lipids on lymphocyte proliferation are consistent with the hypothesis that, in some scenarios, surfactant lipids and proteins may be counterregulatory.

In contrast to whole surfactant, conventional surfactant analogues (Exosurf, Survanta) contain neither SP-A nor SP-D. Consequently, collectin-mediated immune effects are not provided by exogenous replacement therapies. Moreover, the surfactant replacements are generally immunosuppressive, presumably owing to their nonphysiologic lipid/protein ratios. Both Exosurf and Survanta have been shown to inhibit production of the early response cytokines TNF-α, IL-1β, and IL-6 by stimulated alveolar macrophages, putatively by depressing activation of the nuclear transcription factor NF-κB. Similar suppression of fibroblast-derived IL-6 and prostaglandin E_2 (PGE_2) has also been described, suggesting impairment of those inflammatory responses up-regulated by alveolar cytokine networks. Additionally, Survanta has been shown to blunt lymphocyte proliferation, killer cell cytotoxicity, and adhesion molecule expression, possibly through down-regulation of lymphocyte receptors for IL-2. Much of this is in vitro data and must therefore be interpreted cautiously; however, it suggests that available surfactant replacement therapies, although efficacious in normalizing pulmonary compliance, may concomitantly attenuate normal alveolar immune cell responses.

Nitric Oxide

NO is a short-lived, oxyradical-related bioactive mediator with potent vasodilatory actions. It mediates and modulates pulmonary transition from fetal to postnatal life and plays a role in both immune regulation and innate host defense. NO is synthesized by 3 nitric oxide synthase isoforms (NOS-1, -2, and -3), all of which are expressed in the airway epithelium from the bronchus to the distal air space[222]; thus NO is produced along the entire length of the human airway and is present in airway lining fluid at concentrations of about 15 μm.[222] NO isoforms exist in either constitutive (NOS-1, NOS-3) or inducible (NOS-2) forms; cells with constitutive NOS include neurons, vascular endothelium, neutrophils, and platelets. Cells with inducible NOS (iNOS; NOS-2) are recruitable to generate NO and include mononuclear phagocytes, alveolar macrophages, vascular smooth muscle cells, epithelial cells, type II pneumocytes, and hepatocytes.[223-228] All NOS isoforms are detected early in lung development and ontogeny shows no significant changes in abundance or distribution with advancing gestational age.[222]

NO exerts potent cytotoxic and inflammatory effects, which support its role as an antimicrobial molecule during host response to infection. NO production from cells containing iNOS is induced by microbial products such as lipoteichoic acid, endotoxin, and bacterial DNA. Locally produced NO inhibits sodium uptake from the apical surface of airway epithelial cells, increasing airway hydration and enhancing mucociliary clearance. Specific NO products such as nitrosylated thiols exert antimicrobial activity by killing the pathogen directly or by inhibiting the pathogens' replication. NO inhibits the growth of a wide variety of organisms including viruses, bacteria, parasites, and fungi, presumably by targeting microbial DNA and cysteine proteases, which are critical for virulence or replication of these pathogens.[229-231] NO appears to be particularly important in the innate immune response to intracellular organisms such

as *Listeria monocytogenes, Salmonella,* and *Mycobacterium tuberculosis.*[232] NO products also inhibit viral RNA synthesis, inactivate a broad range of microbial proteins by S-nitrosylation, and induce membrane damage through lipid peroxidation. By up-regulating inflammatory cell function; NO augments macrophage motility, up-regulates surface expression of complement and immunoglobulin Fc receptors, and stimulates leukocyte respiratory burst. Concurrent synthesis of NO increases microbicidal activity of the leukocyte respiratory burst by generating additional cytotoxic radicals such as peroxynitrite, which possesses potent activity against bacteria and *Candida.* In vitro, phagocytes demonstrate diminished microbicidal activity after NOS inhibition, and this correlates with in vivo models in which diminished NOS activity impairs microbial clearance.

The role of NO in pulmonary microbial clearance is consistent with recent neonatal data. BAL specimens from preterm (≤32 weeks estimated gestational age) infants exposed to intrauterine infection demonstrated a strong association between inability to express iNOS or up-regulate NO and subsequent development of fulminant pneumonia. Conversely, newborns of similar age and risk that did not develop pneumonia exhibited iNOS up-regulation and increased NO products on BAL analysis, relative to either their pneumonic cohorts or noninfected controls. A separate retrospective analysis of lung tissue from neonatal autopsies yielded similar findings; alveolar macrophage–derived iNOS could not be detected in specimens from fulminant pneumonia patients but was demonstrable in controls. Taken together, these data suggest that delayed or diminished macrophage–derived NO in the setting of neonatal pulmonary infection correlates with increased morbidity. Etiology of this impaired response is speculative, but may result from a paucity of mediators (TNF, IFN-γ) known to up-regulate iNOS expression. Based on the important antimicrobial function of NO, recent studies have evaluated the role of inhaled NO on pulmonary infections in humans. These studies have gerenated mixed results to inhaled NO therapy, however it is likely that infections caused by intracellular organisms will be more likely to benefit from inhaled NO therapy.[233-236]

In general, NO exerts antimicrobial effects; however, not all pathogens are inhibited by NO and in some instances may contribute to infectious disease pathogenesis. In an influenza pneumonia mouse model, targeted deletion of iNOS or treatment with NOS inhibitors led to less severe disease compared with control mice.[237] Similar observations were made in mouse models of *M. avium* pneumonia and herpes simplex virus encephalitis.[238,239] It is postulated that in these disease processes, cytotoxic effects of NO on host cells may contribute to pathogenesis.

Beyond the functions just detailed, NO exhibits additional immunomodulatory activities relevant to pulmonary host defense, which is demonstrated through its up-regulation by immune activating cytokines such as IFN-γ, TNF-α, IL-1, and IL-2. NO has been shown to reduce T helper 1 (Th1) proliferation and IL-2 synthesis while increasing IL-4 synthesis by T helper 2 (Th2) cells.[240-242] Th1 cells produce IFNγ, which is an essential cytokine in viral cell–mediated immune responses. Alternatively, Th2 cells selectively produce IL-4 and IL-5 that participate in the development of humoral immunity. NO-induced imbalance in the Th2/Th1 ratio may promote inflammation in allergic diseases like asthma while inhibiting the inflammatory response to viral and bacterial pathogens.[243] RSV is a common pulmonary infection in infants, which has been linked to a large Th2/Th1 cytokine immune response; it has been associated with the pathogenesis of RSV bronchiolitis and severity of infection in infants.[244-246]

NK cells are specialized cytotoxic lymphocytes and a primary source of IFN-γ within the alveolus; they are normally activated by IL-12. Transgenic iNOS deficiency reportedly induces a phenotype of IL-12 deficiency despite normal IL-12 levels. This phenotype is characterized by diminished IFN-γ and increased transforming growth factor-β (TGF-β), a cytokine milieu suppressing local macrophage function. Analysis of these data subsequently identified iNOS-derived NO as an essential "cosignal" for IL-12 signal transduction and activation of NK cells. In this model, the signaling role of NO was therefore critical in maintaining a cytokine microenvironment that allowed normal immune cell function. NO exerts a biphasic effect on NF-κB activity, which is dependent on local NO concentrations; as such, NO is able to both up-regulate and down-regulate the expression of a number of inflammatory mediators. In this paradigm, immune activation up-regulates iNOS, generating increased NO. Initial NO activation of NF-κB promotes expression of adhesion molecules and proinflammatory cytokines, facilitating immune cell recruitment. NF-κB up-regulation of iNOS amplifies NO production, eventually generating local NO concentrations sufficient to inhibit NF-κB activity. At this point, both inflammatory cytokine and adhesion molecule expression down-regulate, effectively dampening the component of inflammation sensitive to modulation by NO. In summary, the putative actions of NO in immune cell signal transduction suggest a sentinel role for this molecule in both the initiation and suppression of cellular immunity.

Carbon Monoxide

As with NO, evolving data suggest a potential role for carbon monoxide (CO) in pulmonary host defense. Endogenous CO derives from degradation of hemoglobin, myoglobin, NOS, and cytochromes by the enzyme heme oxygenase (HO). HO immunoreactivity is found throughout the airway in respiratory epithelium, alveolar macrophages, seromucous glands, and nose and paranasal sinuses. Like NOS, HO exists in constitutive (HO-2) and inducible (HO-1) isoforms. Whereas HO-2 is basally expressed in most tissues, HO-1 is identified as a heat shock protein inducible by a variety of stimuli including microbial toxins, proinflammatory cytokines, and reactive oxygen or nitrogen species; both isoforms are substantially expressed in human lungs. HO-1 is a cryoprotective and antiinflammatory enzyme that plays a critical role in defending the body against oxidant-induced injury during inflammatory processes.[247] It catalyzes the first and rate-limiting step in the oxidative degradation of heme to CO and two other molecules, biliverdin and ferrous iron, with cytoprotective properties.[248-250] The antiinflammatory properties of HO-1 are related to inhibition of adhesion molecule expression and reduction of oxidative stress, while exogenous CO gas treatment decreases the production of inflammatory mediators such as cytokines and NO.[251] Like iNOS, HO is induced by many infectious agents, and levels of carboxyhemoglobin and/or exhaled CO are reportedly elevated in patients with viral or bacterial respiratory tract infections. Unlike NO, however, the direct effects of CO appear less microbicidal and more antiinflammatory, or host cytoprotective. In vitro and in vivo, CO has been shown to inhibit endotoxin-induced proinflammatory cytokines, including TNF, IL-1β, and macrophage inflammatory protein-1β (MIP-1β) while concurrently up-regulating the antiinflammatory IL-10. Furthermore, in a murine model of influenza A pneumonitis, overexpression of HO-1 resulted in diminished respiratory epithelial cell apoptosis and decreased lung inflammation. Interestingly, HO appears to counteract the cytotoxicity caused by excessive production of NO.[252-255] The up-regulation of iNOS in inducible pulmonary cells upon exposure to LPS results in a burst of NO generation that is essential for bactericidal effects. However, increased amounts of NO are also associated with deleterious effects such as tissue damage.[256] Thus induction of HO-1 significantly reduces NO production and potentially restores cellular homeostatsis.[247,257] It is well accepted that HO-1 and CO play an important role in minimizing injurious inflammatory processes during infection and other inflammatory states. Recent evidence, however, has identified an antimicrobial role for CO during infection. Both in vitro and in vivo studies have demonstrated that CO increases

macrophage and neutrophil phagocytosis of the pathogenic organisms *E. coli* and *E. faecalis,* respectively.[258,259]

Cellular Elements of Innate Immunity

As discussed earlier, effective host response to incipient respiratory infection relies primarily on rapid clearance of the offending pathogen(s). When soluble components of innate immunity are insufficient to achieve organism clearance, cellular immune effectors are invoked. Cellular elements of pulmonary innate immunity include resident alveolar macrophages and DCs, as well as neutrophils and monocytes recruited to the air space. The initial interaction of these cells with microorganisms is mediated by soluble and membrane-bound PRRs that recognize and engage the common microbial motifs characteristic of PAMPs. Soluble factors such as the collectins and LBP utilize specific cell receptors to "present" organisms for phagocytosis and induce cell activation. Once activated, these cells generate signals that either further amplify innate immune responses or, alternatively, initiate transition to adaptive immunity. Of the cellular PRRs, CD14 appears to be the sentinel receptor for activating alveolar macrophages and inducing cytokines that extend pulmonary immune responses.

CD14 recognizes and binds LPS presented as a complex with LBP; however, for this interaction to induce cell activation, CD14 must interact with a second membrane protein, TLR4. TLRs are highly conserved transmembrane proteins, initially recognized in *Drosophila,* that mediate recognition of microbial products. The cytoplasmic domains of the TLRs are homologous to the IL-1 receptor, suggesting common signal transduction pathways. There are 10 TLR genes expressed in humans that are each devoted to recognizing components characteristic of pathogenic organisms. TLR4 confers responsiveness to endotoxin. TLR1 and TLR2 confer responsiveness to gram-positive bacteria, mycobacteria, and yeast. TLR3 confers responsiveness to viral double-stranded mRNA. TLR5 confers responsiveness to flagellin, and TLR9 confers responsiveness to bacterial DNA. TLRs thus provide specificity to the initial recognition of pathogens, even in the setting of "nonspecific" inflammatory cell response. Engagement of these receptors activates signal transduction pathways that overlap with IL-1β–dependent signaling pathways, generating the "early response" cytokines TNF-α and IL-1β. These cytokines enhance the microbicidal activity of alveolar macrophages and also activate other cells within the alveolus. Whereas this results in immediate amplification of inflammation, it also induces elaboration of pleiotropic cytokines that recruit lymphocytes, activate DCs, and eventually orchestrate the transition to an adaptive immune response. Innate immune pulmonary cells can be broadly classified as those that are normal residents of the lung (macrophages, dendritic cells, and mast cells) and itinerant cells that are recruited in response to infection (neutrophils and monocytes).

Macrophages

Macrophages are primary effector cells of innate immunity serving important functions of phagocytosis, microbial killing, accessory immune function, and regulation of local inflammatory processes through cytokine secretion.[260] Within the lung, pulmonary macrophages constitute a cell population of diverse function and origin. Previously thought to be monocyte derived, fetal pulmonary macrophages have been shown to precede formation of myeloid elements in the liver and bone marrow.[261] Initially present as so-called angular cells within the extravascular stroma, these cells lack the morphologic appearance of differentiated phagocytes, but proliferate and differentiate ex vivo in response to colony-stimulating factors such as granlocyte macrophage colony–stimulating factor (GM-CSF) elaborated by lung stromal elements.[262] Postnatally, descendants of fetal macrophages likely constitute a self-renewing portion of the alveolar macrophage population, which may be supplemented by cells of myeloid origin.[263] Under conditions of inflammation, peripheral blood monocytes may be recruited to the lungs where they undergo local differentiation depending on their site of influx.[264] Recruited monocytes, like the angular cells, mature into alveolar macrophages, interstitial macrophages, airway macrophages, or DCs depending on local growth factors and other stimuli; though sharing some functions, each type has slightly different characteristics, perhaps owing to its particular microenvironment.[265,266] The relative number of functional pulmonary macrophages in the neonate is uncertain, however, a recent analysis of 12 term and 11 preterm infants identified 75.8% ± 11.35% of cells in tracheal lavage fluid were alveolar macrophages.[267] Normal resident cell populations are absent from both the airway of stillborn infants and BAL of preterm infants at cesarean section.[268,269] Because true alveolar macrophages are found in infants by 48 hours postnatally, rapid expansion of this cell population clearly occurs. This is consistent with simian models, where alveolar macrophage numbers increase to near adult levels by 48 hours postnatally, temporally associated with increased alveolar surfactant levels.[270] Once macrophage population of the lungs has occurred, the relatively long life span (months) and dual sources of repletion maintain stable lung macrophage numbers, even under conditions of myelosuppression.[271-273]

Phagocytic activity of alveolar macrophages is essential for clearing air stream particulate matter, macromolecular debris (immune complexes, protease-antiprotease complexes, nonfunctioning surfactant, apoptotic cells), and microbes.[260,274] Redundancy and ruffling of the alveolar macrophage cell membrane provide an expanded surface contact area and facilitate the pseudopodal extensions characteristic of phagocytic "engulfing." Alveolar macrophage phagocytosis may be nonspecific or triggered by "pattern-recognition" cell surface receptors that recognize a number of foreign ligands. These include TLRs (TLR2 and TLR4), which bind LPS, and CD14, which recognizes LBP-LPS complexes.[275-277] Other pattern-recognition receptors bind mannose, fibronectin, or surfactant proteins, whereas more specific receptors recognize complement components (C1q and C3b) and the Fc portion of most immunoglobulins.[278-282] If the ingested material is degradable, normal alveolar macrophage surveillance function continues; otherwise, the alveolar macrophage may be removed by upward clearance mechanisms along the respiratory epithelium and mucociliary ladder or through the interstitium by means of regional lymphatic drainage.[283] Studies comparing cord blood monocytes to adult macrophages demonatrate an impaired response to LPS, owing to a decreased expression of the adaptor protein MyD88 rather than TLR4 or CD14.[284] Moreover, recent neonatal studies have demonstrated an impaired monocyte and macrophage response to multiple TLR ligands, further implicating the TLR pathway in contributing to the increased infant susceptibility to pulmonary infections.[285]

After phagocytosis, the plasma membrane–contained particle is internalized by the alveolar macrophage, forming an intracytoplasmic vacuole. This vacuole then fuses with any number of secondary lysosomes to form a phagosome. TLRs may be recruited to phagosomes to sample their contents and initiate an inflammatory response appropriate for the specific organism.[286] Within phagosomes, lysozyme, proteases, and acid hydrolases released from secondary lysosomes will digest most particles; however, because many cell wall–containing bacteria are resistant to enzymatic killing, other mechanisms for their biodegradation must be invoked. In these instances, microbicidal effect is achieved by "oxidative burst" generation of highly cytotoxic reactive species of oxygen and nitrogen, in which electron transfer to the terminal NADPH oxidase in the membrane of the phagolysosome can result in local production of superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, and NO.[287,288] Alveolar macrophages can also generate NO by expression of iNOS.[289] In the

context of these important phagocytic and microbicidal roles, animal and human studies consistently describe depressed levels of neonatal alveolar macrophage function. When neonates of various species are challenged with airway bacterial inoculums in vivo, alveolar macrophage rates of phagocytosis are prolonged, especially in premature subjects; impairment of phagocytosis is also seen in vitro. [290-292] Similarly, neonatal alveolar macrophage antimicrobial activity is also diminished; this may correlate with diminished oxidative burst capacity, deficiency of stored lysosomal materials, or poor TLR4 surface expression.[293]

In contrast to commonly encountered bacteria, viruses and other nonviral intracellular pathogens such as mycobacteria have evolved mechanisms to elude macrophage killing and survive within these cells. Relevant intracellular pathogens of the neonate include *Toxoplasma gondii*, *Listeria monocytogenes*, and herpes simplex virus (HSV); control of infection caused by these organisms therefore relies on mechanisms of enhancing microbicidal activity of the host macrophage. Under most situations of intracellular parasitism, specific cytokines such as IFN-γ, TNF-α, and GM-CSF are generated to stimulate macrophage killing of these organisms.[294,295] Altered signal transduction in response to IFN-γ has been reported for neonatal alveolar macrophages; however, data suggest that these cells can be sufficiently activated by IFN-γ to achieve intracellular killing comparable with that achieved by adult macrophages, and similar data exist from animal models of *Listeria* and HSV infection.[85,296-298] These observations have led to work demonstrating markedly decreased basal and inducible levels of T cell–derived IFN-γ in neonates, attributed to lack of priming of the naive T cell by foreign antigen.[299] Decreased IFN-γ secretion by neonatal monocytes has also been reported.[300] This delay in development of antigen-specific memory T cells, coupled with suboptimal capacity for IFN-γ expression by other cells, may account for greater neonatal susceptibility to these pathogens.

In addition to microbial killing by phagocytosis, alveolar macrophages have accessory roles in multiple other immune responses. Alveolar macrophage may contribute to infection control by direct or antibody-mediated cytotoxicity for cells expressing microbial antigens.[301] Alveolar macrophage can also metabolize complex antigens and "present" small antigen fragments to T cells. There is evidence to suggest that a lack costimulatory signals for T-cell activation reduces their effectiveness in this role; however, other studies suggest that poor up-regulation of such costimulatory molecules and antigen presenting molecules, as well as other, yet unknown, mechanisms may contribute to the increased pulmonary infections in infants.[302-305] Finally, the capacity to generate various chemotaxins and cytokines allows the alveolar macrophage to recruit multiple types of inflammatory cells to the alveolus and regulate the ensuing local inflammatory response. Although macrophage-derived IL-1 production for term and preterm neonates corresponds to that of adults, both TNF-α and IL-12 production are less than seen in adults and are markedly depressed (≤25%) in premature neonates; it has been suggested that the reduced macrophage production of IL-12 is linked to IFNγ deficiency in neonates.[101,306,307] Moreover, neonatal macrophage-derived TNF-α production is less inducible by IFN-γ than in adults. Deficient production of IFN-γ by neonatal T cells, as previously discussed, may further depress neonatal alvelor macrophage TNF-α production, diminishing alveolar macrophage capacity to fully respond to alveolar pathogens, thus creating a cyclic depression of cytokine production in the neonate.

Polymorphonuclear Neutrophils

Unlike macrophages, neutrophils are not tissue-fixed. They have a short life cycle which is spent primarily in the blood until called upon to fend of an invading pathogen. Although resident macrophages are the first to respond to an infectious process in the lungs, they are soon reinforced with large numbers of neutrophils from the pulmonary circulation, a major reservoir for marginated neutrophils. Emigration of PMNs from the local circulation into the respiratory tract is a dynamic process requiring the generation of signals that sustain PMN contact with the vascular endothelium and induce transendothelial diapedesis and chemotactically directed migration to the inflammatory locus. This adherence process is dependent on the expression of specific, corresponding adhesion molecules on both the endothelial cell and PMN surfaces, whereas diapedesis and migration are induced by the local elaboration of specific chemoattractant factors. Once recruited and activated, PMNs, like alveolar macrophages, attempt to contain invading pathogens by means of receptor-mediated phagocytosis. This results in exposure of engulfed pathogens to antimicrobial substances (defensins, bactericidal/permeability-increasing protein, serprocidins, lysozyme) found in primary granules of the PMN and to reactive oxygen species produced by the PMN "oxidative burst."[287,308-310] Unlike macrophages, however, a neutrophil that has phagocytosed a foreign particle will die soon after, whereas a macrophage can repeat this process many times. PMNs may also function locally to generate further chemotactic, proinflammatory mediators such as arachidonate metabolites and IL-8, further amplifying the local inflammatory process.[311]

Several facets of PMN physiology unique to neonates may result in blunted granulocyte recruitment relative to that seen in older children or adults. Systemically, there is diminished capacity to accelerate PMN production in response to infection, owing to both a limited PMN storage pool and already near-maximal baseline levels of PMN production.[312,313] Shorter survival times of neonatal PMNs suggested by in vitro data may contribute to more rapid depletion and hence a smaller storage pool. Specific PMN functions are also compromised in the neonate, possibly secondary to diminished LPS binding capacity and hence less endotoxin responsiveness.[314] In vitro studies suggest dual defects in neonatal PMN adhesion, resulting in only 40% to 45% binding activity to activated endothelium relative to adult PMNs. Mechanisms identified are a relative deficiency of L-selectin on the neonatal PMN surface and inability of activated PMNs to up-regulate expression of the β2-integrins; the net effect is that neonatal PMNs show impaired binding to activated endothelium and bind less avidly than adult PMNs in the presence of local chemotactic factors. Deficient chemotaxis is also consistently reported in neonatal PMNs, especially in premature infants.[292,315-317] Because surface receptor density for specific chemotactic factors appears normal, postreceptor binding factors have been implicated. These factors include a constitutively smaller pool of β2-integrins—whose surface expression is required for PMN binding and diapedesis—and impaired ability of the neonatal PMN to reorganize its cytoskeleton in response to chemotactic stimulation.[316,318,319] Impaired PMN chemotaxis has also been induced in vitro by exposure to indomethacin, suggesting further potential immunologic impairment in premature neonates requiring pharmacologic closure of a patent ductus arteriosus.[320] Finally, important differences in phagocytic activity exist between neonatal and adult PMNs. Although in vitro phagocytic activity is comparable, under conditions of decreased opsonization neonatal PMN phagocytosis is less efficacious; this may be clinically significant in the relatively opsonin-deficient neonatal alveolar milieu.[321] There may also be a diminished capacity to store and release antimicrobial peptides, although other mechanisms (superoxide anion generation, lysozyme release) for intracellular killing of phagocytized organisms appear intact.[308]

Dendritic Cells

Dendritic cells make up fewer than 1% of the pulmonary cell population but serve a critical role as accessory cells in initiation of primary immune responses. Within the lung, DCs exist as

a network of interdigitating cells lying within the alveolar septa and perivascular adventitia and within the airway epithelial cell layer where their contact with air stream particles is enhanced.[322] DCs express certain membrane antigens characteristic of mononuclear phagocytes yet exhibit poor phagocytic and microbicidal activity. Conversely, DCs are exponentially more potent at T-cell activation than are macrophages.[323] This results from their capacity to present antigen in the context of major histocompatibility complex (MHC) molecules and their surface expression of the B7 ligands CD80 and CD86, which are obligate secondary signals for T-cell activation.[324]

As presently characterized, "immature" DCs migrate into the lung where they sample the environment for particles expressing PAMPs.[71,325] If encountered, they "acquire" the antigen by receptor-mediated endocytosis.[326] Antigens are degraded in specialized endosomes, where peptide fragments are loaded onto MHC class II molecules for transfer to the cell surface. Concomitantly, the DC chemokine receptor profile is altered, either by microbial products or locally generated cytokines, inducing DC chemotaxis to tracheal bronchial lymph nodes.[327] During this migration, DCs mature from "antigen processors" to antigen presenters under the influence of locally elaborated GM-CSF.[328] Once in lymphoid tissue, DCs present antigen, stimulate the clonal development of antigen-specific T cells, and activate both cytotoxic and helper T cells; they may also directly interact with B cells to regulate antibody production.[329] Engagement of the DC CD40 surface molecule by the T cell CD40 ligand induces DC-derived IL-12, which is required for T-cell differentiation and IFN-γ production. However, diminished capacity to generate IL-12 may persist among some DC subsets, limiting the range of T-cell responses that may be invoked.[325] DCs can migrate in response to bacterial peptides or LPS, complement cleavage products, early response cytokines such as TNF-α or IL-1, or chemokines; thus inflammation or infection recruits additional DCs from the peripheral blood.[330] In the basal state, pulmonary DC populations undergo complete turnover every few days, consistent with the robust antigen exposure of the pulmonary tree.[323]

Regulation of DC function is multifactorial, consistent with their central role in titrating pulmonary adaptive immune response. One mechanism of DC regulation is anatomic; although immature DCs can acquire antigen, they cannot present it until they have emigrated to regional lymph nodes. Such strict compartmentalization minimizes (possibly spurious) T-cell activation directly within the alveolus and requires the stimulated T-cell clones to recirculate to the lung in response to additional "fail-safe" homing signals.[324] DC function is also regulated by secreted products of alveolar macrophages. Alveolar macrophage–derived NO inhibits GM-CSF–mediated up-regulation of antigen-presenting activity and directly blunts T-cell activation by interfering with signal transduction.[331,332] Alveolar macrophage–derived PGE_2, TGF-β, and IL-10 also suppress antigen processing and MHC class II expression by pulmonary DCs.[333,334] In vivo, the role of alveolar macrophage–mediated regulation is shown in murine models of alveolar macrophage depletion, where hyperresponsiveness to aerosolized antigen results.[335] Finally, cytokines produced during early inflammation, including IFN-γ, TNF-α, and GM-CSF, increase MHC class II expression and allow normalization of DC function.[336]

The prenatal ontogeny of DCs is uncertain; as with alveolar macrophages, animal data suggest early derivation from angular cells of the fetal lung.[337] In humans, two functionally distinct DC precursor subsets, myeloid (m) DC and plasmacytoid (p) DC, occur in peripheral blood. Although both subsets differentiate into efficient antigen presenting cell (APC) after maturation, activated mDC and pDC display intrinsic differences and drive distinct types of T-cell responses. The relative densities of blood pDC and mDC differ between infants and adults, with pDC being more frequent in cord blood and mDC dominating in adult peripheral blood.[81] Adult human airway mucosa contains mainly mDC, whereas the population distrubtion in early life is somewhat different.[338,339] mDCs are almost exclusively expressed by cells in the epithelial compartment, and pDCs are expressed on many cells within subepithelial cellular aggregates; very few pDCs are found in the lamina propria outside these isolated lymphoid follicles.[340] Although previous studies suggested that DCs are present in only small numbers in the respiratory tract at birth, recent and more reliable data demonstrate that these cells can be found in the bronchial mucosa shortly after birth.[324,340-342]

Results from studies evaluating the MHC II antigen processing and presentation pathway in neonates are equivocal, but current evidence suggests neonatal DCs may be defective in this regard and respond poorly to maturation signals of GM-CSF.[343-345] Alternatively, recent data suggest that human neonatal DCs are fully competent in MHC class I antigen processing and presentation.[346] In vitro data suggest that neonatal DCs have less ability to modify their chemokine receptor profiles, impairing normal trafficking.[347] Neonatal DCs also exhibit an altered cytokine expression profile relative to adults; specifically, IL-12 production is markedly impaired whereas TNF-α and IL-10 are preserved, resulting in a skewed effect toward the latter two cytokines.[347] Much of the data comparing neonatal and adult DCs are derived from cord blood–derived DCs or other nonlung origins. A recent study compared the morphology, structure, and phenotype of neonatal and adult ovine lung DCs and found that they were functionally similar in antigen capture and T-cell stimulation.[348]

Immunologically Recruitable Pulmonary Structural Cells

Endothelium

Because of its anatomic location at the interface between the vascular and alveolar compartments, endothelium is uniquely situated to regulate pulmonary immune responses requiring recruitment and facilitated passage of intravascular inflammatory cells. Vascular endothelial cells express MHC class II molecules and may act as antigen presenters to T cells.[301] Endothelial cells also participate in immune processes by elaborating or responding to cytokines in the local microenvironment. For example, TNF-α is an endogenous mediator of inflammatory immune responses secreted by alveolar macrophages in response to bacterial endotoxin. Endothelial cells manifest a pleiotropic response to TNF-α, including increased expression of adhesion molecules, increased prostaglandin production, increased MHC antigen expression, and increased cytokine release including IL-1, IL-6, IL-8, GM-CSF, and monocyte chemotactic protein-1 (MCP-1).[91,349-355] The net effect of these signals is an increased chemotactic recruitment and activation of inflammatory cells at the "gate" from the vascular space to the interstitium; the TNF-induced increase in endothelial cell–leukocyte adhesivity further enhances this response. Endothelial cells also express the TLRs TLR4 (constitutive) and TLR2 (inducible by IFN-γ or TNF-α); this allows their direct response to endotoxin, manifested by up-regulation of adhesion molecule expression and induction of IL-1, IL-8, MCP-1, and GM-CSF expression.[301,354,356-360] By responding either to endotoxin directly or to endotoxin-induced macrophage-derived TNF-α, the endothelial cell may either generate an initial immune response or participate in amplifying a local inflammatory cascade.

Fibroblasts

Whereas most studies of fibroblasts have focused on their production of structural and matrix proteins, it is now evident that fibroblasts may also function as important immune-effector cells at sites of inflammation. When appropriately stimulated, fibroblasts may generate bioactive amounts of multiple cytokines, including IL-6, IL-8, IL-1α, IL-11, colony-stimulating factors, and growth factors such as TGF-β.[361] TNF-α or IL-1 can each induce

fibroblast-derived IL-6, IL-1, IL-8, and IL-11, and their combined action synergistically further up-regulates fibroblast elaboration of these cytokines.[362,363] Although fibroblasts and alveolar macrophages are often capable of elaborating the same inflammatory cytokine(s), maximal production of these cytokines typically occurs in response to different signals. This is illustrated by the distinct response of these cells to endotoxin. Endotoxin is a potent stimulator of macrophage-derived TNF-α and IL-1 that exerts little direct stimulation of fibroblasts. Conversely, pulmonary fibroblasts elaborate inflammatory cytokines in response to IL-1 and TNF-α, whereas macrophage response to these cytokines is more diverse.[364-368] This suggests that although classical immune cells such as alveolar macrophages produce inflammatory cytokines in a stimulus-specific fashion, fibroblasts produce inflammatory cytokines in response to macrophage-derived TNF-α or IL-1, independent of the precipitating stimulus. This response serves to amplify local pulmonary inflammation and illustrates the potential immunologic role of the fibroblast, transcending its putative structural function.

Epithelium

Pulmonary epithelial cells serve multiple functions including maintenance of solute fluxes, production of surfactant, and providing a surface for gas transfer. Beyond these metabolic and barrier functions, pulmonary epithelial cells are capable of augmenting and regulating local innate immunity in response to environmental signals. The airway epithelium senses bacterial exposure and responds by increasing the release of antimicrobial peptides, chemokines, and cytokines. This inflammatory reaction results in recruitment of phagocytes, dendritic cells, and lymphocytes to aid in the innate immune response and prepare an adaptive immune response if necessary. Epithelial cell recognition of these invading pathogens is a key initiating factor in mounting an adequate immune response to microbial pathogens. The mechanism by which this occurs is through the expression of a variety of PRRs including TLR1-10, CD14, and the CFTR.[73,369-375] Additionally, alveolar type II epithelial cells have collectin receptors, allowing them to internalize microbes opsonized by SP-A or SP-D.[206] Endotoxin or the early response cytokines TNF-α or IL-1β each may up-regulate epithelial-derived defensins such as human-β defensin 2 (HBD-2) by binding to cells expressing CD14 and TLR2.[374,376,377] Airway epithelial cells also exhibit tonic, high-level expression of iNOS with cytotoxic NO production.[378] The cyclooxygenase and lipoxygenase pathways are expressed at high levels in epithelial cells, resulting in production of lipid mediators including PGE_2 and the neutrophil chemoactivator leukotriene B_4 (LTB_4).[379-382] Infection with RSV can directly induce epithelial-derived IFN-β, IL-1α, and the chemokines IL-8 and RANTES.[75,383] Alternatively, pulmonary epithelial cells can also secrete several cytokines in response to various stimuli including TGF-β, GM-CSF, IL-5, IL-6, IL-8, and MCP-1; release of these cytokines facilitates chemotaxis and activation of multiple immune cells including PMNs, mononuclear phagocytes, and T lymphocytes.[384-389] Airway epithelial cells can titrate local cytokine signals by shedding soluble TNF-α receptor; this adsorbs available TNF-α and diminishes its bioactivity.[390] Airway and alveolar epithelial cells in vivo also express intercellular adhesion molecule-1 (ICAM-1), a natural ligand for complementary adhesins of PMNs and monocytes induced in response to IFN-γ, TNF-α, or IL-1. Increased epithelial expression of ICAM facilitates inflammatory cell migration along epithelial barriers; moreover, antimicrobial activity of phagocytes in vitro is enhanced by ICAM-mediated interactions with the alveolar epithelium.[391] Finally, airway epithelial cells, in response to specific cytokine stimulation, can express MHC antigens, allowing them to interact directly with T lymphocytes, possibly as antigen-presenting cells.[392] Together, these data suggest that in response to appropriate local stimuli, pulmonary epithelial cells may effect complex immune modulation within the milieu of the alveolus and airway.

CELLULAR MECHANISMS OF ADAPTIVE IMMUNE RESPONSE

Adaptive immunity requires sensitization to individually encountered antigens; once sensitization occurs, these immune responses are characterized by their high antigen specificity and long-lasting memory. Although many soluble and cellular components of host defense may link aspects of innate and adaptive immunity, adaptive immune responses are executed by lymphocytes.

Lymphocytes (T cells, B cells, and NK cells) are normally present at multiple sites in the lungs and may participate in complex immune responses to encountered antigens or act directly as effector cells against local microorganisms. Lung interstitial lymphocytes are plentiful, with numbers comparable to those of the circulating blood pool, and possess a characteristic size, distribution, subset composition, and cytokine production profile.[393] Large numbers of NK cells are also found in the interstitial space.[393]

T Cells

The pulmonary lymphocyte population is made up primarily of T cells.[393,394] Fetal and neonatal T cells express markers found on thymocytes,[395] suggesting that these T cells represent an immature transitional population; alternatively, this may reflect stress-induced release of cortical thymocytes into the circulation.[395] The neonatal T-cell phenotype corresponds closely to that of antigenically naive T cells in the adult.[396] These findings likely reflect a combination of the limited exposure of the neonate to foreign antigen and the limitations of neonatal DC function rather than intrinsic neonatal T-cell dysfunction. T cells recognize foreign peptides that have been modified by antigen-presenting cells and expressed in the context of MHC proteins. CD4+ T cells differentiate upon antigen stimulation in the context of class II MHC and can differentiate along several lineages including Th1, Th2, Th17, or Treg subsets. Important immunoregulatory functions provided by pulmonary T-cell subsets include cytokine production, enhancement of immunoglobulin production, and direct T-cell cytotoxicity.

Cytotoxic T cells mainly express the CD8 coreceptor and act to lyse host cells infected with intracellular pathogens.[397] As opposed to the CD4+ T-cell that recognizes peptide antigen in the context of class II MHC, these T cells recognize small peptide antigens that are presented by MHC class I molecules (signal I). Like other T cells these cells require a second signal to develop effective cytotoxicity, which is performed by costimulatory interaction between the T-cell CD28 molecule and B7 ligands (CD80 and CD86) expressed by the APC. In the absence of costimulation, the CD8 T cell is rendered either anergic or apoptotic.[398] Activated cytotoxic T cells can lyse and kill infected target cells through fas-mediated apoptosis or through the release of cytolytic mediators. The latter process involves the exocytosis of granules containing perforin, granzyme, and granulysin. Perforin is a glycoprotein that induces pore formation in the target cell membrane, facilitating both osmotic lysis and entry of granzymes and granulysin. Granzymes are serine esterases that activate the caspase cascade, inducing target cell apoptosis. Granulysin is an antimicrobial peptide with broad-spectrum activity against bacteria, mycobacteria, and fungi.[399] Cytotoxic lymphocytes are particularly important in host defense against nonlytic viruses such as CMV and also appear to be critical in host response to mycobacteria.[400-402] CD8+ T cells have also been shown to have activity against *Pneumocystis carinii* by up-regulating macrophage-mediated killing of the organism.[403] T lymphocytes from newborns are capable of developing into cytotoxic T cells during natural infections postnatally, and in utero infection may induce profound CD8 cytotoxic response.[404] In general, however, the incidence of neonatal cytotoxic lymphocyte development and the overall magnitude of the response tend to be diminished relative to that in older children.[405]

CD4+ helper T cells, or Th cells, function to orchestrate B-cell and T-cell responses. These CD4 cells recognize antigen presented by the MHC class II–dependent pathway and, on activation, may be induced to differentiate into specific Th phenotypes depending on the concurrent costimulatory signal (signal II) and cytokine stimulation (signal III) they receive. Undifferentiated naive T cells are called Th0 cells and can differentiate along specific T-cell subsets as outlined later.

Th1 cells

Th1 cells differentiate from Th0 cells in the presence of macrophage-derived IL-12p70, a heterodimeric cytokine consisting of Il-12p35 and Il-12p40, which are encoded by separate genes. IL-12 was the first heterodimeric cytokine discovered[406] and is the founding member of the IL-12 family of cytokines that consists of IL-12, IL-23, and IL-27 (Figure 159-6). IL-12 directs Th1 differentiation by the phosphorylation of Stat4.[406,407] Th1 lymphocytes are controlled by the transcription factor T-bet; characterized by their expression of IL-2, IL-12, TNF-α, and IFN-γ; function to activate macrophages and neutrophils; and are critical for host defense against intracellular pathogens such as *M. tuberculosis*.[408,409] In support of this, patients with defects in IL-12p40 production, IFN-γ production, or IFN-γ receptor signaling have an increased risk of TB.[410] Th1 cells can also influence the development of cytotoxic CD8+ T cells as well as facilitating class switching of B cells to produce IgG2a. In this context, Th1 response has been shown to be critical for host resistance against a variety of pulmonary pathogens, including *M. tuberculosis*, *L. pneumophila*, *C. pneumoniae*, and *P. carinii*.[411-413]

IFN-γ activates mononuclear phagocytes, augmenting phagocytosis and inducing production of oxyradical species, NO, and cytokines such as TNF-α, IL-1, and IL-6; IFN-γ up-regulates pulmonary epithelial cell and endothelial cell expression of adhesion molecules, with resultant enhanced PMN adherence.[414-419] This cytokine also increases expression of MHC molecules on epithelial cells and monocytes, enhancing their ability to function as antigen-presenting cells.[420] Finally, IFN- γ promotes proliferation of antigen-stimulated T cells, generation of cytotoxic T cells, and enhanced cytotoxic activity.[421] The net outcome of these effects is enhanced leukocyte recruitment and macrophage antimicrobial activity and amplified inflammatory tone. The enhanced antigen presentation induced by IFN-γ may facilitate later transition from macrophage-driven inflammation to a lymphocyte-executed adaptive immune response. As a sentinel mediator of the Th1 response, IFN-γ is antagonized by the Th2 cytokines IL-4, IL-13, and IL-10. IL-4 suppresses the expression of IFN-γ and other Th1 cytokines. IL-4 and IL-13 each also inhibit the expression of many IFN-γ inducible genes, as well as the ability of IFN-γ to augment microbicidal activities such as superoxide production. IL-10 is similar to IL-4 in its inhibition of IFN-γ synthesis by Th1 cells, NK cells, and macrophages. At the receptor level, Th1 and Th2 cells exhibit different responses to IFN-γ through differential expression of a specific IFN-γ receptor subunit that transduces suppressive signals.[422]

IFN-γ–activated macrophages are capable of restricting the growth of a number of microbes, such as *Legionella*, mycobacteria, *P. carinii*, pathogenic fungi, and *Chlamydia,* that parasitize macrophages [409,423,424] In vivo, IFN-γ replacement or overexpression has been shown to enhance bacterial clearance in murine modela of *L. pneumophila*, *P. aeruginosa*, and *K. pneumoniae.*[425-427] IFN-γ has been demonstrated in the BAL specimens of neonates with RSV, but the pulmonary expression of this cytokine is generally uncharacterized in this age-group.[428] Most likely, the diminished ability of neonatal lymphocytes to generate this cytokine (capacity less than 10% of adult) limits optimal alveolar macrophage activation, compromising neonatal pulmonary immune response.[429]

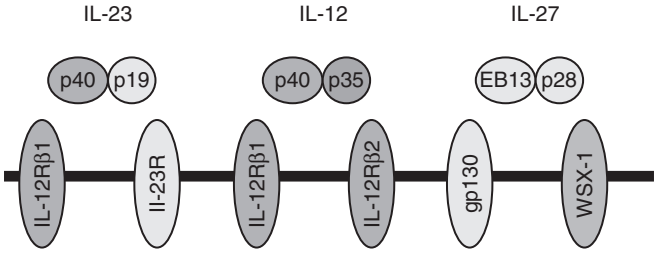


Figure 159-6 IL-12, IL-23, and IL-27 cytokine and receptor complexes. The IL-23 cytokine consists of a heterodimer of IL-12p40 and IL-23 p19, which is similar in structure to the IL-12 p35 subunit. IL-12 comprises of the IL-12p40 subunit and a p35 subunit. IL-27 consists of EBI3 and p28. IL-12p40 and EBI3 are group 3 soluble cytokine receptors. IL-23p19, p35, and p28 are members of the IL-6 subfamily of Type I cytokines. The IL-23 receptor consists of the IL-12Rβ1 and IL-23R subunits. The IL-12 receptor consists of the IL-12Rβ1 and IL-12Rβ2 subunits. The IL-12Rβ2 and IL-23R are structurally similar. The IL-27 receptor consists of a gp130 and WSX-1 subunit.

Th2 cells

Alternatively, in the absence of IL-12, activated Th0 cells are induced by IL-4 to adopt the Th2 phenotype, characterized by secretion of IL-4, IL-5, IL-6, IL-9, and IL-13. Th2 cells develop under control of Stat6 and the transcription factor Gata-3.[430,431] Th2 lymphocytes are required for expulsion of helminth parasites and also function to generate humoral immune responses and antibody production particularly IgG1, immunoglobulin A (IgA), and immunoglobulin E (IgE).[432] The Th1 cytokine IFN-γ acts on Th0 cells to induce Th1 differentiation and inhibit Th2 differentiation. Conversely, the Th2 cytokines IL-4 inhibits Th1 differentiation (Figure 159-7).

Mature T cells are capable of enhancing antibody secretion by regulating the proliferation and immunoglobulin isotype expression of B cells; this regulation is provided both through contact-dependent mechanisms and through secretion of specific cytokines. Contact-dependent interactions include cognate recognition through MHC antigens or receptor-ligand interactions primarily between the B-cell CD40 molecule and the T-cell CD40 ligand.[433] Activated Th cells markedly up-regulate their surface expression of CD40 ligand, a membrane-bound cytokine with homology to TNF.[434] Subsequent engagement with the B cell–surface CD40 molecule triggers B-cell expression of cytokine receptors. At this point, activated Th cells can begin to secrete cytokines in a directional fashion within the "immunologic synapse." Signals delivered to the B cell via CD40 binding markedly enhance immunoglobulin production and promote immunoglobulin class switching in the presence of coactivation signals provided by specific cytokines; relevant examples include IL-2, which promotes IgM secretion; IL-4 or IL-13, which facilitates IgE synthesis; IL-10, which induces IgG1 and IgG3 production; and IFN-γ, which promotes IgG2 production.[435-437] Patients with mutations in CD40L cannot undergo antibody class switching and thus have an abundance of B cells that stain positively for surface IgM. Measurement of antibodies in serum reveals mainly IgM and thus this syndrome is named Hyper IgM syndrome. The patients are at risk for infection from encapsulated bacteria as well as opportunistic fungi such as *P. carinii*.[438]

IL-5 is a major product of Th2 cells.[439] In humans, IL-5 is thought to be specific for promoting eosinophil and basophilic maturation and eosinophil survival and chemotaxis.[440] IL-5 appears to be critical for producing tissue eosinophilia and is thus of primary interest for its role in asthma. The role, if any, of IL-5 in neonatal host response remains uncharacterized. Eosinophilia is reportedly common among premature neonates and considered a marker of occult infection.[441]

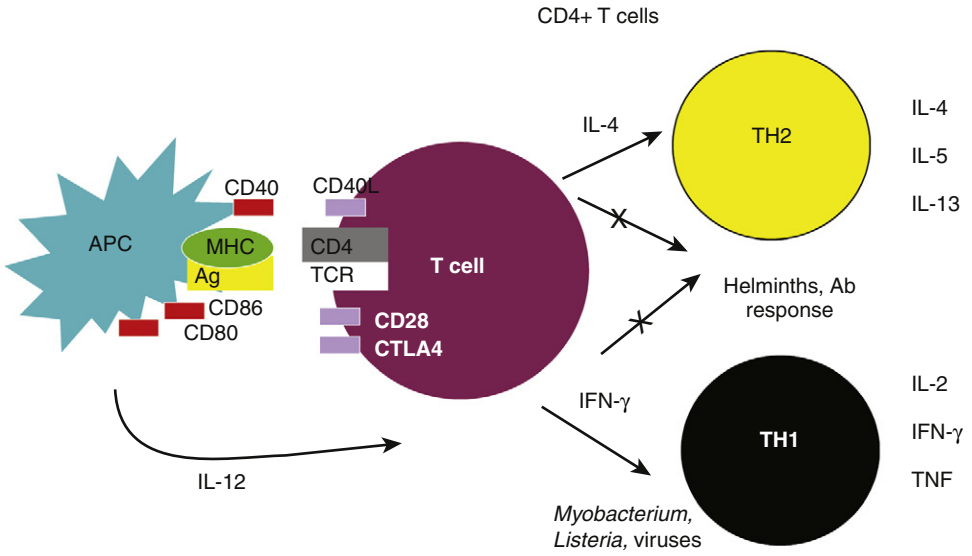


Figure 159-7 Differentiation of CD4+ Th1 and Th2 cells. Th1 cells develop after antigen presentation via class II MHC (signal I), costimulation (signal II), and under cytokine control of IL-12 (signal III). Th1 cells produce IFN-γ and are critical for intracellular pathogens as well as IgG2A production. In the absence of IL-12 and under control of IL-4, Th2 cells can differentiate and produce IL-4, IL-5, and IL-13. These cells are critical for host dense against helminths as well as IgG1 production. Both IFN-γ and IL-4 show reciprocal inhibition of Th2 and Th1 responses, respectively.

IL-4 is an 18-kDa cytokine produced by activated T cells of the Th2 subset, γδ T cells, mast cells, and basophils. IL-4 has two receptors: a high-affinity receptor that shares signaling components with IL-2 and IL-7 and an alternate receptor that shares components of the IL-13 receptor; cells expressing either receptor can transduce the IL-4 signal. IL-4 stimulation of naive T cells induces their differentiation into Th2 cells, initiating the Th2 response. Concurrently, IL-4 exhibits a number of important functions related to humoral immunity, including B-cell activation and induction of B-cell isotype switching necessary for production of IgE and some IgG subclasses.[442] Consistent with the Th2 cellular response, IL-4 facilitates the migration of eosinophils, basophils, and monocytes by up-regulating endothelial expression of vascular cell adhesion molecule (VCAM).[443] Neonatal leukocyte production of IL-4 is estimated to be less than 10% of adult capacity, and this correlates with in vivo data, in which IL-4 is undetectable in tracheobronchial aspirates of preterm and term newborns.[444,445]

IL-13 shares sequence homology (≈30%) and biologic activities with IL-4.[446,447] The activities shared by IL-4 and IL-13 likely result from a shared receptor subunit; however, IL-13-mediated effects are more restricted, and IL-13 is generally less potent than IL-4, owing to competitive inhibition resulting from the higher affinity of IL-4 for its receptor.[448] Although IL-13 does not stimulate T cells as IL-4 does, IL-13 can stimulate B cell proliferation and immunoglobulin isotype switching synergistically with IL-4.[448] IL-13 is also a potent inducer of mucins in lung epithelium.[448,449] Normal pregnancy is associated with the production of appreciable quantities of IL-13, initially by the placenta and subsequently by the fetus; IL-13 is inducible from cord blood cells by 27 weeks gestation.[450] Postnatally, higher levels of inducible IL-13 from cord lymphocytes may identify neonates at risk for later development of atopic or Th2 response-associated disease processes.[451]

Th17 cells

Th17 cells are a recently described lineage that develops from naive T cells under the control of TGF-β and IL-6. Th17 cell development requires phosphorylation of Stat3 and the transcription factors RORγ and RORα.[452,453] Once established, the IL-12 family member IL-23 regulates their proliferation and survival, as well as the expression of specific effector cytokines.[454] The effector cytokines produced by Th17 cells include, IL-17A, IL-17F, IL-22, and TNF-α. In fact IL-23 is required for IL-22 production whereas TGF-β and IL-6–directed differentiation is sufficient for IL-17A and IL-17F expression.[454] Experiments in animal models demonstrate that the Th17 lineage is critical for host defense against extracellular bacteria such as *K. pneumonia,* as well as organisms such as *Mycoplasma pulmonis* and *Citrobacter rodentium*, an enteric pathogen.[455-458] This lineage controls several aspects of mucosal immunity. IL-17R signaling has also been shown to be important for control of disseminated *Candida albicans* infection.[459] IL-17 and IL-17R signaling has been shown to critical for regulating granulopoiesis in response to infection, as well as the regulation of local chemokines such as CXCL1 and CXCL2, which are important for the recruitment of neutrophils to the infected tissue site. IL-22 synergizes with IL-17 to regulate the expression of antimicrobial peptides at epithelial surfaces.[456,460] IL-22 regulation of antimicrobial proteins has been shown to exist at several mucosal surfaces including the skin, gut, and lung.[456,458] Interestingly, although there is some overlap with types of genes induced in skin keratinocytes and lung epithelium, such as HBD-2, IL-22 appears to regulate organ specific genes in the gut. IL-22 regulates the expression of Reg proteins in GI epithelium, which are soluble lectin biding proteins that have direct antimicrobial activity.[458,461] It has recently been shown that patients with Job's syndrome or Hyper IgE syndrome have mutations in Stat3 and thus lack antigen specific Th17 cells in their peripheral blood.[462] These patients develop cutaneous infections with *S. aureus* and *C. albicans*. The individual contributions of IL-17 and IL-22 in this phenotype remain to be determined. This T-cell lineage has also been implicated in diseases of autoimmunity such as rheumatoid arthritis, Crohn's disease, and multiple sclerosis.[454,463,464]

T-cell Immunity in Neonates

As mentioned above, the majority of neonatal T cells are naive cells with reduced expression of the T-cell receptor (TCR), decreased adhesion molecule expression, and diminished

expression of the CD40 ligand.[405] Neonatal T cells also exhibit diminished cytokine production (notably IL-4, IFN-γ, IL-12, IL-15, GM-CSF), putatively owing to labile posttranscriptional regulation and markedly shortened mRNA half-life.[465-467] Although normal levels of cytokine elaboration are inducible by sustained TCR triggering, the decreased adhesion molecule expression by neonatal T cells impairs interaction with antigen-presenting cells, resulting in costimulatory signals insufficient to achieve levels of adult functioning. Similarly, diminished expression of the T-cell CD40 ligand precludes optimal contact-dependent activation of B cells.[468] Finally, neonatal T-cell differentiation appears biased toward a Th2 or Th0 profile under neutral conditions. Whether neonatal T cells are capable of Th17 differentiation remains to be determined.

Factors favoring such a "Th2 bias" include the low MHC-peptide density of neonates (favors priming of Th2 cells), relative dearth of the Th1-inducing cytokines IFN-γ and IL-12, and the greater costimulation required to elicit Th1 differentiation.[469,470] Factors favoring a Th0 state include paucity of the Th2-inducing cytokines IL-4 and IL-10. Interestingly, pregnancy is also associated with a state of T-cell tolerance with several factors (IL-4, IL-10, TGF-β) present at the maternal-fetal interface that can induce a shift from a Th1 to a Th2 profile.[471] Additionally TGF-β can also result in differentiation of regulatory T cells which have been shown to be critical for antigen specific tolerance.[472,473] The importance of this T-cell lineage in humans has been demonstrated in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), which is result of mutations in FOXP3—the transcription factor that controls the development of regulatory T cells.[474-476] This is an X-linked disorder that can present with neonatal diabetes mellitus, immune dysregulation with extremely high concentrations of IgE, and intractable diarrhea.[474,475] These patients also show exaggerated immune responses to viral infections.

γδ T Cells

T cells expressing the γδ receptor are present at mucosal surfaces including the skin, gut, and lung.[477] These cells do not recirculate and appear to be resident pulmonary lymphocytes. γδ T cells can develop by thymic-independent pathways and can recognize small molecules and intact proteins without the requirement for antigen processing that other T cells exhibit.[477] Small molecule recognition by γδ cells requires cell-cell contact, suggesting that non-MHC molecules may present small antigens to these cells or that costimulation from neighboring cells is required.[477,478] A germline-encoded phosphoantigen binding site enables these cells to respond to mycobacterial pyrophosphate, but they are also activated by antigen from disparate pathogens such as *L. monocytogenes.*[479,480] In vitro, γδ T cells can be induced to generate IFN-γ, TNF, IL-17, and IL-4 in stimulus-specific fashion similar to Th0 cells.[481,482] In the skin they regulate epithelial repair,[483] and whether they have a similar function in the lung remains to be determined.[483] The precise function of these cells in host defense remains unclear, but their anatomic locale and potential for cytotoxicity suggest a role in rapid initiation of immune reactions at mucosal surfaces of the airway. They are a rapid source of IL-17 in response to *K. pneumoniae* and *M. tuberculosis*[484] infection, and they are a dominant source of IL-17 in the gut.[484-486] There they may regulate granulopoiesis through IL-17 elaboration, which subsequently controls the expression of G-CSF.[486,487]

B Cells

B cells make up only a small percentage (≤2%) of the lung lymphocyte population, and this proportion of B cells or their precursors is attained in the fetal lung by 18 to 22 weeks gestation.[488] In more mature subjects, B cells of the lung interstitium participate in local humoral responses through elaboration of immunoglobulins in response to specific antigenic stimuli. In the neonate, however, this capacity is limited, attributed in part to the inability of neonatal T cells to provide either the contact-dependent help or cytokine factors required to induce B-cell differentiation into memory B cells.[445] A higher percentage of neonatal B cells are so-called B-1 cells, characterized by production mainly of polyreactive, low-affinity IgM, rather than the specific, high-affinity antibodies generated by B-2 cells.[489] Natural antibodies (nAb) are immunoglobulins existing in individuals before specific antigenic stimulation and include specificities present in cord blood or germ-free mice. The predominant nAb isotype is IgM, and in germ-free mice quantities of IgM in the serum are unchanged whereas IgG and mucosal IgA are significantly diminished, suggesting that production of IgM and IgG-IgA isotypes differ in their requirements for exogenous antigens.[490-492] nAb display low-level broad cross reactivity with self-antigens such as erythrocyte phospholipids and ds-DNA; however, selective pressures on the repertoire may relate to the role of nAb in host defense, as nAb recognize various pathogen molecules, such as bacterial phosphorylcholine, alpha 1,3 dextran, and influenza hemagluttinin. Further, they have been shown to directly neutralize viruses such as varicella zoster virus. As these antibodies are present at the earliest stages of infections and significantly earlier than the adaptive immune response, it is hypothesized that they play a critical role in the limitation of infection. Additionally, as IgM contains unique effector functions, it is thought that the early role of IgM nAb in host defense likely contributes to the quality and quantity of the emerging adaptive host immune response to infection. In addition to direct antimicrobial activity, the functions of IgM nAb appear to regulate disease processes at mucosal surfaces and adaptive immune responses. It has been shown that mice unable to secrete IgM have decreased survival and clearance of infection in response to influenza challenge. Interestingly, these mice had delayed production of influenza specific IgG1 and IgG2a, suggesting that IgM immune complexes with the virus may influence aspects of antigen presentation to B cells.[493]

Receptor ligation of neonatal B cells induces minimal upregulation of MHC class II molecules and fails to induce CD86, impairing antigen presentation by these cells.[494] Neonatal B cells also exhibit diminished expression of the complement receptor CD21, limiting their capacity to be stimulated by complement.[495] Although capable of local immunoglobulin production (primarily IgM and IgA), neonatal B cells remain unable to generate antibodies to bacterial capsular polysaccharides; this results in particular vulnerability to organisms such as *H. influenzae* and group B *Streptococcus*.[496] Neonatal B-cell function and lung humoral defense mechanisms are discussed in further detail later.

NK Cells

NK cells are large granular lymphocytes with cytotoxic function, comprising up to 15% of circulating lymphocytes, but less than 2% of the lung lymphocyte pool. They share a common progenitor with T cells but lack the TCR required for specific antigen recognition.[497] Within the lung, they are found on epithelial surfaces and in the interstitium, and they are also recruitable from the circulation in response to locally generated chemokines.[498] Activated NK cells contribute to early innate defense by lysing infected cells and generating cytokines that stimulate T cells and alveolar macrophages.[499] IL-12p70 is a key cytokine that up-regulates NK-cell perforin and granzyme, enhancing activated NK-cell lysis of infected cells.[497] As opposed to T cells, which recognize their target cells through MHC-restricted peptide expression, NK cells recognize the absence or very low levels of MHC class I antigen expressed on infected cells.[497] This has been termed the "missing self-hypothesis."Activating receptors on NK cells include NKG2D

and DAP10. DAP12 can activate or inhibit NK-cell activation and these negative regulatory adaptor proteins may avoid indiscriminate lysis of noninfected cells.

Early in infection, activated NK cells may be a primary source of IFN-γ within the lung.[500] NK cell–derived IFN-γ and TNF-α from multiple sources synergistically enhance alveolar macrophage killing of intracellular pathogens. In addition, NK cell–derived IFN-γ may initiate alveolar T cell–mediated immunity by inducing differentiation of Th0 cells into a Th1 phenotype.[501] The capacity to produce IFN-γ, augment immunity against intracellular pathogens, and effect cytotoxic responses without prior sensitization all imply a sentinel role for NK cells in neonatal host defense. Indeed, NK-cell precursors appear early in gestation by thymic-independent mechanisms and reach adult levels by term.[502] NK cytolytic function, however, remains diminished (≤50% of adult NK cells) for much of the first year of age, with more pronounced depression in premature neonates.[503] This diminished function corresponds to an immature NK-cell subset with impaired perforin-granzyme delivery[504]; this may predispose the neonatal lung to infections with agents such as HSV and CMV.[504]

Humoral Immune Responses

B lymphocytes bearing surface immunoglobulin of the IgA, IgG, and IgD isotypes appear early in gestation. Although the B-cell immunoglobulin repertoire expands during gestation, at birth it remains limited relative to older hosts.[505,506] Immunoglobulin-secreting plasma cells appear later in gestation than B cells, between weeks 15 and 30.[507] Typically, neonatal B cells can differentiate into IgM-secreting plasma cells as efficiently as in the adult, but do not differentiate into IgG- or IgA-secreting cells until these functions fully mature.[508] As previously discussed, the relative inability to achieve isotype switching in the neonate results at least in part from inadequacy of mutual signaling between neonatal T cells and B cells. The antibody response of neonatal B cells to specific antigens develops sequentially, with responsiveness to antigens requiring contact-dependent T-cell help (for example, protein antigens) preceding the development of responses not requiring such cognate help (for example, capsular polysaccharides). Although infection of neonates elicits a protective response to most protein antigens, the response to polysaccharide antigens is absent or severely blunted. This has been recently postulated to result from decreased surface expression of CD21 and to decreased complement levels, resulting in suboptimal signal transduction via CD21 and inability to achieve the CD21/B-cell receptor synergy required for B-cell activation.[509,510] Alternatively, humoral responses to bacterial capsular polysaccharides are enhanced by specific T cell–derived cytokines such as IFN-γ, IL-12, and GM-CSF, all of which are relatively deficient in the neonate.[511,512] The limited capacity of naive neonatal T cells to provide these cytokines may contribute to the poor antibody responses of neonates to encapsulated bacteria such as group B streptococci. This T-cell immaturity thus combines with differences in antibody repertoire and functional immaturity of B cells to limit the capacity of the fetus or neonate to produce antibodies to certain antigens.

Secretory Immunoglobulins

Secretory component is an epithelial-derived glycoprotein that facilitates transfer of immunoglobulins from subepithelial sites into epithelial-lined lumina by transepithelial transport and secretion.[513] IgA and IgM constitute the primary secreted immunoglobulin subclasses of the lung. IgM is more abundant in secretions of neonates than in adults.[514-516] IgM is the first immunoglobulin class to be produced in a primary response to an antigen and is the only immunoglobulin other than IgG that fixes and activates complement. It is secreted as a pentamer, and the resultant 10 antigen-binding sites render it a superb agglutinin. Whereas serum concentrations are low at birth, postnatal IgM concentrations rise rapidly in the first month, reflecting increased antigen exposure; IgM concentrations in premature infants remain lower for the first 6 months of life.[517,518] Secretory IgA is undetectable at birth but found by 1 to 2 weeks in saliva and nasopharyngeal secretions. The earlier expression of secretory IgA relative to serum IgA presumably reflects increased local production in response to encountered antigen.[519] Serum IgA largely consists of monomeric IgA whereas transported IgA is largely homodimeric. Transport occurs form the basolateral epithelial surface to the apical surface through the basolateral polymeric immunoglobulin receptor (PIGR) (Figure 159-8). Both IL-17 and IL-22 can upregulate the expression of PIGR and may facilitate IgA transport during inflammation.[456]

Other Immunoglobulins

IgG is the predominant immunoglobulin isotype at all ages, and passively derived cross-placental transfer of maternal IgG is the primary source of all IgG subclasses detected in the normal fetus

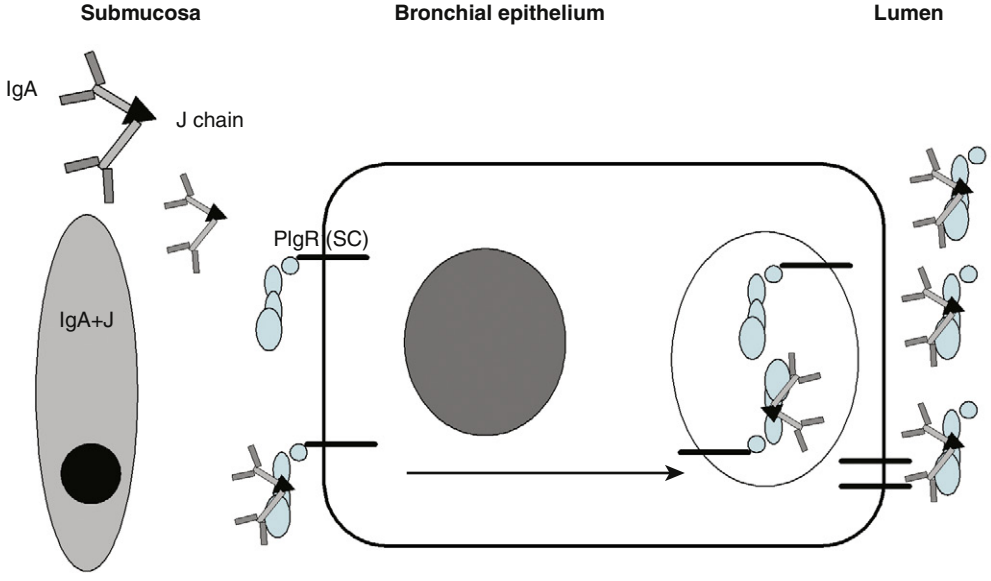


Figure 159-8 Transport of IgA across secretory epithelium. Dimeric IgA binds to the polymeric Ig receptor, also known as membrane secretory component *(SC)*. After transport and export via PIgR, secretory IgA *(SIgA)* consists of dimeric IgA with J chain *(IgA+J)*, which can function as blocking antibodies at mucosal surfaces.

and neonate.[517] These levels fall postnatally, reaching a nadir between 2 and 4 months of age (depending on gestational age) when nascent IgG production by the infant is unable to keep pace with utilization of maternally derived IgG. Preterm infants have proportionally lower IgG concentrations at birth compared with term infants, demonstrate lower serum IgG nadirs, and manifest lower serum IgG levels throughout the first year of life.[518] Although IgG is not actively transported into secretions like IgA, significant quantities of IgG may be found in fluids obtained from bronchoalveolar and airway lavage, presumably by passive transfer.[520] A critical role of IgG in the lung has been shown by patients with various IgG deficiencies such as common variable hypogammaglobulinemia and Bruton's X-lined agammaglobulinemia that suffer from pulmonary infections with encapsulated bacteria. This phenotype is a result of the critical role of IgGs in mediating opsonization as well complement fixation.

Molecules Involved in the Recruitment of Lung Immune Cells

Lipid Mediators

Lipid mediators of inflammation constitute a diverse group of biologically active products liberated from cellular membranes in response to local immune and nonimmune stimuli.[521] As opposed to cytokine synthesis, which often requires transcription, translation, and posttranslational processing steps, lipid mediators can be generated within minutes. Endotoxin directly stimulates leukocyte membrane-associated phospholipase A_2 (PLA_2), which metabolizes adjacent membrane phospholipids to release free fatty acid products such as lysophosphatidylcholine and arachidonic acid.[522] Arachidonic acid can be oxidatively metabolized by cyclooxygenase or lipoxygenase pathways to generate a number of bioactive lipid products including thromboxane A_2 (TXA_2), prostacyclin, prostaglandins, and LTs, while lysophosphatidylcholine may be acylated to produce the potent mediator PAF.[523,524]

PAF is synthesized by a number of cells, including mononuclear phagocytes, neutrophils, endothelial cells, platelets, eosinophils, and mast cells, and has demonstrable biologic effects at nanomolar concentrations.[524,525] Known for its induction of platelet aggregation and degranulation, it also stimulates chemotaxis and activation of PMNs and macrophages and augments PMN adhesion to endothelial cells.[526] Animal models of gram-negative infection suggest a role for PAF in mediating endotoxin-induced inflammation as well as a role in the impaired hypoxic pulmonary vasoconstriction and alveolar capillary leak associated with severe pneumonitis.[527] Other studies have linked PAF to transfusion-associated lung injury and reperfusion lung injury.[528] In neonates, PAF is implicated in the pathogenesis of septic complications such as disseminated intravascular coagulation and necrotizing enterocolitis.[529] Elevated PAF levels are documented in the tracheal aspirates of infants in association with meconium aspiration, pneumonia, and intrauterine infection, suggesting a role in neonatal pulmonary inflammation.[530]

Arachidonic acids are released from cell membrane phospholipids in response to a variety of inflammatory stimuli.[524] Once liberated, they are primarily metabolized by either the lipoxygenase or the cyclooxygenase pathway. The lipoxygenase pathway produces LTs, including the potent PMN chemotactic activator LTB_4, and LTC_4 and its cysteinyl degradation products LTD_4 and LTE_4.[524] LTB_4 is a potent neutrophil chemoattractant whereas the cysteinyl LTs are potent bronchoconstrictors. In addition, the cysteinyl LTs up-regulate macrophage Fc receptor expression and phagocytic activity. The important role of LTs in host response is suggested by a transgenic animal model of LT deficiency. In this model, aveolar macrophage phagocytic function was impaired, intratracheal inoculation of bacteria resulted in increased bacteremia and lethality, and alveolar macrophage function was restorable in vitro by exogenous LTB_4.[531] LT activity in the human neonatal lung has not been characterized, but animal models suggest that neonatal alveolar macrophages may have diminished capacity to synthesize LTB_4 compared with adults. This may result in part from lower inducible levels of IFN-γ and less capacity to up-regulate lipoxygenase pathway enzymes.[532]

Products of the cyclooxygenase pathway include prostacyclin (PGI_2), TXA_2, and prostaglandins. Although TXA_2 increases microvascular tone and activates inflammatory cells, prostaglandin species, particularly the PGE series, are known to block transcription of TNF, suppress leukocyte adhesion, and blunt the generation of other proinflammatory arachidonic acid metabolites.[533] Cellular constituents of the lung appear specialized in their generation of arachidonic acid products, with LTB_4, PGE_2, and TXA_2, the primary ones generated by alveolar macrophages. Because inflammatory actions of these mediators may be conflicting, and because alveolar macrophages may produce varying amounts of these mediators at different stages of the inflammatory process, this suggests another mechanism by which the alveolar macrophage may regulate local inflammatory events.

Proteins

Complement

Activation of the complement cascade by either classical or innate means results in the eventual generation of multiple components with potent inflammatory activity. C3a, C4a, and C5a all have function as "anaphylatoxins," capable of inducing histamine release and increased vascular permeability; there is evidence that C3a and C5a mediate some of these effects by inducing local production of arachidonic acid metabolites.[534,535] C3a and C5a stimulate PMN lysosomal enzyme release, while C5a is also a potent neutrophil chemoattractant (Figure 159-9).[534]

C3a and C5a may regulate a broad range of inflammatory functions by binding their cognate receptors C3aR and C5aR. Previously recognized only on myeloid cells, both receptors have been demonstrated on human alveolar epithelium and alveolar macrophages.[536] In a murine model of sepsis, putative activation of toll pathways by endotoxin increased C3aR and C5aR expression on alveolar epithelium.[537] In this model, C5a activation of C5aR was implicated in increased expression of TNF-α and IL-6. Conversely, C3a has been shown to depress TNF-α and IL-6 secretion.[534]

Neurokinins

The autonomic nervous system may participate in some aspects of airway inflammation because stimulated or injured sensory nerves can initiate or amplify leukocyte responses. Local axonal reflexes play a role through stimulation of afferent nerve fibers, leading to the release of neurokinins. Neurokinins, such as substance P, are stored in unmyelinated nerve fibers (C fibers) as well as intrinsic airway neurons and released as part of a nociceptive response.[538] Airway surface epithelium and submucosal glands are in proximity to sites of neurokinin release. Once secreted, neurokinins activate the neurokinin-1 receptor (NK-1R), mediating local increases in vascular permeability and leukocyte adherence to airway epithelial surfaces; the ensuing plasma extravasation and leukocyte infiltration constitutes neurogenic inflammation.[539] The duration and intensity of this response is determined by the rate of neurokinin breakdown by neutral endopeptidase (NEP), an enzyme produced by epithelial cells.[540] Epithelial damage caused by inflammation, mechanical injury, or viral infection reduces NEP availability, leading to sustained inflammatory effects of the neurokinins.

Beyond the classic model of neurogenic inflammation, recent work has identified other neurokinin sources within the lung. Resident lung macrophages and circulating leukocytes have been found to express neurokinin receptors, as well as the preprotachykinin A (PPT-A) gene encoding neurokinins such as substance P.[541] This suggests that lung immune cells may

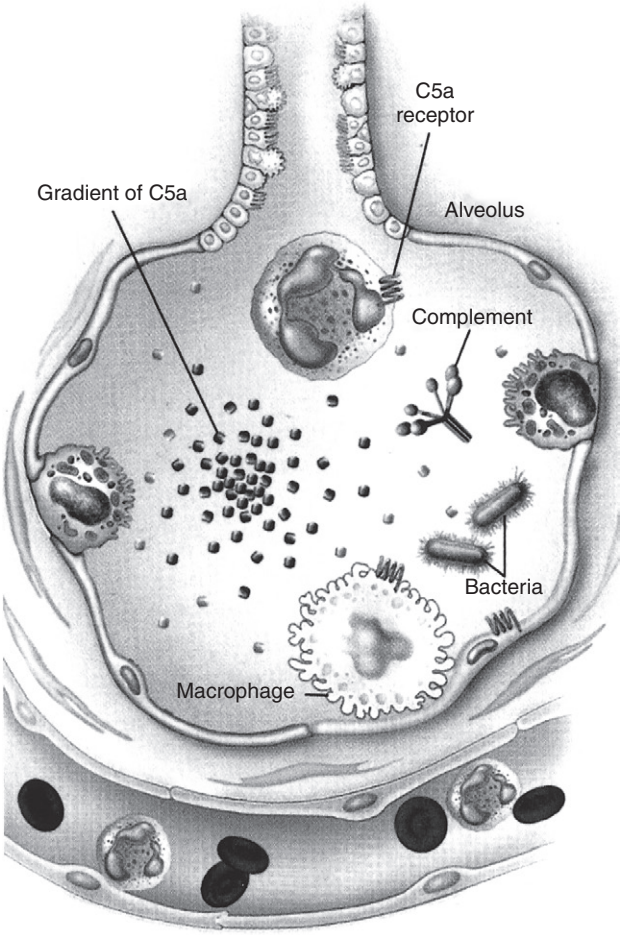


Figure 159-9 Complement components are produced within the alveolus by alveolar macrophages, pulmonary fibroblasts, and type II epithelial cells. Complement activation within this compartment generates a C5a gradient favoring PMN chemotaxis. Increasing local concentrations of C5a arrest chemotaxis and induce PMN respiratory burst and degranulation. Additional local immunomodulation (cytokine induction, IgG receptor upregulation) may occur through C5a interaction with cognate receptors on alveolar epithelium and alveolar macrophages. *(From Gerard C:* N Engl J Med *348:167-169, 2003.)*

use neurokinins for paracrine or autocrine signaling, propagating inflammation beyond the distribution of C fibers and intrinsic neurons. Such a role is supported by animal models of either NK-1R or PPT-A deletion. The injury normally induced by antigen-antibody complexes within the airway is attenuated in a transgenic murine model of NK-1R deletion, suggesting an essential rather than complementary function of neurokinins in this type of inflammation.[542] Similarly, PPT-A deficiency blunts immune complex–mediated inflammation, yielding additional important insights. First, neurokinins intervene proximally in the inflammatory cascade because airway TNF-α levels are essentially undetectable in PPT-A–deficient mice. Second, reconstitution of leukocyte PPT-A expression does not alter the blunted inflammatory response in PPT-A–deficient mice; however, transplantation of PPT-A–deficient bone marrow into "wild-type" mice is protective, underscoring the essential role of leukocyte-derived PPT-A in neurogenic inflammation.[543] These data, taken together, suggest a paradigm in which subepithelial sensory neurons interact synergistically with resident immune cells of the lung to initiate neurogenic inflammation. Secreted neuropeptides are potent chemotaxins and can recruit leukocytes directly or by the induction of cytokines by local interstitial and immune cells.

Apart from their inflammatory actions, other relevant effects of airway neuropeptides relate to alterations in mucus secretion. Substance P stimulates mucin elaboration and increases mucus secretion by stimulating serous cells and goblet cells.[544] Both substance P and vasoactive intestinal peptide (VIP) stimulate epithelial cell chloride secretion, increasing periciliary fluid level.[545] The net effect is increased local production of mucus with increased periciliary fluid secretion to maintain mucus hydration and normal viscosity. Recently it has been shown that neurokinins play a critical role in lung inflammation mediated by RSV infection.[546]

Defensins

Apart from their primary antimicrobial activity, defensins also exhibit properties facilitating pulmonary inflammation. In vitro, α-defensins have been shown to induce the neutrophil chemoattractant IL-8 from airway epithelial cells, and stimulate alveolar macrophage production of LTB_4 and IL-8.[547] Additionally, HNP-1 and HNP-2 are each directly chemotactic for monocytes and can induce monocyte-derived TNF and IL-1.[548,549] Finally, α-defensins have been shown to induce T cell–derived IFN-γ, IL-6, and IL-10, whereas β-defensins have chemoattractant effects on immature DCs and naive T cells; this suggests that beyond their antimicrobial and proinflammatory effects, defensins may also facilitate the local transition from innate to adaptive immune response.[550,551]

Proteases

Phagocytic cells (PMNs, mononuclear leukocytes) contain a number of proteolytic enzymes capable of both microbial killing and proteolytic degradation of lung structural elements.[552] PMN primary (azurophilic) granules contain serine protease homologues with cytocidal activity (serprocidins); these include neutrophil elastase, cathepsin G, proteinase-3, and azurocidin. The serprocidins are structurally related to the granzymes of cytotoxic lymphocytes and exhibit broad-spectrum microbicidal activity against bacteria, fungi, and protozoa. Proteases contained in primary granules have the capacity to degrade a number of extracellular proteins, including collagen (types I-IV), elastin, fibronectin, laminin, proteoglycans, and complement, contributing to microbicidal action; alternatively, many proteases possess antimicrobial activity unrelated to their proteolytic effects.[553,554] PMN secondary (specific) granules contain matrix metalloproteinase 9 (MMP9) and possibly gelatinase, which facilitate PMN migration.[553,555,556] Stored proteases are released extracellularly in response to various stimuli. Migration of PMN through tissues results in release of specific granule contents; this may be caused by the interaction of complement receptors on the specific granule membrane with local complement components directing PMN chemotaxis. The local proteolytic actions of secreted proteases are generally controlled by extracellular antiproteases such as α_1-antitrypsin (α_1-AT), α_1-antichymotrypsin, and α_1-protease inhibito.[557,558] Under conditions of homeostasis, a balance between proteolytic activity and antiprotease activity exists. However, when antiproteases are deficient or overwhelmed by the degree of the inflammatory response, diminished regulation of protease activity may ensue. Subsequent proteolytic generation of chemotactic extracellular matrix fragments and complement components can lead to further recruitment of phagocytic cells and intensified local inflammation.

Reactive Oxygen Species

A key component of pulmonary host defense is the generation of reactive oxygen species including superoxide and hydrogen peroxide. These molecules are often confined to the phagolysosome of PMNs and macrophages to minimize damage to host tissues.[559,560] This process is characterized by a rapid uptake of

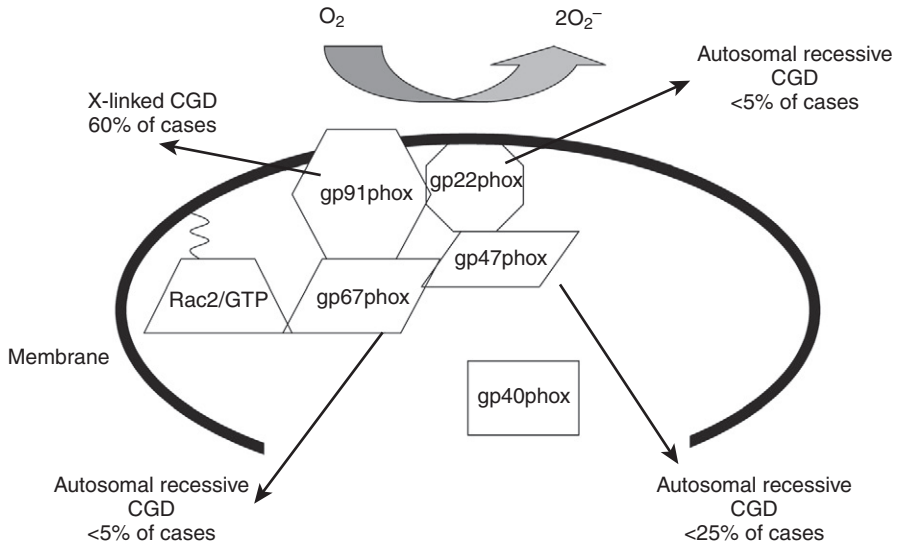


Figure 159-10 Structure of functional NADPH oxidase. NADPH oxidase consists of six subunits that are separated in different locations in the cell. p22phox and gp91phox are integral membrane proteins and form a heterodimeric structure that constitutes the catalytic core of the enzyme. Rac GTPase and p47phox, p67phox, and p40phox are cytosolic members of the complex.

molecular oxygen by the phagocyte (the "oxidative burst") that is triggered by cell membrane signaling before particle ingestion. Activation of cell membrane–associated NADPH oxidase results in shuttling of electrons from cytosolic NADPH to molecular oxygen, initiating a series of reactions responsible for producing multiple oxyradical species. NADPH oxidase consists of six subunits that are separated in different locations in the cell (Figure 159-10). p22phox and gp91phox are integral membrane proteins and form a heterodimeric structure that constitutes the catalytic core of the enzyme.[561] Rac GTPase and p47phox, p67phox, and p40phox are cytosolic members of the complex.[561,562] In the presence of activated-membrane NADPH oxidase, molecular oxygen accepts a single donated electron, becoming superoxide ion (O_2^-). Because this radical can either accept or donate an electron, it subsequently may be oxidized or reduced; when two such radicals interact in the presence of superoxide dismutase, one is oxidized and one is reduced, such that $O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$ generate hydrogen peroxide. Transfer of a second electron to the superoxide ion via NADPH oxidase also generates H_2O_2. Neutrophil granules contain myeloperoxidase, an enzyme capable of generating further antimicrobial oxyradicals. Hydrogen peroxide reacts with myeloperoxidase and available halide species (primarily chloride in vivo) to generate hypochlorous acid (HOCl); HOCl is an extremely powerful oxidant, analogous to bleach.[563] Because it is highly reactive, it does not accumulate and is rapidly consumed in further reactions producing new oxidants. An important interaction may be with primary or secondary amines, to produce chloramines; although chloramines are weaker oxidants than HOCl, they tend to be longer lived and remain able to oxidize multiple potential targets.[564] Mutations in gp91phox or other NADPH oxidase components result in chronic granulomatous disease, and patients with this disorder suffer from both bacterial infections and infections with *Aspergillus fumigatu.*[565]

Cytokines

Cytokines play a sentinel role in the recruitment, proliferation, and survival of inflammatory cells within the lung. Cytokines are soluble proteins transiently synthesized by an appropriately stimulated immune or nonimmune effector cell and whose effects are mediated by binding to specific receptors on target cells. A cytokine may have autocrine effect when it modulates the properties of the cell producing it, paracrine effects when modulating the properties of cells proximally, and endocrine effects when it mediates its effects distally. Some cytokines may remain either cell associated or membrane bound and exert their effects through cell-to-cell contact; this may facilitate more specific regulation of local inflammatory events. A single cytokine may be produced by many cell types, and a single cell type may produce many cytokines. In general, cytokines are multifunctional molecules, participating in a wide spectrum of immune and nonimmune processes, including inflammation, metabolism, morphogenesis, fibrosis, and hemostasis. The action of a given cytokine may vary depending on its dose, receptor availability, the state of activation of the target cell, and the presence of other cytokines in the local milieu. Additionally, cytokines frequently stimulate target tissues to produce other bioactive cytokines. Accordingly, the biologic effects of cytokines result from their direct effects on target tissues and their ability to interact with one another in regulating target cell function; the eventual response of a cell to these mediators will depend on the sum of the signals received and the cell's state of responsiveness to each of these signals.

Tumor Necrosis Factor

TNF is a phylogenetically primitive mediator, based on its high degree of homology across species. It is produced primarily by monocytes and macrophages in response to LPS, as well as in response to enterotoxin, mycobacterial cell wall products, and components of complement.[566] TNF is also produced by T cells and NK cells.[566] Its half-life locale is approximately 6 minutes, with peak levels at 1 to 2 hours after LPS exposure, defining its role as an "early response" mediator in endotoxin-induced inflammatory cascades.[567] It is now clear that TNF is a key mediator of host defense against a wide range of pulmonary pathogens, including *Pseudomonas, Legionella, Klebsiella, S. aureus, S. pneumoniae,* and *Mycobacterium* species.[568-571] Alveolar macrophage–derived TNF remains compartmentalized within the alveolus where it exerts protean inflammatory effects. TNF acts on PMNs to increase phagocytosis, adhesion molecule expression, and respiratory burst capacity.[572,573] TNF acts on epithelial and vascular endothelial cells to similarly up-regulate adhesion molecule expression, thereby facilitating inflammatory cell migration.[572] TNF induces IL-1 generation by both endothelial cells and local mononuclear phagocytes, which augments the initial inflammatory signal and can directly induce production of chemokine IL-8 from multiple cell types, further amplifying inflammation.[574] TNF also induces IL-6 production by fibroblasts, epithelial cells, and endothelial cells, initiating an acute phase response (detailed later).[575] Neonatal monocytes and T cells have diminished (50% to 60%) capacities

to generate TNF relative to adult cells, and these limitations are further pronounced in preterm neonates (25% adult capacity).[576,577]

Interleukins

Interleukin-1

The IL-1 family consists of several proinflammatory agonists however this chapter will focus on two, IL-1α and IL-1β, and the anti-inflammatory IL-1 receptor antagonist protein (IL-1ra). IL-1α is primarily membrane bound whereas IL-1β is secreted. IL-1α and IL-1β are encoded by separate genes but share the same receptor by virtue of their 23% homology and thus can elicit similar effects. These receptors are ubiquitously expressed so the effects of this cytokine are pleiotropic.[578,579] Ligation of the receptor IL-1R1 activates IL-1 receptor-activating kinase (IRAK), which culminates in liberation and nuclear translocation of NF-κB.[579] Of note is that the IL-1R1 signaling cascade shares MyD88 and IRAK with the pathways for TLRs 1,2, 4, 5, 6, 7, 8, and 9 (Figure 159-11).[580] IL-1 shares many properties with TNF, including enhancement of adhesion molecule expression by PMNs and endothelial cells, up-regulation of NOS, and neutrophil priming. IL-1 acts on fibroblasts, epithelial cells, and endothelial cells to induce IL-6 and IL-8 production, thereby modulating both enhanced PMN chemoactivation and initiation of the acute phase response.[579] IL-1 is an endogenous pyrogen and within the central nervous system can elicit fever.[581] Other actions of IL-1 include stimulation of T-cell proliferation, increased IL-2 receptor expression on T and B cells, and increased release of T cell–derived IL-2.[579] IL-1β has recently been implicated in the differentiation of both murine and human Th17 cells.[582,583] Regulation of IL-1 occurs by either direct IL-1ra antagonism or sequestration of IL-1 by its "decoy" receptor, the IL-1R2.[579] Engagement of this receptor by IL-1 does not induce signal transduction; metalloprotease cleavage of this receptor from cell surfaces releases a soluble receptor that blocks IL-1–mediated effects.

Mononuclear phagocytes do not produce IL-1 constitutively, but its production is induced by multiple stimuli including viruses, microbial products (endotoxin, peptidoglycans, techoic acid, yeast cell walls), C5a, TNF, and IL-1 itself. [579] However secretion of IL-1β requires cleavage by caspase-1.[579] This enzyme is activated by proinflammatory stimuli though a series of cytosolic molecules that detect lipopetides. Similar to TLRs these proteins encode leucine-rich repeats which are critical for ligand recognition. These NOD and NALP proteins activate the "inflammasome," which is critical for cleavage of IL-1β.[584,585] Available data suggest that neonates, both term and preterm, possess adult capacity to produce monocyte-derived IL-1 and exceed adult capacity to produce PMN-derived IL-1.[576,586]

IL-1ra is an endogenous IL-1 inhibitor that competitively binds to the IL-1R1 without inducing signal.[587] It exists in two intracellular isoforms found in epithelial cells and as a third isoform secreted by monocytes.[588] IL-1ra is synthesized by alveolar macrophages (and other mononuclear cells) in response to LPS or adherent IgG.[589] Emphasizing the potential role of IL-1 in pulmonary inflammation, animal models of endotoxin-induced pneumonitis demonstrate profound antiinflammatory response to intratracheally instilled IL-1ra whereas murine models of IL-1ra deficiency show an exaggerated and persisting inflammatory response.[590,591] These data, along with known in vitro actions, suggest that IL-1ra may function as an endogenous "down-regulator" of inflammatory cytokine cascade within the alveolar space. IL-1ra is found in tracheal aspirates of preterm infants at risk for bronchopulmonary dysplasia, but this may represent release from injured epithelium rather than *de novo* immune cell production.[592] Serum IL-1ra levels are elevated in healthy newborns, decline within days of birth, and increase in response to infection, indicating capacity for stimulus-specific IL-1ra production.[593]

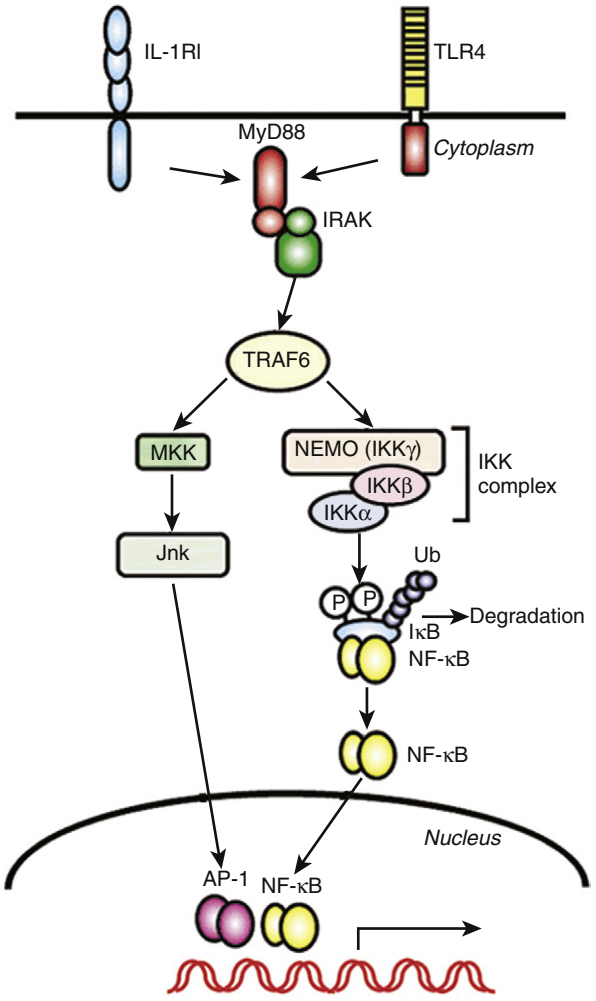


Figure 159-11 IL-1R1/TLR signaling. To illustrate the shares signaling pathway the molecular components involved in IL-1R and TLR4 signaling are shown. Both IL-1R1 and TLR4 (as well as TLR1, 2, 6, 7, 8, and 9) can associate with a cytoplasmic adaptor molecule, MyD88. This interaction occurs between the TIR domains. MyD88 encodes a death domain that mediates association with a serine-threonine kinase, IRAK. TRAF6 serves as scaffold protein can can actvite MAP kinases (MKKs) as well as the IKK complex. MKK can also lead to AP-1 activation via Jnk. The IKK complex induces phosphorylation of IκB, which allows ubiquination of IκB followed by proteasomal degradation. This allows nuclear translocation of NF-κB and gene expression *(From Akira S, Takeda K, Kaisho T:* Nature Immunology 2, *675-680 (2001) doi:10.1038/90609).*

Interleukin-6

IL-6 is primarily produced by mononuclear phagocytes, but it is also produced by T and B cells and may be produced by endothelial cells, epithelial cells, and fibroblasts in response to TNF and IL-1.[594,595] IL-6 production by stimulated neonatal mononuclear cells matches or exceeds that of adults, although diminished production is noted with marked prematurity.[596,597] IL-6 is able to exert effects on a wide range of cells, owing to the unique properties of its receptor. When activated by IL-6, the IL-6 receptor complexes with the gp130 membrane protein, which is required for intracellular signal transduction through the phosphorylation of Stat3. IL-6R also circulates in a soluble (sIL-6R) form, which binds secreted IL-6.[594] The IL-6:IL-6R complex can then activate any cell bearing the gp130 membrane protein, allowing IL-6 to

activate a wide range of cell types. IL-6 provides costimulatory signals to enhance T-cell responses and the differentiation of Th17 cells and stimulates B-cell differentiation and antibody production.[454,472,598] IL-6 also induces the acute phase response, characterized by hepatocyte production of such proteins as fibrinogen, α_2-macroglobulin, and α_1-antitrypsin and also up-regulates local acute phase protein production by mononuclear phagocytes.[599] IL-6 is found in BAL specimens of adults with pulmonary inflammation, as well as in BAL specimens of mechanically ventilated, premature neonates. It is uncertain if IL-6 is a marker or mediator of inflammation in either of these scenarios.[600,601]

Interleukin-10

IL-10 is a critical cytokine produced by T cells, B cells, and mononuclear phagocytes in response to stimuli, including LPS and TNF-α.[602] IL-10 augments proliferation and differentiation of activated B cells while up-regulating their MHC class II expression.[602] IL-10 inhibits the generation of oxyradicals and NO by macrophages and PMNs, impairing their microbicidal functions.[603,604] IL-10 also suppresses macrophage expression of MHC class II and costimulatory molecules CD80 and CD86, diminishing the capacity to present antigen.[602] Finally, IL-10 inhibits macrophage-dependent T-cell proliferation and Th1 cytokine production by macrophages, PMNs, Th1 cells, and NK cells.[602] Mice deficient in IL-10 develop spontaneous colitis.[605,606] These data, along with the fact that IL-10 is expressed by regulatory T cells, suggest that IL-10 is critical as a negative regulator of the immune response.

There is evidence that IL-10 production in the lungs may be protective in some types of inflammatory injuries; animal models of pneumonia have helped to clarify this role of IL-10 in vivo.[607] Exogenous IL-10—preceding intratracheal bacterial challenge—reduced lung TNF, and IFN-γ responses, but increased lung bacterial counts, systemic bacteremia, and morbidity in a murine model of *S. pneumoniae* infection.[608] IL-10 neutralization in this same model resulted in increased lung TNF, diminished lung bacterial counts, and increased survival rates. Similar outcomes have been shown in a murine model of *Klebsiella* pneumonia treated with systemic anti-IL-10 antibody.[609] Given these data, it is thus presumed that in the context of pneumonia, IL-10 acts to modulate the local inflammatory response initiated by the offending pathogen; this preserves alveolar structure, pulmonary gas-exchange function, and host survival. The data regarding neonatal IL-10 generation is somewhat contradictory, but does allow some broad generalizations. First, stimulated neonatal monocytes exhibit only 20% to 40% of the IL-10 production inducible from stimulated adult monocytes.[610,611] Second, available BAL data from mechanically ventilated infants indicates that IL-10 expression in the lung is diminished in preterm infants but increases with advancing gestational age.[612,613] These age-related findings parallel data indicating differential IL-10 production by lung macrophages ex vivo from term and preterm infants.[614] Such differences, if true in vivo, suggest that premature infants may be less able to attenuate alveolar inflammation in the setting of a pneumonitis or other inflammatory insult.

Interleukin-15

IL-15 is a 15-kDa peptide that shares β-and γ-receptor components with IL-2, resulting in overlapping immune functions including induction of T-cell proliferation and increased T-cell cytotoxic activity.[615,616] Like IL-2, IL-15 binding requires a distinct α-chain receptor component (IL-15Ra); this component is expressed in a variety of tissues, conferring pleiotropic immune activities distinct from those of IL-2. IL-15 induces NK cell–derived TNF-α, GM-CSF, and IFN-γ and augments IL-12 up-regulation of T cell–derived IFN-γ.[617,618] IL-15 also induces NK-cell differentiation, activation, and supports prolonged NK-cell survival. Beyond these effects, IL-15 has also been shown to induce IL-8 expression by T cells and monocytes and up-regulate monocyte-derived MCP-1 and MIP-1β, facilitating phagocyte recruitment and a proinflammatory milieu.[619] Finally, IL-15 also directly activates PMNs, inducing membrane "stiffening," enhancing phagocytosis, and delaying apoptosis.[616] Although macrophages are the primary cellular source of IL-15, gene expression of this cytokine has also been identified in lung fibroblast and epithelial cells, DCs, and endothelial cells in response to a variety of bacterial and viral stimuli, indicating a role for IL-15 in pulmonary immune regulation.[616] IL-15 is critical for homeostatic proliferation of T cells and regulates the generation of memory T cells.[620] IL-15 production has not been characterized in the neonatal air space, but stimulated neonatal monocytes in vitro exhibit diminished IL-15 mRNA stability and protein expression.[621] Given this finding, it is reasonable to speculate that attenuated IL-15 production by the neonate may contribute to impaired pulmonary cellular immunity.

Interleukin-17

IL-17 is a proinflammatory cytokine produced by activated memory CD4 T cells of the Th17 susbet that facilitates granulopoiesis and pulmonary PMN recruitment in response to local bacterial infection.[458] It is the prototypical member of a cytokine cohort collectively designated IL-17A-F, which also includes IL-25 (IL-17E).[622] IL-17A signals through the adaptor proteins Act1 to induced NF-κB and AP-1.[623,624] IL-17 stabilizes transcripts for CXCL1 and G-CSF and therefore synergizes with other inducers of NF-κB such as TNF-α.[458,625] The target cells of IL-17s actions are mainly epithelial cells, fibroblasts, and endothelial cells.[458,622] Pulmonary IL-17 has not as yet been quantified in neonatal cohorts, but may be negligible given the relative paucity of memory T cells in this population. IL-17E is the most divergent IL-17 family member showing only 25% homology with IL-17A. Interestingly IL-17E or IL-25 potently induces IL-4 and IL-13 and is involved in Th2 T-cell differentiation.[626,627]

Interleukin-18

Initially identified as IFN-γ–inducing factor (IGIF), IL-18 is an approximately 18-kDa protein that enhances IFN-γ production and exhibits other, proinflammatory, functions.[628] IL-18 is similar to IL-1β both in secondary structure and in its secretion as an inactive precursor requiring cleavage by IL-1β–converting enzyme (caspase-1) to become bioactive.[628] IL-18 also shares receptor components and signal transduction mechanisms with IL-1.[628] Binding of IL-18 to its receptor, the IL-1 receptor–related protein (IL-1Rrp), triggers a signaling pathway that requires IRAK and eventually culminates in the nuclear translocation of NF-γB and gene transcription. IL-18 may also bind to its alternative ligand, IL-18 binding protein (IL-18bp)—an endogenous, soluble, "decoy" receptor that inhibits IL-18 activity; IL-18 bioactivity may thus be titrated either by regulation of cleavage activation or by modulation of IL-18bp levels.[629] IL-18 is present constitutively within the lung, primarily derived from alveolar macrophages, although it is also inducible in vitro from pulmonary fibroblasts.[630] Like other macrophage-derived cytokines, IL-18 is up-regulated in response to microbial products (LPS, gram-positive bacterial exotoxins) and is also induced by exposure to TNF-α, chemokines (IL-8, MCP-1, MIP-1α), and mycobacteria.[628] As suggested earlier, IL-18 has important IFN-γ–inducing activity. By direct activation of the IFN-γ promoter, IL-18 achieves potent synergy with IL-12 for IFN-γ production by T cells, B cells, and NK cells but only modest induction of IFN-γ in the absence of IL-12; this augmented IFN-γ production may favor a Th1 response within the alveolus.[628] IL-18 is protective against fungal, bacterial, and viral pathogens.[631-633] Such a role has been demonstrated in vivo in a transgenic model of IL-18 deficiency, where impaired lung bacterial clearance and fulminant progression to systemic infection resulted from intratracheal inoculation with *S. pneumoniae*.[634] In neonates, however, the pulmonary expression of IL-18 remains incompletely characterized.

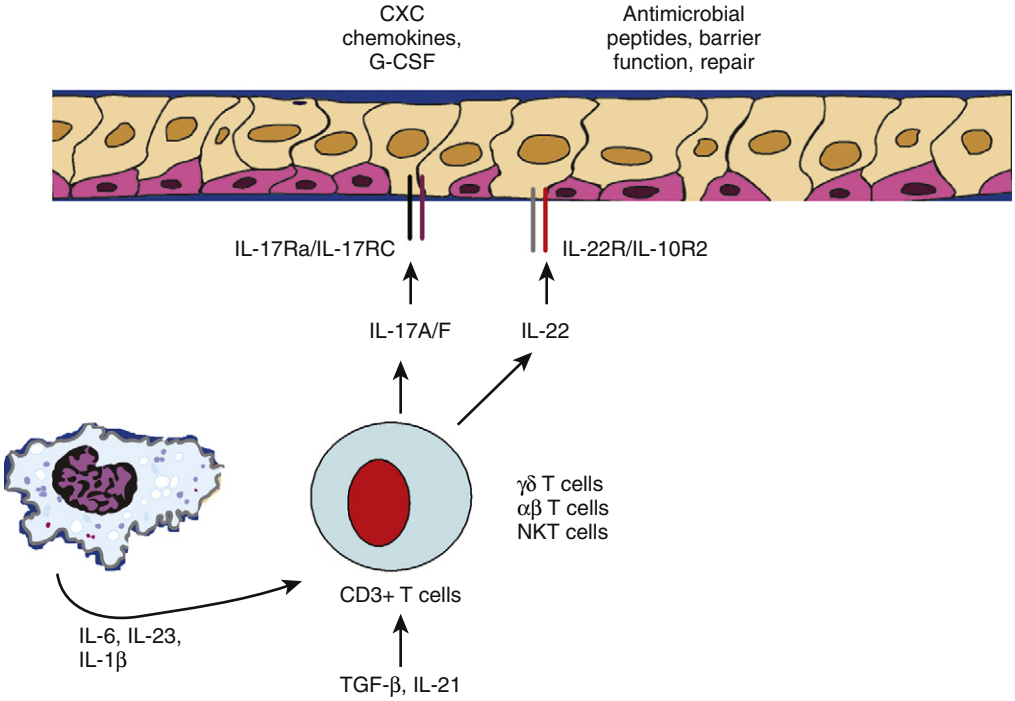


Figure 159-12 Functions of IL-17 and IL-22 during experimental *K. pneumoniae* infection in the lung.IL-17 and IL-22 are induced rapidly in experimental bacterial pneumonia and produced by several T-cell populations in the lung including δ-T cells, NK T cells, as well as effector memory αβ CD4+ T cells. IL-17 signaling regulates granulopoiesis through the regulation of G-CSF, as well as neutrophil recruitment via the regulation of CXC chemokines by epithelial cells. IL-22 along with IL-17 induces antimicrobial peptides from same target cells and can augment epithelial repair.This cooperative induction of neutrophil recruitment and antimicrobial peptide production augments epithelial barrier function and is critical for mucosal host defense against gram-negative bacterial pneumonia.

Interleukin-22

IL-22 is one of the IL-10 family cytokines, which also includes IL-10, IL-19, IL-20, IL-24, and IL-26, as well as more distantly related IL-28 and IL-29. IL-22 was first identified as an IL-10– related T cell–derived inducible factor (IL-TIF) from a lymphoma cell line treated with IL-9.[635] IL-22R and IL-10R2 were soon identified as the heterodimeric receptor complex for the function of IL-22.[458] As mentioned above, one biologic function of IL-22 is that it synergizes with IL-17 to regulate the expression of antimicrobial peptides at epithelial surfaces.[456,460] IL-22 regulation of antimicrobial proteins has been shown to exist at several mucosal surfaces including the skin, gut, and, lung (Figure 159-12).[456,458]

Chemokines

IL-8 and several other structurally homologous proteins make up the family of cytokines known as chemokines. The chemokine family consists of more than 40 small (70-130 amino acids) cytokines divided into four subfamilies based on the position of one or two conserved cysteine residues located near the N-terminus defining four structural motifs: CXC, CC, C, and CX3C.[636,637] Different leukocyte subsets express a unique chemokine receptor profile that defines the response potential of each type of cell. In turn, chemokines can be modified by proteases in the extracellular milieu to become more active or inert.[638] Although most chemokine receptors bind more than one chemokine, CC receptors bind only CC chemokines and CXC receptors bind only CXC chemokines. Chemokines play a critical role in recruitment and activation of inflammatory cells at sites of infection. Both CC and CXC chemokines have positively charged C-terminus residues that bind to negatively charged glycosaminoglycans on matrix proteins in the interstitium and on cell surfaces; this provides a mechanism to stabilize chemokines near sites of generation, achieving relatively fixed local chemotactic gradients. Proteolytic cleavage of glycosaminoglycans such as syndecan can also modify the amount of free chemokine to serve as a chemoattractant.[639] The spectrum of action of a given chemokine is thus a function of its local concentration, its posttranslational processing, and the temporal expression of chemokine receptor(s) on putative target cells. Important representative chemokines implicated in the regulation of pulmonary immune responses include members of the CXC and CC subfamilies.

CXC Chemokine Subfamily

CXC chemokines can be further subdivided into two groups based on the presence or absence of three amino acids (Glu-Leu-Arg: the "ELR" motif) preceding the first cysteine residue.The ELR+ CXC chemokines are potent neutrophil chemoattractants and angiogenic agents, whereas ELR-CXC chemokines are chemoattractant for mononuclear leukocytes and angiostatic. CXCL8 (IL-8), the prototypical ELR+ CXC chemokine, is produced by a number of pulmonary cells, including mononuclear phagocytes, endothelial cells, fibroblasts, epithelial cells, mesothelial cells, and T cells.[640 641] Chemokine production by these cells can be induced by multiple stimuli, including viruses, bacterial products, TNF, IL-1, C5a, LTB_4, IL-17 and IFN.[640,641] Actions of IL-8 on PMNs include potent chemoattraction, upregulation of adhesion molecule expression, and enhancement of PMN respiratory burst and degranulation.[640-642] IL-8 also stimulates PMN production of the inflammatory lipid mediators LTB_4 and PAF.[640,641] It is important to keep in mind that IL-8 is only one ligand for CXCR2 and that CXCL1, CXCL2, CXCL5 and CXCL6 can each serve as ligands for CXCR2. Thus the contribution of CXCL8 in humans compared to these other CXCR2 ligands remains to be defined.

IL-8 is locally expressed in patients with pneumonia, and its critical role in host response is further supported by animal models of bacterial pneumonitis; in these models, depletion of CXC chemokines, or blockade of CXC receptors, attenuates PMN recruitment and blunts pulmonary bacterial clearance.[643-646] A similar role likely exists for IL-8 in neonates; however, data regarding IL-8 production in this population is somewhat contradictory. In vitro stimulation of neonatal immune cells has typically demonstrated IL-8 production well below adult levels.[647,648] However, neonatal IL-8 production exceeding adult response has been shown using flow cytometric analysis in an ex vivo model of neonatal sepsis or neonatal stress.[649,650] Although challenging to previous conventional wisdom, these findings are consistent with other emerging data showing the presence of significant, inducible IL-8 in the BAL specimens of term and preterm newborns with lung insults such as meconium aspiration syndrome or hyaline membrane disease.[651] IL-8 should therefore be considered as a likely relevant mediator both in neonatal pulmonary host defense as well as in inflammatory lung pathology. CXCL9, CXCL10, and CXCL11 are all ligands for CXCR3, which is preferentially expressed on Th1 cells and thus these chemokines have been shown to be critical for recruitment of Th1 cells to the lung and granuloma formation.[652,653]

CC Chemokine Subfamily

CCL3 (MIP-1α), CCL2 (MCP-1), and other members of the CC chemokine subfamily are derived from multiple pulmonary cells including PMNs, mononuclear phagocytes, lymphocytes, NK cells, endothelium, fibroblasts, epithelial cells, and mesothelium.[654-656] Stimuli that induce production of the CC chemokines include viruses, bacterial products, TNF, IL-1, C5a, LTB4, and IFN; IL-10 is a potent CC chemokine suppressor. The CC chemokines have specific chemotactic activity for monocytes, lymphocytes, eosinophils, and basophils and tend to have proinflammatory properties.[657,658] Binding of CC chemokines to their corresponding receptors on monocytes produces an increased respiratory burst and induces rapid release of arachidonic acid metabolites.[656,659] CCL3 and CCL2 regulate monocyte expression of adhesion molecules, preferentially increasing monocyte expression of the β2 integrin CD11/CD18 normally associated with PMNs, rather than the usual monocyte adhesin VLA-4.[660] This enhanced expression of β2 integrins results in augmented monocyte adhesion to endothelium, putatively enhancing monocyte migration to inflammatory loci. The CC chemokines have also been identified in the recruitment of T cells, B cells, and NK cells; however, they appear to lack significant effects on PMNs.[656,661,662]

Although early in vitro work demonstrated neonatal monocytes had decreased inducible CCL3 relative to adults, recent data have challenged these findings.[663] Basal serum levels of CCL3 are found to be similar in neonatal and adult cohorts, and monocyte-derived CCL3 is comparably inducible in these two age groups.[664] Levels of CCL3 are increased in airway fluids of infants with inflammatory pulmonic processes including severe RSV infection, *Ureaplasma* infection, and respiratory distress syndrome, suggesting local inducibility of this chemokine in response to infection or insult.[665-668] Neonatal levels of MCP-1 in both serum and airway fluid are found to be similarly inducible in response to appropriate stress. Taken together, these data suggest that the CC chemokines like IL-8 should be considered relevant mediators of both pulmonary host defense and inflammatory lung injury in the neonate.

Transforming Growth Factor-β

TGF-β is a cytokine primarily associated with tissue repair and fibrosis, but one that also exerts important immunomodulatory effects. This cytokine is secreted as an inactive disulfide bond-linked dimer, and liberation of the 25-kDa active form occurs in response either to proteolytic activation or acidic condition.[669-671] TGF-β is produced by multiple cell lines, including platelets, T cells, PMNs, mononuclear cells, fibroblasts, epithelial cells, and endothelial cells and exerts pleiotropic effects relevant to host immune response.[671] It can directly, or indirectly through IL-6, induce production of acute phase proteins and together with IL-6 control the development of Th17 cells.[472,672] In the absence of IL-6, TGF-β can induce the development of regulatory T cells.[472] TGF-β can also act as a potent chemoattractant for monocytes and macrophages and can induce these cells to produce TGF-β, thereby amplifying its local effects.[673] Although TGF-β can induce monocyte-derived IL-1 and TNF-α, it inhibits LPS induction of macrophage-derived IL-1, TNF-α, and IFN-γ, thus exhibiting antiinflammatory activity in settings of immune activation.[670] TGF-β can also down-regulate macrophage NO production and MHC II expression and suppresses macrophage respiratory burst.[674] Beyond macrophage deactivation, TGF-β also down-regulates IL-2 receptors, inhibiting IL-2–dependent T-cell proliferation and IL-1–dependent lymphocyte proliferation. The net result of TGF-β activity is impairment of IL-2 effect and inhibition of Th1 response. Within the alveolus, this manifests as impaired antigen presentation and a general suppression of inflammation. Data defining the role of TGF-β in neonatal pulmonary defense remain incomplete. In vitro, TGF-β production from stimulated neonatal monocytes is only 35% to 40% of that from adult monocytes; this suggests compromised ability of the neonate to titrate inflammation through TGF production.[663] This observation is supported by in vivo data showing lower TGF-β levels in the BAL specimens of infants progressing to states of chronic lung disease.[675] Finally, additional studies have shown elevated neonatal BAL levels of TGF-β, suggesting that expression of this cytokine may be more inducible than indicated by in vitro data.[676]

Clara Cell Secretory Protein

Clara cell secretory protein (CCSP) is one of the most abundant proteins of the airway lining fluid. It is secreted by Clara cells, as well as by serous and goblet cells of the proximal airways.[677] Structurally, CCSP is a small homodimeric protein consisting of two 8-kDa subunits aligned by disulfide links to form a hydrophobic pocket.[678] This pocket can bind phosphatidylcholine and phosphatidylinositol, and CCSP was originally postulated to participate in surfactant metabolism. More recently, CCSP has been noted to modulate inflammatory responses, suggesting a potential immunoregulatory role within the airways and alveoli. In vitro, CCSP has been shown to inhibit PMN chemotaxis, as well as macrophage phagocytosis.[678] Its hydrophobic pocket allows CCSP to "scavenge" phospholipase A_2 within the milieu, further limiting PMN activation.[678] CCSP has also been shown to inhibit the production and bioactivity of IFN-γ.[678] Murine models of CCSP deficiency have attempted to further clarify the in vivo role of this protein. In a hyperoxic model of lung injury, CCSP-deficient animals exhibited higher lung levels of IL-1β, IL-6, and IL-3, increased lung inflammation, and decreased survival rates.[679] In a model of adenoviral lung infection, CCSP deficiency resulted in both earlier and increased inflammatory response; this was accompanied by increased expression of TNF, IL-1β, IL-6, and chemokines (CCL3 and CCL2).[680] CCSP-deficient animals receiving an antigenic challenge showed increased levels of Th2 cytokines with exacerbated eosinophilic inflammation.[681] Finally, CCSP-deficient mice demonstrate increased expression of IgA.[682] The exaggerated cellular response seen in each of these models is consistent with the putative role of CCSP as an antiinflammatory molecule. LPS depresses CCSP gene expression in a dose-dependent fashion, suggesting that potent signals of infection may dampen CCSP secretion as required to allow an appropriate inflammatory response.[683] In newborns, CCSP levels are detected in tracheal/alveolar aspirates obtained at birth and correlate with gestational age. Neonates with clinical signs of infection have increased CCSP levels, correlating inversely with tracheoalveolar

fluid leukocyte counts.[684] Similarly, infants who subsequently develop BPD demonstrate diminished inducibility of CCSP and generally lower CCSP levels in their airway fluid, possibly contributing to the increased lung inflammation seen in this process.[685]

Colony-Stimulating Factors

Colony-stimulating factors such as GM-CSF are also identified as participants in pulmonary host defense. These factors, including G-CSF and macrophage–colony stimulating factor (M-CSF), are generated by a number of relevant pulmonary cells including endothelial cells, epithelial cells, fibroblasts, mononuclear phagocytes, and T lymphocytes; production of these factors may be induced by an array of microbial products and pathogens.[686,687] Once elaborated at a site of potential infection, these factors exert pleiotropic effects on local immune cells, including increased membrane expression of receptors and adhesion molecules, enhanced motility, increased microbiocidal function of phagocytic cells, enhanced phagocyte survival, and stimulation of cytokine secretion by mononuclear phagocytes.[686,687] GM-CSF also acts on macrophages to stimulate their antigen presentation capabilities and directs DC differentiation and distribution in the lung.[688,689] Within the lung, GM-CSF is produced by alveolar macrophages, bronchial epithelial cells, and vascular endothelium. It is a potent stimulus for PMN degranulation, and its production by endothelial cells has been implicated in PMN-mediated alveolar injury.[690] GM-CSF deficiency results in a phenotype of pulmonary alveolar proteinosis.[691] Although the precise mechanism of this phenotype remains unclear, GM-CSF–deficient macrophages show reduced expression of the transcription factor PU.1 and reduce phagocytic capacity.[692,693] The capacity of neonatal T cells to generate GM-CSF is only 50% of adult T cells; however, neonatal mononuclear cells possess an adult capacity for production of this cytokine and may thus be able to induce local responses dependent on GM-CSF elaboration. This premise is supported by data from newborns demonstrating alveolar macrophage generation of GM-CSF.[694]

Of the colony-stimulating factors, G-CSF appears to be most critical in pulmonary host defense against bacteria. Alveolar macrophages are potent secretors of G-CSF in response to LPS, or when stimulated by TNF or IL-1. Alveolar macrophage–derived G-CSF acts locally to recruit, activate, and delay apoptosis of PMNs while acting systemically to induce granulopoiesis and increase the circulating neutrophil count. The importance of G-CSF in this context is underscored by animal studies where antibody neutralization of G-CSF results in profoundly suppressed PMN recruitment and diminished bactericidal activity against aerosolized *P. aeruginosa.*[695,696] Of note, G-CSF also induces antiinflammatory mediators such as IL-1ra and soluble TNF receptors, locally titrating the inflammatory response.[697] Neonatal monocytes can be stimulated to generate near-adult amounts of G-CSF. Preterm infants, however, appear much more compromised in their ability to up-regulate G-CSF production (10% to 30% of adult response), and this may constitute yet another deficiency of pulmonary host defense in this cohort.[698]

Adhesion Molecules

Leukocyte migration from the intravascular space to an eventual inflammatory locus within the lung is a multistep process dependent on mechanisms directing cell movement to the appropriate site; leukocytes must marginate and adhere to the vascular endothelium followed by diapedesis between endothelial cells and movement from the vascular space. Directed movement along concentration gradients of chemotaxins is one mechanism; another is haptotaxis, or directed migration along gradients of endothelial adhesiveness. This latter process requires the presence of both leukocyte- and endothelial-derived adhesion molecules and is mediated by three families of molecules: the integrins, the immunoglobulin gene superfamily, and the selectins.

The integrins are a family of proteins involved in cell adhesion and motility. Present on leukocyte membranes, these molecules adhere to specific endothelial ligands and various components of connective tissue matrix. The integrins are expressed as heterodimers, each containing one α and one β chain; subclassification of these adhesins is based on specific α/β chain content. The α4 integrins are expressed at high levels on monocytes, lymphocytes, and eosinophils. Although typically absent from neutrophils, α4 integrin is present on immature PMNs or on mature forms under conditions of transendothelial migration or chemokine stimulation.[699,700] The β1 integrins include very late antigen 4 (VLA-4), found on lymphocytes and monocyte.[701] The β2 integrins include CD11a/CD18, constitutively expressed on all leukocyte subsets, and CD11b/CD18 and CD11c/CD18, which are prominent adhesins of PMNs.[702] Because the PMN must deform to navigate the narrow alveolar capillary space, the PMN surface membranes are brought into close contact with those of the vascular endothelium, inviting adherence interactions.[703] CD11/CD18 is therefore maintained in an inactive state until chemotactic activation occurs.[704] PMN activation results in a CD11/CD18 conformational change to an activated form that recognizes its corresponding endothelial ligand.[705] PMN activation also results in mobilization of a significant intracellular stored pool of CD11/CD18, facilitating rapid additional surface expression of this molecule although the constitutively smaller pool in neonatal PMNs may tend to blunt this response.[702]

The immunoglobulin gene superfamily includes intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2), vascular cell adhesion molecule 1 (VCAM-1), and platelet-endothelial cell adhesion molecule (PECAM). ICAM-1, the endothelial ligand for CD11/CD18, is expressed on endothelial cells constitutively and up-regulated in response to endotoxin, TNF-α, or IL-1β; ICAM-1 is also expressed on the surface of fibroblasts and epithelial cells.[706] VCAM is similarly up-regulated by TNF-α and IL-1β, recognizes VLA-4 (and other β1 integrins), and binds monocytes and lymphocytes.[707] PECAM is present constitutively and in the pulmonary vasculature may up-regulate in response to hyperoxia.[708] Its primary counterreceptor is the P-selectin glycoprotein ligand-1 (PSGL-1), found on a variety of leukocytes, including PMNs. PECAM-mediated adhesion may be important in transendothelial cell diapedesis of PMNs from the intravascular space.[709]

The selectin family includes L-selectin, P-selectin, and E-selectin. L-selectin is expressed on the surface of PMNs and is shed shortly after activation in vitro.[710] P-selectin is stored in endothelial cells and rapidly mobilized to the surface after initial endothelial activation.[711] E-selectin (endothelial-leukocyte adhesion molecule [ELAM]) is expressed on the surface of endothelial cells only after activation by bacterial products or mediators including C1q, TNF-α, IL-1β, and IL-10.[712,713] PMN receptors for the endothelial selectins include PSGL-1 and sialyl Lewis X antigen (sLe^x), an oligosaccharide present on many proteins including the PMN adhesins L-selectin and CD11/CD18.[714,715]

The initial step in leukocyte migration to extravascular sites is a slowing of leukocyte movement through the area of inflammation by a series of loose, transient leukocyte-endothelial cell adhesions, characterized as "rolling." This step is mediated through interaction of endothelial selectins with PSGL-1 and L-selectin on the PMN surface.[715] Diminished L-selectin and sLe^x concentrations on neonatal PMN surfaces have been implicated in impaired chemotaxis, but are likely not relevant in the pulmonary circulation where the constrained vascular space obviates the need for this receptor-ligand interaction.[716] The next step entails binding of local chemotactic activators (C3a, C5a, IL-8, PAF) to the "rolling" PMN, inducing expression and activation of CD11/CD18 and shedding of L-selectin.[715] Subsequently, the leukocytes firmly attach to the endothelium via CD11/CD18 to ICAM-1 interaction, PMNs, or by means of VLA-4 to VCAM-1

interaction (mononuclear cells, lymphocytes).[717,718] The final step is diapedesis of the adherent leukocyte through the vessel wall and migration along a chemotactic gradient to the site of inflammation. Although this process remains incompletely characterized, transendothelial migration appears mediated by the adhesion molecules PECAM and the integrin-associated protein CD47.[719] Once extravasated, neutrophils appear to follow a fixed chemotactic gradient laid down in the extracellular matrix, migrating along interstitial fibroblasts and eventually squeezing between type I and type II epithelial cells to reach the air spaces of the lung.[720]

Generation of a Local Immune Response

In animal models of bacterial pneumonia, the influx of inflammatory cells follows a well-orchestrated sequence. First, neutrophils enter the lung within minutes to hours of airway inoculation. This early neutrophil recruitment is partly dependent on TNF-α, and IL-1β, which can upregulate ICAM-1 as well as via the induction CXCL1, CXCL2, and CXCL5. PMN accumulation is maximal within 2 days with cell clearance primarily through apoptosis. Later PMN recruitment is in part regulated by IL-17, which stabilizes CXC chemokine expression.[458,622] By day 2 to 3 monocytes are recruited, with maximal accrual by day 5 to 7 in resolving inflammation. Lymphocyte influx begins by 5 days, often occurring despite clearance of the organism. Although the timing of events is not consistently this rigid, the sequential appearance of each cell type is characteristic, consistent with a transition from acute inflammation to a more specific immune response. Factors unique to the lung that regulate this coordinated host response are now considered.

Intrapulmonary Neutrophil Trafficking

Most marginated neutrophils (up to 50% of intravascular PMN) reside in lung capillaries; thus compared to other organs, the lungs enjoy a large, readily mobilizable granulocyte pool.[721] The narrow caliber of these vessels (diameter 5 to 9 μm) does not allow leukocyte rolling, but rather requires neutrophils (diameter 6.5 to 8 μm) to deform and slowly "squeeze through" this vascular space; this imposes sustained PMN-endothelial contact, independent of adhesion molecules.[722] The small distance between pulmonary capillaries and their alveoli facilitates brisk PMN response to alveolar-derived inflammatory signals. In this setting, mediator activation of neutrophils induces cytoskeletal changes that reduce PMN deformability, increasing duration of capillary transit and neutrophil-endothelial contact.[723] Of note, the impaired deformability of neonatal neutrophils may engender a larger marginated pool of these cells within the newborn lung. Once activated, "stiffened" neutrophils are retained briefly (4 to 7 minutes in vivo) near inflammatory sites until appropriate adhesins can be expressed to facilitate firm attachment and eventual transendothelial diapedesis.[724]

Beyond the rheologic conditions promoting neutrophil margination, neutrophil trafficking in the pulmonary capillary differs from other vascular sites in the repertoire of adhesive interactions invoked. P-selectin is not expressed on pulmonary capillary endothelium, consistent with spatial constraints that obviate selectin-initiated "rolling" as an initial step in PMN recruitment.[725] However, once activated, neutrophils are sequestered only transiently and return to circulation unless firm adhesion to the endothelium occurs. Available data suggest that this adhesion is mediated first by L-selectin then by CD11/CD18.[726] Firm adhesion mediated by CD11/CD18 is temporally associated with resolution of neutrophil "stiffening," allowing sufficient PMN surface area to flatten and engage the endothelium.[723] Once firmly adhered, neutrophil emigration from the vascular space then proceeds in a manner that is either CD11/CD18 dependent or CD11/CD18 independent, depending on the specific stimulus. *P. aeruginosa*, *E. coli* (or its endotoxin), IgG immune complexes, and IL-1 each induce PMN emigration mediated by CD11/CD18; because these stimuli also up-regulate endothelial ICAM expression, CD11/CD18– dependent migration may result from critical availability of this counter ligand.[727] Alternatively, stimuli including hyperoxia, C5a, IL-8, LTB_4, and the organisms *S. pneumoniae*, *S. aureus*, and group B *Streptococcus,* induce PMN emigration, which is CD11/CD18 independent.[727,728]

Alveolar Macrophage Regulation of Inflammation

The ability of the alveolar macrophage to recruit and regulate other inflammatory cells is the major attribute allowing alveolar macrophages to locally regulate pulmonary host defenses. In addition to their phagocytic function, alveolar macrophages can recruit PMNs, eosinophils, interstitial and airway macrophages, and peripheral blood monocytes to the alveolar space; once present in the alveolus, these cells are either activated or suppressed by alveolar macrophage–derived mediators.

Mediators that up-regulate inflammation within the alveolus are not constitutively expressed by alveolar macrophages; their release therefore requires the alveolar macrophages to be in an activated state. Activation typically occurs through engagement of macrophage pattern recognition receptors (PRRs) and can be titrated by competitive interactions with soluble PRRs within the alveolar milieu. One example of this is the interaction between SP-A and the CD14 ligand LBP. In the basal, uninflamed state, SP-A is present at much higher concentrations than the acute phase protein LBP and therefore is the primary ligand for any LPS inadvertently deposited from the air stream into the airway surface liquid. This prevents initiation of the LBP/CD14 pathway and dampens inflammatory tone within the alveolus. However, under conditions of inflammation, SP-A concentrations may fall dramatically, owing to degradation by PMN-derived proteases and elastases, whereas LBP is concurrently up-regulated as an acute phase protein. Under these conditions, LBP becomes the dominant LPS-binding protein in the alveolus; this facilitates alveolar macrophage activation via the CD14 receptor, amplifying the bioactivity of LPS and subsequent inflammation several fold.[729] Alternatively, alveolar macrophage activation can occur in response to GM-CSF elaborated by activated alveolar epithelium or in response to IFN-γ elaborated by local NK cells or T cells.

The activated alveolar macrophage effects PMN emigration to the alveolus through its production of multiple neutrophil chemotaxins including the lipid mediators PAF and LTB_4, complement components C3a and C5a, and IL-8. Activated alveolar macrophages also produce TNF-α and IL-1β, which, although not directly chemotactic, can activate PMN and up-regulate local endothelial cell surface adhesins and may thereby facilitate PMN recruitment from the intravascular space. Additionally, alveolar macrophages have the capacity to recruit and activate other inflammatory cells. Alveolar macrophages can induce chemotaxis and activation of monocytes by secretion of the chemokines MCP-1 and MIP-1α, and in pneumonia this resulting monocyte infiltration typically peaks between days 5 and 7. Mononuclear cell response to either MCP-1 or MIP-1α includes increased respiratory burst, increased chemotaxis, increased monocyte expression of β2 integrins, and increased generation of arachidonic acid products; MCP-1 has also been shown to prime macrophages to respond to LPS, resulting in a proinflammatory amplifying loop. NK cells are also recruited and activated by these chemokines, and alveolar macrophage–derived IL-12 increases NK-cell cytoxicity and induces NK-derived IFN-γ, generating another proinflammatory amplifying loop.

Consistent with its role in regulating intraalveolar immune responses, the alveolar macrophage also induces or expresses several mediators capable of honing or down-regulating local inflammatory processes. IL-6 derived directly from the alveolar macrophage or from alveolar cells (fibroblasts, endothelium,

epithelium), in response to alveolar macrophage-derived IL-1, induces T-cell and B-cell proliferation, but down-regulates subsequent alveolar macrophage production of TNF or IL-1. More directly, alveolar macrophage–derived IL-1ra, produced in response to endotoxin or adherent IgG, inhibits local IL-1β effects by competitive binding of the IL-1 receptor. Alveolar macrophage release of PGE_2 suppresses chemotaxis and release of inflammatory LTs by PMN and alveolar macrophages, further down-regulating TNF and IL-1 production. Alveolar marcrophage–secreted TGF-β blunts further alveolar macrophage response to LPS and serves to dampen the proinflammatory amplifying loops noted earlier. Additionally, alveolar macrophages can be induced to generate IL-10, although the most prominent alveolar source of this cytokine may actually be the recruited peripheral monocytes. This cytokine exhibits protean antiinflammatory activity. It suppresses a variety of proinflammatory cytokines and facilitates transition to a T cell–mediated adaptive immune response. Finally, alveolar macrophages down-regulate inflammation in the air space through clearance of apoptotic neutrophils; this activity is enhanced by the effects of IL-10, which diminishes phagocyte killing capability but up-regulates phagocytic function. This constitutes an antiinflammatory function because failure to clear necrotic PMNs results in protease release, local cell injury, sustained inflammation, and parenchymal destruction, as demonstrated in animal models of pneumonia.[730] Alveolar macrophages are poor at antigen presentation as a result of the fact that they express low levels of costimulatory molecules. Thus it is DCs that play a key role in initiating of adaptive immunity in the lung.

Dendritic Cells and Adaptive Immune Responses

DCs are distributed throughout the airways of the respiratory tract. Moreover, their numbers can be greatly augmented within 6 to 120 minutes by TLR agonists such as LPS.[731,732] Airways contain intraepithelial DCs that can extend dendritc processes into the ASL to sample antigens from the airway surface.[731,732] DCs of the myeloid subset express TLR2, 4, 5, and 9 and are critical for recognizing many extracellular pathogens. Plasmacytoid DCs enriched for TLRs such as TLR3 and TLR7 and TLR8 (that can recognize viral RNAs) have been shown to be critical for the induction of Type I interferons in response to RSV infection.[733] As mentioned earlier, immature DCs are endocytic and upon TLR activation mature and migrate to regional lymph nodes via the upregulation of CCR7. For *M. tuberculosis,* the induction of IL-12p40 is also critical for this migration.[10] Once there, antigen presentation to T cells can occur. Naive T cells differentiate under control of both costimulation and cytokine signals to Th1, Th2, Th17 or Treg cells. It has been recently shown that inhaled antigen that lacks PRR or TLR activation fails to lead to pure anergy in the lung. In contrast, pure antigen that lacks TLR or other PRR activators can induce a population of Foxp3 regulatory T cells that expresses surface TFG-β, and this T-cell population is critical for the development of tolerance in the lung.[734] This mechanism is very important as the lung encounters a lot of inhaled particulate and cannot afford to generate robust immune responses to each particle or risk the development of chronic lung inflammation. Once an effector T cell is generated, it recirculates back to the site of infection along chemokine gradients. This is also the basis of mucosal vaccination to generate a pool of effector memory T cells that can rapidly respond to a pathogen challenge.

The mucosal IgA response is regulated by T cells, which provide an initial contact-dependent signal to the B cell and subsequently secrete TGF-β, resulting in B-cell switching of IgM expression to IgA expression.[735] The T-cell cytokines IL-5 and IL-6 are necessary for final differentiation of surface IgA–expressing B cells to IgA-secreting plasma cells. Similarly, elaboration of other cytokines during T cell–mediated activation induces B-cell switching to other immunoglobulin classes.

In the neonate, however, effective pulmonary adaptive immune response is impaired at multiple levels. As noted previously, the phenotype of neonatal T cells is essentially that of antigenically naive adult T cells, presumably caused by limited antigen exposure. These T cells exhibit decreased surface density of T-cell receptors and decreased adhesion molecules. They require sustained receptor stimulation for activation, as well as greater costimulation to achieve a Th1 phenotype. Additionally, whereas memory T cells tend to "home" to specific organs based on prior antigenic exposure, neonatal T cells preferentially reside in distal lymphoid tissues, resulting in delayed recruitment. The net effect is a neonatal lymphocyte response that is more delayed, less facile in terms of accessory cell activation, less able to rapidly evolve a Th1 cellular immune response, less focused in regard to specific antibody production, and possibly less efficient in terms of dampening inflammatory responses once initiated.

Resolution of Inflammation

Because inflammation caused by cellular responses may induce local tissue injury, the capacity to appropriately down-regulate inflammation is essential to preserve lung function. One mechanism of counterbalancing T-cell immunity is provided by networks involving Treg and Th2 cytokines. As mentioned above, both IL-10 and TGF-β can regulate Th1 immune response. It has recently been shown, for example, that depletion of Tregs markedly enhances lung inflammation and lung injury in response to *P. carinii* lung infection.[736] IL-4 can suppress the development of both Th1 and Th17 cells.[454] Recently it has been shown that another IL-12 family member, IL-27, signaling via Stat1, can suppress Th17 development and thus may also be another critical negative regulator of IL-17–induced inflammation in the lung.[737,738] Many cytokines can also induce suppressor of cytokine signaling proteins such as SOCS3 that can antagonize cytokine receptor signaling to dampen inflammation.

Systemic Cytokine Networking

Pulmonary cytokines generated in response to a local infection ideally, but rarely, remain confined to the alveolar space. Inflammatory disruption of alveolar epithelial integrity allows movement of locally generated cytokines into the intravascular space; this results in systemic cytokine networking, which may be either beneficial or deleterious to the host, depending on the specific cytokine and the context of the infection. For instance, it is likely that lung IL-6 generated during alveolar inflammation reaches the systemic circulation where it stimulates the hepatic acute phase response. Another example of systemic cytokine networking is provided by alveolar-derived colony-stimulating factors, which are generated as part of the amplification response in pulmonary inflammation. GM-CSF and G-CSF are produced by alveolar macrophages in response to LPS, TNF-α, or IL-1β, whereas GM-CSF is also produced by stimulated epithelial cells and endothelial cells. Alveolar macrophages recovered from patients with pneumonia spontaneously release G-CSF, which acts locally to activate recruited PMNs and delay apoptosis.[739] In animal models of pneumonia, levels of G-CSF rise rapidly in the lungs and are measurable in the serum within 24 hours; this response is virtually ablated by antibody neutralization of TNF, underscoring the role of cytokine networking in generating alveolar G-CSF.[740] Quinton and colleagues[740] have shown that in the first few hours of pneumonia the lung is the principal source of G-CSF. Parallel studies have demonstrated that intratracheal deposition of G-CSF induces systemic neutrophilia. Taken together, these data suggest a model in which the lung, when challenged by bacteria, produces G-CSF in response to locally generated early response cytokines. Alveolar G-CSF easily reaches the circulation, stimulates granulopoiesis, and amplifies the PMN recruitment response. This response is associated with higher PMN counts in BAL specimens, more rapid bacterial clearance, and increased host survival.[695]

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SECTION XXI Neurology

160 Development of the Nervous System

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The human brain arises from a few embryonic cells to become, during the brief 280 days of human gestation, one of the most complex systems in the universe. The newborn brain comprises billions of neurons arranged and interconnected in an exquisitely precise three-dimensional network; one in which even minor changes have profound implications for postnatal development and function. The remarkable process of human brain development comprises overlapping genetic and epigenetic events that are tightly regulated in both time and space. This developmental sequence transforms a thin disk of undifferentiated tissue into a multilayered system capable of processing and storing vast amounts of sensory information, and mediating the entire range of human behavior, communication, and emotion. The brain, with its massive prefrontal cortex and ability to investigate and reflect on its own nature and function, primarily differentiates human fetal development from that of other species. In this chapter we review the major temporal events in prenatal nervous system development together with the disorders and anomalies that result from perturbations of these pathways. An overview of significant stages of central nervous system development and the disorders associated with their interruption in fetal life is given in Table 160-1.

EMBRYOGENESIS AND EARLY FORMATION OF THE NERVOUS SYSTEM

The fertilized human ovum divides symmetrically into identical totipotential blastomeres, and within a few cell divisions a blastocyst forms, uterine implantation occurs, and the inner cell mass contains cells committed to formation of the embryo. The progressive specialization of cells including progenitors of the nervous system is highly dependent on intrinsic genomic expression. These critical gene networks often have pleiotropic activity, the same genes often expressed both early and late in neural development, but their functions differing through highly selective temporal and spatial expression patterns. Equally fundamental to neural development are extrinsic signals from neighboring cells and extracellular environment. The nervous system is therefore sensitive to genetic mutation (inherited or de novo) and local, epigenetic disruptions that may lead to similar or overlapping anomalies.

Genetic studies defining the molecular development of the fruit fly *Drosophila* as well as studies in organisms such as the nematode *Caenorhabditis elegans*, the zebra fish, *Xenopus* toad, and avian chick models have defined the key roles of molecular signaling systems that regulate gene expression networks to establish the pattern and polarity of the nervous system. Human molecular genetic studies have defined the highly conserved human homologues of these proteins, suggesting they play a key role in the earliest establishment of the human central nervous system.

Morphologically the key event in further successful development of a brain occurs in the third week of gestation when the inner cell mass, now established as the bilaminar epiblast and hypoblast, undergoes gastrulation to form the three embryonic germ layers. Gastrulation also marks the visible establishment of primary axes of the embryo and nervous system: lateral, anteroposterior (AP) and dorsoventral. This process begins with the appearance of the primitive streak (Figure 160-1) on the epiblast surface and a defined cephalic pole, the primitive node. Epiblast cells migrate toward the streak under control of FGF8, a growth synthesized by streak cells. FGF8 further down-regulates E-cadherin, the molecule responsible for epiblast binding, allowing migrating epiblast cells to invaginate in the region of the node and streak, and delaminate and displace hypoblast cells to form the endoderm and mesoderm. Remaining epiblastic cells differentiate into ectoderm.

TABLE 160-1

Neurology of the Newborn

Development Event	Peak Time	Anomaly
Primary and secondary neurulation	3-4 wk	Cranioraschisis/anencephaly Encephalocele Myeloschisis Myelomeningocele Dysraphic states
Prosencephalic cleavage (ventral induction)	5-6 wk	Holoprosencephaly Agenesis of the corpus callosum Septo-optic dysplasia
Neuronal proliferation		
Cerebral	2-4 mo	Micrencephaly and macrencephaly Hemimegalencephaly Tuberous sclerosis
Cerebellar	2-10 mo postnatal	Dandy-Walker malformation Vermian and cerebellar hypoplasias
Neuronal migration		
Cerebral	3-5 mo	Schizencephaly Lissencephaly-pachygyria Polymicrogyria Heterotopias
Cerebellar	4-10 mo postnatal	Joubert syndrome Rhombencephalosynapsis
Neuronal differentiation		
Axon outgrowth	3 mo-birth	Agenesis of the corpus callosum
Dendritic growth and synapse formation	6 mo-1 yr postnatal	Mental retardation Autism Angelman syndrome
Synaptic rearrangement	Birth-years postnatal	Down syndrome Rett syndrome Fragile X syndrome
Myelination	Birth-years postnatal	Cerebral white matter hypoplasia 18q- syndrome Cerebral white matter disease of prematurity Nutritional and metabolic disturbances

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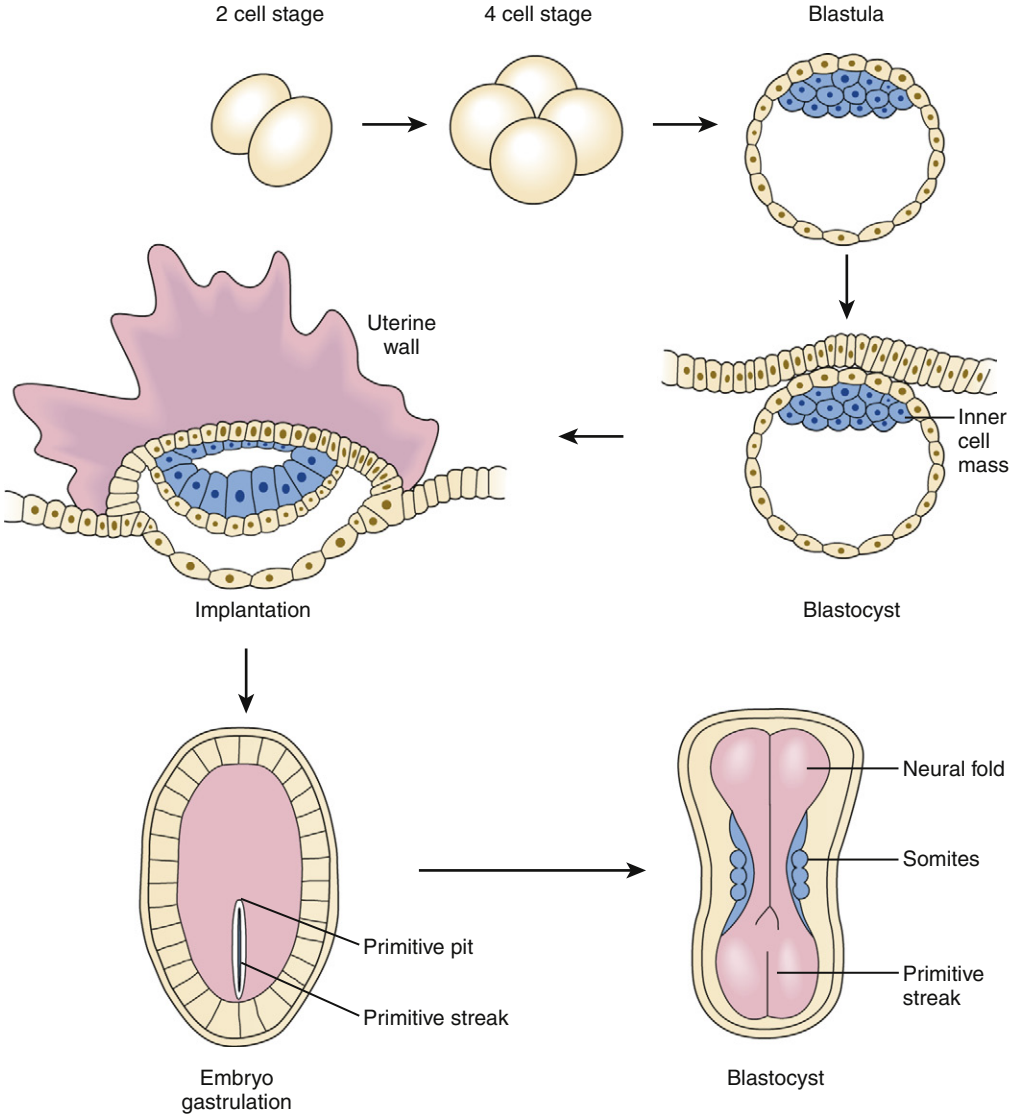


Figure 160-1 Early development of the human embryo. Initial symmetric cell divisions followed by multiple cleavage events to produce a distinct inner cell mass. This is followed by elongation of the embryo and development of the primitive streak. Cells migrate through the primitive streak to form the mesoderm. The overlying ectoderm forms the neural tube that will roll to form the brain and spinal cord. *(Adapted from Sanes DH, Reh TA, Harris WA [eds]:* Development of the Nervous System. *Academic Press, San Diego, CA, 2006.)*

Some of the first cells to migrate through the node in a cephalic direction form the prechordal plate that merges with later migrating cells to form the notochordal plate. Cells within the notochordal plate proliferate and detach from the surrounding hypoblast to form the solid definitive notochord, critical for further development of the nervous system and the basis of the fetal skeleton.

In the fruit fly, maternal effect genes establish the AP axis of the organism and regulate the expression of genes that establish the boundaries of body segments. These genes, gap genes and pair-rule genes, subdivide the embryo into smaller and smaller restricted domains of gene expression. Together with segment polarity genes, these genes subsequently regulate the expression of *homeotic* gene clusters, encoding a family of transcription factors that induce the expression of a battery of genes that induce the development of specific body segments and morphogenetic units. These homeotic *(Hox)* genes have highly conserved homologues in vertebrates with conserved 3′ to 5′ order on chromosomes that recapitulate the AP nature of their expression.

Cephalic and caudal poles of the human embryo are established prior to formation of the primitive streak. The anterior pole is characterized by formation of the anterior visceral mesoderm within the cephalad anterior hypoblast, expressing head-forming genes including *OTX2, HESX1* and *cerebrus*. The dorsoventral axis in mesoderm is established by the ventralizing action of *BMP4* in the presence of *FGF*s. Dorsalization of the mesoderm is regulated by inhibition of *BMP4* in the head region by expression of genes including *chordin*, *noggin,* and *follistatin* and more caudally by genes such as *brachyury (T)*.

The dorsal mesoderm gives rise to the notochord. The appearance of the notochord induces the overlying ectoderm to thicken and form the neural plate and subsequently gives rise to neuroectoderm, marking the end of gastrulation and the beginning of neurulation.

At the end of gastrulation the neural plate consists of the primordia of the fore-, mid-, and hindbrains and spinal cord and initially expresses the *Otx2* gene that later becomes restricted to the fore- and midbrain primordia. Complementary *Gbx2* expressing cells extend from the posterior part of the brain to the midbrain-hindbrain border. Signals from the node appear to activate a labile, preneural and preforebrain state after which

cells receive stabilizing signals from the node or prechordal mesoderm, which also caudalizes surrounding cells to form the mid- and hindbrain and spinal cord.

The default derivation of ectoderm is epidermis, by activation of elements of the bone morphogenetic protein (BMP) pathway such as chordin, noggin, and follistatin that, uninhibited, activate the Smad pathway. If the BMP signal is blocked by one of many extracellular antagonists derived from adjacent cells, the Smad pathway is inhibited allowing expression of the *Sox* proteins that activate transcription of pro-neural *bHLH* class genes. These genes code for a class of more than 20 transcription factors necessary and sufficient for nervous system formation, some expressed throughout the developing nervous system, others specific to regions such as the forebrain and spinal cord. Transcription of these genes eventually leads to transformation of cuboidal primitive ectoderm to columnar neural epithelium.

Similarly, ectodermal cells acquire a neural identity through expression of antagonists to the canonical *Wnt* pathway such as cerebrus, dickkopf, sFrp and Tlc. A second signaling system provides lateral inhibition of neuroblast development. The Notch/Delta signaling pathway allows delamination of ectodermal cells to become neuroblasts, that is, committed neural progenitors while providing lateral inhibition of other ectodermal cells from following this fate. As a result of these signals, cells destined to form the neural plate elongate in an apical-basal direction to form the neural placode, broader at its cranial end and narrowing caudally.

In addition to the differentiation of neural progenitors, the future complex shape and three-dimensional structure of the fetal brain is determined by patterning events that begin with the establishment of the neural placode and plate. Two sets of polarity are required, both under the control of local diffusible factors. The first is AP regional differentiation into future forebrain, midbrain, hindbrain, and spinal cord due to initial clonal restriction and refinement under the influence of local neural plate "organizers." The second is establishment of the dorsoventral polarity that sequestrates motor neurons from sensory neurons in the developing spinal cord, and eventually cerebral cortical regionalization into recognizable functional areas such as motor or visual cortex. This process is dependent on dorsalizing and ventralizing factors secreted from the nonneural ectoderm and notochord, respectively.

Induction of future neural tissue with a posterior identity is under the control of posteriorizing factors such as Fgfs, Wnt, Nodals, and retinoic acid. Local neural plate organizers further refine and maintain the AP identity of the neural plate and determine future cell fate and histiogenesis. These organizers include the anterior neural ridge necessary for maintenance of the forebrain, the midbrain-hindbrain organizer, and the tail organizer.

The *Hox* gene clusters have been most studied in development of the vertebrate hindbrain, which undergoes segmental development into a cluster of rhombomeres similar to the segmental formation of the fly embryo. Paralogous groups of *Hox* genes are expressed in a segmental overlapping pattern to give rise to rhombomeres with unique identities and neurons that go to innervate specific cranial nerves or interconnect within the reticular system of the brain stem. Probably the best studied regulator of *Hox* genes is the human teratogen retinoic acid (RA). RA is synthesized locally in paraxial mesoderm of the notochord, and diffuses locally to bind a cytoplasmic retinoic acid response element (RARE) receptor and then translocate to the nucleus and activate posterior *Hox* genes. Experiments show high levels of RA prevent anterior segment development, whereas low levels prevent caudalization of the embryo. Expression of fibroblast growth factors (FGFs) is similarly able to induce posterior gene expression. A further AP gradient exists of *wnt/beta-catenin* activity. The midbrain-hindbrain border appears to be the key to patterning the important anatomic subdivision of the midbrain and cerebellum. Important gene expression here includes *wnt1, en1,* and *FGF8*.

NEURULATION AND FORMATION OF THE SPINAL CORD

Neurulation is the process of formation of the hollow neural tube by folding of the epithelial neural plate (Figure 160-2). Neurulation occurs in two distinct phases: primary neurulation during weeks 3 and 4 of gestation leading to development of the brain and spinal cord (Figure 160-3) and secondary neurulation during weeks 5 and 6 with formation of lower sacral and coccygeal cord. Defects of neurulation represent the earliest abnormalities of brain development that are clinically detectable in fetal life and extend into postnatal life.

The neural plate elongates into a drop-shaped structure, broader at its cranial end and narrower in the future spinal regions. This morphogenetic event, known as convergent extension, is under control of the noncanonical Wnt/frizzled pathway and is analogous to the process of tissue polarity development in the fly, where genes such as *Frizzled, Disheveled, Strabismus/Van Gogh, Diego, Flamingo,* and *Prickle* appear to be critical. These genes have highly conserved vertebrate homologs that are implicated in the process of convergent extension.

The neural plate is further shaped by bending at the median hinge point overlying the notochord at the future upper spinal cord and at the paired dorsolateral hinge points at the levels of the brain and lower spinal cord. This differential bending appears to be under control of signals diffusing from the notochord including the signal transduction molecule sonic hedgehog. The neural plate rotates by elevation and convergence around the median and dorsal hinge points. This bending appears to be dependent on apical constriction of columnar neural tube cells to become wedge shaped under the control of actin-related genes in particular *p190RhoGap*, a negative regulator of Rho GTPase and the actin-binding protein *Shroom*.

Neural tube closure appears to continue by the development of bilateral folds at its junction with nonneural ectoderm. These folds elevate and appose in the midline with fusion occurring by interdigitation of cellular protrusions from apical cells and the formation of permanent cell contacts. Membrane-bound ephrin ligands and their eph tyrosine kinase receptors have been implicated in this process. Neural tube closure is a discontinuous process occurring at between two and five initiation sites. The open neural folds between initiation closure sites are known as neuropores: as closure progresses, the neuropores gradually shorten and close leading to an intact closed neural tube. This is subsequently covered by ectodermally derived epidermis.

Secondary neurulation is initiated after primary neurulation is complete and the posterior neuropore closes. The tail bud, a pluripotent mass of cells, a remnant of the caudal primitive streak, proliferates and condenses followed by cavitation and fusion with the central canal of the neural tube. As this process of canalization progresses during ensuing weeks, neurons and ependymal cells differentiate to form the caudal end of the spinal cord. Resorption of the tail bud and other cells of the caudal cell mass leave the filum terminale, which often contains ependymal cell nests along its length. These nests of ependymal cells can begin to proliferate later in life as a monoclonal population of glial cells. These ependymomas, uncommon glial cell tumors, are among the most common tumors of the cauda equina and filum terminale.

The closed neural tube consists of a thick pseudostratified epithelium of neuroepithelial cells that begin to divide rapidly immediately after closure and give rise to a second cell type, the neuroblast. These cells form the mantle layer, the future gray matter of the spinal cord. This further gives rise to an outermost layer, the marginal layer that will become myelinated and form the future white matter of the cord. As neuroblast proliferation continues, the neural tube develops dorsal and ventral thickenings that become the alar and basal plates that will give rise

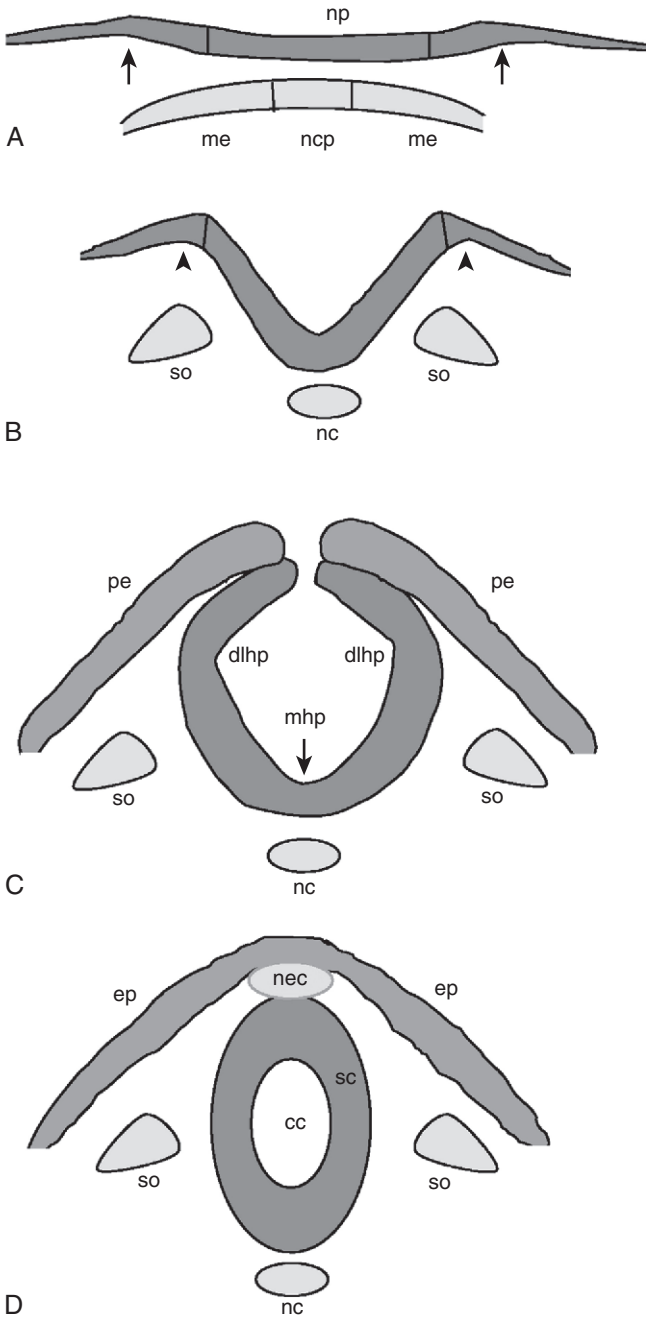


Figure 160-2 A simplified diagram of neural tube closure. **A,** Neural folds form at the lateral extremes of the neural plate (**A,** *arrows*), elevate (**B,** *arrows*) and converge toward the dorsal midline **(C),** and fuse at their dorsal tips to form the closed neural tube **(D).** Bending or hinge points form at two sites: the median hinge point *(mhp)* overlying the notochord and the paired dorsolateral hinge points *(dlhp)* at the lateral sides of the folds **(C).** *cc,* Central canal; *ep,* epidermis; *me,* mesoderm; *nc,* notochord; *ncp,* notochordal plate; *nec,* neural crest; *np,* neural plate; *pe,* presumptive epidermis; *sc,* spinal cord; *so,* somites. *(From Bassuk AG, Kibar Z: Genetic basis of neural tube defects,* Semin Pediatr Neurol *16:102, 2009.)*

to the sensory and motor areas of the spinal cord respectively. A longitudinal groove, the sulcus limitans, demarcates the boundary between the two.

As the neural tube closes, cells at the edge of the neural plate separate from the neural epithelium and migrate into the extracellular matrix to become neural crest cells. This separation is accompanied by a loss of cadherin-mediated cell adhesion from the surrounding neuroepithelium with local decrease in neural cell adhesion molecule (NCAM) levels. It is also accompanied by an increase in the cell adhesion to the extracellular matrix with increased expression of fibronectin. Migrating cells extend cellular motile processes to facilitate migration through the embryo to ultimately reach the final phenotypic sites. Neural crest cells migrate widely to become neurons and glia in dorsal root and autonomic ganglia. The fate of neural crest derived cells is also to provide innervation for the gastrointestinal tract and become neurons in the sensory ganglia of the cranial nerves (also formed in part by ectodermal placodes) and to become melanocytes, cartilage of the bone and face, and a variety of endocrine and structural tissues.

The ultimate phenotypic and developmental fate of neural crest cells is tied to the timing, mode, and pattern of migration and is in large part controlled by the environment in which they reside. There is complex interplay between the expression of inhibitory extracellular matrix components as well as the dynamic ratio between permissive versus inhibitory components. The most important players in the control of neural crest cell migration are fibronectin, laminin isoforms 1 and 8, aggrecan, and PG-M/versican isoforms V0 and V1. In chick quail chimeras, transplanted crest cells tend to assume the phenotype of the location to which they migrate rather than that of their place of origin. Cultured postmitotic crest cells can be induced to change expression of neurotransmitters in myocyte-conditioned medium. From these experiments it can be inferred that the neural crest cells retain a relatively broad developmental potential as they begin migration and that their ultimate fate is strongly influenced by local factors.

NEURAL TUBE DEFECTS

Neural tube defects (NTDs) are collectively the most common malformation of the nervous system with an incidence of 1 to 2 per 1000 births although there is significant geographic and historic variation in prevalence. This variation hints at the complex etiology of these disorders in which both genetic and environmental factors appear to be significant, including maternal age and diet, maternal diabetes and obesity, teratogen exposure, and socioeconomic class. Pathologically NTDs are characterized by a failure of closure of the neural tube at any level. Failures of primary neurulation frequently lead to "open" NTDs in which the neural defect is exposed to the environment, whereas failures of secondary neurulation frequently lead to "closed" defects in which the defect is covered by skin and integuments.

Cranioraschisis and Anencephaly

These disorders represent the most severe failures of primary neurulation. Cranioraschisis is a rare and lethal condition manifested by essentially total failure of neurulation with a neural plate–like structure present but no skeletal or dermal elements overlying it. The incidence is unknown. The equally rare disorder of failure of posterior neuropore closure known as myeloschisis represents the inverse of cranioraschisis, where a neural plate–like structure without overlying vertebrae or dermis replaces large portions of the spinal cord.

Anencephaly is a defect of anterior neural tube closure and comprises failure of formation of elements from the rostral portion of the neural tube anywhere to the foramen magnum

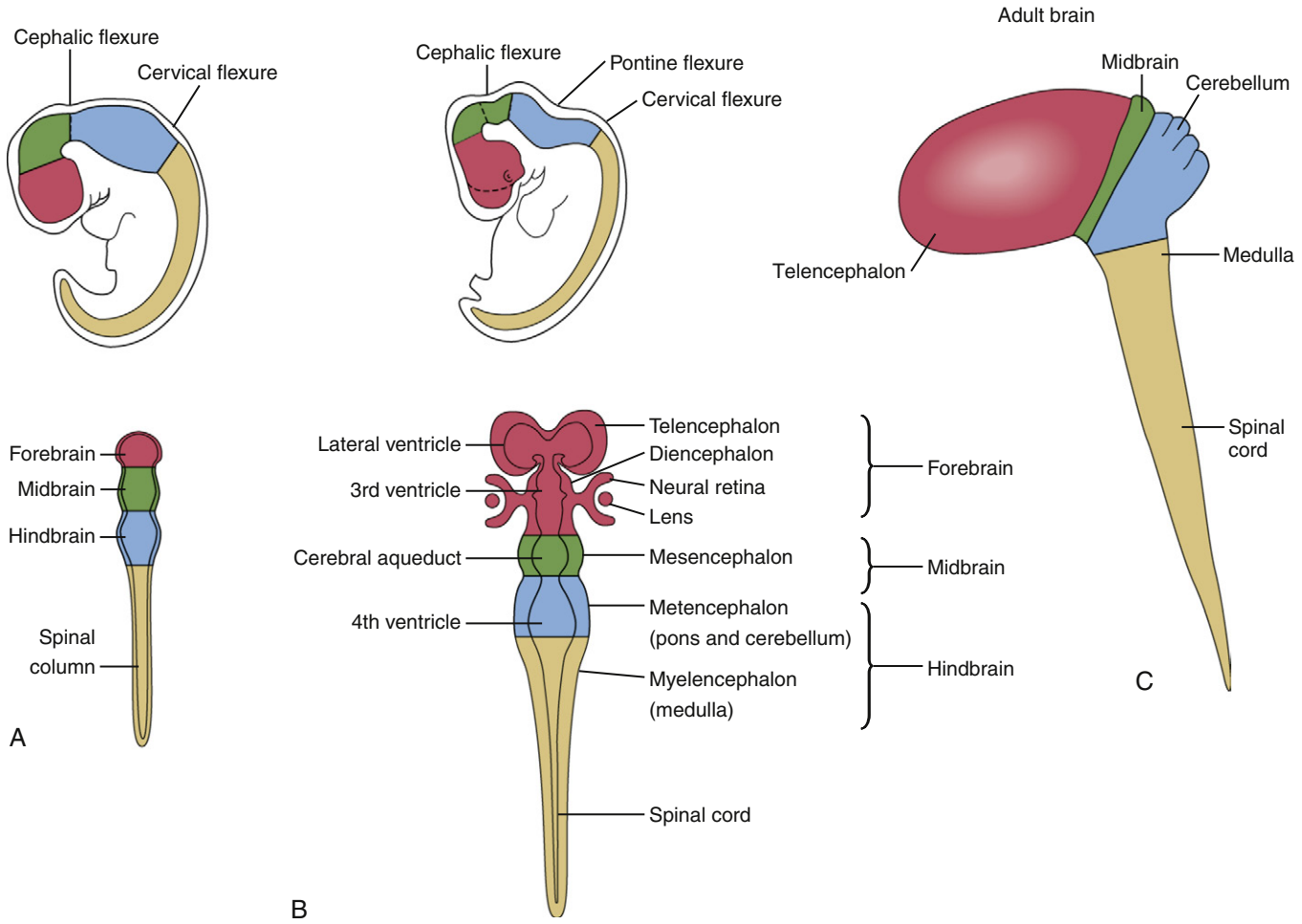


Figure 160-3 Development of the brain and spinal cord from the neural tube. *(Adapted from Sanes DH, Reh TA, Harris WA [eds]:* Development of the Nervous System. *Academic Press, San Diego, CA, 2006.)*

caudally. The forebrain and upper brain stem are usually involved, normal neural tissue replaced by a formless mass of degenerated, hemorrhagic neuronal and glial tissue and absence of the frontal, parietal and squamous occipital bones. The disorder varies considerably between geographic location, race, sex, ethnic group, maternal age, and socioeconomic status. The risk is higher in whites especially those of Irish ethnicity, very young and very old mothers, and in those of lower socioeconomic status or a history of previously affected siblings. The incidence shows a secular decline in the United States from 0.5 per 1000 live births in 1970 to 0.12 per 1000 births in 2011. Polyhydramnios is a frequent prenatal finding and the malformation is now commonly diagnosed in the second trimester during obstetric screening, allowing for elective termination of pregnancy.

Encephalocele

This group of disorders represents herniation of the brain or meninges through the skull. The underlying pathogenesis is unknown but relates in part to the failure of normal closure of the anterior neuropore. It is relatively uncommon with an estimated incidence of 1 to 3 per 10,000 live births and is increasingly detected antenatally. More than 70% of encephaloceles herniate through the occipital part of the skull (Figure 160-4, *A*), although nasofrontal encephaloceles are relatively more common in Southeast Asia. Basilar, temporal, and parietal encephaloceles are unusual. The herniated mass may comprise one or more of meninges, cerebrospinal fluid (CSF) or neural tissue—in some cases surprisingly well organized. Hydrocephalus occurs in 50% of occipital encephaloceles and other malformations are common including agenesis of the corpus callosum, Chiari III malformation, and subependymal nodular heterotopia. Encephaloceles are found in a number of multiple congenital anomaly syndromes and are associated with first-trimester maternal hyperthermia.

Myelomeningocele

Failure of posterior neural tube closure results in a malformation of the spinal cord, myelomeningocele. This is the most commonly encountered NTD in pediatric practice because most infants born with this anomaly survive. The lesion consists of neural plate or rudimentary neural tube tissue herniating through a defect in the vertebra (spina bifida) in the form of a sac containing meninges, CSF, nerve roots, and spinal cord. The dorsal cord is most affected and there may be some dermal coverings present. The vast majority of myelomeningoceles occur in the lumbar region (see Figure 160-4, *B*) and 10% may contain no neural tissue and are known as meningoceles. Hydrocephalus and Chiari II malformation are frequent associations. The incidence of myelomeningocele varies widely by ethnic population and geographic location but ranges from 0.2 to 4 per 1000 live births. Girls are more frequently affected than boys. Recurrence risk is 5%, increasing to 15% if two siblings are affected.

Occult Dysraphisms

Disorders of secondary neurulation involve the caudal spinal cord and its coverings. The occult dysraphic states are commonly associated with vertebral anomalies and are usually covered with skin. Elongation of the conus medullaris and shortening and thickening of the filum terminal with "tethering" of these structures may result in traction injuries to the spinal cord. The occult dysraphic states are commonly associated with vertebral anomalies and are usually covered with skin. A *myelocystocele* is a localized dilation of the central canal in the caudal spinal cord often associated with severe malformations, including cloacal exstrophy and vertebral anomalies. The caudal cord in *diastematomyelia* is divided by a septum arising from a vertebral body. *Diplomyelia* is a divided, and at times duplicated, spinal cord with no septum. *Meningocele* is an extrusion of subarachnoid space with no gross abnormality of either the brain or spinal cord; when associated with a lipoma, the abnormality is referred to as a *lipomeningocele*. *Lipomata* and other tumors such as *teratomata*, *neuroblastomata*, and *ganglioneuromata* may occur and extend into the dural space. A cutaneous dimple in the lumbosacral region may be contiguous with a *dermal sinus* and a *dermal (dermoid)* or *epidermal (epidermoid) cyst* or may extend into the vertebral canal. Lipoma and dermal sinus are the most common of the occult dysraphic states.

ETIOLOGY OF NEURAL TUBE DEFECTS

The etiology of NTDs is heterogeneous; syndromic associations are recognized as are a wide range of aneuploidies. Specific teratogens are associated, in particular thalidomide, carbamazepine, and valproic acid. Other maternal susceptibility factors include hyperthermia, low vitamin B_{12} levels, diabetes mellitus, and obesity. Periconceptual folate supplementation has been shown to significantly reduce the incidence of NTDs (up to 83% reductions with 4 mg of supplemental folate). Additionally, susceptibility factors related to folate metabolism have been identified; defective levels of methylene tetrahydrofolate reductase have been demonstrated in up to 20% of pregnancies affected with NTDs. Nonetheless, primary genetic variants in folate-related genes do not seem to account for the overall genetic contribution to NTD incidences.

Studies in animal models, in particular the mouse, have identified a large list of candidate genes for human NTDs, in particular genes involved in convergent extension and the tissue polarity pathway. These include *ALDH1A2, CYP26A, MSX2, NCAM1, PAX, PDGFRA, PRKCA, SLUG, T box,* and *ZIC2*. Of particular interest is the gene *VANG1*, the human homologue of the mouse gene *Vangl2,* mutated in the severe murine cranioraschisis loop tail *(Lp)* mutant. Sequencing of *VANG1* has identified a number of missense mutations in both sporadic and familial human NTDs.

Disorders of Neural Crest–Derived Cells

Defects in neural crest development are known as neurocristopathies. In Waardenburg syndrome, children have hypoganglionosis, congenital megacolon, abnormalities of the great vessels, sensorineural deafness, and abnormalities of skin, hair, and iris pigmentation. Restricted abnormalities of neural crest development are more common disorder of neural crest cells. Congenital aganglionosis (Hirschsprung disease) is characterized by a lack of neurons in the enteric plexus of the colon as a result of either failure of migration or differentiation of neural crest cells. The anomaly presents in the newborn period as megacolon and the most common cause of neonatal colonic obstruction. A mutation in *L1CAM*, a gene that encodes a neural cell adhesion molecule, has been described in newborn males with congenital hydrocephalus, aqueductal stenosis, and/or agenesis of the corpus callosum and Hirschsprung disease, suggesting a role for *L1CAM*-mediated cell adhesion.

Disorders of the spinal and autonomic ganglia are rare. Congenital insensitivity to pain with anhidrosis is an autosomal-recessive disorder resulting from defective neural crest differentiation. First-order sensory afferents, which are responsible for pain and temperature sensation, are lacking. In addition, there is neuronal loss in the sympathetic ganglia. Immunohistochemistry with antibodies directed against the S100 protein and neuron-specific enolase, markers of glial and neuronal differentiation, fail to show nerve fibers in the vicinity of the eccrine sweat glands. The presence of multiple coincident neurocristopathies has been reported, specifically with the association between congenital central hypoventilation, Hirschsprung disease, and autonomic dysfunction (Haddad syndrome) and

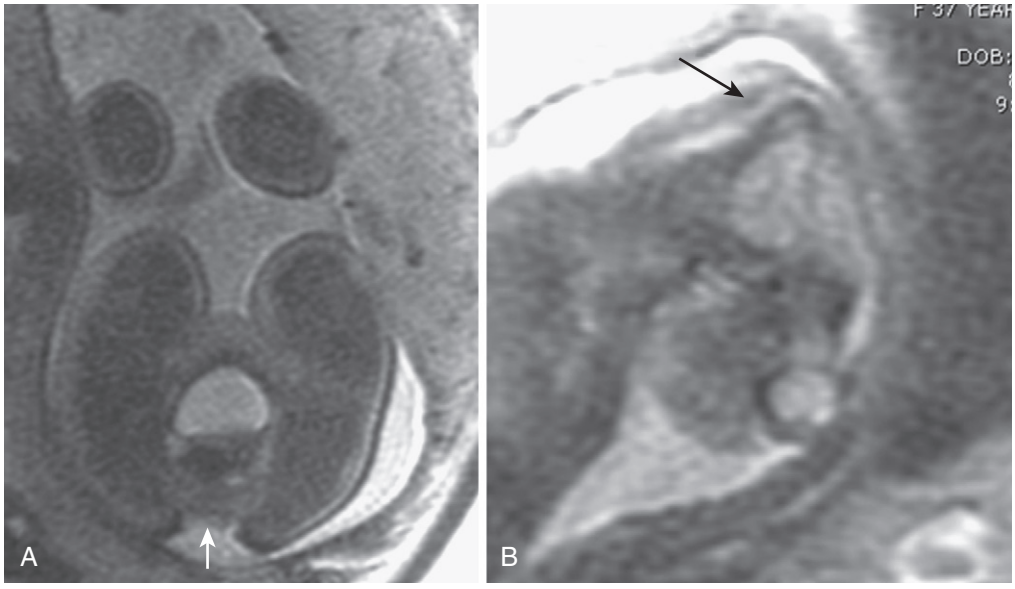


Figure 160-4 Fetal magnetic resonance imaging at 18 gestational weeks. **A,** Occipital encephalocele *(arrow)*. **B,** Lumbosacral myelomeningocele *(arrow)*.

between congenital central hypoventilation, Hirschsprung disease, and neuroblastoma.

EMBRYONIC DEVELOPMENT OF THE BRAIN AND VENTRICULAR SYSTEM

The brain forms from three vesicles that develop in the cranial neural tube: the forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon).

This process occurs at weeks 4 and 6 of gestation shortly after closure of the anterior neuropore. Inductive interactions between the rostral notochord-prechordal mesoderm and the most cranial portion of the neural tube initiate formation of the fetal forebrain. As this process occurs in the ventral surface of the rostral neural tube it is often known as ventral induction. This is followed by the cleavage of the forebrain in three planes to give rise to the paired optic vesicles, olfactory bulbs, and tracts; separation of the diencephalons; and lastly the paired cerebral hemispheres, lateral ventricles, and basal ganglia.

The cerebral hemispheres develop as symmetric evaginations of the lateral wall of the prosencephalon. The median portion of the prosencephalon forms the diencephalon, giving rise ultimately to the third ventricle, thalamus, hypothalamus, and mamillary bodies. The cavities of the hemispheres form the lateral ventricles and communicate with the lumen of the diencephalon through the foramina of Monroe. The basal parts of the hemispheres begin to grow and bulge into the floor of the foramina to form the ganglionic eminence and ultimately the caudate and lentiform nuclei. The medial surfaces of the hemispheres thicken to give rise to the hippocampi. Clusters of neurons along the midline begin to form nuclei of the brain stem, thalamus, and hypothalamus. Axons from the olfactory epithelium extend and establish contact with the cerebral wall, which is followed by development of the olfactory bulbs over the sixth and seventh weeks of gestation. The remarkable expansion of the cerebral hemispheres follows during the remainder of gestation.

The sonic hedgehog signaling pathway is critical to the development of the prosencephalon, with sonic hedgehog protein (SHH) secreted from the prechordal mesoderm. SHH is an intercellular signaling molecule synthesized as a precursor that undergoes cleavage into N- and C-terminal domains. During autoprocessing a cholesterol moiety is attached to the C-terminal of SHH-N allowing the molecule to be secreted. SHH-N binds to the PATCHED-1 receptor to suppress repression of SHH signaling. SHH signaling appears to be mediated through at least three obligate mediators including GLI2. Other major molecular signaling pathways, the Nodal/TGFβ pathway and chordin/noggin/BMP pathways also appear to be critical to forebrain development primarily by maintaining dorsalization.

These events are followed by midline prosencephalic development with formation of the commissural, chiasmatic and hypothalamic plates. These structures give rise to the corpus callosum and septum pellucidum, optic nerve chiasm, and the hypothalamus.

The most prominent of these midline developments is formation of the major commissure of the brain, the corpus callosum, the earliest components of which appear at approximately 9 weeks. By 12 weeks an independent corpus callosum is definable at the commissural plate. Multiple steps are involved in formation of the corpus callosum, all primarily related to axon guidance. These include guidance by preexisting axon tracts formed by pioneering axons expressing neuropilin 1. Glial structures such as the glial wedge, midline zipper glia, and indusium griseum are present at the midline and are necessary for corpus callosum formation. The glial wedge is a symmetric structure composed of radial glial cells that prevent callosal axons entering ventrally and repel already-crossed axons away from the midline. Guidance by the glial wedge occurs through SLIT-ROBO and WNT-RYK signaling. Callosal axons are attracted through Netrin family chemoattactants and then repelled by SLIT activated ROBO molecules. Dorsal to the developing corpus callosum, the glia of the indusium griseum express SLIT2. These glia help guide commissural axons toward their crossing sites. Other signaling molecules involved in callosal axon guidance include NFIA and FGFR1.

The pituitary gland develops from two embryologically separate layers: an ectodermal outpouching of the embryonic mouth known as Rathke's pouch and a downward extension of the diencephalon, the infudibulum. Cells in Rathke's pouch increase rapidly with gestation to form the anterior pituitary gland, and the infudibulum gives rise to the posterior pituitary gland, unsurprisingly containing neuroglial cells including nerve fibers from the hypothalamus.

DISORDERS OF FOREBRAIN DEVELOPMENT

Holoprosencephaly

This group of disorders is the result of different degrees of failure of prosencephalic cleavage and distinguished by the severity of failed cleavage of the cerebral hemispheres and deep nuclear structures. The most severe form is *alobar holoprosencephaly* in which there is a single spherical cerebral structure and monoventricle with fusion of the thalami and deep nuclei together with absence of the corpus callosum and olfactory bulbs. Neuronal migration and cortical organization are severely disordered. In *semilobar holoprosencephaly* there is failure of separation of the anterior cerebral cortex and absence of the anterior corpus callosum (Figure 160-5). The least severe forms are *lobar holoprosencephaly* in which the cerebral hemispheres are fully separated and the deep nuclei are only partially separated. In the *middle interhemispheric variant* only the posterior frontal

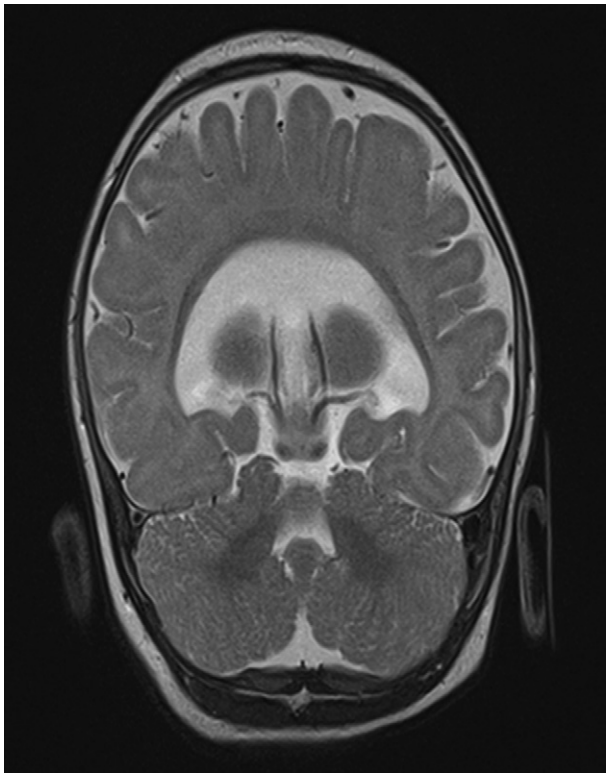


Figure 160-5 Coronal magnetic resonance image of 5-day-old infant demonstrating semilobar holoprosencephaly. Note single ventricle.

and parietal regions fail to separate. Hydrocephalus is present in most infants with alobar holoprosencephaly due to fusion of the thalami and impaired drainage of CSF through the aqueduct. Infants with semilobar and lobar forms are usually microcephalic. Facial anomalies are usual and range from severe cyclopia with proboscis through to ocular hypotelorism with a flat single nostril nose (cebocephaly) to mild hypotelorism with or without cleft palate.

The disorder occurs in 1 per 10,000 live births but is 100 times more common in conceptuses examined after miscarriage or abortion. Causes include both genetic and cytogenetic etiologies as well as teratogens. More than two thirds are related to full or partial chromosomal aneuploidies, in particular trisomy 13. A list of known etiologic factors and genetic loci is given in Table 160-2.

AGENESIS OF THE CORPUS CALLOSUM AND SEPTUM PELLUCIDUM

This group of disorders is characterized by varying degrees of failure of midline prosencephalic development. Agenesis of the corpus callosum (ACC) may be complete or partial and is associated with deformation of the lateral ventricles to give a parallel ventricular arrangement known as colpocephaly (Figure 160-6). This is usually accompanied by longitudinal fibers coursing along the medial aspect of the hemispheres known as Probst bundles. Partial ACC usually comprises absence of the posterior callosum. ACC may be isolated or in up to 40% to 50% of cases associated with other cerebral abnormalities, in particular cerebellar malformation and cortical migration anomalies. ACC is one of the most common anomalies occurring in up to 7 per 1000 live births and 3% of children with developmental delay. It is increasingly diagnosed in fetal life. ACC may be completely asymptomatic or have symptoms only detectable on very refined testing of interhemispheric transfer. In association with other anomalies, however, is a strong risk of cognitive and neuromotor impairment. ACC is associated with well over 65 known genetic syndromes, and the presence of extra-axial abnormalities or cytogenetic abnormalities is a risk factor for impaired neurologic development (Box 160-1).

Absence of the septum pellucidum is another common anomaly and frequently associated with more diffuse cerebral malformations such as schizencephaly, septo-optic dysplasia, ACC, holoprosencephaly and hydrocephalus. Failure of fusion of the septal leaflets is known as cavum septum pellucidum and

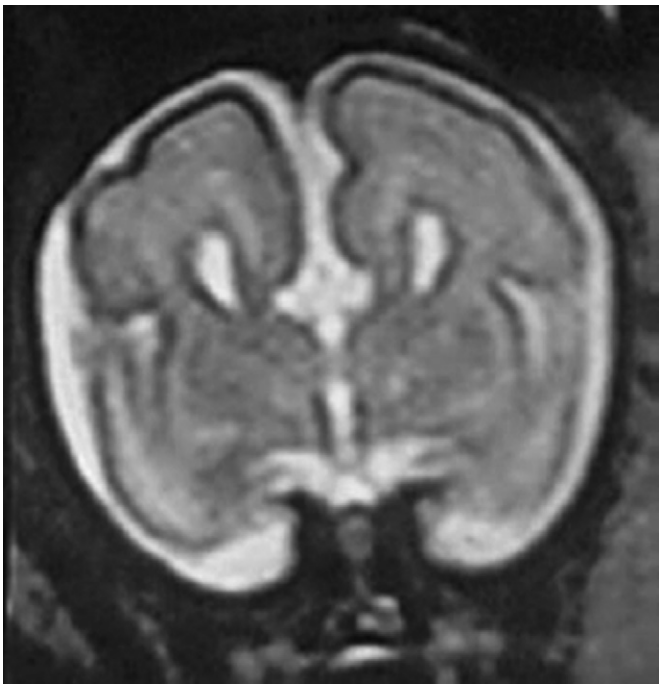


Figure 160-6 Fetal magnetic resonance image at 26 gestational weeks showing complete agenesis of the corpus callosum.

TABLE 160-2

Syndromic and Genetic Loci Associated With Holoprosencephaly

Syndrome	Chromosomal Locus	Gene
CHARGE	8q12	*CHD*
Pallister-Hall	7p13	*GL13*
Rubenstein-Taybi	16p11.3	*CREBBP*
Smith-Lemli-Opitz	11q12-13	*DHCR7*
Meckel		
Pseudotrisomy 13		
Velocardiofacial	22q11	*TBX1*
HPE and fetal akinesia	X	
HPE with ectrodactyly	X	
Loci		
HPE1	21q22.3	
HPE2	2p21	*SIX3*
HPE3	7q36	*SHH*
HPE4	18q11.3	*TGIF*
HPE5	13q32	*ZIC2*
HPE6	2q37.1-q37.3	
HPE7	9q22.3	*PTCH*
HPE8	14q	
HPE9	20p13	
HPE10	1q42-qter	
HPE11	5pter	
HPE12	6q26	
	8q24.3	*FOXH1*
	2q14	*GLI2*
	3p23-p21	*TDGF1*

BOX 160-1

Partial List of Syndromes Associated With Agenesis of the Corpus Callosum (Known Genes in Parentheses)

Aicardi
Andermann *(KCC3)*
XLAG *(ARX)*
Mowat-Wilson *(ZFHX1B)*
Mental retardation-spasticity-adducted thumbs *(L1CAM)*
Chudley-McCullough
Acrocallosal
FG
Toriello-Carey
Marden-Walker
ACC with spastic paraparesis *(SPG11)*
Fryn
Meckel-Gruber
Wolf-Hirschhorn *(4p-)*
Sotos
Craniofrontonasal dysplasia *(CFND)*
Donnai-Barrow
Pyruvate decarboxylase deficiency
Rubenstein-Taybi
Warburg micro
DeMorsier

includes septo-optic dysplasia (SOD, de Morsier syndrome) in which absent septal leaflets are associated with optic nerve hypoplasia and disturbances of the hypothalamic-pituitary axis. Remnants of Rathke's pouch may give rise to the suprasellar tumors, craniopharyngiomas that may cause hydrocephalus, visual field loss, and hypothalamic and pituitary dysfunction.

FETAL BRAIN DEVELOPMENT

The embryonic events of the first 6 weeks of development establish the three-dimensional pattern of the brain and spinal cord and determine the fate of the first neural cells. For the remainder of gestation and well into postnatal life these cells are now committed to the formation and maintenance of the evolutionarily extraordinary human brain. Though capable of only limited capacity for regeneration and repair, neurons must remain dynamic and responsive to the external environment for decades making them among the most remarkable of all cells. Development is characterized by massive proliferation and differentiation of neurons and glia, migration and organization of the cerebral and cerebellar cortex, dendritic growth and synaptogenesis, and finally ensheathment of neurons with myelin. A common plan of cell layers, types and connections is modified by regional variations in cellular architecture. The result is a spectacular laminated structure with a massive diversity of neurons with trillions of local and extrinsic connections that underpin the unique cognitive and behavioral capacity of humans.

NEURONAL AND GLIAL PROLIFERATION

All neurons and glia are derived from the specialized proliferative centers close to the pial surface, the ventricular and subventricular zones (Figure 160-7). Brain growth is characterized between 8 and 15 weeks by neuronal proliferation (Figure 160-8) and generation of radial glia switching to primarily glial multiplication from 15 weeks into postnatal life. Some neuronal proliferation does occur later in gestation, primarily in the external granule cell layer of the cerebellum and the subventricular zone. Morphologic brain growth also occurs secondary to proliferation of the rich cerebral vascular supply, initially through growth of radially arranged vessels and later by proliferation of horizontal branching vessels.

The cortical primordium is composed entirely of dividing neural stem cells that collectively form the ventricular zone (VZ). Prior to neurogenesis these cells have radial processes and divide symmetrically, although are themselves highly polarized. These cells are characterized by receptors at the basal lamina such as integrin-α and apically concentrated proteins such as CD133 and tight junctions. These neural stem cells divide through the unique process of interkinetic nuclear movement, with the nucleus moving toward the ventricular surface shortly before metaphase and migrating to the pial surface during deoxyribonucleic acid (DNA) synthesis. These early proliferative events dramatically increase the thickness and surface area of the VZ, particularly in the forebrain.

Soon after neural tube closure neural stem cells begin to switch to asymmetric division, giving rise to daughter cells, one of which remains a progenitor and reenters the cell cycle, the other a postmitotic cell destined to become a neuron or radial glia and migrate out into the developing cortex. The onset of neurogenesis is marked by down-regulation of the epithelial characteristics of neural stem cells. Among the genes involved in induction of neuroepithelial differentiation are *FOXG1, LHX2, PAX6,* and *EMX2*. Postmitotic cells show regional differences in gene expression that at least partly contributes to regional specification of their progeny.

Following the onset of neurogenesis, intermediate progenitors appear above the VZ known as the subventricular zone (SVZ—conventionally the ventricular surface is considered the top of the proliferative zone and layers described downward. Apical thus refers to the ventricular surface and basal away, toward the pial surface). The SVZ is distinctively large and active in humans compared with other species, retaining the ability to proliferate late into gestation and seemingly acting as a large reservoir of progenitor cells and eventually the principal source of cortical neurons. The cells of the SVZ do not undergo interkinetic nuclear movement but undergo terminal symmetric division to create pairs of neurons. These intermediate progenitors express transcription factors TBR2, NGN2, and CUX1 and CUX2.

Derived from the ventricular zone, radial glia remain more pluripotent and are capable of producing both neurons and glial cells—astrocytes and oligodendrocytes. Radial glial cells are elongated nonneuronal cells that serve two functions; a guidance

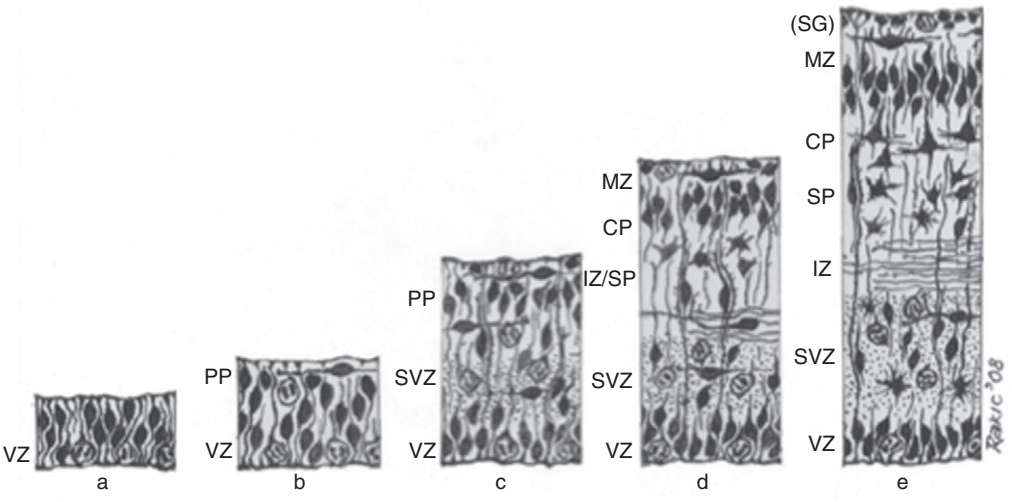


Figure 160-7 Cortical laminar development including transient fetal zones. The panels correspond to the following approximate ages (for the lateral part of the dorsal telencephalon): *a:* embryonic day (E) 30; *b:* E31–E32; *c:* E45; *d:* E55; *e:* gestational week 14. *CP,* Cortical plate; *I & IZ,* intermediate zone; *M & MZ,* marginal zone; *S & SVZ,* subventricular zone; *SG,* subpial granular layer (part of the MZ); *V & VZ,* ventricular zone. *(Modified from Bystron I, Blakemore C, Rakic P: Development of the human cerebral cortex: Boulder Committee revisited,* Nature Rev Neurosci *9:110-112, 2008.)*

scaffold for later migration of neurons and as neurogenic and glial progenitors.

The mechanisms of generation of progenitor cells are largely unclear; however, their maintenance requires molecules along the Notch pathway. Progenitor cells are also depleted in embryonic brains that are deficient for the presenilin 1 *(PS1)* gene, which is a key regulator of Notch signaling. Notch signaling is also thought to allow cells that are in direct contact to adopt different fates. Other genetic and environmental influences are important in the generation and maintenance of progenitor cells. For example, transcription factors such as NeuroD homologues of the helix-loop-helix proteins have been postulated as fate-determination factors. The role of extracellular signals in regulation of progenitor differentiation is poorly understood, but increasing evidence suggests cell adhesion molecules inhibit cell proliferation and induce differentiation to the neuronal lineage, as indicated by an increase in the number of cells staining positively for microtubule-associated protein 2.

There is growing evidence that neuronogenesis occurs into adulthood, at least in the olfactory cortex and hippocampus. Regions of the adult brain and spinal cord ventricular surface appear to have the capacity to generate new neurons. Glial proliferation, in contrast to neuronal proliferation, continues regularly throughout life. Laboratory studies have demonstrated that multipotent cerebral progenitor cells grown in vitro differentiate into neurons when exposed to platelet-derived growth factor (PDGF) and assume glial phenotype when exposed to ciliary neurotrophic factor (CNTF) or fibroblast growth factor (FGF2). Local factors appear to be important because cortical progenitors cultured over embryonic cortical slices differentiate into neurons and those cultured over postnatal cortical slices differentiate into glia, suggesting that there are developmentally and environmentally regulated signals.

The proliferative events involving the radial glial cell as neuronal progenitor are modulated by several key signaling pathways involving the Notch receptor, the ErbB receptor (via the ligand neuregulin), and the fibroblast growth factor receptor and β-catenin. Calcium waves propagating through connexin channels of the radial glial cell also appear to be important in the regulation of radial glial production of neurons.

DISORDERS OF NEURONAL PROLIFERATION

Microcephaly

Disorders related to impaired neuronal proliferation are categorized by the term "primary microcephaly," or "micrencephaly." This distinguishes the disorder from the more common microcephalies secondary to hypoxic-ischemic, infectious metabolic,

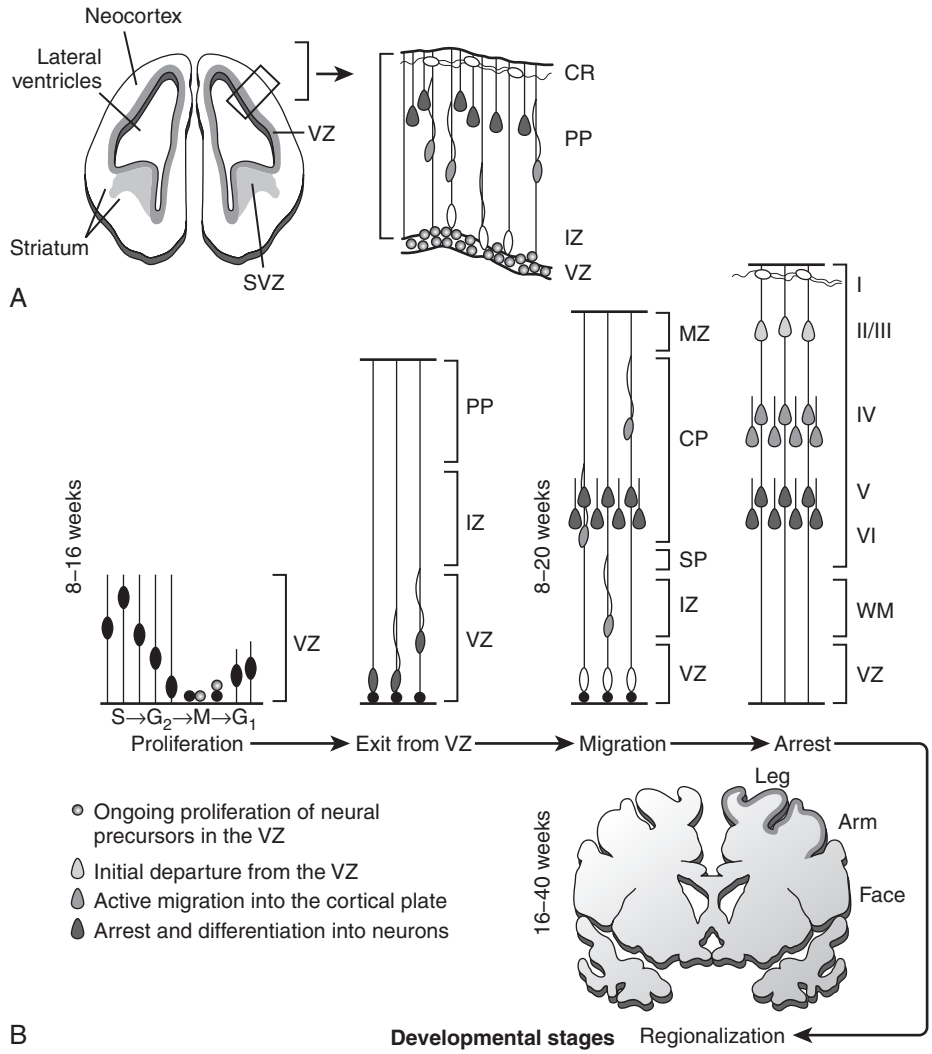


Figure 160-8 Neuronal development. *(From Lian G, Sheen V: Cerebral developmental disorders,* Curr Opin Pediatr *18(6):614-620, 2008.)*

or other destructive events. Micrencephaly vera refers to a group of autosomal recessive disorders characterized small brain size because of a derangement of proliferation (Figure 160-9). Radial microbrain is a rare disorder of particular interest because it appears to provide a clear example of a disturbance in number of proliferative units. There are at least six gene loci identified for autosomal recessive primary micrencephaly (MCPH), or micrencephaly vera. *Microcephalin* is crucial for cell cycle control, chromosome condensation, and DNA repair. *CDK5RAP2* is a centrosomal protein involved in microtubular function necessary for formation of the mitotic spindle. *ASPM* also is necessary for microtubular function at the poles of the mitotic spindle, and *CENPJ* similarly is involved in formation of the mitotic spindle.

Microcephaly can be caused by familial, teratogenic, and syndromic disorders (Box 160-2). In addition to the autosomal recessive group, inherited varieties include autosomal dominant and X-linked recessive types. The best-documented teratogenic agent producing microcephaly is irradiation, for example, by atomic bomb or radiation therapy, particularly before 18 weeks gestation. Maternal alcoholism and cocaine abuse are associated with micrencephaly. Micrencephaly, usually with mental retardation, occurs in as many as 75% to 90% of nonphenylketonuric children of women with phenylketonuria. Other teratogens for microcephaly include anticonvulsant drugs and vitamin A toxicity. Fetal infections that may cause micrencephaly include rubella, cytomegalovirus, and HIV. A vast array of syndromes feature microcephaly as one of a number of fetal anomalies, in particular those associated with aneuploidies and chromosomal rearrangements.

Macrencephaly

Macrencephaly is characterized by a large brain and may relate to either excessive proliferation or abnormally prolonged windows for otherwise normal proliferation during development. Macrencephaly may be isolated or associated with severe neurologic findings, in particular epilepsy and mental retardation, or with other anomalies of somatic growth and some neurocutaneous disorders.

Familial, isolated macrencephaly is an autosomal dominant condition in which the head is unusually large at birth (greater than the 90th percentile in approximately 50%) and continues to grow rapidly in early postnatal life. Neurologic findings are usually minor and parental head size is also usually above the 90th percentile.

Macrencephaly may be associated with generalized disorders of growth, such as Beckwith-Wiedemann syndrome, Sotos syndrome (Figure 160-10), fragile X syndrome, Marshall-Smith syndrome, Simpson-Golabi-Behmel syndrome, and Weaver syndrome. The gene mutated or deleted in Sotos syndrome, *NSD1,* encodes a nuclear receptor binding protein that may be involved in proliferative events.

Several neurocutaneous disorders are associated with evidence of excessive cellular proliferation within the central nervous system (CNS), sometimes with overt macrencephaly, and evidence of excessive proliferation of mesodermal structures. Macrencephaly occurs most consistently in this context in the multiple hemangiomatosis syndromes, and more rarely in conditions such as megalencephaly-cutis marmorata syndrome and megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome.

Type 1 neurofibromatosis is an autosomal dominant disorder characterized by abnormal glial proliferation. The megalencephaly relates primarily to increases in cerebral white matter. The gene for this disorder, *NF1,* has been shown to encode a protein involved in the negative regulation of a key signal transduction pathway, the Ras pathway, which transmits mitogenic signals to the nucleus. Loss of the neurofibromatosis protein neurofibromin leads to increased mitogenic signaling, causing the proliferative abnormalities characteristic of the disorder.

Tuberous sclerosis is associated with abnormal proliferation of both neurons and glia associated with dysfunction of the genes *TSC1* and *TSC2* that encode the proteins hamartin and tuberin. Hamartin and tuberin form a molecular signaling system critical

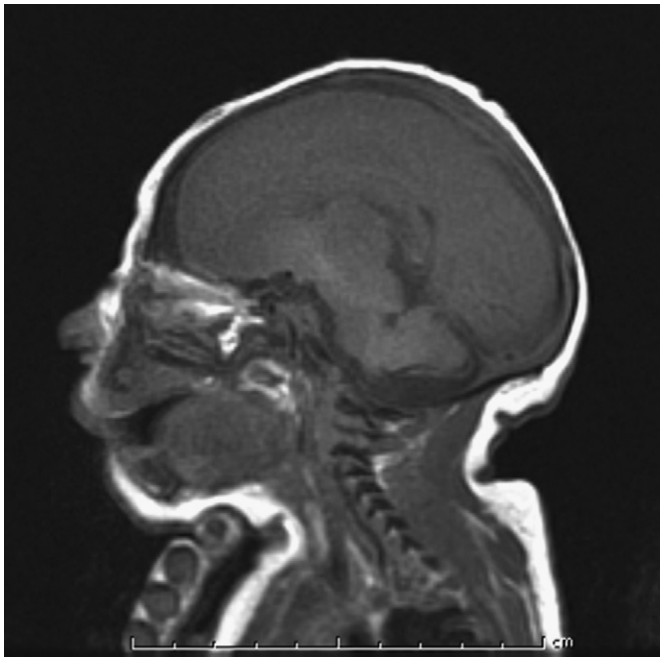


Figure 160-9 Magnetic resonance image of newborn infant with micrencephaly vera. Note disproportion between cranium and face.

BOX 160-2

Partial List of Genetic Causes of Micrencephaly

Isolated
AR micrencephaly
AD micrencephaly
XL micrencephaly

Chromosomal
Trisomy 21
Trisomy 13
Trisomy 18
4p deletion (Wolf-Hirschhorn)
7q11.23 deletion (Williams)
17p13.3 deletion (Miller-Dieker)

Syndromic
Smith-Lemli-Opitz
Cornelia de Lange
Angelman
Rett
Rubenstein-Taybi
Mowat-Wilson
Seckel
Pseudo-TORCH
Fanconi
Cockayne
Cri du chat



Figure 160-10 Patient with Sotos syndrome and macrocephaly. *(From Jones KL [ed]:* Smith's Recognizable Patterns of Human Malformation. *Saunders, Philadelphia, 2006.)*

for cell proliferation, cell growth, and cell adhesion/migration and lie as part of the mTOR and PTEN pathways.

Hemimegalencephaly is characterized by enlargement of part of all of one hemisphere, large and sometimes bizarre neurons, heterotopic neurons in subcortical white matter, and increased number and often size of astrocytes. This abnormality may be syndromic or sporadic. Syndromic causes include epidermal naevus syndrome, Proteus syndrome and hypomelanosis of Ito. A relatively rare neurocutaneous disorder, epidermal nevus syndrome, is associated with hemimegalencephaly in approximately half of cases.

NEURONAL MIGRATION

Between 12 and 20 weeks gestation millions of postmitotic neurons move from their sites of origin in the VZ and SVZ to the developing cortex and deep nuclei, where they will reside for life, taking up specific positions to form the six-layered cortex clearly observable by 28 weeks gestation. The timing and direction of these many simultaneous migrations are tightly regulated, and disorders of this remarkable process are surprisingly uncommon, yet when they do occur have significant consequences for fetal and neonatal well-being.

Before the neural tube even closes, specialized neurons known as predecessor cells, unique to humans, arrive at the pial surface of the forebrain, invading tangentially to form the preplate. These are bipolar cells have a variety of molecular signatures, principally TU20, and lack axons. They have long horizontal processes and may act as a scaffold for axonal navigation. These cells are largely transient and destined to die. The preplate then begins to subcompartmentalize with the appearance of *reelin*-expressing Cajal-Retzius cells. These transient bipolar cells arise from radial migration from the local VZ as well as by tangential migration from more distant extracortical sites. These cells take up a superficial subpial position as the marginal zone. Reelin terminates further migration and these cells therefore mark the pial limit of neuronal migration, the upper boundary of the protocortex, the cortical plate (CP).

Important molecular determinants of migration on radial glia include signaling pathways, involving erb B4 receptors, Notch receptors and brain lipid binding protein (BLBP). Doublecortin is the product of a gene on the X-chromosome, expressed in migrating neurons at the growing end and involved in an intracellular signaling pathway important for neuronal migration. Doublecortin is a microtubule-associated protein, and may be involved in neuronal migration by mediating the cytoskeletal changes required for cellular movement. The gene responsible for the human neuronal migration disorder, periventricular heterotopia, *filamin 1,* encodes a neuronal actin cross-linking protein that transduces ligand-receptor binding into actin again suggesting the importance of cytoskeletal movement in neuronal migration and cortical development (Figure 160-11).

The CP is a critical developmental phase in the formation of the human brain, progressively thickening with a clear temporal gradient. Radially migrating neurons accumulate in the CP in an inside-out sequence, earliest born neurons becoming the deeper layer V/VI and later born neurons becoming more superficial layer II/III. Further GABAergic interneurons migrate tangentially from the basal telencephalon as well as locally from the SVZ. Between the ventricular proliferative zones and the postmigratory neurons towards the pial surface is a heterogeneous compartment, the intermediate zone (IZ). This contains radially and tangentially migrating cells as well as extrinsic axons. Eventually the invasion of corticocortical fibers and myelination transforms the IZ into the white matter.

Between CP and IZ is a distinct neuronal compartment, the subplate (SP), that contains hetereogeneous cell populations. These include radially migrating glutamergic neurons that extend subcortical pioneer axons and express the transcription factors TBR1 and EMX1 and the p75 neurotrophin receptor. Interneurons within SP express transcription factors associated with GABAergic neurons such as DLX and LHX6. The SP expands dramatically during gestation to become four times thicker than the CP by 6 months' gestation, then gradually decreases, leaving only cell-sparse white matter by 6 months of postnatal life. The function of SP remains unclear but silver staining and dye tracing studies suggest that it acts as a waiting compartment for thalamic axons arrested until the CP becomes growth permissive and allows thalamocortical fibers to reach their target. The prolonged existence of this transient fetal zone into the third trimester and early postnatal life have profound implications for perturbations of sensory input related to premature birth or aberrant neonatal experience such as asphyxia or drugs.

As they migrate along radial glia, many neurons within the cerebral wall extend processes that are soon recognizable as axons. The formation and maintenance of axonal and dendritic arbors are largely under control of environmental cues. The tip of each process is composed of a specialized motile structure, a growth cone, which resembles the head of a club, with microspikes (filopodia) extending in all directions. Living growth cones, when viewed in a microscope, are constantly moving by local extension and retraction. When in contact with an appropriate substrate, they crawl forward and leave behind the elongating axon.

In the developing nervous system, a variety of molecules within the extracellular matrix have been shown to provide this substrate for axon outgrowth. Neurons in culture elongate their axons in a substrate-dependent manner. Two of the most active promoters of growth cone migration, laminin and fibronectin, have been shown to be expressed transiently as components of the extracellular matrix in cortical regions that have particularly active axon growth. In addition, responsiveness to laminin may be developmentally regulated and enhanced by treatments that activate integrin function. Administration of exogenous laminin favors neuronal migration and neurite growth, whereas inhibition or neutralization of laminin results in inhibition of neurite outgrowth. Proteoglycans such as chondroitin sulfate, which are macromolecules composed of core polypeptide and glycosaminoglycan chains, can modulate neurite outgrowth along with laminin. Matrix metalloproteinases, proteases that degrade extracellular matrix molecules, are thought to be involved in stabilization of laminin and cell-matrix interactions. Extracellular matrix proteins, such as collagen, also have been shown in cell culture to suppress apoptosis, or programmed cell death, and thus to provide a more nurturing substrate for neuronal survival and differentiation.

Although extracellular matrix substrates promote axon outgrowth, they alone do not provide all the directional signals required during axon outgrowth. As growth cones advance, their filopodia extend and appear to explore the local environment to find the path most favorable for continued axon growth. Growth-associated protein-43 (GAP-43) is a major growth cone protein whose phosphorylation in response to extracellular guidance cues can secondarily regulate microtubule behavior. Mice lacking GAP-43 fail to form the anterior commissure, hippocampal commissure, and corpus callosum. Growth of axons along voltage gradients or diffusible chemical gradients also occurs in the developing brain.

A growing body of evidence indicates that axon guidance is directed by the integrated and summed influence of both attractant and repulsive cues. These cues may be expressed in gradients, and they ultimately lead to the establishment of axon projection maps as found in the projection from the retina to the midbrain tectum. In fact, a family of axon-repulsive molecules, ligands of the *EPH* family of receptor tyrosine kinases, has been identified as the probable positional labels for the retinotectal projection.

Normal Development of the Cerebellar Cortex

The primordia of the cerebellar hemispheres appear in the fifth gestational week as bilateral thickenings in the lateral aspects of the dorsal surface of the rhombencephalon, the rhombic lips. By continued growth of this region the primitive vermis and cerebellar hemispheral anlage become apparent by 12 to 13 weeks. During the latter part of this period progenitor cells in the subependymal germinative zones of the rhombic lip, adjacent to the fourth ventricle, generate (1) neurons that migrate radially to form the dentate nucleus and other roof nuclei and the Purkinje cells, and (2) neurons that migrate tangentially over the surface of the cerebellum to form the external granule cell layer.

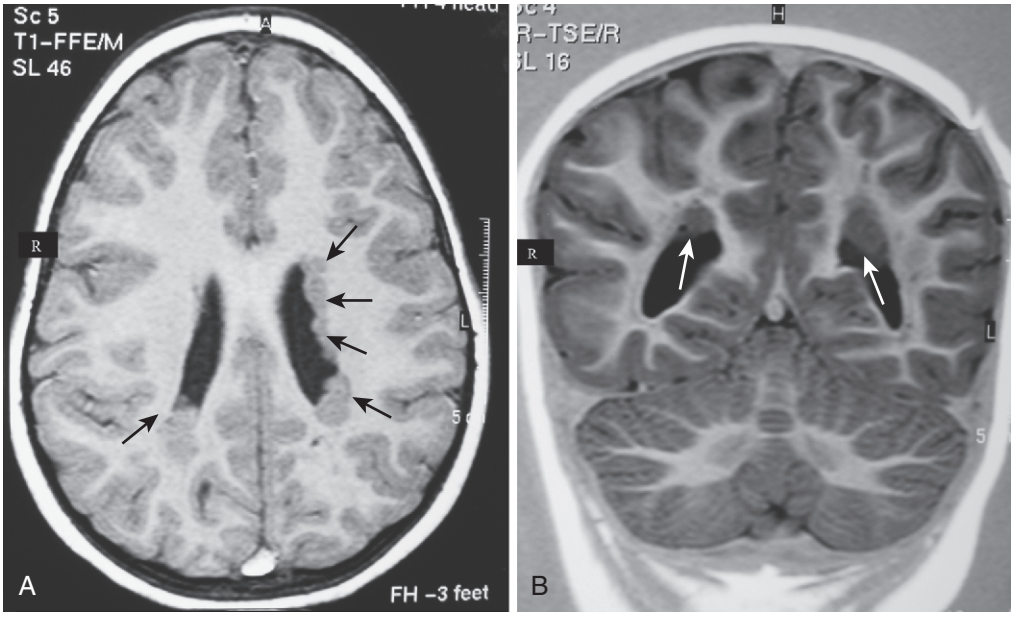


Figure 160-11 Magnetic resonance image showing bilateral periventricular heterotopia in 5-year-old child. *(From Ruggieri M, Spalice A, Polizzi A, et al: Bilateral periventricular heterotopia associated with amniotic band syndrome,* Pediatr Neurol *36(6):407-410, 2007.)*

Over the next 3 months the external granule cell layer increases to a maximum thickness of about nine rows of cells. As postmitotic neurons of the external granule cell layer are generated, they assume a deeper position within the layer. The migrating neurons pass through Purkinje cell dendrites in the molecular layer and then past the Purkinje cell bodies. After crossing the lamina dissecans, a layer just below the Purkinje cells that is rich in axon terminals of cerebellar afferents, such as the mossy fibers, the migrating neurons form the internal granular layer. Horizontal processes establish synapses with Purkinje cells and become parallel fibers, which are ordered within the molecular layer so that more recently generated fibers lie more superficially.

An unusual feature of the external granule cell layer is that it continues to generate neurons for the remainder of gestation and for as long as 7 to 9 months postnatally. This proliferation is remarkable both in terms of pure numbers as well as the protracted period of development; ultimately, cerebellar granule cells outnumber cerebral cortical neurons by more than four to one. The demonstration that medulloblastoma may arise from this layer is perhaps understandable given the long period of differentiation, which leaves neurons vulnerable to disruptions in such closely regulated pathways. Multiple genes are thought to be linked to medulloblastoma formation, including *Ptch*. The Ptch protein is a receptor for Shh, as discussed previously. Ptch normally inhibits signaling, and this inhibition is relieved by binding Shh. This in turn stimulates mitosis of the granule cell precursors. Inactivation of Ptch may therefore lead to the persistent proliferation of granule cell precursors.

Multiple influences on the complex migrations take place in the developing cerebellum. Both genetic and environmental cues are important in the establishment of cerebellar cortical migration and organization. Chemoattractive and chemorepulsive signals influence migrations. For example, netrin receptors are differentially expressed on neurons as they reach successive phases of migration. The Notch signaling pathway is also important for neurogenesis in the cerebellum. Notch1 depletion results in premature onset of neurogenesis in the midbrain-hindbrain and Notch1-deficient cells are eliminated by apoptosis and therefore result in a decreased number of neurons in the adult cerebellum. Sonic hedgehog is expressed in the cerebellum throughout life, though it promotes proliferation of developing cerebellar granule cells only in the early postnatal period, suggesting that downstream regulators may be important gatekeepers to the proliferative environment of the external granule cell layer. There is evidence that zinc finger proteins are involved in the control of cerebellar development. The mouse Zic genes are expressed in developing and mature central nervous system. Mutations in the Zic proteins lead to absent vermis and reduction of proliferation. Lastly, neurotransmitters may be important in the regulation of proliferation and migration of precursor cells via developmentally regulated, ligand-gated ion channel receptors.

Disorders of Neuronal Migration

Migrational disorders are a large and complex group of conditions characterized by relative impairments in the radial migration of neurons into the cerebral cortex and tangential migration of interneurons into the cortex. Imaging by magnetic resonance imaging (MRI) is the most useful initial investigation when a migrational disorder is suspected with the hallmark finding of gyral abnormalities and occasionally abnormalities of the corpus callosum.

Lissencephaly-pachygyria represents a spectrum of disorder marked by underdevelopment of absence of normal gyrification. In type 1 lissencephaly there is absent gyri and an immature cerebral mantle with disorganized and heterotopic neuronal layering (Figure 160-12). Type II lissencephaly is characterized by clustered, whorled arrays of neurons separated by glia and large heterotopic formations of neurons, often migrating into the pia giving a characteristic "cobblestone" appearance. Pachygyria is demonstrated by thickened simple gyri that are underdeveloped and is a continuum of lissencephaly. The known causes of these types of lissencephalies are listed in Table 160-3 together with salient clinical features and diagnostic indicators.

Polymicrogyria is characterized by numerous small folds of gyri that may have a basic four-layered anatomy, or unlayered with a poorly laminated heterotopic collection of neurons below the cerebral mantle. Polymicrogyria is associated with a number

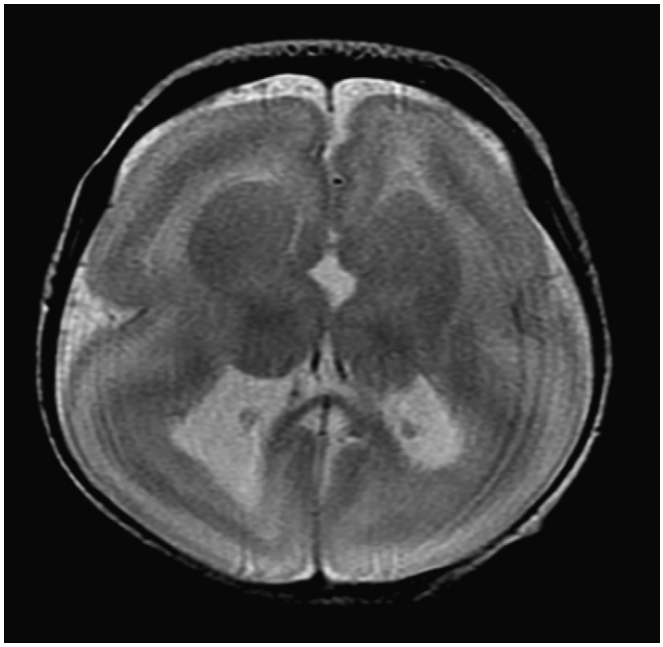


Figure 160-12 Magnetic resonance image of newborn with type 1 lissencephaly and Miller-Dieker syndrome. Note essentially absent gyrification and smooth cerebral surface.

TABLE 160-3

Causes of Type 1 and 2 Lissencephaly With Characteristic MRI or Clinical Findings

Gene	MRI Findings
LIS1	Posterior > anterior gyral abnormality (Miller-Dieker)
DCX (male)	Anterior > posterior gyral abnormality (XLIS)
RELN/DCX	Lissencephaly with cerebellar hypoplasia or male with subcortical band heterotopia
DCX (female)	Subcortical band heterotopia
ARX	Lissencephaly with agenesis corpus callosum and abnormal genitalia (XLAG)
FLNA (female)	Periventricular nodular heterotopia
POMT1	Type 2 lissencephaly with elevated CK and eye anomalies (Walker-Warburg syndrome)
POMGnT1	Type 2 lissencephaly (Muscle-eye-brain disease)
Fukutin	Type 2 lissencephaly (Fukuyama congenital muscular dystrophy)

CK, Creatine kinase; *MRI,* magnetic resonance imaging.

of primary genetic conditions as well as metabolic conditions, in particular disorders of peroxisomal biogenesis such as Zellweger syndrome (Figure 160-13). It frequently accompanies other dysgenetic brain malformations as well after in utero encephaloclastic events such as vascular insults or congenital infection (cytomegalovirus [CMV] in particular).

Neuronal heterotopias and *focal cortical dysplasias* represent arrest of migration of neurons. These may be located close to the ventricular surface in the subependyma following arrest in early radial migration. These are usually nodular and multiple. Other heterotopias are seen in the subcortical white matter and



Figure 160-13 Postmortem appearance of polymicrogyria in the region of the insula and neighboring frontal lobe in newborn with Zellweger syndrome. *(From Ellison D, Lowe J, Harding B [eds]:* Neuropathology, *ed 2. London, Mosby, 2004.)*

may be small rests of cells or more commonly bands. Focal cortical dysplasias represent abnormalities of the later stages of migration and are a cause of intractable epilepsy that may respond to epilepsy surgery. They may comprise collections of abnormally laminated normal neurons or large dysplastic multipotent cells known as balloon cells.

Schizencephaly is a severe restricted disorder of cortical formation characterized by failure of development of a portion of the cerebral wall, leaving a cleft extending from the ependymal surface to the pial surface. The cleft is usually lined by dysplastic heterotopic gray matter, in particular polymicrogyria. The lesion may be unilateral or bilateral, usually in frontal or perirolandic region. The edges of the pial surface may be opposed ("closed-lip") or more commonly widely separated ("open-lip"). Like polymicrogyria the disorder may have a primarily genetic etiology (e.g., mutation in the *EMX2* gene), but vascular disruption and congenital infection such as CMV are also associated with this abnormality.

DISORDER OF CEREBELLAR FORMATION

Cerebellar malformations include malformations of the vermis and hemispheres, typically absence or fusion as well as abnormalities of CSF flow through the aqueduct of Sylvius, draining the third ventricle into the fourth ventricle or through the medial and lateral CSF foramina in the posterior fossa. Hydrocephalus is a frequent association with cerebellar malformations.

Dandy-Walker malformation (DWM) consists of four major abnormalities: (1) complete or partial agenesis of the cerebellar vermis, (2) cystic dilation of the fourth ventricle, (3) enlargement of the posterior fossa with a resulting high position of the tentorium and torcula, and (4) hydrocephalus. Associated abnormalities

of the CNS occur in as many as 70% of cases, with the clinically most important examples being agenesis of the corpus callosum and a variety of defects of neuronal migration. DWM is related to abnormal formation of the cerebellar vermis and the roof of the fourth ventricle. The disturbance in Dandy-Walker malformation appears to be primarily failure of cavitation of the posterior membranous area of the developing roof of the fourth ventricle, allowing a buildup of CSF and development of the cystic dilation of the fourth ventricle (Figure 160-14).

Joubert syndrome is an autosomal recessively inherited syndrome of cerebellar vermis hypoplasia, hypotonia, ataxia, cognitive impairment, abnormal eye movements, and respiratory arrhythmia. The neuroradiologic hallmark of this cerebellar midbrain-hindbrain malformation on axial MRI images, is a triangular ("bat-wing" shaped) fourth ventricle, the small cerebellar vermis and large uncrossed cerebellar peduncles giving rise to the "molar tooth" sign. At least four genetic loci have been identified with multiple overlapping syndromes. The best studied thus far is *JBTS3*. The gene involved is *AHI1*, encoding the Jouberin protein, a presumed signaling molecule expressed in embryonic hindbrain and forebrain. The developmental defect involves cerebral, brainstem, and cerebellar development, perhaps at the levels of patterning, migration, and axon path finding.

Rhomboencephalosynapsis is a rare disorder characterized by hypoplasia of the vermis, fusion of the cerebellar hemispheres, and fusion of the dentate nuclei and cerebellar peduncles.

Synaptogenesis

Synaptic formation differs spatially in the human brain, but the basic principles are formation of earliest synapses in the marginal and subplate zones, an increase in synapses in cortical plate to a peak that exceeds the adult number, and a subsequent period of synaptic elimination. In the cerebrum, synapses are observed initially on neurons of the subplate and of the marginal zone. Initially, dendrites appear as thick processes with only a few fine branches. As development progresses, a great number and variety of dendritic spines appear. Synapse elimination then begins, and a large proportion of synapses is lost.

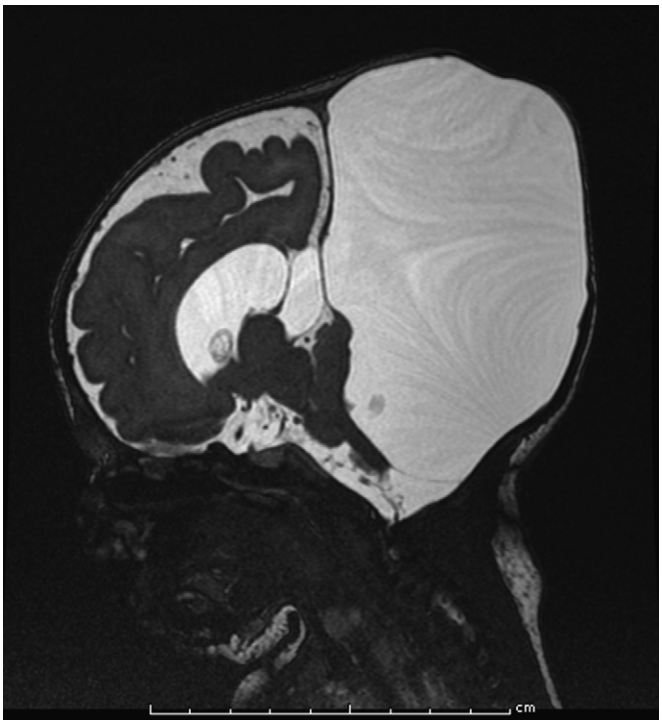


Figure 160-14 Magnetic resonance image of newborn with Dandy-Walker elevation. Note rudimentary cerebellum and massive retrocerebellar cyst.

The factors that stimulate synaptic formation and development in developing brain include both activity-independent events and activity-dependent events occurring after the development of receptors on target neurons and the generation of electrical activity. Many molecules and signaling pathways are involved in dendritic spine development and remodeling. The two principal themes are modulation of ion channels, by neurotransmitters, especially glutamate, and alteration of cell surface receptors by a variety of ligands. These intracellular events lead to effects on actin-binding proteins and the actin cytoskeleton, with resulting changes in spine shape, size, and motility.

CELL DEATH AND SELECTIVE ELIMINATION OF NEURONAL PROCESSES AND SYNAPSES

Cell death and selective elimination of neuronal processes and synapses, or pruning in brain development, are critical to normal postnatal behavior. Typically about half of the neurons in cortical region die before final maturation. This process of programmed cell death, apoptosis, is initiated and sustained by the expression of specific genes. Critical in the final phases of the sequence to cell death is the activation of caspases. Apoptosis appears to be fundamentally triggered by neuronal competition for limited amounts of trophic factors, generated by the target, afferent input, or associated glia, allowing numerical matching of interconnecting populations of neurons and elimination of projections that are aberrant or otherwise incorrect.

Neural organization is refined further by selective pruning of neuronal processes and synapses. This causes the removal of terminal axonal branches and their synapses. Activation of the NMDA type of glutamate receptor appears to be an important step in synapse elimination during development. The observations that cell death and elimination of neuronal processes and synapses occur during the organizational period of development suggest the plasticity of developing brain decreases as this period is completed but similarly has specific implications for the recovery of the neonatal brain, in particular the preterm brain, from injury.

DISORDERS OF ORGANIZATION

Several studies have shown abnormalities of development of dendritic branching and spines in children with idiopathic mental retardation. These findings indicate that a disturbance of cytoskeletal structures can cause severe dendritic abnormality and marked neurologic disturbance. X-linked genes have been shown to be particularly important. Prominent among these are four X-linked genes found mutated in families with mental retardation that encode proteins known as Rho guanine nucleotide exchange factor 6 (ARHGEF6), oligophrenin-1, p21-activated kinase, and guanine dissociation inhibitor 1. These proteins are involved in signaling pathways that regulate the actin cytoskeleton critical for neurite outgrowth, dendritic spine formation and morphology, and neurotransmitter release.

Rett syndrome is one of the most common causes of mental retardation and autism in females. It is characterized clinically by onset of deceleration of rate of head growth in the first months of life, loss of purposeful hand movement near the end of the first year, and development of stereotypic hand movements, autistic behaviors, ataxia, microcephaly, seizures, and mental retardation before the age of 5 years. The underlying molecular defect is principally loss of function of the *MECP2* gene, a transcriptional repressor expressed throughout the brain. The neuropathology consists of a small brain with an apparent disturbance of neuronal

development, characterized by dendritic spine abnormalities (Figure 160-15) and small, densely packed neurons. Animal studies suggest a fundamental perturbation in the ability to form stable synapses despite repeated experience-dependent stimulation.

Abnormalities of dendritic and axonal development have been described in infants with Down syndrome including alterations in cortical lamination, reduced dendritic branching, diminished dendritic spines and synapses, giant spines, and abnormal spine shape. Abnormalities are not apparent before 22 weeks gestation, that is, before the rapid progression of organizational events. In the first months of postnatal life an excess in dendritic branching precedes the consistent decrease observed after approximately 6 months of age. This sequence of excessive initial branching followed by deficits is similar to certain animal models of impaired dendritic development.

Fragile X syndrome is the most common form of inherited mental retardation in males and related to the amplification of a trinucleotide repeat sequence in the *FMR1* gene. The neuropathology is notable for abnormal dendritic spine morphology including long dendritic spines, fewer short spines, more immature-appearing spines, and fewer mature-appearing spines. The brain otherwise has no overt abnormalities. Similarly abnormal dendritic spines are seen in *FMR1* knockout mice.

Increasing evidence from a number of other disorders, both congenital and acquired, suggest the profound influence of structural or functional abnormalities of synaptic and dendritic formation and pruning in the genesis of severe neurologic deficits including mental retardation and epilepsy.

Autism is the leading cause of neurodevelopmental impairment with an incidence of 1 in 110 children in the United States. Evidence from studies of genetic and idiopathic forms of autism continues to accumulate that this disorder reflects fundamental failures of synaptic function or organization. The long understood phenomenologic overlap of conditions such as Rett syndrome, fragile X syndrome, and some forms of mental retardation are increasingly reflected by neurobiologic evidence of common perturbations of dendritic spine morphology, pruning, or stability of synapses. It is likely that this stage of fetal and neonatal brain development will become the most critical in understanding the major postnatal neurologic disorders of childhood.

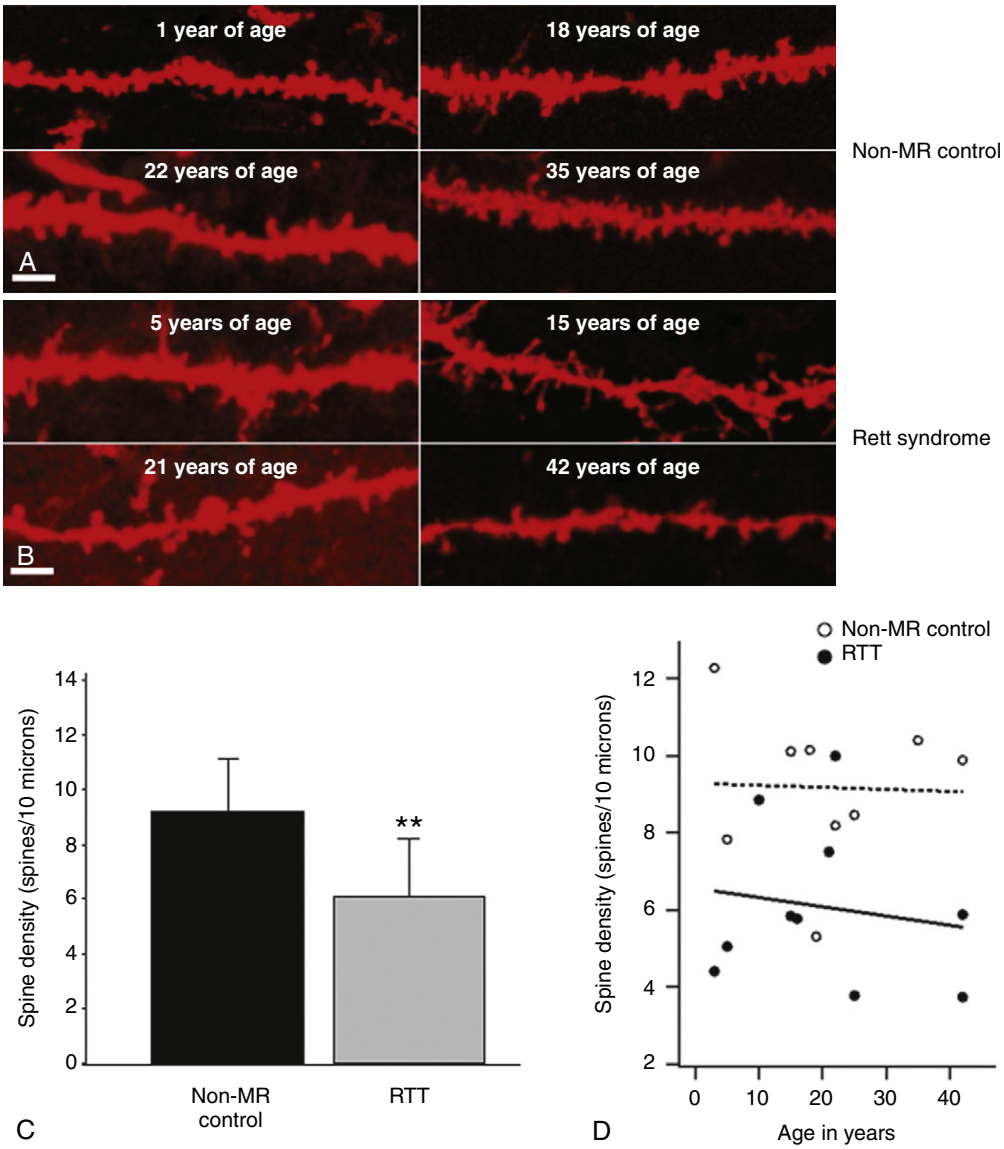


Figure 160-15 Abnormal dendritic spine morphology in patients with Rett syndrome compared with control without mental retardation. *(From Chapleau C, Calfa G, Lane M, et al: Dendritic spine pathologies in hippocampal pyramidal neurons from Rett syndrome brain and after expression of Rett-associated MECP2 mutations,* Neurobiol Dis *35[2]:223, 2009.)*

MYELINATION

Normal Development

Myelination peaks in postnatal life (Figure 160-16). Oligodendrocytes originate from progenitors in the subventricular zone and also from radial glial progenitors. The early phase in oligodendroglial lineage arising from progenitors is a mitotically active migratory cell recognized by the monoclonal antibodies A2B5 and NG2. This cell is generated from midgestation to the early postnatal life. As these precursors migrate they respond to internal cues as well as to the local influences of the other neurons and glia. These cues determine when progenitor cells exit from the cell cycle, begin to proliferate, and differentiate into mature oligodendrocytes. The waves of migration of these cells may be the anatomic correlate of the periventricular bands visualized on magnetic resonance images of the premature infant.

The O4 cell differentiates into the postmitotic immature oligodendrocyte, a richly multipolar cell recognized by a monoclonal antibody to galactocerebroside (O1). The proportion of O1 cells among the entire oligodendroglial population rises from 5% to 10% prior to 28 weeks of gestation to 30% to 40% during the premature period and to approximately 50% at term. In the third trimester of gestation the O1 cells develop linear extensions as they ensheath axons in preparation for myelination. This process is followed by differentiation to the mature oligodendrocyte. This cell becomes the predominant oligodendroglial stage in the months following term and gives rise to myelination.

The molecular determinants of this process include basic fibroblast growth factor (bFGF), neurotrophin-3 (NT-3), platelet-derived growth factor (PDGF), insulin-like growth factors (IGF-1), nerve growth factor, transferrin, iron, members of the interleukin-6 (IL-6) family, thyroid hormone, neuregulin, erbB receptors, semaphorins, neuropilin receptors, ephrin, Eph receptors, Nogo, and Nogo receptors. Cell death is an important feature of oligodendroglial development, with approximately 50% of oligodendroglia undergoing apoptosis during development.

Similar to cortical organization, myelination exhibits pronounced regional variation. These regional features have been defined well in the human cerebrum and anticipate the functional developmental milestones of the human infant. The earliest myelin is detected within the roots of motor and then sensory nerves. Myelination soon occurs within the neuraxis, in which sensory systems generally myelinate before motor systems. In cerebrum, primary projection systems begin to myelinate before associative systems. Finally, the cerebral commmissures and other corticocortical projections begin to myelinate and continue to do so for years postnatally.

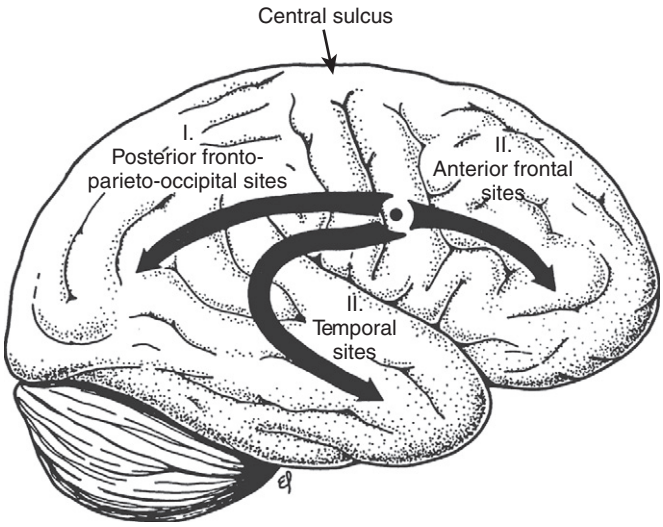


Figure 160-16 Progress of myelination. *(From Volpe JJ [ed]: Neurology of the Newborn, ed 5. Philadelphia, WB Saunders, 2008.)*

Disorders

Animal models of myelination have focused on mice deficient of membrane components. The jumpy mutation of the X-linked proteolipid protein (Plp) gene causes dysmyelination and premature death of mice with resultant hypomyelination and increased numbers of dead oligodendrocytes and astrocytes. The primary abnormality in these mice is the inability of oligodendrocytes to associate with and ensheath axons.

A primary deficiency of myelination and no other apparent neuropathologic condition has been described. Affected children demonstrate primary cerebral white matter hypoplasia, accompanied clinically by severe but nonprogressive intellectual impairment and spastic quadriparesis evident in infancy. The cause of this disorder is unknown, although a genetic abnormality is suggested by several examples of familial occurrence and by the similarity of neuropathologic features in certain mutant mice.

The occurrence of myelin deficiencies in malnutrition suggests that decreased synthesis of myelin lipids and proteins may play a major role in disorders of myelination. Inborn metabolic errors that cause abnormal neuronal storage such as GM1 gangliosidosis, have been associated with dysmature myelination on imaging studies. Evaluation of pathologic specimens from patients with infantile GM1 reveals delayed myelination of later-myelinating structures, suggesting that the metabolic defect has a direct effect on myelin development.

Dysmyelination or delayed myelination has been described in a variety of organic and amino-acidopathies, hypothyroidism, phenylketonuria, some chromosomal anomalies (in particular 18q deletion and Down syndrome) and iron deficiency.

Without doubt the most significant anomaly of myelination in current neonatal practice remains periventricular leukomalacia (Figure 160-17) and cerebral white matter injury of prematurity.

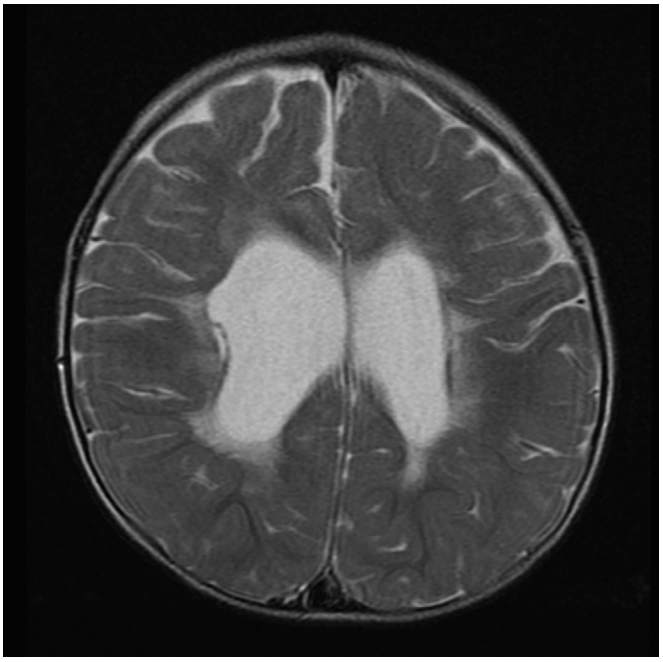


Figure 160-17 Magnetic resonance image of former 28-week premature baby at term corrected age demonstrating periventricular leukomalacia.

This clinical and neuropathologic syndrome reflects the exquisite vulnerability of the myelinating nervous system to external perturbations. Oligodendrocyte injury increasingly appears to have deleterious consequences for neuronal growth and development. The profound effects of the damage to the immaturely myelinated nervous system are reflected in the high incidence of motor, visual, and cognitive-behavioral abnormalities in survivors of preterm birth.

OVERVIEW

The development of the human nervous system is arguably the most remarkable sequence of events observable in nature. Over 40 weeks a few embryonic cells give rise to one of the most complex structures imaginable. Tightly regulated genomic expression interacts with local and distant epigenetic factors to produce trillions of functional connections that continue to grow and develop decades after birth. It is not clear when brain development truly ends because there is evidence of positive morphologic and functional changes well into adulthood.

Inevitably such a complex network of events is sensitive to damage and intrinsic perturbation, the consequences of which are at least in part determined by the phase of development in which they occur. These may represent massive failure as in anencephaly or more subtle aberrations such as cognitive disability and disorders of attention and executive control. Understanding the anatomic scaffold on which the nervous system is built allows us to predict, prevent, and potentially intervene in some of the most challenging disorders in medicine.

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Susan S. Cohen, Shadi N. Malaeb, Daniela Virgintino, and Barbara S. Stonestreet

Development of the Blood-Brain Barrier

The blood-brain barrier is a selective diffusion barrier that maintains central nervous system homeostasis and limits the entry of substances that may potentially alter neuronal function. The main anatomic substrate of the blood-brain barrier is the specialized cerebral microvascular endothelium. Properties specific to this endothelium, in contrast with other endothelial surfaces in the body, include the presence of tight cell-cell junctions between the endothelial cells and the lack of fenestrations across the endothelial surface. The cerebral microvascular endothelium and associated astrocytes, pericytes, and neurons and the extracellular matrix components constitute the *neurovascular unit.*[1] The neurovascular unit is essential to the health and function of the central nervous system throughout development.[1] Although systematic measurements of blood-brain barrier function in the fetus and newborn infant have been limited to date, understanding of the development of the barrier continues to expand.

This chapter reviews the basic concepts of the blood-brain barrier, summarizes the molecular biology of the tight junctional complex, introduces the relatively new concept of the neurovascular unit, and places these concepts in the context of the developing fetus and neonate. Finally, the blood-brain barrier is discussed, with special emphasis on the relevance of the barrier to select aspects of perinatal medicine.

GENERAL CONSIDERATIONS

Historical Perspectives

In 1878, Paul Ehrlich, a German microbiologist, demonstrated in a seminal publication that a barrier exists between the vascular system supplying the peripheral circulatory system and the brain.[2,3] Later experiments by Edwin Goldmann, an associate of Ehrlich, further established that this barrier was truly selective and not a result of the binding properties of the dye.[4] Lewandowsky in 1900 introduced the descriptive term *blood-brain barrier* in this context.[5] Although the concept of the blood-brain barrier has been refined over the past few decades, the current understanding of its basic structure is built on the work of Reese, Karnovsky, and Brightman in the late 1960s.[6,7] Their work confirmed the presence of epithelial-like "tight junctions" in the interendothelial cleft.[6,7] Reese and Karnovsky in 1967 proposed that the capillary lumen bridged by tight junctions formed a continuous, impermeable membrane that constituted the primary anatomic substrate of the blood-brain barrier.[6] Later studies by Brightman and Reese showed that horseradish peroxidase injected into the brain could diffuse through the approximately 20-nm gaps between the astrocyte end-feet to the abluminal surface of the endothelium, indicating that astrocytes do not form the physical barrier.[7]

Key Features of the Blood-Brain Barrier

In peripheral capillaries and the nonbarrier capillaries of the central nervous system, blood-borne polar molecules diffuse across the vessel wall through spaces between adjacent endothelial cells and through transcellular routes—specifically, endothelial fenestrations and vesicular or tubulovesicular structures (Figure 161-1). By contrast, the blood-brain barrier is a selective barrier formed primarily by the specialized endothelial cells that line cerebral microvessels. The blood-brain barrier capillaries have a low nonspecific permeability to polar substances because these diffusional pathways are sparse or lacking.[6-11] The endothelia of the barrier-containing brain capillaries are nonfenestrated and are interconnected by a continuous network of complex tight junctions that functionally fuse the plasma membranes of adjacent endothelial cells. These intercellular junctions were definitively demonstrated to be the site of the barrier to passive diffusion of polar plasma solutes by the classic studies of Reese and Karnovsky[6] and of Brightman and Reese.[7]

The distinct structural characteristics of these junctions were confirmed by both transmission electron microscopy and freeze-fracture studies. Although tight junctions are found in capillaries of all tissues, they are less extensive and less complex in non–barrier-forming endothelia and contain intercellular clefts through which passive diffusion occurs. Such clefts are markedly diminished in brain capillaries. These tight junctions even

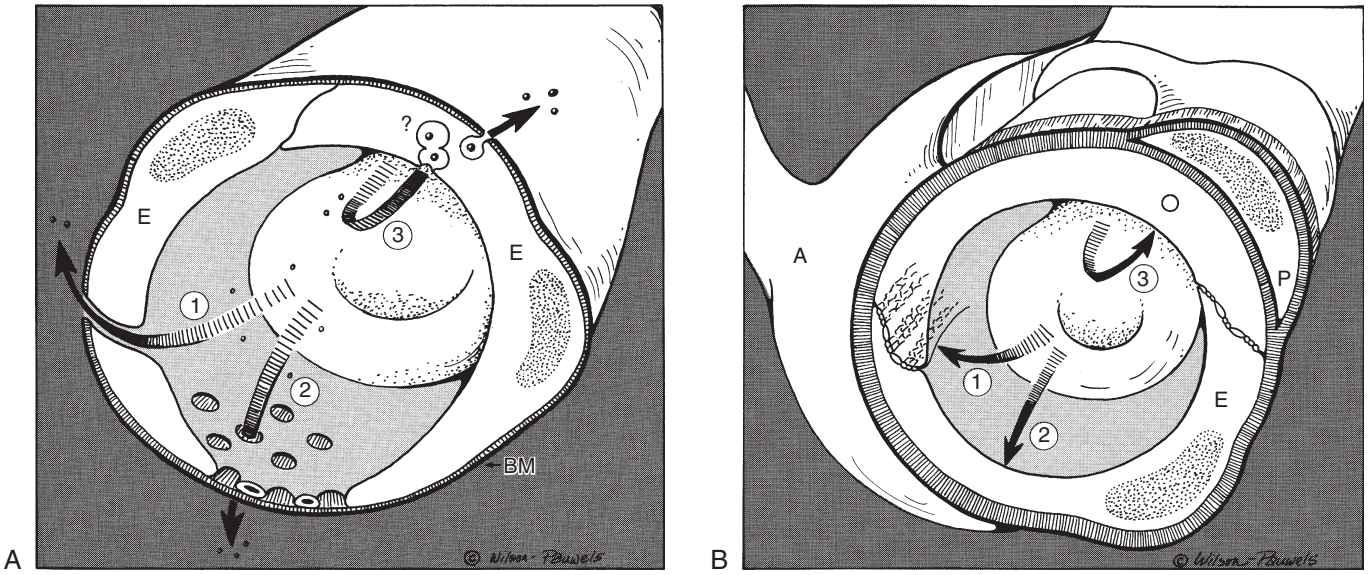


Figure 161-1 Comparative anatomic features of nonbarrier and blood-brain barrier capillaries. A, Peripheral capillaries and nonbarrier capillaries of the central nervous system lack an effective barrier to blood-borne polar molecules because of diffusion between endothelial cells *(1),* diffusion through endothelial fenestrations *(2),* and vesicular-tubulovesicular transport *(3).* **B,** Barrier capillaries are ensheathed by astrocytic foot processes, develop complex interendothelial tight junctions that block paracellular diffusion of polar substances, lack fenestrations, and contain a sparse vesicular transport system. A relatively thick basement membrane is interposed among the endothelial cells *(E),* astrocytic foot processes *(A),* and pericytes *(P).*

restrict the movement of small ions such that the transendothelial electrical resistance, which is 2 to 20 ohm • cm^2 in peripheral capillaries, can be as high as 1000 ohm • cm^2 in the brain endothelium.[1,12] The unique morphology and functional characteristics of the cerebral microvascular endothelium suggest that cerebral endothelial cells are intrinsically unique or that the cellular milieu of the brain induces these distinctive properties.[13]

MOLECULAR BIOLOGY OF THE BLOOD-BRAIN BARRIER JUNCTIONAL COMPLEX

The tight junctions are the primary structures responsible for the properties of the blood-brain barrier[14] (Figure 161-2). The tight junction forms a continuous, circumferential belt separating apical and basolateral plasma membrane domains, working as a barrier within the intercellular space and as a fence within the plasma membrane.[1,14,15] They appear by transmission electron microscopy as focal contacts between membranes of adjacent cells.[7] Freeze-fracture electron microscopy showed that these contacts correspond to branching fibrils of transmembrane particles.[16] These fibrils are formed by at least two distinct transmembrane proteins: occludin and the several members of the multigene claudin family.[16,17] The blood-brain barrier transmembrane proteins, including occludin, claudins, and the junctional adhesion molecules (JAMs) such as JAM-A, JAM-B, and JAM-C, physically occlude the interendothelial space between adjacent endothelial cells (see Figure 161-2).[1,13,14] Occludin and the claudins copolymerize to form the fibrillar heteropolymers, which can be seen on micrographs prepared using freeze-fracture techniques, and are linked laterally to the JAMs.[18] The tight junction–associated proteins, also called *adapter* proteins, are located in the cytoplasm beneath the tight junctions.[16,17,19,20] These proteins are involved in scaffolding the transmembrane proteins and coupling them to the cytoskeleton.[16] This complex array of cytoplasmic plaque proteins includes the zonula occludens (ZO) proteins ZO-1 to ZO-3, symplekin, cingulin, 7H6, ZONAB (*ZO*-1–associated *n*ucleic *a*cid–*b*inding protein), AF-6 (*A*LL1-*f*used gene from chromosome *6* protein), and others (see Figure 161-2).[16,17] The ZO proteins have one or more domains that function as protein-binding modules and have a role in organizing proteins at the plasma membrane.[17] These proteins are located at the cytoplasmic surface of endothelial cells and connect tight junctions to actin, which is the primary cytoskeleton protein for the maintenance of structural and functional integrity of the endothelium.[13] A ring of actin microfilaments underlies the complex and has a role in regulating permeability and structural integrity of the tight junction complexes that are situated on the apical cell membrane.[16]

Occludin was identified in 1993 as the first integral protein localized to the tight junction.[21] It is a 60- to 65-kDa protein with a carboxyl-terminal domain that is capable of linking it to the ZO proteins.[21] The main function of occludin appears to be in tight junction regulation[1,13,22]; however, the family of claudin proteins are 22-kDa phosphoproteins that have four transmembrane domains and contribute to the high electrical resistance of the tight junctions.[1,13,14,23] Twenty-four members of the claudin family have been identified.[1,14,23] Claudin-3, claudin-5, and possibly claudin-12 are the main claudins that contribute to the high resistance of the blood-brain barrier.[1,14,23] Together, claudins and occludins form the extracellular component of tight junctions, and both are required for the formation and proper functioning of the blood-brain barrier.[24] Junction-associated adhesion proteins are members of the immunoglobulin superfamily and also are important for tight junction complex integrity.[14,25] They also contribute to the high electrical resistance of tight junctions and play a role in inflammatory cell transmigration through junctional complexes.[25]

THE NEUROVASCULAR UNIT AND THE BLOOD-BRAIN BARRIER

The association of the microvascular endothelium, astrocytes, pericytes, neurons, and extracellular matrix has been proposed to form a functional neurovascular unit[1,13] (Figure 161-3). The endothelial cells of the brain appear to be important in the modular

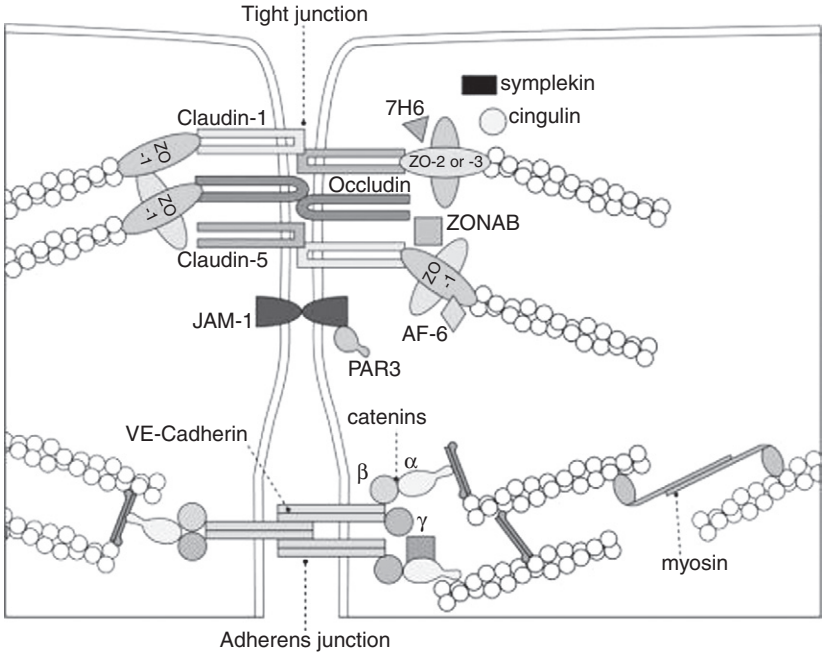


Figure 161-2 Molecular composition of tight junctions. The transmembrane proteins occludin, the claudins, and junctional adhesion molecule-1 (JAM-1) constitute the barrier formed by tight junctions sealing the paracellular space. Claudins adhere to each other in a homotypic tail of occludin and link the tight junction to the actin cytoskeleton. Proteins of the zonula occludens (ZO) family can shuttle to the nucleus to influence transcriptional processes in cellular proliferation and differentiation. The ZO proteins also have been shown to interact with claudins, and to provide molecular scaffolds for tight junction assembly. Cingulin is a 140-kDa tight junction plaque protein that associates with the actomyosin cytoskeleton. Its putative function is transduction of the mechanical force generated by the actomyosin cytoskeleton important for cellular differentiation. The Ras target AF-6 (ALL1-fused gene from chromosome 6 protein) interacts with ZO-1 and serves as a peripheral component of tight junctions in epithelial cells. Symplekin is a 126-kDa protein that occurs and probably functions in the nucleus as well as in the tight junction plaques. Localization of 7H6 tight junction–associated antigen along the cell border of vascular endothelial cells has been shown to correlate with paracellular barrier function. *(Modified from Förster C: Tight junctions and the modulation of barrier function in disease,* Histochem Cell Biol *130:55-70, 2008.)*

organization of this unit.[1,13] All components of the neurovascular unit, including the cellular constituents and components of the basement membrane, can contribute to the dynamic regulation of microvascular permeability and therefore have important interactions that regulate the function of the endothelial *physical barrier.*[1,13]

The neurovascular unit regulates the supply of nutrients to the brain by means of endothelial transporters and acts as a *transport barrier.* Some of these barrier-specific endothelial transporters include the glucose carrier GLUT1, several amino acid carriers (including LAT-1 [system-L large neutral amino acid transporter-1]), and transporters for nucleosides and nucleobases.[1] Other important efflux transporters specialize in eliminating potentially toxic substances from the brain. The *ABCB1/MDR1* gene encodes P-glycoprotein (P-gp/MDR1), which is one of the transmembrane transporters of clinical relevance. It is concentrated on the luminal membrane, constitutes a major barrier to the penetration of drugs into the brain, and serves as a physiologic barrier against the entry of xenobiotics into the brain.[26,27]

Astrocytes are integral elements of the neurovascular unit that regulate several features of the blood-brain barrier, such as the tightness of the interendothelial tight junctions,[1,28,29] and the expression and polarized localization of specialized enzyme and nutrient transporter systems.[1,30-34] The microcirculation of the brain needs to be responsive to the fluctuating metabolic demands of the tissue it supplies, and to adapt to the dynamic nature of neural activity.[13] Tissues with high levels of neuronal activity must communicate with the vasculature not only to increase regional blood flow but also to regulate the regional permeability of nutrients through the blood-brain barrier. Astrocytes are positioned to mediate the moment-to-moment regulation of cerebral microvascular permeability through their interaction with neuronal processes in the neurovascular unit.[13,35]

Pericytes (see Figure 161-3) constitute another important structural element of the neurovascular unit. Pericytes intimately embrace the abluminal surface of the brain capillary endothelium and have a role in the expression, maintenance, and regulation of the phenotypic properties of the barrier.[36,37] The degree of coverage of the cerebral microvasculature by pericytes is greater for brain capillaries than for capillaries of other tissues[38] and appears to correlate with the degree of tightness of the interendothelial junctions.[36,39] Recruitment and integration of pericytes into the endothelial wall are essential for the formation, maturation, and maintenance of the normal microvascular structure and function.[36] Pericyte-derived angiopoietin can induce endothelial expression of occludin, a major protein constituent of the blood-brain barrier.[13,40] Contractile proteins found in cerebral pericytes suggest that they also may regulate capillary blood flow and permeability.[13,41]

In addition to astrocytes, pericytes, and neurons, the extracellular matrix of the basal lamina also interacts with the cerebral microvascular endothelium. Disruption of the extracellular matrix is associated with increased blood-brain barrier permeability in pathologic states.[13,42,43] Recognition of the complex interactions of the components of the neurovascular unit continues to provide a basis for understanding the multiple mechanisms by which cerebral microvascular permeability may potentially be influenced by development, medications, and disease.[1,13,14]

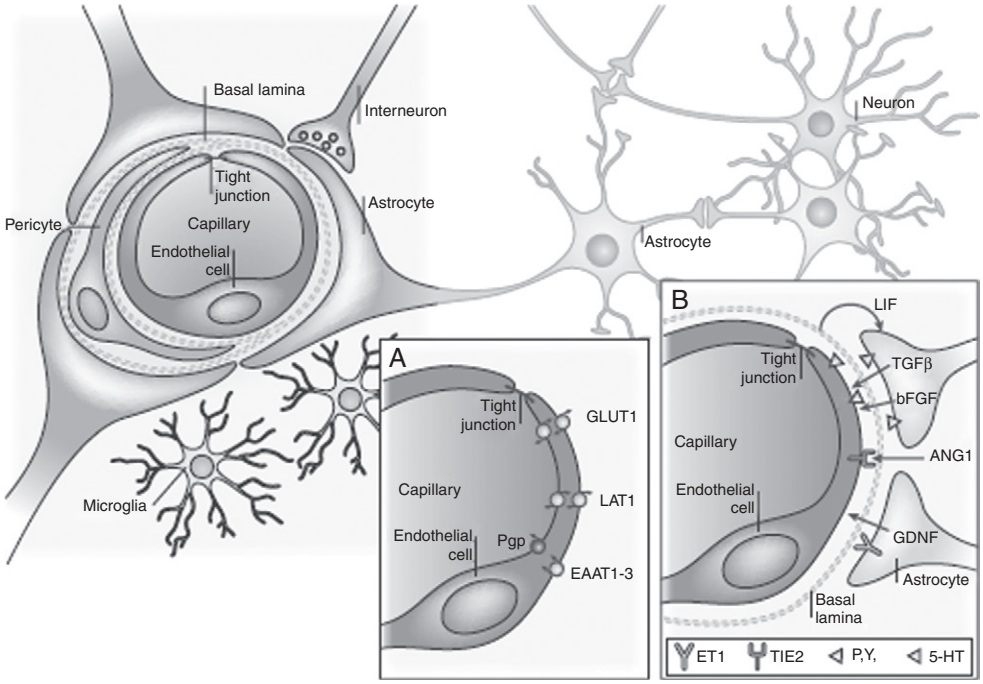


Figure 161-3 The neurovascular unit is formed by microvascular endothelium, astrocytes, pericytes, neurons, and extracellular matrix. **A,** Brain endothelial cell features observed in cell culture. The cells express a number of transporters and receptors, some of which are shown. *EAAT1-3,* Excitatory amino acid transporters 1 to 3; *GLUT1,* glucose transporter 1; *LAT1,* L-system for large neutral amino acids; *Pgp,* P-glycoprotein. **B,** Examples of bidirectional astroglial-endothelial induction necessary to establish and maintain the blood-brain barrier. Some endothelial cell characteristics (receptors and transporters) are shown. *5-HT,* 5-Hydroxytryptamine (serotonin); *ANG1,* angiopoetin 1; *bFGF,* basic fibroblast growth factor; *ET1,* endothelin 1; *GDNF,* glial cell line-derived neurotrophic factor; *LIF,* leukemia inhibitory factor; P_2Y_2, purinergic receptor; *TGF-β*, transforming growth factor-β; *TIE2,* endothelium-specific receptor tyrosine kinase 2. Data were obtained from astroglial-endothelial co-cultures and with the use of conditioned medium. *(Modified from Abbott NJ, Ronnback L, Hansson E: Astrocyte-endothelial interactions at the blood-brain barrier,* Nat Rev Neurosci *7:41-53, 2006.)*

DEVELOPMENTAL ASPECTS OF THE BLOOD-BRAIN BARRIER

Current knowledge regarding the development of the blood-brain barrier in vertebrates is derived from experiments in a wide spectrum of organisms ranging from hagfish to sheep. Although information derived from autopsy material from human fetuses and premature infants more recently has become available,[44-47] data on barrier function in human fetuses and premature infants are lacking. Comparisons among vertebrate classes indicate that the structural and functional aspects of the blood-brain barrier are remarkably conserved.[48] Nonetheless, caution must be exercised in analysis of experimental data on the temporal aspects of prenatal or postnatal barrier development, because the timing of brain growth and differentiation relative to the timing of birth differs considerably among species.[49,50]

Angiogenesis: Capillary Formation

The development of the central nervous system vasculature begins during embryogenesis, when angioblasts arise in the mesoderm of the head region and form a perineural vascular plexus by a process called *vasculogenesis*—in situ differentiation of the endothelial cells.[51] In 2-day-old chick and 9-day-old rodent embryos,[52-54] the perineural vascular plexus covers the entire surface of the neural tube, whereas in the human, it appears at approximately week 8 of embryonic development.[55,56] Vascular sprouts from the perineural plexus invade the proliferating neuroectoderm at 4 and 11 days of embryonic development in the chicken and rodent, respectively, and during the third month of fetal development in the human.[56] The mechanism by which the new vessels are formed from preexisting vessels is called *angiogenesis.* During angiogenesis, newly forming vessels radially invade the neuroectodermal tissue, elongate, give rise to manifold branches, and finally anastomose with adjacent sprouts to form a plexus of undifferentiated capillaries in the ventricular zone of the developing brain.

The endothelial cells later become invested with astrocytic end-feet, at which time a true capillary-astrocytic complex resembling the mature vessel is formed.[57] The basement membrane becomes recognizable concurrent with these events and appears most developed at regions of the closest endothelial-astrocytic contact. Some evidence suggests that the basement membrane forms adjacent to the membranes of each cell type, and that these membranes then coalesce or fuse. Astrocytic investment of endothelial cells and basement membrane formation are associated with marked decreases in the width of the perivascular space and in the fraction of endothelial cells that are surrounded by pericytes or their processes. These events of perivascular consolidation and basement membrane formation are most prominent from birth through the first 3 to 4 postnatal weeks in the mouse and rat. During this period in the mouse, vessel wall thickness decreases by 60%, and the basement membrane thickens from undetectable levels to 71 nm by electron microscopic criteria.[58] Paracellular diffusion diminishes during these latter stages of capillary-astrocytic complex formation, basement membrane formation, and perivascular consolidation. Nonbarrier vessels in the area postrema do not develop these differentiated features.

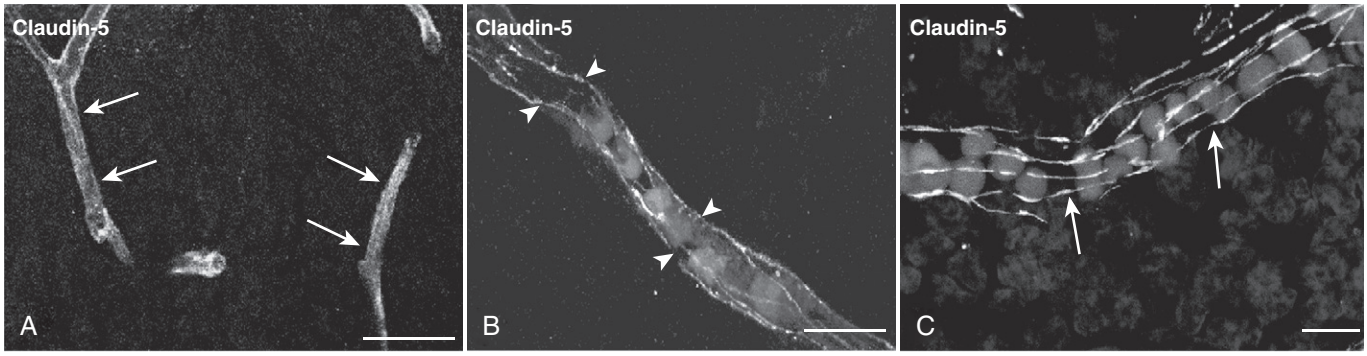


Figure 161-4 Human fetal telencephalon at 12 **(A),** 14 **(B),** and 18 **(C)** weeks of development. At 12 weeks, a claudin-5 punctate staining pattern is detected in the endothelial cytoplasm *(arrows).* At 14 weeks, the punctate pattern starts to form interrupted linear tracks *(arrowheads)* that at 18 weeks (**C**) appear as nearly continuous thick bands *(arrows). Scale bars* = 15 μm in **A** and **B** and 10 μm in **C.** *(Modified from Virgintino D, Errede M, Robertson D, et al: Immunolocalization of tight junction proteins in the adult and developing human brain,* Histochem Cell Biol *122:51-59, 2004.)*

Tight Junction Development

Development of the blood-brain barrier begins during the embryonic period with the formation of tight junction complexes between endothelial cells.[35,59-62] The exact timing of the maturation of these barrier components to macromolecules remains controversial.[57] An important point is that blood-brain barrier does not "switch" to a tight barrier at a specific time during angiogenesis, but rather the tightness of the barrier gradually increases in a region-dependent manner while angiogenesis is ongoing.[63] In rodents, vascular fenestrae disappear, pinocytosis decreases, and vessels decrease in diameter between embryonic days E11 and E17.[62,64] The onset of tight junction formation is detectable by E15, but tight junctions continue to increase in complexity through postnatal day one.[12, 65-68] Results obtained from experiments using horseradish peroxidase and freeze-fracture techniques suggest that the chick, rat, and monkey blood-brain barriers to proteins are tight from the earliest stages of development.[60] Some features of the tight junctions, however, appear to continue to mature with brain development.[59]

Molecular Biology of the Junctional Complex: Developmental Aspects

As described previously, tight junctions are composed of a complex array of transmembrane and cytoplasmic proteins.[1,13,59] As supported by experimental data, the expression of these proteins is developmentally regulated in rodents[67,69,70]: Occludin expression is low in rat brain endothelial cells at postnatal day 8 but clearly increased by day 70,[69] and ZO-1 protein expression increases between fetal days 15 and 19 and after birth in mouse brain microvessels.[67]

In contrast with the rodent, in which the development of the blood-brain barrier occurs primarily after birth, the human barrier and its tight junction protein complex develop mainly in utero.[12,70,71] More recent evidence has confirmed that the proteins of the tight junction complex are present very early during human fetal brain development.[44-47]

The first stage of human fetal intracortical vascularization is characterized by the development of radially penetrating stem vessels in fetuses from 10 to 12 weeks gestation, which is associated with the appearance of vascular basal lamina components including endothelium-specific markers and barrier-specific transporters.[46 47,72] The second stage of intracortical vascularization spans the period of midgestation and is characterized by vascular branching, increasing microvessel density, and barrier differentiation demonstrated by the detection of tight junction molecules, barrier-specific metabolic transporters, and efflux carriers.[46,47,72]

The developmental pattern of tight junctional protein expression in human fetuses and premature infants is somewhat controversial: Two studies reported maturational changes in the expression of these proteins,[44,46] whereas a third study did not find similar changes.[45] The primary endothelial tight junction molecules that have been identified so far in the developing human brain include occludin, claudin-5, JAM-1, and ZO-1.[44,46,47,73] Occludin and claudin-5 are already expressed in the primary vessels of the telencephalon by week 12 of gestation, and by midgestation (18 weeks) they show dramatic changes[46] (Figure 161-4). The most critical change occurs between 12 and 14 weeks of gestation, when occludin and claudin-5 immunoreactivity shifts from the endothelial cytoplasm to the endothelial borders and concentrates in linear, discontinuous tracts that may correspond to simple, incomplete networks of tight junctional strands.[46] Soon thereafter, a near-continuous staining pattern is detectable, and more extended and complex strands form, resembling those observed in mature tight junctions.[46] Therefore human endothelial tight junction development appears to begin earlier and proceed faster than in other vertebrate species.[66,74-77]

Consistent with the maturational changes in the tight junctional proteins during very early in fetal development,[46] occludin, claudin-5, and ZO-1 immunoreactivity was detected in the germinal matrix and cortical vessels of premature infants with postconceptual ages ranging from 24 to 42 weeks.[44] Although the immunoreactivity of occludin, claudin-5, and ZO-1 in the cortical vessels appears to increase with increasing postconceptual age, the immunoreactivity increases for these proteins appear to be greater with maturation in the germinal matrix.[44] Therefore the structural elements of the germinal matrix vascular endothelial tight junctional complexes appear to accumulate from 24 weeks until term, suggesting that the tight junctions in the germinal matrix mature during the third trimester.[44] Nonetheless, in another detailed study, the primary tight junction molecules, occludin,claudin-5, and JAM-1, were shown to be expressed in the blood-brain barrier as early as 16 weeks gestation but did not show significant differences in expression in the vasculature of the germinal matrix, white matter, or cortex between 16 and 40 weeks gestation.[45]

The early embryonic time course for the development of the blood-brain barrier raises the question of what potential inductive factors or cell types promote the early process of endothelial differentiation.[78] Neural progenitor cells are a major cell type in the developing embryonic brain, and endothelial cells have been

shown to influence the differentiation and morphology of neural progenitor cells.[79] In co-culture with endothelial cells, neural progenitor cells show reduced neurogenesis and elevated self-renewal.[79] Neural progenitors also have been observed in contact with early postnatal blood vessels, and, thus, could represent an early stage of astrocyte differentiation.[80] These findings point to the likeihood of important interactions between neural progenitor and endothelial cells. In co-culture studies, neural progenitor cells also have been shown to influence brain endothelial microvascular cells by enhancing barrier properties including increased transendothelial electrical resistance and decreased permeability to a small-molecule tracer, and by affecting tight junctions as indicated by a redistribution of occludin, claudin-5, and ZO-1 to cell-cell borders.[78] In view of these interactions between endothelial cells and embryonic neural progenitor cells, neural progenitor cells probably influence the embryonic brain endothelial phenotype.[78] Thus neural progenitor and brain microvascular endothelial cell interactions may be one of the factors responsible for inducing blood-brain barrier–like properties early in embryonic development.[78]

MATURATION OF THE BLOOD BRAIN-BARRIER

Permeability

The functional development of the fetal and neonatal blood-brain barrier has been the subject of some controversy.[53,81,82] Some investigators have claimed that a barrier is present to proteins as early as blood vessels form in the fetal brain,[81] whereas others consider this concept to be misleading.[82] This controversy[53,81,82] appeared to have been based on a paucity of quantitative blood-brain barrier permeability measurements in the same species from early fetal through later neonatal development up to maturity in adults. As described earlier, comparisons among species are difficult because of differences in rate of brain development.[50] In addition, birth is not necessarily a reliable landmark by which to stage brain and hence blood-brain barrier development.

Blood-brain barrier function was first investigated in developing fetal sheep by examining steady-state brain-to-plasma sucrose ratios in anesthetized exteriorized acutely studied fetal sheep.[83] The brain-to-blood sucrose ratios exhibited rapid decreases in early gestation, followed by smaller decreases later in gestation through the first week of postnatal life.[83] The ontogeny of blood-brain barrier permeability was later quantified with the rate constant for influx (K_i) across the blood-brain barrier using a small-molecular-weight (103-Da) synthetic inert hydrophilic amino acid, α-aminoisobutyric acid (AIB). These studies were done in a nonanesthetized chronically instrumented sheep model for three different points in development: in the last 2 months of fetal life, in the first months of postnatal life, and at full maturation (i.e., in adult sheep)[84] (Figure 161-5). The K_i measured with AIB was significantly lower in all brain regions of the adult sheep, and in most brain regions of newborn and older lambs, than in brain of fetuses at 60% and 90% of gestation. However, the blood-brain barrier was relatively impermeable to AIB in all age groups and brain regions, because the magnitude of the changes from 60% of gestation to maturity was not large.

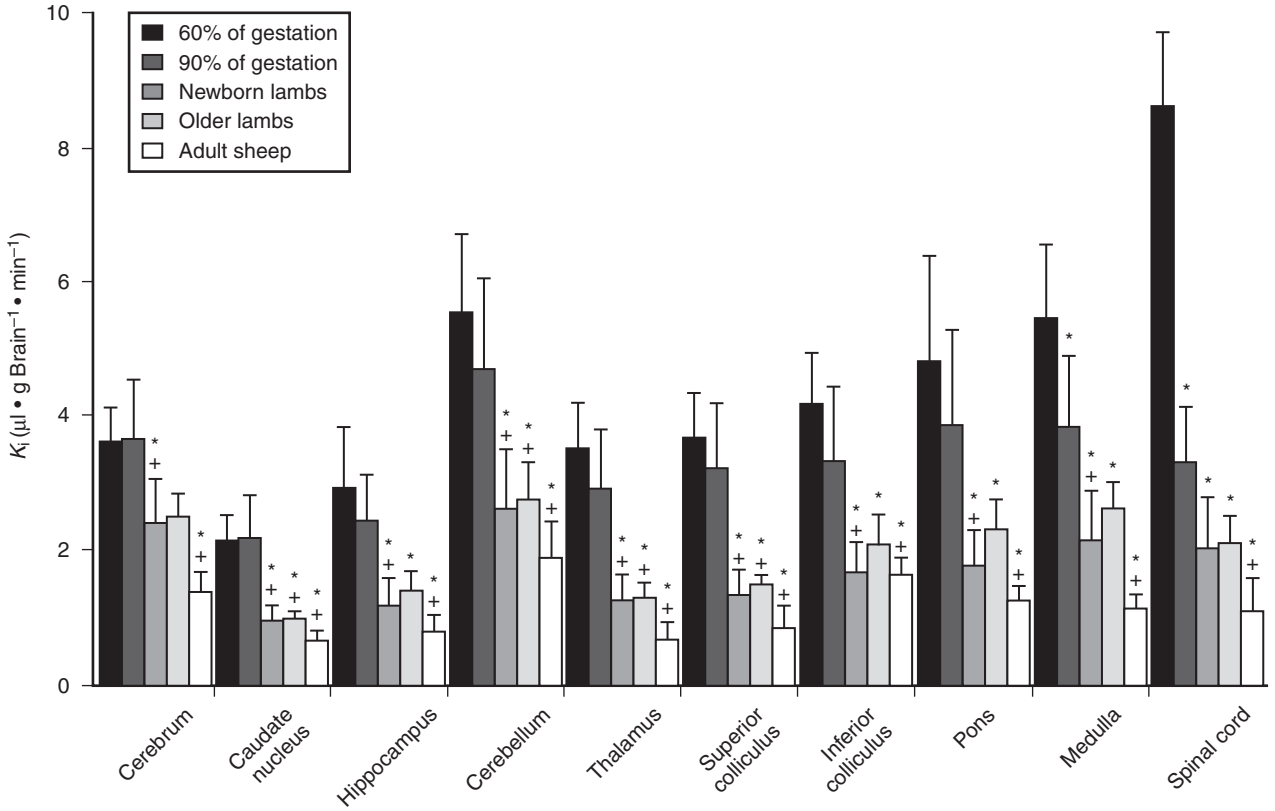


Figure 161-5 Regional blood-to-brain transfer constants for influx (K_i) to AIB, an amino acid used extensively to measure total and regional barrier permeability, decreased with development in sheep. The magnitude of the changes from 60% of gestation to maturity was not large. The low permeability to AIB suggests presence of a functionally tight blood-brain barrier from very early in gestation, which probably serves to isolate the developing brain from systemic blood constituents. *(Modified from Stonestreet BS, Patlak CS, Pettigrew KD, et al: Ontogeny of blood-brain barrier function in ovine fetuses, lambs, and adults,* Am J Physiol *271:R1594-R1601, 1996.)*

Although the permeability of the barrier to AIB was relatively low during fetal and neonatal development, the ovine fetus and neonate exhibited ontogenic decreases in barrier permeability (see Figure 161-5).[84]

The low permeability to AIB suggests that a functionally tight barrier may be present from very early in gestation, which probably serves to isolate the developing brain from systemic blood constituents. Inspection of the regional K_i values for AIB (see Figure 161-5) reveals that the cerebrum exhibits the smallest differences in the K_i between the fetuses at 60% of gestation and the adult sheep. By contrast, the more caudal brain regions, including the cerebellum, medulla and spinal cord, exhibit relatively larger developmental decreases in permeability between fetuses at 60% of gestation and adult sheep. These findings suggest that the cerebral cortex represents a relatively privileged site because of its unique neuronal composition and function. These measures of barrier function early in the sheep gestation are consistent with results suggesting very precocious barrier differentiation in the human fetal cerebral cortex as described earlier.[46,47]

The decreases in permeability to AIB in the sheep[84] were similar to developmental changes in barrier permeability measured quantitatively using potassium in rats.[85] Permeability to potassium was higher in 21-day fetal than in newborn or adult rats.[85] Taken together, the findings in sheep[84] and in developing rats[85] suggest that from fetal to adult life, barrier permeability to both a cation and small-molecular-weight hydrophilic molecules decreases from fetal to adult life.[84]

The ontogenic decreases in permeability in the sheep[84] also were similar to postnatal reductions in permeability to AIB in rabbits[86] and to inulin, sucrose, mannitol, and urea in rats.[87-90] However, in contrast with the findings in rabbits, which exhibited 80% to 90% postnatal reductions in permeability from day 1 of postnatal life to adulthood, the sheep exhibited 39% to 45% reductions in permeability from day 3 to adulthood.[84] The timing and schedule of brain growth probably are critical to maturation of the blood-brain barrier.[85] Comparison of barrier development among species requires consideration of brain growth differences. The sheep brain develops mostly before birth, the rabbit brain develops perinatally, and the rat brain after birth.[50] The prenatal brain development in the sheep may account for the more modest differences in permeability between newborn lamb and adult sheep.

Permeability and Corticosteroids

Evidence in adult subjects suggests that the blood-brain barrier is under hormonal control.[91-94] In adult rats, adrenalectomy increases blood-brain barrier permeability, and corticosterone replacement reverses this effect on the barrier.[92] These findings suggest that the pituitary-adrenal cortical axis may function as a physiologic regulator of barrier function.[92] It is well known that the pituitary–adrenal cortical axis matures during fetal development and that endogenous cortisol concentrations increase particularly in the latter part of gestation.[95] In sheep, maternally administered exogenous antenatal corticosteroids have been shown to reduce blood-brain permeability early (at 60%, 70%, and 80% of gestation) but not late (at 90% of gestation) in fetal development and in the neonatal period.[96-99] In addition, endogenous increases in plasma cortisol concentrations are associated with decreases in blood-brain barrier permeability during normal fetal development.[98] These findings suggest that the blood-brain barrier is hormonally responsive in the fetus and that corticosteroids are important in regulation of barrier maturation.[98] Furthermore, the age-related differential responsiveness of the fetal barrier to maternally administered corticosteroids probably is attributable to the fact that in early gestation, the barrier is still responsive to exogenous corticosteroids and has not yet been exposed to the endogenous increases in corticosteroids.[98,99] Later in gestation[98] and in the newborn,[99] endogenous increases in cortisol will have already accelerated the maturation of the blood-brain barrier, so it is no longer responsive to the exogenously administered corticosteroids.[98,99] These findings are important because during the perinatal period, when the brain potentially may be exposed to systemic elevations in neurotoxic substances, increases in endogenous cortisol serve to protect the fetal and neonatal brain by reducing its permeability.[98,99]

The site of action of corticosteroids on the blood-brain barrier in the fetus[96-98] is most likely on the capillary endothelial cells, because physiologic hydrocortisone concentrations have been shown to increase transendothelial resistance and to decrease permeability in cerebral capillary endothelial cells.[100] The intercellular tight junctions of cerebrovascular endothelial cells are the probable site of action of corticosteroids on the fetal blood-brain barrier, because corticosteroids in vitro have been shown to up-regulate the cerebrovascular endothelial expression of several tight junction proteins.[101,102]

ALTERATIONS IN BLOOD-BRAIN BARRIER DEVELOPMENT AND FUNCTION: CLINICAL IMPLICATIONS

Disruption of the tight junction seal and alterations in blood-brain barrier function are thought to complicate a variety of neurologic disorders.[13,14,103] It is becoming increasingly apparent that failure of the blood-brain barrier is a critical event in the development and progression of several disorders that affect the central nervous system.[13] In some cases, increased blood-brain barrier permeability is a consequence of pathophysiologic changes, such as with ischemic stroke[104] and traumatic brain injury,[105] whereas in other cases, blood-brain barrier opening may be the precipitating event, such as with multiple sclerosis.[13]

The relatively impermeable blood-brain barrier in the fetus and newborn infant serves to protect the developing brain from insults that could impair neuronal function during fetal life, at delivery, and in postnatal development.[84] Emerging evidence suggests that a number of pathologic states, prenatal-perinatal abnormalities, circulating factors, and treatment strategies may be predisposing factors for blood-brain barrier abnormalities at different stages of development.[106] Elucidation of the mechanisms that underlie alterations in blood-brain barrier function during development can be expected to help suggest strategies to protect the brain in both full-term and preterm infants. The entities summarized next represent just a few of the factors that may potentially influence barrier function or contribute to abnormalities of barrier function during development.

Inflammation and Infection

Systemic and local inflammatory mediators can impair the barrier function of the neurovascular unit.[107] Circulating inflammatory ligands such as lipopolysaccharides, locally produced activated complement proteins, and proinflammatory cytokines such as IL-1 and TNF-α can bind to corresponding receptors on various elements of the neurovascular unit and alter the integrity of the blood-brain barrier.[106,108] The expression and localization of tight junction proteins have also been reported to be disrupted during inflammatory conditions.[109]

Some evidence suggests that proinflammatory cytokines may gain access to the fetal brain after being produced during maternal inflammation or chorioamnionitis.[110] The uterus, fetal circulation, and brain represent three compartments separated by boundaries made up of the placenta and amniotic membranes and the blood-brain barrier.[110] It has been postulated that cytokines originating in the uterus, amniotic fluid, or placenta may enter the fetal circulation, or be produced because of inflammatory conditions within the fetal circulation. As a result, proinflammatory cytokines

originating from each of these sources may potentially damage the fetal blood-brain barrier or gain access to the fetal brain by crossing the barrier. Once the cytokines cross the blood-brain barrier, they may directly damage the brain.[110] These concepts are supported by studies in newborn pigs in which intracarotid injections of tumor necrosis factor-α (TNF-α) resulted in increases in cerebral cortical blood-brain barrier permeability.[111] In addition, a single dose of lipopolysaccharide administered intravenously to fetal sheep has been shown to be associated with an increase in albumin concentration in the brain parenchyma, suggesting that blood-brain barrier permeability may increase after exposure of the fetus to systemic endotoxin.[112]

Microorganisms can cause systemic inflammation and gain direct access into the brain parenchyma by breaching the integrity of the blood-brain barrier.[113-116] K1 type *Escherichia coli, Listeria monocytogenes,* and type III group B streptococci are some of the specific bacterial pathogens that represent the leading causes of severe disease in perinatal medicine. These organisms appear to have the greatest capacity to penetrate the cerebrovascular endothelium and to result in the largest increases in blood-brain barrier permeability, when compared with other organisms.[113-117] The K1 type *Escherichia coli* has been shown to have specific genetic virulence determinants that contribute to brain microvascular endothelial cell adhesion and invasion.[117-122] Moreover, increases in TNF-α in cerebrospinal fluid appear to correlate with increases in barrier permeability in experimental meningitis.[114] Inflammation, systemic infections, and meningitis during the fetal and perinatal period have been shown to seriously interfere with normal central nervous system function and development.[123] Abnormalities of the blood-brain barrier probably are important contributors to the brain injury that is associated with systemic inflammation and infection in premature and full-term infants.

Hypoxia, Ischemia, and Reperfusion Injury

Perinatal hypoxic-ischemic brain damage is a major cause of death associated with high-acuity conditions and chronic neurologic morbidity in infants.[124-126] Blood-brain barrier integrity is compromised and permeability increased as a result of hypoxia and ischemia-reperfusion injury to the central nervous system in adult subjects.[13,127] The expression and localization of various tight junction proteins and of nutrient and electrolyte transporters are disturbed, and the nonspecific transport of blood-borne proteins into the brain is increased under hypoxic or ischemic conditions in the brain.[128,129] The mechanisms that underlie these changes include the direct effects of energy failure resulting from oxygen and metabolite deprivation, damaging effects of oxygen free radicals, cessation of shear blood flow with invasion of the neurovascular unit by static leukocytes adhering to the blood vessel walls, and induction of local inflammatory cascades associated with hypoxic-ischemic injury to the tissues.[130]

The need for cardiopulmonary resuscitation is one of the most common scenarios potentially associated with barrier dysfunction and hypoxic-ischemic brain injury. Blood-brain barrier permeability was investigated in young anesthetized pigs during and after cardiopulmonary resuscitation. After 8 minutes of cardiac arrest followed by either 10 or 40 minutes of continuous sternal compression, no increase was seen in the blood-brain barrier transfer coefficient. However, after 8 minutes of arrest, 6 minutes of cardiopulmonary resuscitation, and 4 hours of spontaneous circulation, the blood-brain barrier transfer coefficient increased by 59% to 107% in 10 of 11 regions rostral to the pons. Thus, after an 8-minute period of complete ischemia, the blood-brain barrier remained intact during and immediately after resuscitation, but not after several hours of recirculation. These findings are in contrast with previous work in adult animals in which no delayed increase in permeability was demonstrated.[131]

In a model of cerebral ischemia-reperfusion injury in newborn piglets, the blood-to-brain transfer of urea, sodium, and sucrose was measured at 30 minutes and again after 2 hours of reperfusion.[132] The blood-to-brain transfer constants for the two smaller-molecular-weight tracers, sodium and urea, increased by 30 minutes after ischemia, followed by a subsequent increase for the largest tracer, sucrose, at 2 hours. The increase in blood-to-brain transfer of these small-molecular-weight tracers probably is the result of the opening of water-filled channels or "pores" at the blood-brain barrier, associated with size-dependent changes in tracer influx into the brain. These findings in neonatal piglets are consistent with the effects of hypoxia-ischemia in adults—namely, formation of a paracellular leak across the blood-brain barrier with a pore diameter, which increases with time and the severity of the insult.[132-134] In contrast with the findings just described, the barrier was found to be relatively impermeable to sodium and mannitol in newborn piglets exposed to 60 minutes of systemic hypoxia and hypercapnia and 20 minutes of hypotension, either immediately after the insult or 24 hours later.[134] Taken together, these studies suggest that the amount of damage to the barrier in the newborn probably relates to the duration and severity of the insult, because in the latter study, the brain remained relatively well perfused even though hypoxia and hypercarbia rendered brain blood flow pressure-passive and the mean arterial pressure was reduced below the lower limit of autoregulation for normal piglets.[134] On the other hand, it is likely that global ischemia in the first study resulted in complete tissue ischemia and anoxia, with a consequently relatively more severe insult.[132]

Decreased nutrient supply to neuronal tissue is a major cause of energy failure and subsequent tissue damage after hypoxia or ischemia. Studies in experimental animals and humans indicate that cerebral glucose utilization initially is low in early gestation and increases with maturation. The expression of glucose transporter proteins at the blood-brain barrier and on neuronal and nonneuronal elements in the brain parenchyma shows similar maturational patterns.[124] During nutrient deprivation, alternate energy fuels such as ketone bodies and lactate substitute for glucose, thereby protecting the immature brain from hypoglycemia.[124] These findings suggest that the immature brain may be better able to withstand episodes of decreased nutrient supply during hypoxic-ischemic episodes, because the immature blood-brain barrier may be more efficient than that in the adult at the transport of certain alternative nutrients.[135-137]

Hyperbilirubinemia

Hyperbilirubinemia has been associated with acute bilirubin encephalopathy and kernicterus in both preterm and term infants.[138] Recent evidence indicates that the unbound fraction of bilirubin in the serum is more directly related to bilirubin neurotoxicity than are total bilirubin concentrations.[139] These findings suggest that the higher the level of unbound bilirubin in the serum, the more likely it is for bilirubin to cross the blood-brain barrier and cause bilirubin-related neurotoxicity. At any given unbound serum bilirubin concentration, conditions that decrease blood-brain permeability to bilirubin are less likely to be associated with bilirubin-related neurotoxicity.

Experimental evidence has shown that hyperbilirubinemia induced by infusions of comparable amounts of bilirubin correlated with higher brain bilirubin content and higher brain-to-blood distribution ratios for bilirubin in 2-day-old than in 2-week-old piglets. These ratios were higher in subcortical regions—namely, the cerebellum and brain stem—than in the cerebral cortex.[140] The results of this study suggest that a postnatal maturation of the barrier for unbound bilirubin is likely, and that the permeability for bilirubin is relatively greater in subcortical than in cortical

regions of the developing brain.[139-141] These maturational changes in the permeability to bilirubin during development serve to protect the more mature brain from bilirubin toxicity. They also provide a basis for the clinical observation that newborn infants are at a higher risk for kernicterus at lower bilirubin levels in the first few hours after birth than later in postnatal life.[142]

Conversely, conditions that adversely affect blood-brain permeability to unbound bilirubin could be associated with higher risks for bilirubin-related neurotoxicity. Disruption of the blood-brain barrier by inflammation, or hypoxia–ischemia also could facilitate transport of bilirubin into brain.[143] Premature infants frequently are exposed to systemic fetal and neonatal inflammation, and to recurrent episodes of hypoxia and ischemia-reperfusion. These insults, combined with immaturity of the blood-brain barrier for bilirubin, places very-early-gestation preterm and late-preterm infants at a higher risk for bilirubin-related neurotoxicity compared with full-term newborns.[144] Elucidation of the role of the blood-brain barrier in relationship to the pathogenesis of kernicterus can be expected to help improve assessments of the risks of preterm and full-term infants for bilirubin-induced neurotoxicity.[145]

Hyperosmolar Stress

The phenomenon of hyperosmotic opening of the blood-brain barrier has been investigated extensively in adulthood.[146] Hypertonic solutions can open the barrier by shrinking endothelial cells and widening the intercellular space that normally is sealed by tight junctions. The degree of barrier opening varies with the concentration of the solute and the type of hyperosmotic solution used. Experiments by Cserr and co-workers[147] showed barrier opening with degrees of hyperosmolality comparable to those encountered clinically in sick premature infants.[147-150] More recently, Stonestreet and associates examined the effects of a hyperosmolar stress on blood-brain barrier function measured quantitatively during the last 2 months of fetal development and in the first month of postnatal life in sheep. Their results demonstrated that blood-brain barrier permeability increases with increases in plasma osmolality in fetal sheep and lambs.[151] These experiments also demonstrated that the barrier became more resistant to the effects of hyperosmotic stress during development. The findings suggest that the blood-brain barrier may be more vulnerable to the effects of osmotic stress in the immature fetus than in older subjects.[151]

Intraventricular Hemorrhage

Infants born prematurely are at an increased risk for intraventricular and intraparenchymal hemorrhage, which adversely affects their neurodevelopmental outcomes.[44,152] Extravasation of blood normally contained within the cerebral intravascular space into the parenchymal–ventricular space represents complete breakdown of the blood-brain barrier. Hence, understanding the pathophysiology of impaired blood-brain barrier function is of vital importance in perinatal medicine because premature infants are at high risk for brain hemorrhages.

Germinal matrix hemorrhage progressing to intraventricular or intraparenchymal hemorrhage is the prototype of intracranial hemorrhage encountered in preterm infants.[153,154] Germinal matrix capillaries have large diameters and thin basement membranes and lack direct contact with perivascular structures along a large proportion of their circumference, in contrast with capillaries in the cerebral cortex at same gestational age.[155] The blood vessel density and the percentage of blood vessel area are larger in the germinal matrix vessels than in those in the adjacent gray and white matter in autopsy material from fetuses and premature infants born between 16 and 40 weeks gestation, potentially accounting for propensity for hemorrhage in the germinal matrix.[73] As described previously, controversy exists regarding the structural elements of endothelial tight junctional complexes in the germinal matrix vasculature during development.[44-47] Two reports appear to suggest that the tight junction proteins continue to accumulate over the interval during which premature infants are at highest risk for intraventricular hemorrhage.[44,46] On the other hand, a third study suggested that because the endothelial tight junction molecules were not decreased in germinal matrix vasculature relative to the adjacent gray and white matter, a paucity of these proteins was not likely to be responsible for the fragility of the germinal matrix vasculature and not likely to account for the propensity for hemorrhage in premature infants.[45] Hence the contribution of the tight junction proteins to the structural integrity of the germinal matrix warrants further consideration.

The pericyte is another important component of the neurovascular unit, which functions to enhance the integrity of the blood-brain barrier and to provide structural stability to blood vessels.[36,39,156] The germinal matrix blood vessels have been shown to possess fewer pericytes than the vasculature of the other adjacent brain regions.[156] These findings suggest that a paucity of pericyte coverage of the germinal matrix vasculature may contribute to its fragility and increase its vulnerability to hemorrhage.[156]

Maternal treatment with antenatal corticosteroids has been associated with a decreased incidence of intraventricular hemorrhage in premature infants.[157] As described previously, maternal antenatal corticosteroid administration also results in decreased permeability of the blood-brain barrier early in ovine gestation.[96-98] Taken together, the decreased incidence of intraventricular hemorrhage in premature infants after maternal corticosteroid treatment[157] and the decreased permeability of the blood-brain barrier in lamb fetuses after corticosteroid treatment of the ewes[96-98] suggest that corticosteroids may accelerate microvascular maturation, with a beneficial effect on blood-brain barrier function in the human fetus and premature infant. In addition, endogenous changes in corticosteroids also may account for accelerated maturation of the endothelial vasculature in the fetus and premature infant.[98] Although the contributions of specific alterations in the components of the neurovascular unit to the development of germinal matrix–intraventricular hemorrhage in premature infants still have not been entirely elucidated, alterations in components of neurovascular units, such as the pericyte,[156] probably contribute to the fragility of the germinal matrix vasculature. Moreover, it remains likely that alterations in barrier function in premature infants other than hemorrhage may contribute to brain injury such as that resulting in periventricular leukomalacia, mental retardation, or cerebral palsy.

Mechanical Ventilation

Among premature infants exposed to prolonged ventilation, rates of neurodevelopmental impairment are very high.[158] Abnormalities in the blood-brain barrier may potentially contribute to the associated brain injury by allowing circulating toxic substances greater access to the brain parenchyma. Previous work has shown that elevation in mean airway pressure during positive-pressure ventilation in newborn piglets selectively alters blood-to-brain transport of small water-soluble molecules.[159] It appears likely, therefore, that increased cerebral venous pressures during ventilation cause the increases in the blood-to-brain transport of these small-molecular-weight tracers.[159] Increases in the duration of positive-pressure ventilation also predispose premature lambs to increases in regional blood-brain barrier permeability measured using a small-molecular-weight tracer (i.e.,AIB).These alterations in barrier function were shown to occur over relatively short time intervals (minutes to hours) and were not a result of changes in mean airway pressure.[160] Therefore both increases in mean airway pressure and increases in the duration of ventilation

potentially contribute to altered blood-to-brain transport, which may adversely affect neuronal function during ventilation.[159,160]

SUMMARY

The blood-brain barrier, a complex and dynamic barrier at the level of the microvascular endothelium, has a critical role in maintaining the health and function of the brain. The tight junctional complex plays an essential role in regulating paracellular permeability and can be affected in a variety of perinatal disorders and related therapies. The neurovascular unit provides an interesting model for understanding how the endothelium, astrocytes, neurons, pericytes, and extracellular matrix function together to form a protective barrier for the brain. The development of the blood-brain barrier is an important area for inquiry with a significant impact on the neurodevelopmental outcomes of preterm and full-term infants. Further understanding of this important barrier may lead to identification of clinically useful neuroprotective strategies during development.

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Michael V. Johnston

Development of Neurotransmitters

The function of the immature brain is determined to a considerable extent by the operational state of the specialized chemical synaptic connections between neurons.[1] The capacity of immature synapses to transfer information determines functions as diverse as hormone release, breathing, and vision. Advances in analyzing and understanding the machinery of chemical neurotransmission are providing a more complete picture of the dynamic events that take place in the fetus and neonate.[2,3] Conventional biochemical assays for synaptic activities as well as methods with greater spatial resolution (e.g., receptor autoradiography) have been used to assess development of neurotransmitter pathways. These studies indicate that biochemical

features of neurotransmission are often markedly different in the immature brain compared with those in the adult brain. Distinct neurotransmitter-specific neuronal groups mature at different rates, so multineuronal networks may have a different functional "balance" at different ages.[2] Moreover, although some components of the synapse, such as neurotransmitter synthetic enzymes, are relatively undeveloped or inactive in the fetus and neonate, others, including certain neurotransmitter receptors and second-messenger systems, are expressed at much higher levels than in adults.[4,5] These developmental patterns are relevant to understanding normal physiology as well as the effects of disease in the immature brain.

Chemical transfer of messages across synapses depends on coordinated function of several biochemical processes (Figure 162-1). A neurotransmitter is synthesized by specific enzymes within nerve terminals and may be stored (e.g., catecholamines) in synaptic vesicles awaiting impulse-stimulated release. Specific biochemical uptake processes or "pumps" within axonal nerve terminals may remove transmitter released from the synaptic cleft and may prevent it from having prolonged contact with postsynaptic receptors. Similar uptake processes remove portions of degraded neurotransmitter. For example, acetylcholine is hydrolyzed to yield choline, which is transported into the cholinergic nerve terminal by a high-affinity, sodium-dependent uptake process. Stimulation of neurotransmitter release from axons and reuptake is coordinated with electrical impulse flow. In certain groups of synapses, catalytic enzymes are located within the nerve terminal and on the surface of synaptic endings to degrade released neurotransmitter. Catecholamines are degraded by the cytoplasmic enzyme monoamine oxidase and by the enzyme catechol-*O*-methyl transferase.

Actions of neurotransmitters are expressed in postsynaptic neurons through specific neurotransmitter receptors. These receptors are generally linked to ionophores (ion channels) or second-messenger systems, or both. Stimulation of receptors linked to ion channels generally produces a change in the membrane potential, such as depolarization in the case of excitatory neurotransmitters (e.g., glutamate) or hyperpolarization produced by inhibitory neurotransmitters such as γ-aminobutyric acid (GABA). Second-messenger systems may stimulate a cascade of intracellular events such as protein phosphorylation or modulation of gene expression. The relative influence of a neurotransmitter pathway during development is determined by the sum of these processes, and differential rates of development in each of them modify the relative influence of pathways markedly at different developmental stages. The separate biochemical processes involved in neurotransmission in a single neuronal group may develop at different rates, and these altered relationships (e.g., among transmitter synthesis, release, and reuptake) determine the pathway's influence.[2]

EARLY BIRTH AND DEVELOPMENT OF THE BRAIN STEM NORADRENERGIC PROJECTION TO CEREBRAL CORTEX

Norepinephrine-containing axons project into the cerebral cortex and other parts of the forebrain from the locus ceruleus in the pons.[6] The noradrenergic axonal network within cerebral cortex is thought to be involved in cortical activation and attention, and axons projected into the diencephalon influence neuroendocrine function. The cell bodies in this pathway are among the earliest to undergo neurogenesis in the developing brain.[7] This finding is consistent with the general pattern that neurons originating in the brain stem are "born" or originate before those in more rostral locations. The cell bodies of this projection originate on gestational days 12 to 14 (total gestational period of 21 days) in rats and on gestational days 27 to 36 in monkeys (total 165 days' gestation).[7] The noradrenergic projection appears to be influential in the immature cortex. In the neonatal rat, approximately one third of all cortical synapses possess

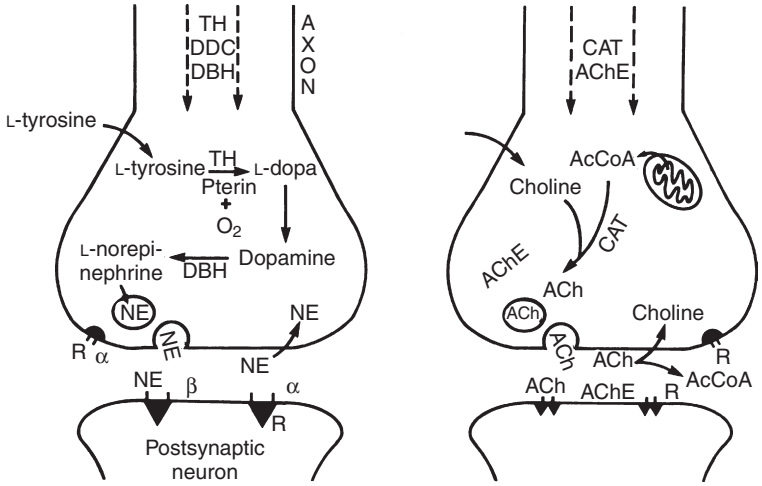


Figure 162-1 Schematic diagrams connected to an axon *(top)* projected from a noradrenergic cell body in the brain stem. The key biochemical markers, which are relatively specific for noradrenergic nerve terminals, are tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH), the biosynthetic enzymes, endogenous norepinephrine (NE) content, and [^{3}H]NE uptake in vitro into sealed-off nerve endings (synaptosomes). The high-affinity uptake pump is shown by the *arrow* directed from the synaptic cleft into the presynaptic terminal. DBH is found only in noradrenergic axons, whereas TH is also found in dopamine nerve terminals. The cortical distributions of noradrenergic and dopamine axons are relatively distinct. β-Adrenergic receptors are found predominantly postsynaptically, whereas α-adrenergic receptors (R) are localized presynaptically and postsynaptically. On the *right,* an acetylcholine synapse is diagrammed, indicating one of the primary markers, choline acetyltransferase (ChAT), the enzyme that synthesizes acetylcholine from acetyl coenzyme A (AcCoA) and choline. Endogenous acetylcholine and [^{3}H]choline uptake into synaptosomes are also specific markers. Acetylcholinesterase (AChE) is relatively specifically localized in cholinergic cell bodies in regions such as the basal forebrain but may be nonspecific in other regions, including the substantia nigra, and in intrinsic cortical neurons. Muscarinic and nicotinic acetylcholine receptors (R) are located presynaptically and postsynaptically. *DDC,* L-Dopa decarboxylase.

an axonal uptake mechanism for monoamines, a proportion that far exceeds the density in the adult.[8] Some experimental work supports the proposal that noradrenergic axons exert a trophic effect on the developing cortex in fetal and early postnatal life. Development of the visual cortex may be regulated by norepinephrine in the "critical period" of visual plasticity.[9]

Development of the noradrenergic system in cortex has been examined by measuring endogenous levels of the neurotransmitter norepinephrine, the energy-dependent, high-affinity uptake of [^{3}H]norepinephrine into pinched-off nerve endings (synaptosomes), and the activity of the neurotransmitter synthetic enzymes tyrosine hydroxylase and dopamine β-hydroxylase during development.[6,10-13] Results of these studies have been compared in rodents and subhuman primates. When biochemical markers for this neurotransmitter system are studied during development (Figure 162-2), investigators have noted that the ability of presynaptic nerve terminals to take up [^{3}H]norepinephrine develops slightly before endogenous levels of neurotransmitters and synthetic enzyme activities rise.

Synaptic effects of norepinephrine are expressed at postsynaptic β-adrenergic and α-adrenergic receptors and at presynaptic α-receptors.[3,14,15] Development of these receptors and of associated second-messenger effects, such as norepinephrine-stimulated cyclic adenosine monophosphate (cAMP) production and phosphoinositide (PI) hydrolysis, increases rapidly in the postnatal period, as shown in Figure 162-2.[4,16] In vitro receptor autoradiography has been used to visualize the location and density of these receptors, and these studies have identified specific laminar and regional differences in their development.[3,14,17]

DOPAMINE INNERVATION

Dopamine, the other major catecholamine in the brain, is contained within neuronal projections extending from the substantia nigra in the midbrain into the basal ganglia, the hypothalamus, and restricted portions of the cerebral cortex such as the cingulum. The neurons from which these projections originate also arise relatively early, at gestational days 13 to 15 in rats and at fetal days 36 to 43 in monkeys.[7] Histofluorescence for dopamine and serotonin has been found in human fetuses as early as 3 months' gestation.[18] The biochemical markers for these axons—the dopamine reuptake system in synaptosomes, the synthetic activity of tyrosine hydroxylase, and endogenous neurotransmitter stores—undergo almost all their maturation in the postnatal period.[1,19] Use of histofluorescence to examine dopamine in the basal ganglia of postnatal animals indicates an early pattern of dopamine islands.[20,21] These islands persist as specialized groupings of axons in the adult brain, although the overall pattern of dopamine is more confluent at that stage. In the adult brain, the specialized compartments extend throughout the anteroposterior extent to form a lamellar or "striasomal" pattern. In rodent studies the turnover of dopamine in the developing brain appears to be relatively high in the postnatal period and exceeds that in the adult. The ratios of homovanillic acid (HVA) and dihydroxyphenylacetic acid, which are products of dopamine oxidation, to endogenous dopamine levels are higher in the brain of immature rodents than in adults. This estimate of turnover suggests that dopamine neurons are active in the early postnatal period.

SEROTONIN PATHWAYS

Serotonin axons emerge from the raphe nuclei in the pons and resemble the widespread projections made by noradrenergic neurons into the forebrain and spinal cord. Serotonin pathways are important for control of hypothalamic and pituitary functions, pain, sleep, appetite, and other behavioral functions. The raphe serotonin neurons also undergo early cell division, with the timing similar to that of catecholamine neurons. Biochemical studies of serotonin turnover indicate that it is relatively high in the neonatal period in rodents, a finding suggesting early establishment of active neuronal impulse flow.[22] In rodents, maturation of serotonergic biochemical markers follows a caudal to rostral gradient with brain stem levels at two thirds of adult levels at birth and hemisphere activity at 20% of adult levels. In the neonatal rodent, a transient, dense serotonin innervation has been found using an immunohistochemical technique to localize serotonin and autoradiography to image serotonin uptake sites on nerve terminals.[23] In somatosensory cortex, dense patches of serotonin innervation are aligned with specialized cellular aggregates, called *barrels,* before 3 weeks of age. Afterward, the patchy areas of hyperinnervation become more uniform and confluent. The ontogeny of serotonin receptors has been studied in the developing brain, and distinct laminar patterns have been identified.[15] These observations suggest that the pattern of serotonin innervation shares features in common with catecholamine projections from the brain stem. Both systems undergo early neurogenesis and appear

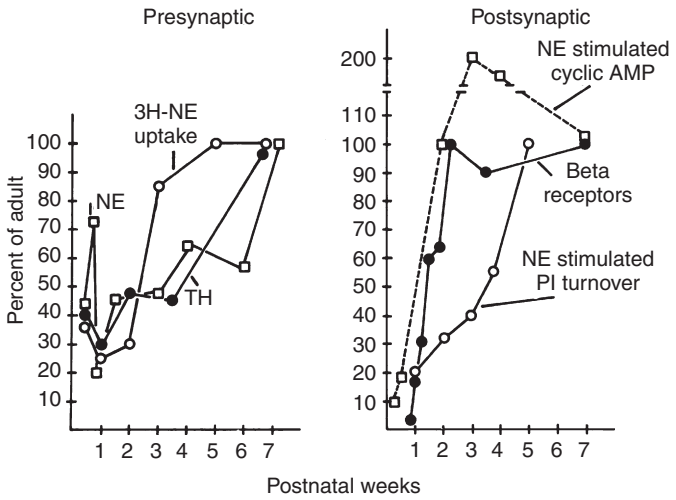


Figure 162-2 Ontogeny of biochemical markers for noradrenergic axons in rodent cerebral cortex during postnatal development. Data for the *left panel* are on presynaptic markers in sensorimotor cortex. The *right panel* shows selected receptor and receptor-stimulated second-messenger activities. *AMP,* Adenosine monophosphate; *NE,* norepinephrine. *(From Johnston MV: Biochemistry of neurotransmitters in cortical development. In Peters A, Jones EG [eds]:* Cerebral Cortex, *New York, 1988, Plenum Press, pp 211-236.)*

to be relatively more densely represented in the neonatal period than later. The axonal arbors of both systems reorganize during postnatal development, and both may influence cortical plasticity. Abnormalities in serotonin metabolism in cerebral cortex have been described in children with autism using positron emission tomography (PET).[24] Drugs that inhibit the reuptake of serotonin into synapses, which are used widely to treat depression, may produce a withdrawal syndrome as well as increased incidence of congenital malformations when used in pregnancy.[25]

DEVELOPMENT OF CEREBROSPINAL FLUID LEVELS OF DOPAMINE AND SEROTONIN METABOLITES IN HUMAN INFANTS

Measurement of levels of cerebrospinal fluid (CSF) metabolites of dopamine and serotonin provides an estimate of functional activity of central dopaminergic and serotonergic neurons in humans. The brain is the major site of HVA synthesis, and although up to 30% of the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA), is produced in the spinal cord, the neurons of origin are in the brain stem. A few studies have examined the maturation of catecholamine and serotonin pathways in the perinatal period.[26,27] In one study of neonates, 35 male and 22 female infants (gestational ages ranging from 28 to 42 weeks) were evaluated.[26] They included 38 premature infants (36 weeks or less) and 19 term infants. Common medical problems in these patients included prematurity, asphyxia, respiratory distress, and infection. Examination of the data, however, did not reveal any relationship of these conditions with the monoamine concentrations. The infants were initially grouped into four age categories for analysis: (1) young premature infants less than 32 weeks gestational age, (2) premature infants 33 to 36 weeks gestational age, (3) infants 37 to 42 weeks gestational age (full term), and (4) older infants up to 6 months of age (Table 162-1). HVA levels increased progressively with maturity ($p < .05$; analysis of variance). Although mean HVA values in girls were slightly higher than those in boys in each age-group, these differences were not statistically significant. In contrast to HVA, 5-HIAA levels were similar in these age-groups, and there were no significant sex differences. Mean 5-HIAA values in all groups of infants were considerably higher than the values in older children.[27]

In Figure 162-3, mean HVA/5-HIAA ratios are plotted for the three groups of neonates and the older infants. The mean HVA/5-HIAA ratio in the most immature infants (28 to 32 weeks gestation) was 1.0 ± 0.12 (mean ± SEM), whereas in the most mature group (1 week to 6 months old), it was 1.98 ± 0.17 ($p < .01$, comparing these two groups by the Student *t*-test). HVA concentrations increased over the period from the last trimester of pregnancy into early infancy, whereas 5-HIAA levels were relatively stable. Thus the HVA/5-HIAA ratio changes markedly over this period. The data suggest that the maturation of human dopaminergic neuronal activity lags behind that of serotonin neurons in the perinatal period.

TABLE 162-1

Homovanillic Acid and 5-Hydroxyindoleacetic Acid Levels in Cerebrospinal Fluid*

COMPARES PREMATURE AND TERM NEONATES AND OLDER INFANTS			
	n	5-HIAA (ng/mL)	HVA (ng/mL)
Group 1, premature neonates	38	96 ± 10	106 ± 10
Group 2, term neonates	19	104 ± 7	129 ± 11
Group 3, infants	13	85 ± 7	159 ± 9†

From Silverstein FS, Donn S, Buchanan K, Johnston MV. Concentrations of homovanillic acid and 5-hydroxyindoleacetic acid in cerebrospinal fluid from human infants in the perinatal period. *J Neurochem* 43:1769, 1984.

*Premature neonates range in gestational age from 28 to 36 weeks; term neonates from 37 to 42 weeks. Infants were 1 to 6 months of age. Values are mean ± SEM.

†$p < .05$ using analysis of variance to compare all HVA values in the three groups.

5-HIAA, 5-Hydroxyindoleacetic acid; *HVA,* homovanillic acid.

ACETYLCHOLINE PATHWAYS

Acetylcholine serves a variety of important functions in the brain. A major projection from the basal forebrain lateral to the hypothalamus into the cerebral cortex is believed to have a role in cortical activation, learning, memory, and cortical plasticity.[28] Cholinergic circuits in the brain stem and the diencephalon influence autonomic functions and form part of the control mechanisms for temperature regulation and hormonal release. In the basal ganglia, cholinergic circuits counterbalance the effects of dopamine pathways and serve an important role in controlling motor tone and movement.[19,21]

The developmental profile of cholinergic neurons has been studied by assaying postnatal endogenous brain concentrations of acetylcholine, [^{3}H]choline uptake into synaptosomes, and the synthetic activity of choline acetyltransferase (ChAT).[11,16,29,30] In the cerebral cortex of rodents, cholinergic markers are present at fairly low levels over the first few postnatal days, and they rise with a sigmoidal pattern during the first postnatal weeks to maturity (Figure 162-4). Labeling studies with [^{3}H]thymidine autoradiography in rodents indicate that the cell bodies from which this pathway originates in the basal forebrain develop on gestational days 12 to 15.[2,31] Therefore the neurons that form the neocortical cholinergic system originate relatively early but differentiate over a more prolonged period than do catecholamine systems.

In the human brain, the nucleus basalis of Meynert in the basal forebrain develops intense acetylcholinesterase (AChE) activity between 12 and 22 weeks gestation.[31] ChAT activity in human cerebral cortex rises after a gradual developmental pattern during the postnatal period that reaches nearly adult levels in adolescence.[32,33] Histochemical localization of the catabolic enzyme AChE is a marker for cholinergic nerve terminals

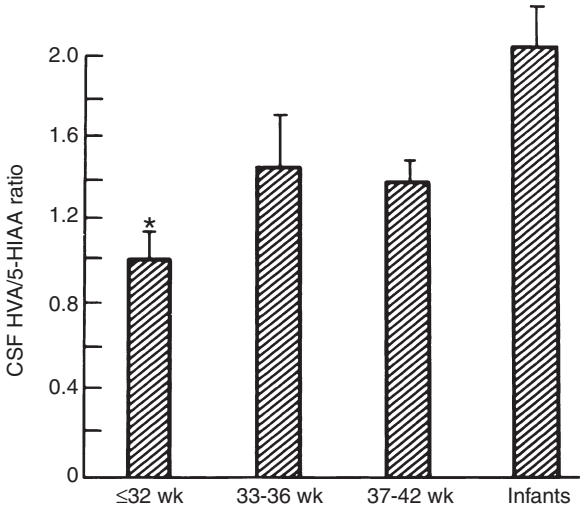


Figure 162-3 Cerebrospinal fluid (CSF) neurotransmitter metabolites in human newborns (32 weeks or less [$n = 12$], 33 to 36 weeks [$n = 26$], 37 weeks or more [$n = 19$]), and infants 1 to 6 months old [$n = 13$]; the *asterisk* indicates $p < .01$, comparing the most immature neonates with the infants using Student *t*-test. *5-HIAA,* 5-Hydroxyindoleactic acid; *HVA,* homovanillic acid. *(From Silverstein FS, Donn S, Buchanan K, Johnston MV,* J Neurochem *43:1769, 1984.)*

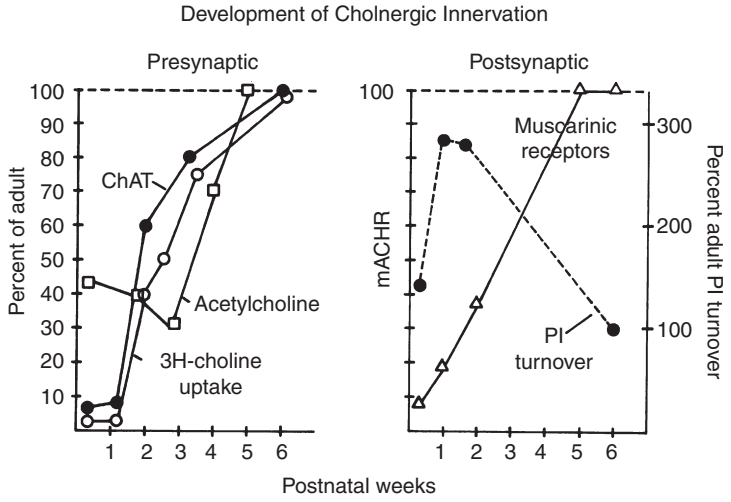


Figure 162-4 Postnatal development of presynaptic and postsynaptic markers for cholinergic synapses in rat neocortex. *Left*, Data for choline acetyltransferase (ChAT), sodium-dependent [^{3}H]choline uptake into synaptosomes, and endogenous acetylcholine are from references 11 and 26. The cortical region assayed was the dorsolateral sensorimotor cortex. *Right,* Data on muscarinic receptors are from references 16 and 26. The ordinate at *left* is for muscarinic receptors; at the *right,* it is phosphatidylinositol (PI) turnover. *mACHR,* Muscarinic acetylcholine receptors.

in cerebral cortex, and this method has been used to examine human postmortem tissue. Kostovic and co-workers[34] found that laminar preferences in AChE staining changed dramatically in the human frontal cortex during the first postnatal year. AChE reactivity of layer three pyramidal cell bodies and the surrounding fibrillar network begins to develop after the first postnatal year and increases gradually to its peak intensity in young adults. Studies of ChAT activity and AChE histochemical features point to the coordinated development of cholinergic pathways in the brain with the relatively prolonged development of cognitive skills in humans.

Muscarinic cholinergic receptors that transmit most of the postsynaptic effects of acetylcholine increase in number during postnatal development in rodents, in concert with the development of nerve terminals.[29] In the adult cerebral cortex a distinct laminar gradient is formed, with more superficial regions relatively enriched.[17] Shaw and associates[17] found that in cats, the laminar gradient is reversed in the postnatal period, with deep layers heavily labeled. During maturation the receptors progressively reorganize into the adult pattern. Human infant neocortex also demonstrates the inversion of the adult pattern of receptor development noted in kitten cortex. In contrast to the rodent, however, muscarinic receptors in human postmortem neonatal cortex are present at a higher density than in the adult.[3]

One population of muscarinic receptors is coupled to stimulation of the PI turnover second-messenger system.[16] Despite the relatively low levels of muscarinic acetylcholine receptors present in the early postnatal period, these receptors are able to stimulate a vigorous PI turnover response. The magnitude of the PI response stimulated by the muscarinic agonist carbachol increases to a level twice that in the adult at postnatal day 7 in rodents, a finding suggesting that the muscarinic receptors are more tightly coupled to phospholipase C at this age than in the adult. Although presynaptic markers for cholinergic axons and muscarinic receptors are less abundant in the immature brain, enhancement of muscarinic-stimulated second-messenger responses may serve to compensate for this. Nicotinic cholinergic receptors also appear to be important in brain development, and transgenic mice with deletions of the β_2-subunit of this receptor are hyperactive.[35]

INHIBITORY GABA-CONTAINING NEURONS

GABA is the predominant inhibitory neurotransmitter in the brain, but it is paradoxically excitatory during fetal life and in the early postnatal period in animal models.[36] Important populations of GABA-containing neurons are found in cerebral cortex, in which they are predominantly intrinsic aspinous stellate interneurons, and in the striatonigral projection in the brain stem. GABA-containing neurons are born in the ganglionic eminence of the ventral forebrain, with neurons destined for the cerebral cortex born in the medial ganglionic eminence and those destined for the basal ganglia originating in the lateral ganglionic eminence.[37] Unlike the predominantly excitatory pyramidal neurons of cerebral cortex that are born in the dorsal forebrain and migrate to their permanent locations radially from the ventricular zone, inhibitory GABA-containing neurons migrate through the cortex following tangential pathways.[37] Development of GABAergic synapses generally precedes the development of excitatory glutamate synapses.[36]

Judged by biochemical assays, GABA-containing synapses reach maturity in rodents at a time midway between the early-developing catecholamine neurons and the later-developing cholinergic systems.[2] The activity of the synthetic enzyme for GABA, L-glutamate decarboxylase, is low in newborn rodent cortex but rises rapidly to achieve adult levels by 3 weeks of age.[38] Maturation of sodium-dependent, high-affinity uptake of [^{3}H]GABA into synaptosomes exhibits a peak at greater than adult levels in cortex at about 3 weeks of age and remains elevated until about 8 weeks of age.[38] The concentration of the neurotransmitter amino acid GABA has already reached approximately 50% of adult levels in neonatal cortex and is at adult levels by 2 weeks of age.

Neurotransmitter receptors for GABA are linked to receptors for benzodiazepine-type drugs, and together they regulate the opening of chloride channels. Stimulation of this ionophore hyperpolarizes postsynaptic neurons in older animals, but depolarizes neurons in the early postnatal period.[36] This occurs because the Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) is active in the immature brain and raises the intraneuronal concentration of Cl^-. Opening of chloride channels then leads to depolarization rather than hyperpolarization.[39] The diuretic and NKCC1 blocker

bumetanide is anticonvulsant in immature rodents.[39] Neurotransmitter-binding studies showed that benzodiazepine receptors are found at relatively high levels in the neonate, and in rodents, levels are almost half the adult level.[40] Significant levels of benzodiazepine receptors have been observed in the human newborn and infant hypothalamus.[41] Binding sites for GABA are present at relatively low concentrations at birth and increase slowly in the postnatal period. Studies of postnatal maturation of $GABA_A$ receptors in children using PET and [^{11}C]flumazenil indicate that the density is 50% higher in cerebral cortex at 2 years of age than in adults, a finding suggesting age-related pruning in synaptic density with age.[42]

NEUROPEPTIDE-CONTAINING NEURONS

Neuropeptide-containing neurons comprise an important, relatively precocious segment of brain circuitry, which is important for a variety of functions in the fetus and newborn. Various neuropeptide-containing neurons found in the brain have been studied from a developmental perspective, mostly in rodents. Neurons containing enkephalin, other opiate peptides, substance P, and thyroid-releasing hormone have been observed in neurons using immunohistochemical techniques in early fetal life in both rodents and humans. Neurons containing cholecystokinin and vasoactive intestinal polypeptide comprise important intrinsic groups of neurons.[43-46] In cerebral cortex, these peptides most often coexist with GABA. In rodents, endogenous levels of cholecystokinin, somatostatin, and vasoactive intestinal polypeptide increase relatively slowly to adult levels over the first 7 postnatal weeks.[43-46] In contrast, endogenous levels of substance P reach the adult concentration in the neonatal period and by 3 weeks of age rise to more than three times that level.[2] Opiate systems appear relatively mature in the neonatal period. Metenkephalin-containing neurons and μ-opiate receptors are found in rat entorhinal cortex and hippocampus in neonates. [^{3}H]Naloxone-receptor binding in newborn rhesus monkeys demonstrates an adult-like pattern in subcortical structures. Much more remains to be learned about the development of these pathways, especially as they relate to important neuroendocrine function and control of breathing.

EXCITATORY AMINO ACID PATHWAYS

Pathways that use excitatory amino acids are prevalent in the brain and appear to convey information in many intracortical, primary sensory, and cortical projection pathways. For example, the pathways from the cortex into the basal ganglia (corticostriatal pathways), the corticospinal pathways, and the pathways from the eyes to the visual cortex appear to use the excitatory amino acid glutamate or a closely analogous compound as their primary neurotransmitter.[2,46-51] Electrophysiologic effects of visual stimuli in cerebral cortex are blocked by specific antagonists for glutamate receptors.[51]

In cerebral cortex, high-affinity, sodium-dependent [^{3}H]glutamate synaptosomal uptake into nerve terminals has been used to study development of the visual pathways.[46] [^{3}H]Glutamate uptake, which reflects development of presynaptic nerve terminals in visual cortex of developing rats, increases continuously from birth and reaches adult levels by postnatal day 15 (Figure 162-5).

Glutamate receptors have been classified into three broad groups determined by their responses to one of three synthetic agonist compounds: *N*-methyl-D-aspartate (NMDA), α-amino3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and metabotropic receptors (Figure 162-6). NMDA and AMPA receptors are linked to ion channels that can admit calcium or sodium, or both, to the neuronal cytoplasm. Ionic receptors that respond to kainic acid are special types of AMPA receptors. Metabotropic receptors

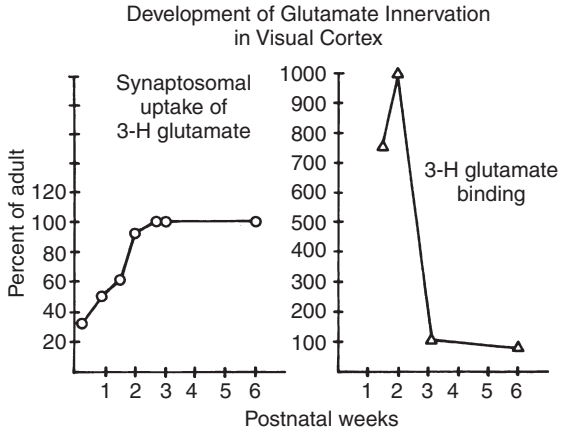


Figure 162-5 *Left,* Ontogeny of sodium-dependent synaptosomal uptake activity for [^{3}H]glutamate in synaptosomes prepared from visual cortex of the rat.[46] *Right,* Ontogeny of sodium-independent binding of [^{3}H]glutamate to visual cortex membranes.[49] Although [^{3}H]glutamate uptake approaches adult levels in the third week, total glutamate binding is 10-fold higher than adult levels. This period includes the critical period for cortical visual plasticity.

stimulated by glutamate regulate levels of the second-messenger molecules cAMP, the polyphosphoinositide IP_3, and diacylglycerol. AMPA receptors mostly mediate the "fast" excitatory activity in the nervous system, whereas the other receptors are reserved for more specialized functions. The NMDA receptor channel (Figure 162-7) opens only when glutamate and glycine stimulate the receptor at the same time that the surrounding membrane potential drops in response to opening of AMPA channels. Under normal circumstances, NMDA receptor–mediated channel opening is reserved for exceptional levels of stimulation mediated by converging stimuli, such as are thought to occur during learning, memory, and other forms of plasticity.

In contrast to the slow rise in biochemical markers for presynaptic, glutamate-containing axon terminals, expression of glutamate receptors and of second-messenger systems stimulated by glutamate is enhanced in the perinatal brain.[4,5,47-52] The ontogenetic profile of glutamate binding in rodent visual cortex has been shown to rise approximately 10-fold higher than the adult level.[49] After day 15 a marked decline in total [^{3}H]glutamate binding corresponds temporally to the pruning of functional synaptic contacts and the time of eye opening and commencement of activity in visual pathways. A similar overshoot in glutamate binding has been observed in developmental studies of the rat hippocampus and human infant temporal cortex.[5,53,54] Receptors that make up the NMDA receptor channel complex are overexpressed in the postnatal period.[53] In addition, molecular genetic studies of NMDA receptor subunits indicate that the types of receptor proteins suppressed in the immature brain create receptors and channels that open more easily than adult channels.[55] This finding corresponds to electrophysiologic studies in the kitten visual system and indicates that stimulation mediated by NMDA receptors is enhanced compared with adult cats.[50]

Binding to non–NMDA-preferring [^{3}H]glutamate sites is also markedly increased in several areas of the immature rodent and human brain in the postnatal period. In the globus pallidus and basal forebrain of the rodent brain, a marked increase in quisqualate-displaceable [^{3}H]glutamate binding in the perinatal period declines to relatively low levels in maturity.[55] Similar areas of transiently increased binding are also seen in areas of human fetal brain. Stimulation of PI turnover, the second-messenger

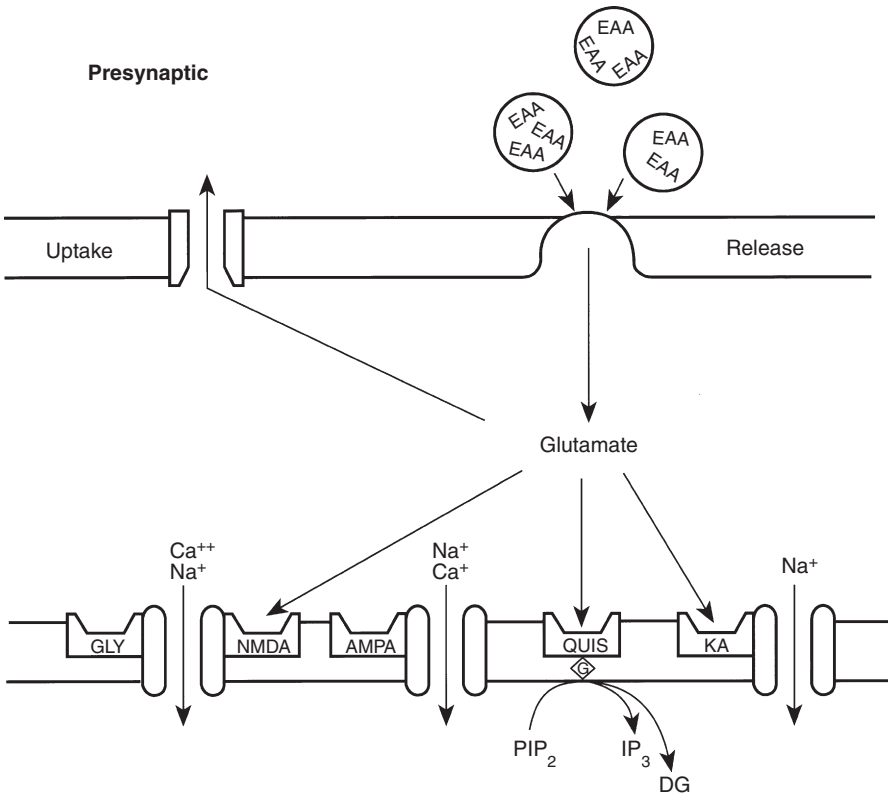


Figure 162-6 Schematic diagram of glutamate synapses. Glutamate released from presynaptic nerve terminals can interact with several postsynaptic receptors for glycine (GLY) and glutamate in the *N*-methyl-D-aspartate (NMDA) conformation linked to a calcium channel. The α-amino3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is linked to a channel that fluxes sodium and some calcium. Glutamate in the conformation of quisqualate (QUIS) can stimulate hydrolysis of polyphosphoinositides to produce the second messengers inositol-1,4,5 triphosphate (IP_3) and diacylglycerol (DG). The kainate receptor (KA) is a special form of the AMPA receptor. *EAA,* Excitatory amino acid; *PIP_2,* phosphatidylinositol 4,5-bisphosphate.

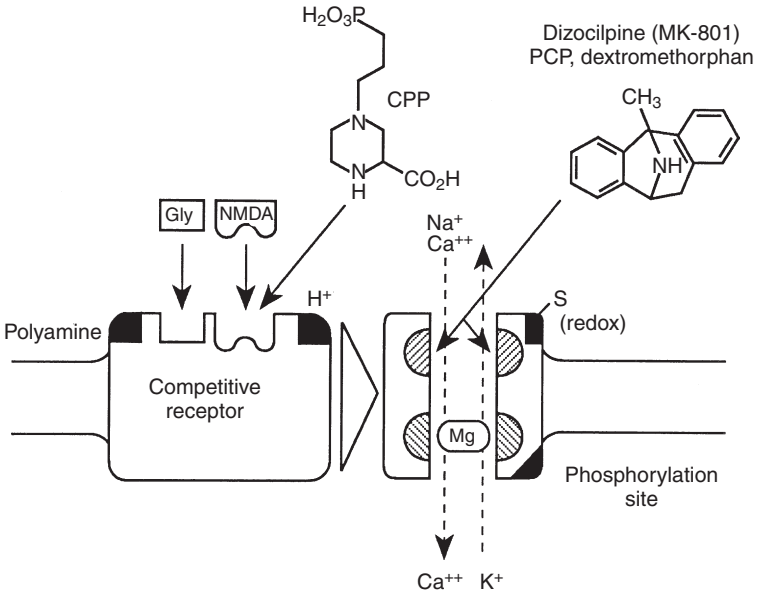


Figure 162-7 The *N*-methyl-D-aspartate (NMDA) receptor channel complex. Competitive binding sites are present for binding glutamate in the NMDA conformation and glycine (Gly). Antagonists are available to block the competitive sites and the channel. The structure of the channel-blocking drug dizocilpine is shown. Modulatory sites are shown for polyamines, hydrogen ions, redox compounds, and phosphorylation. *CPP,* NMDA antagonist; *PCP,* phencyclidine.

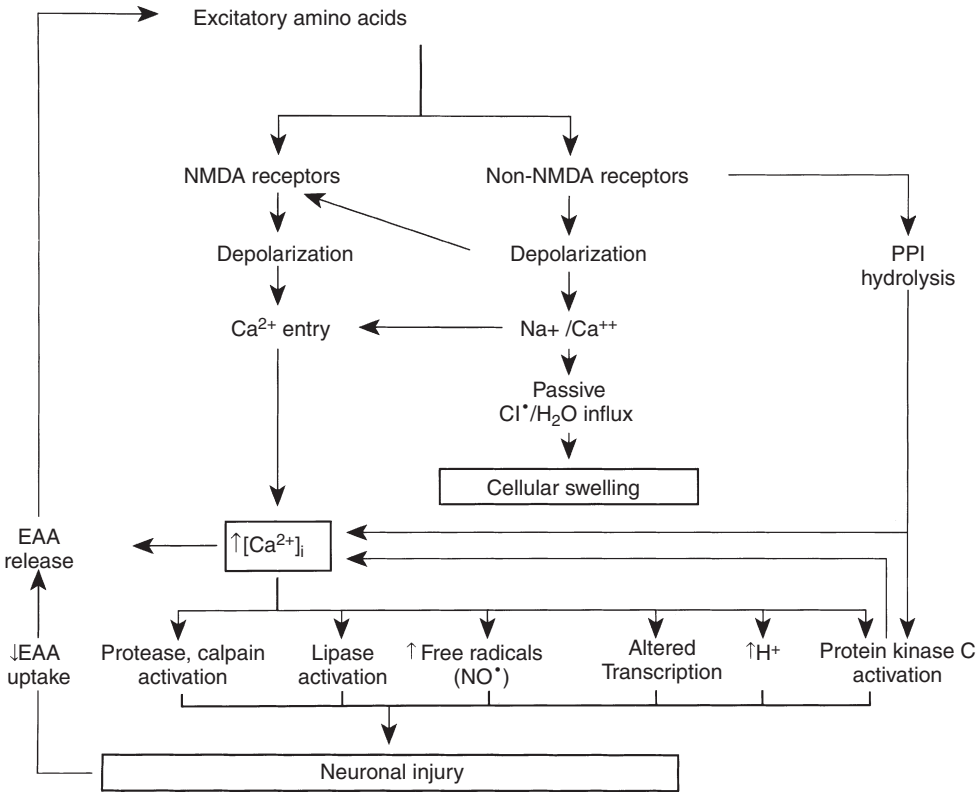


Figure 162-8 Schematic of events thought to be triggered by overstimulation of excitatory amino acid receptors in the brain during hypoxia-ischemia and other disorders. *EAA*, Excitatory amino acid; *NMDA, N*-methyl-D-aspartate; *PPI*, polyphosphoinositides.

system stimulated by a population of quisqualate-preferring glutamate receptors, is also markedly enhanced in hippocampus and striatum in the neonatal period.[4] This response is even larger than the enhanced coupling of PI turnover to muscarinic receptors already described. Immunocytochemical studies of AMPA receptors in brains from premature and term-born human infants demonstrated similar enhanced overexpression in the perinatal period.[56]

The functional enhancement of the effects of the excitatory neurotransmitter systems in developing brain may be related to their role in controlling cortical plasticity.[28] *Plasticity* in this context means the remodeling of neuronal connections, which is directly influenced by excitatory input. For example, in the developing visual system, *cortical columns,* defined physiologically, correspond to alternating inputs of each eye to the visual cortex. The remodeling of *ocular dominance columns* corresponding to one eye can be related to competition among physiologically active axons. Treatment with an NMDA glutamate receptor antagonist can prevent this remodeling in response to amblyopia, a finding suggesting that glutamate receptors mediate activity-dependent plastic changes in developing brain.[57] In this sense, glutamate receptors may mediate a portion of the physiologic activity of sensory stimulation on brain development.[58]

GLUTAMATE-MEDIATED NEUROTOXICITY

Several types of injury in the fetal and neonatal brain are mediated by excessive stimulation of glutamate receptors.[59-61] Hypoxia combined with ischemia impairs the function of reuptake pumps, leading to high levels of glutamate in the synaptic cleft. Resulting overstimulation of glutamate receptors, especially the NMDA receptor, causes influx of calcium and sodium into neurons and other cells in the brain (Figure 162-8). Hypoglycemia has a similar effect.[62] High levels of calcium trigger several intracellular events that can lead to neuronal death.[60] These include activation of nitric oxide synthetase, which catalyzes production of nitric oxide. Nitric oxide, a diffusible free radical compound, can cause death of surrounding neurons. Nitric oxide production stimulated by calcium entering through NMDA channels also appears to be a primary signal for increasing cerebral blood flow associated with increased neuronal activity. Nitric oxide and other free radical compounds can damage mitochondria through *oxidative stress.* Overstimulation of these intracellular events causes cell death by causing necrosis or *apoptosis* (programmed cell death).[60] Glutamate-mediated neurotoxicity seems to be a final common pathway for several forms of injury to the immature brain. Excessive and prolonged blockade of NMDA receptors can also lead to neuronal death, and this may be responsible for the teratogenicity of certain toxins such as ethanol.[63] Excessive stimulation of non-NMDA glutamate receptors is also implicated in damage to immature oligodendroglia-producing periventricular leukomalacia (PVL) in premature infants.[64] The timing of expression of AMPA receptor subunits that facilitate calcium permeability correlates with the vulnerability of oligodendroglia in premature infants, and the vulnerability of neurons in term infants.[56,65]

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163 Perinatal Brain Metabolism

Susan J. Vannucci and Robert C. Vannucci

CEREBRAL OXIDATIVE METABOLISM

The oxidative events that operate in the immature brain appear identical to those in the brain of adult animals and humans, although quantitative differences certainly exist. Cellular respiration is that process whereby an organic substrate is consumed to produce chemical energy (stored in high-energy phosphate bonds, represented by ~P), with carbon dioxide and water as the metabolic byproducts. Respiration is a highly efficient process with minimal energy waste. Under physiologic conditions, 1 mole of glucose, the primary cerebral energy fuel, is catabolized in the presence of oxygen to yield 36 moles of adenosine triphosphate (ATP). Other substrates yield proportionately greater or lesser energy, depending on their molecular carbon structure and their point of entry into the oxidative pathway.

The biochemical machinery that comprises the oxidative pathway is divided into three components—specifically, glycolysis, a cytoplasmic process, and the tricarboxylic (Krebs) cycle and the cytochrome system, both of which operate within mitochondria[1-3] (Figure 163-1). Glycolysis proceeds under both aerobic and anaerobic conditions. In the presence of oxygen, glucose is converted to pyruvic acid, which in turn enters the Krebs cycle with little or no conversion to lactic acid. When oxygen is not available, metabolites of the Krebs cycle are depleted, and glycolysis is accelerated, with the formation of lactic acid in an attempt to maintain cerebral energy stores. However, anaerobic glycolysis is an inefficient method to generate energy (2 moles of ATP for each mole of glucose); thus brain function cannot be maintained on this process alone.

Glycolytic control points—that is, those reactions in which the activities of specific enzymes govern the rate of metabolism—include hexokinase, phosphofructokinase (PFK), and possibly pyruvate kinase. Of these enzymes, PFK is predominantly rate-limiting for glycolysis in both adult and immature brain.[4-6] Metabolites known to inhibit PFK activity and thus glycolysis include ATP, citrate, and hydrogen ions, whereas adenosine diphosphate (ADP), adenosine monophosphate (AMP), and inorganic phosphate (P_i) stimulate PFK activity.[2] These glycolytic regulatory mechanisms are of importance in the cell's response to hypoxia, acid-base imbalance, and other metabolic disturbances.

CEREBRAL HIGH-ENERGY METABOLITES

Metabolites known to exist in brain that can store and transfer energy in the form of anhydride phosphate bonds (~P) include ATP, ADP, and phosphocreatine (PCr).[1,2] Among these three, ATP plays the critical role in the coupling of energy supply to energy demand through substrate and oxidative phosphorylation. PCr

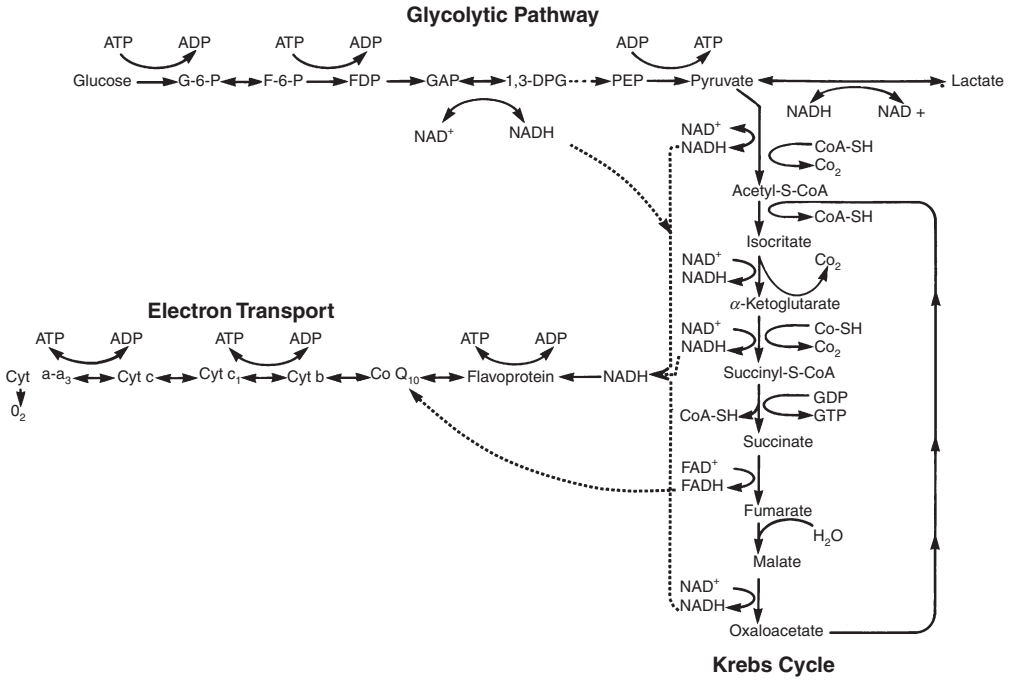


Figure 163-1 Schematic diagram of the oxidative pathway. *ADP,* Adenosine diphosphate; *ATP,* adenosine triphosphate; *1,3-DPG,* diphosphoglycerate; *FDP,* fructose diphosphate; *F-6-P,* fructose-6-phosphate; *GAP,* glyceraldehyde phosphate; *G-6-P,* glucose-6-phosphate; *NAD^+,* nicotinamide-adenine dinucleotide (oxidized form); *NADH,* nicotinamide-adenine dinucleotide (reduced form); *PEP,* phosphoenolpyruvate.

TABLE 163-1

Cerebral High-Energy Metabolites in Developing Rats

	CONCENTRATION (mmol/kg WET WEIGHT)*			
Metabolite	**Fetus[12]**	**Newborn[12]**	**1 Week[12]**	**Adult[13]**
ATP	2.66	2.63	2.64	2.76
ADP	0.43	0.25†	0.53	0.38
AMP	0.03	0.01	0.04	0.03
ATP + ADP + AMP	3.11	2.89	3.21	3.17
PCr	1.74	3.16†	3.33	4.90†
Creatine	5.35	3.83†	4.65†	5.63†
PCr + creatine	7.10	6.99	7.97†	10.53†
PCr-to-creatine ratio	0.33	0.83†	0.72	0.87

*Values represent means for four to seven animals in each age-group. Fetal, newborn, and 1-week values are for forebrain, whereas adult values are for cerebral cortex. Data are from cited references.

†$P < .05$ compared with previous age-group.

ADP, Adenosine diphosphate; *AMP,* adenosine monophosphate; *ATP,* adenosine triphosphate; *PCr,* phosphocreatine.

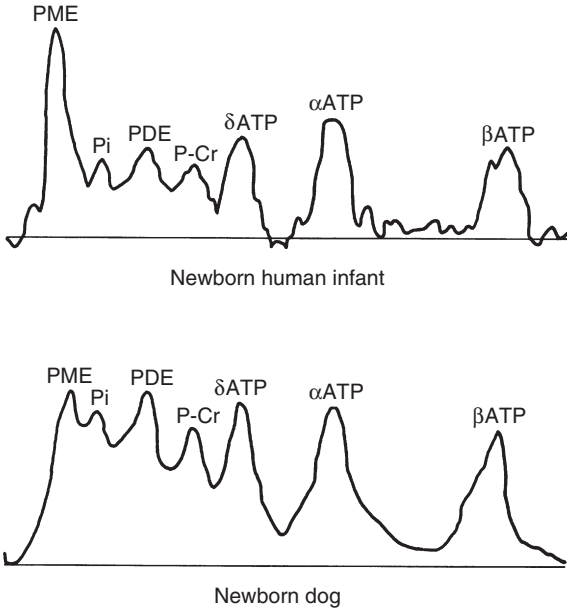


Figure 163-2 Representative phosphorus nuclear magnetic spectra for the brain of a newborn human infant and of a newborn dog. *PME,* Phosphorus monoesters; P_i, inorganic phosphate; *PDE,* phosphorus diesters; *P-Cr,* phosphocreatine; *δATP, αATP, βATP,* δ-, α-, β-adenosine triphosphate. *(Data sources:* infant*:Younkin DP, et al: Unique aspects of human newborn cerebral metabolism evaluated with phosphorus nuclear magnetic resonance spectroscopy,* Ann Neurol *16:581, 1984;* dog*:Young RSK, et al: ^{31}P NMR study of cerebral metabolism during prolonged seizures in the neonatal dog,* Ann Neurol *18:14, 1985.)*

acts as a storage metabolite, whereas guanosine triphosphate (GTP) is important in biosynthetic reactions.

Cerebral high-energy metabolites have been measured in perinatal and adult animals by enzymatic, chromatographic, and magnetic resonance (MR) spectroscopic techniques.[4,7-11] Of the nucleotides, ATP predominates and remains stable from term fetal life through adulthood[12,13] (Table 163-1). Lower concentrations have been found in embryonic brain tissue.[11] PCr concentration is lower in the brains of newborn animals than in adults, as is that of total creatine (PCr + creatine), but the PCr-to-creatine ratio changes little with postnatal development.[12,13] The lower PCr-to-creatine ratio in the fetus presumably is the result of a lower brain pH, owing to increased tissue lactate concentrations and hypercapnia (the creatine kinase equilibrium reaction is pH-dependent).[12]

MR spectroscopy is a technique whereby the concentration of an organic compound in tissue can be determined by the realignment of selected molecules using a supraconducting magnet.[14] Almost any substance can be analyzed according to the strength, duration, and other characteristics of the magnetic field. Commonly examined metabolites in brain are the phosphorus-containing compounds, including PCr, ATP, mono- and diphosphate esters (including AMP and ADP, respectively), and P_i.[15-18] Proton MR spectroscopy provides a measurement of numerous hydrogen-containing metabolites; including lactate and creatine.[19-21] Thus MR spectroscopy is capable of ascertaining the energy status of brain tissue in vivo under both physiologic and pathophysiologic conditions, although usually the measurements are semiquantitative in nature (Figure 163-2).

An investigation using MR spectroscopy in premature and full-term newborn infants confirmed evidence from earlier studies in animals indicating that ATP levels remain relatively stable throughout maturation.[17] A more recent study, however, has suggested that ATP concentrations in the human brain almost double between the newborn period and adulthood.[18] As in animals, levels of PCr, P_i, and the phosphodiesters are low at birth, increasing with advancing age. Calculated intracellular pH, as measured from the chemical shift of the P_i peak relative to the PCr peak, is 7.1 in healthy newborn infants. As anticipated, the PCr-to-P_i and the PCr-to-ATP ratios are lower in newborn infants than in adult animals. Although the reported findings in newborn infants are preliminary in nature, the continued use and further sophistication of MR spectroscopy can be expected to contribute substantially to a full understanding of the bioenergetics of the perinatal human brain (see further on).

QUANTITATIVE ASPECTS OF OXIDATIVE METABOLISM

Measurements of cerebral oxygen consumption give a quantitative estimation of energy production by the brain, since oxygen is required for the oxidative phosphorylation of ADP to ATP in electron transport by way of the cytochrome system (see previous discussion). Oxygen consumption measurements often are referred to as the cerebral metabolic rate for oxygen ($CMRo_2$), expressed as mL/100 g/minute. Estimates of the CMR in developing animals initially were obtained in vitro with the use of the Warburg respirometer to measure oxygen consumption in brain slices or homogenates in nutrient media. Himwich and co-workers[22] found that the oxygen consumption (expressed as wet weight of tissue) of minced cerebral tissue with glucose as substrate is 38% lower in 7-day postnatal rats than in adults. Subsequent in vitro studies of oxygen consumption in either the whole brain or cerebral cortex from other species, including humans, indicated that the CMR is low at birth and increases with maturation[22-25] (Figure 163-3). Changes in the rates of glucose utilization during development tend to parallel the increases in oxygen consumption.[24]

Both immature and adult animals exhibit regional differences in brain oxygen consumption in vitro.[24,26,27] Himwich and Fazekas[27] found that although total oxygen consumption in the brain of 1-week postnatal dogs is 83% of the adult level, significant regional differences exist that change with increasing age. The medulla oblongata exhibits the highest rate of consumption in the immediate newborn period but has the lowest rate for all regions studied in the adult brain (see Figure 163-3). By contrast, the oxygen consumption in the cerebral cortex is the lowest for all regions analyzed in the newborn period but doubles within 5 weeks of development. These in vitro findings of regional

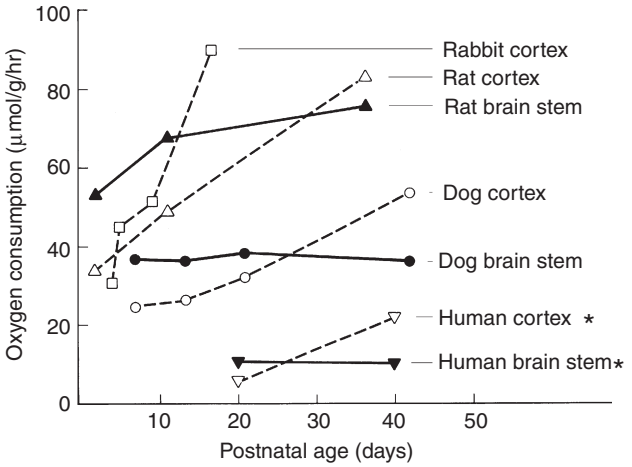


Figure 163-3 In vitro oxygen consumption in brains of developing animals and human fetus. *(Data sources:* dog*: Himwich HE, et al: The respiratory metabolism of infant brain,* Am J Physiol *125:601, 1939;* rat*: Tyler DB, van Harreveld A: The respiration of the developing brain,* Am J Physiol *136:600, 1942;* rabbit*: Swaiman KF, et al: Interrelationships of glucose and glutamic acid metabolism in developing rat brain,* J Neurochem *10:635, 1963;* human fetus*: Himwich HE, et al: Mechanisms for the maintenance of life in the newborn during anoxia,* Am J Physiol *135:387, 1942;* rabbit brain stem*: data not available.)*

TABLE 163-2

Cerebral Metabolic Rate for Oxygen ($CMRo_2$) in Developing Animals and Humans

	$CMRo_2$ (mL/100 g/minute)		
Species*	**Fetal**	**Newborn**	**Adult**
Sheep[28-31]	2.0-3.7	3.9-4.8	4.2
Dog[32,33]	—	1.1	2.8
Monkey[34,35]	—	1.1	3.2
Humans[36-38]	—	1.5	3.2-3.3

*Data from cited references.

TABLE 163-3

Cerebral Blood Flow and Cerebral Metabolic Rates for Oxygen in Newborn and Adult Dogs*

Age-Group†	CBF (mL/100 g/minute)	A – Vo_2 (vol %)	$CMRo_2$ (mL/100 g/minute)
Newborn puppy[33]	24 ± 14	5.6 ± 2.5	1.1 ± 0.3
Adult dog[32]	56 ± 14	5.0 ± ?	2.8 ± 0.8

*Values represent means ± SD for seven newborn and adult dogs.
†Data from cited references.
A – Vo_2, Arteriovenous oxygen difference; *CBF,* cerebral blood flow; *$CMRo_2$,* cerebral metabolic rate for oxygen.

differences in brain metabolism between young and adult animals, including humans, have been substantiated by more recently developed in vivo techniques to measure metabolism (see further on).

Oxygen consumption can be calculated in vivo from the Fick principle by measuring cerebral blood flow (CBF) and the arteriovenous difference for oxygen across the brain:

$$CMRo_2 = CBF(A-Vo_2)$$

With use of this technique, oxygen consumption has been determined in immature animals of several species, including humans.[28-38] As shown in Table 163-2, notable species differences exist for the rates of oxygen consumption by brain, which reflect the age and functional immaturity of the animal at the time of the measurement as well as species variations in the brain's intrinsic metabolic requirements. Specifically, $CMRo_2$ is lower in perinatal animals than in adults of the same species, and the more immature the animal at birth, the lower the rate of oxygen consumption. This assumption is supported by measurements in newborn dogs, which are functionally immature at birth, with values for both CBF and $CMRo_2$ that are 40% of respective values in adult dogs[32,33] (Table 163-3). By contrast, the newborn lamb is relatively mature at birth and already exhibits a $CMRo_2$ that is equal to or even greater than that in adult sheep.

Oxygen consumption of the brain also has been determined in humans, including newborn infants. The $CMRo_2$ in young, healthy adults is 3.3 mL/100 g/minute.[37] Oxygen consumption in unanesthetized healthy children aged 3 to 10 years actually is higher by 23% than it is in adults; Kennedy and Sokoloff[39] reported a value of 5.2 mL/100 g/minute. Settergren and colleagues[38] measured $CMRo_2$ in children aged 11 days to 15 years who were anesthetized with nitrous oxide. The mean value for $CMRo_2$ was 3.2 for the entire group, and no age-related differences could be ascertained. Garfunkel and co-workers[36] found $CMRo_2$ values of 1.1 to 2.1 mL/100 g/minute in three newborn infants anesthetized with barbiturate. These low values may not apply to healthy, awake infants, however, because barbiturates are known to depress the $CMRo_2$,[40] and the subjects had severe central nervous system anomalies.

With the use of positron emission tomography (PET), the cerebral metabolic rate for oxygen as well as cerebral glucose utilization (see further on) can be ascertained in human subjects. PET is based on computed tomography (CT) except that the source of radiation for external detection is transmitted from within the brain outward, rather than passed through the brain as x-rays. In PET, a positron-emitting, rapid-decay isotope, prepared by a cyclotron, is administered to the subject. The substance circulates to the brain, where it is metabolized, during which its positrons combine with electrons to form γ-rays. The γ-rays penetrate the brain, skull, and scalp and are recorded externally on a circular array of scintillation detectors. A computer mathematically reconstructs the spatial distribution of the radioactivity within the brain, which either is quantified or is displayed on a cathode ray screen. Thus PET offers a noninvasive regional assessment not only of $CMRo_2$ but also of many other biologic processes.

Using PET with ^{15}O-labeled water as the positron-emitting isotope, Altman and colleagues[41] measured $CMRo_2$ in 11 newborn infants of gestational ages ranging from 26 to 40 weeks. $CMRo_2$ was low in all of the infants and was beyond the lower range of detection in two. In those infants in whom radioactivity was detectable, $CMRo_2$ ranged from 0.2 to 1.3 mL/100 g/minute, well below the measured value for human adults of 3.3 mL/100 g/minute. The findings indicate that as discussed earlier for perinatal animals, cerebral oxidative metabolism, especially in small premature infants, is low, reflecting the functional immaturity of the brain (as also suggested by the work of Yoxall and Weindling[42]). A less likely proposal, offered by Altman's group,[41] is that the energy requirements of the fetal and prematurely newborn brain are met by nonoxidative metabolism, that is, anaerobic glycolysis (but see further on).

GLYCOLYSIS

As mentioned previously, rates of glycolysis or the glycolytic flux is controlled by specific, rate-limiting enzymes that are either inhibited or activated by changes in the biochemical milieu of the cytoplasm. Thus aerobic glycolysis is stimulated by those

stresses that increase the energy demands of the tissue—specifically, stimulant drugs and hormones, hyperthermia, and seizures. Anaerobic glycolysis is stimulated by hypoxia or cerebral ischemia, or both, in an attempt to maintain optimal cellular energy balance despite the oxygen debt. Glycolysis is inhibited by sedative and anesthetic agents, hypothermia, and acidosis, all of which are associated with reduced energy needs of the tissue. Glycolysis can never cease completely, because at least some energy is always required to maintain ionic gradients across cellular and subcellular membranes, without which cellular integrity is compromised.

The extent to which glycolysis and thus oxidative metabolism can be turned on or off is not entirely known. In adult animals, deep barbiturate anesthesia sufficient to produce an isoelectric signal on the electroencephalogram reduces metabolism by no more than 50%,[43] suggesting that up to 50% of cellular energy production is required for maintenance of biochemical and morphologic integrity, whereas the remainder is devoted to the generation of action potentials and to biosynthetic processes. The extent to which glycolysis can be stimulated (glycolytic capacity) also is conjectural, although studies in experimental animals suggest that glycolytic flux can be accelerated up to 8- to 10-fold during extreme metabolic stress, that is, seizures or hypoxia-ischemia.[2] In this regard, both basal glycolytic flux and glycolytic capacity are lower in immature animals than in their adult counterparts, in keeping with intrinsically lower rates of oxygen consumption and hence metabolic demands. For example, the calculated rate of cerebral glycolysis of the perinatal rat is approximately 10 μmol of glucose/100 g/minute,[44] which is one tenth of the calculated rate for adult rat cerebral cortex.[45] During total cerebral ischemia, produced by decapitation, glycolytic flux in newborn rat brain increases five-fold, compared with an eight-fold increase in adult rat brain.[44,45] Thus the ability of perinatal animals to survive a prolonged period of hypoxia or anoxia compared with that of adults[46] cannot be explained by a heightened capacity of the immature brain to generate energy equivalents through anaerobic glycolysis. Rather, lower cerebral energy demands and hence metabolic requirements underscore the perinatal animal's resistance to hypoxia-ischemia.[9,44]

MITOCHONDRIAL DEVELOPMENT

Because the mitochondria produce the vast bulk of energy utilized for cellular needs, these "powerhouses" have been investigated during maturation of the brain. Investigators have demonstrated a relative deficiency in the oxidative phosphorylating capacity of mitochondria that characterizes the immature brain.[47] Simply put, the immature brain appears to be less able to synthesize ATP per molecule of oxygen consumed. This coupling of ATP to oxygen, expressed in terms of the ATP/O or P/O ratio, is 3 for the oxidized form of nicotinamide-adenine dinucleotide (NAD^+)-linked cytochrome components in adult rat brain.[48] Holtzman and Moore[49] have measured P/O ratios in several regions of rat brain during maturation. Ratios for 1.5 for NAD^+-linked substrates were found in animals 1 to 11 days of postnatal age. Thereafter, ratios increased to 2.4 in pons-medulla and 2.8 in cerebral cortex. The data indicate that the brain cell's increasing efficiency for oxidative phosphorylation (improved coupling) reflects both an increase in the number of mitochondria and an inherent change in their function.

The functional advantage of these mitochondrial changes appears to lie in the pattern of growth of the central nervous system. A striking increase in mitochondrial cell number accompanies neuronal and glial cell hypertrophy secondary to axonal and dendritic expansion.[50] Furthermore, as individual cells enlarge, the cell surface-to-volume ratio increases—a circumstance that raises the energy required to maintain ionic gradients across the membrane and to propagate action potentials.[51]

CEREBRAL ENERGY UTILIZATION

Energy utilization by the brain can be inferred from measurements of oxygen or substrate consumption, because under steady-state conditions, energy demand and supply are equivalent. Cerebral energy utilization also can be measured directly in the experimental animal by a novel decapitation technique.[4] Lowry and co-workers[4] devised this technique to determine the rate of energy use in brain during the total cerebral ischemia that follows decapitation. The investigators assumed that because the isolated head is a closed metabolic compartment (no inflow or outflow), rates of depletion of potential (glucose + glycogen) and endogenous (ATP + ADP + PCr) high-energy stores would be a direct measure of cerebral energy utilization occurring before decapitation. Using this method, various investigators have shown that the calculated rate of cerebral energy use in perinatal animals, like the $CMRo_2$, is substantially less than in adults.[4,9,44] For example, the energy use rate of the 1-day postnatal rat is 1.3 mmol of ~P/kg/minute,[44] 20-fold less than the calculated rate of 27 mmol of ~P/kg/minute for the adult rat brain.[45]

$CMRo_2$ can be calculated from the rate of cerebral energy use if the ADP/O or P/O ratio is known. As already mentioned, P/O ratios for immature rat brain are 1.5 for NAD^+ and 1.2 for the oxidized form of flavin-adenine dinucleotide (FAD^+)-linked substrates.[49] A source of ATP other than that derived from oxidative phosphorylation of ADP is substrate phosphorylation, which occurs twice in glycolysis and once in the Krebs cycle. Substrate phosphorylation yields 6 moles of ATP for every mole of glucose consumed; 2 moles are required for the initial phosphorylation of glucose and fructose-6-phosphate in glycolysis. Thus, for every mole of glucose consumed, 4 moles of ATP are ultimately generated by a source other than oxidative phosphorylation; this amounts to 20% of the total energy production. Assuming a yield of 3 moles of ~P/mole of oxygen reduced (P/O ratio of 1.5), and taking into account the ATP formed by both oxidative and substrate phosphorylation, the rate of cerebral energy utilization for newborn rats translates to a $CMRo_2$ of $[1.3 - (1.3 \times 0.2)]/3 = 0.35$ mmol/kg/minute, or 0.78 mL/100 g/minute. This value is far less than in adult rat brain, for which the measured $CMRo_2$ value is 5.4 mL/100 g/minute[52]—nearly six-fold higher. The fetal rat at term exhibits a rate of cerebral energy utilization comparable with that in the newborn rat pup.[44]

The low cerebral energy requirements of most mammals at birth reflect an immaturity of the central nervous system at this stage of development. It has been suggested that cerebral energy demands in the fetal brain are even less well developed than in the newborn brain[53] and that such demands in embryonic life are met predominantly or entirely by anaerobic glycolysis, with only a small contribution from oxidative metabolism.[23,50] Using the aforementioned Lowry technique to evaluate cerebral energy utilization, Gonya-Magee and Vannucci[11] measured rates of cerebral metabolism in chick embryos at 9, 14, 16, and 19 days of incubation and in newly hatched peeps (Table 163-4). Calculated cerebral metabolic rates were not different in the 9-, 14-, and 16-day-old embryos but doubled between 16 and 19 days and doubled again between 19 days and hatching. The extent to which total cerebral energy utilization could be derived from anaerobic glycolysis (Δ lactate/Δ ~P) increased from a low at day 9 (0.29) to a maximum at day 16 (0.78). The findings in this investigation suggest that cerebral oxidative metabolism becomes progressively lower with decreasing embryonic age, but that a nadir is reached below which metabolism can no longer support cerebral function and growth. Furthermore, the data indicate that despite the low metabolic activity of the embryonic brain, at no time during development is anaerobic glycolysis capable of entirely supporting the energy needs of the tissue.

CEREBRAL GLUCOSE UTILIZATION

In all animal species studied, including humans, glucose is the predominant or exclusive organic fuel for cerebral metabolism under physiologic conditions. This dictum also appears to hold true for the fetus and developing postnatal animal. As with $CMRO_2$, glucose consumption in brain can be measured from a knowledge of CBF and the arteriovenous difference for glucose:

$$CMR_{glucose} = CBF\,(A - V_{glucose})$$

When $CMR_{glucose}$ (or other organic substrate) and $CMRO_2$ are measured simultaneously, the substrate-oxygen quotient, which expresses the relative contribution of the substrate to overall oxidative metabolism, can be ascertained:

$$\frac{\text{Substrate}}{O_2} = \frac{CMR_{substrate}\,(\text{number of C atoms}) \times 100}{CMRO_2}$$

Using these measurements, cerebral glucose utilization has been determined in several perinatal animal species as well as in postnatal human infants[35,38,54-56] (Table 163-5). As in adult humans,[57,58] glucose serves as the major organic substrate to support cerebral metabolism in all species studied. Furthermore, the vast bulk of glucose consumed is aerobically degraded to carbon dioxide and water or is used in biosynthesis, with little or no production of lactic acid (anaerobic glycolysis). Thus, at least under physiologic conditions, glucose is efficiently utilized to produce the maximal amount of chemical energy possible.

In the past, only global estimates of glucose consumption in brain could be ascertained, using the method described earlier. Thanks to the pioneering work of Sokoloff and colleagues,[59,60] regional cerebral glucose utilization (rCGU) now can be determined in both experimental animals and humans. These investigators[60] used radioactive 2-[^{14}C]-deoxyglucose (2-DG) to measure CGU in 30 or more component structures of adult rat brain. The rationale for the use of 2-DG rather than [^{14}C]-glucose is that the radioactive analogue of native glucose is lost from the brain as carbon dioxide and water during the course of an experiment, so calculated CGU would underestimate the true CGU value. Deoxyglucose, like glucose, is taken up by brain and phosphorylated by hexokinase but, unlike glucose, is unable to be metabolized further along the glycolytic pathway. The theoretical basis for this technique and its successful application in the experimental animal together support its usefulness for measuring rCGU in humans by means of PET. Furthermore, because glucose is the predominant cerebral energy fuel, and glucose utilization is stoichiometrically related to oxygen consumption (see previous discussion), the measurement of rCGU is considered to reflect local rates of cerebral energy utilization and intrinsic functional activity.[59]

Using the 2-DG technique, rCGU has been investigated in fetal sheep and in newborn rats, lambs, dogs, and monkeys.[61-65] The studies confirm early in vitro experiments (see preceding discussion) that CGU in the perinatal animal is high in brain stem gray matter structures, declining in a caudal-to-rostral progression through the neuraxis to the cerebral cortex[62,63] (Table 163-6; Figure 163-4). As in adults, CGU is lowest in subcortical and other white matter structures. Duffy and co-workers[62] suggest that the hierarchy of rates of glucose consumption reflects the functional immaturity of the perinatal animal, with its limited, albeit present, sensory and motor capabilities but with little or no memory or learning abilities at this early stage of development.

Using PET with [^{18}F]fluoro-2-deoxyglucose as the positron-emitting isotope, Chugani and Phelps[66,67] measured rCGU in humans from full-term birth through adulthood (Figure 163-5). In infants 5 weeks of age and younger, glucose utilization was highest in the sensorimotor cerebral cortex, thalamus, midbrain-brainstem, and the cerebellar vermis—a distribution of glucose consumption similar to that described in term fetal sheep and in newborn dogs and monkeys (see Figure 163-4).[61-63] By the age of 3 months, glucose metabolic activity in the infants had increased in the parietal, temporal, and occipital cortices and in the basal ganglia, with subsequent increases in frontal and various association regions of cerebral cortex occurring by 8 months. Little further change in rCGU was observed between 8 and 18 months of postnatal age. The investigators emphasized that the maturational increases in rCGU, measured with PET, are in agreement with the

TABLE 163-4

Cerebral Energy Use Rates and Maximal Glycolytic Capacities of Chick Embryos and Newly Hatched Peeps

	RATE OR CAPACITY (mmol/kg wet weight)*		
Age Group	Δ ~P	Δ Lactate	Δ Lactate/Δ ~P
Embryos (days)			
9	2.84	0.79	0.29
14	3.25	1.51†	0.54†
16	2.76	2.04†	0.78†
19	5.76†	3.22†	0.56†
Peeps	8.98†	5.09†	0.56

*Values represent means for four to six animals in each age-group. The ratio Δ lactate/Δ ~P is an indication of the maximal contribution of anaerobic metabolism to the cerebral metabolic rate.

†$P < .05$ compared with the previous age-group.

Data from Gonya-Magee T, Vannucci RC: Ontogeny of cerebral oxidative metabolism in the chick embryo, *J Neurochem* 38:1387, 1982.

TABLE 163-5

Substrate Equivalents and Extent of Anaerobic Metabolism in Newborn Animals and Humans

	% TOTAL CEREBRAL UTILIZATION*			
Substrate	Sheep[56]	Dog[55]	Monkey[35]	Human[38]
Glucose	99.5	93.5	70.4	91.1
β-Hydroxybutyrate	0.5	0.5	14.6	5.5
Acetoacetate	0.0	2.0	4.9	3.4
Lactate	0.0	4.0	10.1	0.0
Totals	100	100	100	100
% Glucose → lactate	2.6%	0.0%	0.0%	5.2%

*Data from cited references.

TABLE 163-6

Regional Cerebral Glucose Utilization (RCGU) in Developing Monkeys

	UTILIZATION RATE (μmol/100 g/minute)*		
Structure	Newborn	Pubescent	% Difference
Frontal cortex	28	50	+44†
Parietal cortex	29	47	+38†
Occipital cortex	32	59	+46†
Hippocampus	25	39	+36*
Corpus callosum	15	11	−36
Caudate nucleus	23	52	+56
Thalamus	35	54	+35†
Substantia nigra	28	29	+3
Inferior colliculus	180	103	−75
Cerebellar hemisphere	22	31	+29

*Values represent means for six to seven animals in each group.

†$P < .01$.

Data from Kennedy C: Energy metabolism of the brain. In Sinclair JC, Warshaw JB, Bloom RS (eds): *Perinatal brain insult: Mead Johnson symposium on perinatal and developmental medicine #17,* Evansville, Ind, 1981, Mead Johnson and Company, pp 30-42.

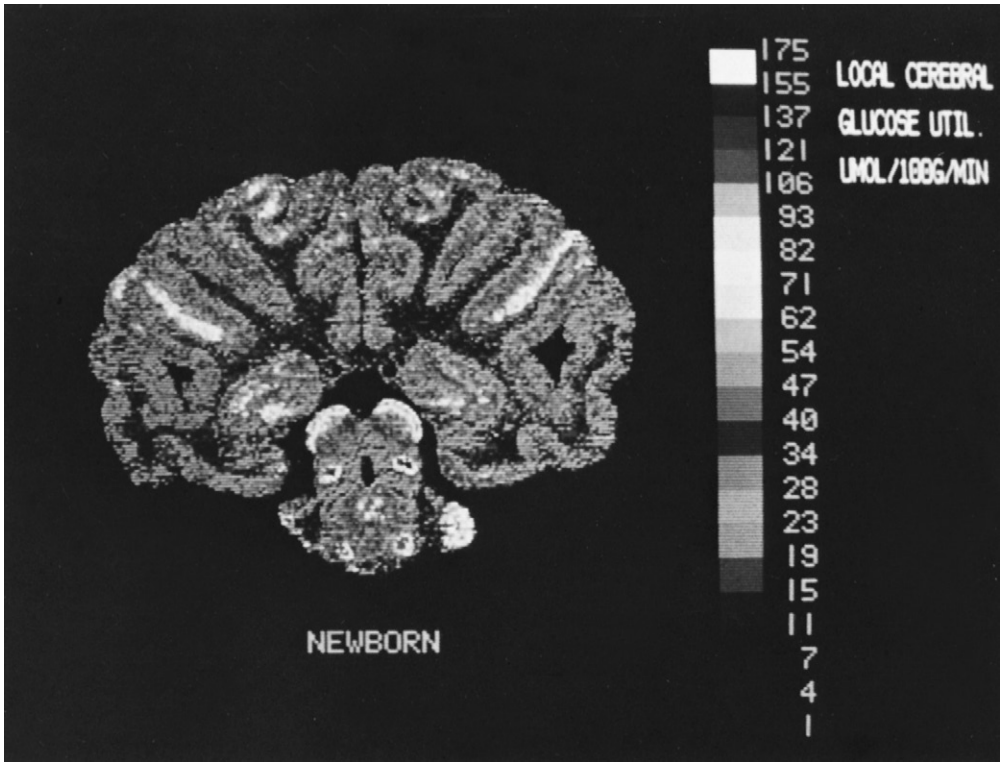


Figure 163-4 Representative ^{14}C-deoxyglucose autoradiogram of brain from a newborn monkey. *Lighter areas* denote regions with higher utilization rates (see scale). Note the high rates of glucose consumption in selected brain stem structures relative to the cerebral hemispheres. *(Courtesy Charles Kennedy, MD.)*

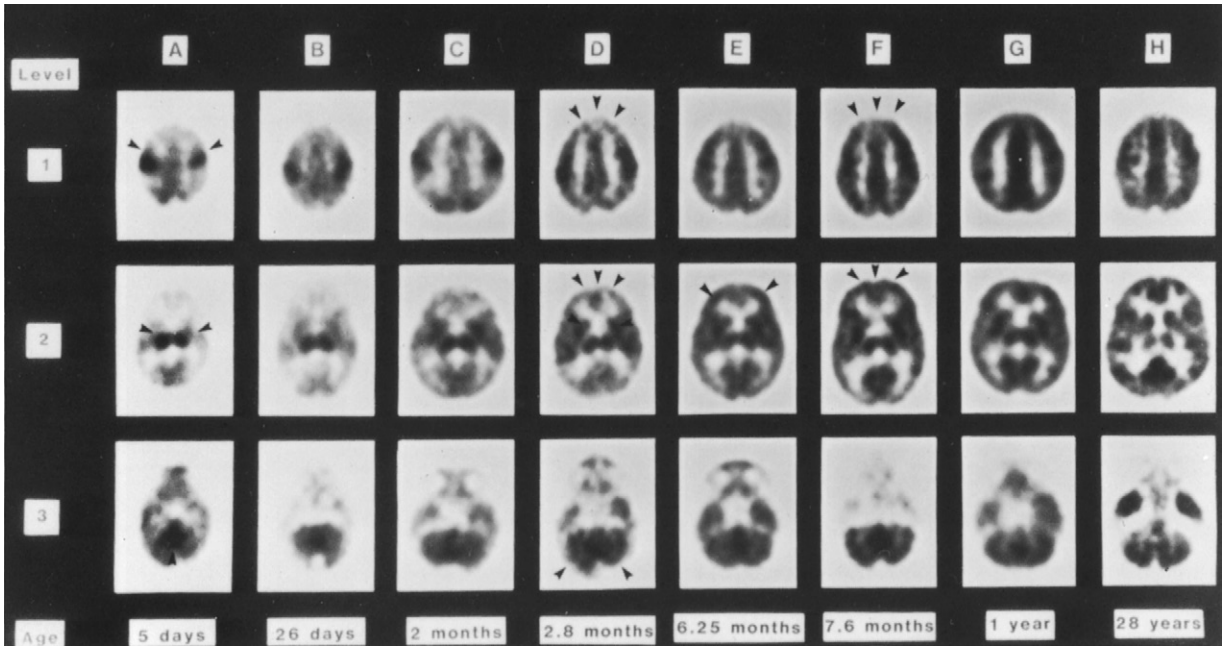


Figure 163-5 Representative [^{18}F]fluoro-2-deoxyglucose positron emission tomograms during human maturation. Shown are three horizontal levels of brain at different ages: postnatal day 5, 1 year, and adulthood. *(From Chugani HT, et al: Positron emission tomography study of human brain functional development,* Ann Neurol *22:487, 1987.)*

behavioral, neurophysiologic, and anatomic changes known to occur during infant development.

Other investigators have used PET to measure global and regional rCGU in premature newborn infants.[68,69] Gestational ages ranged from 25 to 37 weeks. Whole brain CGU was less than that in full-term infants, when the two age groups were compared.[68] As in full-term infants, glucose utilization was higher in brain stem and cerebellar structures compared with cerebral cortex. The lower rCGU in premature infants correlates with the low $CMRo_2$ observed at similar gestational ages.[41]

RELATIONSHIP BETWEEN CEREBRAL BLOOD FLOW AND GLUCOSE UTILIZATION (FLOW-METABOLISM COUPLE)

With the availability of methods to measure CBF and metabolism in animals and humans, investigators were quick to recognize a quantitative relationship between these two important biologic processes in brain. Further investigation revealed that under physiologic conditions, CBF was essentially controlled by intrinsic mechanisms that reflect local rates of metabolism, that is, the extent of tissue carbon dioxide and hydrogen ion accumulation. Thus it is not surprising that CBF and metabolism are tightly linked or coupled. Specifically, when the rate of oxidative metabolism in brain is high, so is CBF, and vice versa. Metabolic diseases are capable of altering this critical relationship, during which neurologic dysfunction occurs and, if the disturbance is severe enough, brain damage supervenes.

The brain flow-metabolism couple appears to be a universal phenomenon that encompasses all animal species of all ages and under all physiologic conditions* (Figure 163-6). In those animals in which CBF and $CMRo_2$ have been measured simultaneously, the relationship has been apparent regardless of age, although differences in the intrinsic rates of metabolism do exist among species. A regional coupling of CBF to metabolism also exists in both adult and immature animals[60,62-64] when rCBF, determined with an inert, radioactive tracer, is compared with rCGU[64] (Figure 163-7). A visual comparison of the published figures depicting rCBF and rCGU, using PET, also suggests the existence of a regional CBF-metabolism couple in the newborn human infant.[66,67,73] Occasional exceptions to the rule have been observed for individual structures (e.g., cerebellum), which may reflect local differences in the contribution of glucose versus alternate substrates to overall metabolism or to the use of glucose in biosynthetic processes (see further on).

ALTERNATIVE SUBSTRATES FOR OXIDATIVE METABOLISM

As mentioned previously, glucose is the predominant or exclusive cerebral energy fuel at all ages under physiologic conditions. In certain clinical situations, however, glucose availability to the brain is limited by hypoglycemia. In such circumstances, the perinatal brain is capable of incorporating and metabolizing substitute organic substrates, the most notable of which are lactic acid and the ketone bodies, β-hydroxybutyrate and acetoacetate. Indeed, the immature brain appears to be capable of consuming a large number of organic metabolites, including free fatty acids and amino acids as well, so long as they are available in reasonable concentrations in blood and the appropriate enzymes for their degradation are present in brain.

The most widely studied substitute fuels for metabolism in perinatal brain have been the ketone bodies and lactic acid. Because all developing mammals are nourished with milk with a high fat content, these animals readily generate ketone bodies by means of the liver, which in turn appear in substantial concentrations in blood. Thus, during suckling, ketone bodies partially substitute for glucose to sustain cerebral metabolism and can account for up to 20% of total energy production.[74-76] As in adults, nutrient fasting induces endogenous ketone body formation, which contributes significantly to overall cerebral metabolism during starvation-induced hypoglycemia.[77]

Hypoglycemia also is encountered in the immediate newborn period before the initiation of sustained oral or parenteral feeding. During this time, as well as during fetal life, ketone bodies are essentially nonexistent in blood and therefore contribute little

*See references 28, 30-36, 38, 39, 52, 58, 70-72.

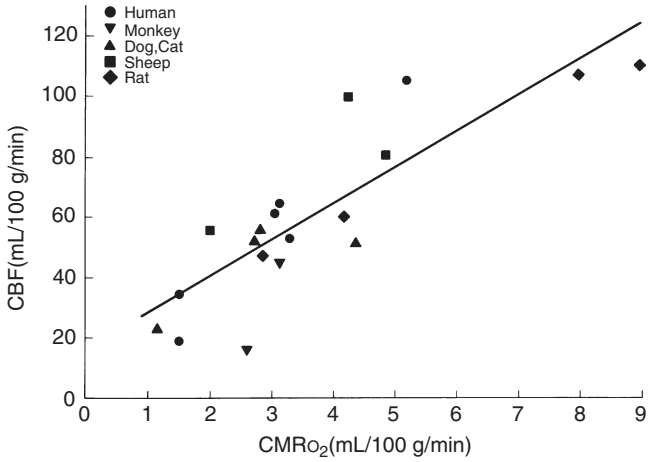


Figure 163-6 Relationship between cerebral blood flow (CBF) and the cerebral metabolic rate for oxygen ($CMRo_2$) in several species of animals. Symbols represent values from animals of varying ages. The *line* denotes the slope of a linear regression analysis, with $y = 12.1x + 16.7$; $r = 0.85$; $P < .001$.

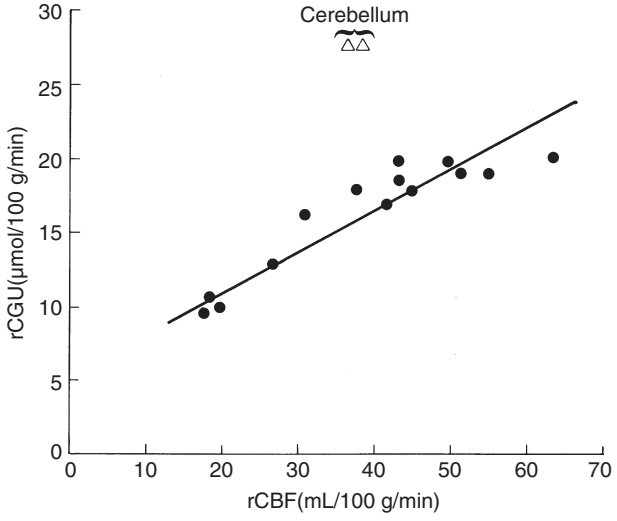


Figure 163-7 Relationship between regional cerebral glucose utilization (rCGU) and regional cerebral blood flow (rCBF) in newborn dogs. *Symbols* represent values from specific regions of brain. The *line* denotes the slope of a linear regression analysis (excluding cerebellum), with $y = 0.24x + 7.0$; $r = 0.93$; $P < .001$. *(Data from Mujsce DJ, et al: Regional cerebral blood flow and glucose utilization during hypoglycemia in newborn dogs,* Am J Physiol *256:H1659, 1989.)*

to cerebral metabolism.[78,79] By contrast, lactic acid is elevated in late fetal and early postnatal life to the extent that its concentration often exceeds that of glucose.[80-82] Studies in newborn dogs have shown that during hypoglycemia, lactic acid not only is incorporated into the perinatal brain but also is consumed to the extent that the metabolite can support up to 58% of total cerebral oxidative metabolism[55,83] (Figure 163-8). The versatility of the immature brain in its capacity to utilize substitute fuels, sparing glucose, undoubtedly contributes to the resistance of the newborn nervous system to the known deleterious effects of hypoglycemia.

The ability of the immature brain to incorporate and metabolize alternate energy fuels resides, at least in part, in the unique

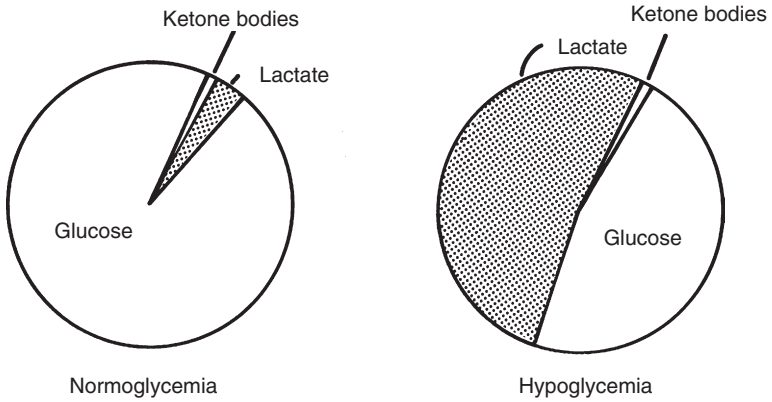


Figure 163-8 Substrate-oxygen quotients as percentage of total cerebral oxidative metabolism in normoglycemic and hypoglycemic newborn dogs. Substrate-oxygen quotients were calculated according to a formula described in the text. *(Data from Hernandez MJ, et al: Cerebral blood flow and metabolism during hypoglycemia in newborn dogs,* J Neurochem *35:622, 1980.)*

characteristics of its blood-brain barrier. All organic metabolites enter the brain from blood predominantly by way of a carrier-mediated transport system. Cremer and co-workers[74,84] first demonstrated the existence of a transport "carrier" for monocarboxylic acids, including ketone bodies and lactate, in the brain of immature rats, the Michaelis constant (K_m) of which is 10 times greater than that demonstrated for adult rat brain. The blood-brain uptake capabilities for β-hydroxybutyrate, acetoacetate, and lactate actually exceed those for glucose at an early postnatal age, facilitated by the high capacity of this transport system. Thus alternative substrates can be readily transported into the perinatal brain to support oxidative metabolism during periods of systemic glucose deficiency.

GLUCOSE AND MONOCARBOXYLIC ACID TRANSPORTERS

Transport of these nutrient substrates from the blood to the brain, as well as into neurons and glia, occurs by facilitated diffusion mediated by specific integral membrane transport proteins. These integral membrane proteins comprise two distinct families: the facilitative glucose transporter proteins (GLUTs) and the monocarboxylic acid transporter proteins (MCTs), the latter of which mediate proton-coupled co-transport. GLUT1 was cloned in 1985, and today, this and other similar proteins with distinct tissue distribution and kinetic characteristics—GLUT1 to GLUT12 and HMIT, the myoinositol transporter—are collectively known as the *SLC2 family* of proteins.[85] Although all 13 of these proteins have been detected in mammalian brain, GLUT1 and GLUT3 are the isoforms primarily involved in cerebral glucose uptake and utilization.[86] The initial transport of glucose across the endothelial cells of the blood-brain barrier is mediated by a heavily glycosylated form of GLUT1, 55-kDa GLUT1; a less glycosylated product of the same gene, 45-kDa GLUT1, is highly concentrated in choroid plexus and the ependymal lining of the ventricles and also in glial cells. GLUT3 is the neuronal glucose transporter.[86] The 14 MCT proteins were cloned a decade later and make up the *SLC16 family*; of these, only MCT1 to MCT4 have been definitively shown to transport metabolically relevant monocarboxylic acids.[87] MCT1, the most widely distributed, is detected in cerebral microvessels and appears to be expressed by all neural cells, whereas MCT2 expression is more restricted and primarily neuronal.

Both the GLUTs and the MCTs are developmentally regulated in brain.[88] GLUT concentrations are quite low in immature rat brain,[89] in keeping with the well-described low rates of cerebral glucose transport into immature rat brain,[84] and increase sharply after the second postnatal week (Figure 163-9). The observation

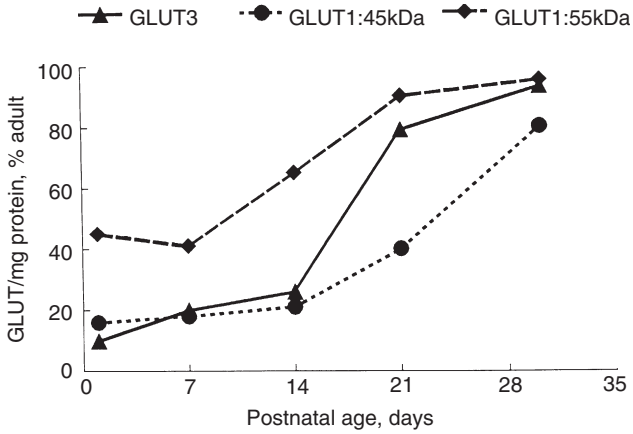


Figure 163-9 Brain glucose transporter proteins (GLUTs) during development in the immature rat. Isolated microvessels and cortical membranes were prepared from postnatal rats between birth and 30 days of age and adults. GLUT1 and GLUT3 membrane protein content was determined by Western blot analysis with specific antipeptide antisera for GLUT1 and GLUT3 and ^{125}I-protein A. Representative autoradiograms are depicted in the *upper part* of the figure. *S,* Brain microsomal membrane "standard" included in all blots for purposes of interblot comparisons and quantification. *Lower part* of the figure represents the quantification of values for four to six animals in each age group, expressed as a percent of the adult value.

of greatly reduced brain glucose concentrations during hypoxia-ischemia (see further on) provides evidence that GLUT concentration is limiting to cerebral glucose utilization at this age. Because the delivery of glucose from the blood into the brain is a function of both the substrate concentration in the blood and the transporter concentration, an increase in substrate concentration produced by glucose supplementation maintains a near-normal or even elevated brain glucose level. In addition, although calculated intracellular glucose concentrations in immature rats become very low during hypoxia-ischemia, the level of glucose in the cerebrospinal fluid—as a reflection of the concentration in the brain extracellular compartments—does not decrease.[90] Accordingly, it appears that an even greater limitation may be imposed at the level of the neurons and glial cells than at the blood-brain barrier.

The developmental expression of the MCTs shows the reverse pattern. Rodent milk is very high in fat, and suckling pups are naturally ketotic, with circulating levels of β-hydroxybutyrate

approaching the millimolar range by the second postnatal week.[88,91] At this time, cerebral ketone body utilization also is maximal,[91] and this is further reflected in the level and extent of expression of MCT1, especially in the cerebral microvessels.[88] Thus the high levels of ketone body transporter in the blood-brain barrier, as well as in neurons and glial cells, not only facilitate ketone body transport into the brain but also can aid in the uptake and clearance of lactic acid. With cerebral maturation and the switch to reliance on glucose as the primary oxidative fuel of the brain, blood-brain barrier MCT1 falls to adult levels coincident with the rise in blood-brain barrier GLUT1 and the nonvascular glucose transporter proteins, especially neuronal GLUT3.[88] Some years ago, Magistretti and colleagues proposed that it is the astrocytes that play the primary role in cerebral glucose utilization by first metabolizing glucose to lactate, which subsequently is transported to the neuron to be oxidized to fuel neuronal energetics.[92] Although this theory, referred to as the *astrocytes-neuron lactate shuttle hypothesis* (ANLSH),[92] gained wide favor for several years, a more recent modeling of cerebral nutrient uptake and utilization that incorporated the concentrations and kinetic parameters of these transport proteins has demonstrated that the neurons are indeed the primary consumers of glucose, and actually produce lactate on activation.[93] Thus, although both lactate and ketone bodies are important fuels for neuronal metabolism in the fetal brain, cerebral development involves the transition to glucose utilization.

CEREBRAL ENERGY METABOLISM IN HYPOXIA-ISCHEMIA

Hypoxia-acidosis or asphyxia is a frequent cause of acute brain damage in human fetuses and newborn infants and contributes substantially to the chronic neurologic sequelae of cerebral palsy with or without associated mental retardation, epilepsy, or learning disability. Experimental and clinical evidence indicates that hypoxia alone does not produce brain damage but that superimposed cerebral ischemia is a necessary prerequisite for tissue injury to occur. Cerebral ischemia in the setting of asphyxia is a consequence of hypoxic-acidotic cardiovascular depression with secondary systemic hypotension. Cerebral ischemia also may be secondary to occlusive vascular disease of the brain (reviewed by Vannucci RC[94,95]).

Brain hypoxia denotes a cellular oxygen debt, owing to inadequate oxygen delivery (CBF × oxygen saturation [Sa_{O_2}]) by nutrient arteries. When the tissue (mitochondria) partial pressure of oxygen falls below a critical value (<0.1 mm Hg), the cytochrome system of mitochondria becomes unsaturated, and reducing equivalents (NADH, FADH) begin to accumulate.[96,97] ATP production by oxidative phosphorylation is curtailed, with concurrent increases in cellular ADP and AMP, because cytosolic ATP hydrolysis continues to drive endergonic reactions.[2] The elevations in ADP and AMP serve to stimulate glycolysis, through activation of its key regulatory enzyme, PFK (see earlier discussion). Unlike oxidative phosphorylation, which produces 36 moles of ATP for every mole of glucose consumed, glycolysis is an inefficient method to generate ATP by substrate phosphorylation, with net production of only 2 moles of ATP for each mole of glucose consumed. To produce the amount of ATP equivalent to that generated by oxidative phosphorylation, glycolysis would need to increase to a rate of 18 times its basal flux. In reality, glycolysis, even when maximally stimulated, is capable of increasing only four- to five-fold, owing in part to the concurrent accumulation of H^+ ions derived from the accumulated NADH, which ions serve to inhibit PFK activity.[4,44] In the immature rat, hypoxia-ischemia leads to a 135% stimulation of glycolytic flux, which equates to 62% of its maximal capacity.[98] Thus glycolysis can never completely substitute for mitochondrial oxidation, although its stimulation can supplement oxidative phosphorylation under conditions of a partial oxygen debt.

Cerebral hypoxia-ischemia severe enough to produce irreversible tissue injury is always associated with major perturbations in the energy status of the brain.[2,99,100] Alterations occur not only in the adenine nucleotides but also in PCr, in which changes actually precede those in ATP, ADP, and AMP. These perturbations have been well characterized in an experimental model of perinatal hypoxic-ischemic brain damage.[101] During hypoxia-ischemia, changes in the tissue concentrations of the high-energy phosphate reserves occur early during the course of the metabolic insult and persist well into the recovery period[102-104] (Figure 163-10). As expected, greater depletions in PCr relative to ATP occur as the cell attempts to maintain optimal levels of ATP through the CK equilibrium reaction driven also by the accumulation of ADP and H^+ ions. With the eventual decline in tissue ATP, ADP and AMP accumulate in proportion to the loss of ATP. Ultimately, the total adenine nucleotide pool (ATP + ADP + AMP) also decreases as AMP is catabolized slowly to adenosine and further breakdown products. The concentrations of ATP and the total adenylate compounds never completely recover after resuscitation, and their persisting partial depletions reflect the presence and severity of tissue destruction[102-104] (see Figure 163-10).

Of necessity, the loss of cellular ATP during hypoxia-ischemia severely compromises those metabolic processes that require energy for their completion. Thus ATP-dependent Na^+ extrusion (efflux) through the plasma membrane in exchange for K^+ is curtailed with a resultant intracellular accumulation of Na^+ and Cl^- as well as water (cytotoxic edema). Equally vital to cellular function is the prompt restoration of high-energy phosphate reserves during and after resuscitation. Without regeneration of ATP, endergonic reactions cannot resume, especially those involving the critical event of ion pumping at plasma and subcellular membranes. Intracellular Na^+ and Cl^- ions and water will continue to accumulate, and electrochemical gradients cannot be reestablished. Just how long the cell can survive under these conditions is not known, but other factors are called into play that prominently influence ultimate cellular integrity; including the secretion of potentially toxic excitatory amino acids into the synaptic cleft, the intracellular accumulation of free calcium, the intracellular production of nitric oxide, and the production of oxygen free radicals, the last especially during the recovery period (Figure 163-11).

The mechanisms by which ATP disruption persists into the recovery period relate to lingering alterations in the function of mitochondria. In this regard, the classic histopathologic studies of Brown and Brierley[105,106] indicate that the earliest morphologic alteration of the neuron arising from hypoxia-ischemia is a dilation of mitochondria with an accompanying separation of their cristae. Biochemical studies support the morphologic alterations to the extent that after hypoxia-ischemia in vitro, analysis of mitochondria reveals a disturbance in substrate oxidation, suggesting an "uncoupling" of oxidative phosphorylation.[107-109] An in vivo investigation in immature rats recovering from cerebral hypoxia-ischemia also suggests the occurrence of an uncoupling of oxidative phosphorylation before or concurrent with the development of morphologically apparent tissue injury.[110] It is assumed that reducing equivalents are oxidized in the presence of oxygen but ATP is not formed from the energy generated. Such energy is consumed internally (not transferred to the cytosol) or is lost as heat. That oxidative phosphorylation is compromised after hypoxia-ischemia is confirmed also by studies showing that the brain can be well oxygenated concurrent with a persistent depletion in ATP.[103,111-113]

The experimental data regarding the persistent depletion of cerebral high-energy phosphate reserves during recovery from hypoxia-ischemia have relevance to the clinical situation in the asphyxiated newborn human infant. Using MR spectroscopy (as described previously), investigators have shown a correlation between lingering alterations between the ratio of PCr to P_i or of ATP to P_i and ultimate brain damage as measured by

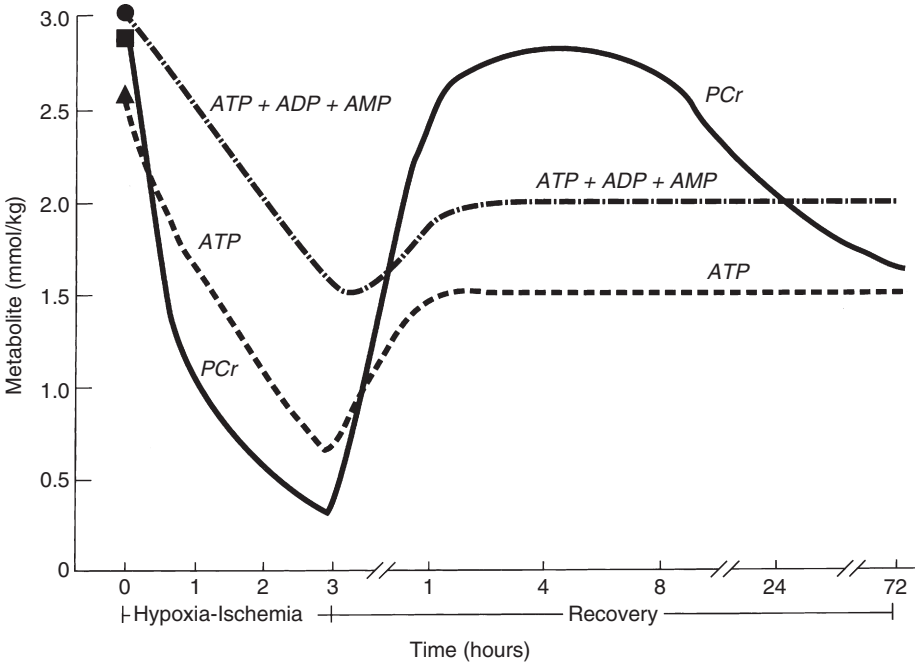


Figure 163-10 Changes in cerebral high-energy phosphate reserves during and after hypoxia-ischemia in the immature rat. Seven-day postnatal rats were subjected to unilateral common carotid artery ligation followed by exposure to systemic hypoxia with 8% oxygen at 37° C. *Symbols* represent means for ATP, PCr, and total adenine nucleotides (ATP + ADP + AMP). *ADP,* Adenosine diphosphate; *AMP,* adenosine monophosphate; *ATP,* adenosine triphosphate; *PCr,* phosphocreatine. *(Data from Rice JE 3rd, et al: The influence of immaturity on hypoxic-ischemic brain damage in the rat,* Ann Neurol *9:131, 1981; Palmer C, et al: Carbohydrate and energy metabolism during the evolution of hypoxic-ischemic brain damage in the immature rat,* J Cereb Blood Flow Metab *10:227, 1990; and Welsh FA, et al: Columnar alterations of NADH fluorescence during hypoxia-ischemia in immature rat brain,* J Cereb Blood Flow Metab *2:221, 1982.)*

neurodevelopmental status or neurologic assessment.[16,114,115] In this regard, PCr/P_i ratios can be normal in the human infant even when brain damage is present,[16,115] as has also been shown in experimental animals.[102,104,116]

A secondary depletion in brain PCr occurs at 18 to 24 hours of recovery from hypoxia-ischemia and beyond in immature experimental animals (see Figure 163-10),[102-104,117,118] which mimics the late changes in PCr/P_i observed in newborn human infants suffering asphyxial encephalopathy.[16,114,115] In newborn pig experiments, PCr/Pi ratios are depressed initially by hypoxia-ischemia only to normalize in the early recovery interval.[117,118] Thereafter, a secondary decrease in the ratio occurs, as indicated by measurements at 24 hours and again at 48 hours of recovery. From the human and animal studies, it has been proposed that the secondary failure in cerebral energy status after hypoxia-ischemia is a significant contributor to the ultimate brain damage and neurologic compromise.

However, the secondary decline in PCr does not necessarily denote a delayed energy failure of the brain but rather may reflect a loss of total creatine from the tissue or its conversion to creatinine. The late reduction in PCr, be it in an experimental animal or a human infant, appears to occur as a mass action effect of the CK equilibrium reaction.[104] Furthermore, the loss of creatine from the brain or its conversion to creatinine, which also would be lost, should result in detectable or increased concentrations of one or both metabolites in cerebrospinal fluid (CSF). The presence of these compounds in CSF may potentially serve as a biochemical marker of previous cerebral hypoxia-ischemia in a manner similar to and perhaps more sensitive than the adenine nucleotide derivatives xanthine and hypoxanthine.[119]

To clarify the issue of whether or not the secondary energy failure causes or contributes to the ultimate brain damage, Vannucci and associates[120] subjected immature rats to hypoxia-ischemia of the brain. During recovery, the brains were individually processed for histopathologic analysis and measurements of high energy reserves and the neuronal protein markers MAP-2 and SNAP-25. PCr and ATP, initially depleted during hypoxia-ischemia, both were partially restored during the first 18 hours of recovery, with secondary depletions at 24 and 48 hours (see earlier). During the initial recovery phase (6 to 18 hours), a significant correlation was observed between PCr and the severity of brain damage determined on histopathologic analysis, but not for ATP. During the late recovery phase (24 to 48 hours), a highly significant correlation was found between all measured energy metabolites and the severity of brain damage. Significant correlation also was observed between the neuronal markers MAP-2 and SNAP-25 and PCr, as well as the sum of PCr and creatine at both phases of recovery. The close correspondence of PCr with histologic brain damage and the loss of the neuronal markers during both the early and late recovery intervals suggest evolving cellular destruction as the primary event, which precedes and lead to the secondary energy failure.

CEREBRAL ACIDOSIS IN HYPOXIA-ISCHEMIA

Hypoxia-ischemia of a severity sufficient to produce brain damage also is associated with a tissue acidosis, owing to the accumulation of lactic acid.[2,99,100] The intracellular lactacidosis results from a shift in glucose utilization from oxidative metabolism to either partial or total anaerobic glycolysis which occurs during the tissue oxygen debt. Indeed, some investigators have suggested that lactacidosis is a major contributor to hypoxic-ischemic injury in vulnerable regions of the brain and that a minimum concentration of 15 to 20 mmol of lactate/kg of brain

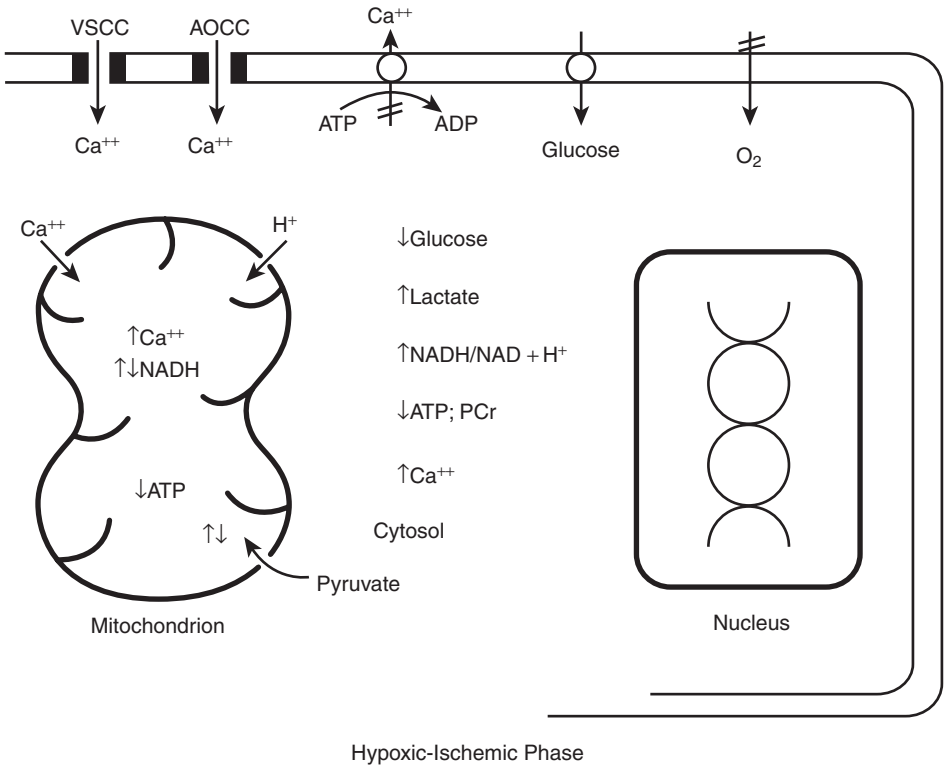


Figure 163-11 Cellular metabolism during cerebral hypoxia-ischemia. During a cellular oxygen debt, anaerobic glycolytic flux is accelerated, resulting in a depletion of intracellular glucose and an accumulation of lactate and H^+ ions (acidosis). The energy generated from anaerobic glycolysis cannot keep pace with energy demand, and ATP and PCr decline in the cytosol and mitochondria. Calcium entry into the cell is accentuated by voltage-sensitive (VSCC) and agonist-operative (AOCC) calcium channels, by which calcium accumulates in the cytosol and mitochondria. These disturbances are only partially reversed during reoxygenation and reperfusion, and continuing metabolic perturbations ultimately result in brain damage. *ATP,* Adenosine triphosphate; *PCr,* phosphocreatine.

tissue is required for irreversible damage to occur.[121-123] Furthermore, it has been shown that the injection of lactic acid into the cerebral cortex of adult rats leads to histopathologic alterations resembling those characteristic of ischemic infarction—an injury that does not occur after the injection of other organic acids of comparable pH.[124-126] In general, the more severe the tissue lactacidosis, the more profound the cerebral blood flow and metabolic alterations that occur after cessation of the insult,[123,127,128] portending greater ultimate brain damage.

It must be emphasized that hypoxia-ischemia leads to cellular acidosis by means of sources of H^+ ions in addition to lactic acid. Major sources of reducing equivalents include products of the acid hydrolysis of ATP and the formation of NADH + H^+, which accumulates during the cellular energy debt.[129] In this regard, Welsh and colleagues[103] examined the oxidation-reduction (redox) state of immature rat brain undergoing hypoxia-ischemia by the technique of reflectance fluorometry (Figure 163-12). Alterations in regional fluorescence, representing the intracellular accumulation of NADH (+ H^+), were prominent in the cerebral cortex and the CA1 sector of the hippocampus during the first hour of hypoxia-ischemia. The pattern of NADH fluorescence mimicked closely the distribution of selective neuronal necrosis observed in this model of perinatal injury. The close correspondence between altered NADH fluorescence and neuropathologic outcome suggests an important role for intracellular acidosis in the pathogenesis of hypoxic-ischemic brain damage. That a cellular acidosis occurs during the course of cerebral hypoxia-ischemia in the immature rat also is indicated by a progressive reduction in intracellular pH, calculated from the tissue concentrations of high-energy phosphate reserves.[130]

Using proton MR spectroscopy, several investigators have demonstrated a persistent elevation in brain lactate after cerebral hypoxia-ischemia in human newborn infants.[20,21,131-133] The persisting elevations in lactate concentration were associated with a brain alkalosis. The previously asphyxiated infants uniformly demonstrated severe neurodevelopmental impairment at 1 year of age. Robertson and associates[133] suggested that the accompanying cerebral lactic alkalosis is the consequence of a prolonged change in the redox state within neuronal tissue, the presence of phagocytic cells, the proliferation of glial cells, or altered buffering mechanisms. Further research can be expected to clarify these lingering biochemical abnormalities after cerebral hypoxia-ischemia in newborn human infants.

CONCLUSIONS AND OUTLOOK

In the overview of current knowledge on the major aspects of perinatal cerebral metabolism presented in this chapter, the emphasis is on substrate and energy transformations under physiologic conditions and during hypoxia-ischemia. These and other disturbances recently have undergone extensive investigation in both experimental animals and human newborn infants. Further characterization of the mechanisms underlying the cerebral metabolic derangement that characterizes hypoxia-ischemia will be fundamental to development of a rational approach to prevention and treatment.

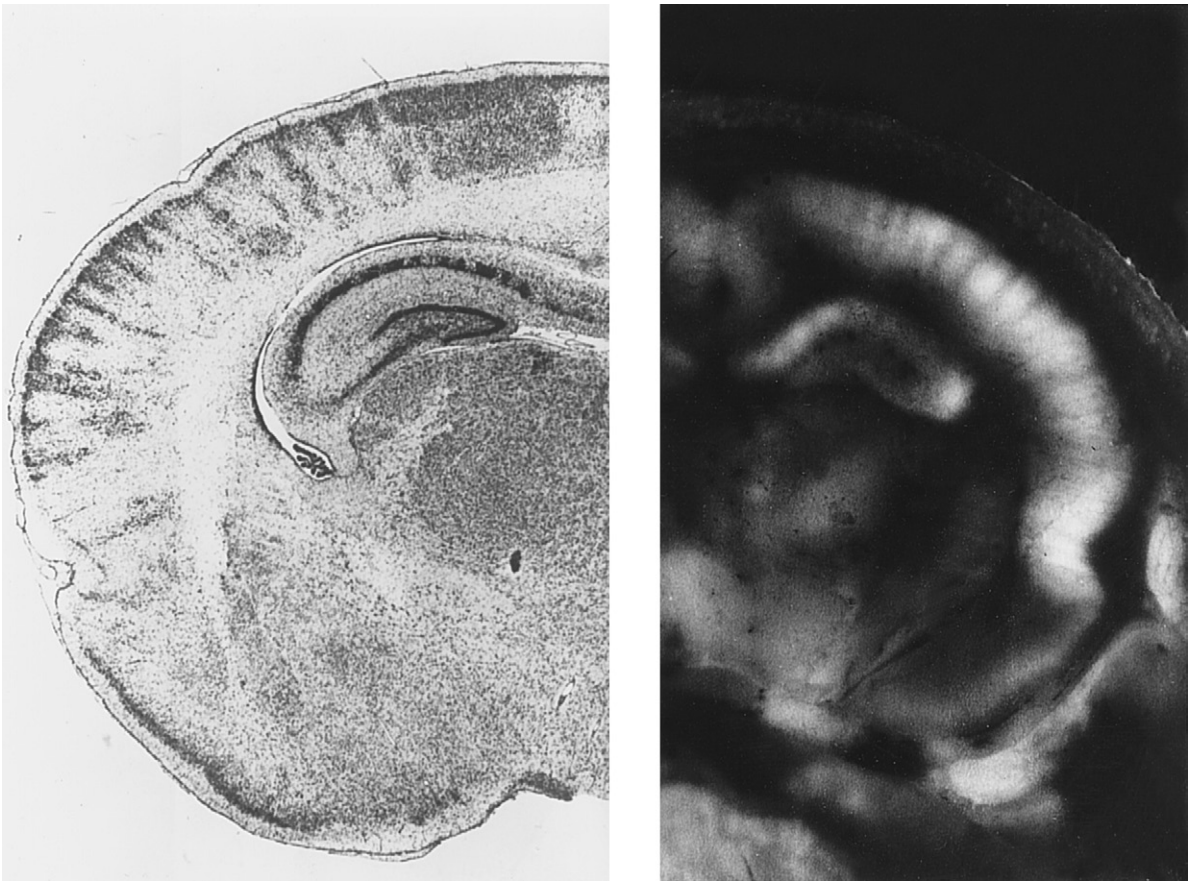


Figure 163-12 Coronal section of immature rat brain showing alterations in NADH fluorescence *(right)* during hypoxia-ischemia and histopathologic alterations *(left)* at 24 hours of recovery. Seven-day postnatal rats were subjected to unilateral cammon carotid artery ligation followed by systemic hypoxia with 8% oxygen at 37° C.[100] During hypoxia-ischemia, the rat brain was frozen in situ and the coronal section illuminated with ultraviolet light; the fluorescent image then was recorded photographically.[102] Note the columnar pattern of NADH fluorescence *(lighter areas)* in cerebral cortex and the enhanced fluorescence in the CA1 and CA3 sectors of the hippocampus, which correspond closely to the distribution of selective neuronal necrosis seen on histopathologic examination. *NADH,* Nicotinamide-adenine dinucleotide (reduced form).

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Robert Ryan Clancy

Electroencephalography in the Premature and Full-Term Infant

The escalation of interest in the electroencephalogram (EEG) of the premature and full-term neonate in the past decade has been remarkable. A lot of attention has been directed to scrutinize the biological underpinnings of EEG in the neurodevelopmental context of brain growth, migration, and neuronal-synaptic connectivity. What is the fundamental basis of the large scale discontinuity that typifies EEG maturation in the immature brain and what gives rise to some of the distinctive patterns of the EEG of premature infants, such as the familiar but short-lived delta brush?[1] Another area that has enjoyed especially robust growth is the use of bedside "cerebral function monitors" intended to be applied and interpreted by nonspecialized caregivers, such as neonatal nurses and physicians.[2-4] Although they represent reduced, simplified EEG formats, their ease of use, greater availability and proficiency to display EEG background trends over very long time periods have sparked much interest in their use across the world. Thus it would be helpful to begin by differentiating the three broad levels of EEG recording.

Conventional EEG recording is the major focus of this chapter[5] (Table 164-1). It refers to the traditional style of EEG examination in which an EEG technologist is summoned to the bedside to carefully measure the scalp to apply about 9 to 11 recording electrodes. A routine EEG runs for 45 to 60 minutes and may capture one or more sleep cycles. The EEG amplifiers filter the "raw" EEG signal, allowing frequencies from 0.3 to 70 Hz to pass. The filtering of the very low frequencies allows for the appearance of a stable signal "platform" that facilitates easier interpretation in the clinical arena. A common recording rate is 1 sec of EEG per 15 mm of display (Figure 164- 1). Long-term EEG monitoring can be conducted by continuous conventional EEG recording at the bedside, often accompanied by time-synchronized digital video. Thus a long-term record that combines a video of the clinical activity of the child with coincident EEG is generated and is very useful to detect and quantify electroencephalographic seizures.

High-fidelity neonatal EEG examinations are currently a research tool intending to provide better spatial resolution by high-density recordings obtained by increasing the number of scalp electrodes using specially developed multielectrode caps and more faithful capture of ultra-low frequency signals that are characteristic of the most immature brains.[6] Because of the limited cortical-to-cortical connectivity of the immature cortex, it is possible that a higher number of electrodes can better measure the degree of electrical connectivity between adjacent or distant cortical areas and how this changes *pari pasu* with underlying brain development (such as the emergence of thalamocortical connections or the appearance of short- and long corticocortical connections). Because very slow EEG frequencies are one of the hallmarks of the immature brain, high-fidelity EEG does not arbitrarily filter out low frequency signals as does conventional EEG. Instead, it attempts to capture even the slowest signal by using direct current (DC)-coupled amplifiers that record down to zero frequency (that is, stationary or DC waves).[7] High-fidelity EEG examinations can be combined with video recordings and are usually recorded for 1 to 2 hours.

Cerebral function monitoring (CFM) was first introduced by Prior and Maynard in the 1960s in adult intensive care unit settings. By the early 1980s, they were used increasingly in neonatal care.[8] CFM is intended to be a simplified method of displaying EEG data over extended time periods that can be applied and interpreted by nonneurology specialists, such as neonatal physicians and nurses. Currently the most popular format of CFM is called amplitude-integrated EEG or aEEG.[2,9-11] It is derived from 1 or 2 channels (2 or 4 *recording* electrodes) placed on the scalp by bedside caregivers. The signal is heavily filtered, removing everything except 2 to 15 Hz frequencies. It is rectified, smoothed, and extremely time compressed with 1 minute of EEG per millimeter of display (see Figure 164-1). The signal is displayed linearly from 0 to 10 μV and semilogarithmically from 10 to 100 μV. This provides a very convenient method to follow the "envelopes" of the upper and lower margins of cerebral electrical activity over extended time periods. In healthy infants, the upper amplitude margin ranges from 30 to 50 μV, and the lower margins range from about 6 to 11 μV. In contrast, an abnormally suppressed aEEG has less than 10 μV for the upper margins and less than 5 μV for the lower amplitude margins.

The CFM, which can be continued for many hours or days, generates a simple output signal that provides useful information of the status of the central nervous system that can be interpreted by nonneurologists. Two competing background classification systems are currently employed by neonatologists to judge the normality of the EEG background. One uses simple voltage

TABLE 164-1

Comparisons of Contemporary EEG Recording Techniques

Type of EEG Recording	Typical Duration of Recording	Number of Recording Electrodes	Low Frequency Cutoff (Hz)	High Frequency Cutoff (Hz)	Recording Speed	Display Length of One Minute EEG
Conventional EEG	45-60 mins	9 to 11	0.3 (AC-coupled amplifiers)	70	15 mm/sec	900 mm
"High fidelity" EEG (research)	60 to 120 mins	20 or more (up to 100)	0 (DC-coupled amplifiers)	70	15 to 30 mm/sec	900 to 1800 mm
Amplitude integrated EEG	hours to days	2 or 4	2	15	1 mm/min (6 cm/hr)	1 mm

criteria[2] and the other a visual pattern recognition system[12] to classify records as normal or abnormal. These two methods are compared in Table 164-2.

The CFM can be used to estimate brain functional integrity quickly in the immediate wake of an acute encephalopathy, such as hypoxia-ischemia (e.g., deciding when a patient is appropriate for enrolment in a neuroprotection trial, which typically must be started within a few hours of the insult), screen for electrographic seizures automatically, and show trends of brain status during serious illness or response to treatment,[9,13,14] but its usefulness in neuroprotection trials has been questioned.[15]

Other Methods to Mathematically Process EEG Signals

Although the EEG "signal" is extremely complex, it is still describable in mathematical terms and subject to signal processing, quantitative analyses, and statistical manipulations. Just as an optical prism can separate white light into its component colors, the neonatal EEG can be decomposed into the sum of its component frequencies in the traditional bandwidths of delta (≤ 3 Hz), theta (4-7 Hz), alpha (8-12 Hz), and beta (≥ 13 Hz) frequencies.[16] EEG signal quantification based on this principle of analysis[17] simply describes the amount of electrical power (measured in picowatts) that is confined to each of these frequency "bins." In healthy neonates, the robust power of the neonatal EEG is largely relegated to the slow (theta and delta) frequency bins (Figure 164-2). Using this relatively simple approach, the EEG of the healthy neonate can be used to develop normative quantitative profiles of electrical power distribution across frequency bandwidths in the awake and asleep states, which serve as a benchmark to compare with those demonstrating acute encephalopathy.[10] The intensity and frequency distribution of the neonatal EEG can similarly be determined by *spectral analysis*. The spectral edge frequency is defined as the frequency below which 90% of the EEG's power falls. It has been shown, for example, that in premature infants, progressively lower spectral edge frequencies predict increasing severities of periventricular leukomalacia.[18]

Mathematicians may choose from an array of other analytical approaches to analyze or quantify the neonatal EEG. The short-time Fourier transform, higher-order spectral analysis (the so-called bispectrum), and autoregressive segmentation are the names of some of the more popular mathematical tools used to analyze and model complex biologic signals, such as the EEG background.

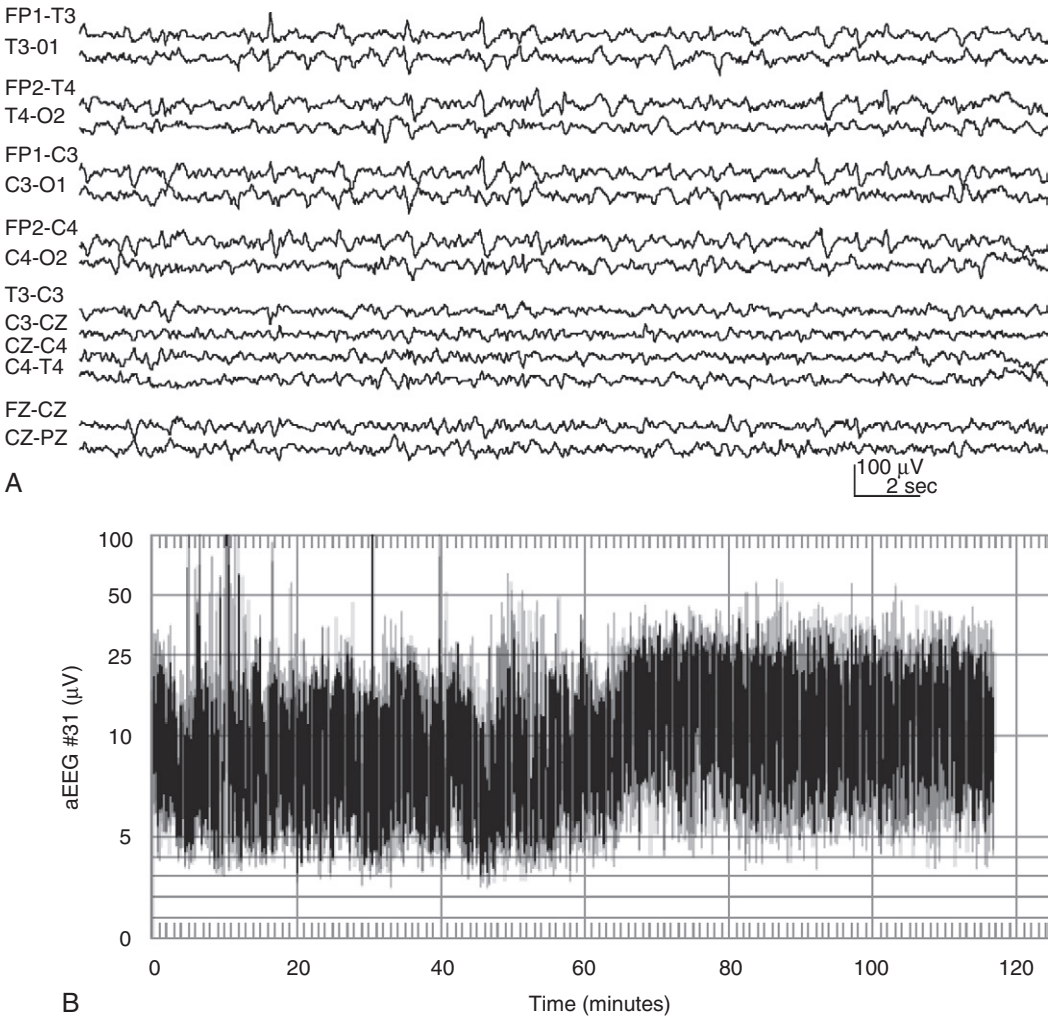


Figure 164-1 A, 30-second segment of a conventional neonatal electroencephalogram (EEG) examination, recorded from a 7-day-old full-term infant during wakefulness. During this portion of the record, the child is alert but calm. The EEG itself is low-to-medium voltage, continuous, and represents an "average" mixture of EEG frequencies (activité moyenne). During active sleep, the EEG has a similar appearance. **B,** Example of nearly 120 minutes of a normal amplitude-integrated EEG recorded from a 5-day-old infant with an estimated gestational age of 39 weeks. The upper margin exceeds 10 µV and the lower margin exceeds 5 µV.

A unique approach is the application of nonlinear dynamics or chaos theory to neonatal EEG analysis. The principle is simple. The normal neonatal EEG is extremely rich in its complexity and inherent "unpredictability." The status or appearance of the background at one moment in time does not predict the next with any probability. The normal neonatal EEG has many "dimensions" of chaos or complexity (unpredictability). In contrast the EEG of a sick neonate with an extremely low voltage, featureless background. or burst suppression (with its regular cycles of "off-signal" and "on-signal") becomes predictable with reduced dimensions of chaos. Finally CFM may analyze the raw EEG signal by neural networks, constructed to achieve an artificial intelligence approach to "decide" whether a novel *test* EEG segment is normal. The network is first instructed to "learn" the boundaries of the normal EEG based on input from a wide assortment of normal EEG samples representing many healthy neonates in a variety of biobehavioral states. After the network has "learned" how normal EEG segments appear, a test EEG sample can be submitted for analysis. If the neural network algorithm recognizes the test segment as comparable with previous normal segments in its memory, the analysis designates the test segment as "normal." Thus this approach does not generate a graphic depiction of EEG function but rather categorical variables, such as a "normal," "moderately abnormal," or "markedly abnormal" EEG.

Analytic algorithms can also be written to recognize EEG seizures. Computer-based EEG seizure detection algorithms are highly sensitive in older children and adults, but they are more challenging to design for use in neonates because of the different ictal waveform frequencies and characteristics.[19-21] Brief, low-amplitude electrographic neonatal seizures are especially difficult to detect. The distinctive clinical motion of some neonatal seizures can even be recognized by algorithms that evaluate the digital video images of babies during their seizures.[22]

TABLE 164-2

Comparison of Two aEEG Classification Systems

Background Classification	Simple System[2] Discrete Voltage Criteria	Advanced System[12] Pattern Recognition Criteria
Normal	Upper margin >10 μV and lower margin >5 μV	"Continuous normal voltage" Continuous EEG activity with amplitudes ranging from lower values of 7-10 μV to upper values of 10-25 μV; sleep-wake cycles evident
Moderately abnormal	Upper margin > 10 μV and lower margin <5 μV	"Discontinuous" Discontinuous background with lower amplitudes <5 μV and upper amplitudes >10 μV
Markedly abnormal	Upper margin <10 μV and lower margin <5 μV	1. "Burst suppression" Invariate, discontinuous background with low amplitude interbursts (0-1 μV) intermixed with higher amplitude (≥25 μV) bursts. 2. "Continuous low voltage" Continuous background of very low voltage (≤5 μV). 3. "Flat tracing" Mainly isoelectric, with some very low amplitude activity

EEG Monitoring for Neuroprotection Trials

One of the goals of contemporary neonatology is the successful completion of neuroprotection trials. Some trials involve obvious selection criteria of suitable candidates. Premature infants are at high risk for the development of periventricular leukomalacia[23] and have been the subjects of pharmacologic trials with allopurinol, magnesium, steroids, caffeine,[24] and other agents to reduce its occurrence. Likewise, infants with complex congenital heart disease, requiring surgery that uses deep hypothermic circulatory arrest, commonly experience postoperative seizures and long-term neurocognitive disorders and are easily recognized for enrollment in neuroprotection trials.[25] On the other hand, neuroprotection trials in hypoxic-ischemic encephalopathy in the term neonate have difficult challenges.[26-30] Currently no universally agreed upon research definition of hypoxic-ischemic encephalopathy is used to select candidates for enrollment. In this setting, the time for identification of truly encephalopathic infants is short. The window of enrollment into a protection trial, such as deliberate hypothermia, must be determined immediately after birth. Both conventional and aEEG have been proposed to assist in the identification of suitable candidates for neuroprotection trials because the quality of the EEG signal shortly after birth is one of the best early predictors of mortality and morbidity.[31,32]

BIOBEHAVIORAL STATES AND CONCEPTIONAL AGE

Normal brain development is intimately connected with the appearance and maturation of sleep cycles. Rather than being a passive period of "rest," sleep is actually an active process that is crucial for neurosensory development, memory, and the preservation of brain plasticity. During wakefulness, our brains are constantly barraged with an array of stimuli from all of our senses. Some of these stimuli have importance for survival and are worthy to consolidate into short- and long-term memory, but many

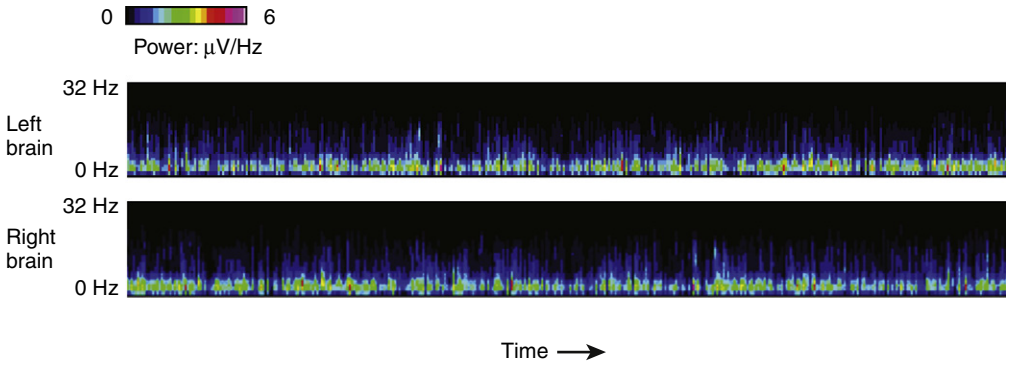


Figure 164-2 Power spectral array display of 12 hours of electroencephalogram from electrodes originating in the left *(upper figure)* and right *(lower figure)* hemispheres. Power, represented as μV/Hz, is displayed as the brighter colors concentrated in the slower (0-5 Hz) frequencies.

others are not. During sleep without rapid-eye movement (REM), the brain somehow selects those experiences to remember that seem advantageous for survival. During REM sleep, these memories are consolidated, organized, and entered into long-term memory, ready for later recall upon the correct cue. Deprivation of REM and non-REM sleep disturbs the development of sensory systems, learning, memory, plasticity in adulthood and even the ultimate size of the brain.

Recognizable patterns of activity that can be assigned as "sleep" (as opposed to wakefulness or drowsiness) first appear between 25 to 30 weeks gestational age (GA).[33,34] Sleep cycles in utero can be inferred by fetal heart rate monitoring. which shows cycles of increased fetal heart variability associated with movements (active sleep), interspersed with periods of diminished fetal heart rate variability and reduced or absent movements (quiet sleep).[35,36] In the infant born with substantial prematurity, sleep cycles can be inferred by direct visual observation of periods of movement and associated changes in the character of the EEG background. The length of the sleep cycle is long in the very premature infant (up to 90 minutes). Thus it is more difficult using conventional EEG (which may only record 45 to 60 minutes) to identify sleep cycles in the very premature baby. Typically sleep cycling can be identified in approximately 50% of premature infants as early as 25 to 30 weeks.[33] On the other hand, aEEG, which is typically recorded for many hours or days, has an advantage of showing periods of sleep cycling at an earlier age than conventional EEG. Thus, using aEEG, some investigators report the onset of definite sleep cycling at an earlier GA and even "acceleration" of EEG maturity compared with conventional EEG.[37]

It is no surprise that the harsh environment of the neonatal intensive care unit may disrupt normal sleep cycles. In contrast to the natural protection of the womb, with no light or pain and less sound and tactile stimulation, the sick neonate is subjected to multiple daily painful procedures, high ambient noise and light, and constant disruption of their sleep cycles. Indeed, it is a wonder that they can establish sleep cycles at all! These observations have lead to philosophies of care in some nurseries that attempt to minimize handing, pain, and sleep disruption. One approach, called "kangaroo care" involves sessions in which the premature baby sleeps peacefully and undisturbed skin-on-skin with the mother. Simultaneous EEG examinations have shown significant improvement in quiet sleep cycles with even a limited number of these sessions.[38]

LABILITY OF BEHAVIOR STATES AND CONCEPTIONAL AGE

The rhythms of our daily lives are not uniform but are woven into a rich variety of activities and behavior. In the early morning we awaken in a relaxed, refreshed, and replenished *state.* Soon we generate activities in preparation to begin the day's work—we are then in a different behavioral *state* of increased activity and heightened physiologic alertness and anticipation. At the peak of the day's work, a *state* of intense concentration, activity, and arousal may exist. By evening, our activity level retraces its steps back through similar behavioral states as we wind down for the day. At night a drowsy state heralding the onset of light sleep approaches. Light sleep surrenders to a deeper sleep state until the crisp onset of our first dream *state.* These *sleep states* are organized into patterns that repeat through the night until we awaken the next day and a new cycle begins.

Despite the superficial appearance of disorganization and anarchy that marks a brief and casual inspection of human newborn infants, they display remarkably predictable and recognizable states: constellations or functional clusters of physiologic, behavioral, and neurologic parameters that describe the net condition of the infant.[39,40] An infant's state is the outward and inward expression of neural activity, which can be modified by the environment. The state of a peacefully sleeping infant can be transformed into an aroused or awake state by a sufficiently potent environmental stimulus (light, noise, or touch). State can also fluctuate in response to an internal trigger—a happy and content infant can quickly engage in a frenzy of crying once the pangs of hunger surface. The ability of an infant to display organized and cyclic behavioral states is a useful yardstick to measure the maturity and health of the neonatal brain.[41] Extremely premature infants lack the physical neurologic sophistication required to harmonize and orchestrate the ingredients of recognizable states. Full-term infants do possess the neural equipment for explicit state organization, but this can be altered by the presence of disease. Drugs, medical disorders, unnatural environmental stresses, or neurologic disease consequently may disrupt and distort the normal predictable flow of infantile behavioral states.[42]

Because the presence and organization of electroclinical states are a reflection of neurologic health and maturity, the *conceptional age* (CA) of the infant should be known. Conceptional age is calculated by adding the estimated gestational age (EGA) to the legal age or chronologic age since birth. Thus a healthy 29-week EGA premature baby with a legal age of 11 weeks has the same CA (29 weeks + 11 weeks = 40 weeks) as a 1-day-old full-term 40-week baby. Although some small neurologic-behavioral differences exist between these 40-week CA babies, they are essentially equivalent in terms of their state of organization and EEG activity.[37,43,44]

Early Development of the EEG in Relationship to Developing Brain Morphology

Early infancy is a period of explosive growth and development unparalleled by any other epoch in the human life span.[45,46] The clinical changes observable are the outwardly visible signs of underlying anatomic, physiologic, and metabolic central nervous system (CNS) maturation.[47] A general description of early brain development, the ontogeny of early infantile states, and associated EEG maturation follows.

The cortex of the human fetus has established its foundation by week 8 of gestation (Table 164-3). Brain function, as measured by the first appearance of an EEG signal, commences shortly thereafter.[46,48] Early fetal movements after 8 weeks gestation are demonstrable by real-time ultrasound. However, at that age, motor activity is almost entirely issued by the brain stem, subcortical structures, and the spinal cord itself.[49]

Correlated neuronal activity plays a pivotal role in cortical development by influencing neuronal differentiation, migration, synapse formation, and integrated neural networks.[50] The relationship between observed EEG patterns that for decades have been recognized purely as empirical phenomena and specific details of neurodevelopment that underlie them have just recently begun to be unraveled.

The earliest cerebral activity appears in the form of spontaneous, high-voltage, random electrical bursts that are believed to arise from pyramidal neurons of the cortex, connected by excitatory synapses.[1] These spontaneous bursts arise before sensory mechanisms have become functional. That is, they are not provoked by arrent volleys but arise intrinsically. The process of cortical folding begins just after 23 weeks gestational age (GA) and continues predictably and sequentially well beyond term. Biagioni and colleagues[51] reported the relationship between cortical folding shown by brain MRI examinations and a number of well-known EEG characteristics in premature babies between 23 and 29 weeks GA. EEG characteristics such as discontinuity and delta brushes were examined for correlation with brain anatomy. The degree of cortical folding was significantly associated with age and the degree of EEG background discontinuity. However, delta brushes and other transient EEG elements were not.

TABLE 164-3

Electroclinical Timetable

Age Since Conception	Behavior	Electroencephalogram (EEG)
<8 wk	Incomplete cortex; intermittent muscle activity generated by the brain stem	No EEG activity
8+ wk	Periods of more sustained motor activity	First appearance of EEG activity
12-14 wk	Onset of intermittent bursts of regular respiratory muscle activity (breathing movements)	
16-20 wk	Quickening	
24 wk	Eyelids unfuse; operationally, wakefulness (eyes open) altermates with sleep (eyes closed)	Invariant, discontinuous EEG
30 wk	First organization of state: consolidation of physiologic and behavioral parameters that distinguish wakefulness and sleep states	EEG relatively continuous during wakefulness or active sleep; more discontinuous EEG during quiet sleep
32-34 wk	Further consolidation of behavioral state: the child spends most of the time in an indeterminant sleep state; sleep cycle times are brief; better concordance between simultaneous behavior state and the EEG	Clear differences are seen in the awake, transitional sleep, active sleep, and quiet sleep EEGs; the tracing is discontinuous (tracé discontinu) mostly during quiet sleep; the EEG can be modified by external stimulation (reactivity)
36-38 wk	At least six distinct behavior states are defined; most of the infant's time is spent asleep; sleep onset begins with active asleep	As concordance improves there is less indeterminant sleep and more quiet sleep; mature continuous slow-wave sleep makes its appearance during quiet sleep; continuous slow- to moderate-voltage mixed frequency activity (activité moyenne) presides during wakefulness and active sleep
40-44 wk	Well-defined behavioral states continue; the infant spends most of the time asleep	Dissolution of many immature EEG patterns (e.g., waning of the Δ brush); emergence of mature quiet sleep states and sleep spindles
4 mo postdelivery	The infant has "settled" through the night and established daytime patterns for eating, naps, and play; onset of deliberate volitional motor patterns such as reaching for items of interest; sleep onset is quiet sleep	The percentage of active sleep approaches adult value; longer sleep cycle time and asynchronous sleep spindles in quiet sleep; during wakefulness precursors of α and μ rhythms appear

These spontaneous bursts of EEG activity serve as the most conspicuous feature of the premature EEG until they eventually yield to the appearance of continuous EEG activity as GABAergic neurotransmission evolves from excitatory to inhibitory. One of the deep mysteries surrounding neurodevelopment is the identification of the factors that select and cement precise neuronal connectivity and produce functionally connected networks. The number of neurons and their synaptic connections vastly dwarfs the number of genes that could conceivably be involved in fashioning them. Because genetics alone cannot account for all of the functionally relevant connections, the process is considered *epigenetic*. The sculpturing process requires spontaneous signals, such as the "self-organizing" bursts of the immature discontinuous EEG, to initiate functional contact with neighbors. The connections within the fledgling network are then reinforced through repeated usage.[1] The operational mantra of these incipient networks is that neurons that "fire together, wire together." Because brain development is so dependent on sensory feedback and the fetus has limited contact with the environment, it has been challenging to understand how both the visual[52] and somatosensory system possess endogenous mechanisms that advance neuronal connectivity.

The actual bursts of spontaneous cerebral activity are themselves complex electrical events. They are comprised of multiple EEG frequencies and several easy to recognize patterns, such as delta brushes,[50,53] rhythmic occipital theta bursts, positive slow waves,[54] rhythmic temporal theta bursts, and other specific named patterns. An elegant neurodevelopment story can now be told of the delta brush (Figure 164-3), which will be discussed again later from a clinical EEG perspective. In the course of normal gestation, expectant mothers report the onset of perceived fetal movements ("quickening") around week 17 to 18 of gestation. Such movements arise spontaneously from discharges in the fetal brainstem or the motor nuclei of the spinal cord. The resulting limb movements, however, trigger a set of functionally associated sensory messages that report back cutaneous sensations and volleys from muscle and joint position sensors. These ascend through sensory pathways to the thalamus, subplate, and overlying cortex, creating a learned, integrated sensori-motor physiologic loop specific to that particular sequence of movement. It is now understood that the familiar "delta brush," an EEG pattern only encountered in the immature brain, is the electrical embodiment of that process.[50] It has recently been shown that the delta brush can appear in response to an endogeneous event (such as fetal kicking) but can also be elicited by gently stroking the skin of a premature baby.[50] Thus sensory feedback from spontaneous muscle activity triggers delta brush oscillations in a somatotopic manner. The delta brush plays a critical role in the development of thalamocortical connections. Delta brushes are consistently seen over the sensorimotor cortex by 24 weeks CA, peak around 30 weeks CA when interneurons begin their extension from the temporary subplate to the overlying cortex, and begin to fade after 35 weeks. In the youngest premature infants, these functional columns behave as isolated islands, answering only to their specific assigned sensorimotor task. Later, however, as long and short range cortical-to-cortical connections appear, functional columns become integrated with and between the hemispheres. With the appearance of corpus callosal fibers around 34 weeks CA, it can be shown that delta brushes appear somewhat synchronously between homologous regions of the two hemispheres, required for sensorimotor coordination (Figure 164- 4). The era of neonatal EEG as a strictly phenomenological description of visual "graphoelements" empirically useful to the bedside clinician has begun to be supplanted by a deeper appreciation of its real message as revealed by the work of developmental neuroscientists.[7]

Fetal EEG

Early attempts in 1970 to record fetal EEG were unsuccessful; investigators attached recording electrodes to the mother's abdomen and the distant and feeble (microvolt) brain electrical activity was not consistently detectable with conventional recording

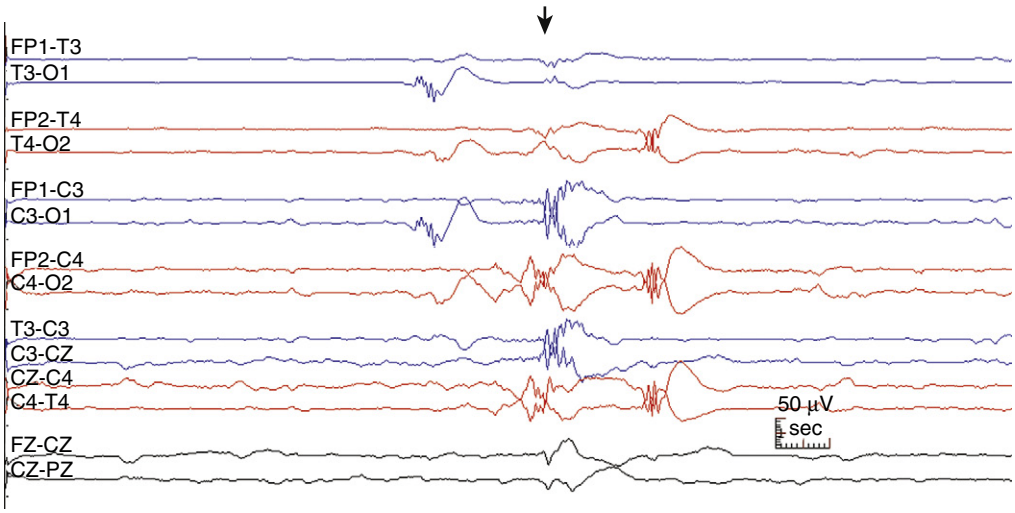


Figure 164-3 The delta brush is a premier example of a distinctive waveform that is a hallmark of the premature electroencephalogram. The delta brush represents a combination of a surface positive slow (delta) transient with an overlying burst of faster (beta) rhythms. It is now believed that delta brushes result from sensory feedback to the cortex from spontaneous limb movements or can be elicited by tactile stroking of the skin. It is believed that such electrical patterns help shape the consolidation of developmental sensory-motor networks.

equipment.[55,56] It is now possible to briefly record fetal EEG during the process of labor and delivery after the maternal membranes have ruptured so that fetal scalp electrodes can be attached. However, this line of investigation simply attempts to measure fetal brain response to the stresses of labor, similar in some respects to using "reassuring" or "nonreassuring" fetal heart rate monitoring to gauge the fetus tolerance to labor. Such brief fetal EEG recordings are not useful in measuring developmental or maturity-based observations. The use of magnetoencephalography (MEG) has opened a new window on examining the developing fetus in situ.[57,58] MEG exploits the fact that synchronized neuronal electrical currents induce very weak magnetic fields that can be measured by extremely sensitive recording devices, such as superconducting quantum interference devices. However, the magnetic strength of the brain is weak compared with fields generated by the general environment or maternal somatic muscle and heart activity. In the case of fetus MEG, specially constructed detection arrays are fabricated to conform to the gravid abdomen and the magnetic fields generated by the fetal heart and the mother's body detected and cancelled. The resulting filtered and processed signal displays the salient features of conventional EEG recorded from prematurely born infants. For example, fetal MEG showed the common features of the premature EEG recognized for decades, such as discontinuity, delta brushes, and sharp EEG transients.

Clinical Recognition of State

In the postnatal arena, the operational definition of the awake state requires that the eyes be open (Table 164-4). This is not possible before week 24 of gestation when the eyelids are fused together. Thereafter, it is possible to distinguish an *awake* infant (with eyes open) from an *asleep* infant (with eyes closed). However, except for this eyes open–eyes closed awake-asleep dichotomy, the behavioral state of the infant remains amorphous and primordial. No distinctive EEG changes uniquely discriminate between the aroused and the sleeping infant.[59] Scher and colleagues[33] reported the appearance of sleep-wake cycles in 33 preterm infants between 25 and 30 weeks CA. Some form of sleep-wake cycles was visible in 24 (73%) with a sleep cycle time ranging from 37 to 100 minutes. By 30 weeks gestation, the first differences between the EEG in awake and sleeping infants emerges, and the notion of "concordance" arises. Concordance describes the agreement between the electrographic and clinical expression of the infant's state: an awake (eyes open) infant displays a more continuous and low-voltage "aroused" EEG background, whereas a sleeping (eyes closed) infant has a different-appearing EEG that is appropriately matched to the clinical sleep state.

Concordance is only marginally and inconsistently established in the 30-week premature infant. At that age, some segments of the tracing demonstrate some agreement between the clinical and electrographic expressions of state. Thus an awake infant who displays eye opening, irregular respirations, and motor activity has a coincident EEG showing a somewhat continuous pattern that is in agreement (concordant) with wakefulness. In contrast, in quiet sleep, the eyes are closed, the baby is relatively still, respirations are more regular, and the EEG assumes greater discontinuity than during wakefulness or dream sleep.[60] In a few more weeks (approximately 32 to 34 weeks gestation), the healthy premature baby demonstrates much better concordance for longer portions of each tracing. Indeed, at that age, a 1-hour EEG recording might capture several electroclinical behavioral states.

At term, a typical sequence of electroclinical states can be demonstrated (see Table 164-3). During wakefulness, the eyes remain open, and the appearance of alert vigilance presides. The level of muscular activity varies from calm quiet to vigorous limb thrashing with crying. The respiratory pattern is irregular. The simultaneous EEG is essentially continuous; interruptions in the nonstop production of low-to moderate-voltage, mixed-frequency cerebral rhythms are few.[61] The infant may next enter a drowsy state as the harbinger of sleep. The voltage of the continuous EEG pattern increases as more slow activity (Δ) appears. As drowsiness deepens, slow, rolling eye movements begin and the eyelids close. The infant may briefly occupy a transitional or indeterminant sleep state. Criteria to unequivocally classify the electroclinical state as fully developed active or quiet sleep are insufficient. However, the infant's state soon crystallizes into definite active sleep. Sleep onset that begins with active sleep contrasts sharply

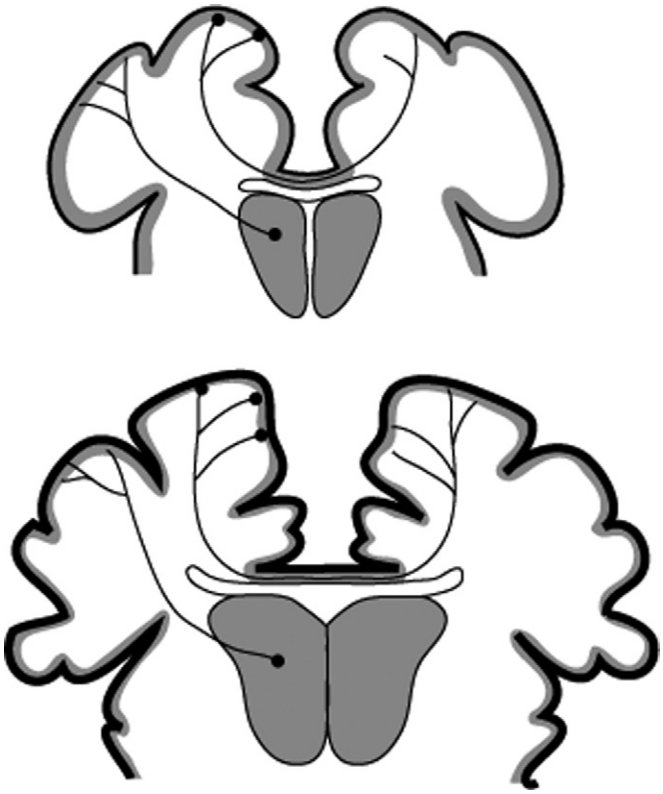


Figure 164-4 Thalamocortical and long corticocortical connections evolve rapidly in early brain development. At 28 weeks gestation *(upper figure),* the thalamocortical projections are just being connected to the transient subplate region below the cortex, and long corticocortical projections through the corpus callosum fall short. By 30 weeks gestation *(lower figure),* the subplate is receding and thalamic projections actually impinge on the cortex while corticocortical projections are just arriving there. *(Adapted from Vanhatalo S, Kaila K: Development of neonatal EEG activity: From phenomenology to physiology,* Semin Fetal Neonatal Med *11:471, 2006.)*

with the onset of sleep in healthy older children and adults who usually enter quiet sleep first. Individuals with the sleep disorder narcolepsy also begin their sleep cycle with active or REM sleep. During active sleep, the eyes remain closed. There are bursts of conjugate, jerky, rapid, predominantly horizontal eye movements. REMs are not homogeneously distributed during active sleep but appear in grouped clusters. The coincident EEG mostly resembles that of wakefulness, that is, a continuous low-to-medium-voltage mixture of EEG frequencies. Indeed, the cerebral cortex is in a similar "aroused" mode whether the subject is awake or engaged in the mental activities of active sleep. The voltage of the EEG signal attenuates in the setting of wakefulness, dreaming, or arousal from quiet sleep because of the removal of the synchronization that exemplifies quiet sleep. The low-voltage EEG is a result of desynchronization of cortical activity.[62]

After the first active sleep period concludes (the first active sleep state may last only 15 to 25 minutes at that age), the infant again enters transitional or indeterminate sleep that culminates in quiet or non-REM sleep. The baby's body is then "quiet" or motionless except for deep, regular, slow excursions of respiration. The face is expressionless, save for occasional bursts of sucking automatisms. The EEG substantially changes its character as the pattern becomes discontinuous. The voltage of the cortical signal then regularly fluctuates between higher voltage segments

TABLE 164-4

Electroclinical States at Term

		ASLEEP	
Electroclinical Parameters	**Awake**	***Active (REM or Dream Sleep)***	***Quiet (NREM)***
Behavior			
Eyelids	Open	Closed	Closed
Eye movements	Present	REMs	None
Tonic EMG	Variable	Off	On
Phasic EMG	Variable	On	Off
Respirations	Irregular	Irregular, brief apneas	Regular
EEG	Continuous (activité moyenne)	Continuous (activité moyenne)	Discontinuous (tracé alternant) and continuous slow-wave sleep

EEG, Electroencephalogram; *EMG,* electromyographic; *NREM,* non-rapid eye movement (sleep); *REM,* rapid eye movement.

of activity and lower voltage periods of quiescence. French electroencephalographers have coined the terms *tracé discontinu* and *tracé alternant* to describe these normal discontinuous quiet sleep patterns (Figure 164-5). Quiet sleep is a delicate electroclinical state. During mild encephalopathies, more of the EEG may appear as indeterminate sleep at the expense of quiet sleep[63]; that is, the percentage of total sleep time classified as indeterminate sleep increases directly as the percentage of quiet sleep diminishes. Although the EEG may appear normal during wakefulness or active sleep in mild encephalopathies, abnormalities may be confined to quiet sleep.

As term gestation approaches (36 to 38 weeks CA), additional signs of electrographic maturity emerge. Although the circadian rhythms at birth are already set at the adult value (24.8 hours), sleep cycle time at term is 50 minutes and gradually lengthens to mature values of 90 minutes.[64] The EEG pattern of quiet sleep is subtly different from the previous tracé discontinu of the more immature infant. Although the pattern remains discontinuous, the quiescent period is not as low in voltage. The record demonstrates repetitive *alternations* between bursts of higher voltage activity and lower voltage quiescent periods. French investigators designate this alternating tracing as *tracé alternant.*

At term, quiet sleep is divisible into two distinct patterns. The immature quiet sleep pattern of tracé alternant is the remnant of earlier quiet sleep patterns that evolved from tracé discontinu. More mature quiet sleep then emerges as continuous slow-wave sleep in which *continuous* high-amplitude θ and Δ activity dominates the record.[43] This resembles in principle the quiet sleep of older infants and children. By 4 to 6 weeks after term, continuous slow-wave sleep also displays, for the first time, the unmistakable sentinel of mature non-REM sleep—the sleep spindle or σ rhythm. Thus, by 6 weeks after term, most healthy infants have cemented the groundwork for the electrocerebral cycles of their future life. By 4 months postterm, the basic architecture of sleep-awake cycles has been transformed from the primitive undifferentiated electroclinical state of the very premature baby to one that basically satisfies the criteria of electroclinical maturity.

By this age, most babies exit wakefulness into non-REM sleep. Furthermore, the proportion of total sleep time occupied by active sleep has fallen from a high value of 50% at term to a mature value of about 20%[43] (Figure 164-6). During quiet sleep, well-formed sleep spindles flourish but do not appear simultaneously in the two hemispheres (i.e., they are asynchronous) until 2 years of age. Poorly developed sleep sharp waves are also visible

from the central vertex (C_z) by 4 months of age and are explicit by 6 months of age.[65] During wakefulness, substantial spatial organization of EEG frequencies and rhythms has appeared. The precursors of the well-known μ rhythm (the "idle" rhythm of the motor cortex) and α rhythm (the "idle" rhythm of the visual cortex) dominate the rolandic and occipital areas, respectively.[66] Although at this age the frequency of these waveforms (around 3 to 4 Hz) is too slow for these patterns to be rightfully labeled as a true μ or α rhythm, their morphologic characteristics, rhythmicity, and reactivity to movement or vision impart them with their fundamental characteristics.

The value of an understanding of the concept of state and EEG ontogeny becomes more tangible when discriminating between abnormal and normal EEGs. The EEG is expected to be temporarily low voltage during wakefulness and dream sleep or after behavioral arousal. However, this normal low-voltage signal is interspersed with higher-voltage EEG segments in quiet sleep. In contrast, the pathologic low-voltage EEG represents a fixed, monotonous, invariant pattern that does not cycle with spontaneous higher voltage activity of quiet sleep. In a similar context, the highly abnormal pattern of "burst suppression" must be distinguished from the normal discontinuity (tracé discontinu and tracé alternant) of immature quiet sleep. The former represents an immutable pattern from which the patient cannot be rescued by the strongest external stimulation (see Figure 164-5). The cerebral activity during the bursts of burst suppression displays an

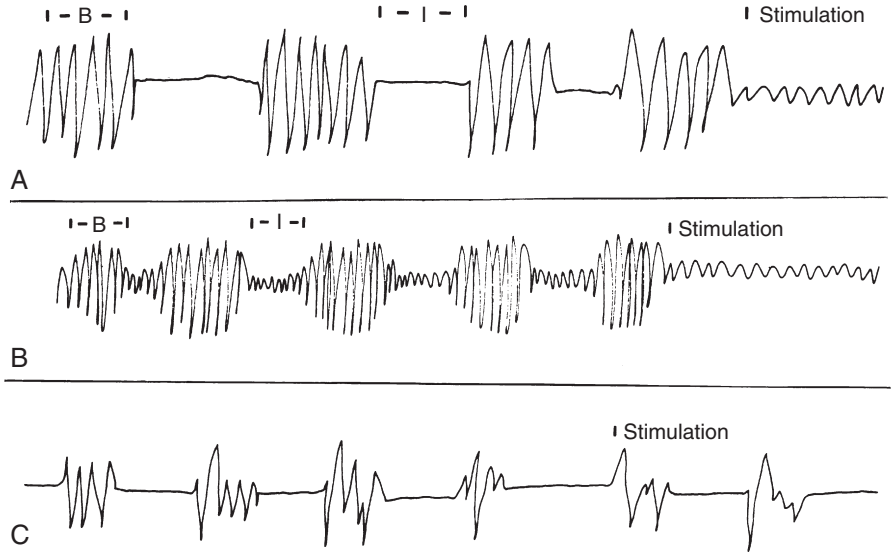


Figure 164-5 Discontinuous electroencephalogram (EEG) activity. A, Normal quiet sleep in a 33-week preterm baby: During tracé discontinu, the bursts *(B)* are composed of normal EEG patterns; the interbursts *(I)* represent low-voltage periods of quiescence. Following stimulation, there is behavioral and EEG reactivity and a transition to a more aroused state. **B**, Normal quiet sleep in a full-term baby: During tracé alternant, the bursts *(B)* are composed of normal EEG signals; the interburst voltage is higher (<25 μV) than tracé discontinu and spectrally resembles the EEG signal during active sleep. Following stimulation, the EEG changes to a continuous, lower-voltage, mixed-frequency signal. **C**, The critically ill premature or full-term infant displays hyporesponsive eye closure signifying lethargy or coma; the bursts of activity are composed of abnormal EEG patterns; the interburst periods are low voltage and tend to be longer than the duration of the interburst during tracé discontinu and tracé alternant. Despite stimulation, there is no EEG reactivity, and the fixed, invariant EEG pattern remains.

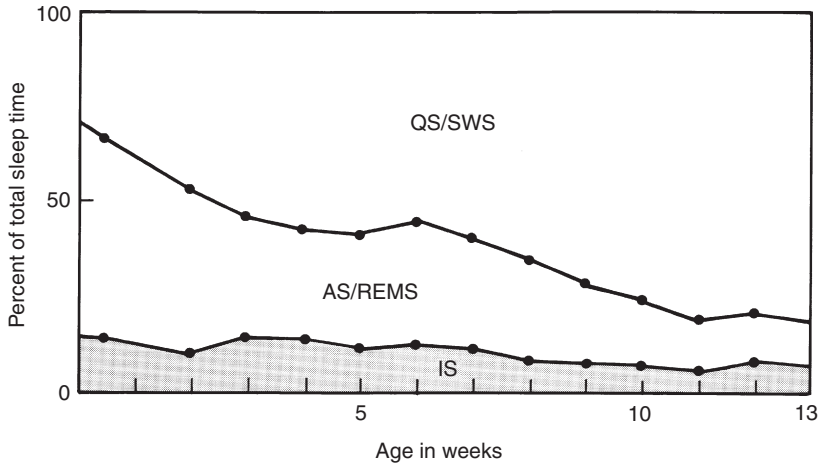


Figure 164-6 Quiet or slow-wave sleep (QS/SWS), active or REM sleep (AS/REMS), and indeterminante sleep (IS) as percentages of total sleep time in normal full-term infants, 1 to 13 weeks old. *(From Ellingson RJ, Peters JF:* Electroencephalogr Clin Neurophysiol *49:112, 1980.)*

abnormal morphologic condition, with admixed spikes, sharp waves, or unusual fast (β) activity.

In just a few months, the anatomic, physiologic, synaptogenic, and electrophysiologic properties of the cortex undergo an unparalleled metamorphosis.[47] This introductory overview paints a broad picture of the electroclinical events that encompass early life outside the womb. The next task is to consider the specific details of recording and interpreting the EEG itself.

TECHNICAL ASPECTS OF CONVENTIONAL NEONATAL EEG RECORDINGS

Conventional EEGs are recorded in the clinical neurophysiology laboratory or at the infant's cribside in the intensive care nursery.[67] The recording technologist must be careful to obtain the most technically acceptable tracing. The EEG is recorded for 45 to 60 minutes to sample EEG activity during several spontaneous state changes. At the conclusion of the EEG, the technologist stimulates the patient with light, sound, and touch to witness arousal patterns that demonstrate reactivity of the EEG signal.

The recording session begins by noting the clinical condition of the baby. The medical and neurologic diagnoses are recorded.[68,69] The infant's level of consciousness, drugs consumed, respiratory status, arterial blood gas values, and other pertinent laboratory data are noted.

The infant's head is carefully measured from standard bony landmarks (e.g., the inion and nasion) (Figure 164-7), and the scalp is marked with a crayon at several key locations.[70] When measured properly, these prescribed locations can be reproducibly located by the same or a different technologist on subsequent EEG recordings. Because the scalp surface area of the neonatal skull is small, a limited number of sites is used rather than the full electrode array employed in older individuals. The EEG recording sites are chosen to provide a fair sampling of cortical electrical activity from several representative brain regions. In the international 10/20 system modified for neonates, surface disk electrodes are applied with electrode paste in the following locations: FP_3-FP_4 (halfway between the conventional frontal polar [FP_1-FP_2] and frontal [F_3-F_4] regions); C_3-C_4 (central, rolandic, or parasagittal areas); T_3-T_4 (midtemporal or paratemporal); O_1-O_2 (occipital); and F_z (frontal vertex), C_z (central vertex), and P_z (parietal vertex) (Figure 164-8). Additional polygraphic monitors are placed to detect eye movements, nasal airflow, chest wall excursions, and the electrocardiogram (ECG). Altogether, at least 20 recording channels on the electroencephalograph are needed for a complete study. Usually a single montage is used for the whole session.



Figure 164-7 Infant undergoing an electroencephalographic examination.

The neonatal EEG is largely composed of Δ and θ activity, with a relative paucity of faster rhythms (see Figure 164-2). The technique of EEG recording is conducted to exploit the rich presence of this slower activity; the usual EEG recording speed is 30 mm/sec, but neonatal tracings are recorded at a slower paper speed (15 mm/sec). This compresses the signal in the time domain and facilitates visual analysis by enhancing the visibility of the Δ activity. The filter settings are also chosen to permit faithful reproduction of the dominantly slow frequency signal. The lower linear frequency is 0.3 to 1 Hz, and the high linear frequency is 70 Hz.

While the tracing is actually being conducted, it is mandatory for the recording technologist to make accurate and copious notations on the unfolding EEG record that describe the patient's behavior and activity. The presence of eye opening or closure, movements, sucking, hiccups, startles, squirming, and so on help the encephalographer to understand the behavioral state and identify possible sources of recording artifact.

GENERAL PROPERTIES OF THE NEONATAL EEG

The neonatal EEG is exceptional in many ways. Not only are the specific bioelectric rhythms that compose the signal unique and age-specific but the fundamental *organization* of the signal is confined to infancy. The next section covers the infrastructure of neonatal EEG: continuity, synchrony, symmetry, and reactivity. Independent criteria have been provided to define the normal parameters of aEEG.[4,11]

Continuity and Discontinuity

The property of continuity confers an uninterrupted voltage level to the tracing (Figure 164-9). No substantial voltage breaks or interruptions occur in a continuous EEG tracing. Continuous EEGs unravel nonstop. Segments of the EEG that show clear-cut interruptions or reductions of voltage are "discontinuous"[71] (Figure 164-10). The "burst" segments of cerebral electrical

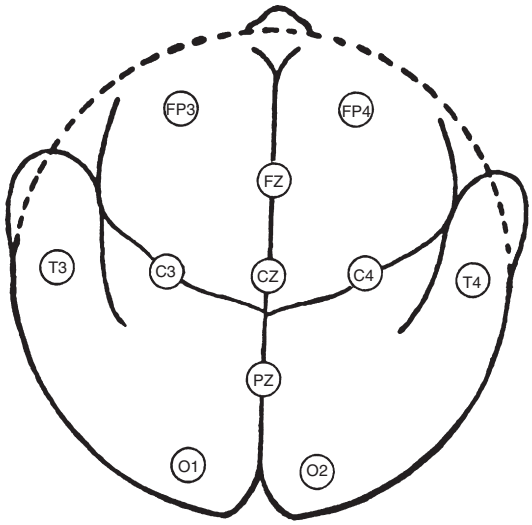


Figure 164-8 Technical specifications. 1. Long interelectrode distances. **2.** Multiple polygraphic variables including ECG, eye movements, nasal air flow, chest wall excursion, movement monitor. **3.** Recording speed: 15 mm/sec. **4.** Recording sensitivity 7 μv/mm. **5.** Filters: HLF: 70 Hz, LLF: 0.3 to 1.0 Hz. **6.** Montage: Channel: (1) $FP_3 \rightarrow T_3$; (2) $T_3 \rightarrow O_1$; (3) $FP_4 \rightarrow T_4$; (4) $T_4 \rightarrow O_2$; (5) $FP_3 \rightarrow C_3$; (6) $C_3 \rightarrow O_1$; (7) $FP_4 \rightarrow C_4$; (8) $C_4 \rightarrow O_2$; (9) $T_3 \rightarrow C_3$; (10) $C_3 \rightarrow C_z$; (11) $C_z \rightarrow C_4$; (12) $O_4 \rightarrow T_4$; (13) $F_z \rightarrow O_z$; (14) $C_z \rightarrow P_z$ (15) $T_3 \rightarrow C_z$; (16) $T_4 \rightarrow C_z$.

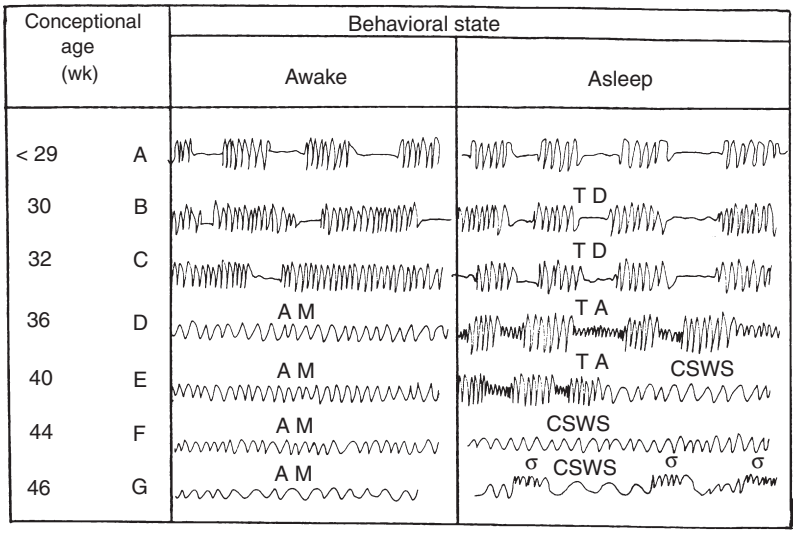


Figure 164-9 Evolution of continuity. *A,* Primordial discontinuity. *B,* Relatively more continuous while awake or during active sleep. Tracé discontinu (TD) during quiet sleep. *C,* Mostly continuous during wakefulness. TD during quiet sleep. *D,* Activité moyenne (AM) continuous low to medium voltage mixed-frequency signal while awake. Tracé alternant (TA) while asleep. *E,* AM during wakefulness. Mixture of immature (TA) and mature continuous slow wave sleep (CSWS) during quiet sleep. *F,* AM during wakefulness. Mostly CSWS during quiet sleep. *G,* AM during wakefulness. Quiet sleep entirely CSWS and sleep spindles (σ) first appear.

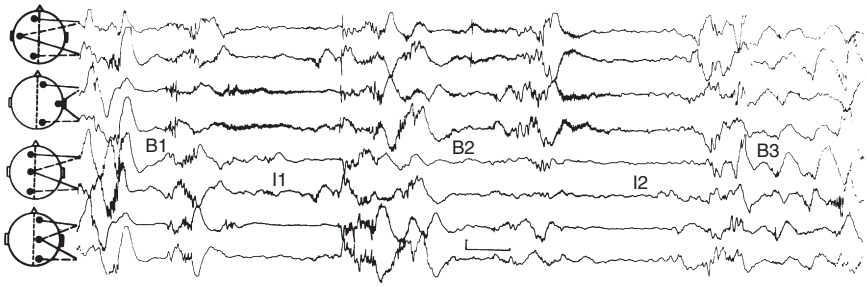


Figure 164-10 Tracé discontinu. Electroencephalogram (EEG) segment recorded from a 1-week-old (29 weeks estimated gestational age [EGA]) premature infant. The infant is in indeterminant sleep. The background activity is discontinuous and characterized by a series of bursts (*B1, B2, B3*) separated by lower-voltage periods of quiescence or interbursts (*I1, I2*). The cerebral electrical activity that makes up the bursts is of normal character, and the duration of the interburst periods is not excessively long for this conceptional age.

activity refer to the active periods in which the EEG voltage is "on." The specific rhythms and patterns that compose the burst activity are then visible. (These specific individual patterns are described in a later section.) Between the bursts of activity are lower voltage interburst periods of relative quiescence. The EEG is "off" in the interburst period. The signal amplitude at a standard recording sensitivity may then fall to zero or a value that is clearly less than the preceding burst activity.

In the extremely young infant (24 to 28 weeks CA), the entire EEG is essentially discontinuous.[72-74] Whether the child is clinically awake or asleep, active or quiet, the EEG itself is comprised almost entirely of a discontinuous background. Stimulation of the patient or spontaneous state changes provoke little qualitative change in the discontinuity.

By 30 weeks of age, more extended periods of relative continuity may appear.[65] The continuous EEG may occasionally persist without interruptions for many minutes as active sleep first materializes and consolidates. By 32 weeks of age, most healthy infants are capable of prolonged, continuous segments of EEG activity during definite active sleep periods. Indeed, this is a landmark CA because for the first time the continuous EEG dominates over the discontinuous portions of the record. Perhaps it is no coincidence that key CNS anatomic and physiologic landmarks mature at the same age.[75]

After 32 weeks of age, EEG discontinuity is mostly confined to quiet sleep. The voltage of the interburst activity, as recorded from the scalp surface with standard recording sensitivity, remains less than 25 μV. This discontinuous quiet sleep tracing (tracé discontinu) resembles the more primitive discontinuity that pervades all states in the very young preterm baby. With advancing maturity, the voltage of the interburst period exceeds 25 μV. The label assigned to this more mature discontinuous quiet sleep activity is tracé alternant, signifying the *alternations* between the higher voltage bursts and lower voltage interbursts[76] (Figure 164-11). The distinction between tracé discontinu and tracé alternant is thus somewhat arbitrary. Except for the higher interburst voltage, little else is fundamentally different. A curious feature of the interburst activity of tracé alternant is that it comprises voltages and frequencies that typify the continuous EEG during wakefulness and active sleep.[77] It is as though tracé alternant quiet sleep were comprised of brief portions of active sleep (i.e., the interburst segments) separated by bursts of higher voltage slowing.

By 1 month after term, the discontinuous quiet sleep pattern has all but disappeared. Tracé discontinu and tracé alternant gradually yield to the more mature, continuous slow-wave sleep, during which nonstop high-voltage θ and Δ activity dominate the tracing. Eventually the more sophisticated trappings of

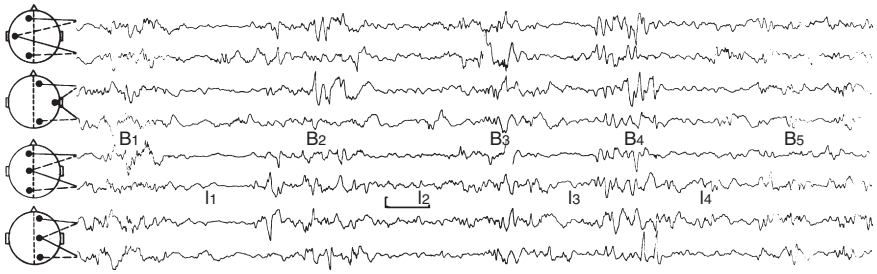


Figure 164-11 Tracé alternant. One-week-old full-term infant in quiet sleep. The cerebral electrical activity is discontinuous and composed of a series of bursts (*B1, B2, B3, B4, B5*) separated by interburst periods (*I1, I2, I3, I4*) of lower voltage and brief duration. Compared with tracé discontinu recorded from younger infants, the interburst periods are briefer and higher in amplitude.

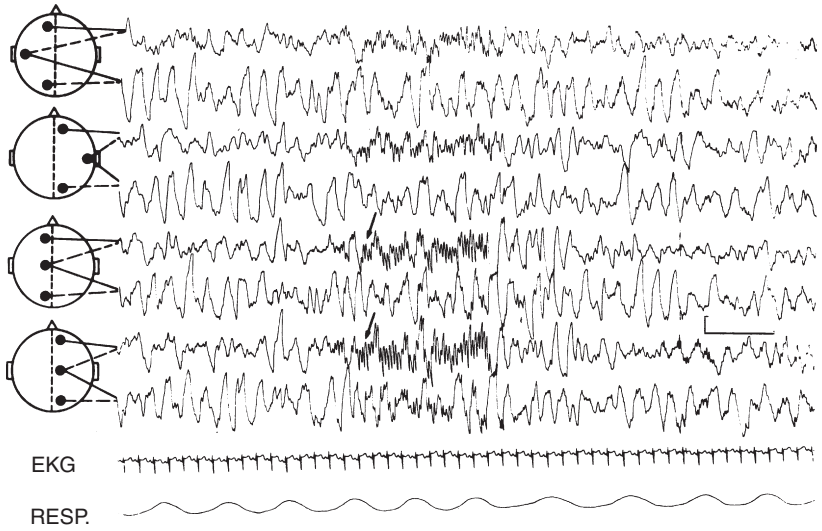


Figure 164-12 Continuous slow wave sleep (CSWS) and spindle. This segment was recorded from a healthy 8-week-old full-term infant during quiet sleep. CSWS is noted with semirhythmic high-amplitude Δ-activity maximally expressed in the bioccipital scalp regions. Twelve-hertz sleep spindles (*arrows*) arise synchronously from the frontal-central regions bilaterally. Although in this segment the spindles arise synchronously, they often do not at this age. Notice that the respiratory pattern is exquisitely regular and that there is a paucity of limb movements during quiet sleep.

non-REM sleep (spindles and vertex waves) punctuate the tracing (Figure 164-12).

Duration of Interbursts

The duration of the burst and interburst periods is maturation (CA)-dependent[59] (Figure 164-13). The length of the interburst is relatively longer in the younger preterm infant and decreases near term). Most investigators have reported a range of values for the duration of interburst as a function of CA.[75,78] The discrepancies in specific reported values reflect differences in technical recording aspects, EEG criteria that define the boundaries between the burst and interburst intervals, and the clinical condition of the babies.[79-81] Tharp[78] has shown that concurrent medical conditions of the baby may transiently influence the interburst duration. With transient hypoxia, the interburst period lengthens in parallel[78] (Figure 164-14). It is probably true that other physically stressful medical conditions (ischemia, hypercapnia, hyponatremia, sepsis, and so on) similarly increase the degree of discontinuity.

Another source of disparity in reported measurements of interburst duration is the selection criteria for individual premature babies. Most "healthy" premature babies have relatively brief interburst periods and eventually mature into developmentally and neurologically normal children. Some sick premature infants have much longer interburst periods and yet fortunately also recover and develop relatively normally. For example, Lombroso[82] historically cited "allowable" interburst periods up to 2 minutes because some of the neonates from whom the measurements were recorded were later developmentally normal. Another perspective is that such excessively long interburst periods are indeed abnormal but do not predict a permanent adverse outcome in all. The situation is not different from the healthy youngster whose EEG is excessively slow and disorganized following a head injury. The pattern itself is abnormal even though the individual can still have a healthy outcome later.

Excessive Discontinuity

Excessive discontinuity is an electrographic abnormality in which bursts of normally composed EEG signal are separated by longer than normal interburst periods. Mildly excessive discontinuity may reflect a temporary metabolic imbalance or mild encephalopathy. Markedly excessive discontinuity usually connotes more serious metabolic or neurologic abnormalities.[83,84] As the acute encephalopathy passes, the excessive discontinuity gradually subsides. In the setting of an acute encephalopathy, the term *burst suppression* denotes a markedly abnormal EEG background in which nonreactive, abnormally composed EEG signals are separated by abnormal low-voltage quiescent periods (Figure 164-15).

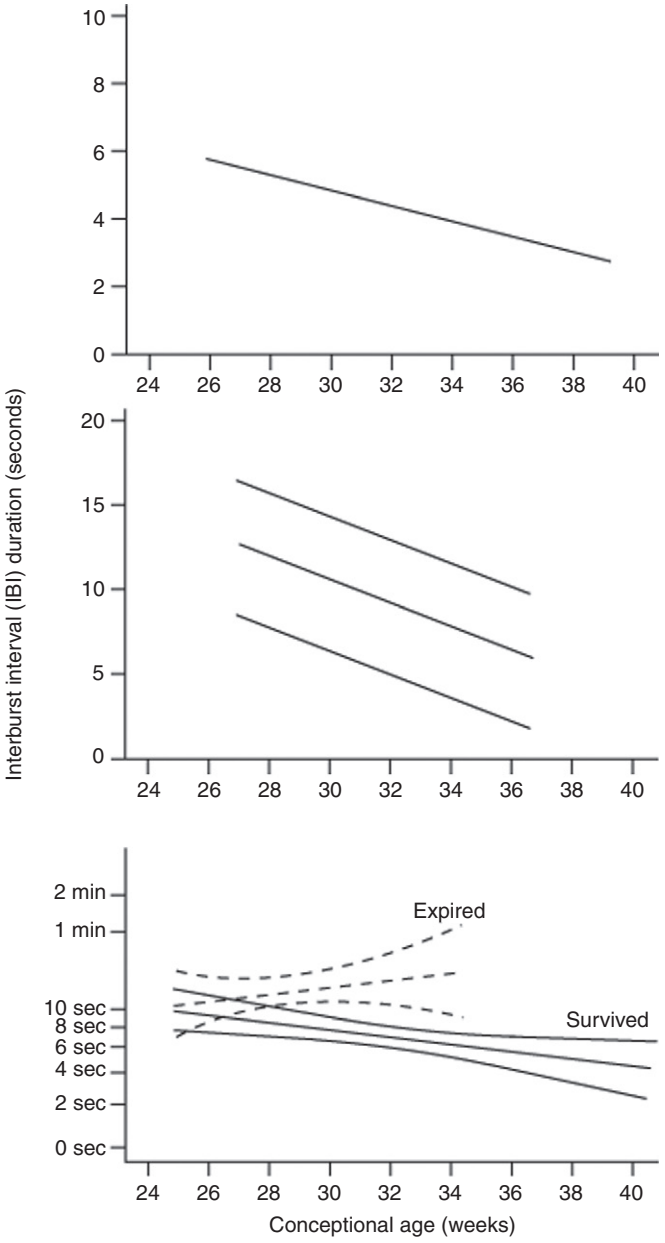


Figure 164-13 The durations of interburst intervals (IBIs) are shown at corresponding conceptional ages (CA), adapted from three separate studies.[79-81] The selection of patient populations, definitions of interburst intervals and recording methodologies differ among the studies. Nevertheless, all show that in healthy or surviving premature infants, the duration of the IBI is relatively brief (mean of 6 to 15 seconds), even at very young CAs, and decreases to a few seconds near term.

NORMAL CONTINUITY

By 30 weeks CA, many healthy babies display relatively protracted periods of continuous EEG activity during which the patient's clinical behavior suggests active sleep. By 32 weeks CA, most records demonstrate definite continuous segments. However, the specific patterns that make up the burst in the discontinuous records appear similar to the continuous segments. As term approaches, the actual composition of the continuous segments differs from the discontinuous quiet sleep record. During wakefulness and the active sleep that immediately follows quiet sleep, the continuous EEG is relatively low voltage and represents a rich mixture of frequencies (β, α, θ, and Δ). This continuous "average" background is called *activité moyenne* by French authors (see Figure 164-1). At term, most quiet sleep still displays the immature tracé alternant pattern, which later yields to the more sophisticated *continuous* slow-wave sleep pattern that is the foundation of adult quiet sleep activity.

Although a progressive substitution of continuous EEG activity to replace discontinuity is desirable, all forms of continuity are not desirable. Some infants electrographically respond to CNS insult with a continuous, but invariant, monotonous EEG that does not display spontaneous-state lability and is unreactive to external stimulation.

Synchrony and Asynchrony

The isolated cerebral cortex generates a discontinuous EEG pattern.[85] Cortex disconnected from deeper pacemakers and remote from coticocortical modulation produces a pattern that inherently fluctuates its voltage (high voltage alternating with low voltage). In the discontinuous portion of an EEG, the two cerebral hemispheres can discharge at the same time (i.e., synchronously) or they can fire independently at different times (i.e., asynchronously). Although the temporal relationship of the signal *between* hemispheres is the usual focus of synchrony, the activity *within* each hemisphere can also be examined. Near term, individual regions within each hemisphere discharge simultaneously. For example, at 38 weeks, signals generated in the left frontal, central, temporal, and occipital areas arise together. In the very young premature baby, separate regions within one hemisphere may discharge independently or out of sync. Thus the temporal regions may be silent while the occipital areas robustly discharge.

Lombroso[86] has proposed an operational definition of interhemispheric synchrony that has been widely adopted. In a synchronous burst of a discontinuous segment of an EEG, the signal begins in both hemispheres within 1.5 seconds. In an asynchronous burst, one hemisphere leads the other by 1.5 or more seconds. Defined in this fashion, synchrony is a measurable property of the background that evolves with maturity.

Curiously, in the very young premature infant with primordial discontinuity, interhemispheric synchrony approaches 100%. By 30 to 31 weeks, when the EEG first demonstrates distinguishable state-dependent patterns, synchrony is only approximately 50% during the discontinuous portions of the recording. It is suspected that the neurologic substrate that underlies synchrony differs in these two circumstances.[82] With advancing maturity, a greater percentage of the bursts are synchronous until term, when nearly 100% of the bursts of tracé alternant begins simultaneously in the two hemispheres.

Excessive interhemispheric asynchrony for CA is an electrographic abnormality (Figure 164-16). It is most commonly seen following diffuse encephalopathies (e.g., intraventricular hemorrhage, meningitis, or asphyxia). No unique type of pathologic condition underlies the EEG expression of excessive asynchrony.[87] Excessive interhemispheric asynchrony can also be seen in cerebral dysgenesis, including agenesis of the corpus callosum or Aicardi syndrome. Although it would be tempting to speculate that the corpus callosum is the anatomic prerequisite for synchrony, the situation is probably more complex because the paired thalami and several commissures also serve as conduits that synchronize activity between the two hemispheres.

Although synchrony is usually intended to describe the temporal unity of the entire hemisphere, some individual EEG signals are also notably synchronous. In the very young premature infant, the occipital regions display remarkably synchronous high-voltage monorhythmic Δ activity. Frontal sharp waves (encoches frontales) and frontal slow transients (anterior dysrhythmia) are also usually synchronous. In contrast, the normal sleep spindles of

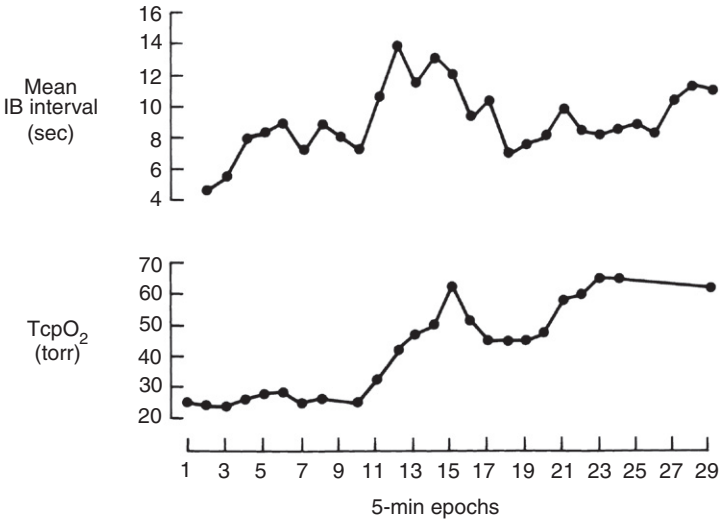


Figure 164-14 The relationship between oxygenation ($TcpO_2$) and mean interburst (IB) intervals/5 minutes in a premature infant with a hypoxic-ischemic encephalopathy and pulmonary hypertension. *(From Tharp BR: Intensive video/EEG monitoring of neonates,* Adv Neurol *46:107, 1986.)*

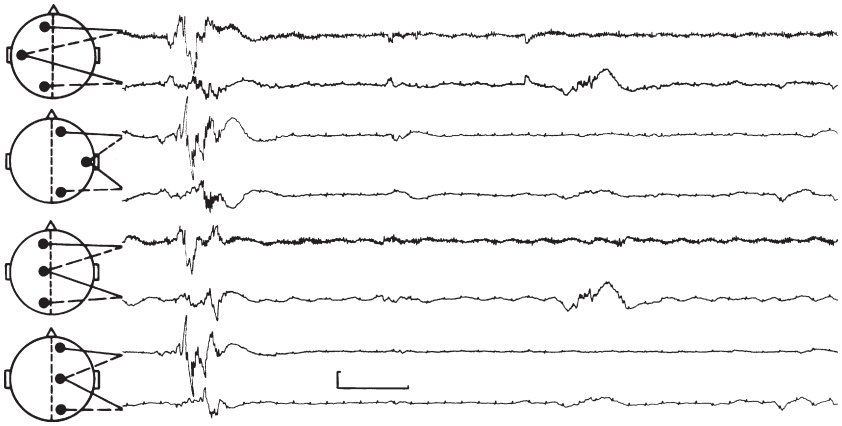


Figure 164-15 Burst suppression. Electroencephalogram (EEG) segment recorded from a severely asphyxiated 35-week estimated gestational age (EGA) infant. During the tracing, the child is comatose. The background shows burst suppression. There is a brief burst of erratic cerebral electrical activity that features an atypical bifrontal sharp wave. The burst of activity is followed by a long period of low-voltage suppressed background. There was no reactivity of the EEG to external stimulation.

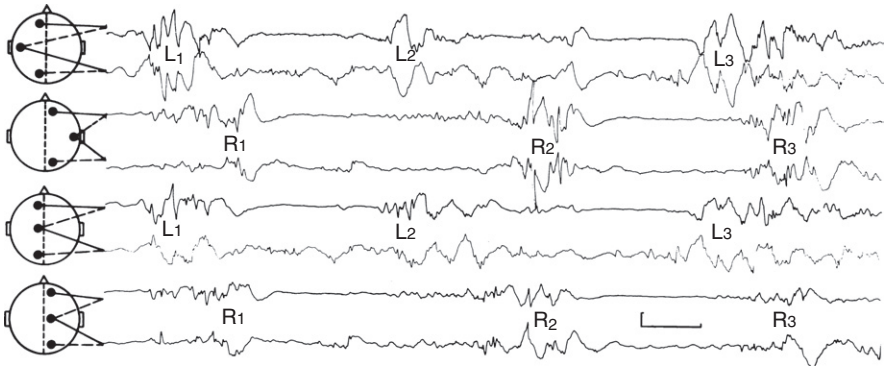


Figure 164-16 Interhemispheric asynchrony. Electroencephalogram (EEG) segment recorded during indeterminate sleep in a 3-day-old (37 week estimated gestational age [EGA]) infant with congenital heart disease. This EEG segment is discontinuous and shows excessive interhemispheric asynchrony. Notice that discharges from the left central and temporal regions (*L1, L2,* and *L3*) are displaced in time from bursts originating from the right central and temporal regions (*R1, R2,* and *R3*).

mature quiet sleep do not achieve full bihemispheric synchrony until 2 years of age.

Symmetry

The average voltage or amplitude of electrical activity in both cerebral hemispheres is generally symmetric.[16] The EEG amplitude and composition are equivalent when a suitably long sample is inspected. Although brief transient asymmetries are common, the overall impression from reading an extended EEG sample should convey the sense that both hemispheres (for that matter, homologous regions of both hemispheres) have equivalent representation of voltage, frequency, and specific background patterns.

The voltage of the background is roughly equal between homologous brain regions and between the two hemispheres. Near term it may be easier to detect voltage asymmetry during quiet sleep because the naturally higher voltage of tracé alternant exceeds that of wakefulness or active sleep. Care must be exercised in labeling a tracing abnormal because of voltage asymmetry alone (i.e., unaccompanied by a change in the frequency composition of the signal). The voltage at a given scalp location can be reduced by improper electrode placement or electrode gel bridging. Two electrodes accidentally placed too close together register the same EEG scalp signal. The differential amplifiers of the electroencephalograph may therefore detect only a modest voltage differential between the two sites, and a low-amplitude output signal results. An artificially low-voltage signal also can result from scalp edema, which removes the recording electrode farther from the cortical surface, the generator source of the signal. This can result from edema caused by a dependent head position or the infiltration of a scalp intravenous infusion. However, similar voltage depression can occur with subdural fluid (blood or cerebrospinal fluid) collections.

A transient voltage-frequency asymmetry may be observed occasionally during the transition between tracé alternant and active sleep in some healthy term babies. As the child enters or exits quiet sleep, one hemisphere desynchronizes into a low-voltage, continuous, mixed-frequency background, whereas the other displays the high-voltage tracé alternant quiet sleep pattern. This obvious asymmetry may persist for several minutes until voltage-frequency equality is restored to the two hemispheres. This phenomenon implies that the cycling of sleep and arousal is modulated independently for both hemispheres.

Although symmetry of voltage is the easiest EEG parameter to detect, symmetry of *composition* is equally important. Localized or lateralized pathologic features may give rise to an asymmetry in the type of EEG signal. There may be a circumscribed paucity of faster or rhythmic waveforms or excessive focal slowing that earmarks a localized cortical functional disturbance (Figure 164-17). Transient patterns such as Δ brushes or frontal sharp waves are also generally symmetric. For example, an equal number of brushes per minute can be counted from corresponding regions of both hemispheres.

Reactivity

EEG reactivity describes a change in the appearance of the cerebral background activity after a specific provocative stimulus (see Figure 164-5). Reactivity is both state- and CA-dependent. EEG reactivity should be distinguished from the clinical neurologic reaction to stimulation. The latter may include eye opening, crying, grimacing, limb movement, or a change in the respiratory pattern. These are not always time-locked to a coincident departure from the baseline EEG.

In the very young premature baby, the primordially discontinuous EEG shows no consistent response to external stimulation. However, by 32 to 34 weeks CA, some change can be induced in the EEG background by an arousing stimulus. As such, the quietly sleeping infant whose EEG shows a tracé discontinu pattern can be redirected into an alert state (wakefulness or active sleep) by external stimulation with a corresponding switch to a continuous EEG background.

By term, healthy, quietly sleeping babies show two basic arousal patterns after external stimulation: a temporary increase in discontinuity (longer duration of the interburst period) and attenuation of voltage or desynchronization of the background signals. During quiet sleep, the discontinuous tracé alternant pattern usually displays interburst periods of 4 to 6 seconds. After a suitably intense stimulus, the discontinuity of the tracing can temporarily increase, with longer interburst intervals and lower amplitude of interburst activity, thus resembling a brief reversion into tracé discontinu. However, unless the arousal persists and a complete state change ensues, the record soon reverts back to the previous tracé alternant pattern. During either tracé alternant or continuous slow-wave quiet sleep, the second major arousal pattern is an abrupt, widespread voltage reduction and the disappearance of the slowest Δ activity.

Reactivity is best visualized in the EEG of a quietly sleeping infant when increased discontinuity or desynchronization is unmistakable. Stimulation of an awake and alert infant or

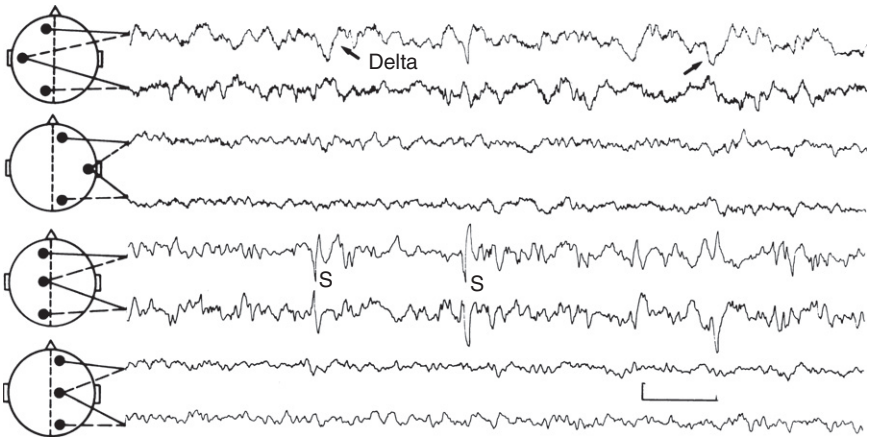


Figure 164-17 Interhemispheric asymmetry. Electroencephalogram (EEG) segment recorded from a 4-day-old full-term infant with an unexplained stroke in the distribution of the left middle cerebral artery. In this rhythm strip, there is a clear asymmetry between the two hemispheres. The left temporal and central regions have abundant high-amplitude Δ-activity *(arrows)* that is not present in similar regions in the opposite healthy hemisphere. Furthermore, excessive sharp EEG transients *(S)* are seen in the left central region.

provocation during active sleep may not produce a dramatic change in the EEG despite a brisk behavioral response.

Nonreactivity

A lack of reactivity is an ominous sign in a suitably mature infant (see Figures 164-5 and 164-15). Indeed, in the healthy infant, spontaneous-state lability and reactivity to external stimulation are expected markers of health. Infants whose tracings are invariant—lacking spontaneous lability or stimulus-provoked state changes—usually harbor an encephalopathy.

SPECIFIC COMPOSITION OF THE ELECTROENCEPHALOGRAM

The EEG background refers to the collective presence of all ongoing moment-to-moment cerebral electrical patterns. The background can be high or low voltage, continuous or discontinuous, normal or abnormal. The prevailing *ingredients* ("graphoelements") that collectively constitute the background emerge and fade at different CAs. The background may be considered a foundation or infrastructure. It serves as a stage that is punctuated by short-lived transients, such as sharp waves, or fleeting events, such as electroencephalographic seizures.

During the perinatal period, the infrastructure of the signal is normally dominated by slow activity (θ and Δ). With maturity, the frequency of the dominant rhythms increases, with a gradual evolution to higher frequencies.

Many of the specific patterns that make up the EEG background exhibit spatial or state-dependent properties, or both (Table 164-5). For example, bursts of monorhythmic θ activity are spatially maximal in the temporal-occipital regions but are independent of state. Sleep spindles exhibit both spatial and state specificity. They originate from the midline vertex-rolandic regions and are confined to quiet sleep.

Monorhythmic Occipital Δ Activity

The posterior regions of the healthy premature baby are dominated by runs of high-voltage, positive-polarity, slow, bioccipital monorhythmic Δ activity (Figure 164-18). The runs of Δ activity can last from 2 to 60 seconds and are often synchronously displayed bilaterally, although minor voltage asymmetries are common. This stereotyped Δ activity serves as the most visible dominant posterior rhythm in this age group. It is present in the youngest viable premature infant, peaks between 30 and 33 weeks of age, and clearly begins to recede by 34 weeks of age. It serves as the Δ constituent of occipital brush activity (see the discussion on Δ brushes). It is a hardy rhythm; sometimes it is among the last of the "named" EEG background patterns to disappear after a significant encephalopathy.

Central-Temporal Δ Activity

The central and central-temporal regions in the young premature infant show sustained trains of semirhythmic Δ activity that is not nearly as bilaterally synchronous as the monorhythmic occipital Δ activity (Figure 164-19). It also serves frequently as the Δ foundation for rolandic brush formation. It also fades from the background by 34 weeks of age.

TABLE 164-5

Summary of General Organization of Electroclinical States

Parameter	27-28 Weeks CA	29-30 Weeks CA	31-33 Weeks CA	34-35 Weeks CA	36-37 Weeks CA	38-40 Weeks CA	41-49 Weeks CA
Behavioral states	Primordial; no difference in behavioral states	Some early behavior organized to suggest early states	Behavioral states easier to define; definite periods of AS/QS	Well-defined states	Well-defined states	Well-defined states	Well-defined states
Continuity/ discontinuity	Entirely discontinuous	Relatively more continuous during AS	Sustained continuity while awake/ASTD; mostly QS, a little in AS 60%	Continuous in awake/AS; TD in QS only	Well-defined TA; traces of CSWS	TA/CSWS	By 1 mo, AS and QS continuous
Synchrony	80%–90%	50%	60%	70%	80%	100%	
Specific EEG patterns							
Rhythmic occipital Δ	+	++	+++	±	—	—	—
Rhythmic temporal θ	+	++	+++	±	—	—	—
Rhythmic occipital θ	++	++	+	±	—	—	—
Δ brushes	+	++ AS > QS	++ AS > QS	+ QS > AS	+ QS > AS	± QS AS	± QS only
Irregular central temporal Δ	+	++	+	—	—	—	—
Anterior dysrhythmia (encoches frontales)	—	—	±	+	++	++	±
Sleep spindles	—	—	—	—	—	—	— (+ after 44 weeks)
Reactivity	Inconsistent	Inconsistent	Variable	Usually present	Reliably present	Present	Present
Concordance	Poor; awake and asleep EEG are similar	Poor; awake and asleep EEG are similar	Fair; EEG relatively continuous during behavioral AS	Good	Good	Good	Good

AS, Active sleep; *CA,* conceptional age; *CSWS,* continuous slow wave sleep; *EEG,* electroencephalogram; *QS,* quiet sleep; *TD,* tracé discontinu; *TA,* tracé alternant; +, present; ++, abundant; —, absent; ±, variably present.

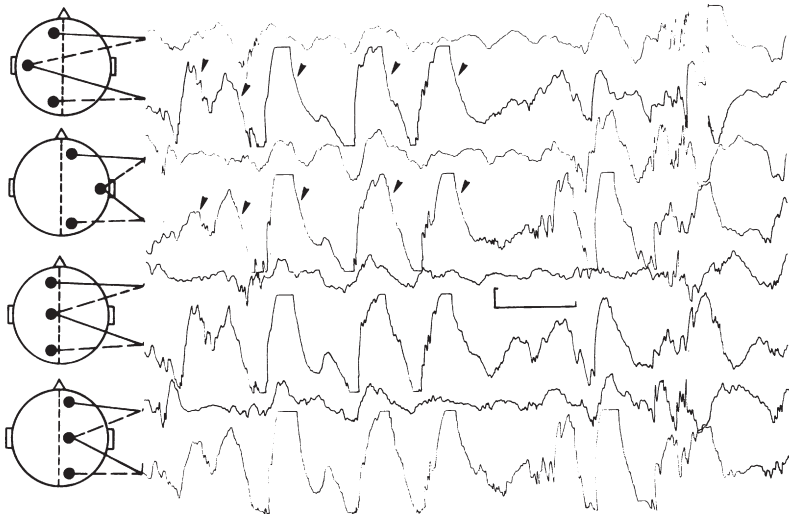


Figure 164-18 Monorhythmic occipital Δ-activity. This electroencephalogram (EEG) segment was recorded from an awake 32-week estimated gestational age (EGA) infant. During this relatively continuous segment, the EEG background is dominated by high-amplitude rhythmic occipital Δ-activity (*arrowheads*). Note that the occipital δ-activity on the left arises at the same time (synchronously) with the occipital Δ-activity on the right.

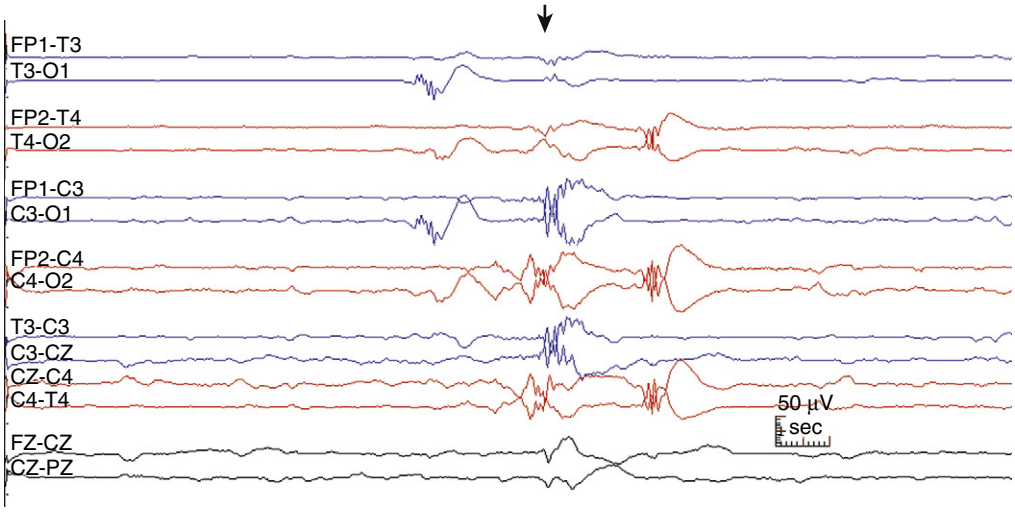


Figure 164-19 Central-temporal Δ-activity. Electroencephalogram (EEG) segment recorded from a sleeping 5-day-old (28-week estimated gestation age [EGA]) infant. Note the series of surface-positive, semirhythmic Δ-transients that are maximal in the central regions *(C_3* and *C_4)*. Many of these are also incorporated into delta brushes.

Rhythmic θ Activity (Sawtooth θ)

Short bursts of very rhythmic θ activity arise in the occipital regions of the premature baby less than 30 weeks of age (Figure 164-20, *A)* and in the temporal-occipital regions in premature infants 30 to 34 weeks of age (Figure 164-20, *B).* It occurs in all behavioral states. Although rhythmic, the waveform may be sharply contoured. The duration of the burst is variable and may last several seconds. If the burst is very brief, a "larval" discharge can occur, resembling a fragment of sharply contoured activity, and mimic "potentially epileptiform activity" in the temporal areas.

Brushes (Δ Brushes, Spindle-Like Fast Rhythms, Ripples of Prematurity)

The quintessential waveform of prematurity is the Δ brush, a pattern that represents the simultaneous fusion of two subunits: an underlying Δ transient and a superimposed, rhythmic, fast activity. Brushes occur during wakefulness, active sleep, and quiet sleep. They are not analogous to or the precursor of the conventional sleep spindle (σ rhythm). Usually no major asymmetries in brush abundance (the number of brushes per hour) occur compared between homologous regions of the two hemispheres.

In the very premature infant, brushes are more common in the rolandic regions[59] (Figure 164-21). When they obtain the highest expression (32 to 34 weeks CA), brushes are seen in the occipital, rolandic, and temporal regions. Frontal brushes are relatively rare at any age.

Even though brushes occur in all states, their abundance is state-dependent. Up to 34 weeks CA, brushes appear more frequently in active sleep than in quiet sleep.[86] By 34 to 36 weeks CA, the number of brushes in quiet sleep exceeds those in active sleep. By term, brushes are infrequent during wakefulness and may only be seen in quiet sleep, especially tracé alternant. By 1 month after term, brushes have largely vanished as greater maturity emerges.

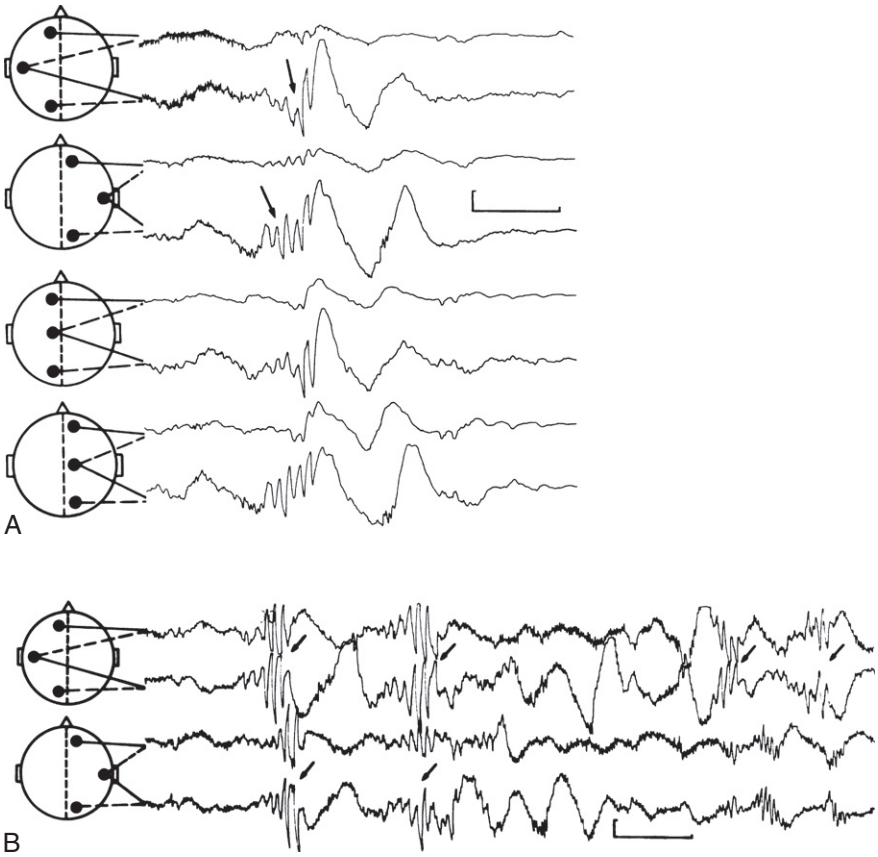


Figure 164-20 Rhythmic θ activity. A, This segment was recorded from a 3-day-old (27-week estimated gestational age [EGA]) premature infant in indeterminate sleep. Notice the appearance of rhythmic bioccipital θ activity (*arrows*) superimposed on high-amplitude monorhythmic bioccipital Δ activity. **B,** Same patient 2 weeks later. The rhythmic θ activity has largely migrated anteriorly and now principally engages the temporal regions (*arrows*). Rhythmic temporal θ activity is frequently asynchronous between the two temporal regions.

Anterior Dysrhythmia (Anterior Slowing)

Brief bursts of high-amplitude, relatively symmetric, and synchronous bifrontal rhythmic Δ activity begin between 33 and 34 weeks CA (Figure 164-22). Despite the usual pathologic connotation of the prefix *dys-*, this pattern is entirely normal. Anterior dysrhythmia appears in all states but is most abundant in transitional sleep preceding quiet sleep. In this context it behaves as a "drowsy pattern." It is also common during quiet sleep but comparatively infrequent in alert wakefulness or dream sleep. Anterior dysrhythmia usually begins to disappear by 1 month after term. Although it is a normal pattern, excessive anterior dysrhythmia is abnormal and may be seen in the setting of a mild diffuse encephalopathy.

Encoches Frontales (Frontal Sharp Waves)

The pattern of encoches frontales consists of a biphasic (negative-positive polarity), notched frontal sharp wave that is inextricably entwined with anterior dysrhythmia[88] (see Figure 164-22). Indeed, the two patterns often comingle and seem harmonically related, implying they share a common neural generator. They are frontally dominant and usually synchronous and symmetric between the left and right frontal regions. Like anterior dysrhythmia, they first appear in immature form at 33 to 34 weeks CA and in complete form by 35 weeks CA. They start to wane by 1 month after term and decay in parallel with anterior dysrhythmia (Figure 164-23).

During mild encephalopathies, encoches frontales mildly increase in frequency, and their morphologic characteristics become more variable. Sometimes the pattern shows a sharper (i.e., briefer duration) morphologic appearance. Encoches frontales convey no

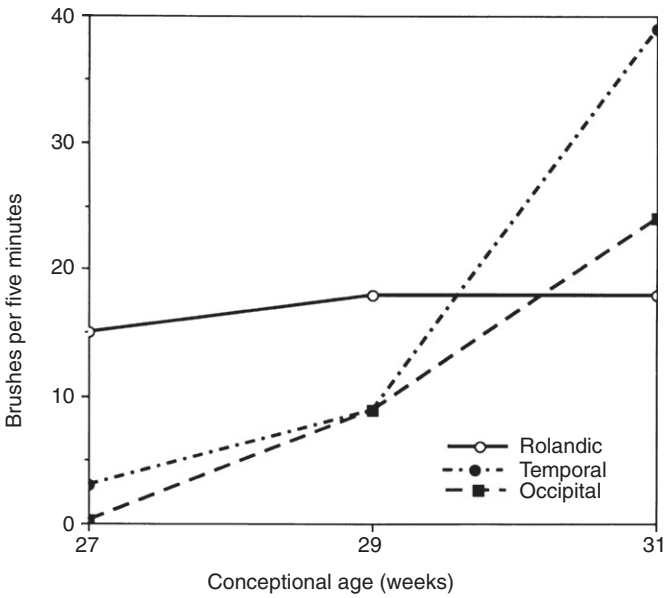


Figure 164-21 Brush abundance. The abundance of brushes is a function of both maturity and location. The graph depicts the abundance (number of brushes/5 minutes of electroencephalogram [EEG] background) seen in the occipital, temporal, and rolandic regions at 27, 29, and 31 weeks conceptional age.[59]

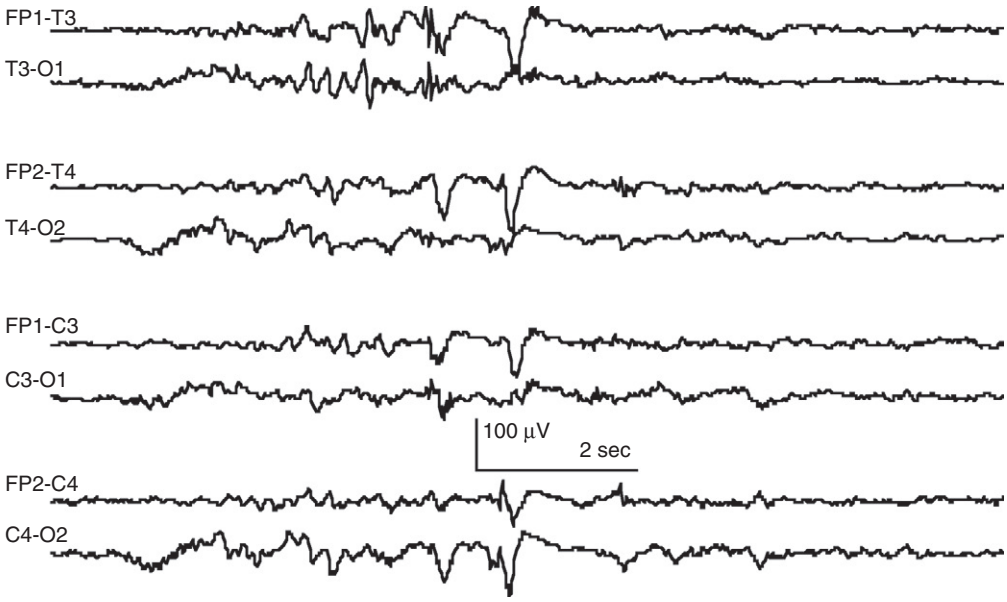


Figure 164-22 Anterior dysrhythmia (frontal delta) evolving into an encoches frontales (physiologic frontal sharp wave) in a quietly sleeping 3-day-old (33-week estimated gestation age [EGA]) infant. Encoches frontales are recognized as synchronous and symmetric biphasic (negative-positive) bifrontal sharp waves. This pattern often appears simultaneously (i.e., synchronously). Frontal sharp waves are most abundant during drowsiness and in quiet sleep and are least evident during alert wakefulness. Sometimes these normal sharp EEG transients are mistaken as "epileptiform" by inexperienced electroencephalographers.

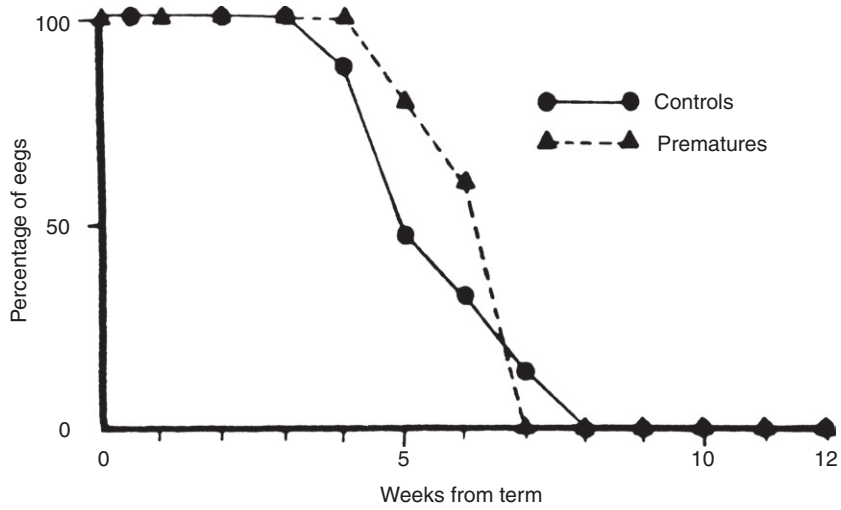


Figure 164-23 Encoches frontales. Proportion of electroencephalograms (EEGs) showing frontal sharp transients in premature and full-term infants. *(From Ellingson RJ, Peters JF: Development of EEG and daytime sleep patterns of low risk premature infants during the first year of life: Longitudinal observations,* Electroencephalogr Clin Neurophysiol *50:165, 1980.)*

implication for epileptogenesis. They are an expected normal EEG signal and do not connote a "lowered seizure threshold."

Other Sharp Waves

Healthy babies have occasional sharp EEG transients in the temporal,[89] central, and occipital regions (Figure 164-24). These transients are most abundant between 37 and 42 weeks CA, gradually decline in abundance by 4 to 6 weeks after term, and entirely vanish from the background by 10 weeks after term[90] (Figure 164-25). They are most common in the temporal areas. They are usually sharp waves rather than spikes, arise solitarily rather than in bursts or consecutive runs, and recur with a frequency of about one sharp EEG transient/minute of active sleep (Table 164-6).

Sleep Spindles

By 44 to 46 weeks CA, quiet sleep sheds its immature tracé alternant pattern and establishes its mature foundation as continuous slow-wave sleep. Sleep spindles (12 to 14 Hz) then appear from the midline vertex (C_z) or the central regions (C_3 and C_4)[65] (see Figure 164-12). Sleep spindles are often asynchronous between the two hemispheres and remain so until 2 years of age.

Dominant Rhythmic Patterns of Early Infancy

Soon after the healthy infant leaves the neonatal period, the organization of the EEG begins to resemble, at least in principle, that of the young child. In early infancy, poorly sustained rhythmic activity first emerges in the occipital and rolandic regions (Figure 164-26). The rhythmic occipital patterns are the precursor

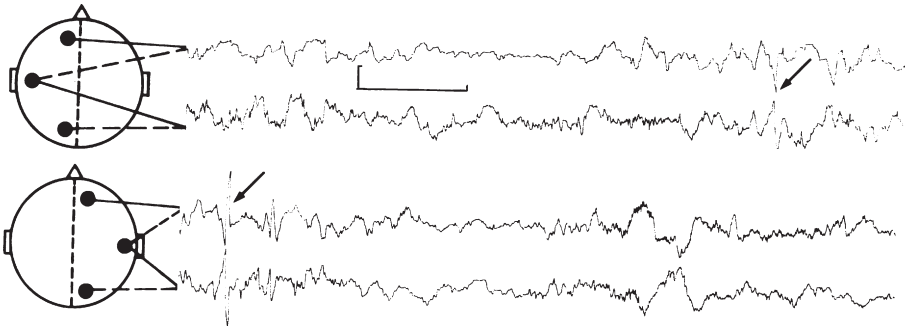


Figure 164-24 Normal sharp electroencephalogram (EEG) transients. This segment was recorded from a healthy, 3-day-old, full-term infant during active sleep. Occasional sharp EEG transients (SETs) are recorded independently from the temporal regions (*arrows*).

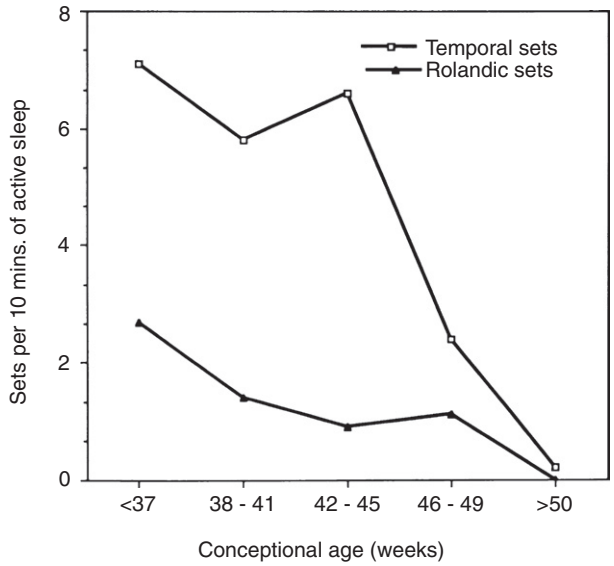


Figure 164-25 The graph depicts the abundance of sharp electroencephalogram (EEG) transients (SETs) in the temporal and central regions during 10 minutes of active sleep. Notice that there are more SETs in the temporal than in the central regions at all ages studied. There is a gradual decline in the frequency of SETs until these patterns disappear from the record near 50 weeks conceptional age.

TABLE 164-6

Comparison of Sharp Electroencephalogram Transients Recorded in Healthy Infants and Those With Confirmed Electroencephalogram Seizures

EEG Parameter	Healthy Infants	Infants with Seizures
Background	Normal	Frequently abnormal
SET abundance	Total SETs from left and right central and temporal regions are maximum near term and recur about 1 SET/1.0 min to 1 SET/1.5 min	
Morphologic characteristics	Usually sharp waves (rarely spikes)	Spikes or sharp waves
Repetitive behavior	Isolated, solitary transients	Solitary; runs or bursts of SETs
Pattern of spatial distribution	Temporal > central > occipital during active sleep; no striking asymmetry between left and right hemispheres	Asymmetries may be present between left and right temporal, left and right central, or left and right hemispheres

EEG, Electroencephalogram; *SETs,* sharp EEG transients.

of the α rhythm. The slow frequency (<8 Hz) of this dominant occipital rhythm precludes labeling it as "true" α, but its location and reactivity to visual stimuli earmark it as an immature version of the genuine article. The frequency of this pattern gradually increases until it is 8 Hz or faster (usually by age 2 years), at which time the label "α rhythm" can be employed with full justification.

Similarly, runs of central rhythmic patterns appear that resemble an immature μ rhythm, the idle rhythm of the motor cortex. This μ rhythm precursor tends to be about 0.5 Hz faster than its corresponding α precursor in each individual and is 8 Hz or faster by age 2 years.

Abnormal EEG Patterns

Newborn infants are exposed to a wide variety of potentially CNS-damaging events. The EEG, a sensitive reflection of cerebral cortical activity, commonly displays an assortment of abnormalities, depending on the injury's severity and topography, and the patient's CA. Most EEG abnormalities are not specific for etiology. Consequently, the tracing does not reveal the cause of the abnormality but rather describes its presence, acuteness, location, and severity. Even though the EEG has little value in revealing the underlying basis of an infant's encephalopathy, it is considered an ideal prognostic indicator.[91-93] Table 164-7 shows common interpretative criteria for mildly, moderately or markedly abnormal EEG backgrounds for near-term and term infants,[94,95] Neonates with normal or only mildly abnormal EEGs generally survive and escape permanent brain damage. On the other hand, those with severe abnormalities, such as isoelectric or burst-suppression recordings, face high mortality and significant morbidity. Those with moderately abnormal EEGs have a mixed outcome: Some do well but others later exhibit a chronic static encephalopathy. The prognostic value of the EEG may be particularly important when the design of a neonatal neuroprotection intervention is contemplated. Infants with well-preserved EEGs (good prognosis) may not be suitable candidates for a protection trial because their outcomes are already favorable, even before the research intervention or placebo. Those with marked EEG abnormalities (dismal prognosis) may be so profoundly injured that they are beyond the reach of benefit from trial enrollment. Those with moderate EEG abnormalities could "go either way," and the balance might be favorably tipped by help from a neuroprotection intervention.

Clinical seizures are among the most common reasons for neonates to undergo an EEG examination. It is a challenge for all involved in the care of neonates to distinguish epileptic-based neonatal seizures from the wide array of nonepileptic "paroxysmal" behaviors, which may occur in healthy or sick infants. A brief discussion of pathologic EEG "sharp waves" and electrographic neonatal seizures follows.

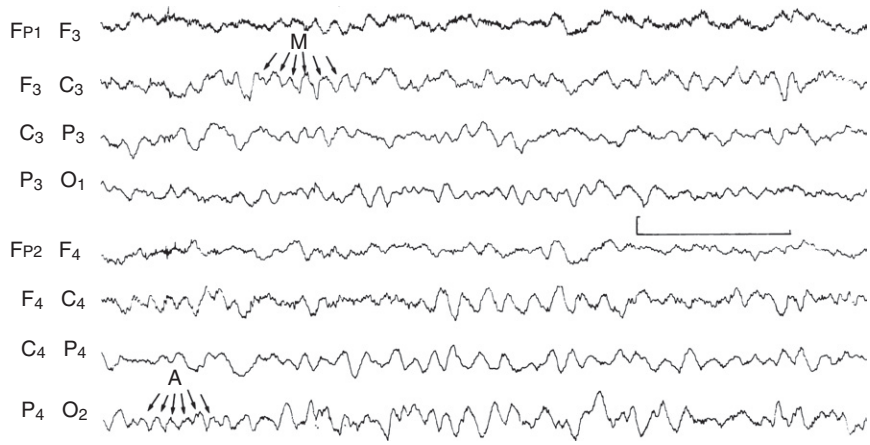


Figure 164-26 Precursors of μ and α rhythms. This electroencephalogram (EEG) segment was recorded from an awake, alert, and quiet 5-month-old infant. Notice the appearance of rhythmic Δ activity in the central regions that are the precursor of μ activity *(M)*. In the occipital regions, rhythmic θ activity is also noted *(A)* and is the precursor of α activity.

TABLE 164-7

Criteria for Conventional EEG Background Interpretation

Interpretive Criteria Near-Term*	Normal	Mildly Abnormal Background	Moderately Abnormal Background	Markedly Abnormal Background
Continuity				
Continuous segments	Mostly continuous in awake (W) and active sleep (AS) states	Continuous in W and AS states	Small percentage of the EEG is continuous	Little or none of the EEG is continuous
Discontinuous segments	Interburst intervals (IBIs) <4 secs in quiet sleep (QS)	IBIs range from 4 to <7 secs in QS	IBIs range from 7 to <15 secs	IBIs ≥15 secs
Amplitude	W or AS: 25-50 μV (p-p)†	W or AS: 25-50 μV	W or AS: 5-15 μV	< 10 μV ("depressed and undifferentiated")
	QS: burst amplitude >50-100 mV	QS: burst amplitude >50-100 mV	QS: 10-25 μV "persistent low voltage"	Some IBIs <2 μV
	QS: amplitude of the IBIs ≥25 μV	QS: amplitude of IBIs <25 μV	Some IBIs <5 to 10 μV	All EEG <2 μV ("isoelectric")
Symmetry	Symmetric	Symmetric	May be asymmetric‡	May be asymmetric
Synchrony	In QS, the bursts of discontinuous portions of EEG are nearly 100% syncronized§	In QS, a few of the bursts may not be syncronized	May show persistent asynchrony in the discontinuous segments of the EEG	May show persistent asynchrony in the discontinuous segments of the EEG
State lability	Spontaneous cycling among W, AS, and QS states and reactive to external stimuli	States cycle spontaneously and react to external stimuli	Poor state differentiation and modest clinical and EEG response to external stimuli	No recognizable states; ("undifferentiated") little or no EEG response to external stimuli
EEG composition graphoelements	Rich variety of frequencies and specific, named patterns such as "delta brushes"	Rich variety of frequencies and specific, named patterns such as "delta brushes"	Poorly developed background with paucity of features	Poverty of all background patterns with few or no specific graphoelements such as "delta brushes"

Modified from Shellhaas RA, Gallagher PR, Clancy RR: Assessment of neonatal electroencephalography (EEG) background by conventional and two amplitude-integrated EEG classification systems. *J Pediatr* 2008:153, 369.

*Near term is defined by a conceptional age of 34 to < 37 weeks; term is a conceptional age from 37 to 44 weeks.

†All voltages presented as peak-to-peak (p-p) values.

‡Asymmetry is present if the ratio of the amplitudes of two homologous brain regions is > 2:1.

§Bursts are synchronized if there is less than 1.5 seconds separating their onset between hemispheres.

Pathologic Sharp Waves

Many forms of sharp waves are an expected, normal feature of the EEG background. However, certain characteristics may render them pathologic[96] (Figure 164-27). For example, contrast the usual behavior of normal temporal sharp waves with pathologic sharp waves recorded *between* seizures in a sick neonate (see Table 164-6). The latter transients are often faster (duration ≤ 100 msec), more abundant, and repetitive and may be unusually distributed spatially in a highly localized fashion. Many times, EEGs recorded to "rule out" seizures in a newborn are not fortuitous to actually capture a seizure in progress. Such interictal tracings may still provide evidence that the patient has a "lowered seizure threshold" by revealing an excessive number of negative-polarity spikes and sharp waves. The EEG spikes or sharp waves are not direct proof of seizures but certainly are consistent with their presence.

Positive-polarity sharp waves are different pathologic EEG transients that do not imply a lowered seizure threshold[97,98] (Figure 164-28), but rather are a marker for injury to the deep

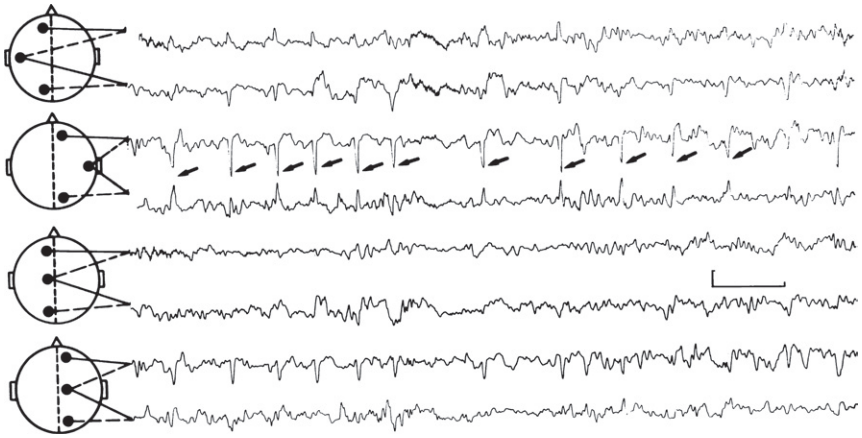


Figure 164-27 Abnormal sharp electroencephalogram (EEG) transients. EEG segment recorded from a 1-week-old (37-week estimated gestational age [EGA]) infant with meningitis and seizures. This segment was recorded between electrographic seizures. Note the excessive abundance of sharp EEG transients (*arrows*), which are virtually entirely confined to the right temporal region except for mild spread into the adjacent rolandic area. This type of electrographic abnormality suggests a lowered seizure threshold and is not usually seen in healthy infants.

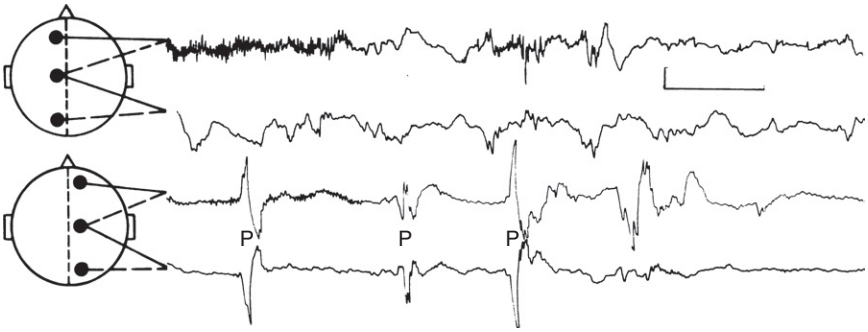


Figure 164-28 Positive rolandic sharp waves. This electroencephalogram (EEG) segment was recorded from a 2-week-old (28-week estimated gestational age [EGA]) infant with a right-sided grade IV intraventricular hemorrhage. On subsequent cranial ultrasound examinations, periventricular leukomalacia was noted on the right. Positive rolandic sharp waves *(P)* are seen as definite sharp EEG transients of positive polarity, which are clearly distinguished from the background activity.

cerebral white matter, such as periventricular leukomalacia. They are usually seen in the midline vertex or rolandic region but can also arise in the temporal areas.[99]

Seizures

In contrast to an isolated sharp wave, which is a fleeting transient EEG pattern, an electrographic neonatal seizure (ENS) unfolds as an *event* with a distinctive beginning and end (Figure 164-29). A typical ENS lasts about 2 minutes and arises focally from a restricted cortical region.[100,101] The knowledge of the relationship between clinical and electrographic seizures has changed over the years, aided by the emergence of video-EEG monitoring. Whereas a routine bedside EEG examination does not capture video and may miss ENSs in a 45-minute recording session, longer-term video-EEG monitoring may be more revealing in evaluating many neonates with seizures.

The valuable role of video-EEG monitoring for characterizing neonatal seizures was established by Mizrahi and Kellaway,[102] who analyzed video-EEGs from 349 neonates between ages 28 and 44 weeks CA with a clinical suspicion of seizures. In 71 patients, 451 paroxysmal clinical events were recorded, but only 29% of the clinical events in 23 patients had a close temporal association with electrographic seizure activity. In other words, some of the abnormal clinical "seizures" were not *epileptic*, but rather a type of abnormal brain stem release or postural reflex. Conversely many electrographic seizures occurred that were not accompanied by any clinical signs of seizure. Such ENSs, termed subclinical or occult, have been identified in other populations. A recent large series from the Neonatal Seizures Clinical Research Centers[103] described 207 infants with video-EEG confirmed seizures. One third of the infants demonstrated only subclinical ENS: Despite the definite electrographic seizures, no obvious clinical seizure occurred at the same time. In 10%, all the patients' abnormal-appearing "seizures" were not accompanied by ENS, even though the EEG background was often seriously abnormal, reflecting their acute encephalopathies. Finally, half of patients had one or more electroclinical seizures that feature simultaneous clinical and electrographic seizures. The clinical events in these instances of electroclinical seizures were frequently recurrent, focal, *clonic* twitching of the face, trunk or extremities.

The hallmark of ENSs is the sudden appearance of a repetitive discharge event containing a definite beginning, middle, and end. ENSs evolve in frequency, morphology, and amplitude. Although no two ENSs appear identical, a few simple criteria aid in their recognition:

(1) An individual ENS arises from a specific focus. ENSs are rarely, if ever, "generalized" (affecting all brain regions in an organized, synchronized, and symmetric fashion). However, after an ENS originates at its focus, it is free to spread or migrate to other locations.

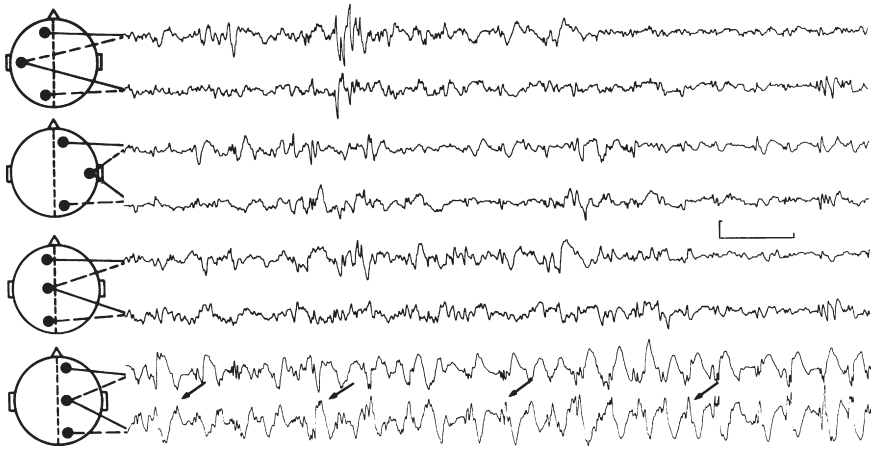


Figure 164-29 Electrographic seizure. This segment was recorded from an asphyxiated 1-day-old full-term infant. An electrographic seizure is in progress (*arrows*).The electrographic seizure is an event with a characteristic evolution of repetitive, stereotyped, rhythmic waveforms or spikes/sharp waves that replace the underlying background rhythms locally. During this particular electrographic seizure, no clinical signs were exhibited by the asphyxiated infant.

(2) The minimum duration of an ENS is conventionally defined as 10 seconds. Most ENSs are shorter than 2 minutes, but it is common to observe multiple, serial, brief ENSs.
(3) The specific shape or morphology of the waveforms that make up the ENS varies widely within and between neonates (see Figure 169-29). Any EEG frequency (e.g., theta activity) may compose the seizure. However, the morphology and amplitude of the ENS evolve; the end of the seizure looks different in amplitude and shape from the beginning.

Laroia and colleagues[104] suggested a strategy to target high-risk neonates for video-EEG monitoring. In their study, 51 neonates with specific intensive care unit admission diagnoses (e.g., asphyxia, meningitis) had a 30 to 60 minute screening EEG, followed by continuous EEG monitoring for 18 to 24 hours. The initial EEG background at admission was classified as normal, immature for age, or mildly, moderately, or severely abnormal. Only 1 of 24 infants (4.2%) with normal or immature EEG backgrounds had subsequent ENS. However, 22 of 27 infants (81%) with abnormal EEG backgrounds had ENS. Thus the initial EEG background significantly predicted the presence or absence of seizures in the subsequent 18 to 24 hours.

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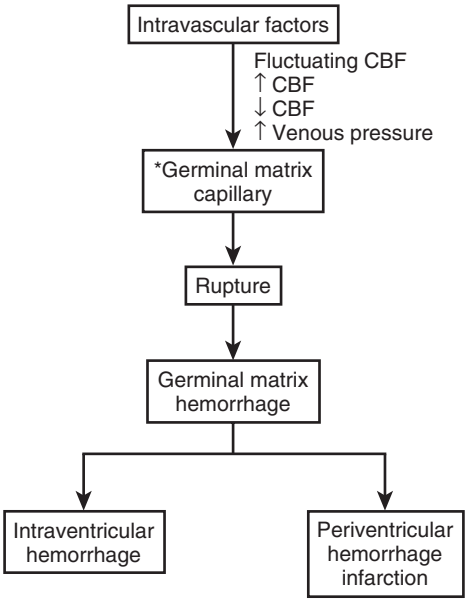


Figure 165-1 Schematic depicting the important vascular changes in the pathogenesis of intraventricular hemorrhage. *Both vascular factors (i.e., involving capillary bed) and extravascular factors (i.e., poor vascular support) likely contribute in the pathogenesis of IVH.

vary has not been determined for newborn infants. Because systemic blood pressure is lower in newborn infants than in older children or adults, the range of pressures at which CBF is constant is expected to be lower than in adults. In newborn animals, the range of blood pressures at which CBF is constant is lower than in adult animals.[20,21] More importance, in fetal lambs, resting blood pressure is only slightly higher than the lower limit of the autoregulatory curve.[21] If a similar situation existed in newborn infants, moderate hypotension would result in reduced CBF and possible ischemic brain injury.

Increasing evidence suggests that the autoregulatory response is intact in human neonates who are experiencing mild asphyxiation or respiratory distress. Thus, in several studies in preterm infants with normal brains, CBF is unaffected by fluctuations in mean arterial blood pressure within the physiologic range of 25 to 60 mm Hg.[22-25] These observations were consistent for infants of various gestational and postnatal ages. By contrast, Lou and colleagues[26] concluded that CBF was impaired in distressed asphyxiated newborns—that is, CBF was significantly related to systolic blood pressure. The conclusions presented by these investigators have been questioned, however, because the study group was heterogeneous with regard to gestational age and birth weight, and some infants had an elevated $Paco_2$ at the time of study.[27] In support of this concern in a recent study using the Doppler technique, the autoregulatory slope was found to increase with progressive increase in Pco_2, suggesting that the cerebral circulation becomes pressure-passive with hypercapnia.[8] Earlier Doppler measurements of flow velocity suggested poorly developed pressure autoregulation in the very premature infant.[28] The limitations of the Doppler technique in this regard are discussed further on.

Systemic Factors and Cerebral Blood Flow

Effect of Carbon Dioxide Tension

The cerebral vessels react to both $Paco_2$ and blood pH. Carbon dioxide has a marked relaxant effect on cerebral vascular muscle, and changes in $Paco_2$ produce a significant change in CBF. Moderate hypocarbia leads to a reduction in CBF, and moderate hypercarbia leads to an increase in CBF without changes in cerebral oxidative metabolism.[29-31] If the $Paco_2$ falls to less than 15 to 20 mm Hg, the reduction in CBF is associated with mild reduction in cerebral oxidative metabolism.[32] Although chronic severe hypercarbia elevates CBF, cerebral oxidative metabolism is not altered. Acute elevations in $Paco_2$, however, may depress cerebral metabolism.[33] Most importance, hypercarbia abolishes the autoregulatory response because of marked vasodilation, and in the premature infant, this effect, not influenced by postnatal age, is noted as the Pco_2 values increase progressively above 45 mm Hg.[8] This disturbance may add to the risk of hemorrhagic brain injury.[34,35]

The cerebral vessels of fetal and newborn animals are responsive to acute changes in $Paco_2$, although the reactivity varies as a function of postnatal age and sickness. As reported in two different studies, spontaneously breathing preterm infants demonstrated appropriate CBF-$Paco_2$ reactivity shortly after birth[22,36] whereas the reactivity was significantly less in mechanically ventilated preterm infants also studied in the first hours of life.[36] In the latter group of infants, CBF-$Paco_2$ reactivity on the second postnatal day was similar to that observed in adults.[36] Attenuated CBF-$Paco_2$ reactivity similarly has been noted in ventilated newborn animals in the immediate postnatal period.[32,37] The mechanism accounting for this attenuated CBF-$Paco_2$ reactivity is unclear, and it is considered perhaps to be a consequence of the hypercapnia state in utero.[23]

Effect of Arterial Oxygen Content

Arterial hypoxia produces cerebral vasodilation and increases CBF.[38,39] The effect of hypoxia is related directly to Cao_2, and not directly to arterial oxygen tension (Pao_2). Cao_2 is determined by the hemoglobin (Hb) concentration in the blood, that is, the oxygen affinity of the Hb and Pao_2. Thus mild to moderate reductions in Pao_2 that are associated with little change in Cao_2 increase CBF only slightly. A reduction in Cao_2 produces a proportional increase in CBF such that cerebral oxygen delivery (CBF × Cao_2) does not change and cerebral oxygen metabolism is constant. The effects of Cao_2 have been studied in detail in experimental animals and in human adults, but data for newborn infants are limited. Both Pryds and Younkin and their colleagues[40,41] found an inverse relationship between CBF and hematocrit or Hb. The increase in CBF with a lower hematocrit or Hb presumably is the result of a reduced Cao_2, although Cao_2 was not measured in either study.

Effect of Blood Glucose

In adult humans, hypoglycemia of mild or moderate degree is not associated with a change in CBF.[42,43] During hypoglycemic coma, however, CBF increases.[44] In experimental animals, CBF increases during hypoglycemic coma and correlates with a loss of electrical activity on the electroencephalogram.[45-48] The increase in CBF appears to result from increased systemic blood pressure and loss of autoregulation.[49] Thus, if the blood pressure is reduced during hypoglycemic coma, CBF returns to baseline.

Pryds and colleagues[50] reported increased CBF in preterm infants with hypoglycemia. CBF was significantly higher at 26 mL/100 g/minute in infants with blood glucose levels lower than 30 mg/dL than in those with blood glucose levels greater than 30 mg/dL, in whom CBF was only 11.8 mL/100 g/minute. None of the infants with low blood glucose levels exhibited clinical signs of hypoglycemia, nor did any progess to coma. Moreover, no abnormalities of the electroencephalogram were apparent. These data suggest that the response to hypoglycemia in preterm infants is different from that in adults. Furthermore, cerebral glucose metabolism may be critically important to provide the energy requirements of the preterm brain. These findings are in agreement with those for a study in which Altman and colleagues[51] reported that cerebral oxygen metabolism in some preterm infants may be minimal, and that cerebral glucose

metabolism may provide the necessary energy for preterm cerebral growth and function.

In summary, the newborn circulation shows many of the same characteristics as those of the adult circulation. The magnitude of the CBF responses and the quantitative relationships of CBF with changes in blood pressure and with a variety of systemic factors have not been fully elucidated. The relationship of these circulatory regulatory responses with the development of IVH and their role in its pathogenesis in sick preterm infants remain to be determined.

MEASUREMENT OF CEREBRAL BLOOD FLOW

Cerebral Arterial Blood Flow Velocity

Cerebral arterial blood flow velocity (CBFV) has been measured in the major intracranial arteries of the developing brain by the Doppler technique for many years. Although this technique does not measure CBF, it has certain advantages: It is both noninvasive and readily available at the bedside.

Principle

The ultrasound technique for the measurement of blood flow velocity makes use of the *Doppler effect*—that is, the frequency of sound waves reflected from a moving object is shifted by an amount proportional to the velocity of the object. When ultrasound studies are used for measurement of blood flow velocity, the sound waves emitted by the transducer are reflected, and their frequency is shifted by moving red blood cells.[52] The Doppler signals are directly related to the frequency of the transmitted ultrasound waves and to the cosine of the angle of incidence of the sound beam to the axis of blood flow, and they are inversely related to the velocity of the ultrasound waves in the tissue. For practical purposes, both the frequency of the transmitted sound waves and their velocity in the tissue are constant from one determination to another; thus, if the angle of incidence is held constant, blood flow velocity will be the main determinant of the Doppler frequency shift.

Attempts to *quantitate* arterial blood flow velocity have used determinations of the pulsatility index (PI) or the area under the velocity curve. The PI is calculated from the formula $PI = \frac{S - D}{S}$, where S is the peak systolic velocity and D is the end-diastolic velocity.[53,54] Changes in probe angle affect the values for S and D similarly, so the PI can be used to minimize the effect of changes in probe angle and to allow meaningful comparisons of serial determinations of CBFV. PI was described initially as a measure of peripheral vascular resistance in large systemic arteries; accordingly, in the current context, PI is assumed to reflect changes in cerebrovascular resistance. Serial measurements of PI also may reflect changes in cerebral perfusion as well as cerebrovascular resistance, especially if either the systolic or diastolic velocity changes individually. If, however, both systolic velocity and diastolic velocity change proportionately, serial determinations of the PI will not reveal a change in absolute flow velocity or tissue perfusion. To avoid this problem, determination of the area under the velocity waveforms (determined automatically by ultrasound machines) has been used. Two types of Doppler systems have been used in the study of the cerebral circulation: continuous-wave and range-gated Doppler systems.

Continuous-Wave Doppler Ultrasound

The continuous-wave system uses a bidirectional probe that is placed over the anterior fontanelle and is oriented toward the pericallosal artery (terminal branch of the anterior cerebral artery) as it courses over the genu of the corpus callosum; alternatively, the right or left middle cerebral artery can be insonated transcranially through the coronal suture. Maximum arterial pulsations of advancing flow are used for measurements.

Range-Gated Doppler Ultrasound

The pulse range-gated Doppler technique selectively detects blood flow in a certain area at a specific distance from the probe and usually is incorporated into a conventional ultrasound imaging instrument, and the vessel to be insonated can be visualized directly. This visualization provides the capability to determine the angle of insonation and to identify specific arteries (e.g., at the circle of Willis) to be insonated. Current technology, however, does not allow for the measurement of the cross-sectional area of the insonated artery, but if the resolution of the ultrasound instruments can be improved to provide this measurement, a major step toward measurement of total volume of arterial blood flow, rather than only velocity of arterial blood flow, will have been accomplished.

Other advantages of this technique are the capability to record the velocity profile of all moving red blood cells in the insonated artery and the capability to compute the instantaneous average blood flow velocity. Because blood flow through a vessel is related to the average velocity and not to the peak velocity, this advantage should help in estimating CBF in situations in which CBF and blood pressure change rapidly.

Relation of Cerebral Arterial Blood Flow Velocity to Cerebral Blood Flow

The degree to which CBFV reflects CBF is of considerable importance, because accurate measurement of CBF involves techniques that are complex, expensive, and invasive, whereas measurement of CBFV is a readily available, noninvasive bedside technique. By convention, CBF is measured as the volume of blood perfusing a mass of brain tissue per unit of time. The volume of blood flowing in a specified artery is related to the instantaneous average velocity of flow (not peak flow velocity) times the cross-sectional area of the vessel. Thus measurements of velocity and arterial caliber are essential to provide an estimate of CBF. If the mass of tissue perfused by a given artery and the cross-sectional area of the artery were to remain constant, CBFV would be the major determinant of CBF. However, both the vessel diameter and the mass of tissue supplied by a given artery may change under normal physiologic circumstances and in pathologic conditions. For example, during autoregulation, CBF is maintained constant over a broad range of perfusion pressures by mechanisms that include change in arterial diameter and resistance. Therefore flow velocity may change inversely relative to vessel diameter even though CBF is unchanged.

In summary, accurate calculation of CBF should be based on average (not peak) CBFV as well as arterial cross-sectional area. Measurements based only on peak CBFV may be misleading. In steady-state conditions in which arterial caliber does not change, such measurements may provide useful information. By contrast, under conditions of changing arterial blood pressure and CBF, estimation of CBF from peak arterial blood flow velocity is of uncertain value and may not reflect changes in CBF.[15]

Experimental Studies

Several studies have examined the relationship of CBFV with CBF in newborn animals and in the human newborn infant. In experimental animals, CBF was raised or lowered by altering $Paco_2$ or Co_2.[55-57] CBFV was measured in the anterior cerebral artery, and CBF was determined by either the microsphere technique or autoradiography. All studies were performed under steady-state conditions, and the results were based on measurement of peak, not average, CBFV. Arterial cross-sectional diameter was not measured but can be assumed to change little in the steady state. Changes in CBFV (i.e., systolic velocity, diastolic velocity, area under the velocity curve, and PI) were significantly related to a change in CBF. This result may have been anticipated, because blood flow velocity is one of the

major determinants of CBF. However, the degree of correlation in the preceding studies, as determined by the correlation coefficient (r), varied, ranging from 0.3 (PI) to 0.9 (area under the velocity curve). In the preceding examples, variation of the PI ($r^2 = 0.09$) accounted for only approximately 10% of the variation in CBF, whereas variation in the area under the velocity curve ($r^2 = 0.81$) accounted for approximately 80% of the variability of CBF.

Therefore changes in CBF produced experimentally are associated with a significant change in CBFV. Furthermore, changes in CBFV may account for up to 80% of the variability in CBF *under steady-state conditions.* Because the PI accounts for only approximately 10% of the variability of CBF (at least under the conditions studied), it is not prudent to use this index as a general indicator of CBF.

Studies in the Human Newborn

Attempts have been made to correlate CBFV with CBF in human newborn infants. Greisen and co-workers[58] compared CBF, measured by the xenon technique, with anterior cerebral artery and common carotid artery flow velocities. All arterial blood flow velocity variables measured were significantly related to CBF. However, the correlation coefficients ranged from 0.4 to 0.8, and diastolic flow velocity exhibited the highest correlation. A similar relationship was found in newborn infants by comparing anterior cerebral artery blood flow velocity with CBF in the frontal region (anterior cerebral artery distribution) measured with positron emission tomography (PET).[59] In 25 premature infants with various neurologic disorders (IVH, asphyxia, hydrocephalus), CBF measured with PET was significantly related to arterial blood flow velocity with a correlation coefficient of 0.7.

Clinical Studies of Cerebral Arterial Blood Flow Velocity and Intraventricular Hemorrhage

Changes in Cerebral Blood Flow Velocity with Respiratory Distress Syndrome and Its Complications

The Doppler technique has been used for many years to study the relationship of changes in the cerebral blood flow profile and the subsequent development of IVH. The most striking association noted was that between fluctuations in CBFV in infants with respiratory distress syndrome and IVH. Thus infants with fluctuations in CBFV in the first 24 hours were significantly more likely to experience IVH than those with a stable pattern of CBFV[60] (Figures 165-2 and 165-3). These fluctuations were attributed to the changes in intrathoracic pressure caused by the infant's breathing out of synchrony with the ventilator.[14] These critical observations led to a study designed to prevent IVH by eliminating the fluctuations in CBFV[56] (Figure 165-4). The infant's respiratory effort was abolished by muscle paralysis with pancuronium; this effect was associated in a significant reduction in IVH and, in particular, severe IVH.[61] Since these initial observations, several changes have evolved in neonatal care that have

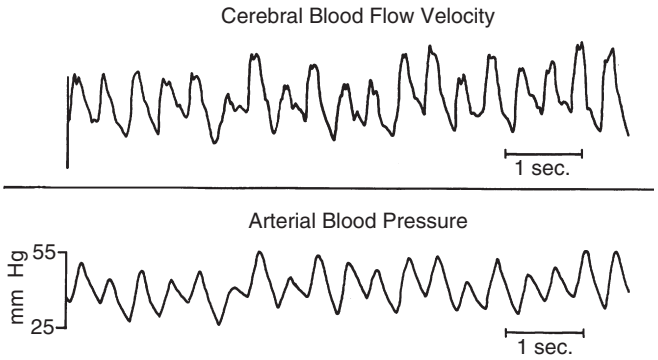


Figure 165-3 Fluctuating arterial blood flow velocity pattern and simultaneously recorded arterial blood pressure in a ventilated premature infant with respiratory distress syndrome. Marked fluctuations are apparent in systolic and diastolic flow velocity and blood pressure. *(From Perlman JM, et al: Reduction of intraventricular hemorrhage by elimination of fluctuating cerebral blood flow velocity in preterm infants with respiratory distress syndrome,* N Engl J Med *312:1353, 1985; reprinted by permission of the New England Journal of Medicine.)*

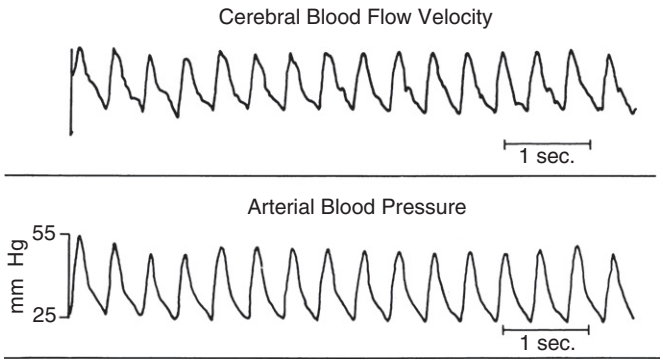


Figure 165-2 Stable arterial blood flow velocity pattern and simultaneously recorded arterial blood pressure in a ventilated premature infant with respiratory distress syndrome. Equal peaks and troughs can be seen in systolic and diastolic flow velocity and blood pressure. *(From Perlman JM, et al: Reduction of intraventricular hemorrhage by elimination of fluctuating cerebral blood flow velocity in preterm infants with respiratory distress syndrome,* N Engl J Med *312:1353, 1985; reprinted by permission of the New England Journal of Medicine.)*

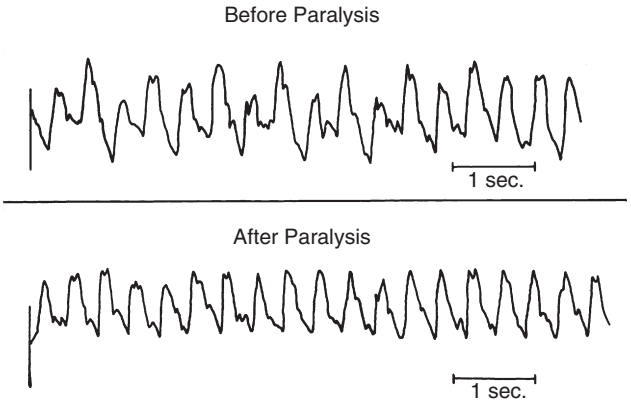


Figure 165-4 Effect of muscle paralysis on fluctuating cerebral blood flow velocity. After paralysis was induced by pancuronium, the fluctuating pattern was eliminated. *(From Perlman JM, et al: Reduction of intraventricular hemorrhage by elimination of fluctuating cerebral blood flow velocity in preterm infants with respiratory distress syndrome,* N Engl J Med *312:1353, 1985; reprinted by permission of the New England Journal of Medicine.)*

modified the impact of these perturbations in CBFV. First is the antenatal administration of steroids, which has been associated with a significant reduction in IVH, perhaps by stabilizing the germinal matrix capillaries.[9,61,62] The second such advance, in neonatal ventilator strategies, is synchronized mechanical ventilation, which has minimized the perturbations in cerebral blood flow.

Additional striking changes in CBFV have been observed in infants in whom IVH developed after pneumothorax.[63] These changes included a significant decrease in the PI, in large part accounted for by an increase in the diastolic flow velocity, which gradually returned to normal over the subsequent 36 hours after evacuation of the pneumothorax. The changes in CBFV were associated with significant changes in systemic blood pressure; that is, mean blood pressure and diastolic blood pressure increased significantly after evacuation of the pneumothorax. It is unclear whether these arterial changes, or alternatively, the increases in systemic venous pressure that have been shown to occur simultaneously in such infants, could have provoked hemorrhage.[64]

Cerebral Blood Flow Velocity Changes Under Other Circumstances

After Surfactant Replacement Therapy

As noted earlier, a strong association has been recognized among respiratory distress syndrome, fluctuations in CBFV, and IVH.[60] However, the introduction of surfactant replacement therapy with improvement in respiratory function has not been associated with a reduction in IVH.[65] The reasons for this unanticipated finding remain unclear. Cowan and co-workers[66] demonstrated acute changes characterized by a marked decrease in CBFV of 36% within 2 minutes after surfactant administration, with almost complete loss of the diastolic velocity pattern. The decrease in diastolic flow velocity is considered to be secondary to shunting across the patent ductus arteriosus (PDA) (see later). In another study, Jorch and associates[67] demonstrated continuous CBFV variability in treated infants, comparable with that seen in untreated infants, at 12-hour intervals after surfactant therapy.[67] Most recently, Kaiser and co-workers demonstrated a striking increase in mean CBFV of 75% ± 52% that peaked approximately 15 minutes after surfactant administration. These changes in CBFV were significantly associated with increasing P_{CO_2} ($P < .0001$) and were not related to changes in mean blood pressure.[68] The significance of these changes to the subsequent development of IVH in infants receiving surfactant therapy has not been determined.

Patent Ductus Arteriosus

Changes in CBFV have been observed in infants who develop symptomatic PDA characterized by a fall in the diastolic flow velocity that reflects the low systemic diastolic blood pressure[69] (a so-called steal phenomenon).

Endotracheal Suctioning

Marked changes in CBFV also may occur in association with routine procedures such as endotracheal suctioning.[70] In particular, a prominent increase in diastolic flow velocity has been documented. Concomitant increases in systolic, diastolic, and mean blood pressures also may be noted.

Summary and Implications

Changes in CBFV and blood pressure have been described in premature infants with respiratory distress syndrome or pneumothorax, after surfactant administration, in babies with PDA, and in association with routine neonatal procedures. In some instances, observed CBFV changes have had a temporal relationship with the subsequent development of IVH. The precise mechanism by which these changes lead to IVH is not clear. Moreover, the changes enumerated earlier have been observed in infants with these conditions in whom IVH did *not* develop. This finding suggests that additional factors are important in the genesis of IVH.

Near-Infrared Spectroscopy

Near-infrared spectroscopy (NIRS) refers to an optical technique that can provide information concerning changes in cerebral oxygen saturation, CBF and volume, cerebral venous oxygen saturation, and cerebral oxygen utilization in the brain.[71] The method is based on two principles: (1) Light in the near-infrared range of wavelengths between 700 and 1000 nm can pass through skin, bone, and other tissues relatively easily and illuminates approximately 6 to 8 cm of newborn brain; and (2) characteristic absorption bands of oxygenated and deoxygenated Hb and of the mitochondrial enzyme cytochrome oxidase are present in the near-infrared range. Changes in absorption bands can be used to monitor quantitative changes in the amounts of oxyhemoglobin (HbO_2) and deoxygenated Hb; total Hb (HbO_2 + deoxygenated Hb) is used as an indicator of blood volume. These changes also can be used to monitor the oxidation reduction state of cytochrome oxidase, a critical terminal enzyme of the respiratory mitochondrial electron transport chain, which facilitates electron passage to molecular oxygen for oxidative phosphorylation and adenosine triphosphate synthesis.

When the near-infrared beam is passed through the tissue, a decrease in signal intensity results from the absorbance of a particular chromophore in the medium. Thus a band of maximum absorption is present at approximately 810 nm for Hb, above which HbO_2 has greater light absorption and below which deoxygenated Hb has greater absorption. A much smaller peak at approximately 830 to 840 nm has been observed for cytochrome oxidase.

Instrumentation

The spectroscope typically contains diodes that serve as a source of near-infrared light.[72] The lasers are pulsed sequentially in a rapid on-off fashion. The light from the lasers is captured in glass fiberoptic strands grouped in a bundle. The end of the bundle is applied to the side of the infant's forehead, commonly against the parietal bone at a point equidistant from the anterior fontanelle and the external auditory meatus. The light traverses the head, where a second fiberoptic bundle attached to the opposite side of the head collects the transmitted light photons and conveys them to a photomultiplier tube. The signals are then amplified, expressed as a ratio of a reference signal, and changed into logarithmic form. To prevent interference from background illumination, the infant's head is wrapped in a light-tight bandage.

The attenuation of light by absorption is a measure of the concentration of the chromophore present. The relationship between the absorption and the concentration of the chromophore is best described by a modified Lambert-Beer law,[73] which, for the purpose of describing optical absorption in a highly scattered medium, can be expressed as follows:

$$\text{Absorption (OD)} = (a \times c \times L \times P) + G$$

where OD = optical density, a = extinction coefficient of the chromophore (mM × cm), c = concentration of the chromophore (mM), L = distance between the point of entry and exit (in centimeters), P = a path length factor accounting for scattering of light in the tissue, and G = a factor related to tissue and optode geometry. If L, P, and G are assumed to remain constant during a study, then changes in chromophore concentration can be expressed as follows:

$$\Delta C = \frac{\Delta OD}{aLP}$$

The chromophore concentration can be calculated because the extinction coefficient, a, has been determined for HbO_2, deoxygenated Hb, and cytochrome oxidase,[73,74] and the value of P in the

brains of premature infants measured after death has been determined by the time-of-flight method and found to be 4.39 ± 0.28 (SD). The latter determination is not straightforward, however, because of scattering of light within the head.[75] This scattering effect may be modulated by several factors including myelination, bone mineralization, and cell concentration. Moreover, path length factor *P* values differ for the various wavelengths used (775 to 903 nm), with a longer path length at shorter wavelengths.[76]

Clinical Application

Attempts have been made with NIRS to quantitate cerebral blood volume (CBV) and to measure CBF. Wyatt and colleagues reported a mean CBV value of 2.22 ± 0.40 (SD) mL/100 g in 12 newborn infants with "normal" brains who were born at 25 to 40 weeks gestation and studied at postnatal ages of 10 to 240 hours—similar to values obtained by PET[77,78] (see later). Mean CBV values were significantly higher at 3.00 ± 1.04 mL/100 g in 10 brain-injured infants ($P < .05$).[77] Similar CBV values were obtained in a subsequent study from the same investigators.[79] In clinical studies, increases in CBV have been observed in association with sharp increases in blood pressure and changes in $Paco_2$[80-83] (Figure 165-5). By contrast, an increase in CBV accompanied by a decrease in HbO_2 has been noted after endotracheal suctioning; under these conditions, the increase in CBV is consistent with elevated central venous pressure.[84] The response of CBV to $Paco_2$ has been shown to increase with advancing gestational age.[82]

Noninvasive measurement of CBF by NIRS uses the Fick principle by determining the rate of increases in cerebral HbO_2 after an increase in arterial oxygen saturation induced by a brief inhalation of oxygen.[85] The mean value of CBF obtained in the 13 premature infants, that is, 18 mL/100 g/minute, is comparable to values obtained by the xenon technique and by PET (see later).

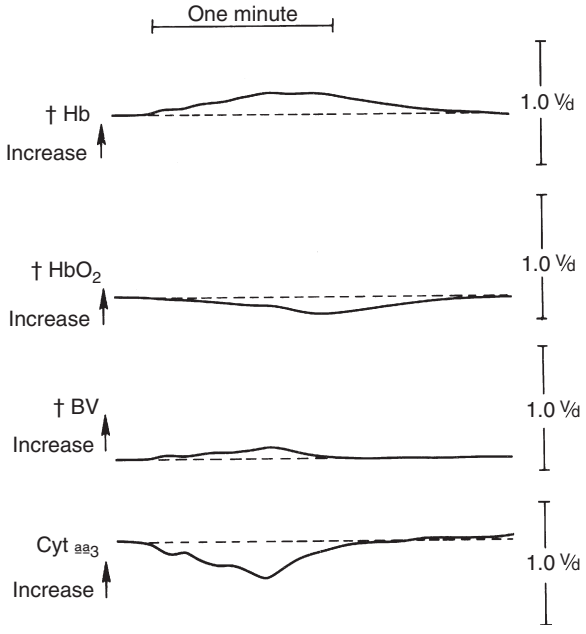


Figure 165-5 Typical response to decreased oxygenation: Oxyhemoglobin ($tHbO_2$) decreases, whereas deoxyhemoglobin (tHb) rises. An overall increase in blood volume (tBV) results, probably as a consequence of increased cerebral blood flow in response to hypoxia. Levels of cytochrome aa_3 (cyt a,a_3) decrease still further, concurrent with the decrease in oxyhemoglobin. *(From Brazy JE: Near infrared spectrophotometry. In Nelson NM, editor:* Current therapy in neonatal-perinatal medicine, *ed 2, Toronto, 1990, BC Decker, pp 439-444.)*

When estimations of CBF obtained using near-infrared spectroscopy were compared with those acquired using ^{133}Xe clearance techniques, a significant correlation was found between the two measurements: $r = 0.80$, $P < .001$ in one study[86] and $r^2 = 0.84$, $P < .001$ in a second study.[77] Certain methodologic problems need to be considered: The test-retest variability in the same subject is reported at 15% to 20%; and in one study, up to two thirds of the NIRS recording was unsuccessful because of inferior quality.[86] NIRS estimation of CBF may be significantly underestimated in the high flow range because of methodologic constraints.

Clinical Studies of Near-Infrared Spectroscopy and Intraventricular Hemorrhage

The relationship between CBF measured in the first 24 hours of life and IVH was determined in 24 premature infants.[87] Infants were divided into three groups on the basis of cranial ultrasound appearance: a group with normal findings, a group with minor IVH, and a group with moderate to severe IVH. Infants with any form of IVH had lower median CBF (7.0 mL/100 g/minute) than those without IVH (12.2 mL/100 g/minute). On subgroup analysis, those infants with severe IVH had the lowest CBF values (median 5.8 mL/100 g/minute). In a second study, quantitative changes in cerebral concentrations of HbO_2 and deoxygenated Hb coupled with simultaneous measurements of arterial oxygen saturation by pulse oximetry and mean arterial pressure were obtained within the first hours of life in 32 premature infants with birth weights less than 2000 g.[24] In addition, an index of cerebral intravascular oxygenation, the continuous hemoglobin difference signal, defined as $Hb_D = HbO_2 - Hb$, which has been shown to be correlated with CBF, was determined as well.[88] Concordant changes in Hb_D and mean arterial pressure consistent with autoregulation were observed in 17 of the 32 infants; either severe IVH or periventricular leukomalacia developed in 8 of these 17 infants. Only 2 of the 15 infants with nonconcordant changes experienced IVH. These observations are akin to the Doppler findings described earlier.[60]

Near-Infrared Spectroscopy Changes Under Other Circumstances

Surfactant

The acute effects of surfactant replacement therapy on cerebral hemodynamics also have been studied with NIRS.[89] Observations were made before and after surfactant administration in 20 sick premature infants. Surfactant administration was associated with acute, small transient perturbations in cerebral HbO_2 concentration and CBV.

Treatment of Patent Ductus Arteriosus with Indomethacin

Acute cerebral hemodynamics and oxygen delivery were studied in 13 preterm infants with a PDA who were treated with indomethacin delivered either rapidly (over 30 seconds) or by slow infusion (20 to 30 minutes).[90] Irrespective of the rate of administration, indomethacin produced a consistent rapid decrease in CBF, oxygen delivery, and reactivity of blood volume to carbon dioxide.

Impact of Chorioamnionitis

Premature infants exposed to chorioamnionitis are at increased risk for the development of IVH.[9,91] The effects of chorioamnionitis on cerebral oxygen delivery as determined by NIRS were assessed in 43 premature infants during the first week of life.[92] Variability in the NIRS tracing was categorized as long-term, intermediate, or short-term. Chorioamnionitis-exposed infants exhibited reduced intermediate variability in cerebral oxygenated and deoxygenated hemoglobin but not in total hemoglobin. Infants with IVH had the lowest intermediate variability on the first postnatal day. No differences in short-term variability were found between chorioamnionitis-exposed

and -nonexposed infants or in those with and without IVH. The significance of these observations with regard to the development of IVH remains unclear.

Quantitative Cerebral Blood Flow Measurements

Positron Emission Tomography

Methodology

The use of PET to measure CBF requires a cyclotron to generate the positron-emitting radionuclides, a PET scanner to measure accurately the regional radioactivity in vivo, and mathematical models of radiotracer kinetics that relate tissue radioactivity measured by PET to a quantitative value, for example, CBF.[93,94] In the early 1980s, this technique was applied for study of the cerebral circulation, but it has not been used since. For completeness, however, the technique and the studies that are relevant to IVH are briefly reviewed next.

Emission tomography is a technique that produces an image of the distribution of a previously administered radionuclide in any desired part of the body (e.g., the head). Certain radionuclides (e.g., ^{15}O) decay by the emission of a positron, which is a small, positively charged particle. After traveling a few millimeters in tissue, the positron interacts with an electron, with destruction of both. The resultant annihilation event produces two high-energy γ-photons that travel in opposite directions (Figure 165-6). These photons can then be detected almost simultaneously by a pair of external detectors placed on opposite sides of the positron-emitting source (i.e., 180 degrees apart). If the detector pair is connected by an electronic coincidence circuit so that a signal is recorded only when two photons arrive virtually simultaneously, only photons originating from annihilation events between the detector pair will be recorded.[95] Thus most scattered photons are not recorded.

To correct for the other problem, that of absorption of photons, a correction factor is derived as follows: Before an individual scan is performed, an external positron-emitting source is placed within the detector field, and the number of coincidences for each detector is recorded. The infant's head is then placed in the detector field (with the external source still in position), and the number of coincidences that each detector now registers is recorded. The difference in recorded coincidences results from absorption (attenuation) of the photons by the infant's head. An attenuation fraction can then be calculated and used to correct data from the subsequent blood flow scans. Failure to correct for attenuation will underestimate the radioactivity present in the sampled tissue, resulting in an erroneously low value.

Positron Emission Tomograph

The PET scanner is composed of rings of external detectors, connected by coincidence circuits, which surround the head. The multiple rings allow simultaneous reconstruction of multiple transverse slices. The information recorded by the detectors in the multiple rings is used by a computerized system to reconstruct an image that is an accurate quantitative representation of regional tissue radioactivity.

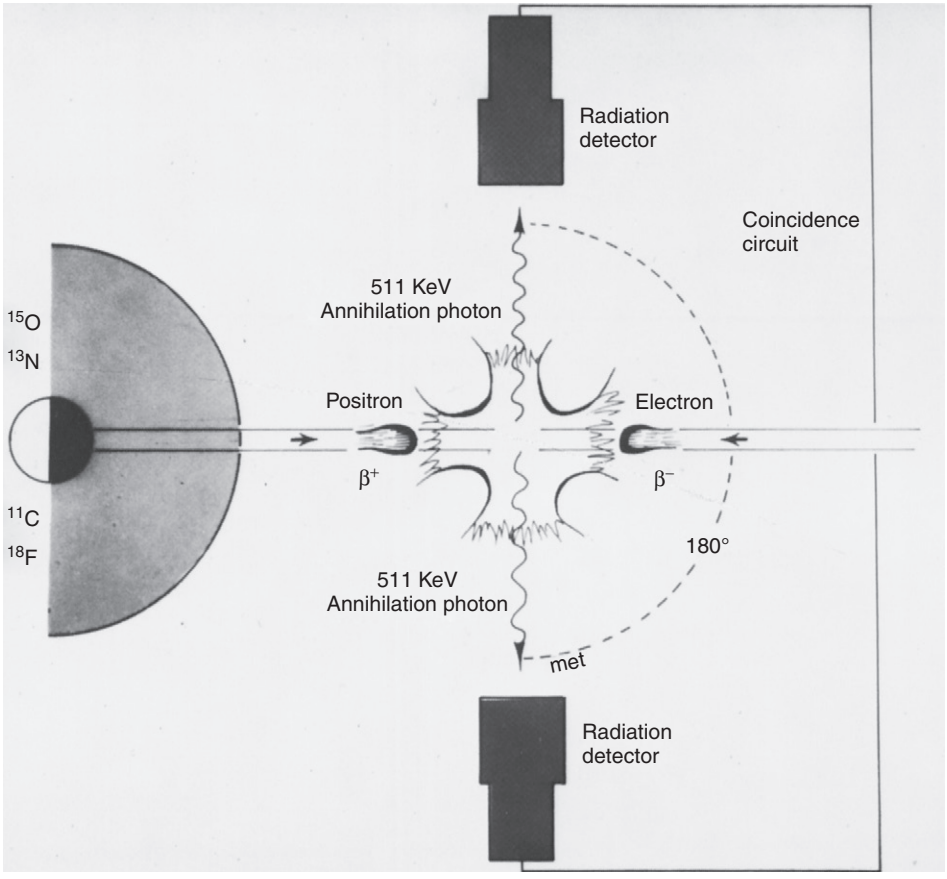


Figure 165-6 Basic principle of positron emission tomography. Isotopes decay by emission of a positron (β^+), which travels a few millimeters before colliding with an electron (β^-), producing an annihilation event. The latter event results in emission of two high-energy photons, which travel at approximately 180 degrees from each other and are detected by radiation detectors, which are linked in coincidence circuits. *(From Raichle ME: Positron emission tomography,* Annu Rev Neurosci *6:249, 1983; reproduced with permission from the Annual Review of Neuroscience, © 1983 by Annual Reviews, Inc.)*

One aspect of PET that is especially important in studies of newborn infants and requires special mention is *spatial resolution.* In the PET image, radioactivity from a given region is redistributed or "smeared" over a larger area. As a result of this redistribution, a given region in the reconstructed PET image contains only a portion of the radioactivity actually emitted from that region in the original structure. The remainder of the radioactivity is redistributed into surrounding areas of the image. Similarly, radioactivity from the surrounding regions is redistributed into the region of interest. Thus the regional radioactivity measurement made with PET represents the combined contribution of the radioactivity actually within that region and a contribution from radioactivity in surrounding regions.[93,96-99] This effect is known as *partial volume averaging.* It is therefore impossible to sample pure cortical gray matter and subcortical white matter because of the small size. In addition, recording in any region that is close to the surface of the brain or to the ventricles will include activity from the cerebrospinal fluid. Inclusion of this physiologically "inactive" fluid will lead to underestimation of the regional measurement for contiguous brain regions.

Measurement of Cerebral Blood Flow with ^{15}O-Labeled Water

The radiotracer used to measure CBF in infants by PET is ^{15}O-labeled water. Accuracy of the method has been described in detail elsewhere[98-100] and is reviewed briefly here: ^{15}O has a half-life of 123 seconds, and this rapid decay of radioactivity allows the absorbed radiation dose to be maintained at a safe, low level. A 40-second PET scan is obtained after the intravenous injection of ^{15}O-labeled water. The relationship between regional cerebral radioactivity and regional CBF is essentially linear, so measured radioactivity in a given region closely approximates mean CBF in that region. Quantitation of absolute values of CBF requires a simultaneous arterial time-activity curve, which is plotted using values for rapid arterial blood sampling obtained either from a peripheral artery (radial, tibial) or from the descending aorta (through an umbilical arterial catheter).

PET Measurements of Cerebral Blood Flow in Newborn Infants with Intraventricular Hemorrhage

A single study used PET methodology to measure regional CBF in six premature infants with IVH associated with a unilateral parenchymal echodensity indicative of hemorrhagic parenchymal infarction.[101] Analysis of the regional CBF images revealed the expected reduction in blood flow in the region of the parenchymal echodensity (Figure 165-7). However, blood flow was apparently reduced more globally in the affected hemisphere in areas distant to and not involved by the hemorrhage. Neuropathologic studies in these infants revealed hemorrhagic infarction in the region of the parenchymal echodensity and nonhemorrhagic infarction in the periventricular regions distant to the hemorrhagic infarction and apparently corresponding with the areas of reduced blood flow. These findings suggest that the hemorrhagic parenchymal infarct is part of a larger lesion that presumably is ischemic. However the mechanisms of pathogenesis andthe timing of the more extensive lesion are unclear.

Measurement of Cerebral Blood Flow by Xenon Clearance Techniques

Methodology

The measurement of CBF by xenon techniques is based on the recording of clearance of ^{133}Xe from the brain by an external detector. The intraarterial xenon method requires introduction of the tracer directly into the carotid artery. Blood flow is calculated from the clearance curve and the partition coefficient of xenon.[102,103] Use of this technique is restricted to patients undergoing internal carotid artery catheterization for clinical indications, and the procedure has not been performed in newborns. Lou and colleagues[104,105] adapted the technique to the newborn by injecting the radiotracer in the aortic arch or the common carotid or innominate artery through an umbilical artery catheter advanced to the appropriate position for the study.

Because of the invasiveness of administering the radiotracer through an arterial line, some investigators introduced the radiotracer by inhalation through an endotracheal tube or mask or by intravenous administration.[106,107] Like PET, this technique was applied for study of the cerebral circulation in the 1980s and early 1990s but has been used infrequently since. For completeness, the technique and relevant studies are briefly reviewed next.

Detection of Xenon

External detectors placed over the infant's head record the clearance of the tracer from the brain, and the windows are set to record the rather low-energy γ-waves emitted by the decay of the ^{133}Xe. Certain characteristics of these detector systems may influence the accuracy of measurements of regional CBF in the newborn infant and particularly in the premature newborn.[108,109] First, the detectors record emissions primarily from a volume of brain that is shaped like a truncated cone. Second, because of Compton scattering of the low-energy γ-waves, radioactive disintegrations from outside the detector field are recorded and may contribute up to 35% of the recorded counts. Third, up to 40% of observed counts in a detector placed over the parietal region will arise from the contralateral hemisphere. This last problem makes it difficult to measure regional CBF accurately and to compare hemispheric differences in blood flow.

Quantitation of Cerebral Blood Flow

Several methods are available to calculate CBF from clearance curves, which are recorded either linearly or semilogarithmically.[110-113] A two-compartment mode of analysis has been used to describe the clearance from a fast-flow compartment

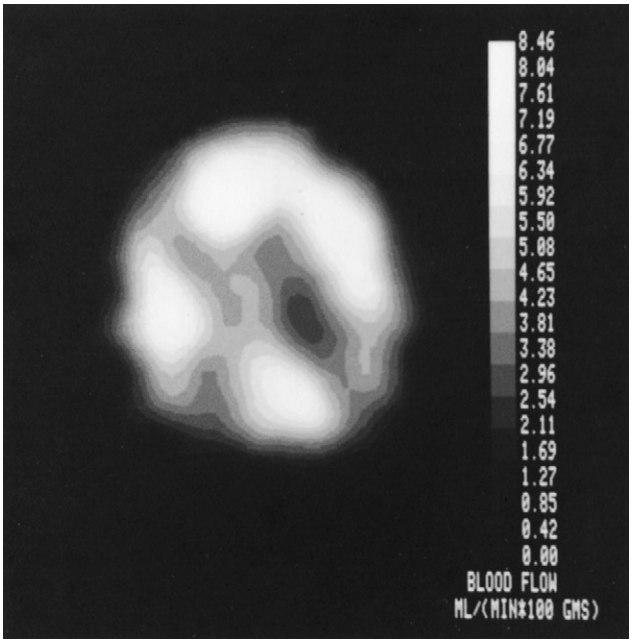


Figure 165-7 Positron emission tomography scan in a premature infant with intraventricular hemorrhage and hemorrhagic parenchymal infarct in the right cerebral hemisphere. Scans represent a horizontal section, with the right side of brain at the *right.* The *scale* is a linear representation of cerebral blood flow (CBF). Extensive impairment of CBF in the hemisphere containing the intracerebral blood is apparent.

(considered to represent gray matter) and from a slow compartment (considered to represent white matter). The fidelity of this relationship, however, can change fundamentally under pathologic conditions, especially with infarcts and intracranial hemorrhage (see later). A solution to this problem is provided by a noncompartmental index that represents global CBF and is based on analysis of the washout curve for relatively long periods (e.g., 15 minutes). An alternative solution is to analyze only the first part of the clearance curve, that is, the initial slope index. Data from the first 2 minutes of the curve are analyzed, and the value obtained is considered to represent mainly cortical flow, with a small contribution from white matter. Further refinements of this index method are based on performing curve analysis at 1-minute intervals during the early part of the washout curve. Theoretical limitations arise for each method when used to detect changes in CBF in infants with cerebrovascular disease.[112,114-115] In addition, the partition coefficient of xenon in the newborn is lower than in adults, probab as a consequence of the fact that the newborn brain contains less myelin, and because xenon is lipid-soluble.[116] This variance renders interpretation of changes in CBF especially difficult and indicates that caution should be exercised in interpreting values when marked tissue injury has occurred.

Clinical Studies of Xenon in Cerebral Blood Flow and Intraventricular Hemorrhage

Measurement of CBF with the xenon clearance technique has been performed in term and preterm infants with perinatal asphyxia and respiratory distress.[104,105,117,118] Ment and coworkers[106] reported CBF measurements in 16 infants weighing less than 1250 g who had evidence of IVH (n = 9); no infant was found to have a parenchymal infarction. At the time of the first CBF measurement (at 24 hours), blood flow changes were unpredictable. Thus asymmetric hemispheric blood flow was documented in five cases: In three of the cases, higher flow occurred on the side of the larger hemorrhage; in a fourth case, higher flow occurred on the side of unilateral subependymal hemorrhage; and in the fifth case, lower blood flow occurred on the side of maximal IVH. In addition, asymmetries in hemispheric flow were noted in four infants with symmetric IVH. These asymmetries in blood flow were not apparent on the subsequent measurements. These conflicting findings in CBF makes it difficult to draw conclusions regarding the relationship of CBF to the development of IVH.

In a follow-up study in 31 infants with birth weights less than 1250 g, CBF measurements were taken at an average postnatal age of 6 hours (range, 4 to 11 hours) and again on postnatal day 5.[119] Cranial ultrasonography, used to document IVH, was performed within 6 hours of birth and then every 12 hours. Infants were divided into three groups on the basis of the ultrasound findings: no IVH, early IVH (i.e., present at 6 hours), and interval IVH (appearing after 6 hours but before 5 days). The early IVH group exhibited hemispheric asymmetries in CBF, and five of the infants had reduced CBF to one or both hemispheres. Three of these infants (who had IVH and no initial parenchymal hemorrhagic involvement) progressed to grade IV hemorrhage (i.e., IVH and a hemorrhagic parenchymal infarct). The following pathogenetic mechanisms may therefore be postulated to account for development of the hemorrhagic parenchymal infarct in these infants: First, infarction of the hemisphere could have occurred perinatally, as evidenced by the reduced CBF at 6 hours, with secondary hemorrhage into this area between 6 hours and 5 days. Thus these data could be used to support the theory that the hemorrhagic parenchymal lesion represents hemorrhagic periventricular leukomalacia. Second, the IVH (and germinal matrix hemorrhage) could have caused the reduced CBF. Reduction in CBF has been observed experimentally after introduction of blood and potassium into the lateral ventricles of animals. Third, the IVH and germinal matrix hemorrhage could have obstructed venous return from the periventricular white matter (see earlier), thereby reducing CBF and resulting in subsequent infarction. These hypotheses must be considered with caution; evidence indicates that functional integrity of the newborn brain can be preserved with very low CBF (as low as 5 mL/100 g/minute).[79] Because the reported CBF values were in this range, occurrence of infarction cannot be established with certainty.

In another report, from Ment and associates,[120] xenon study results in two premature infants included unilateral reduction in hemispheric CBF at 6 hours of age. These infants also exhibited no evidence of IVH. The first infant, on day 5, was shown to have IVH and hemorrhagic infarcts of the basal ganglia (with later cortical atrophy noted on CT scan). The second infant, on day 4, demonstrated bilateral subependymal hemorrhages and an occipital porencephalic cyst. These two cases provide further support for the hypothesis of perinatal cerebral infarction and subsequent IVH.

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The incidence of all grades of IVH remained static over this 5-year period at 25% to 26% with the incidence of the most severe forms of IVH (grade III and IV) approximating 10%. It has

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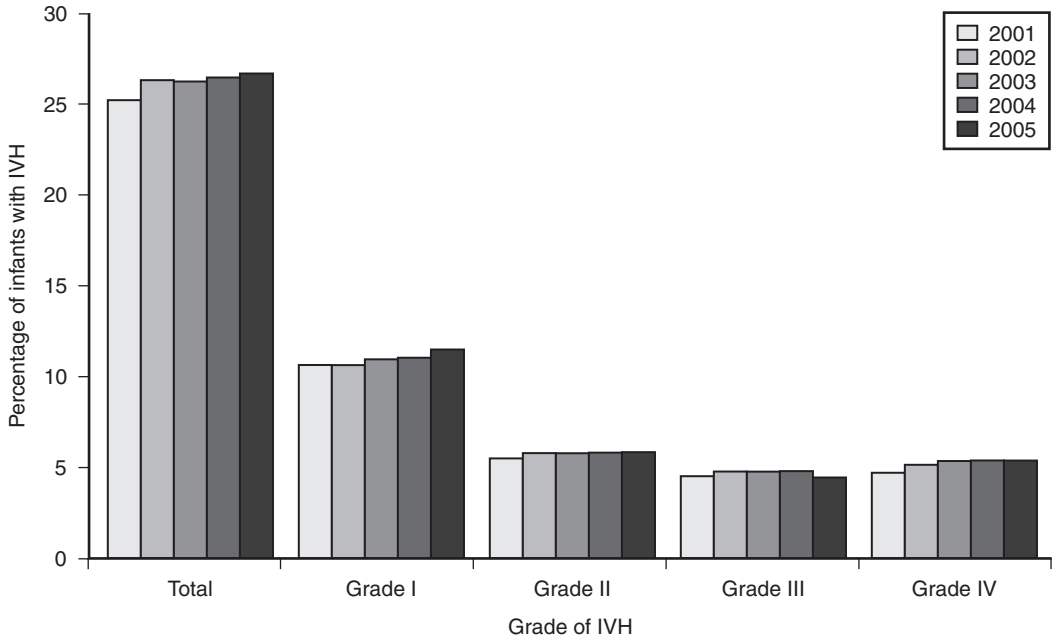


Figure 166-1 The incidence of (percentage of all infants with) all IVH, grade I, II, III, IV IVH, in the Vermont-Oxford Network 2001 to 2005 (n = 160,506). Note that the incidence of IVH is static across all grades and across the 5-year period. *(From Horbar JD:* Vermont-Oxford Network Database Summary. *Burlington, Vermont, Vermont-Oxford Network, 2007.)*

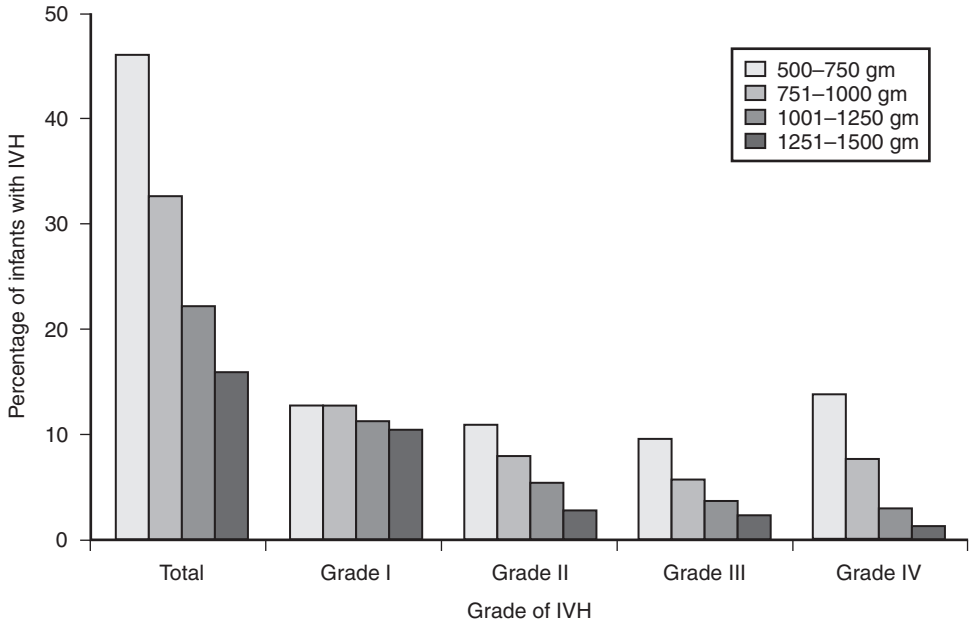


Figure 166-2 The incidence of (percentage of all infants with) all IVH, grade I, II, III, IV IVH, in the Vermont-Oxford Network 2005 (n = 38,383) for each grouping of birth weight from 500 to 750 g, 751 to 1000 g, 1001 to 1250 g, and 1251 to 1500 g. *(From Horbar JD:* Vermont-Oxford Network Database Summary. *Burlington, Vermont, Vermont-Oxford Network, 2007.)*

been hypothesized that the rates of IVH may not be improving because of the greater number of extremely immature infants, with a higher risk of IVH, who are being resuscitated and surviving. However, the data from Vermont-Oxford Database over 2001 to 2005 demonstrate that the percentage of preterm infants less than 750 g across this 5-year period did not alter at 17%, and thus the population of extremely preterm infants remains unchanged in recent years.

Although the percentage of the most immature infants has remained constant over the last 5 years, a very clear increased risk for all forms of IVH still exists in these smallest preterm infants (see Figure 166-2).[7] For infants less than 750 g the risk of any IVH is three-fold higher than for a preterm infant over 1250 g (45% vs. 15%) with a 10-fold higher risk of grade III-IV IVH (22% vs. 2.5%; see Figure 166-2). The most immature infants are also at greatest risk for the more severe forms of IVH. For an infant less

than 750 g an equal risk exists for development of grade I through IV at approximately 10% risk for each grade. In comparison, an infant over 1250 g has a 10% risk for grade I IVH and 1% risk for grade III or IV IVH (see Figure 166-2).

Thus IVH remains a significant problem in the preterm infant, particularly for the most immature infants. Currently in the United States approximately 55,000 very-low-birth-weight infants are born each year, of whom approximately 15,000 will have IVH, and more than 2000 infants will have periventricular hemorrhagic infarction.

In this chapter, we briefly discuss the background of diagnosis and clinical presentation for IVH before a more detailed discussion of the neuropathology and pathogenesis of the lesion, highlighting both experimental and human study findings.

CLINICAL FEATURES

The most common clinical presentation for IVH is a *clinically silent* syndrome occurring in up to 50% of infants with IVH in whom even careful and serial clinical assessments may fail to reveal any abnormal signs indicative of the lesion.[8-10] Thus the timing of the onset of IVH is often limited by clinician recognition. From cranial ultrasound (US) studies it appears that the *onset of IVH* is by the first day of life in at least 50% of affected infants with up to 90% of these lesions being present by 72 hours[5,11] and all lesions being visible by 7 days.[12] However, further *progression* of the lesions occurs in approximately 20% to 40% of the affected infants, with the maximal extent of the lesion seen by 3 to 5 days after the initial diagnosis.[13,14]

DIAGNOSIS

The diagnosis of IVH is most commonly established at the bedside of the sick preterm infant with use of portable cranial US. A vast experience demonstrates the reliability and versatility of cranial US for the diagnosis of IVH in the premature infant.[2,15-21] The determination of the presence of blood in the germinal matrix is best made on the coronal cranial US scan at the level of the caudothalamic notch, whereas the determination of the amount of blood in the lateral ventricles is most accurate on the parasagittal cranial US scan (Figure 166-3).

In comparison with the bedside technique of cranial US, computed tomography (CT) and magnetic resonance imaging (MRI) have the major disadvantages of requiring the sick premature infant to be transported to another site. Historically, CT scanning played a role in the early diagnosis of IVH and clearly demonstrated the site and extent of IVH in the premature infant.[3,4,22,23] However, recent concerns have been raised about the effects of ionizing radiation on the developing nervous system, which, until clarified, should restrict the use of CT scanning to neurosurgical emergencies in the newborn.[24] MRI has been shown to provide excellent images of IVH, but the real advantage of MRI lies in its superior ability to demonstrate the nature and severity of any parenchymal injury[25-28] (Figure 166-4) alongside a more extensive evaluation of the cerebral white matter, deep nuclear gray matter, cerebellum, and gyral development as indicators of additional neuropathology. Finally, in addition to imaging for the diagnosis of IVH, *lumbar puncture* can provide a characteristic cerebrospinal fluid (CSF) profile consisting initially of increased

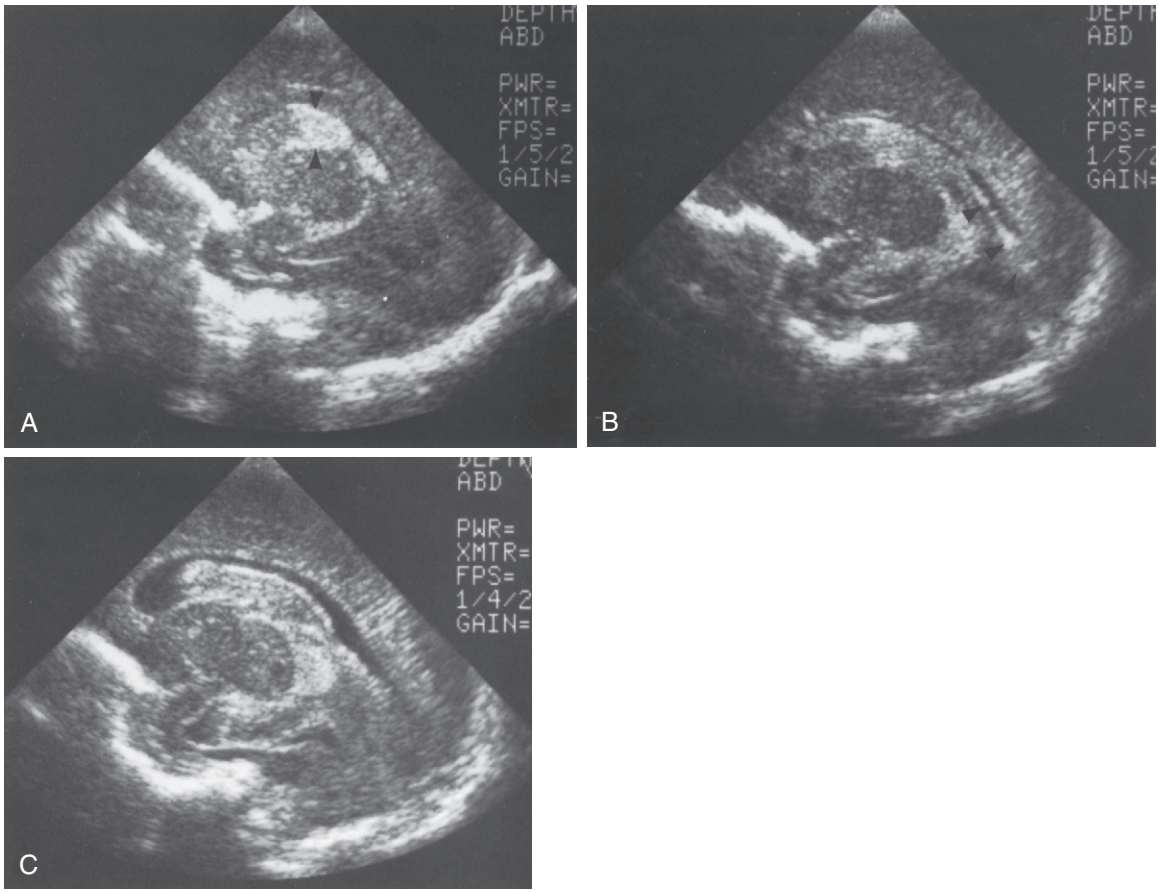


Figure 166-3 Grading of the severity of germinal matrix—IVH; parasagittal US scans. **A,** Grade I. **B,** Grade II. Note the echogenic material filling less than 50% of the ventricular area. **C,** Grade III. Note the large blood clot nearly completely filling and distending the entire lateral ventricle.

red blood cell count and elevated protein content, followed shortly by xanthochromia and depressed glucose content. The degree of elevation of CSF protein correlates approximately with the severity of the hemorrhage.[29]

Classification of Intraventricular Hemorrhage

The grading system most commonly used for IVH in the infant was first reported by Papile and colleagues[4] and is based on the presence and amount of blood in the germinal matrix and the lateral ventricles seen on any form of neuroimaging (Table 166-1 and see Figure 166-3). Grade I represents hemorrhage confined to the subependymal germinal matrix, grade II has hemorrhage within the lateral ventricles without ventricular dilatation, grade III IVH has hemorrhage with ventricular dilatation and/or hemorrhage occupying more than 50% of the ventricle, and grade IV IVH has parenchymal hemorrhage. Although grade IV IVH is now considered as a periventricular hemorrhagic infarction rather than IVH per se, most reports continue to classify the cranial US findings according to this earlier established classification system and because of its common pathway of pathogenesis (see later).

Clinical Risk Factors

The clinical risk factors for IVH have been studied by two scientific approaches: first, by experimental and case-control studies outlining factors related to the pathogenesis in a preterm infant, which will be outlined in more detail later, and, second, by epidemiologic or registry-type studies that may also identify factors related to therapeutic or NICU factors. For example, a recent study of the incidence of IVH across 19,507 preterm infants admitted to 17 NICUs in the Canadian Neonatal Network over an 18-month time period demonstrated that on Bayesian hierarchical logistic regressional modeling 30% of the risk for high-grade IVH was related to baseline population risks (male gender, gestational age, initial severity of illness, and outborn status).[30] A further 14% of the risk was accounted for by admission-day therapies such as treatment for acidosis and hypotension, and the largest fraction of variation, 31%, was related to NICU characteristics. NICUs with high patient volume and high neonatology staffing had lower rates of severe IVH. These findings that NICU structure and care practices influence the risk of IVH are supported by others[31] and suggest that high patient load and neonatal physician experience may be associated with improved care practices and reduction in risk of severe IVH. The variation between NICUs in registry data in the incidence of IVH does not appear to relate to differences in the interpretation of cranial US scan.[32]

NEUROPATHOLOGY

The neuropathology of IVH is considered best in terms of the site of origin, that is, primarily the germinal matrix, the spread of the hemorrhage throughout the ventricular system, the neuropathologic consequences of the hemorrhage, and the neuropathologic accompaniments not necessarily related directly to the IVH.

The basic lesion in germinal matrix–IVH is bleeding into the subependymal germinal matrix. Over the final 12 to 16 weeks gestation, this matrix becomes less and less prominent and is essentially exhausted by term. This region is highly cellular, gelatinous in texture, and richly vascularized (as would be expected for a structure with active cellular proliferation).

TABLE 166-1

Grading of Severity of Germinal Matrix–Intraventricular Hemorrhage by Ultrasound Scanning

Severity	Description
Grade I	Germinal matrix hemorrhage with no or minimal intraventricular hemorrhage (10% of ventricular area on parasagittal view)
Grade II	Intraventricular hemorrhage (10%-50% of ventricular area on parasagittal view)
Grade III	Intraventricular hemorrhage (>50% of ventricular area on parasagittal view; usually distends lateral ventricle)

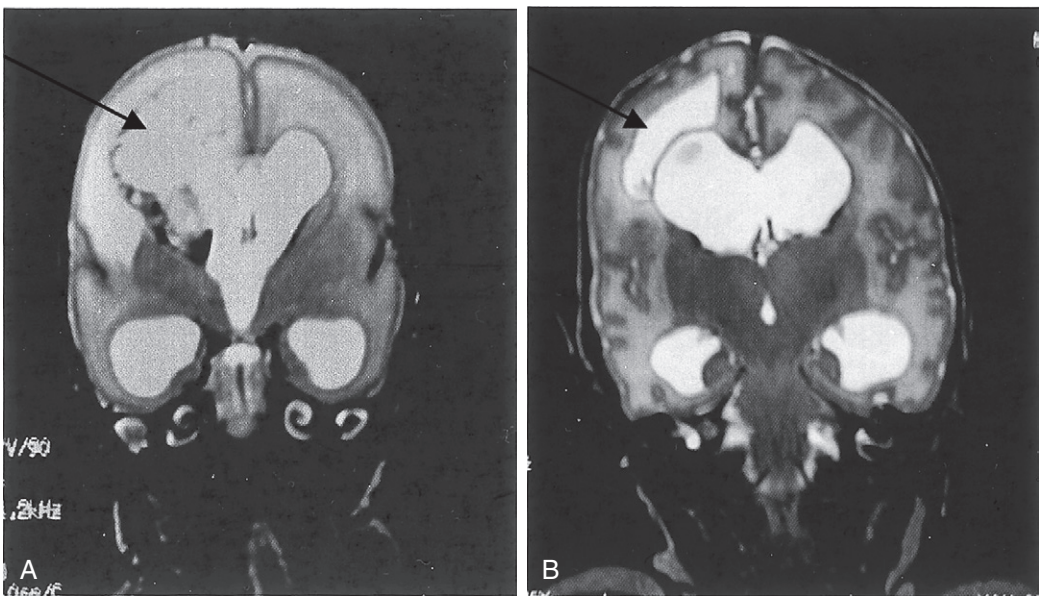


Figure 166-4 Coronal T2-weighted MRI scans of acute periventricular hemorrhagic infarction. **A,** Note the fan-shaped distribution of increased signal in the parenchyma *(arrow)* and the generalized increased signal in the white matter in the area surrounding the infarct. **B,** Later image of the same infant with cystic transformation of the infarct *(arrow).* Note the ventriculomegaly present in both images.

Site of Origin

The site of origin of IVH characteristically is the *subependymal germinal matrix*. This cellular region immediately ventrolateral to the lateral ventricle serves initially as the source of cerebral neuronal precursors between approximately 10 and 20 weeks gestation. Later in the third trimester, the matrix provides glial precursors that will become cerebral oligodendroglia and astrocytes. The matrix undergoes a progressive decrease in size, from a width of 2.5 mm at 23 to 24 weeks, to 1.4 mm at 32 weeks, to nearly complete involution by approximately 36 weeks.[33] The many thin-walled vessels in the matrix are a prime source of bleeding. Hemorrhage from the choroid plexus occurs in nearly 50% of infants with germinal matrix hemorrhage and IVH,[34] and, in more mature infants especially, it may be the exclusive site of origin of IVH.

The vascular *site of origin* of germinal matrix hemorrhage within the microcirculation of this region appears to be the prominent endothelial-lined vessels of the matrix and not the arterioles or arteries.[35-38] The vessels in free communication with the venous circulation appear to be of particular importance, such as the capillary-venule junction or small venules. This has been shown by careful histochemical studies of germinal matrix vessels at the site of hemorrhage[38] and by the emergence of solution into the germinal matrix hemorrhage from postmortem injection into the jugular veins but not from injection into the carotid artery.[37]

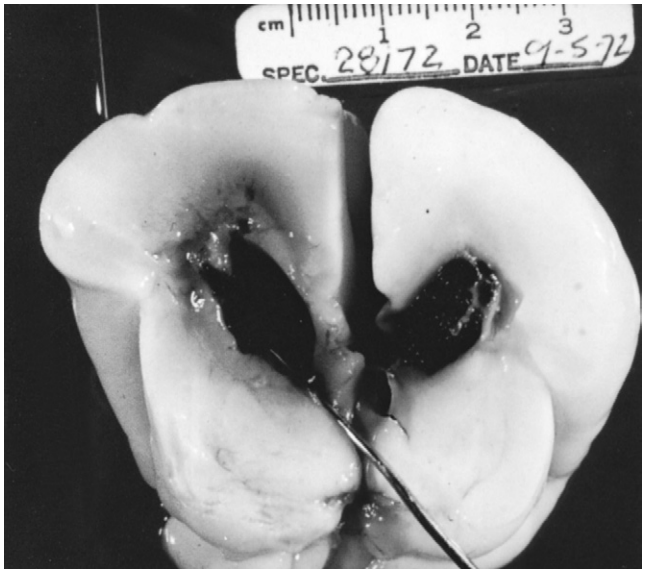


Figure 166-5 Periventricular hemorrhagic infarction with IVH; coronal section of the cerebrum. Note the evolving hemorrhagic infarction on the same side as the larger germinal matrix–IVH.

Spread of Intraventricular Hemorrhage

In approximately 80% of patients with germinal matrix hemorrhage, blood enters the lateral ventricles and spreads throughout the ventricular system.[36,39] Blood proceeds through the foramina of Magendie and Luschka and tends to collect in the basilar cisterns in the posterior fossa. With substantial collections, the blood may incite obliterative arachnoiditis over days to weeks with impairment in CSF flow. Other sites at which particulate blood clot may lead to impaired CSF dynamics are the aqueduct of Sylvius and the arachnoid villi.

Neuropathologic Consequences of Intraventricular Hemorrhage

Several neuropathologic states occur as apparent consequences of IVH, including, in temporal order of occurrence, germinal matrix destruction, periventricular hemorrhagic infarction, and posthemorrhagic hydrocephalus.

Germinal Matrix Destruction

Destruction of germinal matrix and, perhaps of importance, of its glial precursor cells is a consistent and expected feature of germinal matrix hemorrhage.[35,36] The hematoma is replaced frequently by a cyst, the walls of which include hemosiderin-laden macrophages and reactive astrocytes. This destruction of glial precursor cells in the germinal matrix may have a deleterious influence on subsequent brain development and neurodevelopmental outcomes (see later).

Periventricular Hemorrhagic Infarction

Approximately 15% of infants with IVH also exhibit a characteristic parenchymal lesion, *periventricular hemorrhagic infarction.* The incidence of this lesion increases with decreasing gestational age, such that in infants of 500 to 750 g, periventricular hemorrhagic infarction accounts for approximately one quarter of all cases of IVH (see Figure 166-2).[40,41] The neuropathology of periventricular hemorrhagic infarction consists of a relatively large region of hemorrhagic necrosis in the periventricular white matter, just dorsal and lateral to the external angle of the lateral ventricle (Figure 166-5). The necrosis is strikingly *asymmetric*—in the largest series reported, 67% of such lesions were exclusively *unilateral,* and, in virtually all of the remaining cases, they were grossly asymmetric, even though bilateral.[42] Approximately half of the lesions are extensive and involve the periventricular white matter from the frontal to the parieto-occipital regions. Approximately 80% of cases are associated with large IVH, and, commonly (and mistakenly), the parenchymal hemorrhagic lesion is described as an "extension" of the hemorrhage. That simple extension of blood into cerebral white matter from germinal matrix or lateral ventricle does *not* account for the periventricular hemorrhagic necrosis has been shown clearly by several neuropathologic studies.[34,40-44] Microscopic study of this periventricular hemorrhagic necrosis indicates that the lesion is a *hemorrhagic infarction*.[42-45] The careful studies of Gould and associates[43] and Takashima and colleagues[45] emphasize that the hemorrhagic component of the infarction tends to be most concentrated near the ventricular angle where the medullary veins draining the cerebral white matter become confluent and ultimately join the terminal vein in the subependymal region. Thus it appears that periventricular hemorrhagic necrosis occurring in association with large IVH is, in fact, a *venous infarction*.

The pathogenesis of periventricular hemorrhagic infarction appears in most cases to be related directly to the associated germinal matrix–IVH; the association of large asymmetric germinal matrix–IVH and progression to *ipsilateral* periventricular hemorrhagic infarction has been clearly documented.[42,46,47] These data raise the possibility that the IVH or its associated germinal matrix hemorrhage may lead to obstruction of the terminal veins with secondary hemorrhagic venous infarction.[42] A similar conclusion was suggested by a neuropathologic study.[43] This pathogenetic formulation received further support from two imaging studies. Doppler determinations of blood flow velocity demonstrated obstruction of flow in the terminal vein by the ipsilateral germinal matrix hemorrhage during the evolution of the infarction in the living premature infant.[48] In addition, an MRI study of acute periventricular hemorrhagic infarction showed an appearance consistent with a combination of intravascular thrombi and perivascular hemorrhage along the course of the medullary veins within the area of infarction.[49] The pathogenetic scheme that we

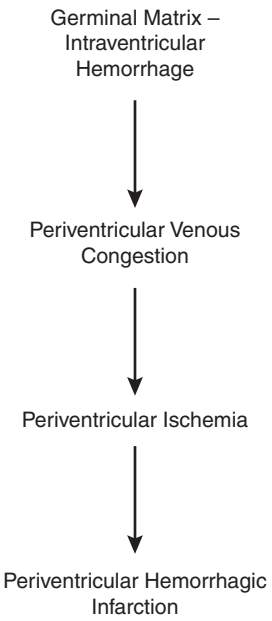


Figure 166-6 Pathogenesis of periventricular hemorrhagic infarction. The formulation indicates a central role for germinal matrix–IVH in causation of the periventricular venous infarction.

consider to account for most examples of periventricular hemorrhagic infarction is shown in Figure 166-6.

Hydrocephalus

A third neuropathologic consequence of IVH is progressive posthemorrhagic ventricular dilation, that is, *hydrocephalus.* The likelihood of and the speed of evolution of hydrocephalus after IVH is related directly to the quantity of intraventricular blood. Thus, with large IVH, hydrocephalus may evolve over days (*acute* hydrocephalus), and, with smaller IVH, the process evolves usually over weeks (*subacute-chronic* hydrocephalus).

Acute hydrocephalus is accompanied by particulate blood clot, which may impair CSF absorption by obstruction of the arachnoid villi. This mechanism may be particularly likely in the newborn with only microscopic arachnoid villi and not larger, later-appearing arachnoid granulations.[50] Other contributing factors to the development of acute hydrocephalus may include an impaired ability to mediate clot lysis with extremely low plasminogen levels and elevations in plasminogen activator inhibitor in the CSF of infants with recent IVH.[51]

Subacute-chronic hydrocephalus relates most commonly either to obliterative arachnoiditis in the posterior fossa resulting in obstruction of fourth ventricular outflow or flow through the tentorial notch or to aqueductal obstruction by blood clot, disrupted ependyma, and reactive gliosis.[35,52,53]

Neuropathologic Accompaniments of Intraventricular Hemorrhage

Several neuropathologic states are commonly associated with IVH but are not directly caused by it. The two most common accompaniments are periventricular leukomalacia and pontine neuronal necrosis.

Periventricular Leukomalacia

Periventricular leukomalacia was observed to occur in 75% of one series of infants who died with IVH.[34] In contrast to parenchymal hemorrhagic infarction, periventricular leukomalacia is a generally symmetric, nonhemorrhagic, and apparently ischemic white matter injury in the arterial end zones (Table 166-2). The frequent association of periventricular leukomalacia with IVH has also been emphasized in three other neuropathologic reports.[45,54] Takashima and colleagues[45] suggested that the two lesions, that is, periventricular hemorrhagic infarction and hemorrhagic periventricular leukomalacia, may be distinguishable in part on the basis of topography. Thus hemorrhagic periventricular leukomalacia has a predilection for periventricular arterial border zones, particularly in the region near the trigone of the lateral ventricles. Venous infarction, especially its most hemorrhagic component, is particularly prominent more anteriorly; that is, the lesion radiates from the periventricular region at the site of confluence of the medullary and terminal veins and assumes a roughly triangular, fan-shaped appearance in periventricular white matter.

TABLE 166-2

Periventricular White Matter Lesions of the Premature Infant

Term disturbance	Symmetry	Gross hemorrhage	Probable site of circulatory insult
Periventricular leukomalacia	Predominantly symmetric	Uncommon	Arterial
Periventricular hemorrhagic infarction	Predominantly asymmetric	Invariable	Venous

Pontine Neuronal Necrosis and Hippocampal Injury

Hypoxic-ischemic neuronal injury may accompany IVH in premature infants, and one subtype of such injury, *pontine neuronal necrosis,* is particularly common. In two carefully studied neuropathologic series,[34,54] 46% and 71% of infants with IVH exhibited pontine neuronal necrosis. Accompanying neuronal necrosis in the subiculum of the hippocampus was also common.[54-56] All the infants with IVH accompanied by pontine neuronal necrosis in the series of Armstrong and associates[34] died of respiratory failure. Previous investigations had suggested that pontine neuronal necrosis is related to hypoxic-ischemic insult, hyperoxia, and hypocarbia.[57,58]

PATHOGENESIS

The pathogenesis of IVH is considered best in terms of intravascular, vascular, and extravascular factors.[1] Clearly, the pathogenesis of IVH is multifactorial, and thus different combinations of these factors are operative in different patients.

Intravascular Factors

Intravascular factors are those that relate primarily to the regulation of blood flow, pressure, and volume in the microvascular bed of the germinal matrix (Box 166-1, Figure 166-7).

Fluctuating Cerebral Blood Flow

Significant fluctuations in *cerebral blood flow* appear important in the pathogenesis of IVH. It has been shown that a *fluctuating pattern* of arterial blood pressure, characterized by marked, *continuous alterations* in both systolic and diastolic flow velocities, was associated with similar trends in the cerebral blood flow velocity tracings and a high risk of subsequent occurrence of IVH. Smaller fluctuations in arterial pressure of less than 10% (coefficient of variation) did not appear to produce this adverse outcome.[59-61] The cause of the fluctuations in both the systemic and cerebral circulations was related to the mechanics of ventilation,[62,63] as well as to the severity of the infant's illness, with contributing factors of hypercarbia, hypovolemia, hypotension, "restlessness," patent ductus arteriosus, and relatively high inspired oxygen concentrations.[60,63-65]

Increases in Cerebral Blood Flow

The close temporal correlation between the occurrence of IVH and abrupt increases in arterial blood pressure, increases in cerebral blood flow, and cerebral blood flow velocity[66-69] supports the earlier hypothesis[69] that increases in cerebral blood flow play an important pathogenetic role in IVH. One major contributing factor to the premature infant's apparent propensity for dangerous elevations of cerebral blood flow is a pressure-passive state of the cerebral circulation.[70-76]

BOX 166-1

Pathogenesis of Germinal Matrix Hemorrhage

Intravascular factors

- Fluctuating blood pressure
- Increase in cerebral blood flow—systemic hypertension in the setting of a pressure-passive circulation, rapid volume expansion, hypercarbia, decreased hematocrit, decreased blood lucose
- Increase in cerebral venous pressure—venous anatomic arrangement, labor and vaginal delivery, respiratory disturbance
- Decrease in cerebral blood flow (followed by reperfusion)—systemic hypotension
- Platelet and coagulation disturbance

Vascular factors

- Tenuous capillary integrity—the germinal matrix is an involuting remodeling capillary bed with deficient vascular lining and a large vascular and luminal area
- Vulnerability of matrix capillaries to hypoxic-ischemic injury—the area has a high requirement for oxidative metabolism and lies at a vascular border zone

Extravascular factors

- Deficient vascular support
- Excessive fibrinolytic activity

Elevations of Arterial Blood Pressure and Pressure-Passive Cerebral Circulation

Investigators have shown that intact cerebrovascular autoregulation is found in clinically stable preterm infants and a pressure-passive cerebral circulation is present in sick preterm infants in whom IVH subsequently develops.[72,77,78] The relationship between a pressure-passive cerebral circulation and the occurrence of IVH was clearly demonstrated in a study in 57 preterm infants[71] in whom US signs of severe IVH developed only in infants with earlier evidence of a pressure-passive cerebral circulation. In those infants with intact cerebrovascular autoregulation either no hemorrhage or only mild hemorrhage developed.[71,74] Consistent with a potential role for arterial hypertension in this setting is the demonstration of a relation between maximum systolic blood pressure above a threshold value and the subsequent occurrence of IVH.[79] The limit for the highest tolerable peak systolic blood pressure was markedly lower for the smaller infants.[79]

Causes of Increased Arterial Blood Pressure in the Human Newborn

Abrupt elevations in arterial blood pressure, which may be accompanied by increased cerebral blood flow velocity, are clearly important to detect and prevent, whenever possible. These causes of elevated arterial blood pressure include the following: such "caretaking" concomitants as inadvertent noxious stimulation, abdominal examination, handling, instillation of mydriatics, and tracheal suctioning; such systemic complications as pneumothorax, exchange transfusion, and rapid infusion of colloid; and such neurologic complications as seizures.[66,67,69,80-86] Pneumothorax has been consistently associated with an elevated risk of IVH.[87,88] In one study in nine infants, pneumothorax was uniformly accompanied by abrupt elevations of systemic blood pressure and cerebral blood flow velocity, and these circulatory changes were followed within hours by IVH.[89] Studies in newborn dogs documented abrupt increases in arterial blood pressure on rapid evacuation of pneumothorax.[87]

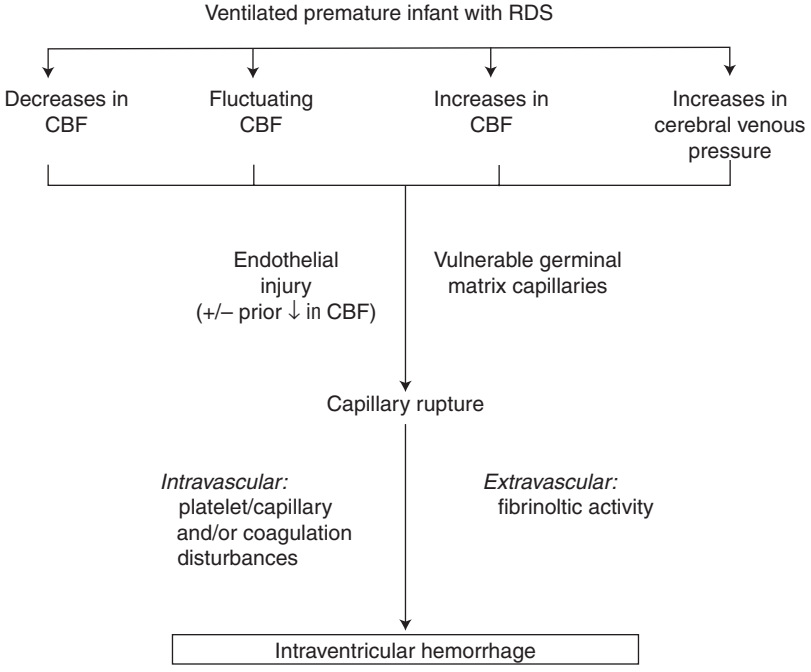


Figure 166-7 Interaction of the major pathogenetic factors for the occurrence of germinal matrix–IVH in the premature infant. *CBF,* Cerebral blood flow.

Relevant Experimental Studies: Role of Hypertension

The importance of abrupt increases in systemic blood pressure and cerebral blood flow in the pathogenesis of IVH was demonstrated in *experimental* studies in the newborn beagle puppy[89-93] and in the preterm sheep fetus.[94] A highly predictable and pattern of germinal matrix–IVH hemorrhage clinically similar to that of the human premature infant is produced in the beagle puppy by a sequence of hypotension and hypertension produced by blood removal and volume reinfusion.[95]

Hypercarbia

Careful studies in preterm infants receiving mechanical ventilation show a pronounced reactivity of cerebral blood flow to changes in arterial carbon dioxide tension ($Paco_2$) with an approximately 30% increase in cerebral blood flow per kilopascal increase in $Paco_2$ after the first 24 hours of life.[72,96,97] However, in the first 24 hours of life, the period of greatest risk for IVH, this reactivity is more attenuated, with an approximately 10% increase in cerebral blood flow per kilopascal increase in $Paco_2$ in well premature infants, and, most strikingly, this reactivity is *absent* in infants with subsequent severe IVH.[71] In contrast, Cooke observed that very-low-birth-weight infants with IVH had significantly higher maximum $Paco_2$ during the first week of life compared with infants without IVH.[98] Van der Bor and colleagues[99] demonstrated that elevated $Paco_2$ preceded the development of IVH in premature infants. Other investigators have observed univariate relationships between $Paco_2$ and risk of IVH, but this has not remained independently significant after controlling for other perinatal variables.[47,61,88,100] Szymonowicz and colleagues[101] were the first to report an association using multivariate analysis between hypercarbia and risk for IVH in a small observational study. After this Kaiser and colleagues documented an association between the maximum $Paco_2$ and risk of grade III-IV IVH in a study in 574 very-low-birth-weight infants between 1999 and 2004.[102] On multivariate logistic modeling controlling for gestational age, gender, 1-minute Apgar score, multiplicity, and inotrope use, maximum $Paco_2$ during the first 72 hours of life was related to the risk of severe IVH in a dose-dependent manner with the risk elevating with a $Paco_2$ >56 mm Hg. The adjusted odds ratio for severe IVH was nearly five-fold greater when the maximum $Paco_2$ was >75 mm Hg. These findings must be considered in light of the trend in newborn medicine toward more permissive hypercapnia.

Decreased Hemoglobin

The role of *decreased hematocrit* as a pathogenetic factor for IVH may be greater than previously suspected. An inverse correlation exists in the human infant between hemoglobin concentration and cerebral blood flow.[72,103,104] In one study in premature infants in the first days of life, cerebral blood flow increased by 12% per 1 mmol/L decrease in hemoglobin.[72] Cerebral blood flow presumably increases to maintain cerebral oxygen delivery constant, but, with increasing cerebral blood flow, risk for IVH will be enhanced in those premature infants born with anemia. These associations and that of total blood volume being related to the risk of IVH are further supported by the recent studies of the timing of cord clamping. Delayed cord clamping (30 seconds) compared with immediate cord clamping (6-7 seconds) is associated with a slightly higher baseline hematocrit (49% vs. 46%), higher mean blood pressure (33.8 vs. 31.9 mm Hg), but a significantly reduced risk for IVH (14% vs. 36%).[105] This reduction in IVH with delayed cord clamping is also supported by a meta-analysis of all randomized trials in preterm infants.[106]

Decreased Blood Glucose

Decreased blood glucose should be considered in the evaluation of pathogenetic factors for IVH in view of the observation that cerebral blood flow increases two- to three-fold when blood glucose declines to levels lower than 1.7 mmol/L in the premature infant.[72,96] Blood glucose levels lower than 1.7 mmol/L in premature infants are not rare in the first days of life in many NICUs. More data are needed on a potential contributory role for low blood glucose concentrations in the pathogenesis of IVH.

Increases in Cerebral Venous Pressure

Elevations of cerebral venous pressure may contribute to the occurrence of IVH. Indeed, potential importance of venous factors is suggested by the demonstration that, with postmortem injection of the carotid artery or jugular vein in infants with germinal matrix hemorrhage, the injected material entered the hemorrhage only by venous injections. The *most important causes* of such increases are labor and delivery, asphyxia, and respiratory complications.

Importance of Venous Anatomy

The importance of increased venous pressure in the pathogenesis of IVH relates in part to the *venous anatomy* in the region of the germinal matrix. The direction of deep venous flow takes a peculiar U-turn in the subependymal region at the level of the foramen of Monro, which is the most common site of germinal matrix hemorrhage.

Labor and Delivery

During labor and delivery, marked increases in cerebral venous pressure may occur. Measurement of "fetal head compression pressure" was determined by a compression transducer positioned between the fetal head and the wall of the uterus during labor, with the overall mean pressure found to be 158 mm Hg.[107] Consistent with this hypothesis of increased venous pressure during labor, an increased risk of the occurrence of IVH appears to exist in the premature infant with vaginal delivery and particularly with prolonged labor (10 to 12 hours).[47,108-111] A more recent study failed to find an association between mode of delivery and risk of IVH in 2674 preterm breech births in Sweden, although the mortality and 5-minute Apgar score were both improved with cesarean section delivery.[112] Head position, both before delivery and in the neonatal nursery, may also alter cerebral venous drainage. One study documented, using near-infrared spectroscopy, that cerebral venous drainage may be impaired in prone or side positions.[113]

Asphyxia

The importance of increased venous pressure in association with asphyxia in the causation of IVH has been shown in experimental studies of preterm fetal sheep.[94]

Respiratory Disturbances

Data suggest that such factors as positive pressure ventilation with relatively high peak inflation pressure, tracheal suctioning, abnormalities of the mechanics of respiration, and pneumothorax may be major causes of increased cerebral venous pressure in the premature infant.[114-117] Doppler measurements of blood flow velocity in the superior sagittal sinus demonstrated a striking sensitivity of the venous circulation to the level of peak inflation pressure with the smallest infants exhibiting the most marked effects.[117]

Tracheal suctioning and pneumothorax have also been shown to produce pronounced changes in venous pressure.[59,118,119] Studies of mode of ventilation in premature infants have raised concerns over associations between high-frequency oscillatory ventilation (HFOV) and increased rates of IVH, particularly high-grade IVH.[120] A meta-analysis of the eight randomized controlled studies comparing HFOV and conventional ventilation demonstrated a significant increase in the risk of grade III or IV IVH (summary relative risk, 1.19 [1.03, 1.38]) with adverse

neurodevelopmental outcome in the two trials with follow-up (relative risk, 1.26 [1.01, 1.58]).[121] An increased risk of high-grade IVH was documented in a randomized controlled trial of HFOV that documented an increased rate of IVH in those infants who were switched from conventional ventilation to HFOV.[122] The potential mechanism of increased IVH in premature infants receiving HFOV may relate to increased intrathoracic pressure with decreased cerebral venous return.[123] However, these findings have led some authors to recommend that conventional ventilation should remain the ventilatory mode of choice in premature infants with respiratory distress syndrome.[121,122]

Decreases in Cerebral Blood Flow

Importance of Pressure-Passive Cerebral Circulation

Decreases in cerebral blood flow may play an important role in the pathogenesis of IVH. The principal consequence of the decreased cerebral blood flow is injury of germinal matrix vessels, which rupture subsequently on reperfusion. As indicated earlier, hemorrhagic hypotension preceding volume reexpansion is the optimal means to produce IVH experimentally in the newborn beagle puppy. In the premature infant, decreases in cerebral blood flow are most likely with perinatal asphyxia and systemic hypotension. Because of the pressure-passive cerebral circulation in sick premature infants, this hypotension can lead to a parallel decrease in cerebral blood flow.

Perinatal Hypoxic-Ischemic Events

Perinatal hypoxic-ischemic events presumably explain, at least in part, the relationship among low Apgar scores, early acidosis, the early use of bicarbonate, and the subsequent occurrence of IVH, particularly cases that develop in the first 12 hours.[124,125] The mechanism for provocation of IVH with perinatal asphyxia is complex and includes increases in cerebral venous pressure; changes in cerebral blood flow associated with impaired vascular autoregulation, such as decreases in cerebral blood flow associated with hypotension; and resulting injury to matrix capillaries. Studies of the concentrations of brain-specific creatine kinase isoenzymes in umbilical cord blood or blood samples obtained early in the postnatal period or of the hypoxanthine metabolite, uric acid, in blood samples obtained on the first postnatal day in preterm infants in whom IVH later developed support the notion that late intrauterine injury (perhaps asphyxia) may be involved in at least some cases of IVH.[126-128] Moreover, the possibility of a perinatal hypoxic-ischemic insult to brain as a predecessor to IVH is suggested also by the finding of electroencephalographic abnormalities before the occurrence of IVH.[129,130]

Postnatal Ischemic Events

The importance of postnatal decreases in arterial blood pressure in the pathogenesis of IVH is indicated by several studies in which arterial blood pressure was monitored continuously from birth or shortly after birth. In a study in approximately 25,000 very-low-birth-weight infants, severe IVH was three times more common in those infants requiring cardiopulmonary resuscitation at delivery (15.3%) than in those not requiring resuscitation (4.9%).[131] A relationship between postnatal arterial hypotension and increased risk of IVH has been frequently documented.[132,133] Near-infrared spectroscopy in the first 24 hours of life in preterm infants has shown that cerebral blood flow is significantly lower in infants in whom IVH subsequently developed (median 7.0 mL/100 g per minute) than in those without IVH (median 12.2 mL/100 g per minute).[134] In addition, supportive of a relation between postnatal ischemic events and the occurrence of IVH is the demonstration of decreased cardiac output in the first 36 hours of life in infants in whom the lesion developed, especially severe IVH.[135] Finally, the potential importance of ischemic injury in pathogenesis is supported further by the demonstration of a beneficial effect of administration of superoxide dismutase in prevention of IVH in the beagle puppy model.[136] This enzyme is critical in the defense against free radicals, the formation of which is provoked by hypoxic-ischemic insult.

Platelet and Coagulation Disturbances

Disturbances of platelet-capillary function and coagulation may contribute to the pathogenesis of IVH, although uniformity is lacking in results of studies designed to investigate the pathogenetic role of such disturbances.

Platelet-Capillary Function

Forty percent of infants of less than 1500 g birth weight in one study[126] exhibited platelet counts less than 100,000/mm^3, and most of these infants with thrombocytopenia had abnormal bleeding times. The incidences of IVH in infants with thrombocytopenia versus those without thrombocytopenia were 78%, versus 48% for those weighing less than 1000 g. The presence of thrombocytopenia appeared to be an independent pathogenetic factor for causation of IVH. Subsequent work has both confirmed and refuted a role for thrombocytopenia in pathogenesis of IVH.[137,138] Some evidence also suggests that premature infants demonstrate elevated levels of prostacyclin before the occurrence of IVH.[139] Because prostaglandins have an impact not only on platelet function but also on factors such as cerebral blood flow and free radical production that also may be important in pathogenesis of IVH, it is not clear to what extent effects on platelet-capillary function were independently related to causation of IVH.

Coagulation

Disagreement exists concerning the role of coagulopathy in the causation of IVH.[137,140-142] Because disturbances of coagulation are not uncommon in preterm infants with other provocative factors for IVH (e.g., serious respiratory distress syndrome, asphyxia) or they may occur *secondary* to major hemorrhage, an independent pathogenetic role for such disturbances has been difficult to establish. Although administration of fresh frozen plasma was shown in one study to decrease the incidence of IVH,[143] no effect on coagulation measures accompanied the apparent beneficial effect. Moreover, a later investigation failed to show a beneficial effect of prophylactic early fresh frozen plasma in premature infants.[144,145]

Several investigators have examined the potential role of mutations in coagulation proteins as possible risk modifiers for IVH. Several common mutations in coagulation proteins are associated with increased tendency to thrombotic events. Those that have been investigated most included factor XIII Val34 Leu mutation, factor V Leiden, factor II (prothrombin) G20210A, and MTHFR C677T.

Factor XIII stabilizes the fibrin clot, and thus it is hypothesized that low levels of factor XIII may predispose to IVH. Low factor XIII levels in preterm infants may be a risk factor for the relatively higher fibrinolytic activity that occurs in preterm infants.[146] Factor V Leiden is a common mutation in the white population associated with thrombotic events with decreased inactivation of factor V leading to greatly increased thrombin generation. The role of factor V Leiden and risk of IVH is unclear. Results have been conflicting as to the role of factor V Leiden in the risk of IVH with some studies suggesting a significant role,[147,148] whereas others suggest no role.[149]

Two mutations (C677T and A1298C) in the 5,10-methylene tetrahydrofolate reductase (MTHFR) lead to hyperhomocysteinemia under conditions of decreased folate or B_{12} concentrations leading to an increased risk of thrombosis. No association has been found with this mutation and IVH.[146]

Vascular Factors

Vascular factors that may predispose the blood vessels of the germinal matrix to hemorrhage include the tenuous integrity of matrix capillaries and the vulnerability of these vessels to hypoxic-ischemic injury.

Tenuous Capillary Integrity

Three lines of anatomic evidence suggest that the capillary integrity is tenuous in the germinal matrix. First, these vessels, like the germinal matrix itself, are in a process of involution. Pape and Wigglesworth[56] characterized the elaborate capillary bed of the germinal matrix as "a persisting immature vascular rete," an immature microvascular network that is remodeled into a mature capillary bed when the matrix disappears. This involuting remodeling capillary bed may be expected to be more susceptible to rupture than more mature vessels. Second, many studies have emphasized that the matrix microcirculation is composed of simple endothelial-lined vessels, often of a larger size than capillaries but not categorizable as arterioles or venules because of absence of muscle and collagen.[35,38,39,56] Careful studies have documented the absence of muscle[150] and of type VI collagen.[151] It has been postulated that such vessels are likely to be more susceptible to rupture. Third, an electron microscopic study of cortical and germinal matrix vessels shows a two- to four-fold greater diameter of both the vessels and the lumina of the vessels of the germinal matrix than those of the cortical plate in infants of 25 to 33 weeks gestation.[152] Thus, in the age range of greatest propensity for occurrence of IVH, the diameters are unusually large, a finding of potential pathogenetic importance because of Laplace's law, which states that the larger the vessel diameter is, the greater the total force on the wall will be at any given pressure.

The possibility should be considered that effects of these maturational deficiencies of germinal matrix vessels underlie the interesting observation that women with a diagnosis of preeclampsia exhibit a much lower risk for an infant with IVH (2.5%) than do women without this diagnosis (17%).[153] This lower risk for IVH has been confirmed.[154,155] Infants born to mothers with preeclampsia exhibit a variety of features suggestive of accelerated maturation of brain and other organs.[156,157]

Vulnerability to Hypoxic-Ischemic Injury

The matrix capillaries have an increased vulnerability to hypoxic-ischemic injury. Takashima and Tanaka[158] showed that a vascular border zone exists between the end-fields of the striate and thalamic arteries at the usual site for matrix hemorrhage. Thus it can be postulated that the matrix vessels could be readily injured by ischemia, and, on reperfusion of these injured vessels, hemorrhage could occur. In addition, matrix capillaries, like other brain capillaries, appear to have a high requirement for oxidative metabolism. These brain endothelial cells have been shown to contain three to five times more mitochondria than do systemic capillary endothelial cells.[159] This intense oxidative activity would further enhance the vulnerability of matrix vessels to ischemic insults.

Extravascular Factors

Extravascular factors are those referable to the space surrounding the germinal matrix capillaries. The most prominent vulnerability in this region is related to deficient vascular support. The germinal matrix can be seen by gross examination to be a gelatinous, friable structure and by microscopic examination to be deficient in supporting mesenchymal elements.[35,160,161] It appears that the extravascular space provides poor support for the large, endothelial-lined capillaries that course through it and that are the site of the hemorrhage. Gould and Howard[161] assessed astrocytic fibrillary development by immunocytochemical staining for glial fibrillary acidic protein showing minimal astrocytic development as late as 27 weeks gestation and prominent glial fibrillary acidic protein staining not until 31 weeks in the matrix region. Because of the role of glial fibers in capillary stabilization, these data suggest that deficient astrocytic development may play a role in the pathogenesis of IVH in the premature infant.

MECHANISMS OF BRAIN INJURY

Major Factors

The principal mechanisms of brain injury in the premature infant with IVH relate to one or more of at least the following six major factors (Box 166-2): (1) hypoxic-ischemic injury that may precede the IVH; (2) destruction of germinal matrix with its glial precursors; (3) destruction of periventricular white matter, that is, periventricular hemorrhagic infarction; (4) periventricular white matter injury resulting from intraventricular blood products; (5) a marked increase in intracranial pressure and concomitant defects in cerebral perfusion at the time of severe IVH; and (6) posthemorrhagic hydrocephalus.

Preceding Hypoxic-Ischemic Injury

Neuropathologic studies indicate that infants who die with IVH exhibit two principal lesions related to hypoxic-ischemic insults: periventricular leukomalacia and a subtype of selective neuronal necrosis, pontine and subicular neuronal necrosis. Periventricular leukomalacia has been reported in neuropathologic series in as many as 75% of cases of IVH, and pontine neuronal necrosis is noted in nearly 50%. Moreover, the finding that ventricular dilation correlates strongly with subsequent neurologic and cognitive deficits after IVH, in the absence of progressive hydrocephalus or periventricular hemorrhagic infarction,[162-165] further suggests that concomitant periventricular leukomalacia and cerebral white matter atrophy are common and important in the outcome of infants with IVH.

Destruction of Germinal Matrix and Glial Precursors

One of the consistent neuropathologic consequences of IVH is destruction of germinal matrix with its glial precursor cells. These glial precursor cells are destined to give rise to both oligodendroglia and astrocytes. The loss of the glial precursor cells may affect subsequent cerebral development through the oligodendroglia or astrocytic cell loss. Loss of oligodendroglia cells may lead to a subsequent impairment of myelination after germinal matrix destruction, although evidence to establish such an influence is lacking. The loss of glial precursor cells may also

BOX 166-2

Mechanisms of Brain Injury With Germinal Matrix—Intraventricular Hemorrhage

- Preceding hypoxic-ischemic injury, especially periventricular leukomalacia
- Destruction of glial precursors in germinal matrix—effect on myelination
- Destruction of periventricular white matter (i.e., periventricular hemorrhagic infarction) and subsequent impairment of cortical organization
- Periventricular white matter injury resulting from intraventricular, parenchymal, or subarachnoid blood products with vasoconstriction and generation of free radical products
- Intracranial hypertension and impaired cerebral perfusion
- Posthemorrhagic hydrocephalus

impair subsequent astrocytic development. Gressens and Evrard and their co-workers[166,167] showed that astrocytes destined for supragranular (upper) cortical layers originate and migrate after the occurrence of neuronal migration and are crucial for normal organizational development of the supragranular cerebral cortex. These astrocytic precursors are likely to be destroyed by germinal matrix hemorrhage, a finding raising the possibility of a subsequent disturbance in cortical development.[167] Such a disturbance would not be detectable by conventional neuropathologic techniques.

Destruction of Periventricular White Matter: Periventricular Hemorrhagic Infarction

Numerous follow-up studies have correlated cranial ultrasonographic findings in the neonatal period in patients with IVH with the occurrence of subsequent neurologic deficits. These studies confirm that the presence of periventricular hemorrhagic infarction is the single most important determinant of neurologic outcome. Of all survivors of IVH, this lesion accounts for the largest proportion of the subsequent severe neurologic deficits. The deleterious neurologic effects of periventricular hemorrhagic infarction may relate not only to destruction of cerebral white matter per se but also to the effects of the white matter injury on cerebral cortical development. Thus careful neuropathologic study of cerebral cortical organization in infants who died with major hemorrhagic white matter lesions has shown marked alterations in neuronal axonal and dendritic ramifications in areas overlying the white matter destruction.[168] These abnormalities are postulated to be caused by disturbances of afferent input to and efferent input from the areas of cortex by disruption of the respective white matter axon. Another potential cause for the cortical abnormalities could be destruction of subplate neurons by the white matter infarction.[169] These neurons are critical for cerebral cortical organization and are abundant in subcortical white matter in the human premature infant. Whatever the mechanism, these *cerebral cortical* abnormalities with periventricular hemorrhagic infarction could be important in determination of subsequent cognitive deficits and seizure disorders.

Periventricular White Matter Injury Caused by Blood Products

The presence of blood products may have a direct pathogenetic role in the evolution of periventricular white matter injury, as demonstrated by the observation that the presence of IVH is associated with a five- to nine-fold increased risk of ultrasonographic correlates (e.g., echolucencies) of white matter injury.[15] Experimental studies suggest that two interacting mechanisms could be operative to produce white matter injury in the presence of IVH: impairment of cerebral blood flow and increased generation of free radicals. *Disturbances of cerebral blood flow* with parenchymal or subarachnoid blood have been demonstrated resulting from local release of vasoconstricting potassium from hemolyzed red blood cells[170] or prostanoids[171] or 5-hydroxytryptamine.[171] Because the walls of cerebral parenchymal vessels do not contain a muscularis necessary for vasospasm in the human premature infant,[150] it seems unlikely that local cerebral blood flow could be affected by this mechanism. However, subarachnoid blood potentially could cause vasoconstriction of major leptomeningeal cerebral vessels, which do contain a muscularis in the human premature infant.

The more likely mechanism of white matter injury with intraventricular or parenchymal blood involves *increased free radical formation*, provoked by local release of iron from the blood.[172] The presence of blood products is particularly dangerous in the premature infant because the expression of heme oxygenase is especially high in the immature cerebral white matter,[173] thus enhancing formation of Fe^{2+} when hemorrhage is present. The Fe^{2+} could then catalyze the generation of the highly reactive and dangerous hydroxyl radical through the Fenton reaction. The early differentiating oligodendroglia has been shown to be exquisitely vulnerable to free radical attack,[174] and thus the enhanced hydroxyl radical generation could accelerate cerebral white matter injury. Although free radical–mediated cellular injury has been proposed as a key pathogenetic factor in cerebral white matter injury in the premature infant, direct evidence heretofore has been lacking. Studies in the premature infant are the first to document an elevation in the early postnatal period in measures of free radical products in the CSF of premature infants with cerebral white matter injury later apparent on MRI at term.[175,176]

COMPLICATIONS OF INTRAVENTRICULAR HEMORRHAGE

Hydrocephalus

Incidence and Definition

Hydrocephalus, which is a progressive ventricular dilatation (PVD) resulting from a disturbance in CSF dynamics, is a not uncommon sequel to IVH (Table 166-3).[2,3,101,177-180] As could be expected, the incidence of posthemorrhagic PVD is related closely to the severity of the initial hemorrhage. A clear clinical distinction between ventriculomegaly resulting from periventricular cerebral atrophy and ventriculomegaly resulting from hydrocephalus with attendant impairment of CSF dynamics can be difficult but is critical for the formulation of appropriate management decisions. In general, the development of ventriculomegaly, resulting from atrophy, occurs slowly, over several weeks; is not associated with the development of increased intracranial pressure or rapid head growth; and evolves to a state of stable ventricular size; that is, ventricular size neither decreases, as in transient hydrocephalus, nor continues to increase, as in persistently progressive hydrocephalus. The evaluation of progressive ventricular dilatation after IVH can be best undertaken with cranial US, particularly for the systematic evaluation of ventricular size. Previously, cranial US images have been typically evaluated qualitatively for ventricular size. Although this strategy has proved useful, it would appear more

TABLE 166-3

Short-Term Outcome of Germinal Matrix–Intraventricular Hemorrhage as a Function of Severity of Hemorrhage

	DEATHS IN FIRST 14 DAYS		PVD IN SURVIVORS 14 DAYS OLD		SURGERY + LATE DEATH			
Grade	<750 g (n = 173)	751-1500 g (n = 75)	<750 g (n = 165)	751-1500 g (n = 56)	<750 g (n = 165)	751-1500 g (n = 56)	Mortality	Surgery in Survivors
I, n = 104 (%)	3/24 (12)	0/80 (0)	1/21 (5)	3/80 (4)	0 + 4/21 (19)	0 + 3/80 (0)	10/104 (10)	0/94 (0)
II, n = 65 (%)	5/21 (24)	1/44 (2)	1/16 (6)	6/43 (14)	0 + 5/16 (31)	1 + 2/43 (7)	13/65 (20)	1/52 (2)
III, n = 45 (%)	6/19 (32)	2/26 (8)	10/13 (77)	18/24 (75)	3 + 2/13 (38)	7 + 4/24 (42)	14/45 (31)	10/31 (32)
IV, n = 34 (%)	5/11 (45)	5/23 (22)	5/6 (83)	12/18 (66)	1 + 3/6 (66)	7 + 4/18 (61)	17/34 (50)	8/17 (47)

PVD, Progressive ventricular dilatation.

informative to evaluate ventricular size in a quantitative fashion, on the basis of established norms.[181] Further, the occipital horn is the first and the frontal horn is the last to enlarge after IVH.[182] Thus measurement of the thalamo-occipital dimension of the ventricle,[181] via the posterior fontanelle, is the most sensitive indicator of ventriculomegaly. Frequent cranial US monitoring of the ventricular dimensions, particularly in the acute period, may assist in assessing the infant's course and determining the need for intervention with ventricular drainage. It may also guide the frequency and volume of CSF drainage necessary to decrease ventricular size. The role of aggressive drainage of CSF with posthemorrhagic ventricular dilatation in preterm infants in improving outcomes remains unproved although retrospective comparisons between NICUs suggest that earlier intervention may be associated with a reduced need for ventriculoperitoneal shunt and improved neurodevelopmental outcomes.[183] This requires further investigation in a randomized controlled trial. The amount of CSF that requires to be drained may also vary greatly between infants with some infants requiring large volumes of CSF to be removed to reduce the pathologic consequences. In a case of posthemorrhagic ventricular dilatation that we studied, the removal of 60 mL/kg over a 12-hour period led to improved volumes of gray matter suggesting improved brain structure but with persisting ventriculomegaly suggesting that very high volumes of CSF may be removed in the subacute period of this disease process.[184]

Pathogenesis

The pathogenesis of posthemorrhagic hydrocephalus can be considered in terms of either the acute process, apparent within days, or the subacute-chronic process, apparent within weeks. Acute hydrocephalus appears to be a result of an impairment of CSF absorption caused by particulate blood clot and demonstrable by US scan (Figure 166-8).[185] Subacute-chronic hydrocephalus presumably is related to obliterative arachnoiditis in the posterior fossa where the blood tends to collect.[35] The ventricular dilatation may begin essentially with the hemorrhage, especially with marked IVH. More often, definite ventricular dilatation and, particularly, the progression thereof begin within 1 to 3 weeks of the hemorrhage.

Relation of Ventricular Dilation to Brain Injury

The precise relation of progressive posthemorrhagic ventricular dilation to brain injury is unclear. This issue is discussed in experimental and clinical studies.

Experimental Studies

The deleterious effects of hydrocephalus on the brain may include disturbances of cerebral white matter, cerebral blood vessels, and cerebral cortex. For *cerebral white matter*, the earliest changes are seen within 1 week after induction of experimental hydrocephalus and involve oligodendrocytes and myelin, with a decrease in myelin-associated enzymes (e.g., ceramide galactosyltransferase) and structural proteins (e.g., myelin basic protein). Later, after several weeks, axonal loss and impaired myelination are apparent. Shunting at 1 week but not at 4 weeks, when axonal loss had occurred, has been shown to allow recovery of myelination.[186] The late occurrence of axonal loss with hydrocephalus was noted in earlier studies.[187-189] Thus intervention must occur before this stage if prevention of such permanent structural deficits is to be achieved.

A potential role for *cerebral vascular changes and ischemia* in the genesis of the white matter injury in experimental hydrocephalus is demonstrated by morphologic studies that show an alteration in the caliber of major cerebral vessels with a decrease in the secondary and tertiary vessels in the cerebral white matter.[190,191] These changes may explain, at least in part, the decrease in cerebral blood flow and energy metabolism in hydrocephalic animal models.[192-194] Further supportive of cerebral ischemia are the findings in cerebral white matter of increased cerebral rates of glucose metabolism (presumably anaerobic glycolysis), elevated lactate, decreased high-energy phosphates, and elevated free radicals.[195-197] Enhanced free radical production is particularly relevant, in view of the particular vulnerability of differentiating oligodendroglia to free radical attack.[198] These biochemical changes and neurochemical signs of axonal loss have been shown to be prevented by early shunting in the various experimental models.[194,196]

Of additional interest are the documented alterations in *cerebral cortical neurons* in hydrocephalic animal models. Disturbances in catecholaminergic and serotonergic neurotransmitter development and in synaptogenesis with evidence of neuronal degeneration have been delineated.[199-209] These effects may reflect disturbance to ascending and descending axon in cerebral white matter with secondary anterograde and retrograde effects on organizational development of cerebral cortex. Again, these deleterious effects have been shown to be reversible when the hydrocephalic state is corrected early.

Human Studies

Data from studies in infants concerning the deleterious effect of progressive posthemorrhagic ventricular dilation relate to cerebral hemodynamics, neurophysiologic function, morphologic disturbances, biochemical indicators of hypoxia, and clinical outcome. The data are difficult to interpret conclusively because such details as rate of progression, degree and duration of ventriculomegaly, intracranial pressure, preceding parenchymal injury, and neurologic outcome are often not provided.

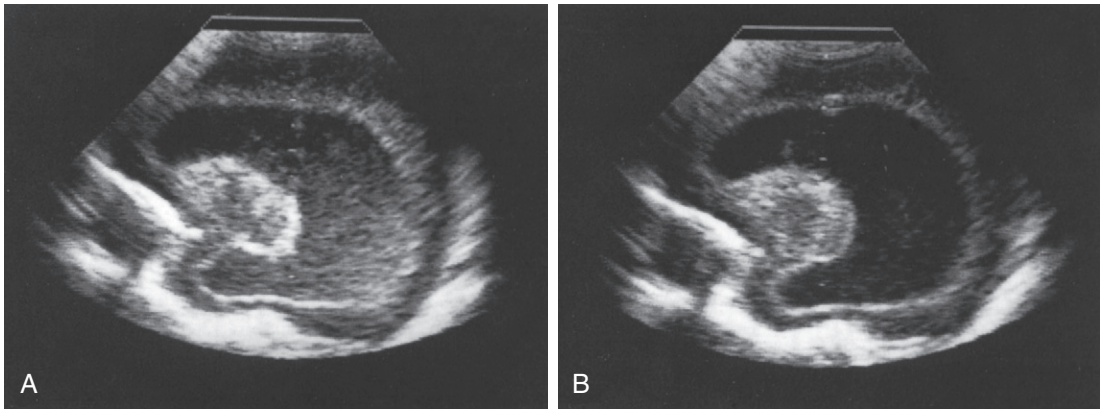


Figure 166-8 **A** and **B,** Acute hydrocephalus with IVH. *(From Volpe JJ:* Neurology of the Newborn, *Philadelphia, ed 4, Saunders, 2001, p 458.)*

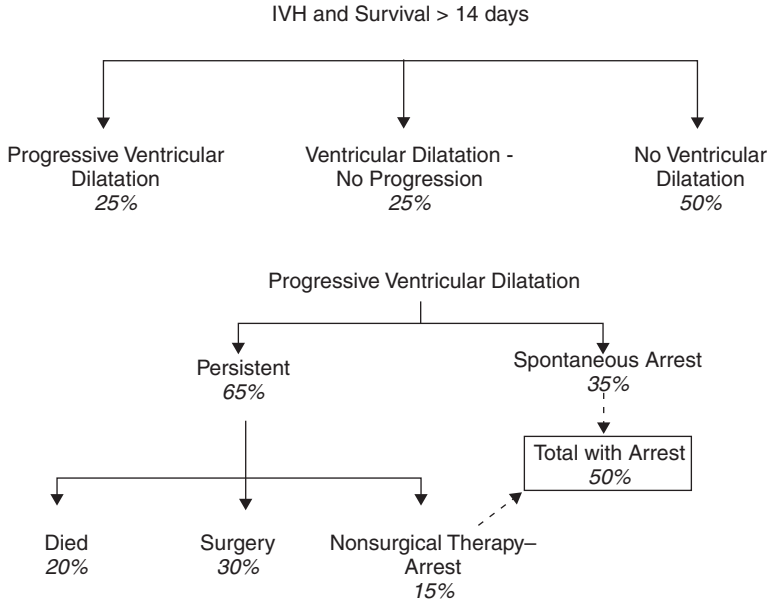


Figure 166-9 Natural history of very-low-birth-weight infants with posthemorrhagic ventricular dilatation.

Concerning *cerebral hemodynamics*, posthemorrhagic hydrocephalus initially has been demonstrated to be associated with *impaired blood flow velocity,*[67,210-213] which can be reversed with therapy to correct the hydrocephalic state.[67] This disturbance of cerebral blood flow velocity appears to correlate more closely with ventriculomegaly than with increased intracranial pressure.

Concerning *neurophysiologic function,* visual evoked potentials, brain stem auditory evoked potentials, and somatosensory evoked responses have all been shown to be altered by posthemorrhagic ventricular dilatation. The studies have demonstrated prolonged latencies of these evoked potentials with posthemorrhagic hydrocephalus in the premature infant.[214-216] Improvement in latencies was demonstrated after CSF removal by placement of a ventriculoperitoneal shunt or direct removal of ventricular fluid.[214-216] These data suggest that an axonal disturbance is caused by ventricular distention, and this disturbance can be reversed by effective therapy.

Concerning *morphologic evidence of brain injury* with posthemorrhagic ventricular dilation, a paucity of data exists from studies of autopsies of human infants who did not have markedly advanced disease. Studies of biopsy specimens taken at the time of ventriculoperitoneal shunt placement have shown four major findings: (1) disruption of ependyma, (2) direct evidence of axonal injury ("axonal ballooning"), (3) lipid-laden microglia, and (4) diminished numbers of myelinated axons.[188,217-220] These findings are consistent with the experimental models of hydrocephalus.

Biochemical evidence of hypoxia has been observed and suggests injury to brain that is reversible by therapy.[221] *Elevated CSF levels of hypoxanthine*, a marker for hypoxic tissue, have been found in infants with posthemorrhagic hydrocephalus that reduced significantly after successful treatment.[221] Although the morphologic effects of hydrocephalus on cerebral vessels and the resulting deleterious cerebral hemodynamic effects are a probable explanation for tissue hypoxia, elevated levels of vasoconstricting eicosanoids (e.g., thromboxanes) or edema-producing eicosanoids (e.g., leukotrienes) appear important. These eicosanoids have been shown to be elevated in CSF of infants with posthemorrhagic hydrocephalus.[222] The source of these compounds remains unclear, and their specific relation to the posthemorrhagic ventricular dilatation remains to be established. Biochemical evidence of oxygen deficiency at the mitochondrial level is found with the demonstration by near-infrared spectroscopy of an increase in *brain levels* of *oxidized cytochrome* aa_3, after lumbar puncture in infants with posthemorrhagic hydrocephalus accompanied by increased intracranial pressure.[223,224] This observation is consistent with the demonstration by positron emission tomography of increases in cerebral metabolic rate for oxygen in several infants (one newborn) after treatment of hydrocephalus.[225] More data are needed on these issues.

Natural History

Management of progressive posthemorrhagic ventricular dilation must begin with recognition of the natural history of the disorder.* We undertook a large study in premature infants to reflect the natural history of PVD. In this cohort of 248 very-low-birth-weight infants with IVH (mean gestational age, 27 weeks), 25% of the infants exhibited PVD (Figure 166-9). Arrest of PVD occurred without treatment in one third of infants with PVD. Of the remaining two thirds of infants who did not have arrested PVD and thus had persistent PVD, most received nonsurgical therapy consisting of pharmacologic therapy or drainage of CSF by serial lumbar punctures only. Half of the patients with persistent PVD received surgical therapy with insertion of a ventriculoperitoneal reservoir or shunt, and one third of the infants with persistent PVD died (see Figure 166-9). The development of PVD after IVH and the occurrence of adverse short-term outcome, such as the requirement for surgical therapy, were predicted most strongly by the severity of IVH (see Table 166-3). These data demonstrate that despite a slight reduction in the overall incidence of PVD, a more aggressive course with appreciable mortality and morbidity appears to exist in the extremely premature infant characteristic of current NICU facilities. The major predictor of adverse short-term outcome, defined as death or the need for surgical intervention for PVD, was the severity of IVH (see Figure 166-9 and Table 166-3).

*References 2, 3, 19, 89, 101, 178-180, 182, 226-241.

TABLE 166-4

Long-Term Outcome: Neurologic Sequelae With Germinal Matrix–Intraventricular Hemorrhage as a Function of Severity of Hemorrhage

Severity of hemorrhage	Incidence of neurologic sequelae (%)
Mild	5-10
Moderate	15
Severe	35
Severe + apparent periventricular hemorrhagic infarction	50-100

PROGNOSIS

The prognosis of IVH is considered best in terms of the short-term outcome (mortality rate and development of PVD) and the long-term outcome (neurologic sequelae). The *short-term outcome* relates clearly to the severity of the hemorrhage. The mortality rates and incidences of progressive posthemorrhagic ventricular dilation (i.e., hydrocephalus) are shown in Table 166-3 as a function of the severity of the hemorrhage, documented primarily by US scan.[3,15,242-245] With small lesions, confined to the germinal matrix or accompanied by small amounts of intraventricular blood, mortality rates are low, and the likelihood of PVD in survivors is rare. In contrast, infants with severe lesions, that is, blood filling the ventricles, have high mortality rates (approximately 20%), and more than half of the survivors exhibit PVD. The *long-term neurodevelopmental outcome* of the infant with IVH depends on the degree of parenchymal injury. A large literature describes associations between cranial US findings with only an approximate relationship existing between the quantity of intraventricular blood and the neurologic outcome (Table 166-4).* The largest, most recent study relating IVH to outcomes is the EPIPAGE study in 1954 infants of <32 weeks gestation.[256] A clear relationship existed between an increased risk of adverse outcome with increasing grade of IVH. However, it is notable that even in isolated grade I-II IVH the rates of cerebral palsy increased substantially for grade I IVH (n = 229) from 5.5% to 8.1% and grade II IVH (n = 168) to 12.2%. Notably, cerebral palsy rates with isolated grade I-III IVH also rose with immaturity from 5% in 31 to 32 weeks to 10% to 15% in 27 to 30 weeks and 33% in 24 to 26 weeks. For school-aged outcomes, Sherlock and colleagues[257] examined 298 preterm infants less than 1000 g at age 8 years to determine the impact of IVH in relation to neuromotor and cognitive outcomes. They documented that no IVH was associated with cerebral palsy rates of 6.7% with no rise in association with grade I IVH (6.4%) but a marked elevation with grade II IVH to 24%. Patra and colleagues[258] also report that grade I-II IVH was associated with a two-fold increase in the risk of lower cognitive performance (Mental Developmental Index) and a 2.6-fold increase in the risk of neuromotor abnormalities (cerebral palsy and tone) after controlling for social and neonatal factors (gender, bronchopulmonary dysplasia, sepsis, necrotizing enterocolitis, maternal marital status, race, and education). Although the studies do differ in their findings, the more recent contemporaneous studies in similar populations of very preterm infants demonstrate similar rates of cerebral palsy with grade I-II IVH approximating a 10% risk, clearly double that of no IVH. It is not clear whether this risk is elevated with grade I IVH alone or whether any elevated risk exists for subsequent cognitive deficit. The major limitation of these studies is the inability to truly distinguish the key neuropathologies that may be leading to the subsequent impairment in neurodevelopmental outcome. Cranial US has been demonstrated to have very poor diagnostic utility for diffuse white matter injury, which has been shown to occur in up to 75% of preterm infants. The cerebral white matter is also the commonest site of neuropathologic impact for IVH, as described earlier. In such a manner, low-grade IVH may be merely a visible marker on cranial US for the true neuropathology of cerebral white matter injury, which on MR imaging has been shown to be highly predictive of both motor and cognitive deficits.[259] Of note, the elevation of risk for poor outcomes with low-grade IVH with increasing immaturity may also be suggesting an independent role for the loss of the astrocytic and glial precursors from the immature germinal matrix.

*References 19, 42, 46, 180, 227, 228, 238, 240, 242, 245-255.

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Pathophysiology of Neural Tube Defects

Neural tube defects are the most common and most severely disabling malformations of the central nervous system (CNS). They include a wide spectrum of malformations comprising open defects, such as myelomeningocele and anencephaly, and skin covered lesions, such as encephalocele and lipomyelomeningocele. The clinical heterogeneity of neural tube defects results from a diversity of underlying mechanisms.[1] In the United States, neural tube defects are one of the most prevalent neonatal anomalies, second only to cardiac malformations. Worldwide, 300,000 or more infants are born each year with neural tube defects.[2] The incidence varies according to geography, ethnicity, and seasonal variation, ranging from 1 to 4 per 1000 live births. The fetal prevalence may well be even higher because neural tube defects detected by screening amniocentesis and ultrasound often result in elective termination of the pregnancy. The highest incidence of neural tube defects is found in Great Britain, Ireland, Pakistan, Northern India, and Egypt. The lowest incidence is observed in Finland, Japan, and Israel. Even within in the United States, a geographic and ethnic distribution of these defects occurs, with the highest incidence in the East and the South, and the lowest in West.[3] There is an increased prevalence of neural tube defects among Hispanics, especially if the mother was born in Mexico.[4] There is also a high incidence of neural tube defects among Sikhs. However, the lesions are frequently restricted to the thoracic level with minimal associated neurologic deficit, suggesting a separate etiology.[5] Women in the United States who have had an infant or fetus with a neural tube defect have a 2% to 3% risk for a subsequent affected pregnancy.[6]

The most common types in humans are anencephaly and myelomeningocele (spina bifida aperta). Anencephaly is almost always lethal, whereas myelomeningocele, although not usually lethal, has enormous personal, familial, and social costs. The life of most affected children is characterized by recurrent hospitalizations, multiple operations, and physical and cognitive impairments. The associated handicaps are dependent on the level of the spinal lesion, the degree of damage to the exposed neural tissue, and the presence of the Chiari type II malformation.[7] Less than 20% of neural tube defects are associated with malformation of nonneural tissues, chromosomal defects, or specific genetic syndromes,[8] such as amniotic bands,[9] trisomies 13 and 18, and the autosomal recessive Meckel-Gruber syndrome.

Despite intensive investigation, the etiology of neural tube defect remains unclear, and the controversy over its pathogenesis continues.

EMBRYONIC DEVELOPMENT

Normal human brain development can be summarized in six basic stages: (1) primary and secondary neurulation; (2) proencephalic development; (3) neuronal proliferation; (4) neuronal migration; (5) organizational events; and (6) myelination.[10] Understanding the process of CNS development is important in understanding the features of the CNS abnormalities in children affected by neural tube defects. Development of the CNS begins prenatally and continues postnatally with overlapping developmental periods (Table 167-1). The embryonic development of the fetal brain and the spinal cord are described in detail in Chapter 160. The embryology, specifically of neural tube formation, is briefly presented here in order to understand neural tube defects in the context of normal CNS development.

Primary Neurulation

The formation of the neural tube begins on the dorsal aspect of the gastrula stage embryo as a plate of tissue differentiating in the middle of the ectoderm. Neurulation occurs between the third and fourth weeks of gestation. According to Smith and Schoenwolf,[11] neurulation can be subdivided into four broad morphologic steps: (1) induction of the neural plate; (2) molding of the neural plate; (3) longitudinal bending of the neural plate; and (4) fusion of the neural folds. The stages of primary neurulation are coordinated with progressive and regressive movements of the primitive streak.[12] The critical event in this process is neural induction, which involves interaction between the ectoderm and the underlying mesoderm (notochord). The notochord and chordal mesoderm induce formation of the neural plate, which occurs between 16 and 18 days gestation. The inductive signals are generated by Hensen node, the avian equivalent of Spemann organizer.[13,14] Cell proliferation and shape change then result in extension and folding of the primordial neural plate, the precursor of both the brain and the spinal cord. As the notochord forms and elongates, the neural plate enlarges and extends beyond the edges of the notochord. The midline, however, remains tethered to the underlying notochord. The neural plate begins to invaginate along the central axis, forming a longitudinal median groove with neural folds on its sides. In the region of the future spinal cord, this process remains, obliterating the groove and bringing the edges of the plate in apposition. At approximately 22 days gestation, the neural tube forms by fusion of the opposed neural folds in the region of the future lower brain stem. Fusion progresses rostrally and caudally. The neural tube remains open at both ends, the anterior and the posterior neuropores, which

TABLE 167-1

Normal Human Brain Development and Time of Occurrence

Normal Human Brain Development	Time of Occurrence
Formation of the brain structure	0-20 weeks gestation
• Primary and secondary neurulation	• 3-4 weeks gestation
• Proencephalic development	• 4-12 weeks gestation
Neuronal migration	5-20 weeks gestation
Neuronal proliferation and brain growth	0 weeks gestation to 12 years of life
Organizational events	12 weeks gestation to 12 years of life
• Differentiation, maturation, synaptogenesis	• 16 weeks gestation to 12 years of life
• Apoptosis	• 24 weeks gestation to 1 month of life
Myelination	13 weeks to 30 years of life

Modified from Volpe JJ: Overview: Normal and abnormal human brain development, *Ment Retard Dev Disabil Res Rev* 6:1-5, 2000.

allow for the fluid of the amniotic cavity to communicate with the neural tube lumen. The anterior end of the neural tube closes at approximately 24 days and the posterior end closes at approximately 28 days gestation.[15]

The mechanisms responsible for neural tube formation are not completely understood, but two theories have been advanced. In the "zipper closure model," the neural tube closure begins in the cervical region, and fusion then proceeds bidirectionally until the neural tube is closed.[16] More recently, the "multisite closure model" has been suggested.[17] This model of multisite neural tube closure may provide the best explanation for neural tube formation (Table 167-2).[18,19] In addition, the complex morphologic events of neural tube formation result from the unity of intrinsic and extrinsic features and may also involve nerve cell adhesion molecules (NCAMs) [20] or other components within the extracellular matrix.[21] Intrinsic processes include microfilament contraction actively changing cell shape[22] and passive folding caused by rapid cell proliferation and underlying membrane tethering.[23] The relationship between the neural plate and the adjacent embryonic tissue constitutes the extrinsic forces in neural tube development.

Primary neurulation results in a closed neural tube with its caudal limits in the upper lumbar spinal cord. Secondary neurulation, the process in which the remaining caudal elements of the spinal cord (i.e., conus medullaris, lower lumbar, and sacrococcygeal segments) are fashioned is related to the future vertebral level S2. An open neural tube defect results if closure is not completed during this developmental window.

EMBRYONIC AND MOLECULAR MECHANISMS OF NEURAL TUBE DEFECTS

Insights From Mouse Genetic Models

Because neurulation in mouse and human embryos appears to be quite similar anatomically, the mouse has been used as a model for investigating the embryonic mechanisms of neural tube defects. Direct genetic analysis of human neural tube defects has been difficult because of the rarity of familial cases and the lack of specific candidate genes involved. To date, more than 80 mouse mutant models have been described (i.e., *curly tail*, *loop tail*, *splotch*, *vacuolated lens,* and *Mlp-knockout* mice) that exhibit a genetic cosegregation of anencephaly, exencephaly, and myelomeningocele analogous to the human condition.[24] However, most of these mutants present additional defects that are never or only infrequently seen in humans with neural tube defects. For example, the malformations in the mouse mutant splotch are similar to those found in human Waardenburg syndrome type I[25,26] and in Greig cephalopolysyndactyly.[27] Absence of the corpus callosum or thinning of the cerebral cortex and retina are rarely found in humans with neural tube defects but frequently present in the Mlp-knockout mouse.[28] Finally, some knockouts, such as *CSK*, *BRCA1*, and *P53*, show neural tube defects but also exhibit growth retardation and other malformations and are associated with a high embryonic lethality.[29-31]

TABLE 167-2

Multisite Closure Model for Neural Tube Formation

Closure	Region of Fusion at the Neural Tube
1	At the midcervical region and proceeds cranially and caudally
2	At the prosencephalon-mesencephalon boundary and proceeds bidirectionally
3	Proceeds rostrally from the stomadeum and convenes cranially with closure
4	At the rhombencephalon and proceeds rostrally
5	Unidirectional closure of the caudal end of the neural tube

Despite these limitations, investigations in two mouse mutants led to two hypotheses to account for the failure of the posterior neuropore to close, resulting in open defects of the spine. The curly tail (ct/ct) mouse mutant, first described by Gruneberg in 1954,[32] exhibits failure of both the anterior and posterior neuropore closures, resembling common nonsyndromic forms of human neural tube defects. The basic defect in the curly tail mouse is not within the neuroepithelium but rather is in the nonneural tissues.[33] In curly tail (ct/ct) mouse mutants, the notochord and the hindgut endoderm demonstrate decreased cell proliferation causing enhanced curvature of the caudal region. This curvature mechanically opposes the closure of the neuropore, leading to the development of open neural tube defects.[34,35] In addition to the altered cell proliferation rates, curly tail mouse embryos exhibit a reduced accumulation of newly synthesized hyaluronan within the extracellular matrix in the notochord and hindgut endoderm.[36] Because hyaluronan significantly contributes to cell proliferation in a variety of cell types, Copp and Bernfield[37] suggested that alteration in hyaluronan synthesis resulting in suboptimal proliferation of the notochord and hindgut endoderm may result in neural tube defects.

Curly tail (ct/ct) mouse mutants demonstrate an increased level of alpha-fetoprotein in the amniotic fluid of fetuses with neural-tube defects,[38] as well as an incomplete penetrance (60%), which varies with genetic background supporting the hypothesis of the presence of modifier genes.[39] Similar to humans, environmental factors (i.e., retinoic acid, mitomycin C, hydroxyurea, and 5-fluoruracil) are important, with alteration in penetrance and expressivity of the curly tail phenotypes.[40] Although treatment with folate and methionine is effective in reducing the incidence of neural tube defects in humans, they have no effect on the incidence or type of neural tube defects in curly tail mice.[41-43] Inositol treatment reduces curly tail neural tube defects by stimulating protein kinase C activity by 70%, suggesting that the curly tail model is a folate-resistant model of neural tube defect phenotypes.[40]

By contrast, homozygous splotch (Sp/Sp) mice were recently found to have a mutation in the paired domain of the *PAX3* gene responsible for the phenotype of these mutants.[44] The failure to express a normal Pax3 protein (a critical embryonic gene involved in neural tube development in mice and humans) in the neuroepithelial cells appears to impair the ability of the neural plate to undergo folding and fusion during the neurulation process.[44,45] Although the cellular mechanisms are unknown, Yang and Trasler[46] and Moase and Trasler[47] have suggested that the failure of Pax3 protein expression affects the cell-cell interaction within the neural plate. Linkage studies of Pax3 and neural tube defects in U.S. and Dutch families, however, have failed to identify the relationship between Pax3 expression and neural tube defects.[48] In addition, mouse models of *HOX*-deficient mice showed delayed closure and aberrant formation of the neural tube.[49] However, analysis of the four known clusters of *HOX* genes (A, B, C, D), which play an important role in neural tube development in humans, found no evidence for their involvement in human neural tube defects.[50] A report of linkage between human neural tube defect and the region of the T locus homologue[51] has not been supported by other studies.[52,53]

In double mutant mice for splotch and curly tail, the incidence and the severity of spina bifida significantly increase, indicating an additive effect in the disruption of the neurulation process.[54] The neural tube defect phenotypes seen in curly tail mutants resemble those of *F52-* (also called MacMARCKS)-deficient mice, and the *F52* gene and ct have both been mapped to distal chromosome 4.[55] The penetrance and severity of ct gene expression, however, are affected by the modifier genes mapped to murine chromosome 3 and 5.[55] About 60% of *F52*-deficient mice are exencephalic, and approximately 10% have spinal neural tube defects.[28] The question that has been raised is whether the curly

tail mouse is a mutation of the *F52*-deficient mouse.[28] However, it is thought that the curly tail mutation addresses cellular defects in the extrinsic forces of neurulation, whereas the MacMARCKS-like protein regulates morphogenesis in various tissues by meditation of cell-cell signaling, changes of actin-cytoskeleton dynamics, and regulation of cytosolic protein expression.[56] Thus modulations regulated by protein kinase C substrates[56] within the neuroepithelial cells play an integral role as intrinsic forces during neurulation.

To summarize, for most mouse models of neural tube defects, there is still a gap between identifying the gene involved in the development of neural tube defects and the morphogenetic mechanism it contributes to the formation of the neural tube. However, it is possible and perhaps likely that neural tube defects in humans result from a combination of common, disease-associated alleles in the population or even from a subtle change of function in specialized neurulation genes.

Insights From Teratogenic Models

Various teratogenic neural tube defect models have been examined in order to understand the mechanisms of abnormal neurulation and to test etiologic and environmental hypotheses suggested by human epidemiologic data. A number of teratogens, such as ethanol, insulin, trypan blue, vitamin A, folic acid antagonists, GABA-receptor agonists, and hyperthermia, have all been used to induce neural tube defects in vitro and in vivo.[57,58] More than 30 classes of chemicals and drugs are listed in the literature that result in neural tube defects.[59] However, the phenotype of neural tube defects, as well as the development of hindbrain herniation, depends on the timing of administration.[60,61]

Administration of valproic acid on day 8 of gestation in the mouse results in high rates of malformations of the anterior neural tube (i.e., exencephaly),[62] whereas a similar dosing regimen of valproic acid on day 9 of gestation results in spina bifida occulta.[63] Having demonstrated the feasibility of creating a spinal defect resembling a human myelomeningocele with valproic acid, Ehlers and colleagues[64] reported that the administration of methionine (70 mg/kg body weight) to valproic acid–exposed mice fetuses significantly reduced the valproic acid–induced spina bifida rate. The interactions of methionine in neural tube formation are not completely understood. However, methionine may play an important role in normal neural tube formation because the absence of methionine causes neural tube defects in cultured rat embryos.[65]

More recently, Danzer and co-workers[66] developed and characterized a novel short-gestational animal model of isolated myelomeningocele in fetal rats by maternal administration of all-*trans*-retinoic acid. Exposure to retinoic acid at the time of posterior neuropore closure (E10 in rats) leads to a pathology that has striking morphologic and clinical similarity to human myelomeningocele in approximately 60% of the fetal rats.[66,67] Prenatal administration of retinoic acid induces a primary defect during the neural tube formation. With respect to fetal surgical intervention (see later), this animal model may facilitate investigations in the very early developmental abnormalities of spina bifida. Furthermore, the fetal rat is large enough for surgical manipulation and has minimal propensity for preterm labor after fetal surgery, allowing the potential use of this model to study various approaches for prenatal coverage of myelomeningocele and the subsequent effect on postnatal neurologic outcome.

Insights From Mechanical Models

In addition to the genetic and teratogenic models of neural tube defects, new models of mechanical disruption of normal neural tube development have been described. These models in particular provide further evidence for the "two-hit hypothesis." The first "hit" is the failure of neurulation early in gestation. The second "hit" is the spinal cord injury resulting from prolonged exposure of the neural tissue to the intrauterine environment. The first was a primate model[68] in which a fetal lumbar laminectomy (L3-L5) was performed late in gestation. One group of animals was repaired immediately with allogeneic bone paste, and a control group had no repair. The unrepaired fetuses showed cystic myelomeningocele-like lesions at birth and showed severe neurologic impairment, including paraplegia, incontinence, and somatosensory loss. The repaired group was neurologically normal at birth. Unfortunately, the experiment did not include a period of exposure to the intrauterine environment before closure.[68] These results nevertheless support the hypothesis that prolonged exposure of the spinal cord to amniotic fluid causes considerable damage to the neural tissue in a myelomeningocele-like lesion. Heffez and colleagues[69,70] reported similar findings in fetal pigs and fetal rats. In both studies, late-gestation fetuses underwent surgical creation of a myelomeningocele-like defect with immediate or delayed (24 hours later) surgical closure resulting in preservation of neurologic function compared with the untreated group. Notably, in some of the animals, dehiscence of their skin closure occurred, resulting in neurologic deficit.[70] Histologic examination of the exposed neural tissue revealed erosion and necrosis, findings similar to those described in children with myelomeningocele. More recently, Correia-Pinto and associates[71] demonstrated in a surgically created rat model that late-gestation exposure to meconium increases damage to neural tissue.

These studies show that exposure of the fetal spinal cord to the intrauterine environment results in significant acquired neural tissue damage with corresponding neurologic deficit at birth. In addition, these studies demonstrate that closure of a surgically created myelomeningocele-like lesion is feasible and improves neurologic function at birth. The model that is most similar to simulating the human disease is the fetal lamb model of myelomeningocele introduced by Meuli and co-workers in 1994.[72] The myelomeningocele-like defect was surgically created in fetal lambs at 75 days gestation (term 145 to 150 days) by excision of skin, paraspinal musculature, and vertebral arches of lumbar vertebrae 1 through 4 exposing the dorsal dura mater. At 100 days of gestation, a reversed latissimus dorsi flap was used to cover the exposed spinal cord, and the animals were delivered by cesarean section just before term.[73,74] As in the primate, rat, and porcine models, the untreated fetuses showed myelomeningocele-like lesions at birth with similar neurologic deficits: complete sensorimotor paraplegia, incontinence of stool and urine, and lack of sensory function of the hind limbs.[73,74] Animals that underwent closure of the defect had healed skin wounds and near-normal neurologic function. Despite mild paraparesis, they were able to stand, walk, perform demanding motor tests, and had no signs of incontinence. Furthermore, sensory function of the hind limbs was present clinically and confirmed electrophysiologically.[73,74]

Hindbrain herniation in children with myelomeningocele is hypothesized to result from leakage of cerebrospinal fluid through the open neural tube at a critical time in posterior fossa formation.[75] Based on this hypothesis, Paek and colleagues[76] added a myelotomy to the surgically created myelomeningocele in fetal sheep. They demonstrated severe hindbrain herniation in lambs with a myelotomy and an unrepaired myelomeningocele. In contrast, the brains of lambs that underwent in utero repair of the lesion were normal. Bouchard and co-workers[77] similarly observed the hindbrain pathology in 85% of fetuses that underwent myelomeningocele creation with myelotomy (75 days gestation). The hindbrain anatomy was restored in 88% of the fetuses that underwent fetal myelomeningocele repair (102 days gestation). In other words, creation of a myelotomy allowed the egress of cerebrospinal fluid from the central hindbrain herniation. Closure of the myelomeningocele defect, by blocking the

leakage of cerebrospinal fluid, is thought restore the hindbrain anatomy.

To summarize, the fetal sheep model of myelomeningocele demonstrated that a spinal cord lesion could be created in utero and repaired at a later time with improved neurologic function after birth. Unlike the previous animal models,[68-70,78] this sheep model more closely resembled that of human myelomeningocele in duration and exposure of the spinal neural tissue to the intrauterine environment as well as in clinical and histologic outcome. Furthermore, brain anomalies such as the hindbrain herniation characteristic of the Chiari II malformation could be replicated with the addition of a myelotomy to the creation of myelomeningocele.

The results of these studies suggest that the intrauterine environment plays a considerable role in secondary neural tissue injury, perhaps as much as the primary defect during the neural tube formation. In addition, these models establish the feasibility of in utero repair and its potential for preserving neurologic function.

NEURAL TUBE DEFECTS AS MULTIFACTORIAL DISORDERS

Family History

A family history of spina bifida or anencephaly is one of the strongest risk factors for these disorders. The risk for neural tube defects in the siblings of affected individuals ranges from 3% to 8% and is consistently higher than that of the general population.[79-81] An increase risk for spina bifida or anencephaly, or both, has also been reported for second- and third-degree relatives of affected individuals.[80,82,83] One report suggested that the risk for having a child with neural tube defect might be increased in couples who have had a child with Down syndrome, and the risk for having a child with this trisomy 21 might be increased in couples who have had a child with a spina bifida.[84] However, subsequent studies could not confirm or rule out any potential link between these two disorders.[85]

Genetic Features

Genetic or inherited features are important to understand the etiology of neural tube defects. However, as with most congenital defects, the role of genetic and environmental factors in the development of neural tube defects in humans is not completely understood. Several lines of evidence support the existence of a genetic predisposition, including (1) variable incidence among ethnic groups and geographic locations; (2) familial recurrence risks; (3) genetic syndromes associated with neural tube defect; (4) alterations in genes involved in folate metabolism associated with an increased risk for neural tube defects; (5) altered sex ratio; and (6) mouse models with at least 80 mutant gene loci that are associated with neural tube defects. This genetic distribution has been interpreted as indicating 60% heritability for human neural tube defects.[86] Neural tube defects, however, have a familial allocation that cannot be accounted for by simple mendelian traits. In addition, the large numbers of mouse models of neural tube defects suggest not only the relative ease with which the neurulation process can be disturbed but also the genetic heterogeneity of these abnormalities.

Supplementation with folate during early pregnancy significantly decreases the risk for neural tube defects (see later).[87] The reason for this effect is unknown.[88] Colas and Schoenwolf[12] suggested that supplementation of folate only neutralizes a genetic defect in folate homeostasis, like a metabolic deficiency in its maternal supply[43] or mutations in the methylenetetrahydrofolate reductase gene in the embryo.[89] These genes, however, account only for a small amount of the total genetic environment of neural tube defects.[90] Additionally, folate supplementation reduces the incidence of several congenital malformations,[91] questioning the specificity of its effect in neural tube formation. It appears that the degree and duration of folate deficiency necessary to inhibit DNA synthesis may be greater than the underlying gene defect that is necessary to produce the neural tube defect. In addition, folate deficiency is not by itself adequate to induce neural tube defects in mice.[92]

In view of the complexity of the developmental processes involved in neurulation, the fact that the identification of genetic variants influencing spina bifida risk has proved to be challenging is not especially surprising. The challenge now is to define innovative hypotheses that address the complexity of neural tube defects, including gene-gene and gene-environment interactions and the potential roles of maternal and paternal genotypes. Such a novel and innovative hypothesis has been recently proposed by Rothenberg and colleagues.[93] They suggested that the development of neural tube defects might be associated with the presence of autoantibodies to folate receptor membrane proteins in genetically predisposed women. Such autoantibodies to the folate receptors were found in 9 of the 12 women (75%) with a pregnancy complicated by a neural tube defect, whereas autoantibodies were detected in only 2 of the 24 (8.3%) controls.[93] However, additional studies of larger populations of women with children affected by neural tube defects are warranted to evaluate whether the observed association of autoantibodies against folate receptors and the development of neural tube defects in this pilot study indeed reflects a causal relation.

Environmental Features

Over the years, environmental studies have been instrumental in elucidating the nongenetic causes of neural tube defects in humans. These data suggested that maternal nutrition is important and that vitamin deficiencies, especially of folic acid, play an essential role in the pathogenesis of neural tube defects.[94-96] A lack of folic acid is a common condition usually caused by a low dietary intake and may be correlated to socioeconomic factors. This may be particularly important during pregnancy, when the folate requirement is increased but the availability of folate may be reduced owing to changes in intestinal absorption and renal clearance.[97] The risks for neural tube defects and rates of anencephaly are usually higher in groups with lower socioeconomic status.[98]

Identifying specific causal environmental features has proved difficult. In a National Institutes of Health–sponsored Collaborative Perinatal Project that reviewed the pregnancies of 53,000 women, Myrianthopoulos and Melnick[99] demonstrated that maternal diabetes mellitus, heart disease, lung disease, and the use of diuretics, antihistamines, and sulfonamides were all associated with an increased risk for neural tube defects. In most of the cases, the neural tube defect was not associated with other malformations or syndromes. In addition, women who had a short interval between the end of a previous pregnancy and the current pregnancy had an increased risk for neural tube defect.[99] Maternal diabetes and other modifications of glucose metabolism such as obesity are well known risk factors for offspring with neural tube defects.[100] The risk ranges from as low as 2% in the United States to as high as 8% in England.[101] The mechanism underlying this teratogenic effect has not been established, but it is clearly related to the degree of maternal metabolic control. Indeed, there is evidence that the risk for congenital malformations in the offspring of diabetic women is associated with first-trimester blood glucose concentrations.[102] Whether increased glucose concentrations are directly teratogenic or whether they serve as a marker for another teratogenic agent (i.e. ketone bodies, free oxygen radicals) is unclear.[103]

In addition to diabetes mellitus, there is substantial evidence that the risk for having a child with spina bifida or anencephaly

is increased in obese women. This risk appears to increase with increasing body mass index.[104-106] Whether the increased risk for neural tube defects in obese women could be connected to hyperinsulinemia is under dispute; because hyperinsulinemia might precede or coexist with obesity and diabetes, this metabolic state provides a potential link between these two maternal risk factors. On the other hand, in women who have undergone gastric bypass surgery for the treatment of obesity, the risk for having a offspring with spina bifida is also increased, most attributable to the reduced absorption of nutrients and vitamins.[107]

Other factors include fever and hyperthermia in early pregnancy,[108] viral infection,[109] parental occupation,[110] and maternal diarrhea.[110] An increased risk for neural tube defects is associated with in utero exposure to valproic acid or carbamazepine, or in combination with each other or other anticonvulsants.[111-113] In infants exposed to valproic acid or carbamazepine prenatally, the risk for neural tube defects might be as high as 2%.[114] Women who use these drugs for indications other than epilepsy (i.e., bipolar disease, migraine, chronic pain) are also at increased risk for having a child with spina bifida if they become pregnant while taking these drugs. The mechanisms by which valproic acid and carbamazepine increase the risk for neural tube defects and whether periconceptional folic acid supplementation can reduce these risks remains speculative.[115] The role of maternal and paternal age in the etiology of neural tube defects is still under debate. For example, McIntosh[116] and Strassburg[117] and their colleagues suggest that both advanced maternal age and paternal age are risk factors for neural tube defects. However, other studies have not confirmed this association.[118,119]

NUTRITIONAL STATUS AND PRIMARY PREVENTION

The relation between nutritional status, especially folate intake, and the occurrence of neural tube defects provides a powerful tool for primary prevention of these congenital lesions. However, based on the wide phenotypic spectrum and the multifactorial etiology of neural tube defects, it is surprising that only a single agent, folic acid, has been demonstrated to reduce the incidence of neural tube defect significantly. Smithells and associates[120] demonstrated that administration of a multivitamin preparation to women who had already one or more affected pregnancies resulted in a marked reduction of recurrence of risk for neural tube defect. A subsequent nonrandomized case study and retrospective observational studies supported these initial findings.[121-123] However, the strongest evidence comes from two randomized, double-blind clinical studies. The British clinical trial conducted by the Medical Research Council UK demonstrated a significant protective effect with periconceptional supplementation of 4 mg folate per day in preventing neural tube defects.[95] The Hungarian trial[87] revealed that periconceptional multivitamins (containing 12 vitamins, 0.8 mg of folate, 4 minerals, and 3 trace elements) prevented not only recurrences but also the first occurrence of neural tube defects. In addition, a recent national study of U.S. birth certificate data indicated a 19% decline in the birth prevalence of neural tube defects and 23% decline in spina bifida incidence among births conceived after folic acid supplementation (October 1998 through December 1999) compared with the neural tube defects before folate administration (October 1995 through December 1996).[124]

However, the underlying mechanisms of prevention of neural tube defects are not completely understood. Folic acid is essential as a cofactor for enzymes involved in DNA and RNA synthesis and vitamin B_{12} and vitamin B_6 in the metabolism of methionine.[125] After it was reported that pregnant women with neural tube defects have an elevated blood level of homocysteine,[126] research has been focused on the homocysteine-methylation cycle. As seen in Figure 167-1, reactions regulating this cycle are dependent on one or more of these vitamins. Inadequate levels of methionine may result from inadequate tissue levels of either the coenzyme, vitamin B_{12}, as methylcobalamin, or folate

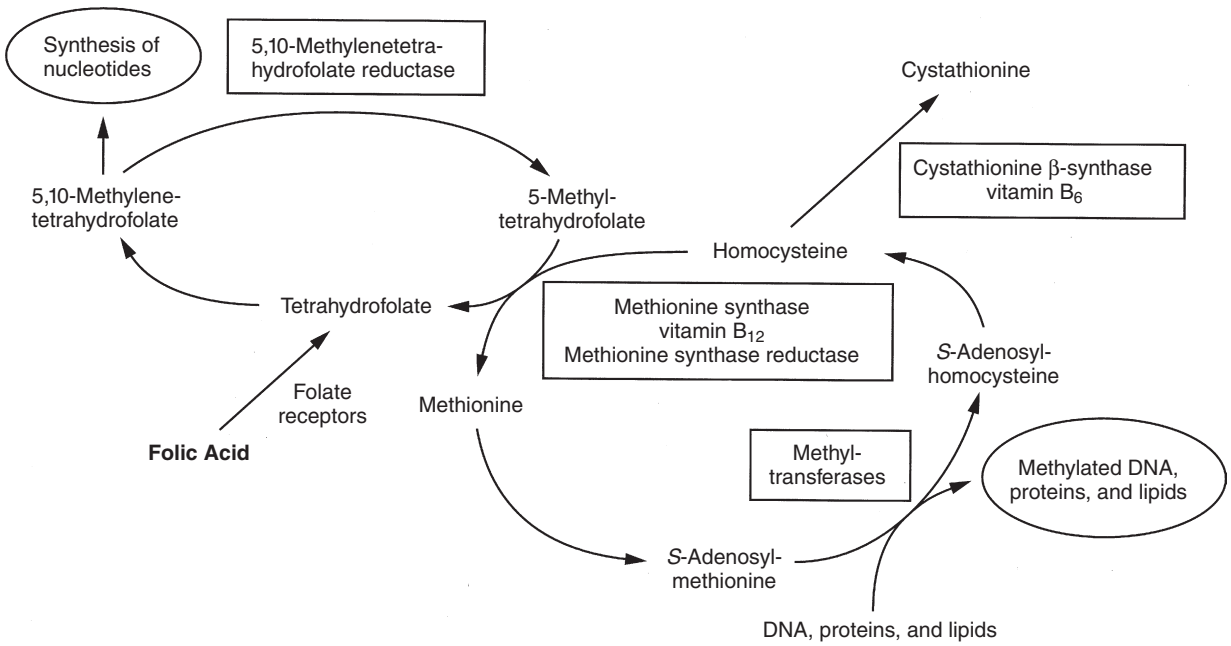


Figure 167-1 The metabolic roles of folic acid. After entering the cell, folic acid, possibly with the aid of folate receptors, is involved in the transfer of carbon atoms that are used to synthesize nucleotides or, through the conversion of homocysteine to methionine, for the methylation of various substrates. These processes are regulated by numerous molecules, including enzymes and vitamins other than folic acid (e.g., vitamins B_6 and B_{12}). The activity of some enzymes, such as methionine synthase, may be influenced by other enzymes, such as methionine synthase reductase. Enzymes and factors that may be important in neural tube development are indicated by *boxes*.

as 5-methyl-THF, of the methionine metabolism. Vitamin B_6 is involved in the first irreversible step in the transsulfuration pathway from methionine to cysteine. Disturbance in the activity of cystathionine β -synthase results in homocysteinemia. Therefore alterations of the vitamin B_{12}–dependent methionine synthase, folate-dependent methylenetetrahydrofolate-reductase and vitamin B_6–dependent cystathionine β -synthase may play a causative role in the development of neural tube defects. Treatment with vitamin B_6 normalizes the hyperhomocysteinemia and hypermethioninemia in approximately 50% of patients[127] and may result in a decreased risk for neural tube defects. These hypotheses are supported by experimental studies. For example, neural tube defects are prevented by methionine and folate supplementation in some rodent models,[43,128] or the effect of homocysteinemia in the pathogenesis of neural tube defects in chick embryo can be ameliorated by simultaneous folate supplementation.[88] However, the link between common mutations of the genes for methionine synthase, methylenetetrahydrofolate-reductase, and cystathionine β -synthase in humans could not be documented.

To summarize, despite the progress in the research on pathogenesis and approaches to primary prevention, the interpretation of these findings remains difficult. Prevention is not possible in all cases of neural tube defects, but supplementation of folate in a periconceptional dose of 0.4 to 4.0 mg/day can prevent the first occurrence and up to 85% of recurrent neural tube defect.[87,95,129,130]

SPECTRUM AND CLINICAL PRESENTATION

Although most studies of neural tube defects deal only with anencephaly and myelomeningocele, the clinical spectrum also includes craniorachischisis totalis, encephalocele, exencephaly, myeloschisis, and iniencephaly (Fig. 167-2). Malformations of the normal midline fusion are often referred to as being dysraphic and vary in clinical significance and outcome. Several different schemata are used to categorize neural tube defects; however, the nomenclature is confusing and even contradictory. Most of the lesions involve a failure of neural tube closure. The term *neural tube defect* is used to characterize this entire group of anomalies. Because the varying forms of neural tube defects probably arise by different mechanisms, it is important to appropriately describe and classify each defect. Based on the clinical significance and embryology, neural tube defects should be subdivided into cranial neural tube defects, open spinal neural tube defects, and closed spinal neural tube defects. Disorders of neural tube formation are reviewed next in order of decreasing severity.

Cranial Neural Tube Defects

Craniorachischisis Totalis

This malformation is characterized by a total failure of neurulation. This is the most severe form of neural tube defects. It occurs before 20 to 22 days of gestation.[131] A neural plate remnant is present throughout, and there is no overlying axial skeleton or dermal covering. Because most cases with craniorachischisis totalis are aborted spontaneously and only few have survived to term, the incidence remains unknown.

Anencephaly

Anencephaly (congenital absence of major portion of the brain, skull, and scalp)[132] is the single most common prenatally detected neural tube defect with an incidence ranging from 1 in 1000 live births and stillbirths in the United States to 1 in 100 live and stillbirths in some of the British Isles.[133] In one study, the prevalence of this neural tube defect was noted to change over time owing to the impact of prenatal sonographic examination and maternal serum screening. Limb and Holmes[134] reported an incidence of anencephaly that varied between 1.0 and 0.4 per 1000 live births and stillbirths during two different study periods (1972 to 1974 and 1979 to 1990). Female fetuses are more commonly affected than males, with a frequency of 3:1 to 4:1.[135] Furthermore, there is an increased incidence of anencephaly in Hispanics, who are 45% more likely than whites to have an affected pregnancy.[136] Anencephaly is associated with maternal hyperthermia as well as with maternal folate, copper, and zinc deficiencies.

In affected fetuses, there is a failure in the development of structures associated with the anterior neural tube closure. The defect is estimated to occur between 24 to 26 days gestation.[137] The anomaly involves variable amounts of the forebrain and upper brain stem. In some cases, the defect extends through the level of the foramen magnum (holoacrania or holoanencephaly are the more severe form,) or may be limited (merocrania or meroanencephaly are the milder forms). In addition to the absent cerebral hemispheres, fetuses with anencephaly demonstrate abnormal patterns in the hypothalamus and cerebellum. The frontal, parietal, and squamous parts of the occipital bones fail to develop; instead, the defect may be covered by a variable amount of angiomatous stroma.[9] The exposed neural tissue appears as a hemorrhagic, fibrotic, and degenerated mass of neurons and glia, with no functional cortex.[138]

Anencephaly is uniformly fatal. Most affected fetuses are spontaneously aborted or stillborn. Through reliable prenatal diagnosis, anencephaly is one of the few universally accepted indications for third-trimester termination.[139] The diagnosis of anencephaly can be confirmed on physical examination when the following criteria are met: (1) a large portion of the skull is absent; (2) the scalp is absent over the skull defect; (3) a hemorrhagic fibrotic mass of degenerated brain tissue is exposed to the environment; and (4) no recognizable cerebral hemispheres are present. Because orbits and eyes in anencephalic fetuses are well developed, this malformation imparts a remarkable froglike appearance to the patients. There is no functioning cerebral cortex, but brain stem function is present in varying degrees. Almost all fetuses born with anencephaly die within the first week, but rarely survival for several months has been reported.[132]

Exencephaly

Exencephaly is a rare fetal anomaly that is incompatible with extrauterine life. This malformation is characterized by partial or complete absence of the calvaria with abnormal development of brain tissue.[140] According to Papp and associates,[141] who screened 36,075 pregnancies for neural tube defects, the incidence of exencephaly is 3 per 10,000 pregnancies. Cox and associates[142] concluded that exencephaly probably represents an embryonic precursor to anencephaly. However, reopening or degeneration of a previously closed neural tube is also possible. Stagiannis and colleagues[143] considered the development of exencephaly related to a failure in mesenchymal migration. The exact pathogenesis remains uncertain.

The absence of the calvaria causes brain destruction by exposing the developing brain to amniotic fluid and repeated trauma. The exposed brain may be destroyed as early as 8 to 10 weeks gestation.[142] In pathologic studies, the exencephalic brain is observed to be covered by a highly vascular epithelial layer. In exencephaly, two relatively equivalent cerebral hemispheric remnants are present within a reddish mass of disorganized tissue, remnants of deep cerebral neural elements, blood vessels, fibrotic tissues, and fluid-filled spaces.[144] In addition, in exencephalic brain tissue, the gyri and sulci are shallow, flattened, and disorganized.

Experimental studies have demonstrated that hyperglycemia can induce exencephaly in mice,[145] and administration of clomiphene citrate before ovulation in female mice increases the incidence of exencephaly.[146] These effects in mice are similar to the inhibition of neural tube closure seen in newborns of diabetic mothers.

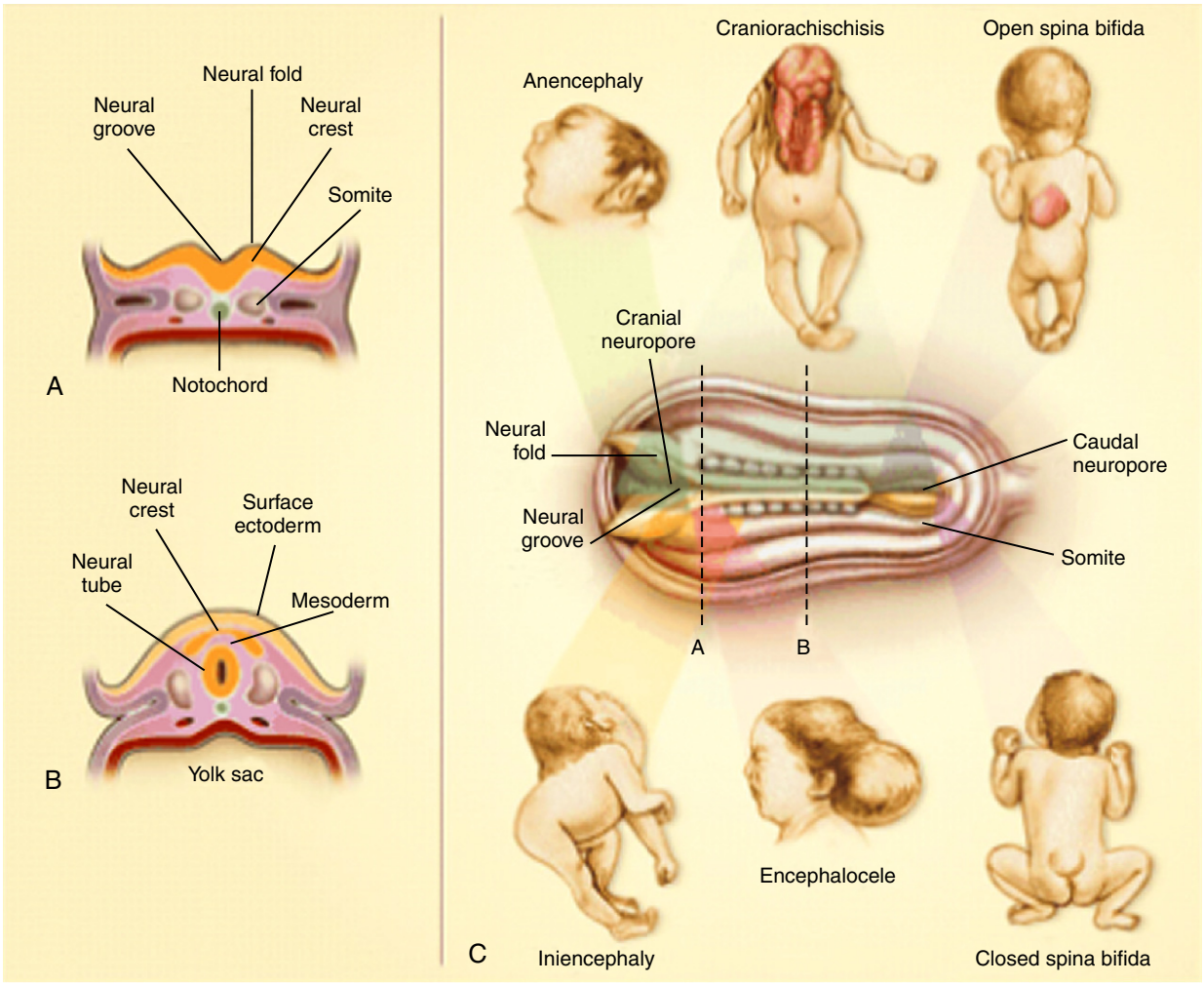


Figure 167-2 Features of neural tube development and neural tube defects. A, Cross section of the rostral end of the embryo about 3 weeks after conception, showing the neural groove in the process of closing, overlying the notochord. The neural folds are the rising margins of the neural tube, topped by the neural crest, and demarcate the neural groove centrally. **B,** Cross section of the middle portion of the embryo after the neural tube has closed. The neural tube, which will ultimately develop into the spinal cord, is now covered by surface ectoderm (later, the skin). The intervening mesoderm will form the bony spine. The notochord is regressing. **C,** Developmental and clinical features of the main types of neural tube defects. The diagram in the center is a dorsal view of a developing embryo, showing a neural tube that is closed in the center but still open at the cranial and caudal ends. The *dotted lines* marked *A* and *B* refer to the cross sections shown in **A** and **B.** *Shaded bars* point to the region of the neural tube relevant to each defect. In anencephaly, the absence of the brain and calvaria can be total or partial. Craniorachischisis is characterized by anencephaly accompanied by a contiguous bony defect of the spine and exposure of neural tissue. In open spina bifida, a bony defect of the posterior vertebral arches (in this case, the lower thoracic vertebrae) is accompanied by herniation of neural tissue and meninges and is not covered by skin. In iniencephaly, dysraphia in the occipital region is accompanied by severe retroflexion of the neck and trunk. In encephalocele, the brain and meninges herniate through a defect in the calvaria. In closed spina bifida, unlike open spina bifida, the bony defect of the posterior vertebral arches (in this case, the lumbar vertebrae), the herniated meninges, and neural tissue are covered by skin. *(From Botto LD, Moore CA, Khoury MJ: Neural-tube defects,* N Engl J Med *341:1509, 1999.)*

Encephalocele

This condition arises as a failure of fusion of restricted parts of the anterior neural tube closure between 26 and 28 days gestation and demonstrates a variable degree of cerebral herniation through a midline defect of the cranium. However, it is difficult to explain the development of encephalocele merely on embryologic grounds, and its precise pathogenesis remains unknown. Additionally, it has been suggested that the anomaly may occur secondary to herniation of brain through the cranium in the second trimester because of hydrocephalus and is not a primary defect of neural tube closure. In a U.S. study comprising a 17-year survey of births occurring in armed forces hospitals, Wiswell and colleagues,[147] recognized 112 cases of encephalocele in 763,364 live births. This yields an incidence 0.15 per 1000 live births. However, the incidence varies within ethnic groups and geographic regions.[148] In the Western world, 75% occur in the occipital region, and frontoethmoidal encephaloceles are more common in Malaysia, Thailand, Indonesia, Burma, and parts of Russia.[149-151] The neural tissue within the defect may be relatively normal, although hemorrhage, necrosis, and cortical dysplasia are present in some cases.[152]

Encephaloceles are frequently associated with microcephaly, maternal rubella, maternal diabetes, and genetic syndromes. In addition, encephaloceles are often found with other

malformations,[150] including agenesis of the corpus callosum, lissencephaly (in Walker-Walburg syndrome) and craniosynostosis (Apert syndrome), or even with renal cystic dysplasia and polydactyly (Meckel-Gruber syndrome). Encephalocele can be produced experimentally by irradiation, trypan blue, or hypervitaminosis.

The prognosis and long-term outcome are dependent on the associated brain malformations and syndromes. Large size is not important prognostically because large encephaloceles may not contain neural elements. Microcephaly and the presence of neural tissue in the encephalocele are associated with a poor prognosis.[153] The surgical challenges in affected newborns include closing the anatomic defect in the cranial vault and achieving as near normal a functional outcome as possible with minimal psychomotor defects.[154]

Myeloschisis

The essential defect in myeloschisis is failure of posterior tube closure. This anomaly arises not later than 26 days gestation. The exposed neural plate–like formation involves large portions of the spinal cord and appears as a flat, raw, velvety structure with no vertebrae or dermal covering. Most of these fetuses are stillborn and merge with the category of more restricted anomalies of neural tube development, such as myelomeningocele.

Iniencephaly

Iniencephaly is an abnormality in cervical vertebrae associated with cervicothoracic spinal retroflexion and neural tube defect. Lewis[155] classified iniencephalus into two subtypes: iniencephalus apertus, in which there is involvement of the brain with an encephalocele present; and iniencephalus clauses, in which no malformation of the occipital bone or brain is present. In iniencephaly, developmental disturbances of the neural tube and spine arise very early in embryogenesis, generally between day 26 and 30 after conception.

Iniencephaly is a rare condition with an estimated incidence between 1 in 1000 to less than 1 in 100,000.[156] Because most iniencephalic fetuses dies in utero, it is difficult to estimate the true incidence accurately. Experimentally, iniencephaly can be produced with administration of vinblastine, streptonigrin, or triparanol.[157] The pathogenesis of iniencephaly in humans, however, remains unknown. It has been noted that in geographic areas where there is an increased prevalence of anencephaly, there is also an increased prevalence of iniencephaly.[158] Different hypotheses for the pathogenesis of iniencephaly have been developed. It is possible that a primary defect in fetal cervical development resulting in extreme dorsal flexion of the head (lordosis) causes a failure of neural tube closure or even dilation and rupture of a previously closed neural tube.[157] Additionally, iniencephaly may be a primary defect in neural tube formation. Gardner[159] suggested that iniencephaly might be a severe form of the Klippel-Feil syndrome or may belong to the spectrum of malformations of the Dandy-Walker syndrome and the Arnold-Chiari malformation.

In the series of David and Nixon,[160] of 50 fetuses with iniencephaly, 84% had multiple associated malformations. The most frequent malformations were hydronephrosis, cardiovascular malformations, single umbilical artery, diaphragmatic hernia, facial clefts, omphalocele, and clubfoot.[160] Associated abnormalities of the CNS include anencephaly, encephalocele, hydrocephalus, hydromyelia, microcephaly agenesis of the cerebellar vermis, and presence of cerebellar cyst.[157,161] The abnormal fetal growth occurring at such an early stage in gestation might be the reason for the multiple associated malformations, owing to subsequent hindering and cramping of structures related to the retroflexed fetal neck.[160] Despite two case reports that discussed the same surviving iniencephalic patient,[156,158] iniencephaly is almost always lethal.

Open Spinal Neural Tube Defects

Myelomeningocele

Myelomeningocele, or open spina bifida, is a nonlethal neural tube defect with sequelae that affect both the central and peripheral nervous systems. The lesion is characterized by the protrusion of the spinal cord and meninges from the spinal canal with an abnormality in the overlying vertebral arches, muscle, and skin. The lesion is generally cystic and contains cerebrospinal fluid that drains when the thin sac is disrupted. It is a severe form of spina bifida affecting about 1 of every 2000 children born worldwide.[162,163] Not included in this figure are the 23% of pregnancies ending in elective termination of affected fetuses.[164,165]

More than 80% of children with a neural tube defect can be detected by maternal serum alpha fetoprotein screening before birth.[166] Although the determination of amniotic fluid acetylcholinesterase can be helpful, ultrasound is the method of choice in the diagnosis of neural tube defects. Direct visualization of the fetal spine can usually be accomplished by 16 weeks gestation. In the past two decades, the sonographic diagnosis of myelomeningocele has been enhanced by the introduction of high-resolution imaging tools and by recognition of specific brain abnormalities.[167] The CNS abnormalities associated with neural tube defects include cerebral ventriculomegaly, microcephaly, abnormalities of the frontal bone, and obliteration of the cisterna magna with anterior curving of the cerebellar hemisphere (banana sign) or an abnormal concavity of the cranium (lemon sign).[168] The abnormal findings of the CNS are thought to be a consequence of the hindbrain herniation (Arnold-Chiari II malformation). First described by Arnold and Chiari at the end of the 19th century,[169,170] the Arnold-Chiari II malformation is defined as the maldevelopment of a small posterior fossa and the herniation of the cerebellar vermis and brain stem (including the fourth ventricle) through an enlarged foramen magnum. Additionally, agenesis of the corpus callosum, enlargement of the massa intermediata, cortical heterotopia, and polymicrogyria can be seen. The etiology of the Arnold-Chiari II malformation remains in dispute. The predominant hypothesis maintains that an imbalance of hydrodynamic forces occurs secondary to loss of cerebrospinal fluid from the lesion.[76,77,171,172] An alternative theory interprets the hindbrain herniation as the consequence of a traction injury caused by cranial growth imbalance.[171,173-175] Overgrowth of the cerebellum and the brain stem, along with a posterior fossa that is smaller than normal, leads to downward dislodgment of these structures and results in Arnold-Chiari II malformation.[176] Despite these controversies about the etiology of myelomeningocele-associated hindbrain herniation, they are identifiable in the embryo from as early as 8 weeks gestation and are established in the fetus by the 12th week.

Pathologic studies of human embryos and fetuses with myelomeningocele in earlier stages of gestation reveal an open neural tube but undamaged neural tissue with almost normal cytoarchitecture.[177] This suggests that neural degeneration occurs at some point later in gestation (the two-hit hypothesis).[178] The first hit is the failure of neurulation early in gestation. The second hit is the spinal cord injury resulting from prolonged exposure of the neural tissue to the intrauterine environment. In theory, this secondary event can be prevented if an adequate prenatal covering of the exposed neural tissue can be provided. To have the best outcome, this repair must be fashioned before the onset of irreversible neural damage. There are several observations in human embryos, fetuses, and infants to support this premise.[178,179]

Osaka and co-workers[180] studied 18 embryos with classic caudal myelodysplasia and found an everted neural plate but preservation of the membranes. Interestingly, Chiari II malformation was not found in any of these embryos, whereas hindbrain herniation was present in two fetuses with caudal myelodysplasia in the same series. Additionally, pathologic signs of ventriculomegaly

were not noted in the embryos but were found in one fetus with Chiari II malformation.[180]

In pathologic examination of spinal cords of stillborn human fetuses with myelomeningocele (19 to 25 weeks gestation), varying degrees of neural tissue loss at the site of the lesion were observed, but the dorsal and ventral horns were normal proximal to the defect.[178] This group was among the first to suggest the two-hit hypothesis because they attributed these alterations to injuries occurring subsequent to the failure of primary neural tube formation. A study of 10 additional fetuses had similar findings.[179]

Marin-Padilla[181] examined a number of human dysraphic malformations and concluded that the primary anomaly was a disturbance of the axial chordomesodermal tissue to support adequate lodging and protection of the developing nervous system and that degeneration and destruction of the neural tissue occur secondarily. Additional support exists for the two-hit hypothesis from recent in vitro studies. Drewek and colleagues[182] reported that damage to open neural tissue appears to be progressive and results from exposure to toxic substances in the amniotic fluid during the third trimester.

Korenromp and colleagues[183] observed that fetuses with myelomeningocele exhibited leg movement at 16 to 17 weeks gestation. They suggested that these affected fetuses did have good function at that point in gestation. No follow-up could be reported in this series because pregnancies were interrupted. Furthermore, Sival and associates[184] compared the leg movements of 13 fetuses with myelomeningocele prenatally and postnatally. Only 1 of the 13 had abnormal leg movement before birth, but 11 demonstrated abnormal leg movements postnatally. Two possible explanations for this phenomenon exist. The prenatal leg movements could be secondary to spinal cord reflex rather than of cerebral origin, thus permitting motion without electrical impulses conducted through the damaged spinal cord tissue. In addition, leg movement early in pregnancy could be due to cerebral function conducted through an exposed spinal cord that is not yet damaged.

Most newborns with myelomeningocele show neurologic impairment of the lower extremities at birth, suggesting that the neurologic injury might occur later in gestation or even at the time of delivery. It is remarkable that patients with lipomeningomyelocele, in which the neural tissue is covered and protected by skin, often have almost normal lower leg and continence function, despite a neurulation abnormality that is nearly identical to that present in newborns with open neural tube defect. These studies support direct injury as the primary cause of damage and loss of function because neural tissue is injured exclusively from the protruding spinal cord.[178,179] As the pregnancy progresses, the volume of the amniotic fluid decreases, which may result in more frequent contact of the exposed neural tissue with the uterine wall. Chick models of oligohydramnios, in which pressure necrosis of prominent areas of the body developed, support this hypothesis.[185]

Closed Spinal Neural Tube Defects

Clinically, closed neural tube defects should be subdivided from open neural tube defects because the embryogenesis appears to differ in most cases. In open defects, there is an essential failure during the primary neurulation, whereas closed neural tube defects appear to result from another form of disturbance during neural tube formation.[186] With few exceptions, the structural malformations of closed neural tube defects are limited to the spinal cord and are not associated with the Arnold-Chiari II malformation or hydrocephalus. In contrast to open defects, newborns with a closed neural tube defect have no exposed neural tissue and do not leak cerebrospinal fluid. Additionally, the prognosis of an infant affected by a closed defect is significantly better than one with an open neural tube defect. Children born with closed defects generally have an intellectual function that is of the same distribution as the normal population, do not require cerebrospinal fluid–diverting shunts, and have considerably fewer problems with lower extremity sensory-motor function and bladder and bowel function.[186] Because the clinical manifestations of closed defects can be undetected for days or even years, the etiology of this group of neural tube defects is unidentified, and no link to genetic, environmental, or dietary factors has as yet been found. The major forms of closed neural tube defect are briefly described below.

Meningocele

This defect is commonly located in the lumbosacral region. Meningoceles are often covered with skin, and the bony abnormality rarely involves more than two to three vertebrae. The sac of the meningocele consists of both arachnoid and dural meninges and contains cerebrospinal fluid. Most meningoceles also contain neural elements. Meningoceles are an infrequent and heterogeneous group of cystic lesions. The accurate prevalence of meningocele is subject to debate because meningoceles are often grouped with myelomeningoceles. Furthermore, the pathogenesis remains unknown. The neurologic outcome of affected newborns is normal, but surgical correction and resection of the herniated meninges are indicated.[187]

Lipomatous Malformations

This abnormality includes all the closed neural tube defects with excessive lipomatous tissue present within or attached to the spinal cord or filum terminale. These lesions are called *lipomyelomeningocele, lipomyelocele, leptomyelolipoma, lumbosacral lipoma,* or *lipoma of the filum terminale*. The origin of lipomatous closed neural tube defects is controversial. Recently, two different theories have been advanced: (1) lipomas arise from cells originating from the somatic mesoderm and (2) lipomatous closed neural tube defects are a true malformation resulting from defective neurulation.[188] Most affected infants have a good prognosis with nearly normal leg and urologic function.

Dermal Sinus

A dermal sinus is a deep squamous epithelium–lined tract found on or near the midline. They are most common in the lumbosacral region (63%),[189] sometimes containing hair or occasionally communicating with the dura. Abnormal adhesions between the ectoderm, which form the neural tube and that which is destined to form the skin, are discussed as the primary cause of dermal sinuses.[187] Surgical removal of the lesions is indicated because of the risk for infection and compression of underlying neural elements by the progressive enlargement of the dermal sinus.

Diastematomyelia

The caudal cord in diastematomyelia (split cord malformation) is divided by a median septum arising from a vertebral body. Each of the hemicords has a single set of dorsal and ventral nerve roots, each contained within its own dural sheath. It has been suggested that the defect results from a failure during neurulation and that a second neuroenteric canal becomes advanced with mesenchyme to form an endomesenchymal tract that splits the notochord and neural plate.[190] A few studies have reported that up to 39% of split cord malformations are associated with myelomeningocele.[191,192] Because of the considerable rate of coexistence of these two neural tube defects, Pang and co-workers[190] proposed a common embryogenesis for split cord malformations and myelomeningocele.

THE BURDEN OF NEURAL TUBE DEFECTS

Anencephaly and myelomeningocele are important contributors to fetal and infant mortality. All newborns affected by anencephaly are stillborn or die shortly after birth, whereas children born

with a myelomeningocele usually survive. However, the risk for death with neural tube defects varies significantly worldwide, depending not only on the severity of the defect but also on such aspects as availability, use, and acceptance of medical and surgical intervention. For example, in some regions of northern China nearly 100%,[193] in the Netherlands 35%,[194] and in the United States 10%[195] of children affected by neural tube defects die.

For those infants affected by myelomeningocele, the significant lifelong disabilities accompanying this malformation include paraplegia, hydrocephalus,[196] pulmonary dysfunction,[197] sexual dysfunction, skeletal deformations and spinal deformities,[198] incontinence, and cognitive impairment,[199] with approximately 15% requiring some form of custodial care.[200,201]

Infants with myelomeningocele almost always have evidence of Chiari II malformation on magnetic resonance imaging.[202] This represents a caudal displacement of the inferior portion of cerebellar vermis, the medulla, and lower pons, and an elongated fourth ventricle through the foramen magnum. Despite aggressive postnatal intervention, nearly 14% of neonates with spina bifida do not survive past 5 years of age, with the mortality rate rising to 35% in those with symptoms of brain stem dysfunction secondary to the Chiari II malformation.[203] Clinical symptoms include swallowing difficulty, apnea, and stridor. Strabismus, a nonlethal Chiari II complication, is found in as many as 61% of patients with myelomeningocele.[204] Furthermore, the hindbrain deformity leads to ventriculomegaly in most cases because of impaired circulation of cerebrospinal fluid. Complications of ventriculomegaly may lead to the impaired cognitive development seen in children with myelomeningocele.[205]

Rintoul and associates[196] performed a retrospective review of 297 patients born with myelomeningocele and described the natural history of ventricular shunting in myelomeningocele patients in relation to radiologic and functional criteria. Most closures (92%) and shunt placements (62%) were performed in the first week of life. The timing of shunt placement and the number of shunt revisions were independent of the level of lesion. The overall incidence of shunting was 81%, which is consistent within the generally accepted range of 80% to 85% for an unselected spina bifida population.[206,207] The rate of ventricular shunting is significantly correlated to the anatomic level of the lesion.[196] All patients with a thoracic functional level required postnatal shunting, compared with 88% of lumbar level and 68% of sacral level patients.

The availability of new medical and surgical treatment modalities has significantly decreased the risk for some long-term medical complications, such as renal failure and severe dermatologic problems, affecting the risk for death among adults with myelomeningocele.[208] For example, Shurtleff and colleagues[209] and Hunt[210] documented that the survival rate to nearly the third decade of life was more than 50% in affected persons in whom the defect was surgically repaired shortly after birth.

The maldevelopment of the spinal cord is generally associated with some degree of weakness and lack of sensation in the lower limbs. Children younger than 8 years can often walk, sometimes without any assistance. However, as the children get older and heavier, their weight-power ratio increases, and walking becomes more difficult; later, many of the affected children need wheelchair assistance.

Another, potentially preventable complication of patients with spina bifida is a disease-associated latex sensitization.[211,212] Up to 43% of children with spina bifida are affected.[211] However, it remains unclear whether this association is a consequence of repeated exposure to latex-containing products (e.g., gloves or catheter material)[213] or is due to a potential genetic contribution and alteration of the human leukocyte antigen phenotype.[214]

In addition to the emotional impact on the family, the financial burden for family and community are enormous. In the United States, the costs of care for infants born with spina bifida in 1988 were almost $500 million per year (in 1992 dollars) or $294,000 for each infant.[215]

TREATMENT OPTIONS

Standard Treatment

Most fetuses with myelomeningocele that are not electively terminated receive no treatment until after birth. Several studies have investigated whether the method of delivery influences the neurologic outcome in infants with myelomeningocele. Although studies by Luthy[216] and Shurtleff[195] and their associates suggest that cesarean section before the onset of labor for fetuses with myelomeningocele may reduces injury to the exposed neural tissue by minimizing contact with the uterine wall and birth canal associated with labor and vaginal delivery, subsequent investigations showed that there is no conclusive evidence that cesarean section improves neurologic outcome in infants with myelomeningocele relative to vaginal delivery.[217,218] However, none of these studies compared vaginal delivery with elective cesarean section in a randomized, controlled fashion.

An initial neurologic evaluation of the newborn by the neonatologist, pediatric neurologist, and neurosurgeon is essential to assess the functional level, the neurologic deficit, and the potential complications (e.g., hydrocephalus, deformities of the lower legs, lower leg movement problems, and future continence problems) and should include a sensory examination to determine the level of the lesion and prognosis for motor ability. Surgical repair of the defect is usually performed within the first 24 to 48 hours of life to minimize the risk for infection.[219] The goal of postnatal myelomeningocele repair is to untether the placode and cover the lesion while preserving function. Because neurologic function typically exists only above the exposed spinal cord, sacrifice of some spinal cord tissue and nerve roots has been considered acceptable.[220]

Fetal Neurosurgical Intervention

Prior to 1997, fetal surgery was considered only for fetuses with life-threatening anomalies.[221,222] However, the severe morbidity and significant mortality associated with myelomeningocele (MMC), combined with the promising results of animal research, led to consideration of prenatal intervention for this disorder. The first case of open fetal MMC (fMMC) surgery was performed in 1994 using an endoscopic technique.[223] This approach at Vanderbilt University proved disastrous (two of four fetuses died) and was abandoned. Percutaneous fetoscopic patch coverage has been tried more recently in a small series of patients, and has also proved enormously problematic.[224,225] Investigators from the University of California, San Francisco[224] (UCSF) reported three patients that underwent fetoscopic MMC surgery. Fetoscopic coverage was successfully completed in one patient. However, the patch partially detached after fetal surgery and the newborn required standard repair and shunt placement postnatally. Due to technical difficulties, the MMC defect in the second fetus was never completely covered and the fetus was delivered prematurely at 31 weeks gestation. Postnatally, the newborn required neurosurgical repair of the lesion and ventriculoperitoneal shunt placement, and subsequently died of urosepsis at one month of age. The third fetus required conversion to an open approach secondary to an anterior placenta and difficulties to appropriately position the fetuses. In the case series by Kohl and associates,[225] endoscopic coverage of the MMC defect in three fetuses resulted in fetal patch detachment in one fetus, successful completion of the operation in the second, and fetal death in the third. Premature rupture of membranes is a substantial problem with the multiple port site fetoscopic approach.

The first successful (that is, improved postnatal neurologic function) fMMC surgery in an early gestation human fetus was performed by our group at the Children's Hospital of Philadelphia

(CHOP) in 1998.[226] A 23-week gestation fetus with T11-S1 lesion, hindbrain herniation, and normal lower extremity movements assessed by preoperative high-resolution ultrasonography, underwent open surgical coverage of the dysraphic defect. Surgery and recovery were uneventful, and the fetus was delivered by cesarean section at 30 weeks gestation after the onset of preterm labor. Postnatally, the infant had a right clubfoot deformity and neuromotor function at the L4 level on the right and L5 level on the left. Whereas hindbrain herniation was documented preoperatively, postnatal magnetic resonance imaging confirmed complete resolution of hindbrain herniation and absence of ventriculomegaly, and as a consequence, a ventriculoperitoneal shunt was not required.

Subsequent studies from CHOP and Vanderbilt University suggest that infants that were treated prenatally had improvement in hindbrain herniation as early as 3 weeks post fMMC surgery and appeared to have a diminished need for shunting relative to infants that underwent standard postnatal neurosurgical repair.[227,228] Compared with historical controls, it was estimated that fMMC surgery may reduce the need for ventriculoperitoneal shunt placement from 80%-90% to 40%.[196,229] The functional significance of the more normal position of the hindbrain, reduced shunt rate, and the restoration of near-normal cerebrospinal fluid (CSF) hydrodynamics[230,231] is that the vast majority of fMMC children demonstrated absent to mild brain stem dysfunction symptoms at 6 years follow-up.[232,233]

Recent evaluation of children that underwent fMMC surgery at CHOP not only showed that the initial improvement in lower extremity function at birth persisted into preschool age, but also revealed that 69% of fMMC children were independent walkers at a mean follow-up age of 66 months.[234] However, toddlers and preschool children often continue to demonstrate coordinative deficits and gait disturbances that are characteristic of children with MMC. Despite these promising findings, recently a few of the patients have developed clinical symptomatic spinal cord tethering in association with dermoid inclusion cysts at the fetal closure site and have required repeat surgery, usually with restoration of function.[235] A possible late decline in neuromotor function owing to tethering with or without a dermoid inclusion cyst underscores the importance of investigating better coverage materials and techniques for fMMC surgery as well as careful, continued, long-term neurologic surveillance of these children.

Regarding preschool neurocognitive outcome, 30 of the initial 54 fMMC survivors (56%) at CHOP underwent standardized preschool neurodevelopmental assessment.[236] At a mean follow-up age of 61 months, the majority of fMMC children had neurocognitive scores in the average to high-average range and are at an age-appropriate point in schooling. Although fMMC children who did not require ventriculoperitoneal shunt placement were more likely to have better scores, for the entire cohort there was a pattern of consistently higher scores in the verbal areas compared with scores for visual-motor or nonverbal learning skills, suggesting the possibility of later learning difficulties.[236] More recently we evaluated the impact of prenatal intervention on daily life functional status in children who underwent fMMC surgery at CHOP prior to the randomized trial.[237] Our results showed that although the majority of fMMC children achieve neurocognitive and neurofunctional independence, nearly one-third continue to require maximal assistance when carrying out self-care tasks (e.g. toileting, bladder and bowel management). These functional studies confirm previous findings by other centers that show only minimal impact of prenatal MMC surgery on continence function.[238,239]

Comparisons of infants with spina bifida who were treated *in utero* with previously reported postnatally repaired controls are subject to substantial bias. Additionally, the medical management of such infants might differ from that of historical controls for reasons unrelated to fetal surgery. For these reasons, the National Institutes of Health sponsored a multicenter, prospective, randomized clinical trial, comparing outcome after in utero and postnatal surgery for MMC. The Management of Myelomeningocele Study (MOMS) was initiated in 2003, and in December 2010 enrollment was stopped because the efficacy of prenatal surgery was demonstrated after recruitment and randomization of 183 of a planned sample size of 200 patients.[240] The study was performed by three fetal surgery units (CHOP, Vanderbilt, and UCSF), the Data Study and Coordinating Center at George Washington University, and the Eunice Kennedy Shriver National Institute of Child Health and Human Development. The objective of the trial was to determine if intrauterine surgery of MMC between 19 and 25 weeks gestation improves outcomes compared with standard postnatal neurosurgical repair. One primary outcome was a composite of fetal or neonatal death or the need for placement of a CSF shunt by the age of 12 months, and another primary outcome at 30 months of age was a composite of mental development and motor function. A variety of secondary neonatal and maternal outcome measures were also examined. Similar to the earlier, nonrandomized results of patients who underwent fMMC surgery, the MOMS trial showed a significant reduction of ventriculoperitoneal shunt placement at one year of age following fMMC surgery (prenatal group: 40% vs. postnatal group: 82%, $P < 0.001$).[240] The trial also demonstrated a substantial improvement in the overall neuromotor function at 30 months of age by a variety of measures including the finding that 42% of fMMC children were walking independently compared with only 21% in the postnatal group ($P < 0.01$). Finally, there was much less hindbrain herniation in the fMMC surgery group than in the postnatal surgery group (no hindbrain herniation in 36% and 4% of the infants, respectively, and severe herniation in 6% and 22%, respectively, $P < 0.001$).[240] The impact on mental capacity, bladder and bowel continence, and sexual function remains to be elicited as these infants advance in age and development.

Despite these promising results, the MOMS trial also revealed that fMMC surgery increases the risks for spontaneous rupture of membranes (prenatal surgery: 46% vs. postnatal surgery: 8%, $P < 0.001$), oligohydramnios (21% vs. 4%, $P = 0.001$), and preterm delivery (79% vs. 15%, $P < 0.001$) including 13% of fMMC children that were born before 30 weeks gestation.[240] The average gestational age at delivery in the prenatal surgery group was 34.1 weeks gestation compared with 37.3 weeks gestation in the postnatal surgery group. Also, at the time of delivery, approximately 25% of mothers in the fMMC surgery group showed evidence of thinning of the uterine wound, and 10% demonstrated variable degrees of dehiscence at the hysterotomy site, but none had a hysterotomy rupture.

Consequently, additional research is necessary to further elucidate the pathophysiology of MMC, to define the ideal timing and technique of fetal closure, and to evaluate the long-term implication of prenatal intervention.

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Xq28, cause X-linked aqueductal stenosis and agenesis of the corpus callosum, occuring about once in 20,000 births.[7,8] The flat dorsal aspect of the embryo develops lateral furls, and then invagination of the neural plate forms a fold. A tube appears along the length of the fetus by fusion of this fold, proceeding both forward and backward.The anterior neuropore closes at day 24, and the posterior neuropore closes at days 26 to 28.[9,10] The neural tube initially consists of a single layer of neuroectodermal cells. These cells proliferate and generate neuroepithelial progenitors for neurons and neuroglial cells. Radial glial cells, which appear early during neural tube formation, are stretched as the neural tube enlarges. Neurons originate at the inner lumen border of the neural tube and then migrate outward along the radial glial cells.Thus the deepest cortical region contains the ontogenically oldest neurons.[11,12]

Neural crest cells, derived from neuroectoderm of the dorsolateral neural tube, form the peripheral and enteric nervous systems.The neural crest cells migrate from the neural tube into the surrounding mesenchyme at about the time of neural tube closure.The cephalic neural crest gives rise to cells that contribute to the formation of the facial skeleton, branchial arches, and, in conjunction with placode-derived cells, the lower cranial nerves. Neural crest cells also migrate from the dorsolateral aspects of the spinal neural tube, there serving as progenitors for the peripheral and enteric nervous systems, the adrenal medulla, and melanocytes.[13-15]

Although the "proper study of mankind is man" (according to D.H. Lawrence), it is difficult to obtain human neural tissue in amounts adequate for complete study. Therefore experimental studies of neural development most often use rodent and other experimental animal models.[16] Mouse CNS at birth is at a developmental stage similar to 5 to 6 months gestation in human CNS. By 10 days post partum, mouse CNS has matured to a stage equivalent to that of human brain at term. Cerebral cortical neurogenesis is largely completed by birth in both human and mouse, although some new neurons continue to be generated from neural stem cells that persist in both mouse and human CNS throughout life, particularly in the hippocampus.[17-19] Differentiated oligodendroglial cells are already present in the human CNS at the time of birth, and CNS myelination in humans commences during the third trimester, whereas in rats and mice, differentiated oligodendroglial cells first appear around the time of birth, and myelination is an entirely postnatal process.[20]

NEUROTROPHIC PROTEINS

Various terms are applied to proteins present in extracellular fluids that modulate the behavior of cells, including *hormone, trophic protein, morphogen, growth factor, cytokine,* and *chemokine*. In general, hormones are secreted proteins and other molecules that circulate in the bloodstream and act on distant tissues, whereas trophic proteins act on neighboring cells (paracrine factors) or the synthetic cells themselves (autocrine factors).[21] Morphogens are proteins, concentration gradients of which provide epigenetic control of cellular specification and commitment during early development, and contribute to axonal guidance at a later stage in development.[22] Examples of neural morphogens are Shh, the Wnts, and the bone morphogenetic proteins (BMPs).[23] The term *trophic,* derived from *trophe,* or "sustenance," should properly be assigned to proteins with a nutritive role, but it is usually used to refer to proteins that influence cell survival and differentiation (e.g., nerve growth factor), rather than to proteins that truly provide nutrients (e.g., plasma lipoproteins). Proteins that enhance proliferation of target cells are referred to as *growth factors,* but many also exert morphogenetic and trophic as well as mitogenic effects. The term *cytokine* initially was applied to bioactive proteins released by lymph node– and bone marrow–derived cells, but it is now clear that other cell types, including those derived from neuroectoderm, can also synthesize these proteins. Chemokines, which are cytokines that exert chemotactic effects on cells, are involved both in recruitment of cells of the immune system from the bloodstream to the CNS and in migration of neural progenitors within the CNS.[24,25] To minimize this semantic complexity, the term *neurotrophic protein* is used in this chapter to refer to any protein present in extracellular fluid that exerts a paracrine or autocrine effect on the proliferation, differentiation, or survival of neurons or neuroglial cells.

In this section, rather than attempting a global description of the many neurotrophic protein classes, we briefly consider three morphogens that govern dorsoventral patterning during CNS development: Shh, the BMPs, and the Wnts. We then focus on three well-characterized neurotrophic protein families that regulate neural cell proliferation, differentiation, and survival: the fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), and neurotrophins.

A concentration gradient of Shh, a morphogen secreted by the notochord and floor plate, signaling through activation of Patched (Ptc) receptors and Gli transcription factors, stimulates production from ventral neural stem cells of motor neurons and oligodendroglial cells. In opposition to this ventralizing action of Shh, members of the BMP and Wnt families, produced by the roof plate and the ectoderm above it, inhibit generation of motor neurons and oligodendrocytes, while stimulating production of sensory neurons and astroglial cells.[26] Mutations in Shh signaling pathways give rise to holoprosencephaly, medulloblastoma, and the nevoid basal cell carcinoma syndrome.[27] Mutations in Wnt signaling pathways may prove to be of etiologic importance in anomalies of forebrain development related to autism and schizophrenia.[28] BMP signaling mutations have been linked to familial pulmonary arterial hypertension and limb malformations, and may participate in cerebral malformations.[29,30]

The FGFs, which exert their effects through activation of plasma membrane protein tyrosine kinase receptors, interact with the retinoids and Wnts during the early development of the posterior aspects of the CNS and later, are important in regulating corpus callosum formation.[31,32] FGF receptor mutations are a cause of craniosynostosis and achondroplasia,[33,34] and mutations in one member of the family of FGFs, FGF14, are a cause of spinocerebellar ataxia.[35] The PDGFs, like the FGFs, signal by activating plasma membrane protein tyrosine kinase receptors. PDGF exerts mitogenic effects of oligodendroglial progenitors, and enhances the specification of neural stem cells to both neuronal and neuroglial lineages.[36,37] Mutations involving PDGF or PDGF receptors are involved in the pathogenesis of various human tumors but have not been linked to human neurodevelopmental defects.

Much of what is known about the regulation of the early stages of neuronogenesis by neurotrophic proteins pertains to neural crest. The neural crest originates as a population of stem cells that arises between nonneural and neural ectoderm. At the neural tube closure stage of development, these stem cells generate migratory progenitors.[38-40] Neural crest stem cells give rise to progenitors restricted to neuronal, glial, and melanogenic lineages.[41-43] The neurotrophins—nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and the specific factors NT-3 and NT-4/5—play leading roles in governing the fates of neural crest neuronal progenitors.[44,45] They are synthesized as precursor proteins (proneurotrophins), which signal by activation of the p75 neurotrophin receptor (p75NTR). However, after they are proteolytically processed to the mature neurotrophins, their primary receptors are members of the Trk family of protein tyrosine kinases. Activation of p75NTR often leads to target cell apoptosis, whereas activation of the Trks enhances neuronal survival and regulates neuronal transmitter phenotype.[46-49] Pioneering in vivo experiments by Hamburger and Levi-Montalcini

demonstrated that excess NGF during development causes sympathetic neuronal hyperplasia, whereas NGF depletion results in partial sympathectomy.[50] Inactivating mutations of the TrkA NGF receptor cause congenital insensitivity to pain with anhidrosis (hereditary sensory and autonomic neuropathy type IV),[51] whereas an inactivating mutation of the TrkB BDNF receptor results in impaired nociception, developmental delay, and hyperphagic obesity.[52]

VITAMINS

Vitamin A and Other Retinoids

Three forms of vitamin A are biologically active: *Retinol* (vitamin A_1 alcohol) maintains mucous membrane structure and function and is the circulating form of the vitamin; *retinal* (vitamin A_1 aldehyde) plays a role in retinal development through conversion to a derivative that incorporates into rhodopsin; and *retinoic acid* (RA) contributes to the regulation of pattern formation in the early embryo.[53-55] At least five natural biologically active forms of RA are known: all *trans*-RA; 9-*cis*-RA; 3,4-didehydro-RA; 14-hydroxy-4,14-retroretinol; and 4-oxo-RA. All can act as dysmorphogens.[56]

Observations in experimental animals and humans that correlated retinoid exposure with CNS malformations led to recognition of the retinoid receptors and homeodomain-encoded transcription factors as effectors for retinoid actions. Megadoses of retinol and therapeutic oral doses of its analogue, isotretinoin (13-*cis*-RA), elicit teratogenic effects in the human fetus, causing both microcephaly and hydrocephalus.[53,57,58] Either vitamin A deficiency or excess can increase intracranial pressure in human infants.[59,60] The teratogenic potential of retinoids is powerful: Malformations resembling RA embryopathy have occurred up to a year after discontinuance of the oral use of the RA analogue etretinate, a form stored in fat and slowly released into the circulation.[61]

The retinoids play important roles during normal neural development at the earliest stages of neurogenesis, at the time of neural plate formation, and, later, when the form of the mature nervous system is established. The neural plate originates from cells in and around Hensen node and the notochord in vertebrate embryos. These three tissues are *zones of polarizing activity*—restricted regions containing signals that determine the anteroposterior axis of parts of the embryo.[62] The neural plate binds retinol and is the site for its most efficient conversion to RA.[62] The region most sensitive to RA is the posterior midbrain–anterior hindbrain area of the neural plate.[54] Later in development, retinoids are found in the developing inner ear[63] and the developing neural retina.[64] In retinal tissue, retinoids play a role in cell fate determination by promoting rod photoreceptor development from progenitor cells.[65] Experiments dramatically demonstrating the effects of retinoids on CNS development were performed in the *Xenopus* embryo, in which RA or 4-oxo-RA treatment at the late blastula to early neurula stage produces microcephaly and absence of eyes, forebrain, and midbrain.[56,66,67] After maternal dietary manipulation to expose rats to RA at embryonic day 10 (E10), isolated myelomeningocoele, with the full range of severity, developed in 60% of the fetuses.[68]

Much of the impetus to pursue RA as an important signaling molecule in the CNS came from observation of the startling and diverse patterns of defects in both deficiency and excess of retinoids. RA exposure, for instance, inhibits migration of cranial neural crest cells in the cultured mouse embryo.[69] Conversely, mice mutants deficient in the RA-synthetic enzyme retinaldehyde dehydrogenase 2 demonstrate failure to develop neural crest derivatives including enteric ganglia elements of pharyngeal pouch outgrowth and also the heart.[70]

RA regulates CNS segmentation by inducing the expression of members of a superfamily of genes that contain a conserved homeodomain, the *HOX* and *HOX*-related genes. These genes occur in four separate clusters, *Hoxa* to *Hoxd*, that encode transcription factors—proteins regulating embryonic segmentation and organogenesis.[71] Their orderly patterns of spatial interaction are regulated by local concentrations of the retinoids.[55,72-74] For example, *Hoxb2*, one of the members of this superfamily, codes for a transcription factor that is expressed in the mouse CNS up to the rhombomere 2–rhombomere 3 border. Studies show that *HOX* genes and RA regulate neural cell adhesion molecule, a key adhesion molecule that modulates cell movement. RA is thought to act in part through increased expression of the target gene, *Hoxa1*. RA has a significant role in cortical development as shown in the postnatal mouse brain (correlating with the second trimester human brain) as RA synthetic enzyme maps to basal ganglia, hippocampus, auditory, visual, and olfactory cortex.[75]

Folic Acid

Neural tube closure defects in the human have been firmly linked to folic acid deficiency. The many studies of folate and the brain in animals were prompted by a sentinel study in 1965 by Hibbard and Smithells[76] that showed folate deficiency in 69% of mothers of children with CNS abnormalities, compared with 17% of control women. Other observations suggesting an important role for folic acid in the prevention of NTDs included the increased occurrence of anencephaly and meningomyelocele in offspring of women of lower socioeconomic status with less than adequate nutrition and the increased incidence of defects associated with the use of folic acid inhibitors in pregnant women.

Folate plays an essential role in methyl group transfer reactions. Methylation is cyclic, involving the interchange of methyl groups between homocysteine and methionine, mediated by the enzyme methionine synthase (5-methyltetrahydrofolate-homocysteine *S*-methyltransferase, designated Enzyme Commission [EC] number 2.1.1.13).[77] Methionine, when activated by adenosine triphosphate, becomes *S*-adenosylmethionine. *S*-adenosylmethionine is a central source for methyl group transfer to proteins, lipids, and nucleic acids. One of the products of methyl transfer is *S*-adenosylhomocysteine, which is then hydrolyzed to homocysteine. In most cells, homocysteine is converted by vitamin B_{12}–dependent methionine synthase back to methionine by remethylation.[77] The latter, which is the only reaction in the human that requires both folic acid and vitamin B_{12} as cofactors, is responsible for the dependency of cellular folate levels on adequate availability of vitamin B_{12}.[77]

Virtually all cells in all organisms contain folate coenzymes, mediating methyl transfer. A small amount of folate is in nuclei, but cellular folate-binding proteins are associated both with surface membranes and, intracellularly, within the cytosol and mitochondria. Most of these intracellular folate-binding proteins are enzymes that use folate as a coenzyme in methyl transfer. The placenta and choroid plexus are sites that exhibit folate receptors.[78,79]

Holmes, more than a decade ago, found the prevalence of NTDs in a New England cohort to be 1.2 per 1000[80]; among the 146 cases in his 15-year survey, 54 infants had spina bifida, 74 had anencephaly, and 19 had encephalocele. Anencephaly, more common in girls than in boys, occurs before 24 days of gestation in the human, and approximately 75% of affected fetuses die in utero. Ethnic differences in the frequency of NTDs also have been reported. For instance, in British Columbia, the incidence of NTDs in the Sikh population was approximately double that in non-Sikhs.[81] Moreover, a recurrence risk of 2.3% between 1958 and 1984 contrasted with 0.24% recurrence risk between 1985 and 1993. This decrease probably was the result of the recommendation of folic acid and multivitamins before conception for

at-risk families in British Columbia.[81] More recently, birth incidence in this province has sharply declined (from 1.16 in 1000 to 0.47 in 1000) thought to be largely secondary to prenatal diagnosis and termination of affected pregnancies.[82]

Both RA and folic acid have been studied in several mouse mutants manifesting NTDs. The effect of RA is not uniform: Although it increases the incidence of exencephaly (precursor of anencephaly) in four different mutant mouse strains, it also decreases the incidence of caudal spina bifida and tail defects in several of the same strains. Other studies have shown that simple maternal folate deficiency in non–genetically predisposed rodents does not produce NTDs,[82,83] and folate treatment does not prevent NTDs in the genetically predisposed *Axd* mouse, whereas methionine does.[84]

Administration of folate, however, does reduce the incidence of valproic acid (VPA)-induced NTDs in the mouse.[85] Arachidonic acid and inositol, which contribute to anchoring of folate-binding protein to the plasma membrane, reduce the incidence of mouse NTDs associated with exposure to phenytoin or hyperglycemia.[86,87] VPA is a potent teratogen in the human, with an estimated 20-fold increase in the frequency of NTDs in offspring. Epileptic mothers with fetal malformations exhibit low folate levels.[88]

VPA's action probably alters the expression of multiple genes, and recently in a rat embryo model,[89] it has been postulated that the mechanism of action of VPA may be through elevated and uncoordinated Hoxa1 expression, the same molecular pathway involved in RA action. Alteration of Wnt-dependent gene expression (see earlier under "Neurotrophic Proteins") and block of histone deacetylase are other postulated mechanisms of action for VPA.

The fetal incidence of NTDs is estimated to be 2.5% in 26-day human embryos just completing neurulation, decreasing to 0.06% at term, as a result of fetal loss.[90] In 1991, the effect of early folic acid supplementation to prevent recurrent NTDs was demonstrated in a multicenter, randomized, double-blind trial. Administration of 4 mg of folic acid daily before conception and during early gestation resulted in a six-fold diminution in risk (0.6% versus 3.6%) of recurrent NTDs.[91] First occurrences of NTDs also are prevented with folic acid supplements.[92]

Since the early 1990s, folic acid has been recommended at 0.4 mg daily before conception and until 3 months gestation for the general population and 4 mg daily for women who have already had children with NTDs.[93]

In 1998, when an estimated 300,000 infants with spina bifida or anencephaly were born yearly throughout the world, a next step in prevention was begun in the United States with mandatory folic acid fortification of cereal grain products. This was accomplished by the minimum addition of 0.14 mg of folic acid to 100 g of cereal grain, calculated to increase intake by an average of 0.1 mg/day. Subsequently, the prevalence of NTDs in the United States dropped by 36%, from 10.8 per 10,000 population in 1995-1996 to 6.9 per 10,000 in late 2006. Similar results followed cereal fortification in several other countries.[94] This approach was shown to be effective in a Southern California study, which demonstrated that levels of serum and red cell folate greatly exceeded minimal acceptable levels in both socioeconomically disadvantaged and advantaged women.[95] In a survey of multiple states, the NTD prevalence before and after fortification was shown to decrease by 19%, to 30.5 per 100,000 live births[96]; however, no drop occurred among patients with either no or only late prenatal care, confirming the major role of early specific folate prophylaxis.

Most but not all cases of spina bifida and anencephaly are preventable with folate supplementation.[97] To investigate such "resistance" to folate, a study of the response to folate loading in women who had had two pregnancies with NTDs was undertaken.[98] The women in the group with two documented NTD pregnancies and the control subjects with no affected pregnancies attained similar serum and red cell folate levels, but no correlation between levels and intake existed in the women with two affected pregnancies, implying altered folate metabolism in the women whose folate levels were unpredictable. In other studies, homocysteine levels also were reported to be elevated in women who had had children with NTDs, although classic homocystinuria is not associated with such anomalies. Mothers of children with NTDs also were shown to have impaired metabolism of homocysteine when their plasma vitamin B_{12} levels were in the low-normal range.[99-101] Thus a defect was postulated in the enzyme requiring both vitamin B_{12} and folic acid as cofactors, methionine synthase. Further implication of this enzyme derives from the delay in incorporation of 5-methyltetrahydrofolate into DNA (another methionine synthase–catalyzed reaction) in trophoblast cells of women who gave birth to NTD-affected infants.[101-103]

Abnormality of another enzyme, 5,10-methylenetetrahydrofolate reductase (MTHFR), estimated to be present in 5% to 15% of the general population, is associated with defective transfer of a methyl group to homocysteine to form methionine. Finally, two mutations in the *MTHFR* gene, C677T and A1298C, which lead to increased homocysteine levels, also significantly increase risks for NTD in children. Homocysteine levels in women with these *MTHFR* variants can be normalized with large doses of folate.[104]

FATTY ACIDS AND CHOLESTEROL

The brain is rich in lipid. Research in animal models shows that brain lipid can be changed by alterations in intake of essential fatty acids.[105] The essential fatty acids of the diet are linoleic (C18:2*n*-6) and linolenic (C18:3*n*-3) acids. Premature and growth-restricted infants are born with essential fatty acid deficiency, and studies in experimental animals indicate that such deficiency can be associated with cognitive and visual impairments. Studies of essential fatty acid supplementation in humans have included analyses of the effects of *n*-3 fatty acid supplementation or breast milk feedings on retinal[105] and mental[106] function, the latter as reflected by intelligence quotient (IQ), in premature infants. Maternal supplementation with these same acids during pregnancy and lactation may have a beneficial effect on the mental development of breast-fed healthy term infants,[107] but significant effects on development from supplementation of formula thus far seem unlikely.[108]

Inborn errors of cholesterol metabolism have pointed to many links to embryonic development. Smith-Lemli-Opitz syndrome, the prototypic and first-described disorder of cholesterol biosynthesis, is caused by any of more than 100 inherited mutations of the 3β-hydroxysterol-δ7 reductase (DHCR7) gene, resulting in deficient enzyme and hypocholesterolemia in tandem with high 7- and 8-dehydrocholesterol levels. Clinical manifestations consist of dysmorphic facies, mental retardation, hypotonia, and various anomalies of the heart, lungs, intestine, kidneys, genitalia, and limbs; cardinal signs are hypospadias, microcephaly with agenesis of the corpus callosum and holoprosencephaly, small or proximally placed thumb, and second-third toe syndactyly.[109] The effectors in this and other sterol-related brain developmental disorders are postulated to lie within the Shh signaling cascade or its receptors, because this protein requires covalently bound cholesterol for its function.[110] Ptc and Smoothened (Smo) are receptors that mediate the early developmental effects of Shh on morphogenesis and commissural axon growth in the ventral and lateral telencephalon.

Most aspects of synaptogenesis and maintenance of synapses require cholesterol as well.[111] Roux had demonstrated, with the use of teratogens, that varying the maternal supply of cholesterol to the developing rat fetus alters the frequency and severity of Shh-related malformations.[112] More recently, Kelley found

a significantly lower mean cholesterol level in mothers (but not fathers) of children with isolated holoprosencephaly, and other workers have found a correlation of maternal apoE genotype and the severity of Smith-Lemli-Opitz syndrome.[113] Apolipoprotein E plays an important role in regulation of intracellular cholesterol trafficking, especially in transport of CNS lipids. Reliable early identification of the affected fetus is possible, and prenatal maternal dietary treatment has been tried only once, without success.[114] Postnatal dietary cholesterol treatment, although not been remedial for all aspects of the disorder, is recommended; therapy with hydroxymethylglutarate coenzyme A (HMG CoA) reductase inhibitor therapy may be detrimental.[115]

HORMONES

Although the effects of hormonal deficiency or excess on neural function in the adult may be profound, they are in general reversible. By contrast, perturbations in availability of hormones during the critical periods of neuronal and glial cell development often result in permanent and profound abnormalities in synaptogenesis and myelination. This section focuses on thyroid and steroid hormones, with emphasis on the special vulnerability of the immature nervous system to deficiencies of these hormones.

Thyroid Hormones

Thyroid disorders are the most prevalent endocrine derangements in the world, even more common than diabetes mellitus. In estimates from 1990, endemic hypothyroidism primarily resulting from low dietary iodine intake affected approximately 750 million people in the world, largely in Asia and Africa, and approximately 43 million were affected by brain damage secondary to low iodine intake either prenatally, in infancy, or in early childhood.[116] Recently, it was estimated that as many as 1.9 billion people worldwide have inadequate iodine nutrition.[117]

In a major inland region of Guinea, in West Africa's goiter belt in the 1990s, 70% of the adults had goiters. Environmental variables responsible for this high incidence included the low iodine content of the water and soil, excessive intake of goitrogens such as thiocyanate (in cassava) and flavonoids (in millet), and protein malnutrition. Many infants had goiter at birth, and 55% of preschoolers were affected. At puberty, 58% of boys and 80% of girls had goiter.[118] Although the incidence of myxedematous cretinism, manifested by retardation and deafness, was only 2% in this population, this figure is likely to have been spuriously lowered due to early deaths of severely neurologically impaired children in this environment. Introduction of iodized salt has decreased the incidence of hypothyroidism and gradually decreased the incidence of goiter in western Africa in the last decade.[119]

Debate is ongoing over optimal iodine intake, with the World Health Organization (WHO) recommending intakes of 200 μg daily in pregnancy and lactation, 150 μg daily in other adults, 120 μg daily in 6- to 12-year-olds, and 90 μg daily in children younger than 5 years of age. These intakes should lead to iodine sufficiency as manifested by urinary iodine excretion of approximately 100 to 200 μg/L.[115] Studies of iodine supplementation in China, however, have suggested that more than adequate or excessive levels of iodine intake may actually increase the incidence of hypothyroidism, probably secondary to autoimmune thyroiditis. These studies imply that the addition of iodine must be carefully adjusted for the baseline iodine intake of the population to be supplemented.[120]

The neurologic consequences of early hypothyroidism vary with the degree of maternal, fetal, and neonatal hypothyroidism. Maternal thyroxine (T_4) is not available to the fetus for brain protection when the mother is iodine-deficient, and this lack accounts for the severe CNS damage associated with endemic goiter, This contrasts with minimal to no neurologic deficit in the hypothyroidism that may be present in a child born to a euthyroid mother.[121]

Sporadic hypothyroidism occurs in approximately 1 in 4000 live births. Screening programs for this abnormality are widespread among developed countries. Knowledge gained from these programs has shown that the fetus usually is unaffected by intrinsic in utero hypothyroidism, even when athyrotic. Preservation of brain thyroid status in the hypothyroid rat and human fetus is effected by maternal T_4 that crosses the placenta.[122]

Experimentally elicited neonatal hypothyroidism in rodents causes deficiencies in CNS neuronogenesis, synchronization of axonal and dendritic outgrowth, synaptogenesis, and myelination.[123] Thyroid deficiency also can interfere with the responsiveness of neural tissues to neurotransmitter-mediated and neurotrophic signals. For example, adequate supplies of triiodothyronine or T_4 are necessary for normal expression of NT-3 and its receptor, TrkC, by immature rat cerebellar granule cells.[124]

Thyroid hormone deficiency also alters the metabolism of the thyroid hormones themselves. The conversion of T_4 to triiodothyronine in the brain of hypothyroid rat fetuses is accelerated, owing to the induction of astroglial type II deiodinase, probably secondary to an elevation in thyrotrophin levels in the fetuses.[125] This homeostatic mechanism helps to preserve brain function in the presence of hypothyroidism in the fetus.

Important interactions between thyroid hormones and retinoids are recognized.[126-128] Thyroid hormone receptor-α_1 (TR-α_1) inhibits RA-responsive gene expression but also cooperates with retinoids in supporting the formation of embryonic mouse cerebral neurons. Moreover, retinoids cause a 200-fold, specific induction of type III deiodinase (the enzyme responsible for degradation of brain T_4) in astroglial cells in culture.

The effects of thyroid hormones on neural and other tissues are transduced by nuclear thyroid hormone receptors TR-α and TR-β, encoded by genes on chromosomes 17 and 3, respectively.[129-131] These nuclear receptors bind both to thyroid hormone and to thyroid response elements, which are specific DNA sequences in noncoding regions of thyroid hormone--responsive genes, often encoding neurotransmitters and cytoskeletal proteins. Thyroid-responsive genes are numerous and are identified frequently.

TR-α and TR-β are homologous to nuclear receptors for steroids, RA, and vitamin D_3 and are detected as early as 10 weeks in the human fetal brain.[132] They are expressed in a spatially and temporally regulated pattern in the CNS. This pattern is of some clinical significance. For example, the anlage of the cochlea is particularly rich in these nuclear receptors. The role of these receptors at a critical period for auditory end-organ development is demonstrated by frequent abnormalities in auditory brain stem responses in hypothyroid children (which tend to persist despite early replacement therapy),[133-135] and it also is shown by the syndrome of isolated deafness in humans with a chromosomal deletion syndrome involving most of the thyroid receptor-β gene.[136] The nuclear thyroid receptors also are subject to mutations that result in inherited thyroid hormone resistance.[137]

Environmental exposure of pregnant animals to dioxins and polychlorinated biphenyls (PCBs) leads to behavioral changes similar to those associated with fetal hypothyroidism.[138] These substances are industrial pollutants that have persisted despite their ban in the 1970s. This continued presence in the environment is especially concerning because of their concentration in fatty tissues and human milk.[139] Several mechanisms for the hypothyroid effects of these toxins have been postulated: reduction of thyroid hormone at low levels of PCBs and thyroid receptor–mediated thyromimetic effects at high levels of both classes of toxins.[140] Structural similarity between these toxins and thyroid hormones permits them to bind to the same transporters and nuclear receptors, thereby interfering with the effects of thyroid hormone on the fetal brain.[141]

Steroid Hormones

Glucocorticoids, estrogens, progesterone, androgens, and vitamin D metabolites influence CNS development. A common feature of all these steroids is that their actions are transduced by cytosolic receptors that, on binding of their ligand steroid, are released from complexes with other cytosolic proteins and move to the nucleus, where they modulate transcription of target genes.[142,143] The biologic effects of the steroid hormones on neural tissues are dictated by the distributions of expression and patterns of gene activation of their receptors. These biologic effects include regulation of the secretion of neurotrophins by neuroglial cells and of the incidence of apoptosis of nascent neurons.[144-146]

Type I adrenal corticosteroid receptors bind both mineralocorticoids and glucocorticoids with high affinity, and they are concentrated largely in the hippocampal system. Type II corticosteroid receptors, which bind glucocorticoids with high affinity, are distributed throughout the brain, but they are most abundant in the anterior pituitary and the preoptic region of the hypothalamus.[143-147] In the developing nervous system, glucocorticoids regulate the frequency with which nascent neurons undergo apoptosis.[148] Excess glucocorticoid has complex effects on the immature brain, by stimulating cholinergic neurotransmission through a neurotrophin-dependent mechanism,[149,150] but also by eliciting neuronal loss in the hippocampus, which is associated with lifelong impairment of memory.[151]

In the relatively recent past, antenatal glucocorticoid was injected weekly for threatened preterm labor to induce fetal lung maturation. Often this regimen was continued for several weeks until birth. Subsequent magnetic resonance images of the brains in these children revealed simpler gyral patterns and reduced surface area when compared with those in brains of control children.[152]

Receptors for estradiol, progesterone, and androgens are widespread in the developing CNS. Their activation, by maternal steroid hormones and by progesterone, estrogens, and androgens synthesized endogenously in the CNS, influences the motility of neuroblasts and the development of neurons in sexually dimorphic motor nuclei of the hypothalamus, brain stem, and spinal cord. These receptors also have effects on synthesis of neurotransmitters.[153-156] Endogenous synthesis of progesterone in the neonatal rat CNS promotes the maturation of cerebellar Purkinje cells.[157]

Activation of CNS neuroglial cell receptors for the vitamin D metabolite, 1,25-dihydroxyvitamin D_3, stimulates neuroglial neurotrophin synthesis.[158] 1,25-Dihydroxyvitamin D_3 also acts on hippocampal neurons and, by down-regulating their expression of L-type calcium channels, protects them from excitotoxic injury.[159]

CONCLUSIONS AND FUTURE PROSPECTS

The 1970s and 1980s were marked by substantial advances in knowledge of environmental influences on neural development. These advances, generated largely by epidemiologic studies, included recognition of the ill effects of various toxins, such as methylmercury and lead,[160] and the validation of iodized salt– and folate-supplemented flour as prophylaxis against gestational hypothyroidism and neural tube defects.[161,162] The decreases in incidence of neonatal hypothyroidism and spina bifida that followed introduction of these dietary manipulations helped investigators to focus during the 1990s on kindreds in which mutations, rather than environmental influences, were responsible for abnormalities in neural development.[163,164] Research today is entering yet a third phase of analysis of the interplay between environment and genome, using array-based technologies and comprehensive atlases of neural gene expression to elucidate how the interplay between environmental factors and the neural transcriptome dictates development and function of the nervous system.[165,166]

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Anne M. Ades and Margaret Myers

Developmental Aspects of Pain

Within the past several decades the field of neonatology has made vast strides in the reduction of infant morbidity and mortality. This progress, however, comes with increasing exposure to painful and stressful stimuli.[1,2] A study of neonates in a neonatal intensive care unit revealed that patients underwent a mean of 14 invasive procedures per day with a range of 0 to 53 procedures per day.[3] Yet the assessment and treatment of pain has been included only relatively recently in the management of these critically ill neonates.

Until the 1980s, newborns frequently underwent invasive procedures, including surgery, with no analgesic or anesthetic. There were several reasons for this. It was widely believed that the immature neonatal brain could not respond in a manner that perceived or localized painful stimuli.[4,5] In addition, clinicians frequently assumed that neonates neither experienced pain as severely as adults nor remembered painful experiences.[6] Concerns about the potential adverse side effects of analgesic and anesthetic agents in the neonate overwhelmed the perceived benefits.[7] It was not until the literature revealed the relative safety of anesthetic agents and the improved outcomes of patients undergoing surgery with anesthesia that the field of neonatal pain research, both clinical and basic science, evolved.[8,9-12]

This chapter reviews the development of pain sensation in the fetus and neonate as well as the clinical markers of pain and the impact of pain in this vulnerable patient population.

DEFINITION OF PAIN

The International Subcommittee for the Study of Pain defined pain in 1979 as "an unpleasant sensory and emotional response associated with actual or potential tissue damage, or described in terms of such damage."[13] Schechter[4] proposed that pain perception is dependent on two components. The first, nociception, is a sensory component that is neurophysiologically determined. The second is an emotional component based, among many factors, on affective state, past experience, and development. Understanding of both of these components in the infant continues to evolve with ongoing research.

DEVELOPMENT OF NOCICEPTION

Pain may be described as a process in which stimulation of peripheral sensory nociceptors produces responses conducted via the spinal cord neurons to sensory areas of the cerebral cortex. The basic mechanisms of pain perception in the fetus and neonate are similar to those of adults though differences exist as a result of neuroanatomic, neurophysiologic, and neurochemical developmental immaturity.

Neuroanatomy, Neurophysiology, and Neurochemistry

Cutaneous sensory receptors first appear in the perioral area of the human fetus in the seventh week of gestation and spread throughout the fetus by the twentieth week of gestation, as outlined in Table 169-1.[14] Evidence suggests that the density of cutaneous nociceptor receptors in the late fetus and neonate is comparable or may even exceed that of adult receptor density.[15] Nociceptors may be divided into two broad types: high-threshold mechanoreceptors that respond to pressure and polymodal nociceptors that respond to heat or chemical irritation as well as to pressure. The mechanoreceptor axons, A-delta fibers, are large and myelinated and conduct rapidly, providing the rapid onset of sharp, localized pain following injury. The polymodal receptor axons, C fibers, are small and unmyelinated, conducting relatively slowly and yielding slow, prolonged, poorly localized pain.

Cutaneous nociceptor afferents have cell bodies in the dorsal root ganglia of the spinal cord, and there is evidence that sensory fiber–to–dorsal-horn interneuron synapse development occurs as early as the sixth week of gestation.[16] Development and differentiation of dorsal horn cells begin at around 13 weeks gestation, and the laminar arrangement, as well as synaptic interconnections and neurotransmitter vesicles, mature in some regions of the spinal cord by 30 weeks gestation.[17] The A-delta and C fibers divide and make connections at the entering spinal cord level as well as at dermatomes above and below. Muscle spasm, the

TABLE 169–1

Development of Cutaneous Sensory Receptors in the Human Fetus

Week of Gestation	Location of Receptors
7th	Perioral area
11th	Face, palms, and soles
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20th	All cutaneous and mucous surfaces

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withdrawal reflex, is produced with spread of the nociceptive signal to the anterior horn cells, and vasoconstriction of the skin and splanchnic circulation occur with spread of the signal to the preganglionic sympathetic neurons of the autonomic nervous system. Reflecting this development, the cutaneous withdrawal reflex, mediated through the spinal cord, can be evoked at least as early as 25 weeks gestation.[18]

Most nociceptive signals to the cerebral cortex travel via second-order neurons in the spinothalamic, spinoreticular, and spinomesoencephalic tracts to synapse in the thalamus. The cerebral cortex neurons develop by 20 weeks gestation but undergo a process of dendritic branching, developing synaptic targets for thalamocortical fibers. Synaptogenesis of thalamocortical connections is established between 20 and 24 weeks gestation. Myelination of the pain pathways of the spinal cord and brain stem is completed during the second and third trimesters of gestation.[19] Overall development and myelination of pain pathways occur simultaneously with fetal cortical maturation, dendritic arborization, and thalamocortical fiber synaptogenesis (see Table 169-1).[20]

Impulses from A-delta fibers are transmitted to the somatosensory cortex, and C fibers are transmitted to the limbic cortex. Fibers also go to the periaqueductal gray area, reticular activating system, and hypothalamus with subsequent further cortical spread. Thus there is no one focal cortical pain center. Near-infrared spectroscopy has been used to demonstrate cortical activity in response to nociceptive-inducing stimuli in neonates studied as premature as 25 weeks postmenstrual age.[21,22] This cortical response became more robust as postmenstrual age advanced. In addition, facial responses to painful stimuli can be provoked as early as 24 weeks gestation, suggesting the development of these functional pathways from the spinal cord to the brain.[18]

The complex nature of the pain response is modified by dozens of neurotransmitters within the nervous system. Pain-related neurotransmitters including the neuropeptide, substance P, thought of as the primary afferent nociceptive neurotransmitter, appear in the spinal cord dorsal horn early in gestation. Release of these neurotransmitters activates dorsal horn interneurons and thus the ascending pathways. Inhibition of the pain response at the level of the spinal cord, the periaqueductal gray matter, and medulla occurs through the descending corticospinal, raphe spinal, and reticulospinal tracts. This inhibition is mediated through release of neurotransmitters—serotonin, dopamine, and norepinephrine—that inhibit excitatory neurotransmitters.

Several lines of evidence suggest that neonates are actually more sensitive to pain—with lower pain thresholds, poor discriminative abilities, and increased tendencies to sensitization— than adults. Anatomic and physiologic mechanisms in rat pups supporting this increased sensitivity are shown in Table 169-2.[23] Maturation of these pain pathways is reflected in the decreasing cutaneous withdrawal response, increasing pain threshold, and decreasing receptive field size with increasing postnatal age.

The foregoing neuroanatomic and neurochemical evidence, in addition to the well-established behavioral and physiologic responses to pain, leads one to conclude that both the fetus and infant have nociceptive systems capable of communicating painful stimuli from the periphery to the cerebral cortex and regulating the response via efferent inhibitory pathways.

CLINICAL AND PHYSIOLOGIC MARKERS ASSOCIATED WITH PAIN

The physiologic changes associated with pain encompass responses that include those of the cardiovascular, respiratory, endocrine, metabolic, and immune systems.

The hemodynamic and respiratory changes associated with pain frequently have been used as indices of reactivity to assess painful events in preterm and term infants. These responses occur through activation of the autonomic nervous system by nociceptive impulses reaching the periaqueductal gray matter. An increase in an infant's heart rate and blood pressure during and following a painful procedure such as circumcision or heel lance has been well documented.[24-27] This presumably reflects increased sympathetic modulation as well as parasympathetic withdrawal.[28] In many cases this increase can be prevented or attenuated by the use of local or topical anesthetics.[29-32] Whereas infants of all gestational ages demonstrate heart rate reactivity in response to painful stimuli, both gestational age and postmenstrual age appear to be factors in determining the magnitude of this physiologic response.[33,34]

Neonates undergoing surgery have been observed to experience large fluctuations in measured transcutaneous oxygen tension.[35,36] Decreases in transcutaneous oxygen tension also were observed during circumcision and could be attenuated or prevented by the use of local anesthetic agents.[32,37] Tracheal intubation, another example of a noxious procedure, caused decreased transcutaneous oxygen tension measurements as well as increased arterial blood pressure and intracranial pressure in both term and preterm infants.[38,39] These cardiovascular responses could be diminished by the administration of anesthetic agents.[40,41]

Stress response is a term used to describe the wide range of hormonal and metabolic changes associated with trauma, both physical and emotional.[42-44] This encompasses the release of pituitary, pancreatic, and adrenal hormones that affect metabolic

TABLE 169-2

Mechanisms Supporting Increased Pain Sensitivity in Neonates

Components of Pain System	Developmental Processes in Newborn Rat Pup
Cutaneous receptors Polymodal nociceptors High-threshold mechanoreceptors	Mature soma membrane properties (membrane thresholds, firing frequencies, neurotransmitter expression) in the late fetal rat and at birth immature soma membrane properties (low thresholds, low frequencies)
Cutaneous nerve terminals	Superficial plexus of nerve terminals becomes subepidermal with development of the stratum corneum, density regulated by NGF expression
Skin or tissue injury	Marked hyperinnervation of neonatally injured skin, decreased threshold
C fiber terminals in the dorsal horn	Delayed entry into the substantia gelatinosa, synaptogensis at 3 weeks
A fiber terminals in the dorsal horn	Early synaptogensis in laminae 3-5, collaterals to substantia gelatinosa
Nociceptive neurons in the substantia gelatinosa (laminae 1,2)	Large receptive fields, marked overlapping of adjacent neurons, low thresholds, early expression of excitatory neurotransmitters/receptors delayed development of descending inhibition (5-HT, NE, dopamine); single stimuli evoke long-lasting excitation, which lowers the threshold to subsequent stimuli, repeated stimuli cause immense background activity
Dorsal horn substance P receptors	Sixfold greater density as compared to the adult, diffuse localization
NMDA receptors in the dorsal horn	Peak density at 6 to 10 days age, increased affinity for NMDA and [Ca^{++}] release
Inhibitory transmitters: GABA, glycine	Fast inhibitory transmitters in the adult spinal cord actually depolarize immature spinal neurons and cause increases in intracellular [Ca^{++}]
Somatosensory cortex pyramidal cells	Large receptive fields, which may be caused by a lack of surround inhibition

5-HT, 5-Hydroxytryptamine, GABA, γ-aminobutyric acid; NE, norepinephrine; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartate.

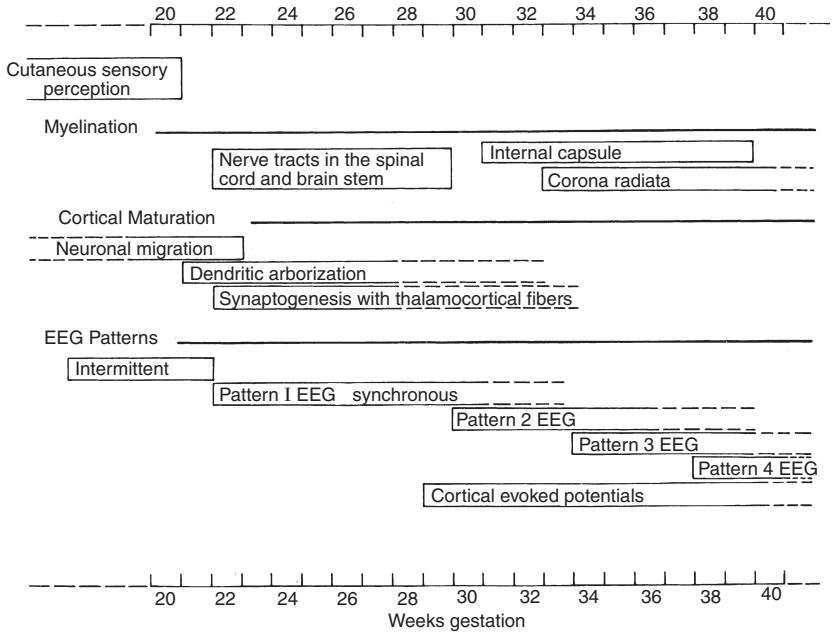


Figure 169-1 Depiction of the maturation of nociceptive pathways and functions during the third trimester of fetal gestation. *(From Anand KJS, Hickey PR: Pain and its effects in the human neonate and fetus,* N Engl J Med *317:1321, 1987, with permission.)*

balance and once may have played a part in allowing injured mammals to catabolize their own body stores, thereby improving survival.[43] The hormonal responses to surgically induced stress in infants have been shown to include increases in catecholamines (both epinephrine and norepinephrine), cortisol, β-endorphins, insulin, glucagon, renin-aldosterone, growth hormone, and prolactin.[45-47] Giannakoulopoulos and colleagues[48] demonstrated that fetuses of 24 to 34 weeks gestation have shown increases in cortisol and β-endorphin levels in response to intrauterine abdominal needling.

As mentioned earlier the stress response in neonates has been most closely investigated in response to surgery and relatively minor procedures such as circumcision and venipuncture. Anesthesia generally has been shown to attenuate this stress response, both intra- and postoperatively.[9,11,12] It is interesting that in one study the neonatal cortisol response to circumcision was not significantly reduced by the administration of a local anesthetic nerve block.[49] Further investigation is needed to confirm this phenomenon. The investigators theorize about the potential problem of awake procedures with only regional afferent neuronal blockade causing a stress response via cortical and adrenal pathways in infants because of their fear, anxiety, and inability to respond to verbal reassurance.

BEHAVIORAL MARKERS ASSOCIATED WITH PAIN

Anand and Hickey[20] described the behavioral changes associated with pain and divided them into four general categories: simple motor response, facial expressions, crying, and complex behavioral responses. Simple motor response includes flexion and adduction of extremities in response to a painful stimulus. Much of this response is mediated through spinal cord reflexes. Distinct facial expressions have been associated with pain and are the basis for portions of many pain assessment techniques.[50,51] Facial response is also thought to be mediated through the periaqueductal gray matter. These facial reactions become more pronounced with increasing postnatal age in contrast to the withdrawal reflex, which decreases in intensity with increasing postnatal age. The cry response to painful stimuli has been shown to have specific characteristics and acoustic features that can be differentiated by adult listeners from other crying behavior such as hunger.[52] Complex behavioral responses include altered sleep-wake cycles and irritability following painful stimulus. Changes in complex behavior were noted following circumcision in boy infants; these effects were absent in infants given local anesthesia before the painful procedure.[53]

PAIN ASSESSMENT IN THE NEONATE

Assessment of pain in infants is a difficult task given that their preverbal developmental state prohibits communication of their pain in words. Research in the late 1980s and early 1990s established that pain could be assessed in infants by means of cardiorespiratory, neuroendocrine, and behavioral responses to painful stimuli.[54,55] Refinement of infant pain assessment scales continued throughout the 1990s and into the new millennium. Although more than three dozen scales have been developed, no single tool has emerged as the "gold standard" for assessment of neonatal pain.[51,56,57] However a multidimensional pain scale should be used with limitations recognized in infants who are pharmacologically paralyzed or have severe neurologic impairment. It is also important to understand that the behavioral and physiologic responses to pain are modified with increasing gestational age at birth, increasing postnatal age, and previous experiences thus making any one pain scale difficult to use in all neonatal populations.

EFFECTS OF PAIN

There is a growing body of evidence that pain is associated with more than the immediate effects such as elevated heart rate, blood pressure, intracranial pressure, and the endocrine- metabolic stress response. Andrews[58] and colleagues showed that in infants with unilateral hydronephrosis and presumed referred visceral pain, abdominal skin reflex thresholds did not increase and reflex radiation did not diminish with postnatal age when compared with controls. This suggests that the presence of pain may interfere with the normal maturation of pain pathways. In

addition, other long-lasting pathophysiologic effects occur with exposure to pain-inducing stimuli in the neonate.

Procedures that result in tissue injury produce a local inflammatory response. This inflammation elicits nociceptor recruitment and sensitization resulting in primary hyperalgesia and increased sensitivity to noxious stimuli. Lasting hyperinnervation can occur as the result of release of neurotrophic factors by the damaged tissue, resulting in a dendritic sprouting of local sensory nerve terminals. This response seems to be most pronounced with tissue damage that occurs in the immediate perinatal period. This phenomenon is demonstrated in preterm neonates who have lower reflex withdrawal thresholds in the foot exposed to repeated heel sticks.[59] This hypersensitivity was ameliorated and in some cases totally abolished by the use of a topical analgesic cream. In addition, secondary hyperalgesia may occur with allodynia—pain caused by a stimulus that is nonnoxious—in the surrounding area. This secondary hyperalgesia and allodynia is caused by increased excitability of the sensory neurons in the dorsal horn of the spinal cord and brainstem with repetitive inputs by A-delta and C fibers, the so called wind-up phenomenon.

Bhutta and Anand[60] described an enhanced vulnerability of immature neurons to degenerative changes and noted that repetitive pain during the neonatal period might have a significant impact on neuronal survival. They proposed two mechanisms that lead to neuronal cell death in the immature brain. The first was excitotoxic cell death initiated by an *N*-methyl-D-aspartate (NMDA) receptor–mediated neuronal cell calcium influx. The second was enhanced neuronal apoptosis potentially caused by maternal separation or metabolic stressors.

The hormonal response to stress-inducing stimuli also seems to be altered in neonates exposed to painful procedures. Extremely low-birth-weight infants studied at 8 months corrected age had higher cortisol levels both at baseline and after exposure to a visual novelty than term and very low-birth-weight neonates.[61] The number of skin-breaking procedures correlated with higher basal cortisol levels in the extremely low-birth-weight infants. Morphine exposure was not associated with the cortisol response.

The pain associated with neonatal circumcision and its effect on future behavioral changes has been the focus of several studies. Neonatal circumcision was shown to increase the pain response to vaccination at 4 to 6 months of age.[62] Further studies confirmed that there was a stronger pain response to subsequent routine vaccination in circumcised versus uncircumcised infants.[63] Treatment with a topical anesthetic before circumcision attenuated this enhanced pain response during 4- and 6-month routine immunizations. Major surgery performed with preemptive analgesia within the first 3 months of life was not associated with an increased response to pain at later (14- or 45-month) immunization.[64] This implies either that the enhanced pain responses noted earlier diminish over a relatively prolonged period following exposure to the noxious stimulus or that preemptive analgesia prevented the memory-associated response.

Altered behavioral and physiologic reactivity to later painful stimuli has been demonstrated in extremely low-birth-weight infants and has been correlated with increased exposure to painful procedures.[65-67] Although term infants show increased behavioral response to vaccination after neonatal exposure to a painful procedure (circumcision),[63,64] preterm infants show dampened behavioral responses even up to 8 months corrected age with exposure to a pain-provoking stimulus.[65] In addition to the alteration in behavioral response, lasting variations in heart rate reactivity to painful stimuli have also been found.[65,66] These alterations are dependent on gestational age at birth, postnatal age, number of painful procedures, and exposure to analgesia.

SUMMARY

Infants have the capacity to perceive pain. The neuroanatomic, neurophysiologic, and neurochemical substrates for pain perception are developed by midgestation and continue to mature throughout late gestation and infancy. Term and preterm infant responses to pain, both physiologic and behavioral, have been well described and are used to perform multidimensional pain assessments. Research supports the concept that exposure to pain in the neonatal period has both short- and long-term effects. Prevention or treatment of pain may reduce the potential sequelae of intense, repetitive, and/or prolonged pain and improve outcomes in the vulnerable fetal and neonatal populations. However, further research is needed to define the best way to treat pain to prevent these effects without introducing detrimental effects from the analgesics themselves. Several organizations have developed consensus statements to help the practitioner approach and treat pain and to define gaps in knowledge and research that should be conducted to fill these gaps.[68-70]

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SECTION XXII

Special Sensory Systems in the Fetus and Neonate

170

Helen A. Mintz-Hittner

Retinal Development and the Pathophysiology of Retinopathy of Prematurity

Retinal development occurs in an orderly process after the formation of the optic cup (vesicle). The differentiation of the cellular components of the retina occurs from the optic disk (posterior portion of the eye) toward the ora serrata (anterior extent of the retina) and from the internal (vitreal) surface toward the external (scleral) surface of the eye. Differentiation of the most highly specialized region of the retina, the macula, is not complete for the first decade of life. The choroidal vasculature is present on the external side of the retina by 6 to 7 weeks gestational age. As the retina thickens, inner retinal vessels develop on the internal side of the retina from the optic disk at 14 to 16 weeks gestational age to the ora serrata at approximately week 40.

Interruption of normal inner retinal vascularization by premature birth is the initiating event of retinopathy of prematurity (ROP). The timing of the occurrence of observable clinical and histological changes and of measurable growth factor levels is very predictable. Modulation of the infant's extrauterine environment can reduce or prevent the development and severity of ROP.

In clinical practice, knowledge of the classification of ROP, the rules for screening for ROP, and the recommendations for treating ROP is essential to optimize visual outcomes. ROP is one of the most common causes of blindness in both developed and developing nations of the world today and remains a focus of intense clinical investigation.

DIFFERENTIATION OF THE CELLULAR COMPONENTS OF THE RETINA

The ganglion cells are the first identifiable cells in the developing retina and establish the innermost layer of the retina by 20 weeks gestation. Shortly thereafter, cellular migration progresses, and the inner plexiform and inner nuclear layers differentiate. This event is followed by differentiation of the outer plexiform and outer nuclear layers. The nuclei of the outher nuclear layer are the nuclei of the photoreceptors, which develop radially from the optic disk to the ora serrata beginning at approximately 26 weeks gestation.[1,2] Photoreceptor maturation occurs on a gradient, with the most mature photoreceptors near the optic disk and the more immature photoreceptors toward the ora serrata. During stage I of photoreceptor development, the photoreceptor has no outer segment. In stage II, a small, balloon-like outer segment develops. By stage III, disk development can be seen. By stage IV, the outer segments are better developed, with stacks of disks evident to the ora serrata by 29 weeks gestation[1,2] (Figure 170-1).

As the photoreceptor matures, an increasing amount of interstitial retinol-binding protein is present. This protein binds both fat-soluble antioxidant vitamins A and E. Thus, by week 29, the protein is secreted by photoreceptors from the optic disk to the ora serrata, allowing the transport of these antioxidant vitamins to the inner layers of the retina.[1,2] The water-soluble antioxidant vitamin C does not require this protein for transport and thus can traverse the neurosensory retina freely.

The development of the foveal region takes much longer and is not complete until several years after birth.[3] The fovea can first be identified around week 14 of gestation. A central migration of photoreceptors, with remodeling of cones toward the foveal region, is a key event. Maturation proceeds very slowly, with the ganglion cells and glial cells thinning in this area. This process is not complete until after the age of 10 years. This area becomes the region of the retina with highest visual acuity and color perception.

The neuronal elements and their connections and signal processing in the retina are complex, with many types of bipolar, amacrin, and ganglion cell connections to more than 120 million rods and 6 million cones.

NORMAL VASCULARIZATION OF THE RETINA

Vasculogenesis

Vasculogenesis is a process defined by the appearance of angioblasts arising from the optic disk forming a superficial plexus of inner retinal vessels located just beneath the inner limiting

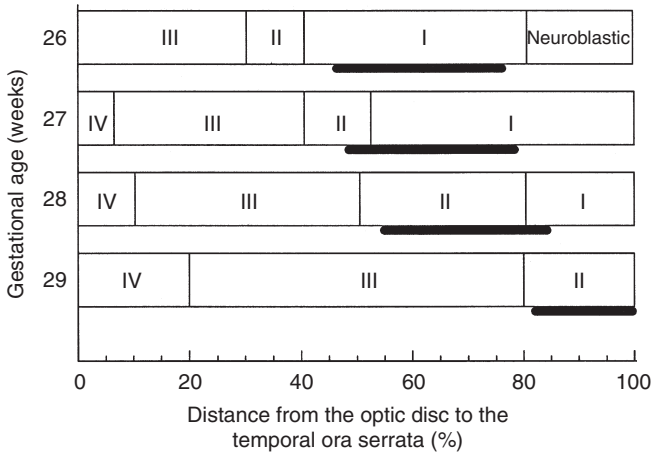


Figure 170-1 Gradient of photoreceptor development from 26 to 29 weeks gestation. *Roman numerals* indicate the stage of photoreceptor development, and the *solid black lines* mark the location of the embryonic vascular elements. *(Modified from Kretzer FL, Hittner HM: Pathogenesis and antioxidant suppression of retinopathy of prematurity. In Miguel J, Quintanilha AT, Weber H, editors:* CRC Handbook of Biomedicine of Free Radicals and Antioxidants, *Boca Raton, FL, 1989, CRC Press, pp 315-332.)*

membrane (on the vitreous surface) and progressing toward the ora serrata from 10 to 20 weeks gestational age.[4] VEGF is normal at this time, and the fetus is not yet viable. However, VEGF is secreted by these superficial astrocytes after preterm birth in response to hypoxia. Embryonic retina has a pool of precursors that enlarge centrifugally during fetal development and migrate anteriorly into the nerve fiber layer, with ultimate canalization of new vessels regulated by various factors.[5]

Angiogenesis

Angiogenesis is the process defined by the appearance of vascular precursors arising in the area of the future macula forming a deeper plexus of inner retinal vessels located in the inner nuclear layer and progressing toward the ora serrata from 20 to 40 weeks gestational age.[6] This plexus normally secretes relatively low levels of VEGF. However, VEGF is secreted at higher levels by the Müller cells in the inner nuclear layer after preterm birth in response to hypoxia.

These two processes, vasculogenesis and angiogenesis, probably constitute a continuum, with growth factors orchestrating the formation of final vessel formation of the inner retina while differentiation with thickening of the retina acts as a driving force[7] (Figure 170-2).

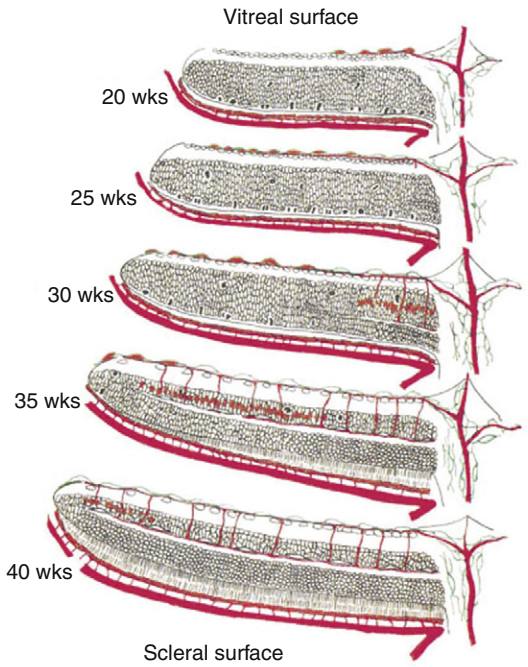


Figure 170-2 Differentiation of the cellular elements and development of the vascular components of the retina during late gestation. *(Modifiea from Stone J, Itin A, Alon T, et al: Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia,* J Neurosci *15:4738-4747, 1995.)*

ABNORMAL VASCULARIZATION OF THE RETINA: RETINOPATHY OF PREMATURITY

Histologic Changes in Vascular Precursors and Predictions of Angiogenic Factors

Both Michaelson in 1948[4] and Ashton in 1970[8] had proposed that an angiogenic factor was released from the retina causing retinopathy of prematurity. However, it was not until 1986 that Kretzer, et al. identified an angiogenic factor in the avascular retina in preterm infants and quantified this factor at different time points after birth in infants with and without retinopathy of prematurity. The presence of an angiogenic factor was indicated in peripheral angioblasts by (1) an increase in the gap junction area between altered angioblasts in the avascular retina indicating cross-chatter between adjacent cells; (2) an increase in the surface area of rough endoplasmic reticulum in these altered angioblasts denoting production of a large amount of an unknown substance; and (3) the ability of retinal homogenates of the peripheral avascular retina to induce neovascularization on chorioallantoic membrane substrates (Figure 170-3).

It has been shown that vascularization may not proceed normally in infants born prematurely in whom ROP develops subsequently. With ROP in an infant born at or before 28 weeks gestation, vascularization may never extend to the ora serrata.[9] The extent of vascularization is dependent on the location of vascular precursors at the time of birth. The initially avascular fovea may revascularize under the influence of abnormally increased angiogenic factors, with new vessels growing toward its center, making the fovea appear to be small or absent.[10]

Additionally, it has been shown that both rod and cone differentiation and function may be delayed or interrupted in preterm infants as a result of environmental changes.[11,12]

Growth Factors and Pathologic Vascularization

Phase I

Vasculogenesis is the formation of normal inner retinal vessels in utero in an environment of relative hypoxia with physiologic levels of VEGF. At the time of premature birth, the infant retina becomes hyperoxic (even in room air) with decreased levels of VEGF. For a period of time, vasculogenesis is halted at the interface between the vascular and avascular retina, usually with some loss of capillaries in the posterior retina (phase I—in clinical terms, between 22 and 30 weeks postmenstrual age). The retinal vessels often vasoconstrict; however, the vessels of the choriocapillaris do not, thereby allowing environmental oxygen free radicals ready access to the external retina (Figure 170-4).

Phase II

As the eye grows, the avascular retina continues to increase both in thickness and in area, without accompanying inner retinal vessels, and areas of capillary fallout remain. This creates a peripheral as well as a posterior area of hypoxic retina, resulting in increased levels of VEGF, which stimulate pathologic neovascularization at the interface between the vascular and the avascular retina (phase II—in clinical terms, 31 to 45 weeks postmenstrual age) (see Figure 170-4).

This two-phase process leads to vision-threatening ROP, most frequently in extremely immature infants with other comorbid disease of prematurity (i.e., risk factors for ROP).[13] Thereafter, VEGF becomes less important. Either intravitreal fibrosis with membrane formation and resultant retinal traction becomes the determinant of ROP outcome or regression of ROP with resumption of relatively normal vessel growth occurs (if laser ablation of the peripheral avascular retina has not been performed).

PATHOPHYSIOLOGY OF RETINOPATHY OF PREMATURITY

ROP is manifested clinically by the abnormal growth of developing retinal vessels. The requisite pathophysiologic mechanisms include both incomplete vascularization of the retina and

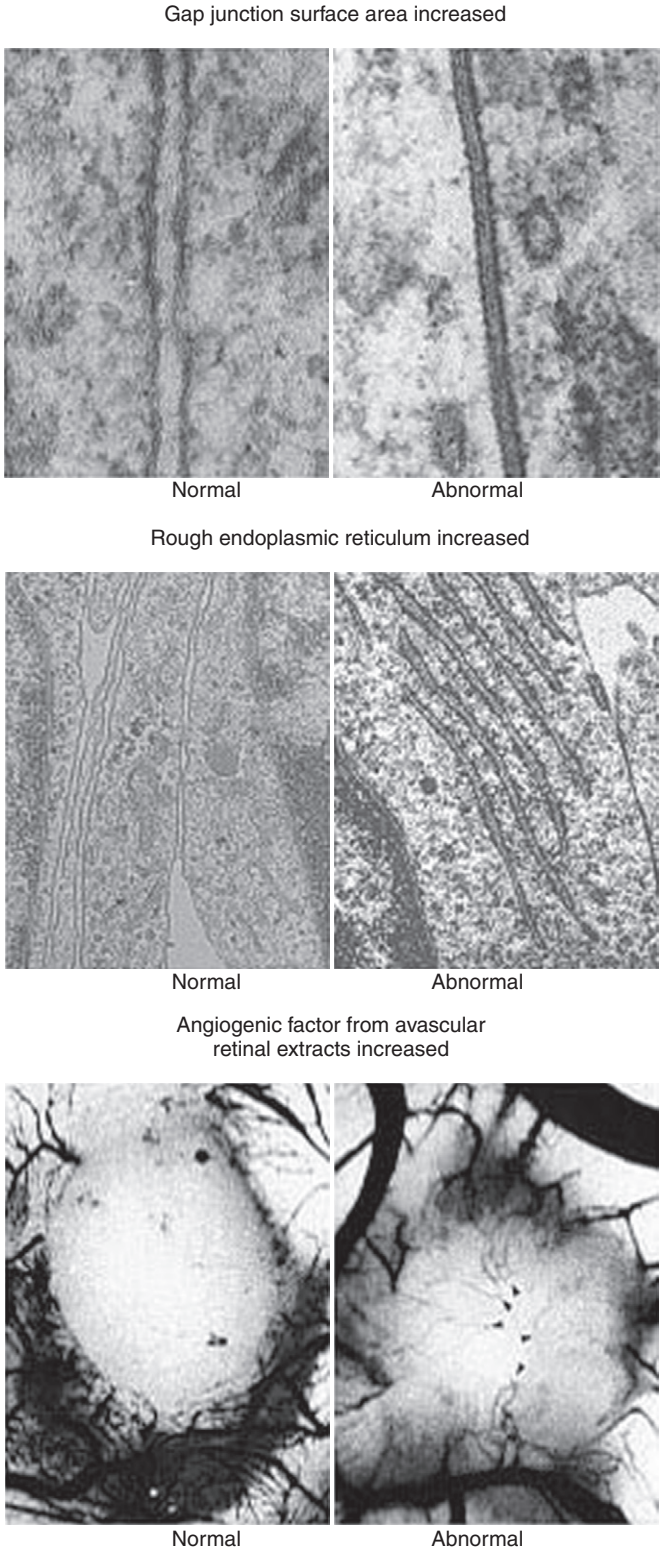


Figure 170-3 Evidence of the production of angiogenic factors by angioblasts. *(Modified from Kretzer FL, Hittner HM: Initiating events in the development of retinopathy of prematurity. In Silverman WA, Flynn JT, editors:* Retinopathy of Prematurity, *Boston, 1985, Blackwell Scientific Publications, PP 121-152; and Kretzer FL, Hittner HM: Pathogenesis and antioxidant suppression of retinopathy of prematurity. In Miguel J, Quintanilha AT, Weber H, editors:* CRC Handbook of Biomedicine of Free Radicals and Antioxidants, *Boca Raton, FL, 1989, CRC Press, pp 315-332.)*

exposure of this immature retina to an abnormal environment[13] (Table 170-1). Children who are born very prematurely are at higher risk for the development of ROP, especially in the presence of multiple other risk factors.

In determining timing for the first ROP screening examination, a predictable relationship has been recognized regarding the onset of ROP and gestational age at birth, postmenstrual age, and postnatal age (see Table 170-2).[15,16] It is clear that in terms of postmenstrual age, ROP first develops and then progresses to more serious stages at a predictable time in type I ROP (i.e., classic ROP). The severity of ROP progresses in an orderly fashion from stage 1 to stage 2, and so on, with "plus" disease ("prethreshold" and "threshold") developing after stage 2 (see Figure 170-4).[14] The development of type II ROP, also called aggressive posterior retinopathy of prematurity (AP-ROP) and formerly known as RUSH disease, is quite different. The plus develops early in the disease process, and the traditional stages of ROP, which occur earlier than in type I ROP and usually in zone I (see Figure 170-4), are replaced by the development of "flat" neovascularization.[15]

Modulating the Environment of the Developing Retina

Modulating the environment of the developing retina to recreate the intrauterine environment of the third trimester should be the goal of all pharmaceutical interventions to suppress retinal neovascularization. Retinopathy can be suppressed by preventing vessel loss, which produces hypoxia and stimulates neovascularization, or by direct inhibition of neovascularization. If these measures fail, then intervention with drugs that oppose neovascular stimulants may be the only option. These approaches are summarized in Table 170-1, which lists the multiple methods to prevent, screen, and treat ROP by attempting to restore balance to the premature infant's retinal environment.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a hypoxia-induced growth factor (HIF) that is produced primarily in the retinal Müller cells and endothelial precursors in response to hypoxia and is essential for normal retinal vascularization. Hyperoxia in phase I creates areas of capillary fallout with decreasing retinal levels of VEGF.[16-20] Hypoxia in phase II stimulates neovascularization with increasing VEGF levels. A small case series has used anti-VEGF therapy (bevacizumab [Avastin]) for monotherapy of vision-threatening (stage 3 with plus disease) ROP in zone I and posterior zone II,[21,22] and a multicenter clinical trial of anti-VEGF therapy (intravitreal bevacizumab) with conventional laser to the avascular retina as a control group (BEAT-ROP [Bevacizumab Eliminates the Angiogenic Threat of Retinopathy of Prematurity]) is now being conducted to establish safety and efficacy of this therapeutic approach to ROP[23] (see Table 170-1). Bevacizumab also has been used in combination with laser therapy for treatment of stages 3, 4, and 5.[24]

Erythropoietin

EPO, another HIF, is produced primarily in the kidney in response to anemia and hypoxia and subsequently stimulates both cellular and vascular elements. EPO activates the production of red blood cells as well as retinal angiogenesis. Systemic administration of recombinant human erythropoietin (rhEPO) in phase I has a beneficial effect, not only in treating anemia but also in preventing ROP by limiting vessel loss (by increasing TNF and decreasing capase).[25-27] Systemic administration of rhEPO in phase II has an adverse effect, however, and should be avoided after 30 weeks gestational age (see Table 170-1).[28-30]

Recent studies have demonstrated that inhibiting EPO mRNA with EPO small interference RNA (siRNA) by intravitreal

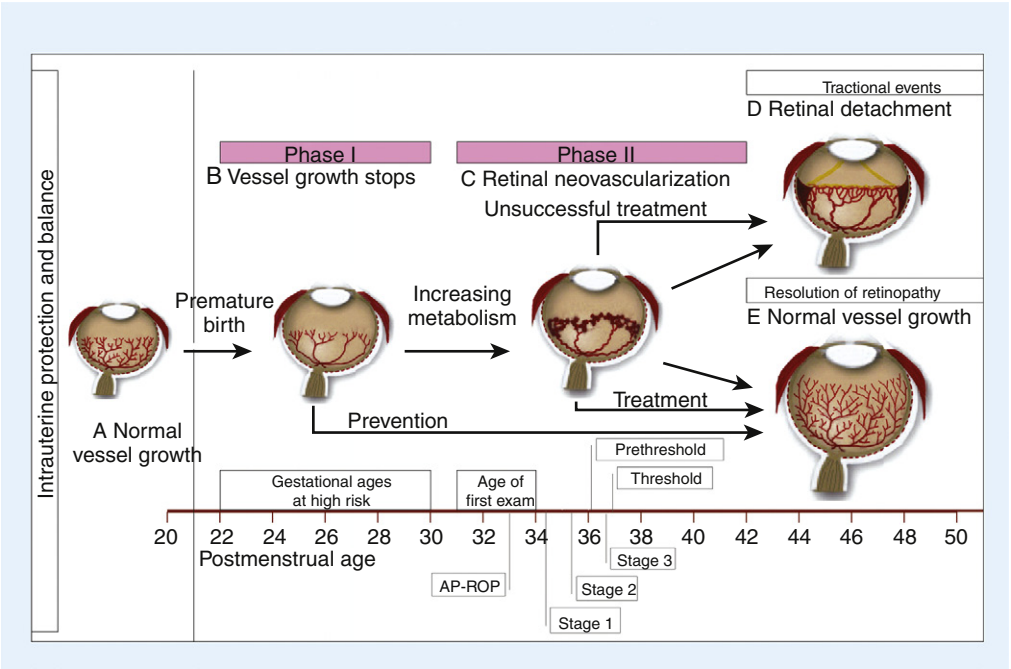


Figure 170-4 Pathogenesis of retinopathy of prematurity. Timeline of intrauterine protection and balance. *A,* Normal vessel growth—phase I. *B,* Vessel growth stops—phase II. *C,* Retinal neovascularization—tractional events. *D,* Retinal detachment and resolution of retinopathy. *E,* Normal retinal growth. The gestational ages of highest risk and age at first examination are indicated. *(Gestational ages at high risk and age of first exam data from Section of Ophthalmology AAoP, American Academy of Ophthalmology, American Association for Pediatric Ophthalmology and Strabismus: Screening examination of premature infants for retinopathy of prematurity [policy statement],* Pediatrics *117:572-576, 2006 [erratum in* Pediatrics *118:1324, 2006]. Data on the development of stages 1, 2, and 3 and of prethreshold and threshold from Palmer EA, Flynn JT, Hardy RJ, et al: Incidence and early course of retinopathy of prematurity. The Cryotherapy for Retinopathy of Prematurity Cooperative Group,* Ophthalmology *98:1628-1640, 1991. Data on the development of aggressive posterior ROP [AP-ROP] from Vinekar A, Trese MT, Capone A Jr., for the Photographic Screening for Retinopathy of Prematurity [Photo-ROP] Group: Evolution of retinal detachment in posterior retinopathy of prematurity: impact on treatment approach,* Am J Ophthalmol *145:548-555, 2008. Phases I and II from Smith LEH: Through the eyes of a child: understanding retinopathy through ROP—the Friedenwald Lecture,* Invest Ophthalmol Vis Sci *49:5177-5182, 2008.)*

injections during phase II decreases neovascularization in the mouse model of ROP.[31,32] This promising area of clinical research can be expected to lead to development of a second therapeutic drug for use as an adjunct to bevacizumab.[33,34]

Insulin-Like Growth Factor

Insulin-like growth factor (IGF) is a growth factor provided by the maternal circulation until premature birth. Thereafter IGF levels drop precipitously, and supplementation of IGF may be an early treatment that may completely prevent ROP. More recently, observation of early weight gain and IGF decrease has been shown to predict ROP in 100% of infants requiring treatment and correctly identified a majority of infants with ROP who did not require treatment.[35,36] The simple measurement of early postnatal weight gain can markedly reduce the repeated, stressful, and costly ocular screening examinations in all infants with birth weights of 1500 g or lessthat benefit only 10% of the infants actually needing treatment. Thus prediction rather than diagnosis is the goal, with the caveat that postnatal factors that are likely to increase the risk of ROP, as provided by use of the WINROP algorithm (*w*eight, *in*sulin-like growth factor, *n*eonatal *r*etinopathy *o*f *p*rematurity), may be appropriate for identifying vision-threatening ROP.[35]

ω-3 Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs), both ω-3 and ω-6, have been studied for possible benefit in ROP. The major PUFAs found in the retina are docosahexaenoic acid (DHA) (i.e., C22:6 ω-3) and arachidonic acid (C20:4 ω-3), found in the vascular cell membrane phospholipids, and eicosapentaenoic acid (EPA) (i.e., C20:5 ω-3), the precursor to DHA found in the retinal vascular endothelium. Vascular regrowth after injury was increased by ω-3 PUFAs, and neovascularization was reduced by ω-3 PUFAs and their derived mediators neuroprotectionD1, resolvinD1, and resolvinE1. This effect was mediated partly by suppression of tumor necrosis factor-α, whose major source is monocyte-derived cells near blood vessels in the retina.[37] This antiinflammatory cytokine is located in a subset of microglial cells that are associated with retinal vessels.[38]

Oxygen

The role of inspired oxygen has long been established as a contributing factor in the development of retinopathy of prematurity. Monitoring blood gases after birth has been a standard of care for decades. More recently, the strict control of oxygen saturation throughout the newborn infant's hospitalization has significantly decreased the incidence of ROP.[39-41] Oxygen stresses have recently been identified as a trigger for ROP through reactive oxygen species (ROS) by way of the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway and NAPDH oxidase signaling.[42]

Antioxidant Defenses

Multiple mechanisms serve as antioxidant systems, including those mediated by vitamins (C and E), and several nonenzymatic systems to maintain an effective environment for scavenging of

TABLE 170-1

Methods to Restore Balance to Premature Infant's Retinal Environment

Approach	Principle/Rationale	Clinical Intervention/Comment
Prevention*	Growth factor administration	Administer insulin-like growth factor-I (IGF-I)
	Fatty acid administration	Administer ω-3 polyunsaturated fatty acids (ω-3 PUFAs)
	Prevent angiogenesis with VEGF	Administer vascular endothelial growth factor (VEGF) but NOT after 30 weeks PMA
	Prevent angiogenesis with erythropoietin (EPO)	Administer EPO in first few weeks for anemia but NOT after 30 weeks PMA
	Oxygen monitoring	Utilize blood gas analyzer and cutaneous probes
	Antioxidant administration	Administer antioxidant multivitamins (and minerals): vitamins A, C, E (plus selenium)
	Avoid activating NADPH oxidase and JAK2/STAT2 pathways	Oxygen supplementation and fluctuations may create intravitreal neovascularization
	Prevent sepsis	Avoid oxygen surge secondary to macrophage ingestion of bacteria
	Minimal stimulation	Decreases intraventricular hemorrhage (IVH)
	Delay feedings	Decrease necrotizing enterocolitis (NEC)
Screening†	WINROP	Measure body weight and IGF-I
	Indirect ophthalmoscopy	Gentle examination: without speculum and scleral depression
	Telemedicine	Primarily for remote sites and research documentation
Treatment‡ Early stage 3 ROP (before membranes form)	Laser ablation of peripheral retina	Current standard of care: decreases VEGF by destroying angioblasts and Müller cells in peripheral retina
	Treat neovascularization with intravitreal anti-VEGF	Administer anti-VEGF for neovascularization—but *only* after 31 weeks PMA: bevacizumab (Avastin) as *monotherapy* (NOT with laser): 0.625 mg in 0.025 mL; sterile; small needle (31 gauge)

**Prevention* is best achieved by simulating third-trimester events in Phase I. (See references 16-44.)

†Screening should improve the specificity and sensitivity of early recognition of Type I ROP (described in ETROP). (See references 35, 36, 50, 51.)

‡Treatment is best given early in Phase II: early Stage 3 (before significant membranes form) rather than in late Stage 3, or in Stages 4 and 5, when thick membranes have formed and retinal detachment is occurring. (See references 15, 51.)

PMA, Postmenstrual age; *ROP,* retinopathy of prematurity; *WINROP,* weight, insulin-like growth factor, neonatal retinopathy of prematurity.

oxygen-derived free radicals.[43] In addition, glutathione peroxidase and glutathione-*S*-transferase covalently bind or initiate peroxidation of electrophilic sites of compounds that could damage cellular membranes.[44]

Clinical Standards

Classification: Plus Disease, Zones, Stages

The International Classification for ROP (ICROP) was published in 1984 to define ROP in terms of location (zones I to III) (Figure 170-5), severity (stages 1 to 5), extent (hours 1 to 12 on a clock face), and vascular dilatation and tortuosity (so-called plus disease).[45] This classification was revisited in 2005 to include aggressive posterior ROP (AP-ROP) (formerly called "rush disease"), or pre-plus disease, and is a practical way to estimate the extent of zone I in clinical practice using indirect ophthalmoscopy[46,47] (Figure 170-6).

TABLE 170-2

Timing of First Eye Examination Based on Gestational Age at Birth*

Gestational Age at Birth (wk)	AGE AT FIRST EYE EXAMINATION (WK)	
	Postmenstrual Age	Postnatal Age
22[a]	31	9
23[a]	31	8
24	31	7
25	31	6
26	31	5
27	31	4
28	32	4
29	33	4
30	34	4

*This guideline should be considered tentative rather than evidence-based for infants with a gestational age of 22 to 23 weeks because of the small number of survivors in this gestational age category.

Data from Section of Ophthalmology AAoP, American Academy of Ophthalmology, American Association for Pediatric Ophthalmology and Strabismus: Screening examination of premature infants for retinopathy of prematurity (policy statement), *Pediatrics* 117:572-576, 2006 [erratum in *Pediatrics* 118:1324, 2006].

Screening Rules

Who needs to be screened: All infants whose birth weight is 1500 g or less *or* whose gestational age at birth is 30 weeks or less should be screened for ROP,[48,49] that is, the "15(00) or 30" rule.

When initial screening should be performed: Infants should attain 4 weeks of chronologic age *and* at least 31 weeks of postmenstrual age before the initial screening examination is performed (see Table 170-2)[48,49]—the "4 and 31" rule.

How screening should be performed: Any of several methods of screening can be used. In teaching centers and other locations in which pediatric or retinovitreal ophthalmologists are readily available, conventional indirect ophthalmoscopy is the usual method of screening. With the advent of RetCam imaging systems (Clarity Medical Systems, Plesanton, California), an additional means of screening has emerged, especially useful if a pediatric or retino-vitreal ophthalmologist is not available.[50] The most recent and simplest method of monitoring longitudinal postnatal weight and IGF-I may prove to be extremely valuable in limiting the number of indirect ophthalmoloscopic examinations and RetCam imaging sessions.[36]

How frequently follow-up examinations should be performed: Follow-up examination should be performed from twice a week to every 3 weeks, in accordance with recommendations published in 2006 (Figure 170-6).[48]

Treatment Rules

Who needs to be treated: Retinas that develop plus disease or stage 3 in zone I need to be treated (Figure 170-6).[51]

When treatment needs to be done: Since 2003, emphasis has been placed on early treatment, expecially in cases of AP-ROP.[15,52] Treatment before the extensive formation of membranes is necessary if vitrectomy is to be avoided.

How infants should be treated: Early laser therapy to destroy VEGF by ablation of the peripheral retina has generally replaced (as standard of care)[51] the use of cryotherapy which is more damaging and painful. A point worthy of emphasis is that both laser and cryotherapy are basically destructive processes, invariably resulting in some loss of peripheral retina and often the development of myopia. This loss of peripheral retina and development of myopia is generally more extensive in zone I cases.

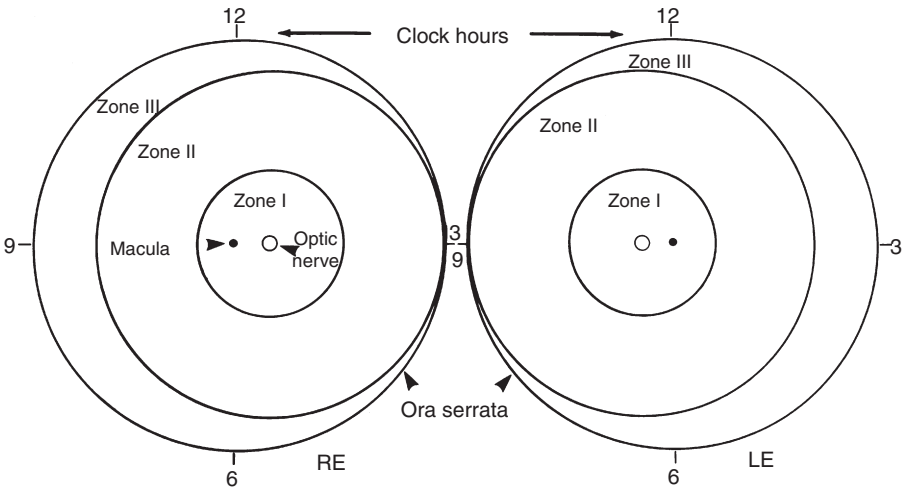


Figure 170-5 Zones of the International Classification of Retinopathy of Prematurity. Scheme of right and left eyes showing the zone of vascularization to indicate the location of retinopathy of prematurity. *(Diagrammatic representation from: An international classification of retinopathy of prematurity. The Committee for the Classification of Retinopathy of Prematurity,* Arch Ophthalmol *102:1130-1134, 1984. Copyright © 1984, American Medical Association.)*

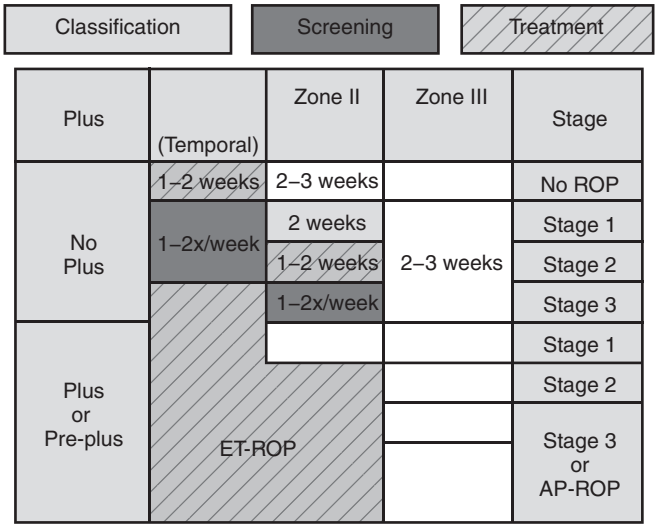


Figure 170-6 Schematic approach to classification, screening, and treatment for retinopathy of prematurity. Use of this summary will allow nurseries to comply with current standards of care for classification *(light shading),* screening *(darker shading),* and treatment *(cross-hatched)* for retinopathy of prematurity. It also permits better communication between neonatologists and ophthalmologists regarding the initiation and frequency of screening and indications for treatment for retinopathy of prematurity. *(Modified from ICROP Committee:* Arch Ophthalmol *123:991-999, 2005 and* Arch Ophthalmol *124:1669-1170, 2006; Section of Ophthalmology AAoP, American Academy of Ophthalmology, American Association for Pediatric Ophthalmology and Strabismus: Screening examination of premature infants for retinopathy of prematurity [policy statement],* Pediatrics *117:572-576, 2006 [erratum in* Pediatrics *118:1324, 2006]; Early Treatment for Retinopathy of Prematurity Cooperative Group: Revised indications for the treatment of retinopathy of prematurity: results of the Early Treatment for Retinopathy of Prematurity randomized trial,* Arch Ophthalmol *121:1684-1694, 2003.)*

With the more recent use of an anti-VEGF drug (bevacizumab [Avastin]) as monotherapy for treatment of ROP in several case reports and case series, and with the publication of the early results of a prospective, randomized, controlled, multicenter trial (BEAT-ROP), another weapon can now be suggested for use against the most devastating cases of ROP (especially severe zone I cases).[21,23] Additionally, anti-EPO treatments may be additive to anti-VEGF drugs and may ultimately become available.[33,34] The development of multiple other treatments can be expected in future research.

How often laser treatment should be performed: Although no standards of care have been established by evidence-based medicine, it is clear that the return of plus disease with the advance of vessels onto retina previously treated with laser is an indication for retreatment. If recurrence is identified early and additional laser therapy is applied, vitrectomy often can be avoided.

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171 Early Development of the Human Auditory System

Lynne A. Werner

In all vertebrates, the auditory system begins to function before the visual system (Figure 171-1). In many mammalian species, hearing begins only after birth. In humans, the first responses to sound probably occur by the 24th week of gestation, and, by birth, hearing is quite functional if not adultlike. The fact that human hearing begins before birth suggests that hearing is very important to human function and survival. The early onset of human hearing also implies that experience with sound has the potential to influence auditory function and behavior, even in the prenatal period. Because development may capitalize on universally occurring experience with sound to guide its course,[1] it is especially important to recognize the possible effects of early abnormal auditory experience, as in the case of premature birth.

The prenatal onset of hearing in humans also means that many aspects of early auditory physiology have not been well described in humans. It is difficult to elicit and interpret responses in utero. Because of the medical complications associated with premature birth, basic research on premature infants' auditory function is not often a high priority, and, of course, there is no guarantee that the development of the auditory system in this special population follows the same course as it would in utero. The major landmarks in auditory morphologic development are quite similar in all vertebrates, however. Where structure-function correlations have been established in other species, it is possible to make reasonable predictions about the functional status of the human auditory system even in the prenatal period. At the same time differences are bound to exist among species, and each auditory capacity depends on many structures, any of which may develop differently in different species. Thus any conclusions about human auditory physiology based on development in nonhuman species must be recognized as tentative.

This chapter begins with an overview of the morphologic and physiologic development of the human auditory system. Where physiologic data are not available for humans, data from nonhumans are described. The gold standard of hearing, however, is auditory behavior; the limited information about the fetal and neonatal development of auditory behavior is reviewed in the second section, and the likely anatomic and physiologic substrates of early auditory behavior are discussed.

EARLY MORPHOLOGY AND PHYSIOLOGY

This section presents in broad outline the course of morphologic development of the auditory system and the physiologic correlates of these events as measured in both humans and nonhumans. In studies of nonhumans, the function of the auditory system is primarily measured as potentials recorded at the site of generation, such as single-unit responses. Gross, scalp-recorded evoked potentials are less frequently used (but see, e.g., Blatchley and colleagues,[2] Smith and Kraus,[3] and Walsh and colleagues[3-6]). Many of the same auditory capacities—sensitivity, frequency resolution, temporal resolution—have been measured in human fetuses and neonates. In the case of humans, however, these measurements are largely based on gross, scalp-recorded evoked potentials. Although the same capacities can be measured in this way, it is obviously more difficult to understand the source of immaturity when instruments such as evoked potentials are used. And, of course, a satisfactory approach to assessing auditory function in fetuses in utero has yet to be developed. Despite these difficulties, many of the developmental trends that have been observed in nonhumans have also been described in human fetuses and neonates.

Peripheral Auditory System: The Ear

The ear is generally divided into three sections: the external ear, including the pinna and ear canal; the middle ear, including the tympanic membrane and ossicles suspended in the tympanic cavity; and the inner ear, including the semicircular canals, vestibule, and cochlea (Figure 171-2). Functionally, the external and middle ears comprise the conductive component of the ear. They shape the spectrum of sound arriving at the inner ear. The cochlea is the site of auditory transduction: displacement of the stereocilia of auditory hair cells resulting from the mechanical displacement of the basilar membrane begins a chain of electrochemical and biochemical events that ends with the generation of an action potential in the neurons of the spiral ganglion (Figure 171-3). A detailed account of current understanding of sound conduction and transduction in the auditory system can be found in Moller[7] and Musiek and Baran.[8] Several classic texts describe the embryology and later morphologic development of the peripheral auditory system.[9,10] Recent texts on this topic are widely accessible.[11,12]

Conductive Apparatus

The ear canal forms at the dorsal end of the first branchial groove. The cells known as the meatal plug can be identified by 6 gestational weeks; these cells subsequently degenerate to form the ear canal. The distal end of the ear canal closes as it elongates and does not reopen until approximately the time that responses to sound can first be recorded. In addition at approximately 6 gestational weeks, six mesenchymal protuberances, the auricular hillocks, appear at the edges of the first branchial groove. The auricular hillocks fuse to form the pinna. Both the ear canal and the pinna continue to grow in size well into childhood, although in form they are well developed by the time that responses to sound can first be recorded.

The mesenchyme that will form the middle ear ossicles is first identifiable at approximately 5 weeks of gestation. As the ossicles grow and begin ossification, the tympanic cavity expands around them. Eventually the ossicles will be suspended in the cavity. The tympanic cavity continues to increase in size well into childhood. The linear dimensions and mass of the ossicles are still increasing between 21 and 40 weeks gestation and, in fact, continue to increase somewhat in the postnatal period.[13] The tympanic membrane is derived from the membrane of the first branchial groove. Mesenchyme that grows between the endoderm lining the presumptive tympanic cavity and the surface ectoderm eventually differentiates into the collagenic fibers that give the membrane its elasticity. As development proceeds the tympanic membrane thins in cross-section and expands in surface area. The tympanic membrane area is more or less adultlike by approximately 28 weeks gestation.[14] It is the ratio of the area of the tympanic

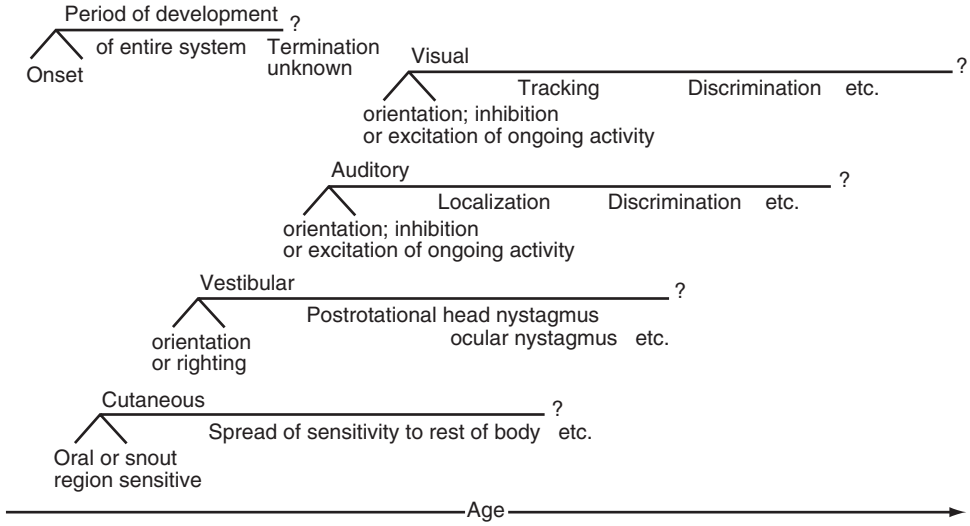


Figure 171-1 The ontogenetic sequence of development of four sensory systems. *(From Gottlieb G: Ontogenesis of sensory function in birds and mammals. In Tobach E, Aronson LR, Shaw ES [eds.]:* The Biopsychology of Development, *New York, 1971, Academic Press, p 69.)*

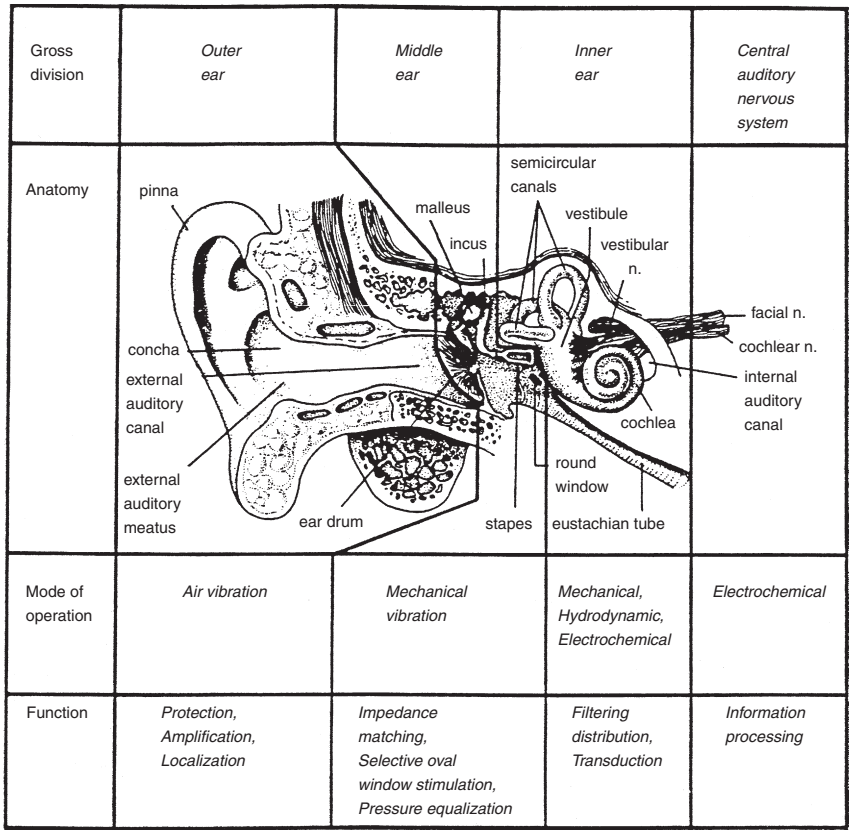


Figure 171-2 Cross-section of the human ear, showing the external, middle, and inner ear and the major functions of each subdivision. *n,* Nerve. *(From Yost W:* Fundamentals of Hearing, *New York, 1994, Academic Press, 1994, p 62.)*

membrane to the area of the stapes footplate that allows the middle ear to overcome the impedance mismatch between air and the fluids of the inner ear. Although this ratio has not been followed during development in humans, in cats the ratio changes little over the period when hearing function begins.[15]

Around the time that responses to sound can first be recorded, the conductive mechanism has several characteristics that appear to be common to all mammals.[16] The ear canal and pinna are small. At term birth, the human ear canal is still only 14 to 15 mm long, compared with the adult length of 23 mm.[17-19] The ear canal may not be completely open. The ear canal walls are more compliant in young animals than in adults. The middle ear cavity is also small. The ossicles are not completely ossified, and they are somewhat smaller and lighter than in the adult. The tympanic membrane appears less stiff than in the adult. Many of these characteristics persist in the term neonate.

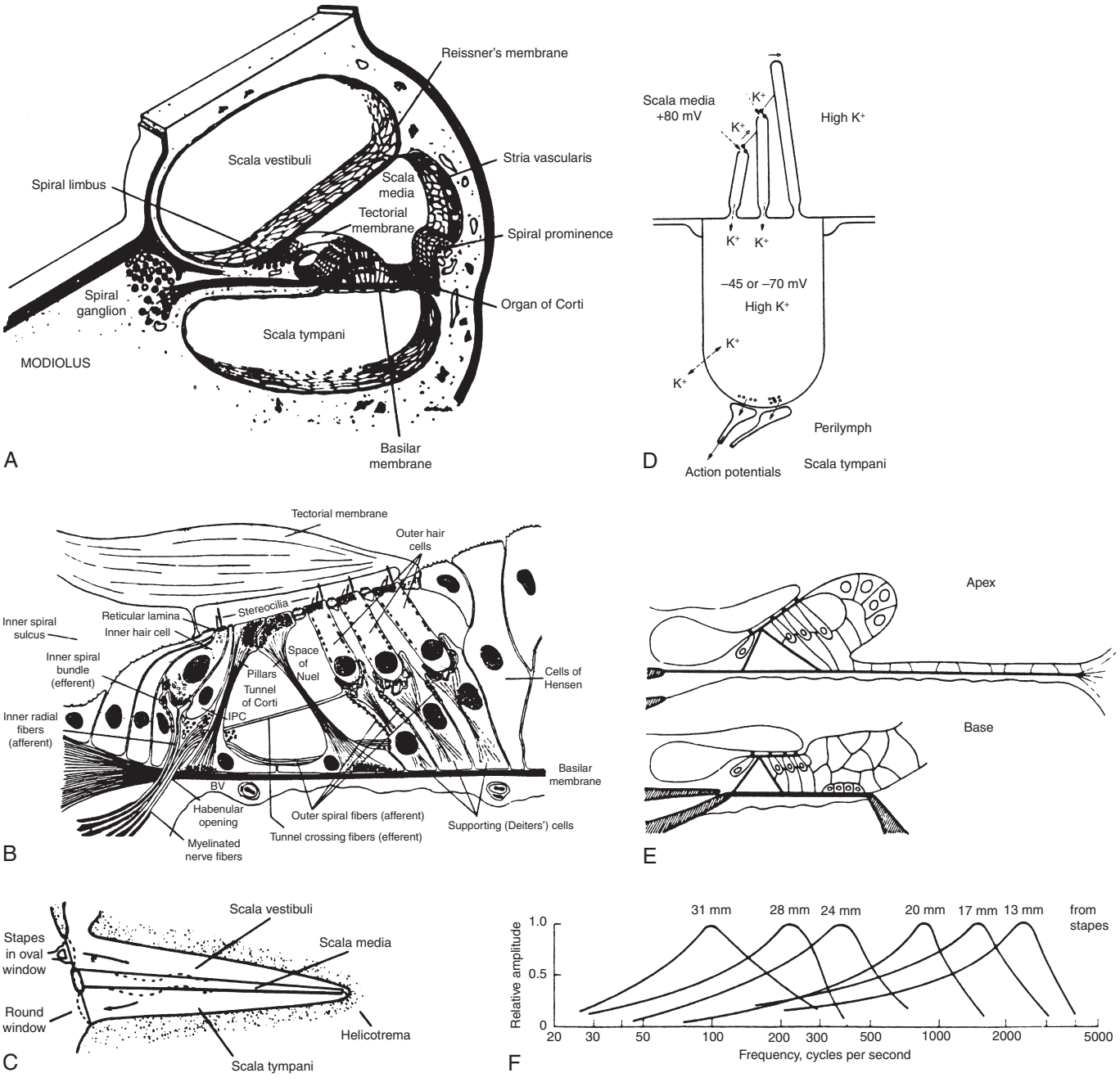


Figure 171-3 Series of drawings illustrating the process by which sound energy is transduced into a neural response by the inner ear. **A,** Cross-section of the cochlear duct. **B,** Schematic cross-section through the organ of Corti. The hair cells are the auditory receptors. **C,** Longitudinal cross-section through the cochlear duct, here represented as if uncoiled. Sound coming through the external and middle ear causes movement of the stapes in the oval window, which results in a traveling wave along the cochlear partition. **D,** Movement of the basilar membrane causes a shearing action across the hair cell stereocilia, opening ion channels in the stereocilia tips, depolarizing the hair cell, and leading to a response in the auditory nerve fiber. **E,** Schematic cross-sections through the organ of Corti near the apex and the base of the cochlea. The basilar membrane is wider and floppier near the apex and narrower and stiffer near the base. **F,** The amplitude of basilar membrane displacement as a function of frequency at various locations along the basilar membrane, specified in distance from the stapes (cochlear base). The structural differences along the length of the basilar membrane have the results that higher frequencies produce maximum displacements near the base of the cochlea, whereas lower frequencies cause maximum displacements near the apex of the cochlea. Thus the position of maximum activity along the length of the cochlea provides information about a sound's frequency. *APs,* Action potentials, *cps,* cycles per second. *(From Pickles JO:* An Introduction to the Physiology of Hearing. *New York, 1988, Academic Press, pp 28-30, 39, 60.)*

The functional implications of conductive development are quite different for animals that begin to hear in utero than they are for animals that begin to hear after birth. If the neonate first begins to hear airborne sounds, then the external and middle ear will determine the spectrum of the sound reaching the inner ear. Obviously the situation in utero is quite different. The normal conductive route to the inner ear becomes irrelevant in a fluid environment, and it is likely that little of the sound that reaches the fetus is transmitted through the external and middle ear. (How much sound reaches the fetus is a separate issue considered in a subsequent section.) If sound reaches the inner ear, it is most probably conducted through the skull, that is, by bone conduction. The response of the cochlea is the same whether a sound reaches it by conduction through the middle ear or through the skull. However, the way that the skull shapes the spectrum of sound reaching the inner ear will be different from the way that the external and middle ears do. When sounds are transmitted to the adult inner ear by bone conduction, with use of a vibrator placed on the mastoid, much greater sound energy is required to elicit a response. The difference is on the order of 40 dB at 1000 Hz. Furthermore, the response of the skull has a "high-frequency emphasis": Compared with air conduction, high frequencies are transmitted relatively more efficiently than low frequencies. For example, for adults, the detection threshold at 1000 Hz is approximately 15 dB lower than that at 250 Hz for air-conducted tones, but approximately 22 dB lower for bone-conducted tones. Of course, the fetal skull is not as well ossified as the adult skull, and in utero sound is transmitted to the skull from the fluid surrounding the fetus, not from a mechanical vibrator. Thus, although it is clear that the route of sound transmission to the fetal inner ear will have substantial effects on what the fetus hears, the nature of these effects is not entirely predictable from the data on hearing by bone conduction in adults. One clear implication of the differences between air-conducted and bone-conducted sound is that infants who are born prematurely will be exposed to a qualitatively different set of sounds than they would have been in utero.

The acoustic consequences of the structural characteristics of the immature conductive apparatus are predictable after birth. The resonance characteristics of a small ear canal and pinna will mean that for sounds of equal amplitude approaching the listener, high frequencies will reach the middle ear at relatively higher amplitude than low frequencies. If the ear canal is not fully open, of course, significantly less sound energy will reach the middle ear. Immaturity of the tympanic membrane and ossicles may decrease the efficiency of the middle ear in transmitting sound to the inner ear, and the response of the middle ear may be biased toward lower frequencies. Studies of the acoustic response of the conductive apparatus in nonhuman species confirm these predictions.[20-22]

The acoustics of the external and middle ear have not been described in premature human infants. Data exist for 1-month postterm infants. The external ear data are in the form of sound-field–to–ear-canal transfer functions, which show the level of sound in the ear canal as a function of frequency, when the level of sound in the surrounding field is equal at all frequencies. In adults, this function shows a broad peak of approximately 10 dB between approximately 2000 and 5000 Hz as a result of the resonance of the ear canal and pinna (Figure 171-4, *A*). The peak resonance frequency of the adult external ear is typically given as 2700 Hz. Keefe and colleagues[19] characterized the acoustic response of the infant external ear. The youngest infants included were 1-month-olds. The average resonance frequency in this age group was 4000 to 5000 Hz (see Figure 171-4, *B*), consistent with physical measurements of ear canal length. Thus, relative to adults, the level of sound reaching the neonatal middle ear at frequencies in the 1000 to 3000 Hz range will be lower. Keefe and colleagues[19] also found that the sound-field–to–ear-canal transfer function is not mature by 24 months postnatal age, but the age at which the transfer function becomes adultlike is not known.

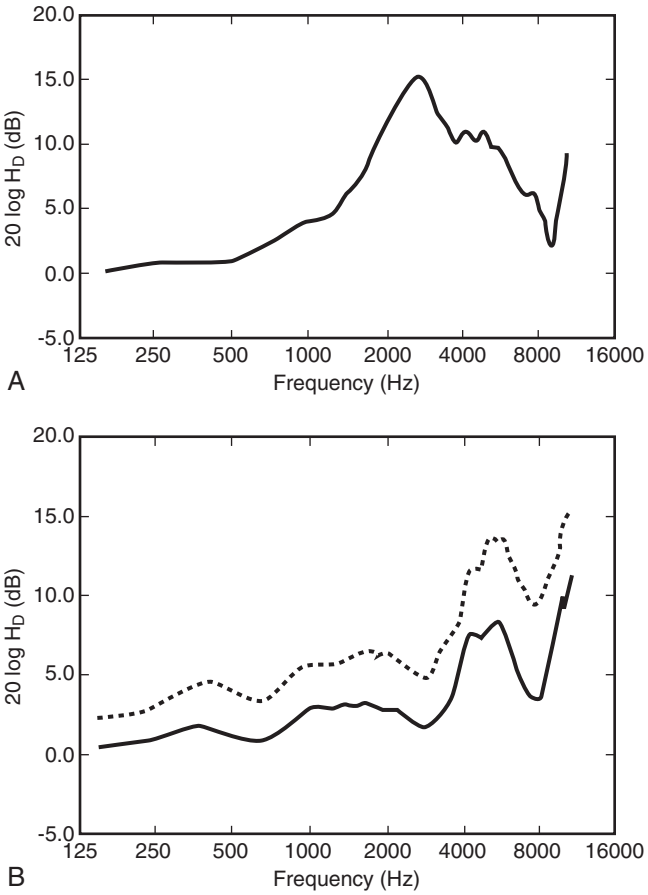


Figure 171-4 Diffuse field transfer functions measured in a KEMAR human adult manikin **(A)** and for the average 1-month-old infant **(B).** The *solid line* is the average; the *dashed line* is the average plus 1 SD. H_D, Diffuse field transfer function magnitude. *(From Keefe D, Burns EM, Bulen JC, et al: Pressure transfer function from the diffuse field to the human infant ear canal,* J Acoust Soc Am *95:355, 1994.)*

Keefe and colleagues[23,24] have also published the only comprehensive studies of the acoustic properties of the infant middle ear. Keefe and colleagues[23,24] estimated the acoustic conductance, or flow of acoustic energy, looking into the middle ear in the ear canals of newborns and 1- to 24-month-olds. The results for newborns, 1-month-olds, and adults are shown in Figure 171-5. The conductance of the infants' middle ear is smaller than that of adults for frequencies above 500 Hz, and the greatest difference is at the higher frequencies. For frequencies above 1000 Hz some improvement appears to occur in conductance in the first postnatal month, but at approximately 4000 Hz infants would be expected to lose 10 to 15 dB relative to adults. The combined effects of external and middle ear immaturity could lead to a relative loss of 15 to 20 dB in the higher frequencies. At frequencies below 500 Hz, both neonates and 1-month-olds actually appear to generate higher levels of sound in the middle ear than adults do. In fact, conductance appears to decline between birth and 1 month and again between 1 month and adulthood in the low frequencies. This result is consistent with the presence of a low-frequency resonance in the neonatal ear canal, perhaps resulting from the greater compliance of the ear canal walls. This

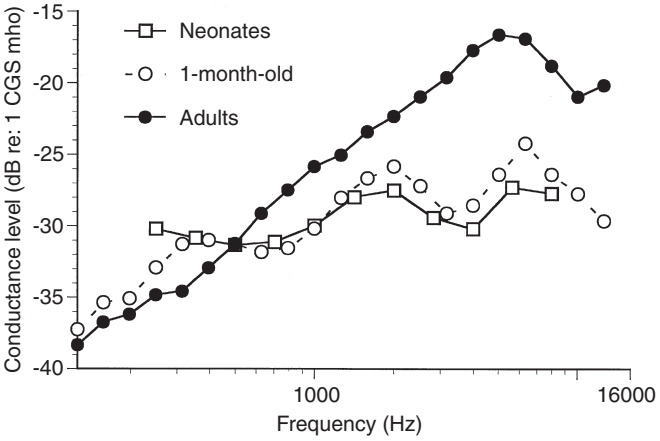


Figure 171-5 Ear canal conductance level, indicating energy flow into the middle ear, as a function of frequency for adults, neonates, and 1-month-old infants. *CGS,* Centimeter-gram-second. *(From Keefe D, Bulen JC, Arehart KH, et al: Ear-canal impedance and reflection coefficient in human infants and adults,* J Acoust Soc Am *94:2617, 1993; and Keefe D, Folsom RC, Gorga MP, et al: Identification of neonatal hearing impairment: ear-canal measurements of acoustic admittance and reflectance in neonates,* Ear Hear *21:443, 2000.)*

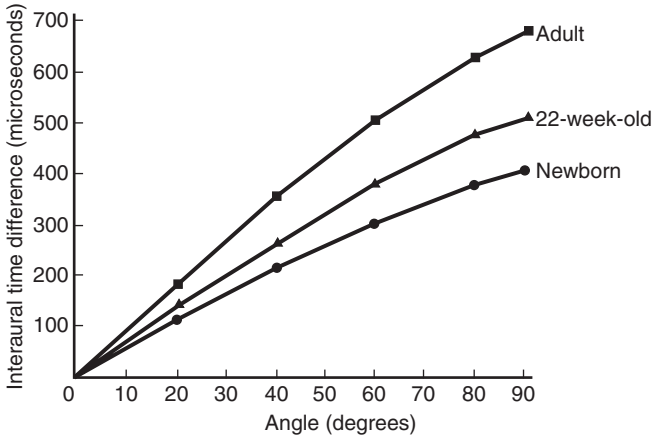


Figure 171-6 Interaural time differences produced by sounds as a function of their position in azimuth, for adults, 22-week-old infants, and newborns. *(From Clifton RK, Gwiazda J, Bauer J, et al: Growth in head size during infancy: implications for sound localization,* Dev Psychol *24:477-483, 1988.)*

could further explain why tympanometry may not be a useful diagnostic tool in young infants.[25]

It is not often appreciated that growth of the pinna, ear canal, and head also has important implications for sound localization. The primary cues to the location of a sound source in the horizontal plane are differences in the phase and intensity of the sound arriving at the two ears.[26] Small heads would produce smaller interaural differences. Clifton and colleagues[27] measured head diameter longitudinally in a group of infants from birth to 22 weeks of age. From these measurements, they estimated that the largest interaural time difference that an infant's head could produce increased from 420 microseconds at birth to 520 microseconds at 22 weeks of age. The average adult head, on the other hand, could produce an interaural time difference on the order of 700 microseconds. Thus, for any sound source location, the infant must work with a smaller cue (Figure 171-6). Similar effects would be expected for interaural intensity differences. Interaural differences would also vary with sound frequency differently when the head is small: interaural phase differences would be useful to fairly high frequencies, but interaural intensity differences in the midfrequency range would be relatively poor.[28] Finally, the sound-field–to–ear-canal transfer function changes with the position of the sound source relative to the ear. These "spectral cues" are used to localize sounds in elevation and to distinguish sound source locations behind the head from those in front of the head. One would expect that smaller pinnas and ear canals would produce poor spectral cues for the localization of sound sources in the midfrequency range. However, the relative contributions of conductive, cochlear, and neural maturation to the development of sound localization remain to be established.

Cochlea

The earliest structures that can be identified as external, middle, and inner ear arise in close temporal proximity. The otic placode, a thickening of the surface ectoderm at the level of the caudal hindbrain, can be seen early in the fourth week of gestation. The placode invaginates, closes off, and detaches from the epidermal surface to form the otocyst, or otic vesicle, a few days later. Shortly thereafter the precursors of the spiral ganglion migrate out of the otocyst and come to lie ventromedial to the developing inner ear. As the precursors of the specialized support cells and hair cells are born, the otocyst elongates and the older cells are "pushed" toward the distal end of the presumptive cochlea.[29] These undifferentiated cells form a mass along the dorsal wall of the tube. The otocyst coils at it elongates, eventually forming two and a half turns by the 25th week of gestation. These events are illustrated schematically in Figure 171-7.

The bony labyrinth that contains the cochlea begins to ossify at approximately 16 weeks gestation. This process must be closely coordinated with cochlear growth and innervation. In fact Van De Water and colleagues[30-33] have demonstrated that a complex series of interactions between epithelial and mesenchymal tissues regulates the growth, ossification, and differentiation of the inner ear structures. The otic capsule appears to be largely ossified by approximately 20 weeks gestational age.[34]

In a series of papers, Lavigne-Rebillard, Pujol, and colleagues[35-43] have described the early development of the human cochlea using electron microscopy. Their results are quite similar to those reported for nonhuman mammals.[43] As described in other vertebrates, the afferent processes of the spiral ganglion grow into the presumptive cochlea and make contact with undifferentiated cells by 9 weeks gestation. These cells then separate from their basement membrane and migrate toward the luminal surface of the epithelium. Once the cells have reached the luminal surface, stereocilia form, and other features of hair cells become evident. Efferent processes are not identified in the cochlea until some time later, approximately 12 weeks gestation in humans.[38,44-46] The stria vascularis, which is responsible for maintaining the high resting potential of the cochlea, appears at 9 weeks gestation, matures slowly between 14 and 20 weeks, and by 34 weeks appears adultlike.[37] The tectorial membrane also appears at approximately 9 weeks gestation. Inner hair cells tend to differentiate and mature ahead of outer hair cells. Innervation and differentiation of support cells and hair cells tend to occur earlier at or near the base of the developing cochlea.[38,47] These developmental gradients have later functional implications, because inner and outer hair cells play quite different roles in the transduction process and because the frequency eliciting the greatest response decreases progressively along the length of the cochlea from base to apex.

When responses to sound can first be observed, the inner ear is immature in several respects. The fluid spaces within the cochlea are not well developed, the basilar membrane is thick,

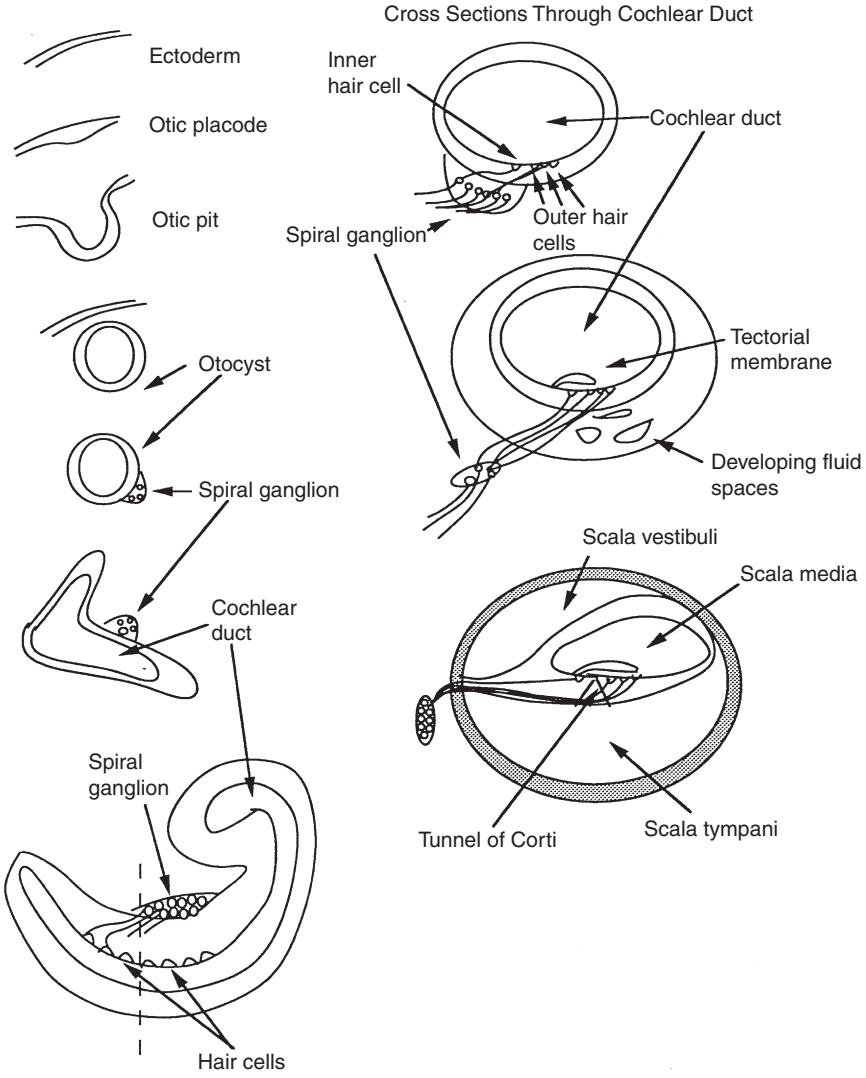


Figure 171-7 Major events in the development of the inner ear.

and the physical arrangement of the support cells and hair cells is immature (Figure 171-8). Such factors are likely to limit the mechanical response of the cochlea. In addition, the innervation of the hair cells is immature.[36,38,42,48] In mature mammals, afferent endings predominate on inner hair cells, and efferent fibers synapse on afferent fibers, not on the inner hair cell membrane. Around the time that cochlear responses can first be recorded, both afferent and efferent synapses can be found on the inner hair cell membrane. Mature outer hair cells feature massive efferent endings encapsulating their basal membrane and a few small afferent endings. At the onset of hearing, the number of afferent and efferent endings contacting outer hair cells is more nearly equal, and both types of ending are rather small and bouton-shaped (Figure 171-9). The stria vascularis still differs from the adult in several respects, suggesting that the endocochlear potential, the "battery" that drives the transduction process, is immature. In recent electron microscopic studies, Lavigne-Rebillard and colleagues[35-37] have shown that these features are characteristic of the human cochlea approaching 20 to 22 weeks gestational age.

An obvious implication of the structural immaturity of the cochlea at this stage of development is that sensitivity to sound will be quite poor. More acoustic energy will be required to set the basilar membrane in motion, and energy will be transferred to the stereocilia less efficiently. Inner hair cells undoubtedly transmit their message to the central nervous system. The outer hair cells, however, are known to act as effectors, mechanically amplifying the basilar membrane response and increasing the frequency specificity of the response. To the extent that the outer hair cells are immature, sensitivity may be poorer by 30 to 40 dB.

It is well documented in nonhumans that the combination of conductive and cochlear immaturity is reflected in cochlear responses[49]; in single-unit responses in the auditory nerve, brain stem, and cortex (e.g., Woolf and Ryan[50] and Brugge and colleagues[51-53]); and in gross evoked potentials.[3,6,54] When responses to sound are first observed, thresholds are quite high, on the order of 100 dB sound pressure level (SPL). Although the precise time course varies across species, sensitivity improves quite dramatically in the subsequent days or weeks. In humans, this rapid maturation of auditory sensitivity probably occurs between 24 and 35 weeks gestational age. Unfortunately, no data document the course of auditory physiologic development before approximately 28 weeks gestational age.

Otoacoustic emissions (OAE) are sounds generated by the action of the outer hair cells that can be recorded in the ear canal that provide a direct measure of cochlear function.[55] OAE can generally be recorded in normally functioning cochleas, as long

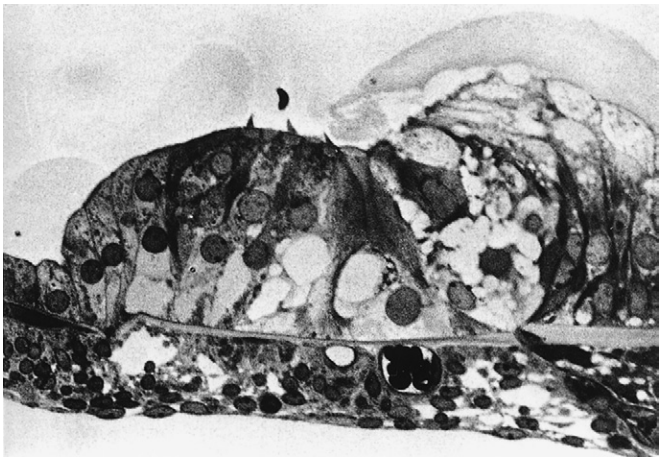


Figure 171-8 Cross-sections through the cochlea of a 20-week-old human fetus, near the upper basal coil. The fluid spaces and mechanical arrangement of cells are underdeveloped. *(From Igarashi Y, Ishii T: Embryonic development of the human organ of Corti: electron microscopic study,* Int J Pediatr Otorhinolaryngol *2:51, 1980.)*

as their transmission through the middle ear is not blocked. OAE have been recorded in infants as young as 30 weeks conceptional age. Click-evoked OAE have been recorded in nearly all infants by 33 weeks conceptional age, at a threshold 10 to 15 dB higher that that of adults.[56] The amplitude to OAE to a fixed-level click also increases between 30 and 40 weeks conceptional age.[57-59] A frequency-specific OAE can also be recorded when two tones close in frequency, f_1 and f_2, are presented to the ear. In this case, the emission is a pure tone with a frequency equal to $2\ f_1\text{-}f_2$ and is known as a distortion product OAE (DPOAE). Lasky[60,61] recorded DPOAE in preterm infants, term infants, and adults. The minimum stimulus level required to elicit a DPOAE was similar across age groups, at least for stimulating tones at approximately 4000 Hz. Lasky concluded that nearly all of the observed age-related differences in DPOAE could be accounted for by conductive factors.

The *auditory brain stem response* (ABR) is a scalp-recorded potential reflecting the aggregate response of neurons in the auditory nerve and brain stem. Wave I of the ABR is known to arise from the auditory nerve; the inferior colliculus makes a major contribution to wave V, though it is not clear that it is the sole generator.[62] Adult ABR thresholds for a click are typically approximately 10 dB normal adult hearing level (nHL).[62] The ABR can first be reliably elicited from preterm infants at approximately 28 weeks postconceptional age, although a few

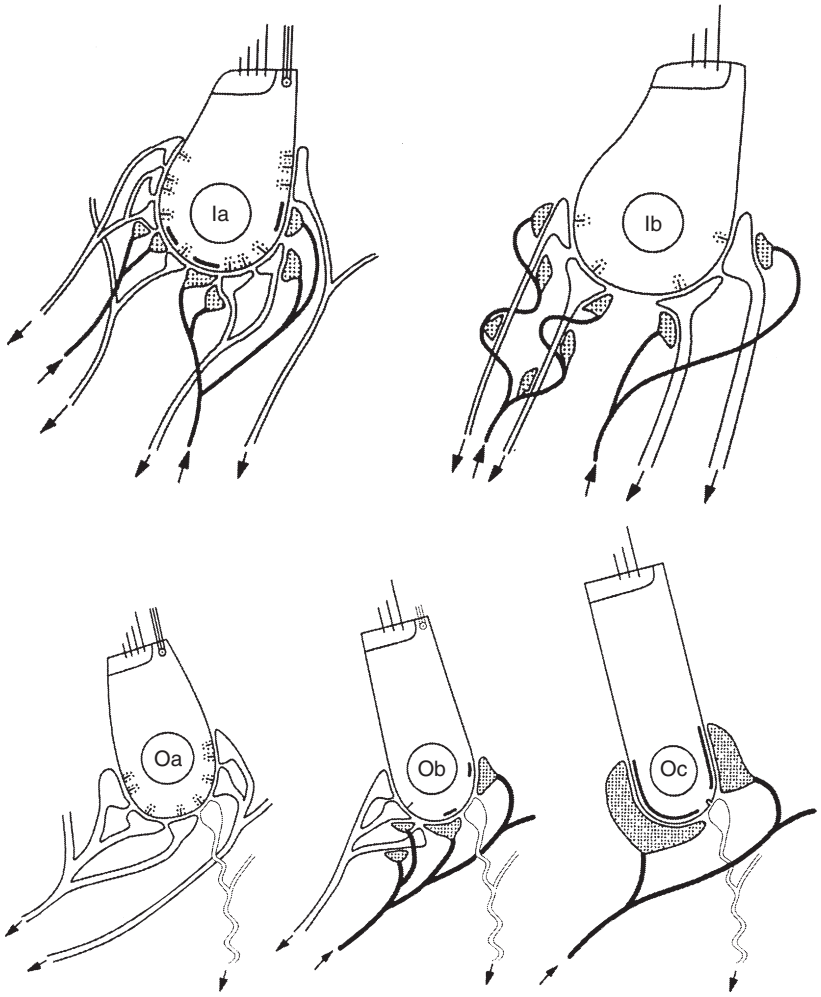


Figure 171-9 Changes in the innervation patterns of inner and outer hair cells around the time that the cochlea begins to function. Efferents are filled fibers with vesiculated profiles and *inward arrows*. Afferents are open fibers with *outward arrows. I,* Inner; *O*, outer. *(From Pujol R, Lavigne-Rebillard M, Lenoir M: Development of sensory and neural structures in the mammalian cochlea. In Rubel EW, Fay RR, Popper AN, et al, editors:* Development of the Auditory System, *New York, 1998, Springer Verlag, pp 146-192.)*

reports exist of responses at 26 or 27 weeks.[63,64] Lary and coworkers[64] reported that the average click intensity required to elicit an ABR decreased from approximately 55 dB nHL at 27 weeks conceptional age to 21 dB nHL at 38 weeks conceptional age. Subsequent studies have confirmed this result.[65,66] This pattern is consistent with an improvement in auditory sensitivity on the order of 25 to 30 dB between the onset of the response and term birth, but the relative contributions of conductive, cochlear, and neural sensitivity to the ABR threshold are not known. The latency of wave I decreases between 33 and 44 weeks conceptional age,[63] but latencies may be prolonged without concomitant sensitivity loss.[67]

One result that seems to be consistent across studies is that by the time of term birth, thresholds for cochlear responses elicited by broadband sounds are within 10 or 15 dB of adult values. This is the case for wave I of the ABR,[62] OAE,[56] and electrocochleograms (scalp-recorded cochlear potentials).[68] Because a threshold shift of that magnitude can easily be accounted for by conductive factors,[23] these reports suggest that the cochlea is probably mature by 40 weeks postconceptional age.

Coincident with the improvement in sensitivity, a dramatic improvement in the frequency selectivity of the cochlear response occurs in nonhumans just after hearing begins. In the mature mammal, each inner hair cell and the afferent fibers connected to it respond over a range of approximately one third of an octave. When the cochlea begins to respond to sound, many auditory nerve fibers are essentially "untuned," and the best fibers respond over a range as broad as three octaves (Figure 171-10).[69] Frequency specificity is closely correlated with sensitivity during development, and its maturation is likely the result of both mechanical maturation of the basilar membrane and the outer hair cells. Many functional consequences of reduced frequency selectivity would be expected, although few of these expectations have been put to the test. Sensitivity in noise is known to be especially poor.[70] The internal representation of the spectrum of a complex sound will also be "fuzzy" to the novice listener, and similar frequencies would be difficult to distinguish.

Frequency selectivity at the cochlear level has been studied with DPOAE. The amplitude of DPOAE can be reduced by presenting external sounds that are close to the emission in frequency; the emission is said to be "suppressed." In fact, the better the cochlear frequency selectivity, the narrower the range of frequencies that will suppress the emission. The curve that shows the level of an external tone required to reduce the emission by a criterion amount, as a function of the frequency of the tone, is termed a *suppression tuning curve*. Abdala[71-73] has found that preterm and term infants demonstrate sharper suppression tuning curves, or better frequency selectivity, than adults (Figure 171-11). Abdala argued that early in development, the human cochlea "overshoots" the amplification required, as observed in young gerbils by Mills and colleagues.[74] However, it is also true that suppression tuning curves measured at lower intensity are generally sharper than those measured at higher intensity. Thus attenuation of the sound reaching the infant cochlea by an immature middle ear could be responsible for the age difference in tuning. Three articles now support the hypothesis that apparent differences in cochlear tuning in neonates result from immaturity of the middle ear.[75-77] Of course, neural limitations may effectively limit the frequency selectivity of the system, as discussed later.

As noted earlier, many events in cochlear development tend to occur in structures located at or near the base of the cochlea before they occur in structures located at the apex. Because the mass and stiffness of the basilar membrane vary along its length, in mature vertebrates high-frequency sounds produce greater responses than low-frequency sounds at the base of the cochlea, whereas low-frequency sounds produce greater responses at the apex. Thus one might predict that responses to high-frequency sounds might appear and mature earlier than those to low-frequency sounds. In fact, just the opposite is typically observed: the initial responses to sound are limited to the low or low-to-middle part of the mature animal's range of hearing. The range of frequencies that elicit responses gradually increases as sensitivity improves during development. The most likely explanation for this apparent paradox is that the basal cochlea is most responsive to relatively low frequencies at the time that it begins to respond.[78-81] Recent studies suggest that the immature frequency response of the basal cochlea is the result of mechanical properties of the basilar membrane.[74] Although such effects have yet to be demonstrated in humans, they would be predicted to occur between 24 and 37 weeks gestation.

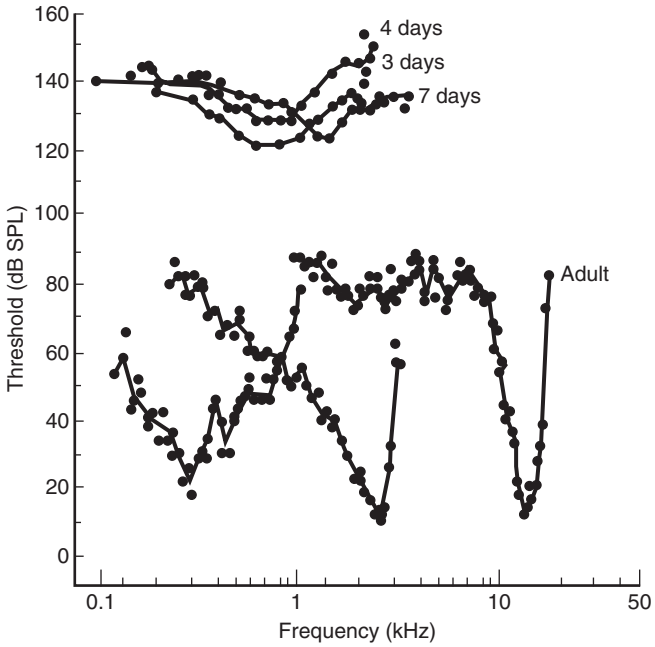


Figure 171-10 Level required to produce a threshold response in fibers of the cat auditory nerve as a function of frequency. Each curve represents the response of a single nerve fiber. Adult responses tend to be "tuned." The fiber tends to respond to very low sound levels within a restricted frequency range. Responses of 3- to 7-day-old kittens require very high sound levels and are not well tuned. *SPL,* Sound pressure level. *(From Walsh EJ, McGee J: Postnatal development of auditory nerve and cochlear nucleus neuronal responses in kittens,* Hear Res *28:97-16, 1987.)*

Congenital hearing loss occurs in 2 or 3 per 1000 neonates.[82,83] Because cochlear response can be readily assessed in neonates with use of OAE and ABR and because the vast majority of congenital hearing losses originate in the ear, these measures are commonly used in newborn hearing screening.

Early Hearing Detection and Intervention (EHDI) Programs

Click, or *transient*, evoked OAE, DPOAE, and ABR have been shown to be equally sensitive screening measures, with similar referral rates.[84,85] Concurrent measurement of middle-ear function may allow distinction between transitory or permanent conductive impairment and cochlear impairment.[24] Ideally, infants who fail the initial screen are evaluated before 3 months of age, and early intervention services are implemented before 6 months. Early intervention is associated with near-normal language development by the preschool period.[86,87] Early cochlear implantation is now a good option for infants with severe-to-profound hearing loss. It should be noted, however, that children with mild-to-moderate or unilateral hearing loss may be missed by EHDI programs. Furthermore, if OAE alone are considered, hearing

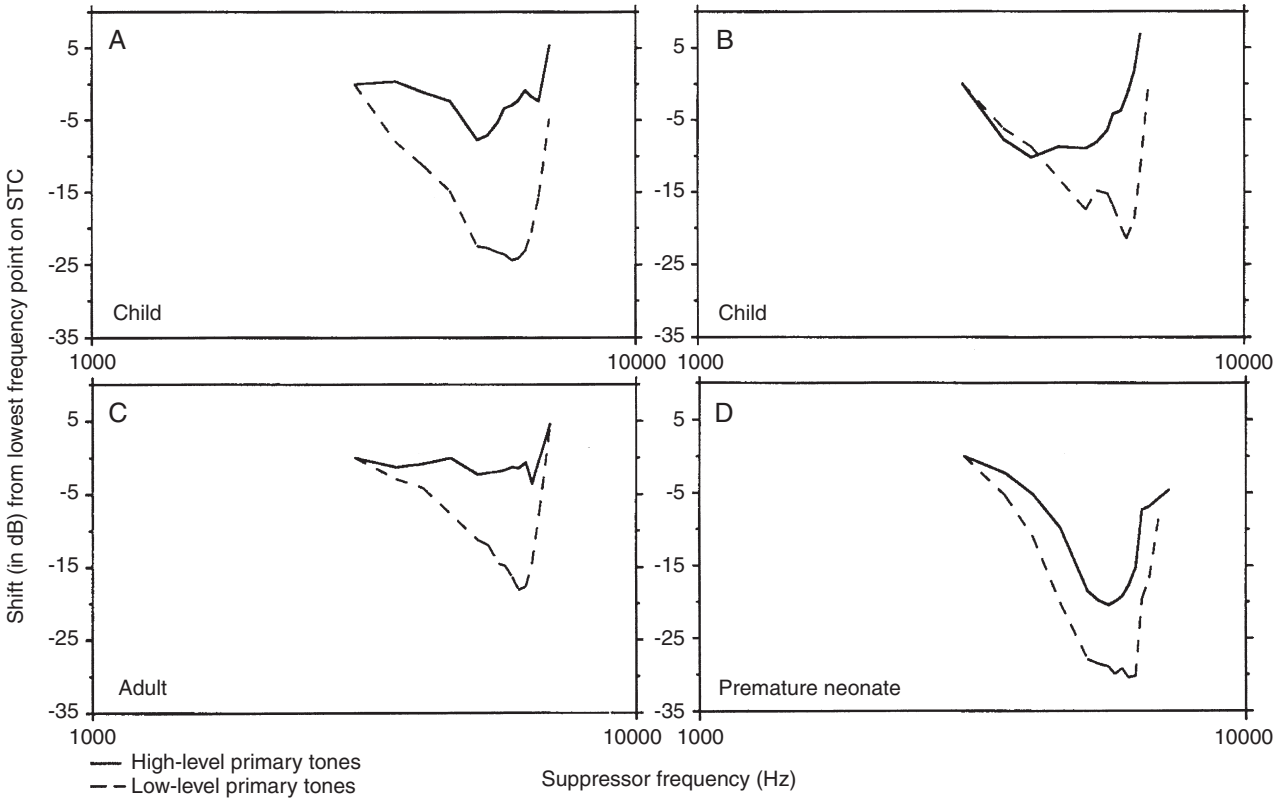


Figure 171-11 Relative level of a suppressor tone required to suppress a distortion product otoacoustic emission, as a function of the frequency of the tone, so-called suppression tuning curves *(STC),* for high- and low-level stimulating ("primary") tones. Each emission tends to be suppressed with low-level tones close to its own frequency. **A** and **B,** Responses from children 8 to 12 years of age. **C,** Response from an adult. **D,** Response from a premature infant. The premature infants' response is sharper than those of other subjects, and the sharpness is maintained at high levels of stimulation. *(From Abdala C: DPOAE suppression tuning: cochlear immaturity in premature neonates or auditory aging in normal-hearing adults?* J Acoust Soc Am *110:3155-3162, 2001.)*

disorders that result from so-called *auditory neuropathy* will also not be identified.[88]

More than half of congenital hearing losses have genetic causes, with the remainder resulting from infection, exposure to ototoxic drugs, prematurity, or other environmental causes.[89,90] Hearing loss is associated with several hereditary syndromes, including Waardenburg, branchio-oto-renal, Stickler, Pendred, and Usher among others. The majority of genetic deafness, however, is nonsyndromic. Tremendous progress has been made in the last decade in identifying the genes responsible for many types of nonsyndromic hearing loss. More than 70% of nonsyndromic congenital hearing losses are autosomal recessive; approximately half of these result from mutations of *GJB2* and/or *GJB6.*[91] Approximately 1 in 33 people of northern European descent in the United States is a carrier of a recessive deafness-causing mutation of *GJB2.*[92] *GJB2* and *GJB6* encode Connexin 26 and Connexin 30, respectively. These proteins form the gap junctions that provide intercellular bridges for the recirculation of potassium ions into the cochlear endolymph. The other cases of nonsyndromic congenital hearing loss result from mutations of numerous other genes, and some of these occur in only one or two families.[93,94] Some types of genetic hearing loss, particularly syndromic and autosomal dominant types, have their onset later in childhood or in adulthood.[95]

Central Auditory Nervous System

The limited data concerning the development of the human auditory nervous system are often found in articles dealing with the development of the whole brain.[96] Although this situation has begun to be remedied,[97-104] the available information with respect to human auditory neural development remains sparse. What data are available suggest, however, that substantial differences do not exist between humans and nonhumans in the development of the primary auditory pathways. In all vertebrates the neurons of the auditory pathways undergo final mitosis, migrate to their mature locations, and form appropriate connections in parallel with developmental events at the periphery; by the time that the cochlea begins to respond to sound, gross evoked responses can be recorded in primary auditory cortex.[105,106]

Early sensitivity to sound, however, may be limited by brain stem immaturity. Sininger and Abdala[107] estimated newborns' frequency-specific ABR threshold, calibrating their stimuli in the ear canal to control for age differences in ear canal size. Their data show that newborns are close to adultlike in sensitivity at 500 Hz but that they are 20 to 25 dB less sensitive than adults above 4000 Hz. The high-frequency age difference is greater than can be accounted for by middle-ear immaturity alone.

Neural immaturities appear to limit the frequency selectivity of the auditory system early in human development. Two recent studies show that the ABR to a high-frequency sound is affected by a broader range of interfering frequencies, or "maskers," in 3-month-old infants than in adults.[108,109] This suggests that the frequency selectivity of the infant brain stem response is poorer at high frequencies. Because the cochlear response appears to be mature by this age,[110] the source of the immaturity in frequency selectivity is likely to be neural. Presumably, these immaturities will be more pronounced for still younger infants, and the immaturities may extend to lower frequencies as well.

Of course, at younger ages, cochlear immaturities may also be present, and it is not known how cochlear and neural immaturities may interact.

The work of Sanes and his colleagues has been important to understanding how neural maturation and experience with sound contribute to the development of frequency resolution. Sanes and Constantine-Paton[111] reported that exposing young mice to repetitive clicks resulted in a reduction in neural frequency selectivity that could not be explained by peripheral effects. The selectivity of neural connections, then, can affect frequency selectivity, and normal experience with sound appears to be necessary to induce or maintain the nervous system's ability to accurately transmit the selective response established at the cochlear level. Sanes and Rubel,[112] moreover, noted that fine frequency selectivity was not characteristic of all neurons in the auditory brain stem. Within a single frequency region, neurons with mature selectivity and neurons with very immature selectivity could be found. Because all of these neurons receive their afferent input from the same cochlear location, it is difficult to see how cochlear factors could produce such variability in response. In other studies Sanes and co-workers[113] directly measured the increased specificity of neural connections in one auditory brain stem nucleus, and Sanes[114] showed that maturation of structural specificity was reflected in maturation of neural response specificity. Finally, it should be noted that although some neural response patterns appear mature when described on a relative scale, they appear quite immature on an absolute scale. Thus it is becoming increasingly evident that neural immaturities impose a fundamental limitation on early auditory function, although a detailed description of these immaturities is not yet possible. Certainly in humans, structural maturation of auditory cortex continues until 11 or 12 years of age.[102,103]

A commonly reported characteristic of immature auditory neurons is that they cannot sustain a continuous response.[115] In addition, their maximum response rate is quite low (summarized by Sanes and Rubel[116]). Such response characteristics would have profound influences on all aspects of acoustic coding. A reduction in the range of intensities that could be processed would be expected, the ability to distinguish a change in intensity would differ, and, of course, sensitivity would be reduced as well. The "fatigability" of young neurons is almost certainly not a result of cochlear limitations, although the reduction in dynamic range may well reflect similar limitations in the cochlear response. Although no data are available in this regard for preterm infants, Durieux-Smith and colleagues[117] have reported a similar limitation in the neonatal ABR: increasing the intensity of a click results in a smaller increase in response amplitude for term neonates than it does for adults (Figure 171-12). Cornacchia and colleagues[118] reported that this characteristic of the ABR is adultlike by 6 months after term.

Neural maturation is also clearly reflected in the development of temporal aspects of the neural response. Progressive myelination of neural pathways is noted during development in all vertebrates. It is often stated that myelination of the brain in general, and of the auditory pathway in particular, proceeds in a peripheral-to-central gradient.[96] More recent studies suggest that this gradient holds only on a coarse scale. Moore and colleagues[101,119] have found that, in the human auditory system, the brain stem pathway is myelinated at approximately the same time along its length but that the brain stem pathway is well myelinated before myelin is observed in the brain stem–to–midbrain pathway. Increased myelination would certainly increase the speed with which information is transmitted through the auditory system. In fact, the latency of both single-unit responses and of gross evoked potentials progressively decreases during development, and the age-related change in response latency is greater in more central nuclei.[5,53] Increased myelination might also be expected to produce improvements in auditory functions that depend on a precise temporal representation of the stimulus, such as sound localization. Variability in the latencies of action potentials, both within and between neurons, would be reduced in well-myelinated nerve fibers.

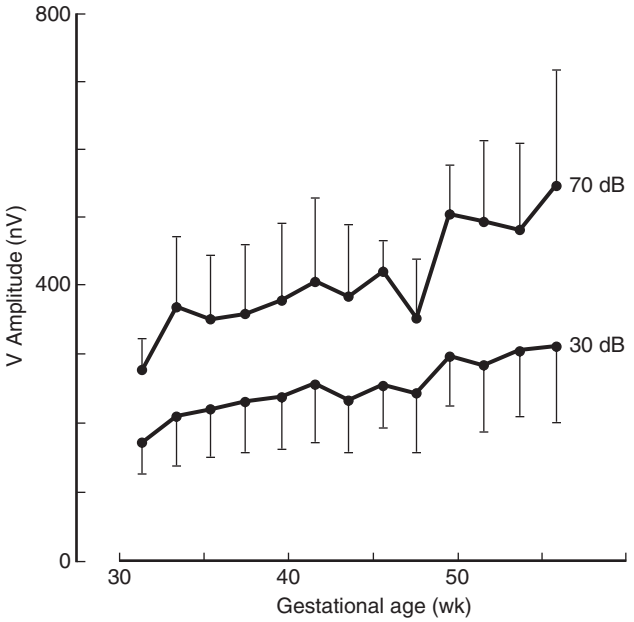


Figure 171-12 Amplitude of wave V of the auditory brain stem response as a function of gestational age for two sound intensities. The effects of increasing intensity are greater at later gestational ages. *(From Durieux-Smith A, Edwards CG, Picton TW, et al: Auditory brainstem responses to clicks in neonates,* J Otolaryngol *14:12-18, 1985.)*

Response latency is the most frequently studied characteristic of evoked potentials in humans, and it is one of the few measures that has been made extensively for both preterm and term neonates. Gorga and colleagues,[63] for example, reported that between 33 and 44 weeks conceptional age, wave I latency in response to 80 dB nHL clicks decreased from approximately 1.8 milliseconds to approximately 1.6 milliseconds. Wave V latency decreased from 7.0 milliseconds to 6.5 milliseconds over the same period. The implication of the fact that wave V latency decreases more than wave I latency is that most of the decrease in wave V latency stems from neural maturation. One interesting aspect of these results is that the effects of increasing intensity were no different for the youngest (33-34 weeks conceptional age) and oldest (43-44 weeks conceptional age) infants (see also Zimmerman and colleagues[120]). In fact, the effects of level on wave V latency were adultlike in the youngest preterms examined (Figure 171-13). By 40 weeks conceptional age wave I latency is close to adult values, but wave V latency continues to decrease into the second postnatal year (e.g., Hall[62] and Eggermont and Salamy[121]).

Two issues concerning the infant ABR have both developmental and clinical implications. First, some reports exist of rapid improvements in ABR detectability, amplitude, and latency in the first 48 hours after birth.[122-125] Explanations for these rapid postnatal changes vary. The typical explanation is that early postnatal changes result from clearing of fluid or other material from the middle ear,[125-127] although some argue that cochlear adaptation to extrauterine life is responsible.[127-131] Yamasaki and colleagues[122] found that the wave I-V interwave interval, the so-called central conduction time, also decreased in the immediate postnatal period. How differences in central conduction time could result from either conductive or cochlear factors is not

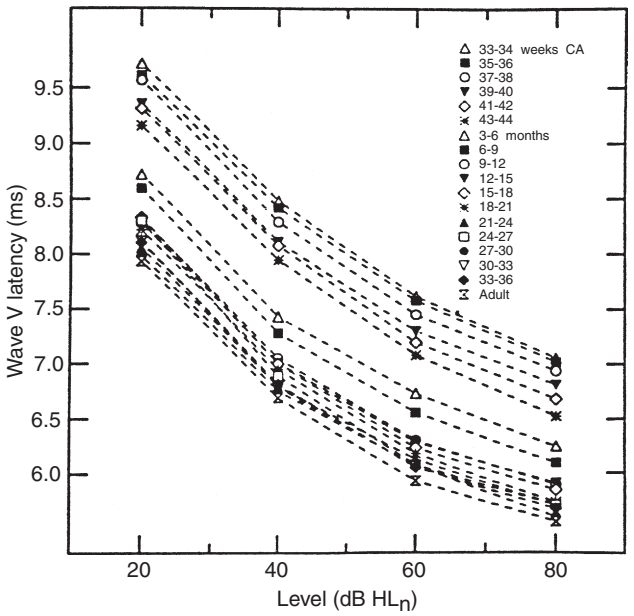


Figure 171-13 Latency of wave V of the auditory brain stem response as a function of sound intensities at various ages. The curves are more or less parallel. HL_n, Normalized hearing level. *(From Gorga MP, Kaminski JR, Beauchaine KL, et al: Auditory brainstem responses from children three months to three years of age: normal patterns of response II, J Speech Hear Res 32:281-288, 1989.)*

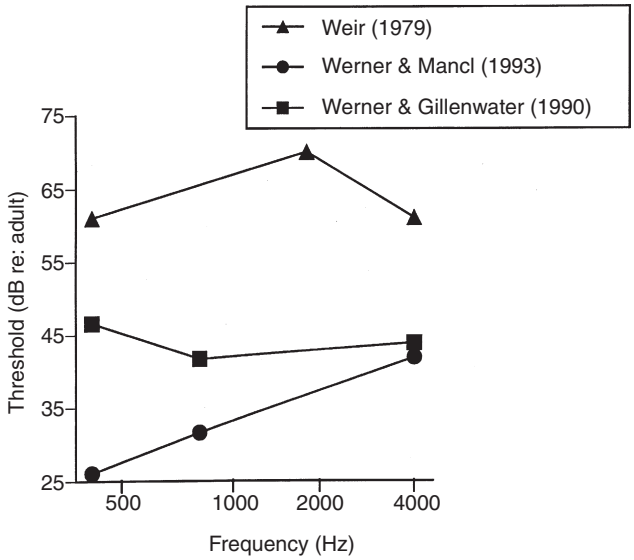


Figure 171-14 Comparison of detection thresholds, expressed relative to adult threshold, at three frequencies from 0- to 1-month-old infants in three studies. *(From Werner LA, Marean GC:* Human Auditory Development, *Boulder, CO, 1995, Westview Press.)*

clear. Thus, although the reason for rapid changes in the ABR after birth are not settled, a consensus seems to exist that stable ABRs are most readily obtained after 48 postnatal hours.

The second issue concerns the use of the ABR in the preterm population. Essentially the question is whether extrauterine experience affects ABR parameters independently of conceptional age. In other words, is the ABR of a 40-week conceptional age infant whose gestational age is 32 weeks different from the ABR of a 40-week conceptional age infant who was born yesterday? Some studies report no differences between preterm and term infants of equal conceptional age.[132,133] Eggermont and Salamy[121] found that preterm infants tended to have longer wave latencies but equivalent interwave latencies. Eggermont and Salamy[121] propose that this difference arises from the higher incidence of otitis media in the preterm population. Collet and colleagues,[134] in contrast, report shorter interwave intervals among preterm infants and conclude that the smaller head size of preterm infants is responsible. In any case, the differences between term and preterm infants of the same conceptional age are small, and little evidence exists of a direct effect of extrauterine experience on the development of the ABR.

At least three factors influence the latency of evoked potentials and are, thus, potential explanations for developmental changes. First, sensitivity at sites peripheral to the generator of a given wave contributes to its latency. For example, latencies of all ABR waves are longer for lower intensities of sound and in cases of conductive or sensorineural hearing loss.[62] The changes in wave I latency with conceptional age are consistent with approximately a 20-dB conductive immaturity at 33 to 34 weeks conceptional age and a 10- to 15-dB conductive immaturity at term birth, as suggested by the middle-ear data.[23] To achieve a wave V latency of 7.5 milliseconds, however, the data of Gorga and colleagues[135] indicate that a term infant requires a level approximately 25 dB higher than an adult (Figure 171-14). It is clear that neither conductive nor cochlear factors can account for such a large difference. Second, myelinization of the auditory pathway would increase conduction velocity; it is well established that myelinization of the auditory pathways progresses through the postnatal period and into childhood.[96,119] This is the most clearly established contribution to ABR latency development; by some accounts, conduction time improvements can completely account for changes in the latency of the ABR during development.[136] Third, synaptic efficiency may play a role. The number of synapses in the auditory brain stem continues to increase throughout childhood and into adulthood,[137] although the efficiency with which each synapse operates, an unknown quantity, may be more relevant. One finding that suggests that synaptic efficiency may be important is that the wave I-V interwave interval is positively correlated with behavioral thresholds in early infancy.[138] No evidence exists that neural latency directly affects sensitivity; in fact, latencies can be very abnormal while sensitivity remains normal.[67] A loss of information resulting from inefficient synaptic transmission could well affect both sensitivity and evoked response latency. Rotteveel and colleagues[65] also note an association between ABR threshold and interwave intervals among preterm infants.

Other evoked responses, such as the middle and long latency responses that originate from the midbrain and cortex, have not been studied extensively in neonates (see Hall[62] for a review). It appears that the difference between infants and adults in threshold for the middle latency response (MLR), for example, is greater than the difference in ABR threshold,[139] and MLR latencies take longer to reach adult values.[140-142] Cortical responses have been recorded, even in preterm infants,[106] but again the developmental course of such responses is prolonged. Although the factors that contribute to the delayed maturation of MLRs and late responses have not been delineated, their later development is consistent with the later myelinization of the midbrain and cortical pathway. Other factors may also be involved.

Of course, latency is not the only temporal characteristic of neural responses. Because of the characteristics of the transduction process, auditory neurons tend to fire at the same phase of the stimulus on each cycle of a periodic sound; the neural response is said to be "phase-locked." Phase locking is strong

enough and the neural transfer of the phase-locked response is precise enough that the period, and thus the frequency, of a sound could be estimated quite well on the basis of the interval between action potentials. The precision of neural phase locking is likely to depend on both the efficiency of synaptic transmission and the extent of myelination of nerve fibers. Phase locking is believed to contribute to pitch perception, among other things, and it is the basis of interaural phase difference processing in sound localization. Of the basic auditory neural response properties, phase locking is the one that takes longest to develop. Brugge and colleagues[51] reported that phase locking in the cat's auditory nerve matured over a 2-week period, with fibers responding to low frequencies achieving mature phase locking earlier. The development of phase locking at the level of the cochlear nucleus followed the same frequency gradient but took as long as 2 months to complete.[143] The course of its development in nuclei central to the cochlear nucleus has not been described. The relative contributions of synaptic maturation and increased myelination (see later) to the development of phase locking have not been assessed. The frequency-following response (FFR) is a scalp-recorded response that has the same frequency as the tone used to elicit it. The FFR is believed to reflect the aggregate phase-locked responses in the auditory nerve and brain stem.[62,144] Two studies have examined the FFR in term neonates[145-147]; in general, the response appears to be adultlike. Similar measures of phase locking at more central locations in the nervous system have not been developed. Again, no data on the development of temporal processing are available for preterm infants.

Another property of neurons that has implications for temporal coding is adaptation. Auditory nerve fibers show a characteristic high response rate at stimulus onset, followed by a rapid decrease in response rate to a lower, steady rate. This decrease in response over time is known as adaptation. After the offset of the stimulus, a recovery period occurs during which it is difficult to elicit responses from the neuron; even spontaneous activity is absent during recovery. Evidence exists that the immature auditory system is more susceptible to adaptation than the mature system and that it takes longer to recover from the effects of adaptation. In human infants increasing the rate of presentation of clicks used to elicit the response has a greater effect on response latency for newborns.[126,148-150] The size of the latency shift with increasing stimulus rate decreases progressively between 28 and 50 weeks conceptional age,[151] and the effect is more pronounced for wave V than it is for wave I, suggesting a neural contribution to the effect on wave V. By 3 months of age, the ABR shows "rate effects" similar to those seen for adults. Responses originating at more central locations, such as the MLR and cortical responses, continue to be more susceptible to increased stimulation rate well into childhood.[140,152]

In a series of studies summarized by Altman and Bayer,[153] the first neurons to undergo their final mitosis, in nearly all auditory nuclei, were the neurons that in adulthood would respond to high-frequency sounds. The same gradient has been reported for at least some aspects of neural differentiation.[154] Although the first cochlear hair cells to undergo final mitosis are apparently the ones that respond to low-frequency sounds,[29] the gradient in neural development mirrors the near basal to apical gradient of cochlear differentiation and innervation. Thus the most mature cochlear hair cells would appear to be connected to the most mature neurons in each auditory nucleus. Interestingly, two recent studies of the frequency-specific ABR of human infants reveal a similar frequency gradient. ABR wave I-V interwave interval appears to mature first at frequencies of 2 to 4 kHz and later at both lower and higher frequencies.[155,156] In other words, maturation begins in the middle- to high-frequency range and proceeds to the highest and lowest frequencies simultaneously with development. The first response to mature does so between 1 and 2 years of age, whereas the last is not mature at 2 years.

BEHAVIORAL DEVELOPMENT

An obvious and important question is how the morphologic and physiologic properties of the fetal and neonatal auditory system are reflected in auditory behavior. It is also one of the more difficult questions to answer.

Sensitivity

In the last 45 years many studies of fetal motor or cardiac responses to external "sounds" have been published.[157-172] Numerous methodologic difficulties exist with these studies. Not the least of these is that a frequently used stimulator is an artificial larynx applied to the maternal abdomen. Much of the energy delivered by such a device will be in the frequency range to which tactile receptors are sensitive.[173] Frequently the mother is aware of when stimulus presentations are occurring, so that fetal responses to sound may actually represent a response to maternal physiologic state. In any case, the actual sound intensity delivered to the fetus can be estimated only in a very broad fashion, and it is also difficult to estimate the level of intrauterine noise.[167]

Nonetheless a general consensus is that the first fetal motor responses to sound occur at approximately 26 weeks gestation. This is the case even in studies that use tighter controls on the stimulus and stimulus delivery system.[162] The proportion of fetuses demonstrating responses increases progressively with gestational age,[157,162] and some indication is seen that older fetuses (34-39 weeks gestation) respond to lower sound intensities than younger fetuses (26-28 weeks gestation) do.[157,161] In general, the external SPL required to elicit a fetal response is on the order of 90 to 110 dB SPL. Lecanuet and colleagues,[165] however, reported late-gestation heart rate responses to a syllable played at an external level of only 80 to 90 dB SPL. Lecanuet and colleagues[164] showed that late-gestation fetuses responded most often to high-intensity, high-frequency (5000 Hz) sounds. Although responses were recorded to 500- and 2000-Hz stimuli, these were less frequent and tended to occur only at the highest intensities presented. This finding is puzzling in that mammals that begin to hear after birth respond first to low- to middle-frequency sounds.[174] Differences in the transfer of sound through the maternal abdomen cannot account for the difference: the maternal abdomen appears to attenuate frequencies above 1000 Hz rather dramatically.[167] The spectrum of intrauterine noise, however, is weighted toward low frequencies[167] and so may mask other low-frequency sounds. Probably the most important factor is that the spectrum of sound delivered to the fetal inner ear is shaped by the skull, rather than by the external and middle ears (see discussion earlier), so that the effective level of the high-frequency sound entering the system is higher than that of low frequencies. The apparent sensitivity differences across frequency could be easily accounted for by the acoustic properties of the skull.[175]

If the frequency dependence of the fetal response to sound is determined by how sound is transmitted to the inner ear, then one might predict that preterm infants' responses would be different. However, Gerber and colleagues[176,177] reported that both term and 29- to 37-week conceptional age neonates respond motorically to approximately only 25% of the presentations of a wide-band noise of 90 dB SPL centered at approximately 3000 Hz. Although Schulman[178] showed that preterm infants' heart rate changed in response to a doorbell with a level estimated at approximately 80 dB SPL, no information about the frequency dependence of the response is available.

Our knowledge of the fetal behavioral response to sound, then, is easily summarized: fetuses begin to respond to intense

external sounds at approximately 26 weeks gestation. Late-gestation fetuses appear to respond to somewhat lower intensities, particularly for an appropriate stimulus, and to be more sensitive to high-frequency external sounds. Rather high sound intensities are also required to elicit a motor response from preterm infants at the same conceptional age, but the frequency dependence of the preterm response is unknown.

A few studies have estimated thresholds for frequency-specific sounds among term neonates. These data are summarized in Figure 171-14. Weir[179] used a combination of respiratory and motor responses to judge 1- to 8-day postterm infants' sensitivity to pure tones. She found that few infants would respond consistently to the stimuli, but those who did respond consistently had thresholds that would be on the order of 30 to 70 dB higher than those of adults. Thresholds were more adultlike at lower frequencies. Subsequent studies in 2- to 4-week-olds using more sensitive measures of behavior have reported more realistic thresholds, 25 to 35 dB higher than those of adults between 0.5 and 4 kHz.[180,181]

Early threshold immaturity is at least partly accounted for by conductive immaturity. Immature brain stem pathways are also involved, as suggested by the similarity of the behavioral thresholds to those reported for the ABR by Sininger and Abdala.[107] Furthermore, Werner and colleagues[138] demonstrated a moderately high correlation (approximately 0.7) between behavioral threshold and ABR wave I-V interwave latency among 3-month-olds, suggesting that some factor such as synaptic efficiency is at least partially responsible for threshold immaturity in early infancy.

Regardless of the mode of sound transmission in utero, it is clear that fetuses hear and are affected by the sounds they hear. For example, newborn infants recognize their mother's voice very soon after birth[182,183] and even recognize sounds and speech patterns to which they were exposed only in utero.[184-186]

Sound Localization

Among the most widely studied of infant auditory capacities is the ability to localize a sound source. Interest in this topic stems from the observation that sound localization must change after birth as the head and external ears grow, as well as the recognition that neural development could well involve maturation of the ability to make comparisons between ears and across frequencies. In fact, the precision with which changes in sound source location can be detected improves progressively between term birth and approximately 5 years of age.[187,188]

Despite some early controversy on this topic,[189-191] it is now well established that term neonates will turn their head and eyes to the left or right toward a sound source.[192-196] Neonates orient toward a sound source slowly, and their heads must be supported to allow the response to occur.[193,194] The frequency, duration, and repetition rate of the stimulus are important in determining whether a response will occur.[197-201] Muir[202] reported that the orienting response was present in infants as young as 32 weeks conceptional age.

Sound location is typically specified in terms of angle in azimuth—the horizontal plane intersecting the ears—and angle in elevation above or below that plane. Although nearly all studies of newborns' orientation to sound sources have asked infants to make only a left-right discrimination, Muir[202] reported that the extent of the neonate's head turn toward a sound source was related to the position of the sound source in azimuth. The precision with which a newborn can assign a location to a sound source along either dimension has not been established; however, by 1 month of age infants respond to a change in azimuth on the order of 27 degrees.[203] This figure decreases progressively through infancy and early childhood to a value of 1 to 2 degrees.[187,188] Similarly, Muir[202] reported that neonates would turn their heads up or down appropriately toward sound sources above or below azimuth. Morrongiello and colleagues[204,205] have reported that infants can detect a change in elevation on the order of 15 degrees at 6 months of age; this value decreases to a value of 4 degrees among adults, along a developmental time course similar to that seen for localization in azimuth. Finally, although neonates are able to discriminate left and right sound source locations, they are unable to suppress echoes as adults do[26] and thus would have difficulty in identifying sound source locations in a reverberant environment.[197,206] Even 5-year-olds are less able than adults to localize in a reverberant environment.[188]

The sources of early immaturity of sound localization are only beginning to be understood. Although physical limitations on head and ear size modify and limit the cues available for localization, it is clear that other factors are involved. According to head size measurements by Clifton and colleagues,[27] a newborn would need a change in sound source azimuth of 20 degrees to produce an interaural time difference similar to that produced by a 10-degree change for adults. Recall that adults can detect changes in azimuth of 1 to 2 degrees, whereas newborns cannot detect a change in azimuth as small as 20 degrees. Ashmead and colleagues[207] found that infants could detect interaural time difference changes smaller than those produced by a change in sound source spatial location that they could just barely detect. Both of these findings could be taken to mean that the limitation is neither in the physical cue available nor in the coding of the physical cue but in the assignment of a spatial location to the physical cue. Until additional information on infants' utilization of interaural intensity differences is available, however, it cannot be determined whether interaural intensity cues or their coding also play a role.

To summarize, neonates are capable of distinguishing the location of a sound source on the basis of auditory cues alone to an accuracy of approximately 25 degrees in both azimuth and elevation. The age at which sound localization can first be observed is not known. Although the physical size of the neonates' head and ears limits the acoustic information available about sound source location, both the accuracy with which acoustic cues are coded in early infancy and the precision with which values of acoustic cues are mapped onto locations in space are probably greater limitations.

Speech Perception

Some of the most interesting studies of infant auditory perception are those describing the infant's ability to discriminate between speech sounds. Eimas and colleagues[208] demonstrated that 1-month-old infants discriminated between /b/ and /p/, speech sounds differing in only one phonetic feature. Since that demonstration, the development of speech perception has been one of the most active areas of infant research. The results of these studies are easily summarized, although their interpretation may be more complicated than originally thought. A major finding is that even newborn infants appear to be capable of discriminating between all of the pairs of speech sounds that have been examined to date.[209,210] Furthermore, this ability appears to extend to the sounds of all spoken languages.[211] Second, although it is clear that the newborn can represent speech sounds sufficiently well to discriminate between sounds under many conditions, the newborn's representation of speech sounds may be less detailed than that of adults or older infants.[212-215] The ability to make fine distinctions among speech sounds probably continues to develop into childhood.[216-224] Abnormal experience with sound may retard this process of refinement.[225] Third, infants appear to be sensitive to many aspects of speech besides phonemic distinctions. For example, they are sensitive to changes in intonation that signal clause boundaries.[226,227] Newborns are capable of distinguishing between different languages, although this ability depends on the native language to which the infant was exposed before birth, as well as the specific languages under consideration.[228]

The major events in the development of speech perception during the first year of life involve a progressive specification of native language sounds, sometimes at the cost of losing the ability to discriminate nonnative speech sounds. By 2 months of age, infants appear to recognize the predominant stress pattern that occurs in their native speech.[229] By 6 months of age, they tend to categorize the vowels of their native language in a broader way than they categorize the vowels of other languages.[230,231] The extent to which these tendencies depend on prenatal experience with speech sounds is not clear; the age when infants first demonstrate these aspects of language-specific perception has not been determined.

By 12 months of age, infants have lost the ability to discriminate among many nonnative consonants.[232-235] Some nonnative consonants appear to be discriminable by infants and remain discriminable into adulthood, such as Zulu "clicks" by American speakers of English.[236] This appears to occur when the nonnative sound is so different acoustically from native sounds that the specification of native speech sounds has no effect on the nonnative speech category. Other speech sounds are discriminable by 6-month-olds, not discriminable by 12-month-olds, and remain very difficult for adults to discriminate, such as Nthlakampx (a native American language) glottal stops by Canadian speakers of English.[235] In this case it appears that, as native speech sounds are specified, native speech categories "absorb" acoustically similar nonnative speech categories. Finally, some nonnative vowel sounds are discriminable by 6-month-olds and not discriminable by 12-month-olds but can be discriminated by adults, such as German back rounded vowels by Canadian speakers of English.[237] Apparently the refinement of speech perception that occurs during childhood can have the effect of redefining a native speech category in such a way that it no longer includes nonnative speech sounds that it may have "absorbed" early in development.

An important issue for future research in this area is the importance of specific language experience on both the initial specification of native language sounds and of the subsequent refinement of speech sound categories. What experience is necessary, how much experience is necessary, and when experience is necessary are not known. Although these issues are difficult to address, they are very important ones for certain populations—preterm infants, infants with congenital hearing loss, infants with chronic otitis media—whose early experience with sound is abnormal to varying degrees. Of high interest in this regard are recent studies that demonstrate that adults exaggerate phonetic differences in the speech they direct to infants.[238-240]

A final note with respect to the development of speech perception is that despite the young infant's sometimes amazing ability to process speech, immature primary auditory processing remains an important constraint on speech processing. Speech is not privileged over other sounds. Six-month-olds require just as much of a boost in SPL to detect and discriminate speech sounds as they do to detect and discriminate pure tones.[241-243] Newborn infants are less sensitive to sound. They may be particularly vulnerable to interference from noise, and their representations of the spectral shape of speech sounds may be "fuzzier" than those of older infants and adults. They may be less capable of following rapid changes in a sound (but see Jusczyk and colleagues[244]). Thus it is not surprising that their representations of speech are not detailed[213] or that adults exaggerate many acoustic properties of speech when they speak to infants.[245] Another important issue for future research will be to determine precisely how immature sensory processing limits early speech perception.

SUMMARY AND CONCLUSION

Between the time that the cochlea begins to function during gestation and the neonatal period, the auditory system undergoes dramatic development. Although few data from humans exist, all indications from the available data are that the auditory system of a 28-week fetus is immature indeed: sensitivity is known to be poor, and both spectral and temporal representations of suprathreshold sounds are likely to be fuzzy. By term birth, hearing has improved dramatically; neonates are capable of discriminating among many complex sounds. Nonetheless the term neonate is still less sensitive and still less able to process the details of a stimulus than an adult. During very early development, the major contributor to improvements in hearing is probably the maturation of the cochlea. The middle ear and the auditory nervous system, however, continue to develop over a longer time period. These are probably the major limitations on hearing in the neonatal period. Beyond the neonatal period, as the middle ear and auditory nervous system mature, higher-level processes such as attention and memory still limit auditory perception. At some point during infancy, it is likely that these higher-level processes become more important than sensory limitations in determining what infants hear. At present, we do not know when that transition occurs.

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Beverly J. Cowart, Gary K. Beauchamp, and Julie A. Mennella

Development of Taste and Smell in the Neonate

This chapter provides a critical overview of the literature on human taste and smell function in the prenatal and neonatal periods, with some consideration of later infancy and early childhood, by integrating and updating our previous reviews and those of our colleagues.[1-7]

Taste and smell share a common stimulus class—chemicals. Functionally, taste is intimately connected to the ingestion or rejection of potential foods and perhaps plays a role in the utilization of ingested foods.[8] Smell also contributes to the identification and acceptance of foods and may be involved in regulating human social interactions (e.g., individual and sexual identification and attraction), as it is in many other animals.[9] Flavor, as an attribute of foods and beverages, is defined as the integration of multiple sensory inputs from the gustatory (taste), olfactory (via retronasal olfaction), and trigeminal (touch, temperature, irritation) systems that a substance elicits in the oral and nasal cavities. In particular, retronasal olfaction, the aromas of substances we put in our mouths, contributes significantly to the complexity of flavor, and a discussion of the ontogeny of flavor perception and preferences is highlighted in the section on smell.

Taste and smell have much in common, but major differences exist as well. Most striking are the apparent differences in the ranges of qualitative experiences aroused by these two senses. Although it is still somewhat controversial, most investigators believe that taste is composed of a restricted set of qualities or categories, namely, sweet, salty, sour, and bitter, with umami, the taste associated with monosodium glutamate (MSG), having more recently gained widespread acceptance as a fifth basic taste after the discovery of taste receptors for L-glutamate.[10,11] It has been hypothesized that this small number of qualities evolved because of the functional importance of the primary stimuli (e.g., sugars, sodium chloride, organic acids, bitter toxins, amino acids) in nutrient selection. A practical consequence of this classification scheme in developmental (as well as other) taste research has been to limit the range of stimuli examined.

In contrast, no widely accepted classification scheme exists for smell stimuli, and this situation has resulted in the absence of an agreed-on set of stimuli to use in sensory tests. Thus olfactory stimulus selection tends to be based on the availability and presumed safety of single compounds or on ecologically significant odor sources that are not chemically specified (e.g., amniotic fluid, mothers' breast odors).

Human studies can provide data relevant to two aspects of chemical sensation: the sensitivity of the system to chemical stimuli and the hedonic valence of the sensation. In studies in adults, the distinction between these two is usually unambiguous. Measures of sensitivity include thresholds, just-noticeable differences, intensity judgments, sensory adaptation, and so forth, whereas measurements involving a hedonic dimension usually consist of ratings of pleasantness or preference. In studies in infants and young children, these two classes of response to chemical stimuli can be more difficult to distinguish. For taste, very few studies have attempted to evaluate sensitivity specifically; almost all response measures are associated with acceptance or rejection and thus presumably involve a hedonic component. Although published developmental studies on olfaction historically focused

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more on sensitivity and discrimination, more recent studies have also investigated the hedonic aspects of odors.

TASTE

Prenatal Development

Taste receptors or buds are composed of modified epithelial cells and are found throughout the oral cavity, on the hard and soft palates, the tonsils, the pharynx, the larynx, the epiglottis, and the esophagus[12]; however, they are most numerous on the papillae of the tongue. Peripheral innervation of these cells is via branches of three different cranial nerves: the facial (VII), glossopharyngeal (IX), and vagus (X). Four morphologically distinct types of lingual papillae can be identified in humans, but only three bear taste buds. These are the fungiform, the foliate, and the circumvallate papillae. Fungiform papillae are mushroom-shaped buttons found mainly at the tip and along the front edges of the tongue; many of these may contain no taste buds at any given time, but, of those with buds, most have one to three.[13] Most taste buds are found in the foliate and circumvallate papillae. The former consist of parallel folds along the rear edges of the tongue; the latter are flat mounds surrounded by a trench on the posterior dorsal border between the oral and pharyngeal tongue (typically 8 to 12 arranged in an inverted V in humans).

Specialized taste cells first appear in the human fetus at 7 to 8 weeks of gestation, and morphologically mature cells are recognizable at 13 to 15 weeks.[14,15] The taste buds of fetal sheep, the development of which parallels that observed in the human fetus, have been shown to respond to chemical stimulation as early as the 80th day of gestation.[16,17] Fetal taste receptors may be stimulated by chemicals present in the amniotic fluid. The fetus begins to swallow episodically at approximately the 12th week of gestation[18] and may swallow 200 to 760 mL/day by term.[19] Amniotic fluid contains glucose, fructose, lactic acid, pyruvic acid, citric acid, fatty acids, phospholipids, creatinine, urea, uric acid, amino acids, polypeptides, proteins, and salts.[20] Moreover, the chemical composition of the amniotic fluid varies over the course of gestation and may be abruptly altered by fetal urination.[16,21] Reported differential fetal swallowing after injections of sweet or bitter substances into the amniotic fluid of pregnant women suggests that fetuses may show a preference for sweet and a rejection of bitter,[20,22] but a strong demonstration of preferential responding by human fetuses to taste stimuli is lacking.

What may the organism gain from in utero taste experience? Swallowing amniotic fluid appears to be necessary for the proper development of the gut, but gustatory stimulation is probably not essential.[23] Neural development of the central and peripheral taste systems could be influenced by the intrauterine chemical milieu, such that acceptance and rejection of substances after birth may coincide with prior amniotic contents and concentrations.[16,24] Animal model studies support this hypothesis.[25]

Direct studies of taste in the preterm infant are rare, partly because of methodological limitations. As discussed later, in term infants, investigators have monitored facial expressions, autonomic responses, consummatory intake, and sucking behavior when the infants are offered flavored solutions. This is problematic for preterm infants who risk fluid aspiration as a result of immature suck-swallow coordination.

To avoid this problem, nonnutritive sucking was studied in preterm infants (33 to 40 weeks after conception) exposed either to brief, intraoral presentations of very small amounts of glucose solution[26] or to sucrose embedded in a nipple-shaped gelatin medium that released small amounts when mouthed or sucked.[27] Both approaches demonstrated that these infants, who had little or no extrauterine taste experience, possessed the ability to detect sweet and showed an avidity for it by producing stronger and more frequent sucking responses than they did when presented with no-taste controls.

This ability on the part of the preterm infant to respond to sweet gustatory stimulation behaviorally, and presumably physiologically, suggests a possible intervention aimed at stimulating these responses to augment development of the infant. In other words, it may be possible that taste stimulation presented during tube feedings would enhance growth efficiency beyond that achieved by allowing infants to suck on a pacifier during this time.[28-32]

Palatable, sweet-tasting stimuli also promote "calming," analgesic-like reactions in preterm, as well as more mature, infants during heelstick and other invasive procedures. When sweet-tasting sugars are present in the oral cavity, crying is reduced, heart rate increases are attenuated, and facial expressions of pain are blunted in preterm infants as young as 25 weeks after conception.[33] Moreover, this analgesic effect appears to be the result of taste per se, because the same sweet solution is ineffective in producing analgesia when administered intragastrically in preterm (32 to 36 week) infants.[34]

These studies indicate that not only are taste buds capable of conveying gustatory information to the central nervous system by the last trimester of pregnancy but also this information is available to systems organizing changes in sucking, facial expressions, and other affective behaviors.

Sensitivity and Discrimination: Newborns

The principal response measures that have been used to assess neonatal taste perception are tongue movements, autonomic reactivity, facial expressions, and differential ingestion and sucking patterns. With one exception, all these responses are believed to reflect preference or acceptance or pleasure. When infants exhibit affective responses to taste stimuli, it can be inferred that they are sensitive to them; however, failure to observe such responses does not indicate whether the infant failed to detect the stimulus or was simply hedonically indifferent to it.

The single, clear exception to hedonically motivated taste responses is the *lateral tongue reflex*.[35,36] This reflex is elicited by the application of microdrops of fluid to the newborn's tongue, and neither its form nor frequency is affected by qualitative changes in the fluid. Thus the occurrence of the response appears to be solely dependent on sensory mechanisms. Studies using this measure suggest that newborns possess distinct functional taste receptors for sweet and salty; however, differential responding has only been reliably observed with concentrated taste solutions, for example, 2 mol/L salty, which is undoubtedly a trigeminal, as well as a gustatory, stimulus, producing irritant sensations.[37-39] Consequently, the utility of this response as a measure of absolute gustatory sensitivity is questionable.

Autonomic reactivity may also provide information on gustatory sensitivity. Crook and Lipsitt[40] and Lipsitt[41] published the only modern studies of infant taste perception using such measures, and their research was limited to sweet taste responsivity. In both studies, heart rate was found to be positively correlated with the concentration of sucrose presented to infants. Lipsitt proposed that the heart rate response is a signal of "the joy that the infant derives from [the] 'pleasure of sensation'"[41]; however, the possibility cannot be discounted that the heart rate response reflects arousal rather than pleasure and could provide a measure of gustatory sensitivity, as opposed to preference, in infancy.

Hedonics: Newborns

Facial expressions, suggestive of contentment and liking or discomfort and rejection, were used to assess the newborn's responsiveness to taste stimuli in some of the earliest investigations of human taste development.[42-44] Steiner and colleagues[45-49] reported that consistent, quality-specific facial expressions can be elicited in the first few hours of life by sweet (sucrose, 0.73 mol/L), sour (citric acid, 0.12 mol/L), and bitter (quinine sulfate,

0.003 mol/L) stimuli. Because the stimuli used to elicit these responses were concentrated, differences among the responses to each taste could be at least partially reflective of differences in the extent of trigeminal involvement. In one study, these investigators examined responses to less concentrated taste stimuli and provided data on the frequency of occurrence of specific facial features.[48] High concentrations of tastes (0.0001 mol/L quinine, 1.0 mol/L sucrose, and 0.25 mol/L urea) elicited more responses than the other stimuli (distilled water, 0.1 mol/L sucrose, and 0.15 mol/L urea), and the patterns of response to the quinine and more concentrated urea differed from that elicited by 1.0 mol/L sucrose. However, considerable overlap existed in the frequency distributions of facial features elicited by all the stimuli, and responses elicited by the weaker concentrations of sucrose and urea were similar to those observed after water presentations. Finally, infants displayed distinctive positive facial expressions to soup to which MSG was added when compared with unadulterated soup.[49] MSG alone (i.e., in water) did not appear to elicit these facial responses, however, a finding that raised the question of exactly what it is about the soup-MSG mixture that is of apparent positive hedonic valence.

The most detailed study of newborns' facial responses to taste stimuli was conducted by Rosenstein and Oster.[50] Consistent with the findings of Steiner and colleagues,[45-49] analyses of the patterns of full-face expressions indicated that the newborns discriminated sweet from nonsweet solutions and, among nonsweet solutions, between the sour and bitter taste stimuli. However, such analyses also indicated that taste-elicited facial expressions are not highly stereotyped. Overlap appears to be considerable in the distribution of not only discrete facial actions but also full-face configurations elicited by the different taste stimuli, as well as considerable individual variation in facial responses. Moreover, although hedonically negative facial components were more common in responses to all three nonsweet tastes than in responses to sweet, untrained observers found only the responses to the bitter tastant to be highly negative. Even with their fine-grained analysis and a concentrated salt stimulus (0.73 mol/L), a distinctive facial response to salt was not identified.

Differential ingestion has been used more frequently than any other response to study taste preference during infancy. Several limitations, however, are associated with this measure. First, because multiple stimuli cannot be presented simultaneously to human infants, thereby providing a true choice situation, the measure should probably be viewed as one of relative acceptance rather than of preference. More critically, the number and range of stimulus concentrations that are either feasible or ethical to present to infants in an unrestricted feeding situation are limited. Finally, the strength of the sucking reflex in young infants is quite strong, and this may mitigate clear rejection of a taste stimulus.

Intake studies have repeatedly demonstrated strong acceptance of sweet tastes by infants at birth and have shown that newborns respond to even dilute sweet tastes and can differentiate between varying degrees of sweetness.[51-53] However, they have generally failed to demonstrate differential responding to sour or bitter tastes, as well as to salt. In the single exception to this generalization, Desor and colleagues[54] used responses to a mild sweet solution rather than plain water as the baseline measurement (ensuring the infants were capable of responding at levels both higher and lower than baseline) and presented salty (NaCl), sour (citric acid), and bitter (urea) tastes in this sweet base. Under these circumstances, relative suppression of intake was observed with the addition of citric acid concentrations at and above 0.003 mol/L, but no significant changes in intake occurred in response to the addition of either NaCl (0.05 to 0.20 mol/L) or urea (0.18 to 0.48 mol/L). The failure to observe rejection of urea (0.12 to 0.24 mol/L) by newborns, even when presented in a sweet base, has been replicated.[55] In the same study, however, significant rejection of all concentrations of urea was observed in slightly older infants (14 to 180 days, with no difference between responses of infants less than or more than 3 months of age), a finding suggesting an early developmental increase in sensitivity to bitterness.

Absence of differential responding to salt in newborns is consistent with results from the studies of facial expression described earlier, but the apparent indifference to bitter is not. Three points should be noted, however. First, for adults, urea is a much less intensive bitter stimulus than quinine and seems to fall into a separate class of bitter stimuli that may be coded by a different receptor mechanism.[56] Second, although 0.25 mol/L urea elicited facial actions that included apparently negative components similar to those elicited by a quinine stimulus, the urea also elicited more apparently positive facial actions, such as sucking and licking, than did the quinine.[48] Third, when taste stimuli are mixed, the intensity of each of the component tastes tends to be suppressed; sour taste intensity may be less susceptible to suppression than the other primary tastes.[57] Thus the discrepancy among findings concerning bitter responsivity in newborns could partially reflect differences in the type or intensity of bitter stimuli presented.

Finally, the study of sucking patterns has been used as an alternative to the measurement of intake in full-term newborns, as well as premature infants. Techniques for recording multiple parameters of sucking in response to small stimulus volumes can potentially allow for the study of responses to sapid substances one would be reluctant to have infants consume in any quantity. Conversely, the relative significance of individual parameters of the sucking response and the relationships among different parameters are not well understood. Presentation of minute amounts of sweet solution leads to concentration-dependent increases in the number and length of bursts of sucking activity and to decreases in the time between bursts, all of which are intuitively consistent with a positive hedonic reaction and thus with the results of studies using measures of intake and facial expressions,[58,59] although, seemingly paradoxically, the rate of sucking within bursts after sweet stimulation has been found to decrease.[40,58] Similarly, the number and strength of sucks in response to urea are relatively depressed in infants older than 2 weeks of age, but not newborns, a result paralleling that seen with intake measures (see earlier).[55]

Responses to sour stimuli have not been formally evaluated by using this kind of procedure. In the case of salt, however, sucking parameters seem to reveal a subtle response by newborns not evident in measures of intake or facial expression. Crook[59] reported that even a weak salt solution (0.1 mol/L) significantly decreased the length of sucking bursts. Similarly, Beauchamp and associates[60] found that, relative to water, both 0.2 and 0.4 mol/L NaCl significantly depressed the number of sucks and of sucking bursts produced by newborns, although relative intake of the moderately salty solution (0.2 mol/L) was not significantly depressed.[54]

Each of the measures used in studies of taste responses in very young infants has its limitations, but the convergence of results from different measures of sweet taste perception in newborns gives confidence in the conclusion that infants are quite sensitive to this taste and display an innate preference for it. Similarly, even though sour taste perception has been studied less extensively, measures of intake and facial expression suggest that sour taste perception may be reasonably well developed at birth and that newborns reject substances having this taste. The highly concentrated sour stimuli that have been used to elicit facial expressions do, however, pose some interpretive problems, and additional studies of sour taste responsivity are necessary before strong conclusions can be drawn with regard to sour perception in early development.

Neonatal responses to bitter are more problematic. Newborns respond with highly negative facial expressions to concentrated quinine but not necessarily to urea. They also do not reject

moderately strong urea solutions relative to water as assessed by either intake or sucking measures, although significant rejection seems to appear within a few weeks of birth. As noted, evidence exists of multiple bitter taste transduction sequences.[56,61] In addition, sensitivity to different bitter compounds may be differentially affected by aging.[62] Thus the early developmental course of bitter taste perception may also be compound specific.

The newborn's response, or lack thereof, to salt is perhaps most puzzling. Given the subtlety of the responses that have been observed, and the evidence for postnatal development of salt taste reception in other mammalian species,[63-66] it seems likely that humans are relatively insensitive to salt at birth. However, to the extent that this stimulus is perceived, the newborn's hedonic response to it appears to be negative.

Hedonics: Older Infants and Young Children

Relatively few studies have been done of taste perception in older infants and children as compared with neonates, and many of those that have been reported were based on small samples and not described in detail. For all practical purposes, the measures obtained have been limited to intake (i.e., differential ingestion) and paired comparison or rank ordering of taste stimuli to determine preference, and, with few exceptions, only responses to sweet and salty tastes have been assessed.

Babies beyond the neonatal period (1 to 24 months) have been most neglected in studies of taste development. In part, this is probably because of the lack of clearly appropriate measures of either preference or sensitivity for subjects in this age range. Differential ingestion has been the default option in almost all studies. However, certain problems are encountered with older infants that do not arise with neonates. For example, older infants may be less willing to accept unfamiliar bottles or foods from an unfamiliar person, and, as Filer noted, the amount of any particular food eaten in a natural feeding situation may depend as much on the mother's "mechanical skill… and determination to feed her infant"[67] as on the infant's preference. Finally, because intake has been the only measure obtained from older infants, no cross-validation of the meaningfulness of this measure has been done. Nonetheless, a few notable findings, suggestive of developmental or experiential changes in taste preferences during infancy, have been reported.

In the context of studies on effects of protein-calorie malnutrition on taste, sucrose, citric acid, urea, and salt acceptability was evaluated in well-nourished and malnourished Mexican infants from 2 months to 2 years of age.[68] Relative to water, infants less than 1 year of age preferentially ingested 0.1 and 0.2 mol/L NaCl. A preference for sucrose was observed for infants at all ages. The bitter (urea, 0.12 and 0.24 mol/L) and sour (citric acid, 0.006 and 0.012 mol/L) stimuli were tested with the same mild sucrose solution as diluent as used by Desor and colleagues[54] (see earlier). Consistent with findings in newborn infants, citric acid was rejected relative to the diluent. Both concentrations of the bitter urea were also rejected by these infants, as was also found by Kajiura and associates[55] in their group of older infants.

In addition to the classic taste stimuli, a sample of Mexican infants was also tested with a mixture of amino acids and peptides (casein hydrolysate) and with MSG.[69] For these two taste substances, a low-glutamate soup was used as the diluent. Consistent with Steiner's report on newborns,[49] both malnourished and well-nourished infants preferred the soup with MSG (0.4%) to the soup alone. This preference was observed even in the youngest infants tested (2- to 4-month-old infants). In contrast, nutritional state influenced responses to the casein hydrolysate. Infants who were malnourished preferred soup with added casein hydrolysate when compared with soup alone; the opposite response was observed in well-nourished infants[68] and in recovered malnourished infants.[69]

Newborn infants have been found to be indifferent to or to reject salt relative to water, whereas Vazquez and colleagues[68] reported that older infants found salt solutions more acceptable than water. This suggestion of a developmental shift in salt acceptability was supported in studies in a sample of children from the United States. Preferential ingestion of salt water relative to plain water emerged at approximately 4 months of age. Beauchamp and associates[70] and Cowart and Beauchamp[71] argued that experience with salty tastes probably does not play a major role in the shift from apparent indifference to (or rejection of) salt at birth to acceptance in later infancy; rather, this change in response may reflect postnatal maturation of central or peripheral mechanisms underlying salt taste perception, allowing for the expression of a largely unlearned preference for saltiness.

Conversely, some evidence exists that, at least by 6 months of age, frequency of dietary exposure to high-sodium foods (although not total dietary sodium) may affect the degree of preference for salted versus unsalted cereal. In a study in 10 6-month-old infants, Harris and Booth[72] determined the difference between the amount of salted cereal eaten during feedings on 2 days and the amount of unsalted cereal eaten on 2 different days and used that as a measure of salt preference. These investigators found relative preference to be significantly correlated with the number of times the infants were exposed to foods containing at least the amount of sodium in the salted cereal during the week preceding testing; none of the infants actually rejected the salted cereal relative to the unsalted one, however, and all but one exhibited some preference for it by consuming more salted than unsalted cereal.

Effects of early experience on the preference for salt taste are also suggested by the finding that 16-week-old infants of mothers who had had extreme morning sickness during pregnancy consumed larger volumes of 0.2 mol/L NaCl than did infants whose mothers had not exhibited this symptom.[73] Moreover, adult children (college students) of mothers who had considerable morning sickness during their pregnancies had greater salt preferences than did students whose mothers had little or no morning sickness.[73,74] The proposed mechanism is that morning sickness leads to transient fluid and sodium depletion in a manner analogous to that reported in animal model studies. Consistent with these findings, 12- to 14-year-old children who had been exposed to a chloride-deficient formula during early development showed heightened preferences for salty (but not sweet) foods relative to their unexposed sibling controls[75]; chloride deficiency leads to hormonal alterations that mimic those occurring during sodium deficiency.[76]

Similarly, evidence exists of an effect of experience on the expression of sweet preferences in 6-month-old infants.[77] Intake of sweetened versus unsweetened water was assessed in the same infants at birth and at 6 months. Although the infants preferred sweetened water at both ages, only the 6-month-old infants who had received sugared water as part of their diet continued to exhibit the same degree of preference for sweet in water that they had at birth.

Dietary experience—in particular, familiarity with specific foods and with tastes in specific food contexts—has also been shown to play an important role in the preferences of preschool children.[78-81] The other major finding concerning taste preferences in early childhood has been that children prefer more concentrated sugar and salt than do adults, although the bases for these extreme preferences have yet to be elucidated.[71,82,83]

SMELL

Prenatal Development

The human olfactory epithelium lines the roof of the nasal cavity, part of the nasal septum and the superior turbinates, and may extend to the middle turbinates. The olfactory receptor cells are bipolar neurons, with dendrites that bear motile cilia lying

in a layer of mucus covering the epithelium. The axons of these cells comprise the olfactory nerve (cranial nerve I) and travel in unmyelinated fascicles through the bony roof of the nasal cavity into the olfactory bulb, where they terminate and synapse with second-order cells. The nasal cavity is also innervated by the nasopalatine branch of the trigeminal nerve (cranial nerve V), which mediates pain, touch, temperature, and proprioception and the receptors of which also respond to many odorous volatiles.

The ethmoid and nasopalatine branches of the trigeminal nerve have differentiated and respond to tactile stimulation by approximately the 10th gestational week.[84,85] The olfactory bulbs and receptor cells have attained adultlike morphology by the 11th week of gestation.[86,87] Olfactory marker protein, which appears to be a biochemical correlate of olfactory receptor functioning in fetal rats,[88] has been identified in the olfactory epithelium of human fetuses at 28 weeks gestation.[89] Because the epithelial plugs that obstruct the external nares resolve between gestational weeks 16 and 24,[90] a continual turnover of amniotic fluid takes place through the nasal passages. Even in air-breathing organisms, volatile molecules must penetrate the aqueous mucus layer covering the olfactory epithelium to reach receptor sites on the cilia. Thus no fundamental distinction exists between olfactory detection of airborne and waterborne stimuli.

In fact, amniotic fluid provides a natural, ever-changing olfactory, as well as taste, environment for the fetus.[91-95] In particular, the fetus may learn about the dietary choices of the mother through odorants found in the amniotic fluid that derive from foods eaten by the pregnant mother and reflect their flavors. This was demonstrated experimentally in a study in which amniotic fluid samples were obtained from 10 pregnant women who were undergoing routine amniocentesis and who ingested either garlic or placebo capsules approximately 45 minutes before the procedure.[92] The odor of the amniotic fluid obtained from the women who ingested the garlic, as judged by adult human evaluators, was stronger or more like garlic than amniotic fluid from the women who did not consume garlic.

Several lines of evidence suggest that, at approximately 28 weeks, the olfactory system is capable of detecting chemical stimuli. Studies in premature newborns demonstrated reactions to odorants (odorous chemicals) in infants as young as 28 weeks gestation.[42,96,97] For example, 8 of 11 sleeping preterm infants (29 to 32 weeks) initiated sucking or displayed arousal-withdrawal within 30 seconds of the presentation of a peppermint extract odorant.[98] Although similar responses were observed in one infant who was 28 weeks gestation, this was not characteristic of other infants (n = 5) that age. All infants younger than 28 weeks gestational age failed to respond differentially to the peppermint and control trials, whereas all infants between 32 and 36 gestational weeks did.

Although these data appear to support the hypothesis that functional olfaction has its inception at approximately 28 gestational weeks, three caveats must be put forth. First, the failure of infants who were less than 28 weeks gestation to respond to an odor stimulus in a reliable manner may reflect a response limitation as opposed to an inability to detect the stimulus. In studies of term neonates, change in respiratory rate provides a more reliable measure of the ability to detect odorants than motoric responses,[98,99] and therefore it may provide a more sensitive test of the preterm infant's ability to detect odorants. Second, it is not clear whether responses in the aforementioned studies were mediated by the olfactory or trigeminal system. Finally, these studies tested only a small subset of odorants, and it is possible that different odorants could elicit responses in younger preterm infants.

Sensitivity and Discrimination: Newborns

Numerous studies have shown that newborn infants can detect and discriminate various qualitatively distinct odorants.[98,100-109] In one of the earliest controlled studies, Engen and colleagues[98] compared newborns' responses to odor pairs, the members of which varied in intensity and hedonic valence: 100% acetic acid and phenylethyl alcohol (experiment 1) and anise oil and tincture of asafetida (experiment 2). Odors on cotton swabs were presented to infants during sleep, and general activity, leg withdrawal, respiration (experiments 1 and 2), and heart rate (experiment 2) were monitored. The investigators concluded that newborns were sensitive to the odors and that they discriminated among them. The most reliable elicitor of a response was acetic acid, followed in descending order by asafetida, anise, and phenylethyl alcohol. Because the odors were not equated for perceived intensity, it is unclear whether these differences are best accounted for by differences in quality, intensity, hedonicity, or trigeminal component. For example, 100% acetic acid surely stimulates not only the olfactory receptors but also receptors sensitive to irritation in the nose and in the eyes.

Some evidence suggests that the neonate may be remarkably sensitive to other nonbiologic odors, such as the homologous alcohols.[110-112] The observation that preschoolers[113] and prepubescent school-aged children[114] are more likely to be sensitive to a biologic odor, androstenone, than are older children and adults, lends further credence to the possibility that newborns' olfactory acuity may equal or exceed that of adults. Finally, comparisons of olfactory neuroepithelium in fetuses and physiologically normal adults reveals degeneration in adults and a higher ratio of olfactory to respiratory epithelium in fetuses.[115]

Nonetheless, Lipsitt and colleagues[116] suggested that olfactory sensitivity may increase over the first few days of life. They tested 10 infants' responsiveness to increasing concentrations of asafetida over the first 4 days of life. Because the concentration step required to produce a response, relative to no-odor control trials, decreased for each of the 10 infants over test days, it was concluded that olfactory threshold decreases during the first 4 days of life.

It is not clear whether this change in response is best explained in terms of (1) a stimulus-independent postnatal maturation of peripheral or central neural processes including those that may underlie a shift to more nasal—relative to mouth—breathing, (2) the induction of sensitivity contingent on odor exposure, or (3) merely the development of a more coordinated response system. Although the first and third alternatives are probably the most likely, three findings are consistent with the second alternative. First, newborns tested in a longitudinal design over the first 3 days of life showed more reliable respiratory responses to several odors on the third day of testing than did 3-day-old infants who had not received any prior exposure to the odorants.[100] Second, repeated exposure to one odorant (i.e., androstenone) can induce the ability to detect that odorant in adults.[117] Finally, changes occur in the olfactory system of rodents exposed to odors as either neonates[118-121] or adults.[122] These findings are merely suggestive, and human neonatal studies that directly manipulate odor exposure conditions in relation to age and developing olfactory sensitivity are necessary to discriminate among these alternatives.

Certain other aspects of olfactory function have been examined in infants. Engen and associates[98] reported that exposure to an odorant may result in decreased responsiveness to the odor when assessed as a change in respiratory rate. A subsequent study by the same group provided evidence that this phenomenon is best explained in terms of response habituation, as opposed to sensory fatigue.[99] In infants who showed a decrement in response to a mixture of two odorants (anise oil and asafetida or amyl acetate and heptanol), the response recovered when they were tested with a component of the mixture at the same concentration as in the mixture. Moreover, the magnitude of the recovery was a function of how perceptually similar (as rated by adults) the component was to the mixture, even when

the components were equated for perceived intensity. This finding suggests that perceived similarity among odors is much the same in the newborn as in the adult, although it would be premature to endorse this view without demonstrating similar effects with other odorants. The possibility that odor categories are not dependent on either a language system or much experience with odors is intriguing and is worthy of further study. As yet, there is no agreed-on method for categorizing odors, and the extent to which different schemes of categorization are dependent on experience and language is unclear. Studies of odor similarity in infants could aid in determining "basic" categories for odorous chemicals.

The relationship between perceived intensity and molecular structure of homologous alcohols appears to be much the same in adults and newborns. The likelihood of newborns' responding (using change in respiratory rate as a dependent measure) to each in a series of full-strength homologous alcohols (ethanol to decanol) decreases as the chain length increases, in much the same way as the perceived intensity of these alcohols decreases with increased chain length for adults.[112] Although the short-chain alcohols elicit the greatest response when tested at full concentration, the long-chain alcohols elicit responses more systematically when threshold concentrations are evaluated. Adult psychophysical data suggest an explanation, showing that the effect of dilution on perceived intensity of these alcohols varies as a function of the chain length: for example, a 50% dilution of a long-chain alcohol results in a smaller change in perceived intensity than the same dilution of a short-chain alcohol.[112] A subsequent study in which newborns' responses were scaled, with use of degree of general movement as a measure, revealed a similar relationship between intensity functions and alcohol chain length,[111] a finding confirming an essential similarity between olfactory processing in adults and that in newborns.

The ability to localize an odor source appears to be another perceptual capacity of the newborn. Newborns (1 to 5.5 days old) systematically turn their heads away from a source of ammonium hydroxide located to either the right or left of midline, just below the nostrils.[101] As with many of the foregoing phenomena, it is unclear whether this ability is mediated by the olfactory nerve, the trigeminal system, or both, because ammonium hydroxide is a potent stimulant of the trigeminal nerve. Evaluation of neonates' responses to odorants that appear selectively to stimulate the olfactory nerve, such as phenylethyl alcohol or vanillin,[123] could help to distinguish among these possibilities.

Finally, several adult studies have demonstrated that exposure to certain olfactory stimuli can inhibit perception of structurally related chemicals. It is presumed that the interaction of the first odor with many of the same receptors responsive to the second results in a decrement in responsiveness to the second odor. Cross-adaptation studies, like similarity judgments, have been taken as a way to explore basic odor categories. The opposite effect, facilitation of one odor by prior exposure to another, is less common but has been reported.[124] Both cross-adaptation and facilitation have also been observed in human neonates,[111,125] a further indication of the similarity between infant and adult olfactory function.

In addition to studies of newborns' responses to the odors of arbitrarily chosen, single-chemical compounds, a growing body of literature now exists that characterizes the neonate's response to odors associated with the mother and her amniotic fluid and breast milk. Most of these studies have used preferential orientation as a response measure and are discussed in the following section on odor hedonics. In this context, however, it is worth noting that, within hours after birth, infants can recognize their mothers through the sense of smell alone.[102-104,106-109] Like much of the work discussed earlier, this finding suggests exceptional olfactory acuity in the newborn.

Hedonics: Newborns, Older Infants, and Young Children

Given the striking resemblance between the neonate and the adult in sensitivity and discrimination, as outlined earlier, it is somewhat surprising that the origin of hedonic responses to odors has not been unequivocally traced to the neonatal period; in fact, controversy on this issue is substantial. This is particularly puzzling because hedonic valence is the most salient psychologic attribute of an odor for adults,[126] and, anatomically, olfactory structures have relatively direct access to brain structures that govern emotion.[127] Moreover, innately determined hedonic reactions to odors could be hypothesized to play a role in mediating components of food selection and social interaction.

The strongest evidence of affective odor reactions in very early infancy comes from neonatal studies of preferential responses to maternal and milk odors, alluded to earlier.[102-104,106-109] That these maternal odors have both a calming and feeding preparatory effect on the infant is demonstrated by the findings that newborns spend more time orienting toward a breast pad previously worn by their lactating mothers than one worn by an unfamiliar lactating woman,[106] and infants move the head and arms less, mouth more, and cry less during exposure to their mothers' body odors.[106,128] Moreover, shortly after birth, infants prefer their mothers' breast unwashed as compared with when it is thoroughly washed and is less odorous.[102] Thus, as for other mammalian young, the recognition of and preference for maternal odors may play a role in guiding the infant to the nipple area and facilitating early nipple attachment and breast-feeding. Both early experience and genetic determinants may underlie these preferences.

Several findings suggest that newborns' affective reactions to orthonasally presented odors are influenced by experience. First, unlike breast-feeding infants, bottle-fed infants do not preferentially orient toward their mothers' axillary odors when paired with those of either a nonparturient woman or an unfamiliar lactating woman.[104] Similarly, breast-feeding infants evidence no recognition of or preference for the axillary odors of their own father relative to another father's.[104] Third, 7-day-old, breast-fed newborns turn toward a perfume that has been worn on the mother's breast during feedings in preference to perfumes worn by other mothers.[129,130] Fourth, after a 20- to 24-hour period of exposure to an artificial odor (wild cherry or ginger) in the ambient air, female newborns less than 2.5 days old turn their heads in the direction of that odorant (presented on a gauze pad) as opposed to an equally pleasant (to adults) but unfamiliar odorant.[105] A similar preferential response to the familiar odor has been observed in both sexes when testing was delayed for 2 weeks after the initial exposure, thus indicating that neonates retained a memory trace of the exposed scent throughout this period.[131]

Studies also indicate that infants' preferences for retronasally presented odors are mediated by experience. Various volatile flavors (e.g., garlic, alcohol, vanilla, carrot, mint) enter human milk via the mother's diet, altering its smell and, presumably, flavor.[132-136] Both garlic[132] and vanilla[135] in the mother's milk stimulate increased sucking and ingestion by the nursing infant. Enhanced sucking is also observed when the flavor of vanilla is added to bottle-fed infants' formula.[135] Repeated consumption of garlic by nursing mothers modifies their infants' response to garlic-flavored milk, eliminating the enhancement in intake (relative to a day on which a bland diet is consumed) that is seen when mothers have maintained a bland diet for a period of time.[111] Thus, in the case of foods, infants' odor or flavor preferences may largely reflect a positive response to novelty or variety in milk flavor.

Experience with flavor cues in amniotic fluid, mother's milk, or both can lead to enhancement of preferences for the flavors of

foods eaten by the mother during pregnancy and lactation. First, day-old infants born to mothers who regularly consumed anise-flavored beverages during pregnancy preferred the odor of anise relative to a control odor, whereas those infants whose mothers did not consume anise displayed aversive or neutral facial reactions to this odor.[94] Second, weaning-aged infants, who had had several weeks of exposure to the flavor of carrots in either amniotic fluid or mothers' milk, consumed significantly more of a carrot-flavored cereal and were perceived by their mothers as enjoying the carrot-flavored cereal more than plain cereal.[95] In contrast, control infants, whose mothers avoided carrots during pregnancy and lactation, exhibited no such preference. Thus these very early flavor experiences may provide the foundation for cultural and ethnic differences in cuisine.

Early experiences may also result in the generation of olfactory memories through associative learning processes, as suggested by a study in children who were between the ages of 3 and 6 years.[137] Children's hedonic responses to the odor of alcohol were related to the emotional context in which parents experience alcohol and the parents' frequency of drinking. In other words, children of a parent or parents who drank alcohol to change their state of mind or to reduce dysphoric feelings ("escape drinking") were significantly more likely to judge the odor of beer as unpleasant than were similarly aged children whose parents did not drink to escape. This difference between the groups was odor specific: children in the two groups were similar in their preference for the bubble gum and rejection of the pyridine odors. These data support the hypothesis that associative learning in the context of emotionally salient conditions is a powerful mechanism by which odors acquire personal significance and that the emotional context in which children experience an odor can influence subsequent behaviors.

Clearly, experience is important in mediating affective and behavioral responses to odors in infancy and childhood. It has even been argued that all odor preferences and aversions are learned through associational learning processes; even the smell of a skunk would be perceived as pleasant if experienced under appropriate conditions.[130,138] This view is primarily based on several studies that failed to find adultlike odor preference patterns in children younger than 5 years of age.[139,140] For example, 3- to 4-year-old children have been reported to be as likely to say they like the odors of synthetic sweat and feces as they are to say that they like the smell of amyl acetate (resembling banana or pear).[139] Even 6- to 7-year-old children may differ from adults in their hedonic experience of odors. Although adults and 6- to 7-year-old children have been shown to exhibit similar rank-order preferences for odors,[141-143] children this age were less consistent than the adults in (1) selecting preferred odors in a pair-wise comparison paradigm[141] and (2) classifying odors as "liked" or "disliked."[142]

Methodologic difficulties could contribute to these apparent age-related differences. For example, children as old as 4 years tended to answer questions about odors in the affirmative, saying "yes" whether they were asked whether an odor was pleasant or unpleasant.[131] Perhaps some of the foregoing developmental findings, based on opinion polling, reflect this response bias in young children, rather than developmental changes in olfactory preferences. Furthermore, any method relying on sustained attention or placing demands on memory could yield spurious age differences.

Studies using more age-appropriate methods provide partial support for this hypothesis. Essentially adultlike odor preference patterns were obtained for a set of nine odorants (carvone, phenylethylmethyl carbinol, methyl salicylate, C-16 aldehyde, eugenol, amyl acetate, androstenone, butyric acid, and pyridine) when 3-year-old children were asked to give "good smells" to a likeable television character (Big Bird) and "bad smells" to a grouchy character (Oscar the Grouch).[113] Although children and adults in this study did differ in their distributions of hedonic ratings for some odorants, the similarities outweighed the differences. Using a similar dichotomous forced-choice task in which children pointed to a smiling face or a frowning face to indicate their odor preferences, Strickland and associates[144] also demonstrated adultlike hedonic classifications of two other odorants (benzaldehyde and dimethyl disulfide) in children as young as 3 years. Together, these studies do not support the conclusion that all odor preferences are learned relatively late in the preschool period and suggest experimental reconsideration of the view that some odors may be inherently pleasant or unpleasant.

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SECTION XXIII Orthopedics

173 The Growth Plate: Embryologic Origin, Structure, and Function

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Skeletal formation and growth occur as a process of sequential morphologic and biochemical events that occur during fetal development. During development, bone tissue is formed and grows through one of two processes. Bone can form directly from mesenchymal tissue, which is called *intramembranous ossification.* This occurs at the periosteal surfaces of all bones as well as in parts of the pelvis, scapula, clavicles, and skull. Alternatively, bone tissue can form by replacement of a cartilaginous model, which is referred to as *endochondral ossification.* Endochondral ossification occurs at the base of the skull, the vertebrae, and at the growth plates of the appendicular skeleton.

Proliferating cartilage constitutes the basis for much of the size increase of bones as organs because bone tissue itself does not grow interstitially. In the long bones of the limbs the proliferating cartilage is located at the ends, in the form of a *growth plate,* or *physis.* The cartilage in the growth plate has a unique zonal structure, biochemistry, process of matrix mineralization, and blood supply that differs markedly from hyaline or articular cartilage. Other areas of the body that have proliferating cartilage of various configurations include the skull (sutures and the base), the spine (end plates and synchondroses), the pelvis (triradiate cartilages), and the carpus and tarsus. Discussions of the growth plate and cartilage growth usually focus on the typical long bone growth plate, such as the distal femur because of its highly organized histology and great activity.

DEVELOPMENT OF THE GROWTH PLATE

Limb development has been studied in various organisms. Chick studies provided a wealth of information in the past because of the accessibility of the limb bud in ovo. However, molecular studies have focused largely on the mouse embryo. In addition, cross-species transplants have indicated that many of the same signals control limb formation in both organisms. Additional information about limb patterning has been derived from the study of regenerating amphibian limbs.

The formation of the developing limb bud in vertebrates is initiated by the mesenchyme. Somites give rise to all the limb muscle cells, whereas the lateral plate mesoderm gives rise to connective tissue and cartilage and thus determines the primary limb pattern.[1,2] In the absence of somites, the lateral plate mesoderm forms a limb with normal skeletal structure and tendons, devoid of any musculature.

The embryonic limb bud formation is initiated during the fourth week of gestation by the lateral plate mesoderm as a small projection on the ventrolateral body wall.[3] This mesenchymal projection is covered by ectoderm, the tip of which thickens and becomes the *apical ectodermal ridge* (AER) (Figure 173-1), which drives limb outgrowth and proximodistal patterning. Underlying the AER are rapidly proliferating, undifferentiated mesenchymal cells that form the *progress zone* (PZ). Proliferation of these cells causes limb outgrowth. The events in upper and lower limb buds are similar in character and sequence. There is, however, a slight craniocaudal time gradient, with the upper limb bud appearing and progressing 1 to 2 days before the lower limb bud. The molecular interaction between the AER and the underlying undifferentiated mesenchymal cells (PZ) drives limb development. Once the process of differentiation of all limb elements is complete, the AER disappears.

As the limb bud grows, three major limb axes appear at different times and by different mechanisms (Figure 173-2). The limb bud develops asymmetries along the proximodistal (flank to digit tip), anteroposterior (thumb to small finger), and dorsoventral (back of hand to palm) axes. Some of the important signals for initiating and maintaining these axes have been worked out in detail. The genes that initiate and potentiate signals in different axes often interact and feed back with each other, resulting in effects in multiple planes of growth.

Limb Bud Outgrowth (Proximodistal Patterning)

Limb bud outgrowth (proximodistal patterning) is dependent on signals emanating from the AER. Fibroblast growth factors (FGFs) produced in the AER stimulate cell proliferation in the underlying mesenchyme in the progress zone. The expression of FGF-10 and FGF-8 is necessary to *initiate* limb bud outgrowth, and the expression of each supports and promotes the expression of the other.[4] This FGF-8/FGF-10 loop is mediated by Wnt-2b, a mammalian homologue of *Drosophila* segment polarity gene-wingless and WNT-8c proteins, through a β-catenin (WNT/β-catenin pathway).[5] FGF-2, FGF-4, and FGF-8 are expressed in the AER, and each is able to *sustain* limb bud outgrowth. A graft of an AER to an ectopic site on the bud leads to extra limb growth in the ectopic location. Removal of the AER stops limb bud growth and leads to limb truncation. However, limb bud outgrowth can be sustained after excision of the AER, if FGF-impregnated beads are inserted in place of the AER.[6,7]

Cell growth and proliferation of the mesenchyme are maintained through a regulatory loop, with FGFs and a protein called sonic hedgehog (shh), which maintains the integrity of the AER. The formin gene, which encodes several proteins that function as cytokines, is required to establish this Shh/FGF-4 feedback loop. Bone morphogenic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, play a complex role in the regulation of the AER (also in dorsoventral patterning).[8,9] BMPs are necessary to the induction of the AER.[8] However, BMP signaling must moderated by Gremlin

(a BMP antagonist) in order to maintain the Shh/FGF-4 feedback loop necessary for limb outgrowth and patterning.[10] The activation of Gremlin is dependent on the expression of the formin gene.[11]

Anteroposterior Axis Determination

Anteroposterior axis determination is under the control of the *zone of polarizing activity* (ZPA), which is an area of tissue in the posterior aspect of the limb bud (see Figure 173-1). Early chick studies showed that when tissue from this posterior area was transplanted to the anterior portion of another wing bud, a mirror image digit pattern developed.[12] The number of these additional digits developed depended on the strength (number of cells) and duration of transplant (16 hours for 2 digits, 20 hours for 3 digits). This observation led to the development of the morphogen gradient model, whereby anteroposterior patterning was determined by diffusion of a signal. The sonic hedge hog *(Shh)* gene is an important regulator of anteroposterior patterning.[13] Although early studies showed that exogenous application of retinoic acid to the anterior border of a normal limb could mimic results from ZPA transplant studies, this effect is now thought to be via activation of the *Shh* gene.[14] *Shh* represses Gli3R activity in the posterior limb bud, thus affecting the precise balance of Gli3A and Gli3R, which may be the basis for the graded response to Shh signaling.[13]

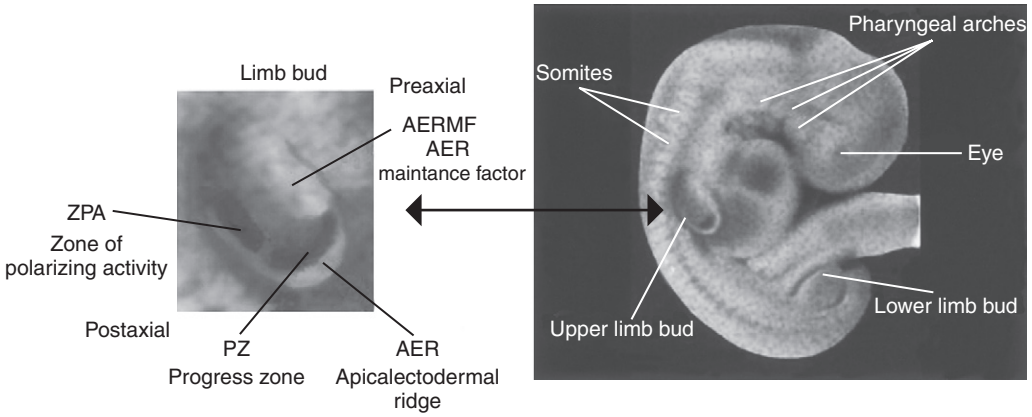


Figure 173-1 A 5-week-old human embryo and enlargement of the upper and lower limb buds. The limb bud at this time is a jacket of ectoderm (apical ectodermal ridge [AER]) covering undifferentiated mesenchymal cells (progress zone [PZ]). The zone of polarizing activity (ZPA), which is involved in anteroposterior patterning of the limb, is localized in a small area of mesoderm along the posterior border of the limb bud.

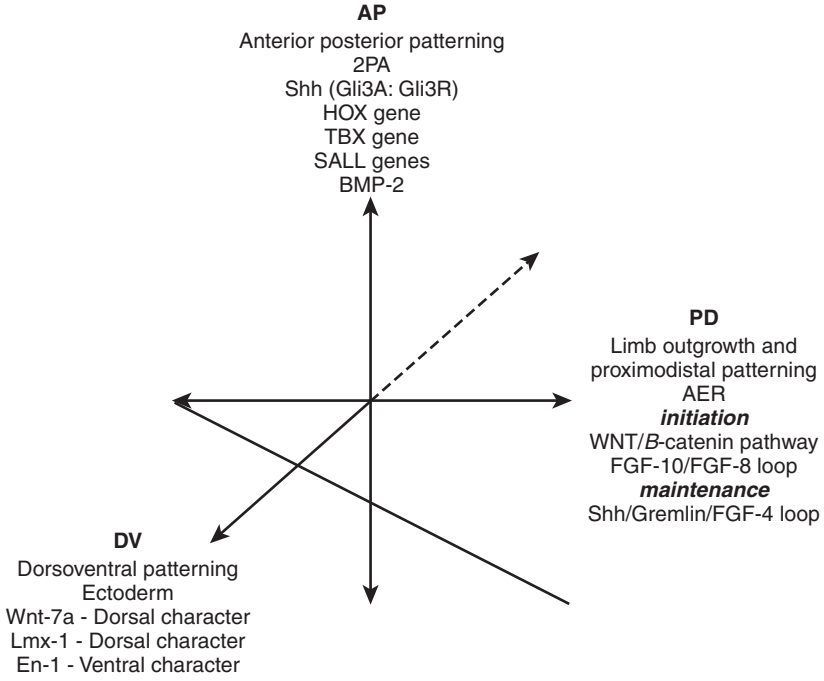


Figure 173-2 Three major limb axes. As the limb bud grows it develops asymmetries along the proximodistal (PD), anteroposterior (AP), and dorsoventral (DV) axes. The genes encoding the important signals that initiate and maintain these axes often interact and feed back, resulting in effects that are not confined to one direction of growth. *AER,* Apical ectodermal ridge; *BMP,* bone morphogenic protein; *En,* engrailed; *Fgf*, fibroblast growth factor; *GLI3A,* GLI3 activate; *Gli3R,* GLI3 repress; *Hox,* homeobox-containing gene; *Shh*, sonic hedgehog; *SALL,* Sal-like gene; *TBX,* T-bBox genes; *Wnt,* wingless; *ZPA,* zone of polarizing activity.

Other genes important in AP patterning under investigation include the 5'Hoxd genes, Tbox, and Sall family genes (specifically Tbx2, Tbx3, Sall1, and Sall4),[15,16] and defects in these genes cause digital alterations in humans.[17]

Dorsoventral Axis Determination

The dorsoventral axis is under the control of both the mesoderm and ectoderm of the limb bud at different stages of development. The mesoderm specifies the axis initially, but very early after limb bud formation, the ectodermal orientation becomes prominent. Evidence suggests that WNT-7a, a secreted signaling protein, confers the dorsal character to the ectoderm and stimulates Lmx-1.[18] Lmx-1 is a HOX gene that encodes a transcription factor (LMX-1) that dorsalizes mesoderm. The ventral limb expresses a transcription factor called engrailed (EN-1), which suppresses Wnt-7a expression, thereby limiting such expression, and consequently LMX-1 activity to the area of the dorsal mesenchyme.[19] Studies on BMP function have shown that BMP signaling is both necessary and sufficient to regulate EN1 expression, and consequently plays a role in dorsoventral patterning.[8]

Both the AER and the ZPA activate *HOXA* and *HOXD* genes. *HOX* genes have specifically been shown to affect the growth of cartilage and precartilaginous condensations. They are likely important in determining the length, segmentation, and branching of limb elements. *HOX* genes are not expressed strictly along anteroposterior or dorsoventral axes, and their position of expression differs in various areas of the limb.

Bone and Growth Plate Development

The first sign of future potential bone formation occurs in the early embryonic period as a localized condensation of the mesenchyme. Cellular condensations arise as a result of either increased mitotic activity or an aggregation of cells drawn toward a specific site.[20] As the mesenchyme begins to condense, the cells become more rounded, concomitant with a reduction in the amount of intercellular substance. This stage is referred to as the *precartilage blastema.* As the tissue continues to mature, the mesenchymal condensations differentiate into chondrocytes that form a template, or anlagen (skeletal anlagen), of the future bone (Figure 173-3). This transformation occurs during the sixth week of gestation. The expression of some specific molecular markers, such as aggrecan and type II collagen, distinguishes differentiating chondrocytes from the undifferentiated mesenchymal cells remaining at the periphery of the skeletal element that form a structure called the *perichondrium.*[21] During the seventh week of gestation, the innermost chondrocytes of the anlagen further differentiate into hypertrophic chondrocytes. At the same time as the chondrocytes hypertrophy, perichondrial cells start to differentiate into osteoblasts to form, around the cartilaginous core, a mineralized structure, termed the *bone collar*.[22,23] Once fully differentiated, hypertrophic chondrocytes become surrounded by a calcified extracellular matrix that, through a vascular endothelial growth factor–dependent pathway, favors vascular invasions from the bone collar. This process brings in chondroblasts that will degrade the extracellular matrix surrounding the hypertrophic chondrocytes and provides progenitors of osteoblasts derived from the bone collar.[24] The osteoblasts produce an osteoid matrix on the surfaces of the calcified cartilaginous bars and form the primary trabeculae; this process is called endochondral ossification. This initial site of ossification is known as the *primary ossification center,* and most of these appear in the late fetal period (Figure 173-4).

This sequential process of chondrocyte proliferation, hypertrophy, and replacement by osteoblasts becomes organized into the growth plate at each end of the expanding bone. The growth plates are apposed to the metaphyses at either end of the long bone. The histologic elements of the growth plate are well defined. Chondrocyte division is well organized into zones that can be demonstrated autoradiographically using tritiated thymidine.[25-27] Once the growth plate has been formed, longitudinal growth of the bone occurs by appositional growth of cells from within the growth plate, and new bone is formed in the metaphyseal side of each growth plate through a process that recapitulates the stages that occurred in the central portion of the original cartilaginous anlage. The rate at which this growth occurs is different at different anatomic positions; proliferating cartilage is arranged differently at different anatomic locations.[28] This process continues until closure of the growth plates at skeletal maturity.

At specific times in the postnatal development of each long bone, a secondary center of ossification (epiphysis) forms at the end of each bone. The secondary center of ossification is another zone of proliferating cartilage beneath the articular cartilage and is responsible for growth of the chondroephiphyseal cartilage

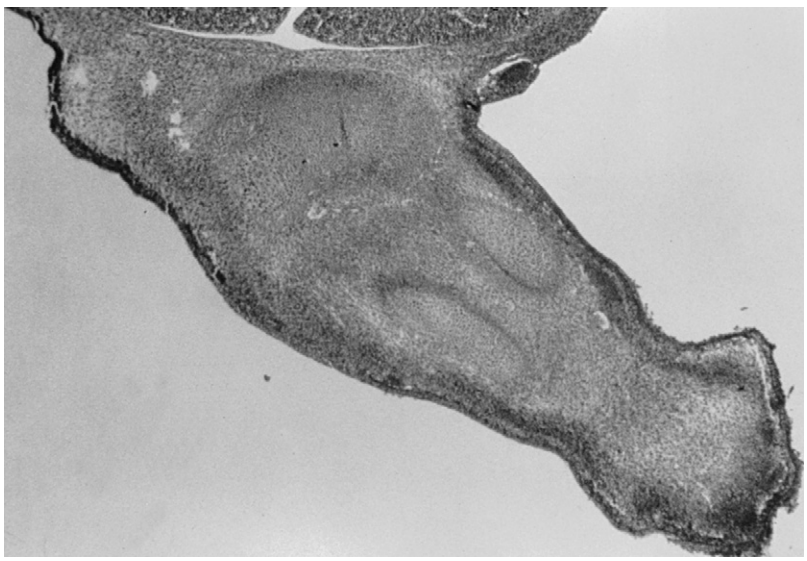


Figure 173-3 Longitudinal section of the developing hindlimb from a 13-day mouse embryo (H&E, ×40). Mesenchymal condensations of the skeletal anlagen have begun to form.

surrounding the epiphysis (Figure 173-5).[29] The processes of chondrocyte proliferation and hypertrophy, matrix calcification, vascular invasion, and osteoblastic new bone formation occur in the same sequence as in the growth plate associated with the primary ossification center. However, the epiphyseal end of the long bone enlarges as a result of radial apposition of cells around the secondary ossification center.

The Molecular Biology of Chondrocyte Differentiation

The growth plate is made up of highly organized chondrocytes at different stages of differentiation. Nonhypertrophic chondrocytes reside in the resting and proliferative zone and contribute to the replenishing of stem cells and growth through cell division. Hypertropic cells in the hypertropic zone contribute to longitudinal growth by an increase in their size and excrete factors that lead to the transformation of cartilage and matrix into bone.[30] Several growth factors control nonhypertrophic chondrocyte differentiation. The FGF receptor-3 (FGFR3) inhibits growth by restraining chondrocyte division.[31] A genetic mutation leading to constitutive activation of FGFR3 is the cause of achondroplasia, a dwarfing condition in which short stature is the result in FGFR3 continuous inhibition of chondrocyte proliferation in the growth plate.[32,33] Indian hedgehog (Ihh) promotes nonhypertrophic chondrocyte proliferation.[22] An interesting link between the Ihh pathway and the FGF pathway is indicated by data showing that FGFR3 signaling can inhibit Ihh expression.[31]

The hypertrophic chondrocytes comprise two subpopulations: the prehypertrophic chondrocytes and the fully differentiated hypertrophic chondrocytes. Parathyroid hormone (PTH)–related peptide (PTHrP) binds to the PTH/PTHrP receptor present in prehypertrophic chondrocytes, and it plays a critical role in

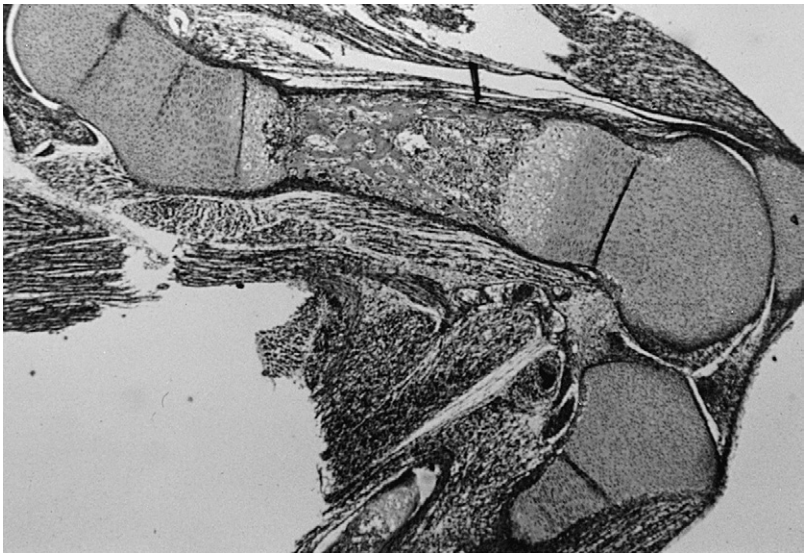


Figure 173-4 Longitudinal section of the femur and proximal tibia from an 18-day mouse fetus (H&E, ×40).The primary center of ossification has formed at the midshaft of the femur.The proximal (*left,* hip) and distal (*right,* knee) chondroepiphyses are visible, as are part of the cartilaginous patella and the proximal tibial chondroepiphysis.

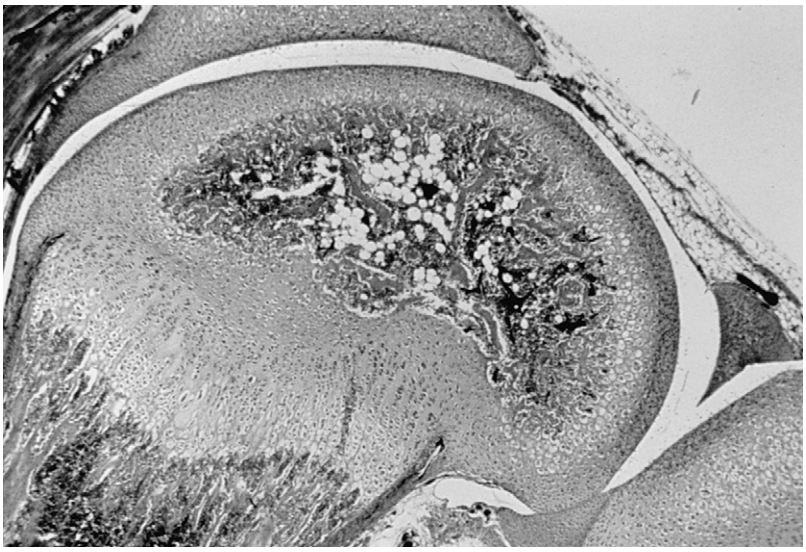


Figure 173-5 Longitudinal section of the distal femur from a 10-day postnatal mouse (H&E, ×40).The secondary center of ossification has formed within the distal femoral chondroepiphysis.The growth plate or physis is clearly defined between the secondary center of ossification and the metaphysis.

regulating hypertrophic chondrocyte differentiation, by delaying chondrocyte maturation and in doing such ensures the supply of proliferating chondrocytes necessary for skeletal growth. PTHrP expression is under the control of Ihh; Ihh up-regulates the synthesis of PTHrP by prehypertrophic chondrocytes, thereby indirectly slowing down the process of chondrocyte hypertrophy, and promoting chondrocyte proliferation.[22,34] Wnt signaling also plays an important role in the regulation of hypertrophic chondrocyte biology. Studies have shown that misexpression of Wnt-4 accelerates the transition from nonhypertrophic chondrocyte to hypertrophic chondrocyte, and in doing so results in slightly advanced ossification, whereas misexpression of Wnt-5a causes a delay in the transition from prehypertrophic to hypertrophic chondrocyte and results in a mild delay in ossification.[31]

Runx2, also called Core-binding factor-α subunit 1 (CBFA1), is a member of the runt family of transcription factors, and is necessary for osteoblast specific gene expression.[34] Runx2 is expressed in prehypertrophic chondrocytes and its constitutive expression in nonhypertrophic chondrocytes induces differentiation to hypertrophic chondrocytes, Ihh expression, and, eventually, endochondral bone formation.[35] These functions, along with its role during osteoblast differentiation and vascular invasion, identify CBFA1 as the most pleiotropic regulator of skeletogenesis.

GROWTH PLATE

The growth plate of a long bone provides an exceptional correlation among histology, ordered cytodifferentiation, molecular biology, and physical function. The growth plate is composed of a cartilaginous component that is surrounded by a fibrous component and bounded by a bony metaphyseal component. Each component has a unique structure, biochemistry, and function; together, these result in longitudinal and latitudinal growth and remodeling of the developing skeleton. The vascular supply of the growth plate results in unique biochemical properties and is integral to normal function.

Vascular Supply

The normal growth and development of a bone are inextricably linked to its vascular, and in particular its arterial, supply. In fact, the onset and maintenance of ossification depend on a constant supply of nutrients. There are three major vascular suppliers to the growth plate: the epiphyseal arteries, the nutrient arteries, and the perichondrial arteries (Figure 173-6). The epiphyseal arteries are derived from periarticular vascular arcades that form on the nonarticular bone surfaces. These arteries send branches through the cartilaginous epiphysis within cartilage canals to supply the reserve and proliferative zones of the growth plate.[36] Thus the overall viability of the growth plate to maintain normal bone growth and development depends on the integrity of these vessels. Any damage to these arteries will result in growth arrest.

The metaphyseal supply is primarily derived from the nutrient arteries, also known as diaphyseal arteries. The nutrient arteries are generally derived from an adjacent major systemic artery. A nutrient artery enters a bone through its nutrient foramen, which leads into a nutrient canal, and once the vessel reaches the medullary cavity, it divides into ascending and descending medullary branches.[36,37] The ascending and descending medullary branches anastomose with the metaphyseal collaterals at the metaphyseal zone. The metaphyseal supply induces blood-borne precursors that promote ossification and remodeling at the hypertrophic zone (HZ) of the growth plate.[36,38] The physeal cartilage itself maintains a separation between these two circulations throughout normal human growth. The perichondrial arteries supply the ring of LaCroix and the groove of Ranvier. Capillaries from this system communicate with the epiphyseal and metaphyseal capillaries in addition to the vessels of the joint capsule.

Cartilaginous Component

Within the growth plate the various subpopulations of chondrocytes (zones)—reserve (resting), proliferative (proliferating), hypertrophic—are arranged in columns (see Figure 173-6). These

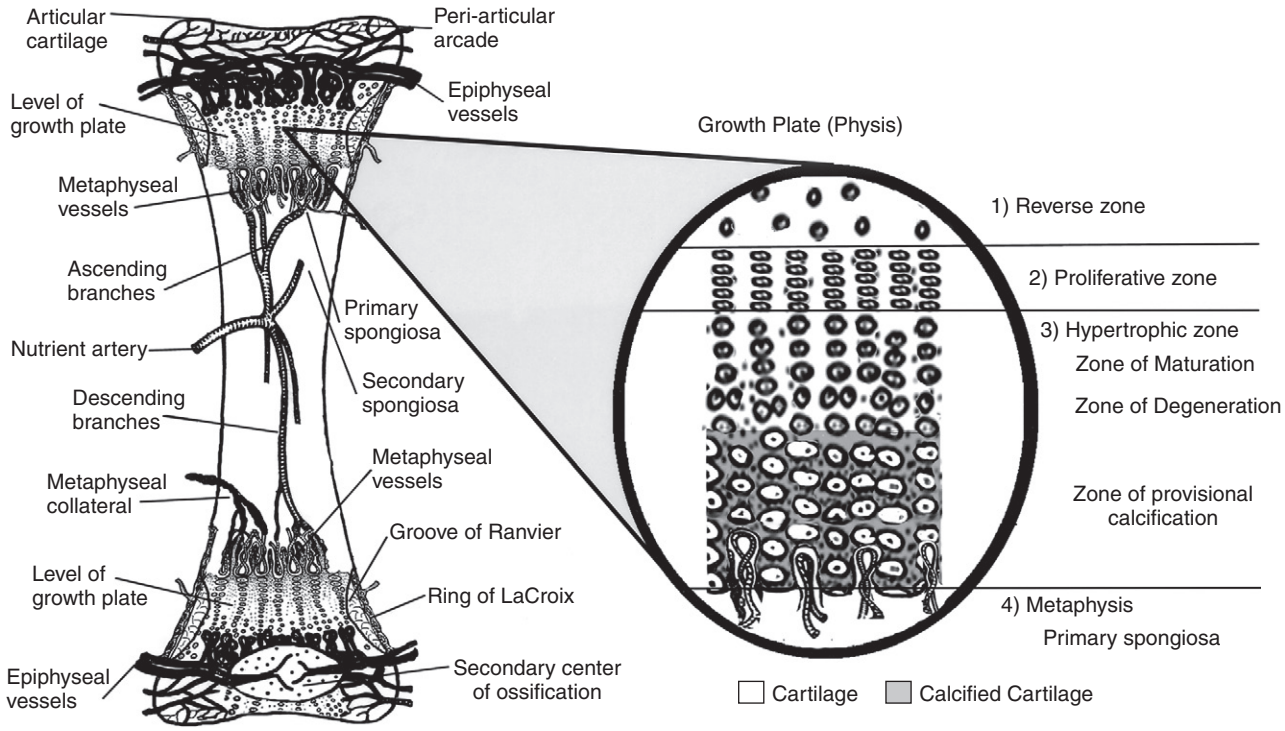


Figure 173-6 Diagrammatic illustration of the blood supply and zones of the growth plate. *(Modified from Scheuer L, Black S: Bone development. In Scheuer L, Black S [eds]:* Developmental Juvenile Osteology. *London, Academic Press, 2000, pp 18-31.)*

chondrocytes regulate longitudinal growth of the skeleton until their disappearance at the end of puberty in humans. Each zone has characteristic histologic and biochemical features that define its formation. The zone farthest away from the diaphysis and closest to the epiphysis is the *reserve zone* (RZ). In this zone the chondrocytes are small, round, and randomly distributed. The chondrocytes in the RZ replicate rarely; the ratio of matrix volume to cell volume is the highest of any layer of the growth plate.[39] The matrix contains randomly oriented fibrils of type II collagen, which inhibits calcification and acts as a barrier to the advancing front of the secondary center of ossification.[21] The chondrocytes in this zone receive a vascular supply from the epiphyseal vessels; however, the epiphyseal vessels do not actually arborize in the RZ, and hence the oxygen tension in this zone is relatively low. The precise function of the RZ remains ill defined. Two likely functions are (1) physical separation between the osseous tissue of the secondary center and the remainder of the growth plate and (2) synthesis and storage of nutrients and raw materials (i.e., glycogen, lipid, and protein) that will be used by other chondrocytes in the lower zones of the growth plate.

In the adjacent *proliferative zone* the chondrocytes are tightly bound in columns parallel to the axis of the length of the bone. The cytoplasm contains glycogen stores and is rich in endoplasmic reticulum, features suggesting a rich source of nutrients for aerobic glycolysis and a high capacity for protein synthesis. Of the three zones this zone has the highest rate of proteoglycan synthesis and turnover. The proteoglycans (aggrecans) in this zone are large aggregates consisting of glycosaminoglycans attached to core proteins.[40] Such aggregates inhibit calcification. The chondrocytes replicate rapidly in the proliferative zone, and they become flattened and slightly irregular in shape. The rate of duplication of cells is closely regulated and usually constant.[41] The binding matrix is contiguous with the matrix of the RZ and has the same biochemical composition. The type II collagen fibrils are oriented longitudinally, surrounding the columns of chondrocytes. The epiphyseal vessels that crossed the RZ terminate in the proliferative zone, thereby resulting in the highest oxygen concentration in the growth plate. The presence of rich glycogen stores and a high oxygen tension supports aerobic metabolism in the proliferative zone chondrocyte. The functions of the proliferative zone—matrix production and cellular division—together contribute to longitudinal growth.

The HZ has traditionally been divided into two parts: the upper, or zone of maturation; and the lower, or zone of degeneration. The lowest portion of the HZ is also called the zone of provisional calcification. In the zone of maturation the chondrocytes start to hypertrophy. There is a change in the proteoglycans of the matrix in this zone, with the size of the aggrecan decreasing. This decrease results from an active degradation mediated by physeal enzymes, including metalloproteases (i.e., collagenase, stromelysin, and neutral growth plate proteases).[42,43] There is also a marked decrease in type II collagen synthesis. In moving down the cellular column and away from the epiphyseal vessels, the chondrocytes are exposed to an environment of increasingly lower oxygen tension.

The major energy source for production of adenosine triphosphate (ATP) in the HZ chondrocytes is anaerobic glycolysis of glucose from endogenous glycogen stores. Glycogen is stored in the RZ and proliferative zone and is then consumed in the HZ. Metabolic activity in the HZ cells differs from that in most animal cells. In most cell types, glucose is oxidized to pyruvate in the cytoplasm, and the pyruvate then enters the mitochondria (by glycerol phosphate shuttle) to be further oxidized by the Krebs cycle. The Krebs cycle is an important additional energy source for most cells. In the growth plate pyruvate continues to enter the mitochondria but at a much reduced rate, and glycerol phosphate does not enter the mitochondria because the growth plate zones lack the glycerol phosphate shuttle. Thus even in the presence of oxygen, the growth plate cartilage metabolizes glucose relatively anaerobically. The mitochondria of the chondrocytes in the proliferative zone are capable of conducting the Krebs cycle and generating ATP. However, in the HZ, owing to lower oxygen tension, there is a switch in the function of the mitochondria from ATP production to calcium accumulation. These mitochondrial stores allow buffering of the ionized intracellular calcium in the already high concentrations of the HZ.[44]

In the zone of degeneration is a further diminution in proteoglycan size. The proteoglycan, which in aggrecan form is an inhibitor of calcification, no longer serves such a role because it is increasingly degraded.[42,43] Biochemical analysis shows that the chondrocytes in this zone are very active metabolically. They synthesize alkaline phosphatase, neutral proteases, and type X collagen, thereby participating in matrix mineralization. The mitochondria of the chondrocytes here begin to disgorge the calcium previously accumulated. This calcium apparently is packaged in vesicles of cellular membrane and is deposited into the surrounding matrix.[45] The hypertrophic chondrocytes do not synthesize type II collagen in the lower HZ, but instead synthesize type X collagen, a unique protein associated with endochondral ossification.[46,47] Active changes in chondrocytic activity occur at this site and lead to degeneration and cell death at the last intact transverse septum forming the interface with the metaphysis.[48] The longitudinal septa begin to calcify in this region of the growth plate; hence this zone is also termed the *zone of provisional calcification.* This is where the last transverse septa are ultimately degraded.

Metaphysis

The metaphysis begins distal to the last intact transverse septum of each cartilaginous cell column of the HZ (see Figure 173-6). It functions in the removal of the mineralized cartilaginous matrix of the HZ and in the formation of the primary spongiosa. Bone formation begins with the invasion of the hypertrophic lacunae by vascular loops, bringing with them osteoblasts that begin the synthesis of bone. The osteoblasts progressively lay down bone on the cartilage template. Subsequently, the initial woven bone and cartilage bars of the primary spongiosa are resorbed by osteoblasts and are replaced by lamellar bone to produce the secondary spongiosa.[49]

Perichondrial Ring of LaCroix and Groove of Ranvier

Surrounding the periphery of the physis are a wedge-shaped structure, the *groove of Ranvier,* and a ring of fibrous tissue, the *ring of LaCroix* (see Figure 173-6). The cells in the groove of Ranvier are active in cell division and contribute to an increase in the diameter, or latitudinal growth, of the growth plate. The basic structure of the perichondrial ring of LaCroix is a fibrous collagenous network that is continuous with the fibrous portion of the groove of Ranvier and the periosteum of the metaphysis. The perichondrial ring functions as a strong mechanical support at the bone-cartilage junction of the growth plate.

GROWTH AND ITS CONTROL

The endocrine control of the growth plate is a complex and important aspect of growth. Numerous factors have been identified as important regulators of bone and cartilage (Table 173-1). Some of these factors (systemic hormones, vitamins, and growth factors) are produced at a site distant from the growth plate and therefore act on the chondrocytes through a classic endocrine mechanism. Other factors both are produced and act within the growth plate and therefore function as paracrine or autocrine factors.[30] Each zone of the growth plate may be specifically targeted by one or more agents to affect a particular stage in the cell maturation phenomenon.

Growth hormone (GH) is a peptide hormone secreted by the pituitary gland, and its absence leads to pituitary dwarfism. GH acts throughout the growth plate and primarily stimulates cellular proliferation. GH does not directly stimulate the growth plate; rather, it stimulates production of a group of peptide growth factors termed *somatomedin* or *insulin-like growth factor* (IGF). The effects of GH mainly result from the production of serum-derived IGF by the liver, but there is also a direct GH effect to induce chondrocytes to produce IGF locally (paracrine or autocrine effect). IGF-I (somatomedin C) is the potent stimulator of postnatal growth mediating chondrocyte proliferation and matrix synthesis, whereas IGF-II is the fetal somatomedin.[50] Immunohistochemical studies have localized the highest concentration of IGF-I receptors to the PZ of the growth plate.[51] IGF-I increases cellular proliferation, probably by two mechanisms: by direct IGF-I effects; and, perhaps more important, through the modulation of other growth factor effects. The synergistic interactions of IGF-I with FGF and TGF-β are specific for deoxyribonucleic acid (DNA) synthesis, a finding that confirms the central role of IGF-I on mitogenesis in the growth plate.[50] Insulin can crossreact with the IGF-I receptors and therefore may have some minor anabolic effect at physiologic insulin levels.[44]

TABLE 173-1

Growth Plate Control

Agent	Effect
Specific endocrine hormones	
Thyroid hormones	↑ Chondrocyte proliferation, ↑ chondrocyte maturation
Growth hormones	Secreted by pituitary, converted to somatomedin (IGF-I); IGF-I interacts with local factors
IGF-I	↑ Chondrocyte proliferation
Insulin	Interacts with IGF-I receptor, ↑ chondrocyte proliferation
PTH-related peptide	Binds to PTH receptor → ↑ cAMP → ↑ chondrocyte proliferation and matrix synthesis, also works through inositol triphosphate and protein kinase C
Androgens	Anabolic but pharmacologic doses may promote growth plate closure
Estrogens	Inhibit bone resorption but may be important agent in plate closure
Local agents	
Growth factors	
Cytokines	
Other small molecules	
Autocrine, paracrine	
Fibroblast growth factor	↑ Chondrocyte proliferation, interacts with IGF-I
Transforming growth factor-β	Relatively weak and variable effects depending on other agents present, ↑ chondrocyte proliferation and maturation
Interleukin-1	↑ Matrix degradation through metalloproteinases
Prostaglandin	↑ Proteoglycan, ↓ collagen, alkaline phosphatase
Vitamins	
Vitamin A	Required for chondrocyte maturation
Vitamin C	Necessary cofactor for collagen synthesis
Vitamin D and metabolites	Required for calcification of matrix, deficiency leads to failure of calcification and marked elongation of the hypertrophic zone (rickets)

↑, Up-regulates; ↓, down-regulates; *cAMP,* cyclic adenosine monophosphate; *IGF-I,* insulin-like growth factor-I; *PTH,* parathyroid hormone.

The thyroid hormones, thyroxine and triiodothyronine, are peptide hormones produced by the thyroid gland. The thyroid hormones act on the PZ and upper HZ chondrocytes through a systemic endocrine mechanism and promotes chondrocyte maturation.[34] Thyroxine enhances cartilage growth by two functions: (1) it increases DNA synthesis in cells from the proliferative zone and (2) it increases glycosaminoglycan and collagen synthesis and alkaline phosphatase activity. Its effect on cartilage growth is mediated by synergy between thyroxine and IGF-I. These two agents together restore both growth and maturation. Triiodothyronine also increases cartilage growth by interacting with IGF-I. It promotes the cytodifferentiation from a proliferative to a hypertrophic chondrocyte, independently from IGF-I, however.

PTH is produced by the parathyroid glands. It acts primarily on the proliferative zone and upper HZ chondrocytes, and it has a direct agonist role in the cellular metabolism of the growth plate. There is a complex relationship among PTH, the intracellular calcium concentration, and regulation of growth plate.[52] In the growth plate chondrocyte, PTH stimulates the production of inositol triphosphate from phosphoinositol 4,5-biphosphate. Inositol triphosphate induces the release of calcium from an intracellular store, which causes a transient increase in the cytosolic ionized calcium concentration. PTH also mediates an increase in proteoglycan synthesis through the breakdown of membrane phosphoinositides and subsequent activation of protein kinase C. The regeneration of membrane phosphoinositides can result in the stimulation of prostaglandin synthesis, which also appears to have a small stimulatory effect on proteoglycan synthesis.

The effects of sex steroids (androgens and estrogens) on proliferating cartilage are complex. Although androgens and estrogens may increase chondrocyte division, they may also interact with adrenal steroids and many other factors. Androgens function primarily in the lower portion of the growth plate to stimulate mineralization. Their anabolic effect is manifested as an increased deposition of glycogen and lipids in cells and an increase in proteoglycans in cartilage matrix.[44] Androgens can also stimulate DNA synthesis in chondrocytes. Supraphysiologic doses of androgens depress growth and accelerate growth plate closure. Estrogen signaling is necessary for normal skeletal maturation and growth plate closure, but premature exposure to high levels of estrogen (precocious puberty) causes premature growth plate closure and short stature.[30]

Besides FGFs (see the earlier discussion of the development of the growth plate), several other growth factors have been demonstrated to contribute to the regulation of growth plate development. Platelet-derived growth factor stimulates DNA synthesis and cell replication of chondrocytes as well as protein synthesis. TGF-β is a multifunctional peptide that controls cell replication and differentiation. It is involved in cartilage formation during the first step of endochondral bone formation. Release of TGF-β is stimulated by PTH, but the overall effect of TGF-β appears to depend on constituent growth factors in the cell. Therefore its effects may be stimulatory or inhibitory, depending on the hormonal environment when TGF-β is introduced to the chondrocytes.[44] For example, TGF-β can inhibit progression of growth plate chondrocytes to the full hypertrophic phenotype, although this can be overcome by the thyroid hormones which drive maturation.[28] On the other hand, TGF-β is a potent inhibitor of a lymphocyte-activating factor called *interleukin-1,* which induces degradation in growth plate chondrocytes.[53]

Several vitamins are required for normal growth plate function.[54] The active metabolites of vitamin D (1,25- and 24,25-dihydroxyvitamin D) are produced in the liver and kidney. Vitamin D deficiency results in an elongation of the cell columns of hypertrophic zone. This effect is cause by a vitamin D–induced

decrease in systemic calcium and phosphorus and the subsequent inhibition of mineralization. The vitamin D metabolites are bound to cells in all growth plate zones except the HZ. The highest levels are found in the PZ. A deficiency state leads to rickets, which at the histologic level of the growth plate manifests itself as a failure of calcification of the matrix and marked elongation of the HZ. Histologically the RZ and PZ are normal, but the HZ is greatly expanded, and this results in widening of the growth plate.[30,55] Vitamin C is a necessary cofactor for the synthesis of collagen. Deficiency of vitamin C leads to scurvy. The greatest deficiency in collagen synthesis is seen in the metaphysis. The microscopic appearance of the cartilaginous portion of the growth plate is normal, but that of the metaphysis is quite abnormal. The physeal-metaphyseal junction is particularly disrupted, with persistence of calcified cartilage and sparse bony trabeculae. Vitamin A is essential for epiphyseal cartilage cell metabolism. A deficiency state results in impairment of the chondrocyte maturation.

CORRELATED CLINICAL CONDITIONS

It is interesting to review the causes of some skeletal dysplasias (osteochondrodysplasias), a group of genetic disorders characterized by generalized disorders of growth and development of bone and cartilage. The generalized disturbance in the development of the skeleton affects the skull, spine, and extremities in varying degrees resulting in disproportionate short stature (short-trunk or short-limb dwarfism). The pathogenesis of many of these conditions is slowly being discovered, teaching us little by little about the growth of the skeleton (Table 173-2). Future research in this area may lead to the development of therapeutic agents that target the genetic abnormalities that cause the previously mentioned pathologies.[56]

TABLE 173-2

Molecular Basis of Some Abnormalities of the Growth Plate

Condition	Molecule	Human Chromosome Location
Achondroplasia	Fibroblast growth factor receptor-3 (FGFR3)	4
Hypochondroplasia		
Thanatophoric dysplasia		
Diastrophic dysplasia	Diastrophic dysplasia sulfate transporter	5
Stickler syndrome	Type II collagen	12
Spondyloepiphyseal dysplasia congenita		
Kniest dysplasia		
Pseudoachondroplasia	Cartilage oligomeric matrix protein (COMP)	19
Multiple epiphyseal dysplasia		
Jansen metaphyseal chondrodysplasia	PTH-PTHrP receptor	3

PTH, Parathyroid hormone; *PTHrP,* parathyroid hormone–related protein.

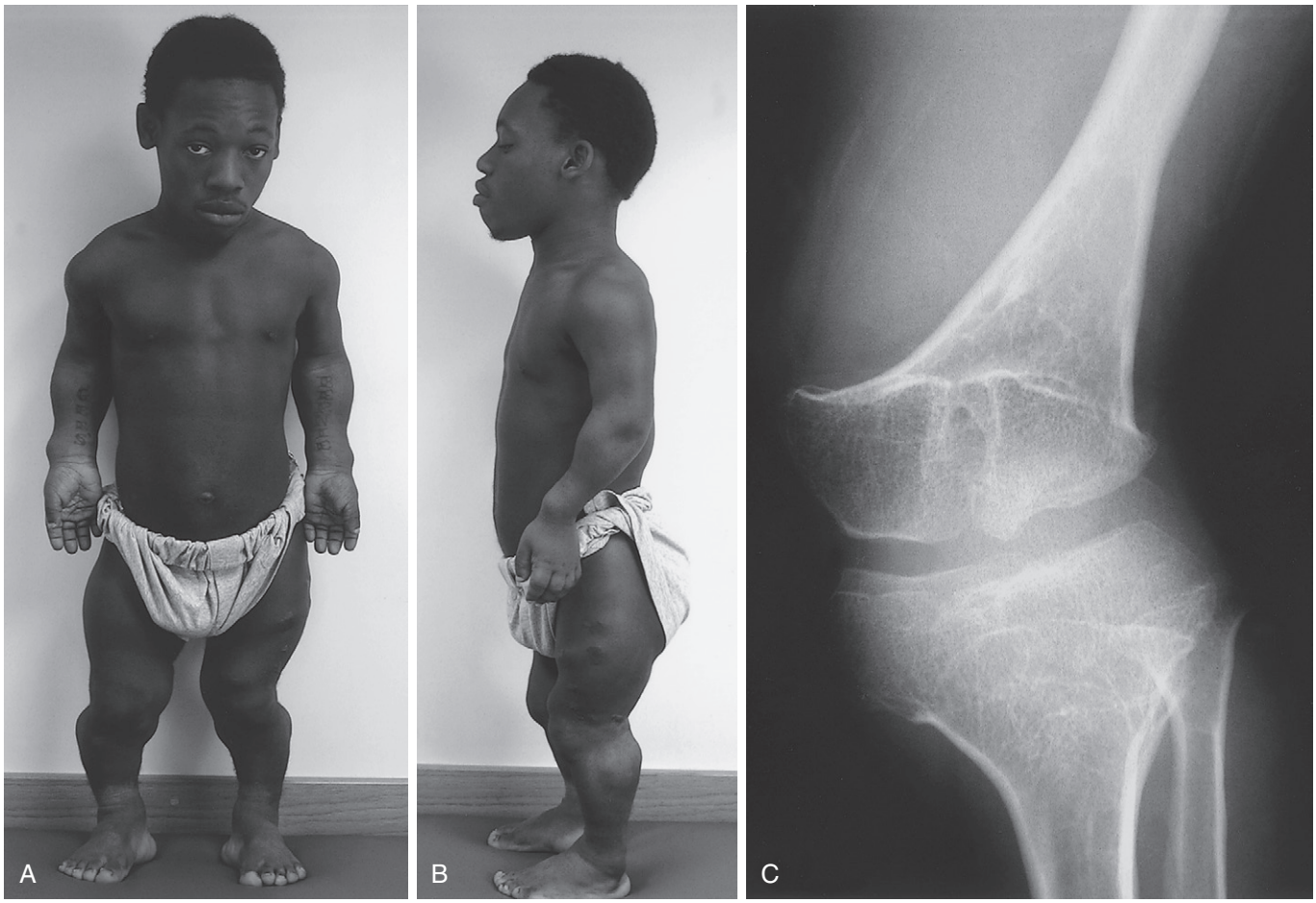


Figure 173-7 A 19-year-old patient with spondyloepiphyseal dysplasia congenita. **A** and **B,** Note the markedly short stature with short trunk and limbs. There are angular deformities of the lower limbs. **C,** An anteroposterior radiograph of the knee shows abnormally flattened and broad femoral and tibial epiphyses, causing angular deformities.

Achondroplasia, the most frequent form of short-limb dwarfism, is an autosomal dominant condition, although two thirds of cases arise by spontaneous new mutations that have been mapped to chromosome 4, in a region encoding *Fgfr-3*.[32,33] The activating mutations in the *Fgfr-3* gene lead to a retardation in chondrocyte proliferation resulting in abnormal endochondral ossification. *Hypochondroplasia* is an autosomal dominant allelic variant of achondroplasia with similar phenotypic and genotypic features, and the mutation in the *Fgfr-3* gene occurs in a different region (in the tyrosine kinase domain, in contrast to the transmembrane domain in achondroplasia).[33] A mutation in *Fgfr-2* underlies the *Crouzon syndrome,* in which the cranial sutures are disrupted.[57] In the *Pfeiffer* and *Jackson-Weiss syndromes* (both of which are characterized by craniosynostoses and limb defects) are mutations in both *Fgfr-1* and *Fgfr-2*.[58] The coordination of the various *Fgfr*s and receptors appears to be responsible for proportional growth.

Diastrophic dysplasia is a rare autosomal recessive condition characterized by short-limb dwarfism with spinal deformities and specific hand, foot, and ear abnormalities. The responsible gene has been mapped to chromosome 5 and encodes a sulfate transporter protein (diastrophic dysplasia sulfate transporter).[59] Impaired function of this protein is thought to lead to undersulfation of proteoglycans in the cartilage matrix, thus affecting hydration of cartilage.

Mutations in the gene encoding type II collagen, the predominant protein of cartilage, underlie several skeletal dysplasias: Stickler syndrome, spondyloepiphyseal dysplasia congenita, Kniest dysplasia, and Langer-Saldino achondrogenesis.[60,61] These conditions are characterized by short-trunk dwarfism and severe involvement of the vertebrae and epiphyseal centers (Figure 173-7). The gene defect has been linked to a deletion in the *COL2A1* gene on chromosome 12.

Pseudoachondroplasia and *multiple epiphyseal dysplasia* are frequent autosomal dominant disorders characterized by short-limb dwarfism and epiphyseal and metaphyseal changes. The gene responsible for these disorders has been localized to chromosome 19 and appears to encode the cartilage oligomeric matrix protein, a glycoprotein found in matrix surrounding chondrocytes.[62]

Jansen metaphyseal chondrodysplasia is a rare autosomal dominant disorder characterized by abnormal growth plate maturation and laboratory findings that are indistinguishable from hyperparathyroidism. This disorder is caused by activating mutations in PTH/PTHrP receptors.[63] This receptor is important both in calcium metabolism and in regulation of chondrocyte maturation.[64] By somatic cell hybrid analysis, the mutations in the PTH receptor gene have been mapped to chromosome 3 in humans.

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Lee Niswander and Damien Bates

Developmental Basis of Congenital Limb Differences

OVERVIEW

The phenotypic manifestations of abnormalities in limb development previously have been referred to as *deformities* or *anomalies,* but the term *congenital limb differences* is now considered more appropriate.[1,2] Although the focus of this chapter is on the developmental basis of congenital limb differences in the upper limb, similar principles and pathways apply to the lower limb.[3] The molecular regulation of limb identity is discussed next.

The embryonic upper limb begins as a small bud protruding from the body wall (somatic lateral plate mesoderm) at approximately 26 days gestation.[4] Over a period of 25 days, a complex of genetic signals controls the molecular pathways that result in a limb with the correct size, orientation, and number of digits.[5]

The bud initially is composed of morphologically homogeneous mesenchyme cells that are covered by a layer of ectoderm.[6] The mesenchymal cells are of two separate origins: Cells from the somites migrate into the limb to form muscles,[7] whereas the mesenchymal cells derived from the lateral plate mesoderm give rise to the skeleton (cartilage then bone), tendon, ligament, dermis, and other connective tissues.[8] The ectoderm gives rise to the epidermis.

Bone formation begins when mesenchymal cells form condensations.[9] This process requires bone morphogenetic protein (BMP) signaling.[10] Mesenchymal condensations in the limb differentiate to form chondrocytes, which are characterized by the expression of *Sox9.*[9] A subset of chondrocytes, the hypertrophic chondrocyte, signals to adjacent perichondrial cells to become osteoblasts. Hypertrophic chondrocytes then undergo apoptosis leaving a cartilage matrix for subsequent osteoblast migration and ultimately replacement by bone.[9]

Initial formation of tendons occurs autonomously with respect to muscle; however, further tendon development requires the presence of muscle.[11] The development of tendons also occurs in close association with that of cartilage and bone. Early in mouse limb development, tendon precursors (expressing *scleraxis*) have been observed in the mesenchymal tissue surrounding skeletal primordia, but with continued limb development, *scleraxis* expression segregates to the interface between muscle and skeletal primordia.[12]

Blood vessels are present in the limb bud from the very earliest stages except for an avascular zone subjacent to the ectoderm.[13] The vascular system undergoes extensive remodeling characterized mainly by regression and branching.[14] With time, the initial capillary network transforms into a segmental arterial supply. Regression of all but the major axial artery (called the primary subclavian artery) is followed by branching of this vessel to form the mature arterial pattern. Veins undergo two main phases of development: Initially,

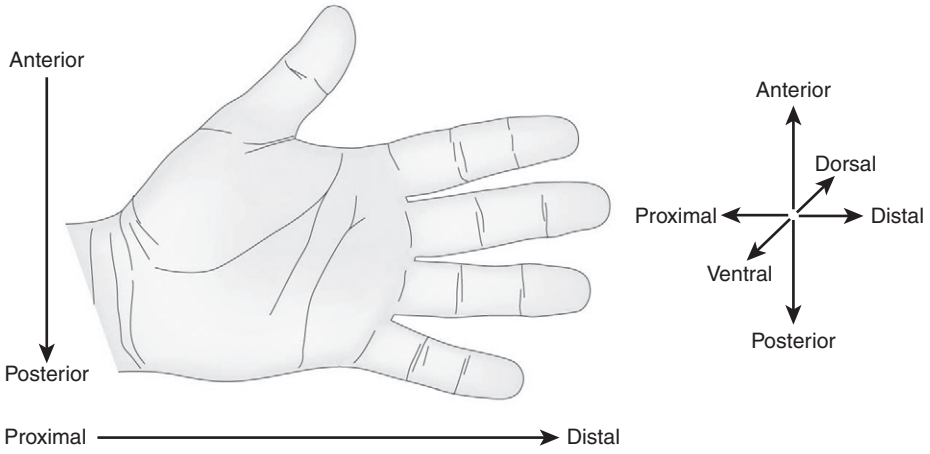


Figure 174-1 The three main axes of the human hand: proximodistal (P-D), anteroposterior (A-P), and dorsoventral (D-V). Tightly regulated processes during embryonic development ensure that the thumb arises at one edge of the hand, whereas the little finger arises at the other.[20]

the veins are located superficially along the anterior border, posterior border and apex of the limb (anterior marginal vein, posterior marginal vein, and marginal sinus). The marginal sinus subsequently regresses, and the anterior marginal vein becomes the cephalic vein and the posterior marginal vein becomes the basilic vein. Later in limb development (at approximately week 6), the deep veins that accompany the arteries become more prominent.

Nerves enter the upper limb in week 5 of development along the same planes as for the blood vessels and are in close physical proximity to them.[14] Positional signaling from the mesoderm appears to control the pattern of blood vessels and nerves.[15-17] Although not required for nerve ingrowth into the limb, muscles and skin are required for subsequent peripheral nerve branching.[18,19]

The limb bud grows along three primary axes: proximodistal (P-D), from shoulder to finger tips; anteroposterior (A-P), from thumb to little finger; and dorsoventral (D-V), from the back of the hand to the palm[20] (Figure 174-1). By day 33 of development, a paddle-shaped hand plate has formed; it is not until day 56, however, that the major morphologic features of the upper limb are complete.[4]

Limb growth is predominantly along the P-D axis during the early stages of limb development and is characterized by the sequential condensation of mesenchyme along this axis.[21] These mesenchymal condensations segregate into three main areas: the stylopod (from which will form the humerus, or upper arm), the zeugopod (from which will form the radius and ulna, or forearm), and the autopod (from which will form the carpus, or wrist, and hand).[6]

The concept of *positional information* has been used to explain how the limb pattern arises. According to this concept, mesenchymal cells are informed of their position in the three-dimensional matrix of the limb bud, and correct interpretation of this positional information results in differentiation of that cell to form the appropriate structure.[20] The pervading view of limb development was that this cellular differentiation occurred sequentially along the P-D axis.[22] In this view, the progressive specification of different parts of the limb was controlled by a timing mechanism that exerted its effect on a group of undifferentiated mesenchymal cells located in a region subjacent to the distal ectodermal cap called the *progress zone.*[23] The length of time cells spent in this progress zone specified the P-D information, and the positional value acquired as a result was fixed at the time the cells leave the zone.[22] More recent evidence, however, has challenged this concept, suggesting instead that cell fate may be specified earlier than was previously thought and by a different mechanism.[24] Differences between the early specification and progress zone models are illustrated diagrammatically in Figure 174-2.

Limb initiation is dependent on the activity of fibroblast growth factor 10 (FGF10) in the lateral plate mesoderm. *Fgf10* expression is itself downstream of WNT signaling (WNT-2b in the prospective upper limb region and WNT-8c in the prospective lower limb region). The precise craniocaudal location of limb outgrowth from the lateral aspect of the body wall is thought to be determined by the physical expression pattern of *Hox* genes.[25] Deletion of *Fgf10* in the mouse results in the absence of limbs[26,27] because FGF10 signaling in the limb mesenchyme is required to induce and maintain normal *Fgf8* expression in the *apical ectodermal ridge* (AER), which is essential for normal limb outgrowth. FGF8 and FGF10 reinforce each other through a positive feedback loop.[3]

In both *early specification* and *progress zone* models, further limb outgrowth is essential for normal patterning in the P-D axis.[22] Growth in the P-D axis is mediated by the AER. The AER is a strip of specialized epithelium, which forms at the junction between the dorsal and ventral ectoderm.[6] The key signals that mediate AER function are members of the FGF family.[22] Disruption of the AER results in limb truncation. Earlier disruptions to the AER during limb development result in more proximal limb truncations.[28] Of note, although the AER provides a permissive signal (through FGFs) enabling the realization of the P-D pattern,[6] the information for P-D patterning is intrinsic to the mesenchyme.[29] Removal of the AER is associated with cell death in the subjacent mesenchyme,[24] whereas persistence of the AER results in additional tissue outgrowth (e.g., extra phalanx).[30]

A specialized zone of mesenchyme located along the posterior border of the limb called the *zone of polarizing activity* (ZPA) is responsible for limb patterning along the A-P axis.[6] The activity of the ZPA is mediated by a molecule called Sonic hedgehog (*Shh*). If *Shh* is inappropriately expressed in the anterior portion of the limb mirror image, digit duplications result.[31] Conversely, loss of *Shh* results in loss of limb structures along the A-P axis.[32] The three GLI proteins, GLI1, GLI2, and GLI3, are the transcriptional effectors of SHH signaling.[20] In the absence of SHH, GLI2 and GLI3 repress SHH target genes. In the presence of SHH, GLI1 and GLI2 function as activators to induce the expression of SHH target genes.[20] Normally the anterior portion of the limb has high levels of the repressive GLI3,[33] which acts to prevent radial polydactyly.

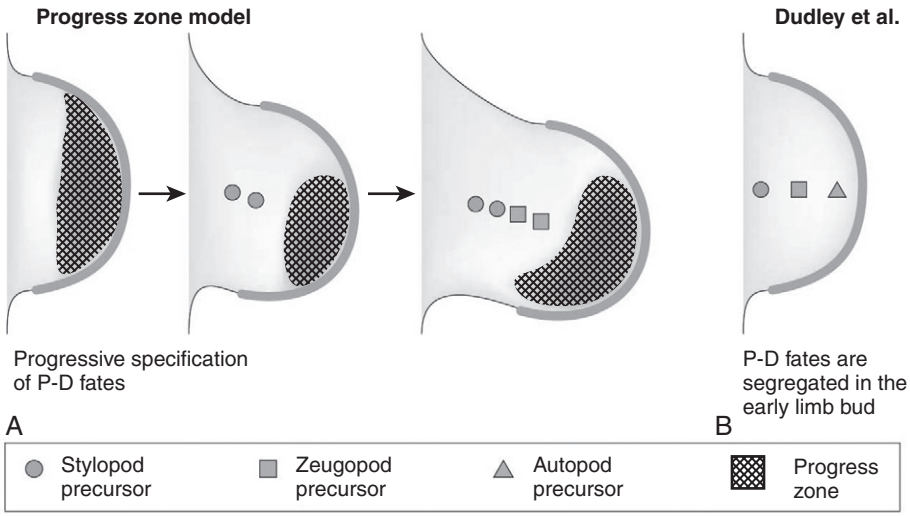


Figure 174-2 Graphical comparison of the progress zone and early specification models. The progress zone model proposes progressive proximodistal (P-D) specification that depends on the apical ectodermal ridge (AER), in which proximal fate is specified first, followed by progressively more distal specification. The early specification model stipulates that all P-D fates are established within the early limb bud.[6]

It is thought that the main role of SHH signaling is to derepress GLI3 in the posterior portion of the limb to allow digit formation to occur.[20] SHH is particularly important in autopod development.[6] High concentrations of SHH specify more posterior-type digits (e.g., little finger).[22] This property of SHH may be mediated by BMP-2,[34] because SHH signaling persists only until the digit primordia appear.[22] BMP activity in the interdigital mesenchyme appear to be the downstream vestiges of SHH activity.[22] For example, the phenotype of the individual digits appears to be determined by BMP signaling in the tissue of the interdigital space posterior to it.[35] Although BMPs appear to act in concert with SHH with regard to digit identity, they appear to antagonize SHH function with regard to digit number.[36] The number of digits is directly related to the A-P length of the apical ridge,[37] and AER length can be increased by BMP antagonists such as Noggin and Gremlin.[36] Of interest, expression of *Gremlin* has been shown to be dependent on SHH signaling.[38]

Patterning of the limb in the D-V axis is intrinisic to the mesenchyme before limb bud formation. This information subsequently is transferred to the overlying ectoderm.[39] Expression of *Wnt-7a* in the dorsal ectoderm induces the mesenchymal expression of *Lmx1b*, which confers dorsal characteristics to the limb.[40] *En-1* is expressed in the ventral ectoderm and promotes ventral limb characteristics.[40]

Apoptosis and skeletal segmentation also play important roles in further sculpting and refining, respectively, the developing limb.[41,42] Interdigital cell death occurs by caspase-dependent[43,44] and caspase-independent[45] mechanisms around 6 to 8 weeks of development.[46] WNT-14 has an inductive role in joint formation,[47] and growth and differentiation factor-5 (GDF-5) has been shown to repress chondrogenesis in regions of prospective joint formation.[48] BMPs play an important role in regulating differential phalangeal growth.[49]

Normal growth of the limb is dependent on reciprocal signaling between the ectoderm and the underlying mesenchyme.[22,50] For example, if the AER is absent or defective, SHH signaling is not induced in the mesenchyme.[51] SHH signaling maintains *Fgf4* expression in the AER.[38] FGFs, BMPs, and SHH all regulate Hox gene expression,[52] and expression of specific sets of *Hox* genes correlates with the formation of specific segments of the developing limb.[53] *Hoxa13* and *Hoxd13* play important roles in digital development, and *Hoxa11* and *Hoxd11* in forearm development.[22] The relationship between congenital limb differences and human Hox genes has been summarized by Goodman.[54]

Gene deletion and misexpression studies have demonstrated that *Tbx5,Tbx4,* and *Pitx1* have fundamental roles in the specification of limb type identity (e.g., upper limb versus lower limb).[3] *Tbx5* is required for upper limb development, and *Tbx4* is required for lower limb development. *Tbx5*$^{-}$ mutant mouse embryos demonstrate a spectrum of deformities ranging from absent upper limbs[55] to absent digit I.[56] Ectopic expression of *Tbx5* in the developing chick lower limb can partially transform its identity to that of an upper limb.[57,58] TBX5 is thought to achieve this transformation by repressing the expression of lower limb–specific genes such as *Tbx4* and *Pitx1*.[57,58] Conversely, misexpression of *Tbx4* or *Pitx1* in the upper limb transforms its identity to a lower limb.[58,59]

Congenital limb differences can be classified in many ways.[60-62] The classification system most commonly used by reconstructive surgeons treating these conditions initially was proposed by Swanson[63] and was revised by the Congenital Anomalies Committee of the International Federation of Societies for Surgery of the Hand (IFSSH).[64] The following section incorporates current knowledge about the developmental biology of the limb within the relevant parts of this classification system. A summary of the molecular basis of the many syndromes that manifest with congenital limb differences, as described next, is presented in Table 174-1.

FAILURE OF FORMATION OF PARTS

Transverse Arrest

The most common levels of transverse arrest are at the proximal forearm and the midcarpal, occurring in 1 in 20,000 live births.[2] These deficiencies commonly are associated with hypoplasia of structures more proximal in the limb. This pattern is consistent with a disruption to the AER early in development causing cell death of the underlying mesenchyme and failure of further P-D outgrowth.[24] Complete amelia is consistent with a failure of FGF10 or WNT-2b signaling in the prospective upper limb region.

TABLE 174-1

Molecular Basis of Limb Patterning

Gene		Associated Human Condition(s)
Genes Encoding Cell-Cell Signaling Molecules in the Limb and Molecules Involved in Signal Transduction		
Fibroblast growth factor (FGF) genes	FGF receptors	
Fgf8	*Fgfr*1*	Pfeiffer syndrome
Fgf4	*Fgfr2**	Apert, Pfeiffer, Jackson-Weiss syndromes
Fgf9	*Fgfr3**	Achondroplasia
Fgf10		
Wnt (wingless/Int) genes	Frizzleds (receptors)	
Wnt7a		
Wnt3a		
Wnt5a		
Hedgehog genes	*Patched* (receptor)	
Shh: Signal transduction	*Smoothened*	
Ihh: Cartilage		
	Hip (Hedgehog-interacting protein)	
	Gli3*	Greig cephalosyndactyly, Pallister-Hall syndrome
Bone morphogenetic protein (BMP) genes†	BMP receptors	
Bmp2		
Bmp4		
Bmp7		
Genes Expressed in Response to Signals in the Limb		
Hox genes		
Hoxa9 to *Hoxa13**		Hand-foot-genital syndrome
Hoxd9 to *Hoxd13**		Polysyndactyly
*Spalt**		Townes-Brockes syndrome
Meis		
*Lmx1**		Nail-patella syndrome
T-box genes		
*Tbx3**		Ulnar-mammary syndrome
Tbx4		
*Tbx5**		Holt-Oram syndrome

Data from Tickle C: Molecular basis of vertebrate limb patterning, *Am J Med Genet* 112:250-255, 2002.

*Direct association with named pathologic condition(s).

†Part of the TGF-β superfamily.

TGF-β, Transforming growth factor-β.

Longitudinal Arrest

Generally, longitudinal arrest may result from local absence of the AER at some point along the A-P axis or attenuation of the ZPA (so that posterior structures are missing).[25]

Preaxial

Radial Deficiency

Longitudinal radial deficiency occurs in 1 in 30,000 to 1 in 100,000 live births.[2] The most common syndromic associations are Fanconi anemia, thrombocytopenia–absent radius (TAR) syndrome, Holt-Oram syndrome, and the VACTERL association (*v*ertebral anomalies, *a*nal atresia, *c*ardiovascular anomalies, *t*racheoesophageal fistula, *e*sophageal atresia, *r*enal and/or radial anomalies, *l*imb defects). With the exception of that for Holt-Oram syndrome, the pathogenic mechanism for this deficiency is not known. Mutations in *Tbx5* cause the autosomal dominant form of Holt-Oram syndrome, which is characterized by a variety of thumb abnormalities (e.g., hypoplastic thumb) and heart defects (e.g., atrial septal defect [ASD]).[65] In severe cases, radial aplasia may be a feature. *Tbx5*-knockout mice have defects in digit I.[56] TBX5 activity is thought to be upstream of *Wnt2*b and *Fgf10*; accordingly, down-regulation of *Tbx5* is associated with a concomitant down-regulation of *Fgf10* and *Fgf8* in the anterior limb.[3]

Central

Central Ray Deficiency

Ectrodactyly is a central reduction of the hands and feet that occurs in approximately 1 in 18,000 births.[66] It can be associated with the ectrodactyly–ectodermal dysplasia–cleft lip or palate (EEC) syndrome, limb-mammary syndrome, or nonsyndromic split hand–split foot syndrome (SHFM). SHFM is a congenital limb difference involving the central rays of the autopod and manifests with syndactyly, median clefts of the hands and feet, and aplasia or hypoplasia of the phalanges, metacarpals, and metatarsals.[67] Digit loss can result from failure to maintain the AER locally, as occurs in the mouse mutant dactylplasia (Dac), with central loss of digits.[68] Dac homozygotes therefore lack hands and feet except for malformed single digits, whereas heterozygotes lack phalanges of the three middle digits. Dac resembles the human SHFM. The area of mapping of SHFM3 on 10q24 shows conservation of synteny with the Dac region on mouse chromosome 19; thus SHFM3 may be due to disruption of the human homologue of Dac. Mutations in p63 have been found to be associated with EEC syndrome and rarely in SHFM, which is more commonly associated with mutations in the *dactylin* gene.

Postaxial

Ulnar Deficiency

Tbx3 is one of the targets of ZPA signaling. *Tbx3* haploinsufficiency has been linked with ulnar-mammary syndrome, in which predominantly posterior defects occur in the upper limb.[22] Loss of the most posterior digit occurs as a consequence of loss of ZPA signaling. Such deficiency is a feature of mouse mutations involving *Wnt-7a,* a molecule that usually is involved in maintaining the ZPA.[69,70]

Intercalated

Phocomelia is characterized by the absence of proximal limb elements to various degrees combined with the presence of distal limb structures (e.g., autopod). Historically, this condition was associated with thalidomide ingestion during pregnancy. However, many congenital syndromes can manifest with phocomelia (e.g., Holt-Oram, TAR, Roberts, and Schinzel syndromes).[2] The *progress zone* model of limb development attempts to explain the pathogenesis of this condition by stating that proximal limb structures are relatively more sensitive to global insults than distal limb structures.[71] The *early specification* model counters this view with the notion that the progressive determination of distal fates limits the regulative ability of undifferentiated tissue in the developing limb.[24] Other theories suggest that thalidomide mediates its teratogenicity by inhibiting blood vessel growth.[72]

FAILURE OF DIFFERENTIATION (SEPARATION) OF PARTS

Soft Tissue

Cutaneous Syndactyly

Cutaneous syndactyly is described as *complete* when no regression of the interdigital tissue has been achieved (i.e., the tissue extends to the tips of the fingers) and *incomplete* when some regression of the interdigital tissue has occurred.[73] Interdigital mesenchyme normally is eliminated by apoptosis during embryonic

development. The regulation of cell death is complex. In summary, BMPs induce cell death[74,75] inhibitors of BMP signaling (e.g., Gremlin, Noggin, overexpression of dominant-negative BMP receptors) inhibit cell death,[75-77] and FGF signaling antagonizes BMP-induced cell death.[78] As stated earlier, molecules involved directly in caspase-dependent apoptosis (e.g., Bak, Bax, Dad (*d*efender *a*gainst *d*eath), APAF1 [*a*poptotic *p*rotease-*a*ctivating *f*actor-1]) also are crucial for interdigital cell death.[43,44,79]

Mouse models of cutaneous syndactyly involve either interference with cell death[43,44,77,79-82] or physical disruption of the ectodermal-mesodermal junction.[83] Most disruptions to the ectodermal-mesodermal junction result in acrosyndactyly, which is described in more detail further on. Mouse mutants with cutaneous syndactyly may have concomitant abnormalities in the AER (e.g., widening or delayed regression) secondary to inhibition of BMP signaling.[77,81,84]

Connexin 43, encoded by *GJA1,* is a gap junction protein. At later stages of limb development, *GJA1* is expressed in the AER. Mutations in *GJA1* are found in type III syndactyly (involving digits IV and V) in association with oculodentodigital syndrome.[85]

Skeletal Involvement

Osseous Syndactyly (Complex)

Interdigital tissue has the capacity to form cartilage. This observation has led to the concept that the chondrogenic potential of interdigital tissue before the onset of cell death must be actively repressed.[86] This repression may be mediated by FGF-FGFR2 signaling from the overlying ectoderm.[87] Mouse models suggest that osseous or complex syndactyly can arise as a result of abnormalities in the AER,[88,89] abnormal patterning in the mesenchyme (e.g., synpolydactyly caused by mutations in *HoxD13*),[90] or failure of bone growth (e.g., brachydactyly), described further on. Again, many of these defects trace back to defective AER maintenance. This is observed in the limb deformity *(ld)* mouse mutant, in which Gremlin activity is absent, allowing unrestrained BMP activity, which in turn prevents *Fgf4* expression in the AER.[38]

Acrosyndactyly

Acrosyndactyly is a particular type of syndactyly in which the distal portions of the fingers are joined.[73] Acrosyndactyly can arise sporadically as a result of constriction ring syndrome (secondary to physically encircling of the part by a band of amnion). It also is commonly associated with Apert syndrome.[91] Apert syndrome is an autosomal dominant condition caused by an activating mutation of FGFR2—either S252W or P253R. The syndactyly in both the hands and the feet is more severe in patients with the P253R mutation. An activating mutation of FGFR2 is consistent with the notion that FGF signaling is antagonizing BMP-induced cell death. Acrosyndactyly also is observed in a number of mouse mutants characterized by disruption of the ectodermal-mesenchymal junction.[92,93]

Synostosis

The secreted polypeptide Noggin binds to and inactivates members of the transforming growth factor-β (TGF-β) superfamily of signaling proteins, such as BMP4. Dominant mutations in human *noggin* have been found in a family segregating multiple synostoses (joint fusion) syndrome.[94]

Radioulnar synostosis is a feature of certain chromosome abnormalities, notably the triple X–Y syndrome (XXXY).[95] Mouse models of synostosis are characterized by an inhibition of BMP signaling. Murine Noggin (an inhibitor of BMP signaling) is expressed in condensing cartilage and immature chondrocytes, as are many BMPs. In mice lacking *noggin,* cartilage condensations initiate normally but subsequently become hyperplastic, and initiation of joint development fails as measured by the expression of *Gdf-5*. Excess BMP activity in the absence of Noggin antagonism may enhance the recruitment of cells into cartilage, resulting in oversized growth plates. Chondrocytes also are refractory to joint-inducing positional cues, producing synostosis.[96] The relationship of the triple X–Y syndrome to the BMP signaling pathway is not known.

Symphalangia

Symphalangism is characterized by failure of joint formation at the proximal interphalangeal joint. Proximal symphalangism syndrome (SYM1) also is associated with fusion of the carpal bones and sometimes the metacarpophalangeal joints. Dominant mutations in the gene encoding Noggin (the BMP antagonist) have been found in SYM1.[94]

DUPLICATION

When ZPA activity is abnormally present along the anterior border of the limb (e.g., as demonstrated by grafting posterior limb bud tissue to the anterior limb margin), mirror-image symmetric limbs develop.[97] Factors such as the location, concentration, and duration of the ectopic signal (e.g., Shh) and the stage of limb development will determine the extent to which duplication occurs (e.g., whole-limb duplication versus digital duplication).

Polydactyly

Polydactyly has been associated with mutations in 39 genes, not all of which fit into the current understanding of limb development. However, abnormalities in the GLI3 transcription factor are responsible for four or five phenotypes. A summary of disorders that include polydactyly as a clinical feature and their genetic basis is available in a review by Biesecker.[98]

Ulnar Polydactyly

As previously discussed, GLI3 has a repressive role in the SHH pathway. Missense mutations in *GLI3* (R625W) are associated with Greig cephalopolysyndactyly syndrome, which is characterized by, among other features, postaxial polydactyly.[99]

Central Polydactyly

Synpolydactyly (SPD), a condition of autosomal dominant inheritance, is characterized by syndactyly between digits III and IV with an extra digit in between.[90] The correlation between phenotype and the size of the polyalanine expansion tract suggests that expansion of the tract leads to a specific gain of function in the mutant HOXD13 protein.[100] These severe phenotypes are correlated with abnormal *Hoxd13* expression during the second phase of *Hoxd13* expression in the developing limbs of chicks and mice,[53] which occurs during specification of the autopod. During this phase, *Hoxd13* has the largest expression domain relative to the other 5 *Hoxd* genes and also is the first to be expressed. Mice homozygous for a triple deletion of *Hoxd11, Hoxd12,* and *Hoxd13* have phenotypes similar to those of SPD heterozygotes (presence of an extra central digit fused to digit III or IV) and SPD homozygotes.[101] HOXD13 is thought to play a dominant role by controlling mesenchymal proliferation,[102,103] and it is this that appears to be perturbed in SPD.

Mice homozygous for a mutation in *Gli3* show central polydactyly.[104] This finding is consistent with the central polydactyly phenotype in Pallister-Hall syndrome.[105] The distribution of polydactyly contrasts with that found in Greig cephalopolysyndactyly, as described.

Radial Polydactyly

Radial polydactyly may arise from abnormalities of the BMP pathway (repression) or the hedgehog pathway (activation). This is because Gremlin (BMP antagonist) usually is repressed by GLI3 (inhibitor of hedgehog pathway).[38] Expansion of the AER occurs with repression of BMP signaling. Mouse embryos null mutant for

Bmp7[106,107] or heterozygous for a null allele of *Bmp4*[108] display radial polydactyly. This observation is consistent with the concept that anterior expansion of the AER mediated by BMP repression may cause radial polydactyly. Signaling by ectopic Indian hedgehog (IHH)—which is functionally similar to SHH—in the distal anterior part of the limb results in radial polydactyly,[109] and *Gli3*$^{-/-}$ mouse mutants also have radial polydactyly.[110] Intraflagellar transport proteins have been implicated in the hedgehog pathway, and mutants of these proteins also are associated with radial polydactyly.[111]

UNDERGROWTH

Brachydactyly

Brachydactylies are a group of congenital limb differences characterized by shortening of the digits secondary to abnormal development of the phalanges or the metacarpals.[112] IHH is a member of the hedgehog family of secreted ligands and is closely related to SHH.[9] IHH activity is expressed in both prehypertrophic and early hypertrophic chondrocytes and is a regulator of chondrocyte proliferation. *Ihh*$^{-/-}$ mice have abnormalities of bone growth secondary to premature exit of chondrocytes from the proliferative pool. *Ihh*$^{-/-}$ mice fail to synthesize parathyroid hormone–related protein (PTHrP). PTHrP acts primarily to keep chondrocytes proliferating, and its signaling is downstream of IHH.[9] Mutations in the gene encoding *Ihh* are present in patients with brachydactyly type A1.[113,114] This type is associated with hypoplasia or aplasia of all middle phalanges, sometimes with joint fusions between the middle and proximal phalanx (i.e., symphalangism). These findings are consistent with experimental data showing that ectopic SHH induces ectopic phalanges.[36] In this scenario, SHH may interfere with an endogenous signaling system, possibly based on IHH, that controls the morphogenesis of each individual digit.

Receptor tyrosine kinase ROR2 is required for proper expansion of chondrocytes and growth plate organization in distal limb bones.[115] Brachydactyly type B is associated with aplasia or hypoplasia of the terminal phalanges and nails and is caused by mutations in the receptor tyrosine kinase gene *ROR2*.[116]

BMPs have multiple roles during bone formation.[9] GDF-5 is necessary and sufficient for both cartilage development and the restriction of joint formation to the appropriate location.[117] *GDF5*$^{-/-}$ mice have abnormalities in digit formation that involve failure of extension of mesenchymal condensations that lead to digit formation. Brachydactyly type C is characterized by shortening of the middle phalanges (brachymesophalangia) of digits II, III, and V. Heterozygous frameshift or nonsense mutations affecting *GDF-5* have been identified in persons with brachydactyly type C.[118]

BmpR1b is expressed in cartilage condensations during limb development. The formation of cartilage condensations is inhibited by overexpression of the BMP antagonist Noggin in chick limbs.[10] Overexpression of the protein encoded by a mutant *BmpR1b* in the chick embryo also causes shortening or aplasia of the phalanges.[112] A mutation in this gene has been found to be responsible for brachydactyly type A2, which is characterized by shortening and lateral deviation of the index fingers.[112]

GENERALIZED SKELETAL ABNORMALITIES

In addition to their role in limb patterning, FGFs are critical to subsequent bone growth.[119] Proliferating chondrocytes express *Fgf* receptor-3 (FgfR3), prehypertrophic or hypertrophic chondrocytes express *FgfR1,* and perichondrial cells express *FgfR2*.[9] In vitro data support the notion that FGF signaling is mediated by the suppression of *Ihh* expression.[120] FGF signaling through FGFR3 inhibits proliferation of chondrocytes. This mechanism is supported by gain- and loss-of-function mutation mouse models.[121,122]

Apert syndrome is characterized by craniosynostosis with complex syndactyly. It is caused by increased FGFR2 affinity for FGF ligands in the mesenchyme.[119] Pfeiffer syndrome is characterized by broad first digits and craniosynostosis and is due to a mutation in *FgfR1*. Variable mutations in *FgfR3* give rise to achondroplasia, hypochondroplasia, thanatophoric dysplasia, and a syndrome of severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN syndrome). All of these conditions are characterized by limb shortening.

As described earlier, SHH signaling from the ZPA is the primary determinant of A-P polarity in the vertebrate limb field. An active signal is produced by an autoprocessing reaction that covalently links cholesterol to the amino-terminal (N-terminal) signaling moiety N-SHH(p), tethering it to the cell membrane. Thus cholesterol modification is essential for the normal range of signaling.[123] Smith-Lemli-Opitz syndrome is caused by defects in 7-dehydrocholesterol reductase (DHCR7), an enzyme catalyzing the final step of cholesterol biosynthesis. It is characterized by generalized skeletal abnormalities including short, proximally placed thumbs and postaxial polydactyly.[124] SHH not modified by the cholesterol moiety has an increased propensity to spread long distances, causing ectopic SHH pathway activation in the anterior limb mesoderm.[125] These findings suggest that the congenital limb differences found in Smith-Lemli-Opitz syndrome may result from defects in DHCR7 leading to derepression of SHH signaling.[126]

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175 Ontogenesis of Striated Muscle

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HISTORICAL BACKGROUND

Embryology is the basis for understanding the intimate relation between structures in different organ systems such as the nervous system and muscle and is primordial for understanding disorders of development that in the human may present as one of the congenital myopathies. The timing and sequence of striated muscle maturation are as precise and predictable as in the nervous system. Interest in neuromuscular ontogeny began with the studies of MacCallum in the late 19th century.[1] The account of histologic changes in developing human muscle published in 1917 by Tello in Spain[2] remains as accurate and valid today as any subsequent studies by light microscopy. Ultrastructural studies of developing muscle by transmission electron microscopy began in the 1950s and were supplemented by the use of the scanning electron microscope two decades later. Histochemical techniques to demonstrate biochemical constituents and enzymatic activities in developing muscle were introduced in the 1960s and 1970s; the 1980s were a decade for the introduction

of immunocytochemistry to identify other molecules of subcellular components. The late 1980s and early 1990s saw a major breakthrough in the understanding of muscle differentiation by the discovery of myogenic regulatory genes and their transcription products. Studies of the complex interactions of these genes, their translated proteins, and the role of various trophic factors continue to be the focus of current investigations in muscle ontogenesis. Modern embryology, an integration of classic descriptive morphogenesis and the molecular genetic regulation of myogenesis, is the foundation for understanding the pathogenesis of congenital myopathies.[3]

EMBRYONIC ORIGIN OF MUSCLE

Muscle originates even before the three definitive germ layers are differentiated from the gastrula. The epiblast, the uppermost layer of the bilayered blastula, contains primordial mesodermal cells. Epiblastic cells migrate toward a line formed between parallel ridges that converge at one end; this line is known as the *primitive streak,* and the convergence of the parallel ridges is designated "anterior" and termed the *primitive node.* The axis thus formed establishes bilateral symmetry and a cephalocaudal gradient as the fundamental body plan.

From the primitive node and streak, epiblastic cells stream into the space between the two layers to form paired columns of prospective mesoderm. The primitive node is the future notochord. It is positioned beneath the future neural plate, which is of ectodermal origin. The mesodermal columns are composed of undifferentiated mesenchymal cells extending the entire length of the primitive streak. These cells migrate laterally and rostrally to fill the space between the overlying surface ectoderm, which includes the neural plate, and the deeper endodermal layer, except for an area on either side of the notochord beneath the neural plate.

Further development subdivides the layer of primitive mesenchyme. A thickened band of mesoderm condenses on either side of the primitive streak, from which primordial somites will differentiate. The myotomal plates within these still-unsegmented somite columns are anatomically distinct from the broad lateral expanse and a smaller intermediate strip of mesoderm. Elongation of the embryo is accompanied by regression of the primitive node (notochordal process) and streak caudally. As the node moves posteriorly, paired blocks of somites become segmentally condensed from the originally continuous somitic plate on either side of the developing neural tube. In addition, the lateral mesoderm splits into two layers: the upper or *somatopleure* forms the body wall, and the lower or *splanchnopleure* forms the mesenteries of the internal organs. A lateral palisading of paraxial mesenchyme against the lateral aspects of the notochord precedes overt segmentation, but, once formed, the boundaries between somites are stable and provide no opportunity for cellular mixing.[4]

Segmentation of the myotomal plate occurs along a rostrocaudal gradient. As the more caudal segments are still in the process of separating, segregated anterior somites are already changing in size and arrangement. In transverse section, the somites are composed of high columnar cells arranged radially around a small central cavity. The dorsal part of the somite, the *dermatome*, retains this columnar structure and forms a flat plate beneath the surface ectoderm. Cells of the ventral portion of each somite disperse to form a loose network known as the *sclerotome*. The small myocele cavity of the somite is transitory and becomes obliterated by cellular proliferation.

The myotome that differentiates at the medial end of the dermatome as a ventral extension near the neural tube differentiates into the axial (i.e., paraspinal) muscles and also into the muscles of the extremities as the limb buds form. The contractile elements of the developing muscle are of somitic origin, whereas the tendons, interstitial connective tissue, blood vessels, and epimysial sheaths are somatopleural derivatives.

The notochord and floor plate ependyma of the neural plate synthesize retinoic acid[5] and express a gene known in vertebrates as *Sonic hedgehog (SHH)*.[6] These factors are important in the dorsoventral patterning not only of the developing neural tube but also of the somites. Overexpression of these factors has a powerful ventralizing influence so that an ectopic length of notochord or a section of neural tube floor plate transplanted ventral to the newly formed segmental somite causes the excessive differentiation of sclerotome (the ventral part of the somite) and deficient formation of the myotome and dermatome, the dorsal part of the somite.[6-9] As the fetus matures, the loss of balanced antagonism between ventralizing genes, such as *SHH*, and dorsalizing genes, such as those of the *bone morphogenetic protein (BMP)* and *paired homeobox (PAX)* families, may result in segmental amyoplasia at the level of the notochordal or floor plate implant, a disturbance of *somitic induction*. Notochordal signals also regulate the transcriptional cascade of myogenic bHLH genes in the somite for myoblastic differentiation.[10] In a genetic mutant mouse in which somites form after notochordal degeneration, apoptosis of epaxial myotomes is accelerated.[11]

MYOGENIC REGULATORY GENES

A family of four proto-oncogenes directs the differentiation of striated muscle from any undifferentiated or incompletely differentiated mesodermal cell by blocking the methylation of DNA.[12] The molecular mechanism of myoblast differentiation requires the activation and suppression of specific genes in a temporal and spatial sequence controlled by a complex array of regulatory transcription factors. Each of these genes can activate the expression of another and, under certain circumstances, can autoactivate as well.[13-16] The four myogenic regulatory genes are a "family" not only because of their closely related functions in myogenesis but also because they all share encoding transcription factors of the *basic helix-loop-helix* (bHLH) protein, a molecular structure so fundamental in evolution that it is even found in some bacteria. These four genes or *myogenic regulatory factors (MRFs)* that control the specification and differentiation of striated muscle lineage are *Myf5, myogenin, MRF4* (also known as *herculin* and *Myf6*), and *myoblast-differentiating factor*, also known as *MyoD* or *MyoD1*.[13,17-25] Several other genes influence these MRFs or interact with them in ways that augment or impair their expression. *Myogenin* mainly mediates myoblast fusion, and, without this factor, an abundance of myoblasts may form but they do not fuse to form myotubes. The expression of *Myf5* and *MRF4* is transient in early ontogenesis and returns much later in fetal life to persist into adult life.[23]

One of the most remarkable and complex features of all myogenic genes is their intimate interactions with one another. After the discovery of this family of genes, initially it was thought that they all were redundant and any could substitute for another if one were deleted. Some indeed do have overlapping functions, such as *Myf5* and *MyoD*, that allow normal muscle development if one, but not both, of these two is defective.[26,27] A similar overlap or redundancy exists between *MRF4* and *MyoD*.[28] In general, despite these partial redundancies, each gene regulates a different aspect of myogenesis, however, and they do not easily compensate for each other either in the embryo and fetus or in myoblast precursor cells in adults for the regeneration of muscle.[29] *MyoD* cannot compensate for an absence of myogenin expression.[30] *MRF4* has functions similar to those of myogenin but not of *MyoD*[31] and can substitute for myogenin early in development.[32] *Myf5* similarly may be redundant with myogenin but less efficiently.[33,34] In *MRF4*- or myogenin-deficient mice, particularly severe impairments exist in the development of the thoracic myotomes for intercostal muscle development, and rib

fusions and sternal defects result, but these are probably secondary because neither of these two genes is expressed in rib cartilage or sternum.[35] Immunoreactivity of *MyoD* is now available for application to human fetal and neonatal muscle and is useful for some diagnoses of congenital disorders such as segmental amyoplasia.

Myogenic factor 5 (Myf5) is the earliest muscle-specific bHLH gene activated in the developing embryo.[14,19] Its expression is restricted to the medial aspect of the myotome, adjacent to the least mature myocytes.[9] *Myf5* alone cannot support myogenesis without the other three bHLH genes, however.[36] *Myf5* also seems to be mitogenic in inducing the proliferation of myoblast precursors. *Myf5* requires some activation by *MRF4*, and murine *MRF4*-null mutants show extremely reduced expression of *Myf5*, similar to *Myf5* knockout mice.[27] *Myogenin* can substitute for *Myf5* but is less efficient in this function.[33]

Myogenin does not serve a proliferative function in the genesis of myoblasts but is essential to myoblast fusion in the formation of myotubes, and it is expressed before fusion begins.[37] *Myogenin* inactivation in mice results in a normal number of myoblasts, but they become arrested in terminal differentiation.[27] Wild-type myoblasts can rescue *myogenin*-null myoblasts and cause them to fuse in vivo.[38] *Myogenin* is a specific transcriptional target of mitochondrial activity, unlike *Myf5* and *MyoD*.[39] *Myogenin* can be demonstrated by immunoreactivity in sections of human fetal and neonatal muscle (Figure 175-1).

MyoD1 is the last of the genes to become activated.[23,24] *Myf5* expression is normally suppressed by *MyoD*.[26] Despite its late expression, fetal mouse myoblasts express both *myogenin* and *MyoD* on day 1 in culture, but adult myoblasts (satellite cells) have a delay in expression of these genes and of myoblast fusion.[40] However, satellite cells coexpress proliferating cell nuclear antigen and *MyoD* on entering the mitotic cycle, and *MyoD* is critical for the programming of satellite cells into *myogenin*-differentiating cells.[41] The transition from proliferation to differentiation is delayed in mice lacking *MyoD*. Though coexpressed, *MyoD* and *Myf5* act at different times in the cell cycle: *Myf5* is maximally expressed in the G2 phase and is minimal in G1; *MyoD* is maximal in G1 and minimal in S-phase; it is reexpressed in G2 but not as strongly as in G1.[42] Another bHLH muscle–restricted transcription factor, *MyoR*, which antagonizes *MyoD*, has been recently identified.[43] *MyoD* and *myogenin* are expressed not only during fetal ontogenesis but also in adult life and are essential to the function of myogenic stem cells (i.e., satellite cells) in normal adults and also in the regenerating muscle in muscular dystrophies.[44,45] The myogenic regulatory genes, particularly *MyoD*, are so dominant that they are able to convert fibroblasts, chondroblasts, and other partially differentiated mesodermal lineages into myoblasts.[46]

The human locus of the *MyoD1* gene is on chromosome 11, very near the domain associated with embryonal rhabdomyosarcoma.[25] The genes encoding *Myf5* and *MRF4* are on chromosome 12 and that for *myogenin* is on chromosome 1. *MyoD* and *myogenin* are strongly expressed in human rhabdomyosarcoma cell lines.[47-49]

In addition to the family of four bHLH genes, *Pax3* has important interactions. It is redundant with *Myf5*: an absence of both *Pax3* and *Myf5* in embryonic mice results in generalized total amyoplasia and death; an isolated *Myf5* mutation causes milder, partial amyoplasia. Up-regulation of *Pax3* in chick embryos, by contrast, leads to precocious muscle development and arrested migration of myoblast precursor cells. *Pax3* and *Lbx1* both induce myoblast proliferation.[50] Other genes that *Pax3* induces include *Dach2, Eya2*, and *Six1*.[51,52] *Myogenin* is another key regulator of *Six1*.[53] *Pax3* is essential for the migration but not differentiation of limb muscle precursors.[54] Additional important functions of *Pax3* are the development of the eyes and the differentiation and maintenance of Bergmann glial cells of the cerebellum.

Other important genes or gene families that influence the bHLH myogenic genes are BMP2, which inhibits terminal differentiation of myogenic cells by suppressing *MyoD* and *myogenin*,[55] and LIM protein, which promotes myogenesis by enhancing the activity of *MyoD*.[56] Wnt signaling also plays a role in regulating the functions of *MyoD* and *myogenin*.[57]

Myostatin is a negative regulator gene of muscle maturation.[58,59] It is a member of the transforming growth factor β (TGF-β) family, similar to BMP. It has the latest expression in somites, after all other myogenic genes and desmin are already expressed. It also is coexpressed with *MyoD* but has a wider extent, also involving nonmuscular tissues such as the dermis. *Myostatin* inhibits transition from proliferation to differentiation but does not destroy myoblasts or alter mitotic cycling. *Myostatin* has recently

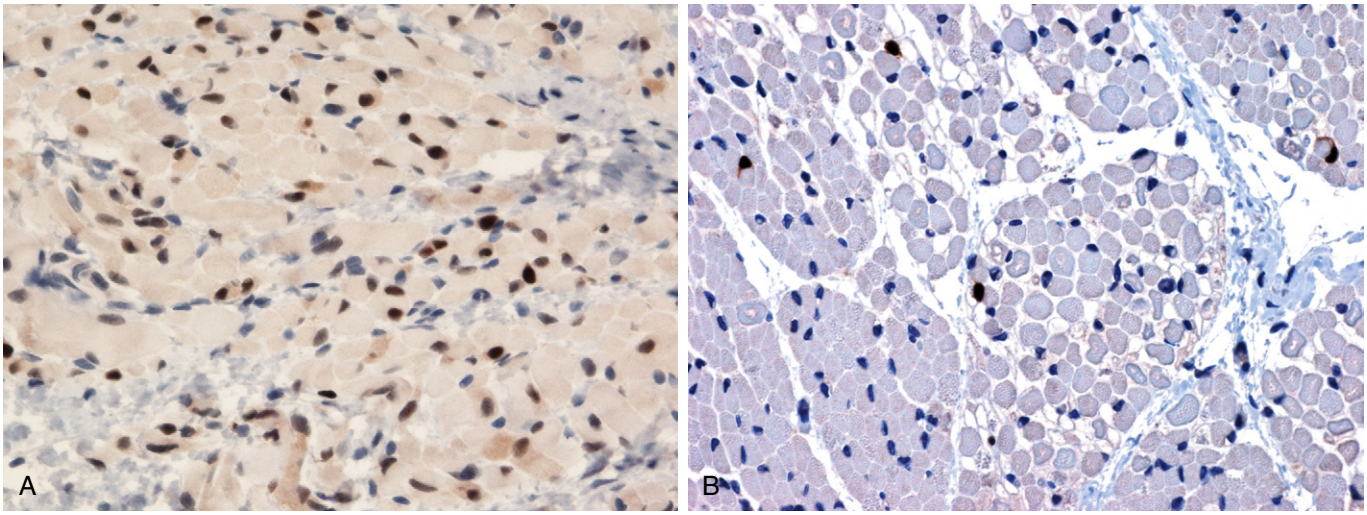


Figure 175-1 Intranuclear *myogenin* immunoreactivity in **(A)** full-term human neonate, and **(B)** 5–month-old boy with severe Duchenne muscular dystrophy. Normal neonatal muscle exhibits only occasional scattered nuclei that are marked; these are myoblasts or resident stem cells enclosed within the same basal lamina as their associated myofibre, also known as satellite cells in adult muscle. In muscular dystrophy, these myoblasts increase to attempt to regenerate muscle; such extensive distribution as in this child resembles an 8-10 week fetus. (Original magnification ×250.)

received attention in the lay press because of its potential use to enhance athletic performance.[60,61] A commercial interest in it to augment the muscular bulk of cattle and swine also exists.

In later stages of muscle ontogenesis, the selective accumulation of *MyoD1* and *myogenin* messenger RNA transcripts is not purely under genetic control but is modulated and modified by innervation of the muscle and by hormonal influences.[62-64] These effects begin earlier than the stage of histochemical differentiation of muscle fiber types at midgestation. Insulin-like growth factor (IGF) regulates normal *myogenin* expression[65] and influences muscle differentiation both upstream and downstream of *myogenin*.[66] IGF also augments proliferation that precedes differentiation.[67] Basic fibroblast growth factor (BFGF) similarly has a regulating influence on myoblast proliferation and differentiation.[68] Tumor necrosis factor-α, a cytokine, and BFGF differentially inhibit IGF-I–induced expression of *myogenin* in myoblasts.[69,70] IGF-I has early inhibitory and late stimulatory effects on *myogenin* transcription.[71] Other families of genes the transcription factors of which influence myodifferentiation include the POU homeodomain,[72] RHO proteins,[73] and SOX proteins.[74] A small number of neuroepithelial cells of the mouse neural tube coexpress neuronal markers and also *Myf5* and myosin in vitro, suggesting a potential for myogenesis from the neural tube itself.[75]

Micro-RNA expressions to detect the posttranscriptional modulation of cellular phenotypes in muscle and particularly in some of the congenital and degenerative diseases of muscle (muscular dystrophies) in humans are now feasible and promise to resolve many questions about the genetic regulation of muscle development, as well as the pathogenetic mechanisms of several developmental myopathies.[76] RNA interference (RNAi) libraries are being established in simple animals, such as the fruit fly *Drosophila*, which have genes that are phylogenetically conserved and include those of sarcomere formation in mammals.[77]

Other genes essential to muscle development and maintenance are the several genes that program sarcolemmal ion channels, particularly calcium channels. These channels form before innervations of the myofibre at 9 weeks gestation.

CYTODIFFERENTIATION OF MUSCLE CELLS

Structural and Contractile Proteins

The presumptive myoblast is a morphologically undifferentiated mesenchymal cell that divides mitotically, lacks the distinctive myofilaments of muscle cells, and does not fuse with neighboring cells of the same type.[78] The myoblast differs from its precursor, the presumptive myoblast, by synthesizing myosin and actin and assembling these proteins into thick and thin myofilaments, indicating a commitment to further differentiation as a muscle cell.[79] Although sarcomeres do not form in myoblasts, irregularly scattered clusters of myofilaments are loosely organized into interdigitating arrays. The cytoplasm of myoblasts is sparse. Adjacent myoblasts fuse by extending their contractile myofilaments across the plasma membranes of both cells and aligning the myofilaments produced by a row of several fused myoblasts (Figure 175-2). Myoblast fusion is enhanced by isoforms of the neural cell adhesion molecule that mediates interactions of apposed plasma membranes.[80]

The multinucleated syncytium of the row of fused myoblasts forms the myotube. Early myotubes continue to fuse with myoblasts and also with other early myotubules[81] (Figure 175-3). Circumferentially distributed myofilaments align themselves parallel to the longitudinal axis of the row of fused myoblasts as the filaments grow in length to extend through several fused myoblasts. Myotubular nuclei are enclosed within the center of the newly formed myotube as a single row within a central core of cytoplasm containing the membranous organelles and cytoplasmic particles. Cytoplasm is abundant, and contractile myofilaments are sparse in myotubes in comparison with more mature myocytes, but myofilaments and myofibrils are much more numerous in late myotubes than in early myotubes. Early myotubes predominate until the time of innervation at approximately 11 weeks gestation, and late myotubes are dominant during the second half of the myotubular stage from 11 to 16 weeks gestation.

Intermediate filaments, thinner but longer than the contractile myofilaments, appear in fusing myoblasts and myotubes.[82] These intermediate filaments, such as vimentin and desmin, are

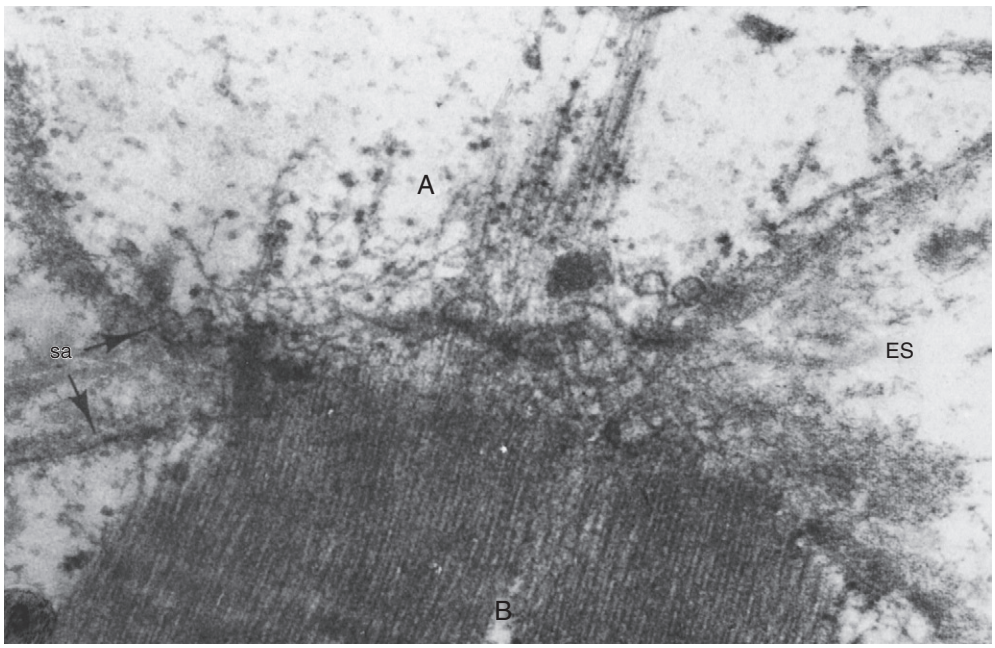


Figure 175-2 Myoblast (A) fusing with early myotube (B) in the vastus lateralis muscle of a 12-week fetus. Filaments from the myotube are seen extending into the neighboring cell. *ES,* Extracellular space; *sa,* sarcolemma. (Original magnification ×50,000.) *(From Tomanek RJ, Colling-Saltin AS: Cytological differentiation of human fetal skeletal muscle,* Am J Anat *149:227, 1977.)*

cytoskeletal proteins that help organize the cytologic structure of the muscle cell and spatial relationships of organelles, including the orientation and alignment of myofilaments (see later).

A basal lamina (i.e., basement membrane) appears as an indistinct, electron-dense thickening around the developing myotube. It usually encloses several myotubes and a number of less-differentiated myoblasts and presumptive myoblasts in the vicinity.[78,82,83] Mast cells, fibroblast-like cells, and other "unclassified" cells also may be included within the basal lamina.[84] Isolated myoblasts lack a basal lamina.[83] In this regard, they are similar to isolated fibroblasts and mast cells within the perimysium.

Further maturation of the myotube to become a myocyte involves the synthesis of more contractile myofilaments and more numerous myofibrils, displacing the central nuclei to the peripheral subsarcolemmal region and dispersing the sarcoplasm (i.e., cytoplasm) and its organelles between myofibrils. The Z-bands of several myofibrils within each maturing myotube are not "in register" with each other and do not become aligned consistently until approximately 30 weeks gestation.[78] The sarcotubular system begins forming in the late myotube but is not mature until after 30 weeks gestation.

After the myofiber achieves morphologic maturity in the relative positions and orientations of the various subcellular components, the next phase consists of the myofiber achieving greater length, an increase in the number of myofibrils in a cross-sectional field of the myofiber, and the development of histochemical fiber types. Although the number of myofibers and their morphologic maturation are mainly genetically determined, metabolic differentiation into fiber types is principally under neural control. Metabolic fiber types also exhibit subtle morphologic differences in the relative number of mitochondria and glycogen granules and in variation in the width of the Z-band.

Spindle-shaped mononucleated cells with abundant polyribosomes and endoplasmic reticulum persist in close apposition to the myotube and myocyte (i.e., multinucleated myofiber) enclosed within the same basal lamina. Such cells are presumptive myoblasts (Figure 175-4). In mature muscle, they are redesignated *satellite cells* (Figure 175-5). Although they are dormant and inactive, they are capable of mitotic proliferation and myogenesis even in the adult and are the basis of regeneration of injured muscle.[85-89]

No stage of fetal muscle development is "pure" in the sense of muscle cells exhibiting the same degree of maturation uniformly. Transitions among periods of development are gradual. At 5 to 8 weeks gestation, presumptive and true myoblasts predominate, but scattered myotubes are already evident at 6 weeks gestation. The period of 8 to 16 weeks gestation is regarded as the myotubular stage of development, and myotubes are indeed the most numerous types of muscle cells. However, many myoblasts are still identified, presumptive myoblasts frequently undergoing mitosis are encountered, and a few maturing myocytes beyond the myotubular stage are scattered during the latter weeks of this period. From 16 to 20 weeks gestation, a progressive maturation of myotubes to the cytologic structure of the mature myocyte takes place, but a few myotubes persist. Histochemical differentiation of muscle occurs mainly between 20 and 30 weeks gestation. From 30 weeks gestation to term, myofibers undergo several more subtle morphologic adjustments to maturity, such as alignment of Z-bands of adjacent myofibrils to attain "register" and further development of the sarcotubular system.

A 427-kilodalton cytoskeletal protein known as *dystrophin* is encoded by a large gene at the Xp21.2 locus. This subsarcolemmal protein attaches to the sarcolemmal membrane overlying the A-band and M-band of the myofibrils and consists of four distinct regions or domains: the amino terminus (N terminus) contains 250 amino acids and is related to the binding site of α-actinin; the second domain is the largest with 2800 amino acids and contains many repeats, giving it a characteristic rod shape; a third domain is rich in cysteine and is related to the carboxyl-terminus (C terminus) domain of 400 amino acids, unique to dystrophin and to a dystrophin-related protein encoded by chromosome 6.[90] Dystrophin may be demonstrated in striated muscle by its immunoreactivity, either by immunoperoxidase reaction (Figure 175-6) or by fluorescence microscopy, and is detected in developing human fetal muscle at 11 weeks gestation.[91,92] Dystrophin messenger RNA normally is detected in cardiac and smooth muscle in striated muscle and in the brain. The dystrophin gene encodes a 14-kilobase messenger RNA of approximately

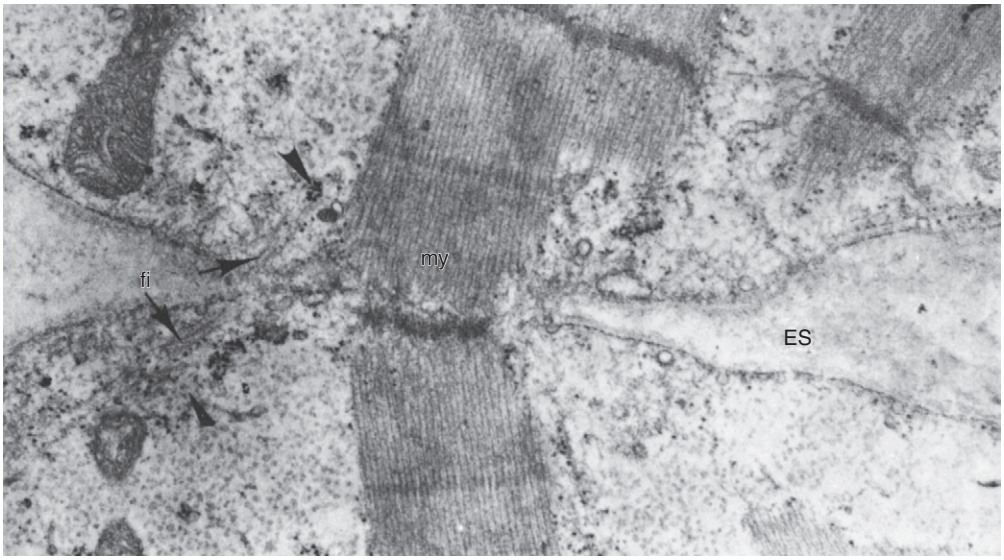


Figure 175-3 Fusion of two myotubes, more advanced in development than the myocytes shown in Figure 175-1, from the soleus muscle of a 16- to 17-week fetus. A well-formed myofibril *(my)* extends between two cells, which are joined by a narrow channel of sarcoplasm. Polyribosomes *(arrowheads)* and unassembled filaments *(fi)* are present in both cells. *ES,* Extracellular space. (Original magnification ×19,500.) *(From Tomanek RJ, Colling-Saltin AS: Cytological differentiation of human fetal skeletal muscle,* Am J Anat *149:227, 1977.)*

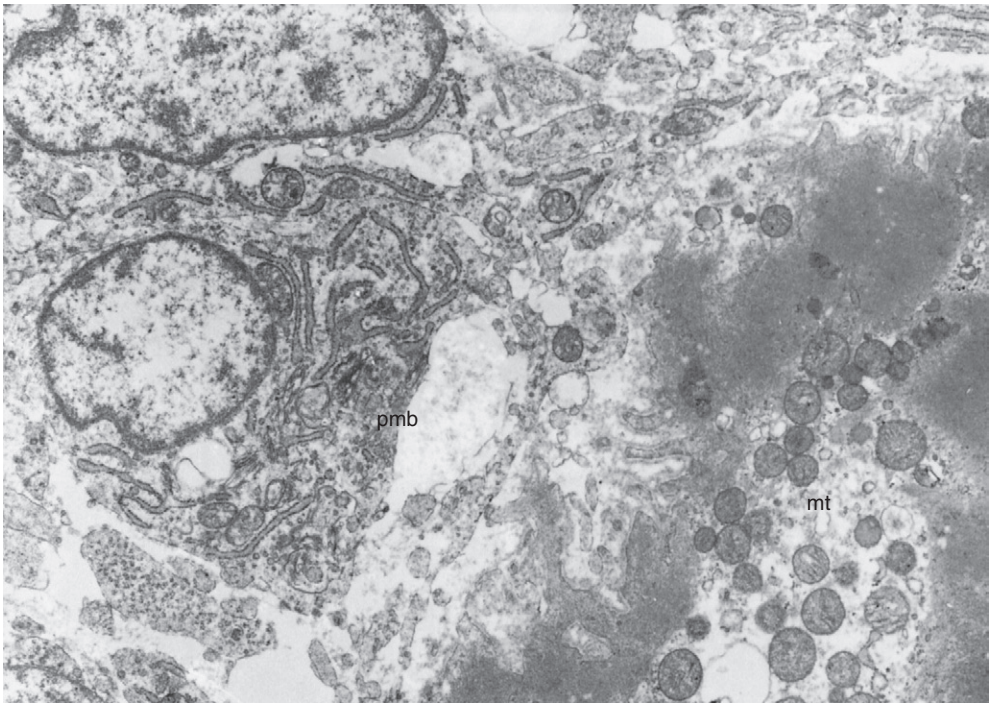


Figure 175-4 Presumptive myoblast *(pmb)* associated with a myotube *(mt)* in the quadriceps femoris muscle of a 14-week human fetus. An extensive rough endoplasmic reticulum and many free ribosomes are present, but no contractile filaments or T tubules are developed. Mitochondria also are seen, both in the presumptive myoblast and in the central core of sarcoplasm of the adjacent myotube. (Electron micrograph, original magnification ×12,000.)



Figure 175-5 Satellite cell of fully mature myofiber from quadriceps femoris muscle in a 7-year-old girl. As with the presumptive myoblast of fetal muscle (see Figure 175-3), the satellite cell contains mitochondria, rough endoplasmic reticulum, and free ribosomes but no contractile proteins or T-tubules and is enclosed within the same basal lamina *(arrowheads)* as the adjacent myofiber. The satellite cell is capable of mitosis and differentiation to become a true myoblast and then mature further and is the cell responsible for regeneration of injured muscle at any age. (Electron micrograph, uranyl acetate and lead citrate, original magnification ×20,000.)

80 exons. Defective dystrophin is the biologic basis of Duchenne and Becker muscular dystrophies.

Several dystrophin-related glycoproteins also form in this same general period. Alpha-sarcoglycan (formerly *adhalin*) becomes expressed before 14 weeks gestation. The other sarcoglycans (β, γ, δ) are expressed shortly thereafter. Dysferlin and caveolin-3 are other sarcolemmal membrane proteins at different sites from dystrophin and the sarcoglycans. Dysferlin deficiency is the basis of a well-documented progressive myopathy in children and adults, but dysferlin overexpression in striated muscle also can produce a progressive myopathy; hence the genetic regulation of expression, including its binding proteins, must be precise.[93] Caveolin-3 is first detected on day 10 in developing human somites.[94]

α-Dystroglycan is another sarcolemmal region protein. O-glycosylation of this protein may be altered by mutations of several genes during development, and hypoglycosylation is the molecular basis for some of the congenital muscular dystrophies.[95]

The precise timing of earliest expression of most sarcolemmal region proteins is uncertain, but collagen VI, which is deficient in a congenital muscular dystrophy known as Ullrich disease, probably is first detected at approximately 10 to 12 weeks,[96] similar to other proteins that attach to the sarcolemma. The principal source of collagen VI synthesis in striated muscle is the interstitial resident fibroblasts in the muscle.[97] It is noteworthy that this timing of appearance of sarcolemmal regional proteins coincides with the time of innervation of muscle at 9 to 11 weeks gestation. Knowledge of this timing is important in medicine because fetal muscle biopsies may now be performed during the course of pregnancy. Therefore, if a fetus is at risk for one of these diseases because of involvement of a sibling, for example, and the family wishes to know whether the present fetus is similarly involved, an accurate diagnosis can be confirmed as early as possible by immunoreactivity of sections of the fetal muscle or by haplotype analysis.[96] The various sarcolemmal regional proteins of striated muscle and their genetic defects in various myopathies were recently reviewed by Sewry.[98]

Cadherins are another group of transmembrane proteins that mediate calcium-dependent cell-cell adhesion by means of homophilic binding; M (muscle)-cadherin is a recently detected cell type–specific member of this family. In regenerating muscle and in developing fetal muscle, reactivity for M-cadherin is restricted to the plasma membrane of myoblasts and satellite cells and is most intense at the membrane areas facing adjacent myotubes or myofibers.[99-101] M-cadherin is genetically expressed but appears to be neurally regulated[102]; it is not expressed in denervated muscle.[100] The mechanism by which increased cadherin promotes early steps in muscle differentiation is by up-regulation of *myogenin*.[103,104]

Merosin is an extracellular matrix protein forming the heavy chain of the skeletal muscle basement membrane protein *laminin* and is important for maintaining the integrity of the sarcolemma. Defective merosin is implicated in the pathogenesis of one form of congenital muscular dystrophy,[105-107] and the chromosomal site of the disease gene is at the 6q2 locus.[107] The merosin (laminin-M) gene maps to chromosome 6q22-23.[108] Merosin may be detected before birth in the human placenta.[109] Type XIX collagen is a transient extracellular matrix protein that contributes to the formation of fetal basal lamina and closely follows the expression of *Myf5*.[110] Extracellular matrix is required for muscle differentiation and for the fusion of myoblasts to form myotubes, independent of *myogenin*.[111] Inhibition of fibrillar collagen production in avian and mammalian embryos results in abnormal somite formation and perturbed myogenesis.[112-114]

The innervation of muscle fibers occurs at approximately 11 weeks gestation, in the middle of the myotubular stage. Muscle spindles and Golgi tendon organs form after the myotubular stage as the general fascicular organization of the muscle evolves.

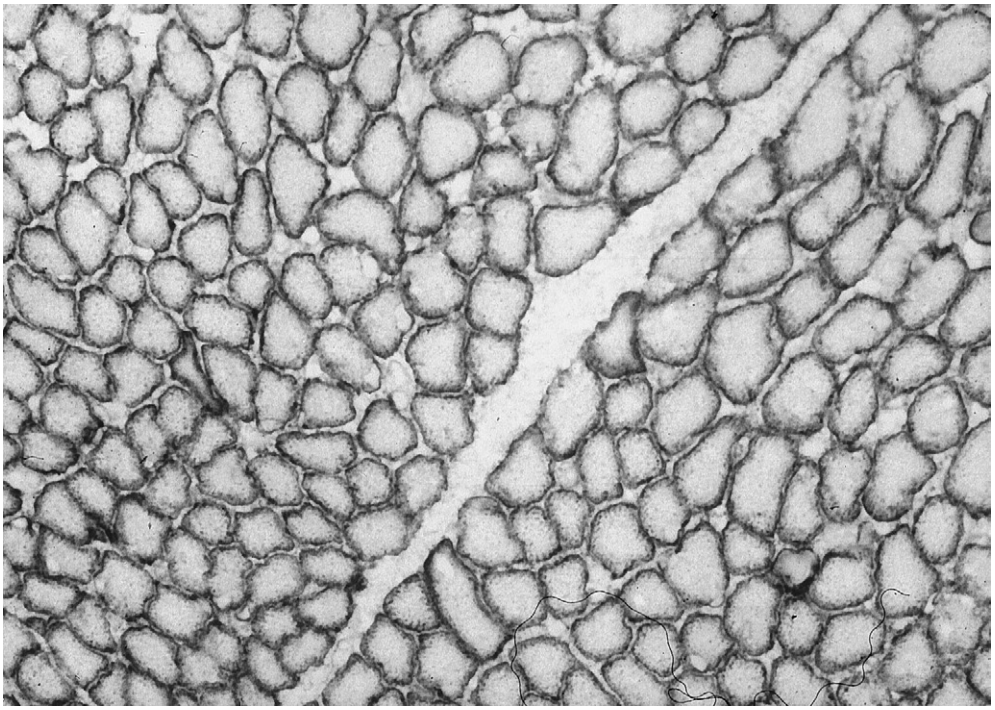


Figure 175-6 Immunocytochemical stain demonstrating dystrophin outlining the sarcolemma of normal quadriceps femoris muscle fibers in a biopsy specimen of a term male neonate. The monoclonal antibodies shown are specific for the rod domain of the dystrophin molecule; other antibodies demonstrate only the C terminus or N terminus. Dystrophin immunoreactivity is first detected in developing human muscle at 11 weeks gestation. (Original magnification ×250.)

The development of joints in the extremities begins at approximately 5 weeks gestation within the early mesenchymal condensations of precartilaginous bone. Joint spaces are already apparent by 7 weeks. Motion is probably essential for the development of joints, and some articular motion is evident as early as 8 weeks, before myotubes are innervated. These small-amplitude movements probably result from spontaneous contractions of newly formed sarcomeres. Active fetal movements are well established after 11 weeks, with the innervation of skeletal muscle, and increase progressively in both force and range of motion around articulations until birth.

Chaperone Proteins in Muscle Development

In addition to the various structural proteins of the myofiber and especially of the sarcolemma, certain "chaperone proteins" are essential for intracellular folding and assembly of polypeptides to achieve the final molecular structure of cellular structural proteins. However, they remain chaperones because they do not themselves become incorporated into the molecular structure of the protein they regulate in development. One of the most recognized of these chaperone proteins is the small heat shock protein α-B-crystallin, closely related to heat shock protein-27, which is found in all tissues and is phylogenetically very old, expressed even in bacteria and simple worms. It is demonstrated in both cytoplasm and nucleoplasm. It inhibits aggregation and precipitation of denatured proteins[115] and is up-regulated in a variety of tissues during various adverse or stressful conditions, such as fever, chronic ischemia, acidosis, x-irradiation, and exposure to toxins, and in epileptic activity in the brain.[116,117] A close association of α-B-crystallin with the Z-band of striated muscle implies that it is particularly important in protecting the Z-band from the disintegration that results in myofibrillar breakdown and also protects myosin from thermal denaturation.[118]

In muscle, the natural form of α-B-crystallin is overexpressed in myofiber atrophy, associated with a decrease in the amount of the cytoskeletal microtubule protein tubulin.[119] It also is overexpressed, but as a mutant form, in certain diseases of muscle known as myofibrillar myopathies[120] and may cause severe weakness and muscle stiffness leading to early infantile death.[121] The α-B-crystallin may have an additional role in muscle in regulating connective tissue proliferation, as it does in the dermis of the skin during wound healing.[122] In normal human fetal muscle, this protein is not demonstrated by immunoreactivity at any gestational age, perhaps because concentrations are too low to detect by this method (Sarnat HB, unpublished observations).

MORPHOLOGIC STRUCTURE OF MYOTUBES AND MYOCYTES

Sarcolemmal Nuclei

Myoblasts and myotubes have large, vesicular, ovoid nuclei. The chromatin is finely dispersed throughout the nucleoplasm, with a thin condensation against the nuclear membrane. One or often two prominent nucleoli are present (Figure 175-7). Ultrastructurally, the ribonucleoprotein of these nucleoli forms coarse, electron-dense strands (nucleolonemata) rather than the more massive nucleolar sphere of the mature sarcolemmal nucleus (Figure 175-8). These features of the nucleoli and of the finely dispersed DNA are characteristic of active transcription of RNA needed for the synthesis of contractile proteins. The nuclei of regenerating myofibers of children and adults have a similar appearance. The nuclear membrane of fetal myotubes contains pores, similar to the mature state. In the term neonate, some sarcolemmal nuclei are still vesicular and ovoid as are seen in less-mature stages, but most nuclei have acquired the elongated shape with dense chromatin that characterizes the mature state (Figure 175-9). The nuclei of myotubes are arranged in a single-file row within the central core of sarcoplasm. Nuclei may be tightly packed and in contact with neighboring nuclei, or they may be separated by intermediate segments of sarcoplasm of variable lengths (Figure 175-10). Both arrangements

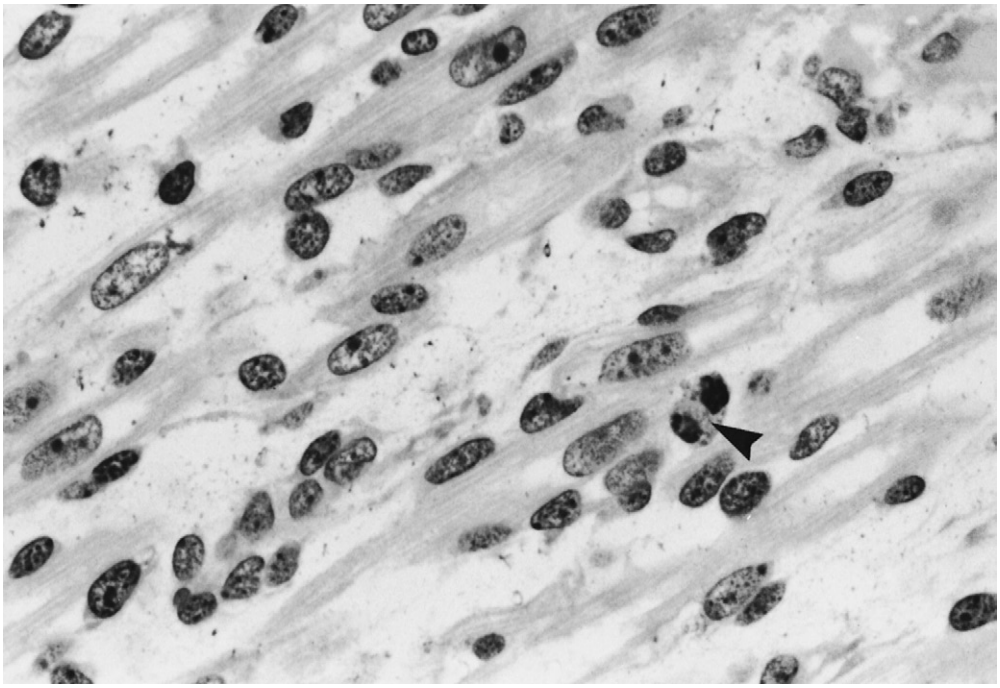


Figure 175-7 Myotubes in the quadriceps femoris muscle of a 14-week human fetus. Nuclei are large and vesicular with finely dispersed chromatin and one or two prominent nucleoli. Mitotic figures of presumptive myoblasts *(arrowhead)* are common. (Hematoxylin-eosin, original magnification ×1000.)

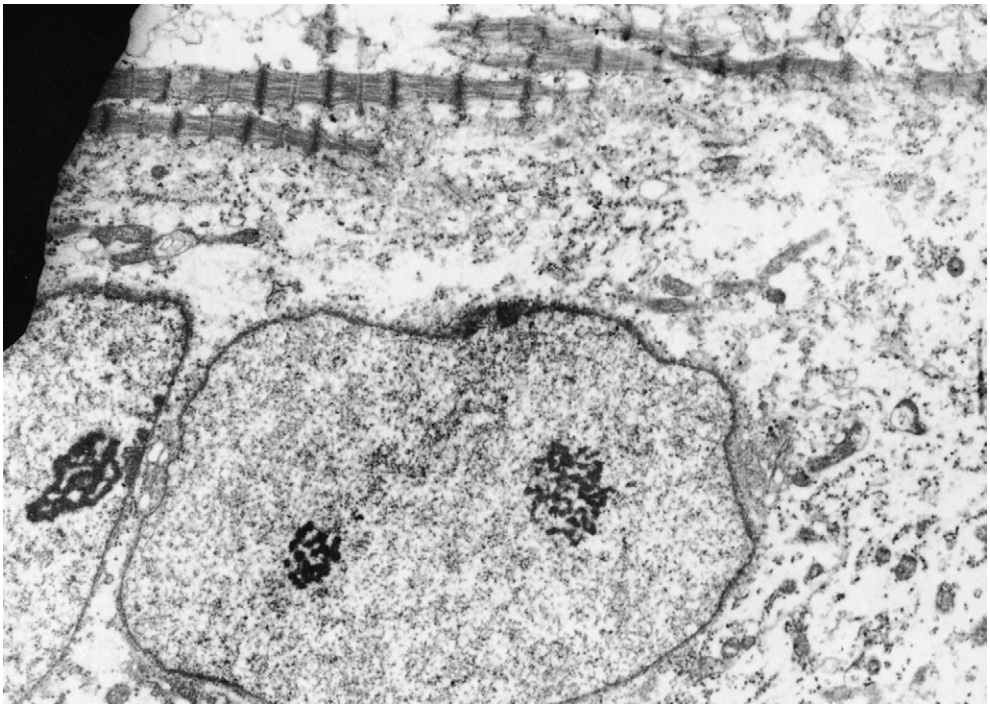


Figure 175-8 Myotube in the quadriceps femoris muscle of a 14-week human fetus. Nuclei exhibit finely dispersed chromatin and strands of ribonucleoprotein-forming nucleoli. Newly formed sarcomeres of contractile proteins are seen at the *top* of the figure. The sarcoplasmic core contains many mitochondria. (Electron micrograph, uranyl acetate and lead citrate, original magnification ×10,000.)

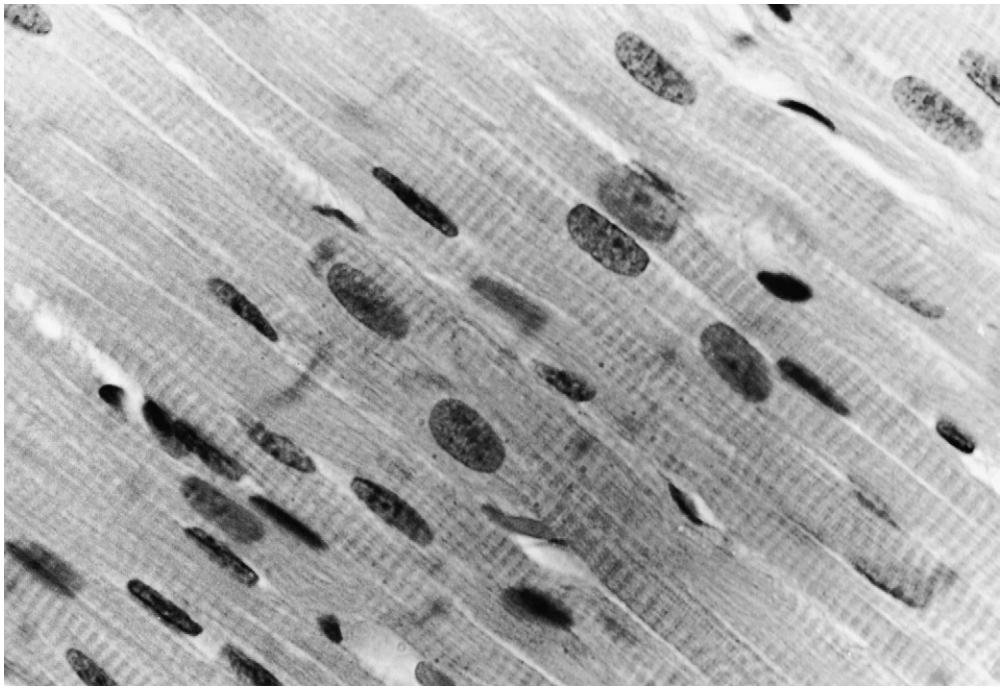


Figure 175-9 Longitudinal section of the quadriceps femoris muscle from a term neonate. Many sarcolemmal nuclei retain the ovoid, vesicular appearance of the immature myofiber, but others are elongated and hyperchromatic, characteristic of mature myocytes. (Hematoxylin-eosin, original magnification ×1000.)

occur simultaneously within the same muscle, and contiguous as well as widely spaced nuclei occur within different segments along the same myofiber. Closely spaced nuclei are generally more characteristic of early myotubes than of late myotubes. Intranuclear lamin A/C is expressed in differentiating myoblasts.[123] Lamin A/C, lamin B1, and emerin are defective in certain hereditary myopathies, highlighting the importance of myonuclear membrane proteins, as well as sarcolemmal plasma membranes, in the integrity of normal muscle development and maintenance.

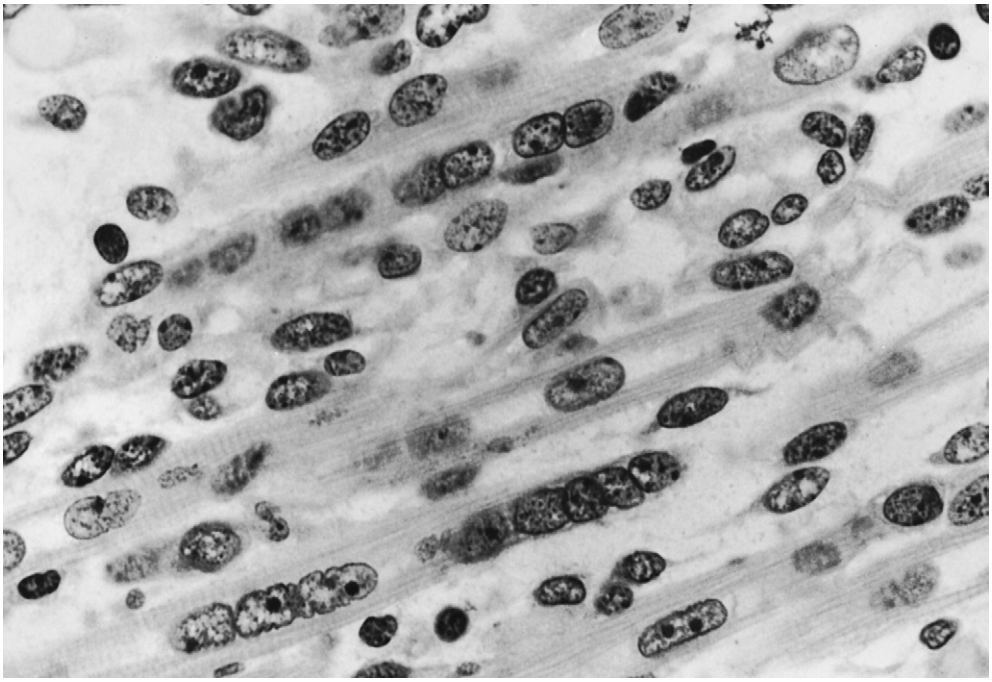


Figure 175-10 Myotubes in the quadriceps femoris muscle of a 12-week human fetus. The spacing of central nuclei is variable. Rows of closely spaced, contiguous nuclei without intervening sarcoplasm are seen, or segments of internuclear sarcoplasm, often several nuclear diameters in length, are found. (Hematoxylin-eosin, original magnification ×1000.)

Sarcoplasmic Organelles

The abundant sarcoplasm (i.e., cytoplasm) within the core of the myotube between and around nuclei contains membranous organelles. These are mitochondria with well-formed cristae, Golgi apparatus generally at the end of a nucleus, and single-membrane vesicles. Most vesicles have dense cores and probably are lysosomes. Notably lacking from the core of sarcoplasm in myotubes are transverse tubules and sarcoplasmic reticulum (SR) (i.e., endoplasmic reticulum), including their diads and triads that are so common in the intermyofibrillar sarcoplasm of more mature myocytes. Mitochondria are almost the only organelles found in the subsarcolemmal sarcoplasma of myotubes. The septae of sarcoplasm that separate the myofibrils of myotubes contain few organelles, unlike the intermyofibrillar sarcoplasm of mature myofibers, although mitochondria may already begin to redistribute themselves between myofibrils of late myotubes.[83]

Glycogen

Free glycogen particles are numerous in the central core of sarcoplasm and in the subsarcolemmal region of myotubes but are less concentrated between myofibrils. In older fetuses (after 30 weeks gestation) and in the first postnatal month, double-membrane vesicles or membranous fragments containing glycogen are commonly seen in the intermyofibrillar sarcoplasm. These vesicles are probably derived from lysosomes. Free glycogen particles are notably lacking from the regions of vesicle-enclosed glycogen.[78]

Contractile Myofilaments

In the myoblast, randomly oriented thin and thick myofilaments occur sparsely in the cytoplasm without parallel alignment or evidence of even rudimentary sarcomere formation. Z-band material is not condensed. The early myotube shows poorly aligned thin myofilaments in the periphery of the cytoplasm, near the inner cell membrane; clusters of thick myofilaments with their associated ribosomes are in close proximity.[78] Lattice formation involving alternating thick and thin filaments in a hexagonal array and Z-bands become evident with the synthesis of even a small number of myofilaments. Haphazardly oriented thin filaments are peripheral to the lattice structure, suggesting their assembly even as new myosin filaments are synthesized. However, filament assembly and lattice formation may not depend on the presence of Z-band material because clusters of thin and thick filaments without associated Z-material are commonly encountered. The Z-bands that are seen always show fine filaments extending in either direction, and the earliest appearance of Z-bands is consistently observed in foci of thin filament formation.[78] Z-material may form when thick myofilaments are either present or lacking, and it appears to be unrelated to myosin.

Thick filaments are synthesized in different sites of the early myotube cytoplasm than are thin filaments. Although thin filaments first appear in the periphery of the cytoplasm, thick filaments appear nearer the central core of sarcoplasm. Thin filaments also appear earlier than thick filaments.[124] Ribosomes are attached along the thick filaments but decrease in number as these myosin filaments are incorporated into the lattice structure of the sarcomere.

As more thin and thick myofilaments are assembled into a lattice structure, and repeating sarcomeres form as the myotube lengthens, the contractile filaments form a denser tubular structure enclosing the core of sarcoplasm and central nuclei (Figure 175-11). The wall of this tube of contractile filaments thickens, and the diameter of the myotube increases. Progressive conversion of the myotube to a mature myofiber involves continued synthesis and arrangement of myofilaments, encroachment on the central sarcoplasmic core, and squeezing of the sarcoplasm between groups of myofilaments as the septa that isolate groups of myofilament lattices from adjacent groups. In addition, the central nuclei "migrate" between the forming myofibrils to the subsarcolemmal position that they occupy in the mature myofiber.

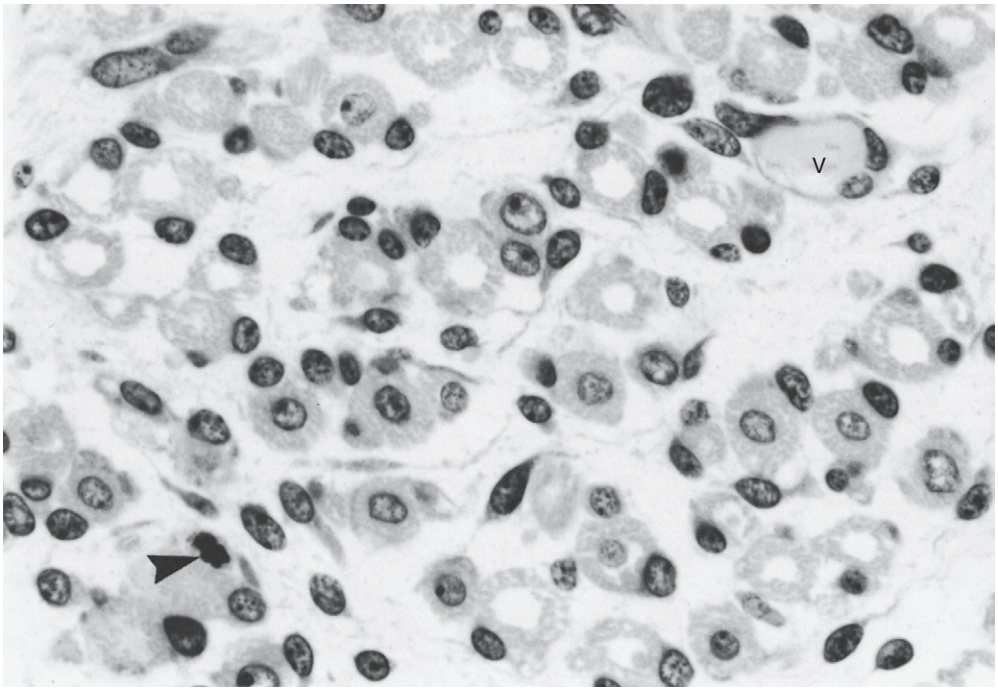


Figure 175-11 Transverse section of the quadriceps femoris muscle in a 12-week human fetus. Late myotubes are a thick cylinder of myofibrils, surrounding central nuclei and the central core of sarcoplasm, seen as a clear space at levels between nuclei. Early myotubes have much thinner cylinders of myofibrils. The peripheral nuclei associated with some myotubes are myoblasts or presumptive myoblasts; one of the latter is undergoing mitosis *(arrowhead)*. Blood vessels *(V)* are a thin endothelium without smooth muscular walls. (Hematoxylin-eosin, original magnification ×1000.)

Growth of the myofiber near term and after birth involves mostly a continued synthesis of contractile proteins and an increased number of myofibrils, associated with a corresponding increase in the amount of sarcoplasm and organelles, as well as the addition of more sarcomeres as the muscle grows in length.

Intermediate Filaments

Intermediate filaments are fibrous cytoskeletal polymers intermediate in size between 6-nm actin filaments and 23-nm microtubules that form the structural framework of nearly all eukaryotic cells. These scaffold proteins anchor cells against mechanical stress and function as bridges between linkage proteins and other cytoskeletal components.[124,125] Perinuclear regions of myotubes characteristically include nonoriented filaments, intermediate in diameter to thick 15-nm and thin 8-nm contractile filaments and measuring 10 to 12 nm.[78,82] Muscle intermediate filaments also are found distal to regions of sarcomere formation. These intermediate filaments are cytoskeletal proteins that help maintain the positions of nuclei and organelles in all cells and help organize the alignment of contractile elements but are not contractile themselves. They may be important in determining the polarity of the early myotube.

Intermediate filament proteins have specific immunoreactivity in differentiating cells and mature cells of various types. The development of intermediate filaments has been less studied in muscle than in the nervous system, and most studies have focused on maturing muscle in tissue culture rather than on animal or human fetuses in vivo. Vimentin is largely a transitory fetal intermediate protein that disappears from most cells with maturation. In the fetal central nervous system, vimentin is demonstrated in primitive neuroectodermal cells but is replaced by either neurofilament protein or glial fibrillary acidic protein in cells differentiating along neuronal or glial lines, respectively. Vimentin is prominent in myoblasts,[126-129] and some investigators find that it disappears after myoblast fusion.[128] It continues to be expressed in fetal myotubes and has stronger immunoreactivity in late myotubes than in early myotubes (Figure 175-12), but it is not demonstrated in mature striated muscle (see Figure 175-12). However, even in adult life it persists in smooth muscle cells, including the muscular walls of blood vessels.

Another important muscular intermediate filament, desmin, is responsible for maintaining the register of Z-bands between adjacent myofibrils by forming links between Z-bands.[130] Desmin is thus the third protein, in addition to actinin and actin, to be localized at the Z-band with use of immunocytochemical techniques. Desmin synthesis is initiated at the time of myoblast fusion and is prominent in fetal myotubes, especially late myotubes (Figure 175-13, *A*), having the same distribution as vimentin.[126,127] Early myotubes contain three times as much desmin as vimentin,[129] but this is difficult to demonstrate in mature muscle by immunocytologic reaction (see Figure 175-13, *B*). Desmin filaments begin to redistribute transversely at the time of sarcomere formation.[128] Nevertheless, desmin is still demonstrated in adult muscle by electrophoretic and special immunologic methods.[127-133] The sparse strands of desmin in adult muscle connect myofibrils at the level of the Z-bands to adjacent sarcolemma, mitochondria, and nuclei to maintain the relative positions of these structures, but they do not attach to the T-tubule systems.[133] Desmin also is associated with the neuromuscular junction site.[134] Immunocytochemical analysis demonstrates both vimentin and desmin in human striated muscle fibers until approximately 36 weeks gestation, although neither is as strongly expressed as in myotubes.[135] In the term neonate, vimentin is not normally demonstrable in myofibers but is prominent in the endothelial cells of blood vessels. Desmin shows only trace or no immunoreactivity in myofibers unless incubated with highly concentrated antibodies (1:50 dilution used for tumors is not appropriate; 1:1200 dilution should be employed).[136] As with vimentin, desmin continues to be strongly expressed by immunocytochemical methods in mature smooth

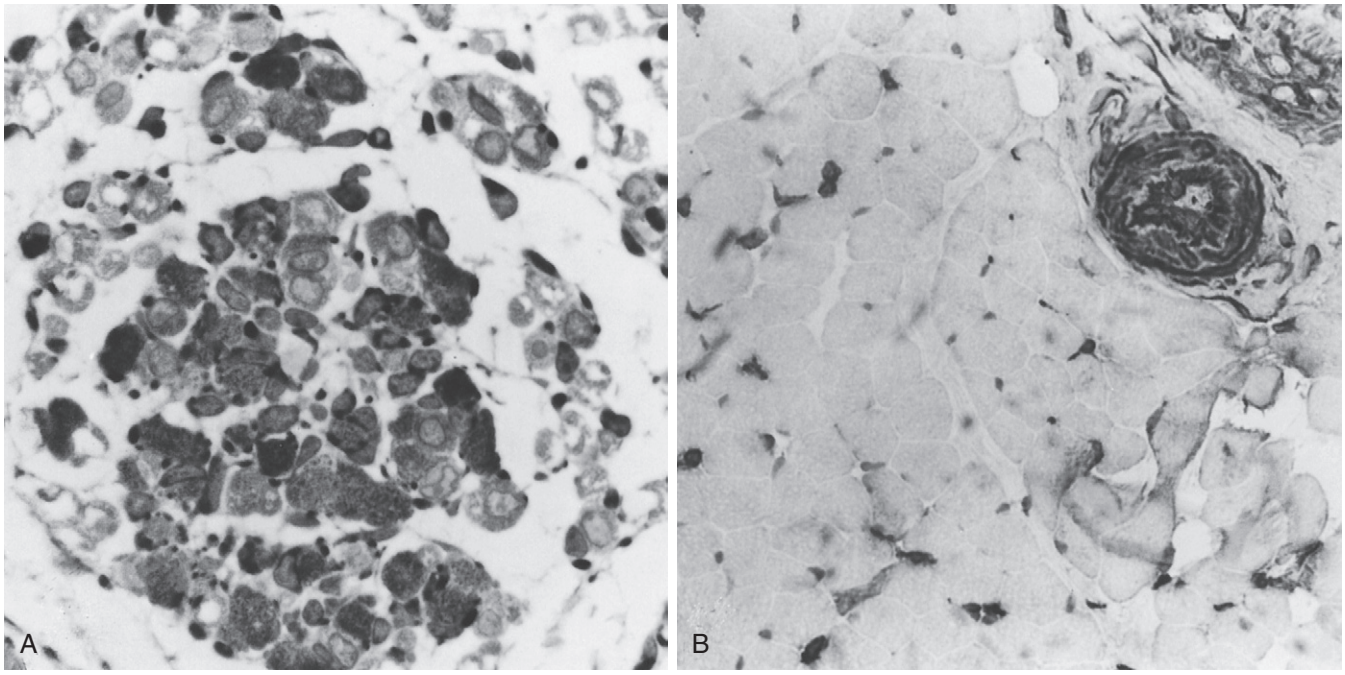


Figure 175-12 Vimentin, a transient intermediate filament protein, is strong in fetal myotubes (dark-staining immunoreactive product), particularly late myotubes, but is no longer seen in striated myofibers of the term neonate, although it is still strongly reactive in smooth muscle of vascular walls and also in endothelial cells. **A,** 14-week fetus. **B,** Term neonate. (1:200 dilution; original magnification ×1000.)

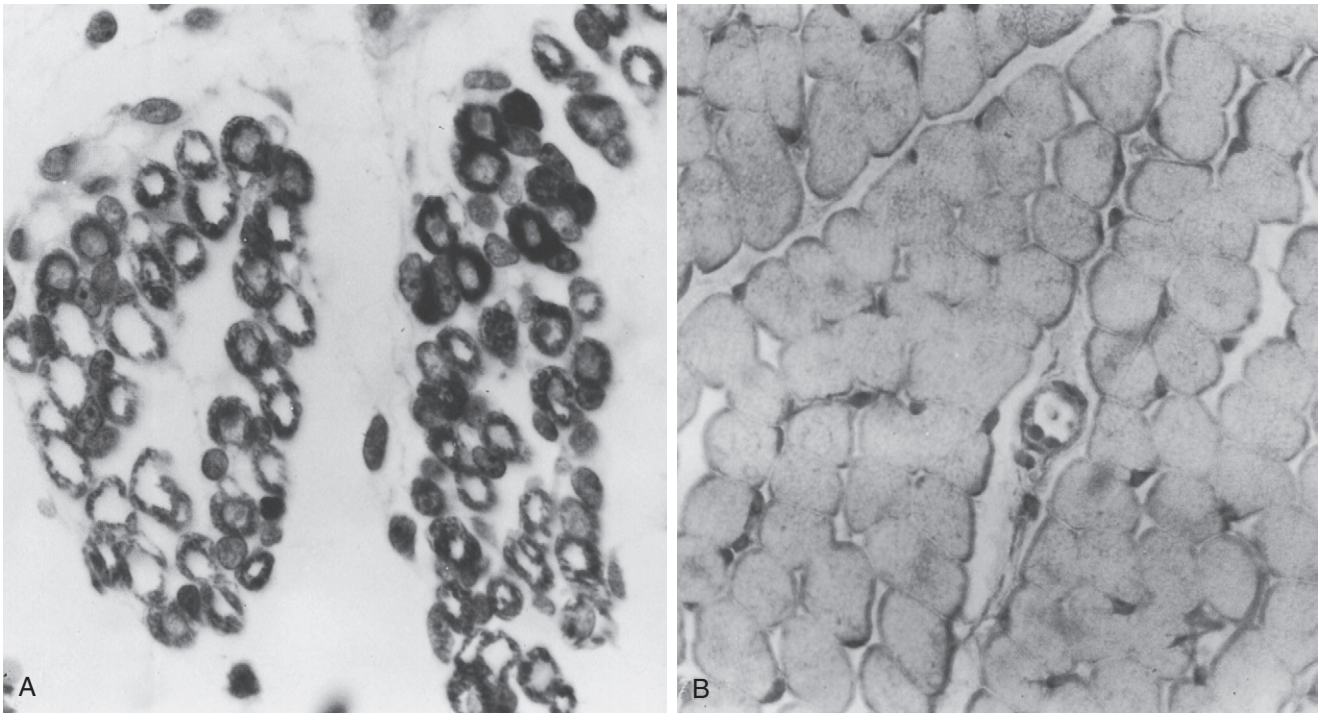


Figure 175-13 Desmin (dark-staining product) is abundant in myotubes and has the same distribution as vimentin. It is no longer demonstrated by immunoreactivity in term neonatal muscle, although it persists in small amounts to maintain the registry of adjacent Z-bands. **A,** 14-week fetus. **B,** Term neonate. (1:800 dilution; original magnification ×1000.)

muscle cells, in which these intermediate filaments appear to be associated with the plasma membrane but are not connected to either the actin or myosin filaments.[137] Desmin is closely associated with α-B-crystallin and is augmented in certain human myopathies in a general category as "myofibrillar myopathies."[138]

Microtubules

Microtubules that are approximately 25 nm in diameter are characteristic in regions of myofilament synthesis and early assembly, but they are lacking in regions of more advanced myofibril

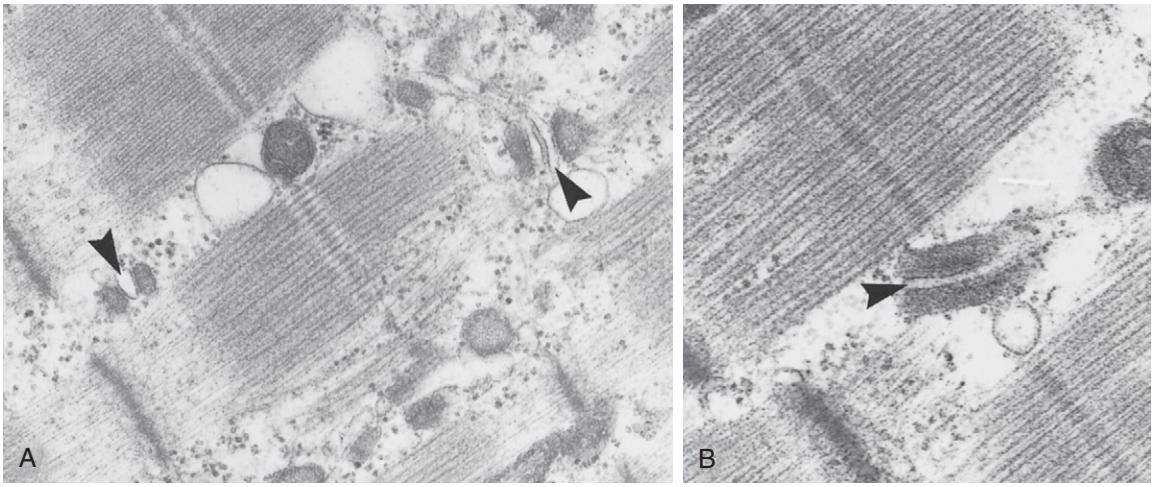


Figure 175-14 Triad formation in term neonatal muscle. The triads *(arrowheads)* are formed by a T-tubule with a pair of dilated terminal cisternae of sarcoplasmic reticulum. They generally occur at or near the Z-band and are perpendicular to the long axis of the myofiber in the mature state **(A),** although they initially have a more parallel orientation in relation to the myofiber axis **(B)** until approximately 28 weeks gestation. (Electron micrographs, uranyl acetate and lead citrate. **A**, Original magnification ×25,000. **B**, Original magnification ×35,000.)

formation.[78] Their orientation is usually oblique, and they are situated near the ends of developing sarcomeres. A microtubular network is present in both mononucleated myoblasts (in which they radiate from a juxtanuclear center toward the cell periphery) and multinucleated myotubes, in which microtubules are numerous and are arranged in parallel longitudinal orientation.[130,131]

Ribosomes

Ribosomes are abundant in regions of myofilament assembly and sarcomere formation.[82] They may be single and free in the cytoplasm, may occur in clusters as polyribosomes, or may be membrane associated. Ribosomes are structurally related to the formation of thick myosin filaments but not to the formation of thin filaments. Polyribosomes of approximately 15 individual ribosomes are consistently clustered near both ends of the thick filaments, nearest to the I-band, and single ribosomes also occur along the length of the myosin filament.[64] Ribosomes decrease in number and virtually disappear as the sarcomeres form, and they are rare in mature muscle. They reappear in regenerating myofibers at any age. The high RNA content in fetal myotubes and regenerating myofibers of injured mature muscle and the lack of demonstrable RNA in mature normal myocytes are well demonstrated by acridine orange fluorochrome studies.[139]

Sarcotubular System

In myotubes and mature myofibers, the endoplasmic reticulum of less-differentiated myoblasts and presumptive myoblasts is called the SR. Early myotubes exhibit multiple vesicular structures that probably are a primordial stage in the formation of SR. Irregular tubules show branching as the myotube matures, and these tubules are found in regions of cytoplasm containing myofilaments and forming sarcomeres. Numerous Z-bands are in continuity with the SR, but this is not a constant feature. Some studies suggest that the SR tubules completely encircle the Z-band,[83] but my observations agree with those of Tomanek and Colling-Saltin[78] that this is an inconsistent feature. The SR does not appear essential in the assembly of myofilaments; however, the alignment of Z-bands in register with those of adjacent myofibrils, a late development after 30 weeks gestation, may be a function of SR tubules associated with Z-bands.[78] The SR of both developing and mature myocytes is mostly smooth, without attached ribosomes. Short segments of rough endoplasmic reticulum are also encountered in myotubes but not in mature myofibers.

The T-system develops by invaginations of the sarcolemma (i.e., cell membrane) of the myotube in cultured chick embryonic muscle.[140] Observations of human fetal muscle suggest a similar process of T-tubule formation.[78] Diads, a close association of an SR tubule and a T-tubule, and triads, a T-tubule between a pair of dilated terminal cisternae of SR, are seen only in the periphery of myotubes, often associated with the sarcolemmal membrane[78,140-142] (Figure 175-14). Diads and triads occur in deeper regions of the myofiber only after sarcoplasmic septa separate the myofibrils as the central core of cytoplasm becomes dispersed with advanced maturation of the myotube. Membranes of SR and T-tubules first become apposed with no visible structure between them; tenuous connections next traverse the space between apposed membranes, and well-developed bridges finally form.[142] Cisternae of the SR expand to form their characteristic structure after contacting T-tubules.[140] SR cisternae contain electron-dense material that consists of mucopolysaccharides or glycoproteins to provide sites for calcium binding.

The observation that during early fetal development SR cisternae seldom show connections to other SR components suggests either that they develop independently or that they form on short fragments of SR that eventually unite with other SR tubules.[78] Triads have a predominantly longitudinal orientation relative to the long axis of the myofiber until approximately 28 weeks gestation, after which time they rotate to the mature transverse orientation (see Figure 175-14).[140-143]

Spherical vesicles with bristles on their outer surface in the periphery of the myotube are probably components of SR terminal cisternae rather than T-tubules because they also are found in satellite cells. Presumptive myoblasts of fetal muscle and satellite cells of mature muscle possess an extensive endoplasmic reticulum, but T-tubules are not evident and do not appear until the myotubular stage of development.[78]

Mitochondria

The mitochondria of striated muscle are similar to those in other types of cells but generally larger than in most cells and are very numerous because of the need to continuously generate energy.

In the myotube, mitochondria are located in the central core of the myofiber between the row of central nuclei. With reorganization of the fiber after 15 weeks gestation and relocation of the nuclei to the subsarcolemmal region, mitochondria become redistributed into the intermyofibrillar sarcoplasm throughout the fiber and in the subsarcolemmal region. They become particularly numerous and tightly clustered in the regions of motor end plates. Little difference is seen ultrastructurally between the cristae of mitochondria in immature and mature muscle. Because muscle is such a rich source of mitochondria, muscle biopsy often is performed as the tissue of choice for the study of mitochondrial histochemical and ultrastructural features, for biochemical assay of respiratory chain enzymes, and for the study of mitochondrial DNA in suspected systemic mitochondrial diseases.

PHYSIOLOGIC CELL DEATH OF MYOTUBES

Degenerating myotubes occur singly or in small groups among normal myotubes in fetuses of 10 to 14 weeks gestation.[144-146] Mononuclear phagocytic cells may be numerous around degenerating myocytes. Electron microscopy reveals nonartifactual extensive necrotic and degenerative changes in both nuclei and sarcoplasm, loss of cell membranes, amorphous condensation of myofibrils, and extrusion of cellular contents into the endomysial extracellular space. This process of physiologic cell death is similar to that observed in the central nervous system and other body systems. An even earlier phase of apoptosis occurs among myoblasts still in the somitic myotome, before migrating into the sites of axial and appendicular muscles.[11]

GROWTH OF MYOFIBERS

The size of an individual muscle is determined by the number and size of its constituent myofibers and also by the amount of connective tissue that forms septa separating fascicles (i.e., perimysium). The diameter of individual muscle fibers is affected by many factors, including age, nutrition, exercise, and the effects of hormones, drugs, and disease. However, the number of muscle fibers in a particular muscle is relatively constant after birth and is genetically programmed.[147,148] A few studies have suggested a postnatal increase in total myofibers of particular muscles such as those of mastication.[147] Protein-calorie malnutrition during fetal development may alter the final number of muscle fibers in animals,[149] but this phenomenon has not been adequately studied in human infants who have had intrauterine growth restriction or who are born to undernourished mothers. In contrast, the ratios of histochemical fiber types within the muscle are under neural control and are altered in either fetal or adult life by abnormal innervation patterns (see later). The average cross-sectional diameter of muscle fibers in the term neonate is 15 μm; the adult mean diameter of 65 μm is achieved at approximately 13 years of age. A standard curve plotted in 1969 for quantitative measurement in muscle biopsies of children remains valid and widely used by pathologists.[150]

Between 9 and 16 weeks gestation in the human embryo, myotubes contain approximately 35 myofibrils in a transverse section of the tube of contractile filaments. Mature muscle fibers each contain approximately 80 to 110 myofibrils in transverse section. Initially, myotubes are larger in cross-sectional diameter than other myofibers, but the situation is later reversed; as myotubes lengthen, their diameter actually decreases. Although the ratio of myotubes to maturing myofibers reverses, hypertrophy of individual myofibers replaces hyperplasia (i.e., increased number of myofibers) as the principal factor contributing to the total increase in the transverse area of the whole muscle.

Myofibril number is not related to myotube size, although it does increase at a declining rate. The total proliferation in number of sarcolemmal nuclei with longitudinal growth of the myofiber is accomplished by mitosis of presumptive myoblasts and their conversion to true myoblasts, rather than by proliferation of existing sarcolemmal nuclei, because the latter are incapable of mitosis. This process slows in late gestation.[146]

Between 20 and 24 weeks gestation, approximately 10% of total myofibers in a cross-sectional field are noted to be approximately twice as large as the remaining 90%. These hypertrophic fibers are scattered uniformly throughout the fascicles and are never grouped. They were first identified by Wohlfart in 1937 and were designated by him as b-fibers; the majority population of smaller fibers were termed a-fibers,[151] a terminology still used. The Wohlfart b-fibers were also later shown to be distinguishable from the a-fibers (see later) by histochemical reactivity. Between 24 and 30 weeks gestation, as histochemical differentiation proceeds, the Wohlfart a-fibers increase in diameter to achieve the same size as the b-fibers that do not grow as fast, until the two populations of myofibers can no longer be distinguished by size after 30 weeks.

Growth of myofibers during late gestation is mainly in length rather than diameter, but the latter accelerates in the postnatal period. Some increase in the number of myofibers in late gestation and after birth may occur because of longitudinal splitting; an explanation of this splitting down the middle of the growing myofiber is that, when a fiber achieves a critical size, the oblique pull of peripheral actin filaments is strong enough to cause the Z-band to rupture.[152] After birth, linear growth of skeletal myofibers is greatest in the first 2 years and is accomplished mainly by adding sarcomeres rather than by extending the distance between Z-bands.

METABOLIC DIFFERENTIATION OF MYOFIBERS

After nearly all fetal myotubes have become myocytes with peripheral sarcolemmal nuclei and an intermyofibrillar distribution of sarcoplasm and organelles, the next major maturational development is the differentiation of the uniform population of myofibers into a heterogeneous population of two distinct metabolic types and at least one subtype. Neural influence is first expressed in muscle maturation in the further growth and morphologic reorganization of myotubes and plays no role in the fusion of myoblasts to become myotubes. However, the expression of metabolic differences between muscle fibers is strongly controlled by innervation, and myofibers retain the capacity to change metabolic type with changes in innervation, as with denervation-reinnervation of muscle. This mutability is retained in human striated muscle not only during fetal life and infancy but also in the adult.

Metabolic differences between myofibers are expressed as relative differences in various enzymatic activities of the oxidative and glycolytic pathways, differences in calcium-mediated myofibrillar adenosine triphosphatase (ATPase) isozymes that are expressed maximally at various pH ranges, in heavy-chain myosin, and in relative numbers or concentrations of mitochondria, lipid droplets, and glycogen particles within the sarcoplasm. These features are well demonstrated in frozen tissue section by histochemical methods and, more recently, by immunocytochemical reactivity for heavy-chain myosin that distinguishes fiber types in formalin-fixed, paraffin-embedded sections.[153]

The period of histochemical differentiation is between 20 and 30 weeks gestation in humans, but some differences among myofibers in their metabolic profiles may be seen as early as 18 weeks. Before that time, all myofibers appear histochemically uniform with all enzymatic stains; with use of myofibrillar ATPase stains, they correspond to IIc fibers, which are considered the least differentiated subtype in mature muscle.

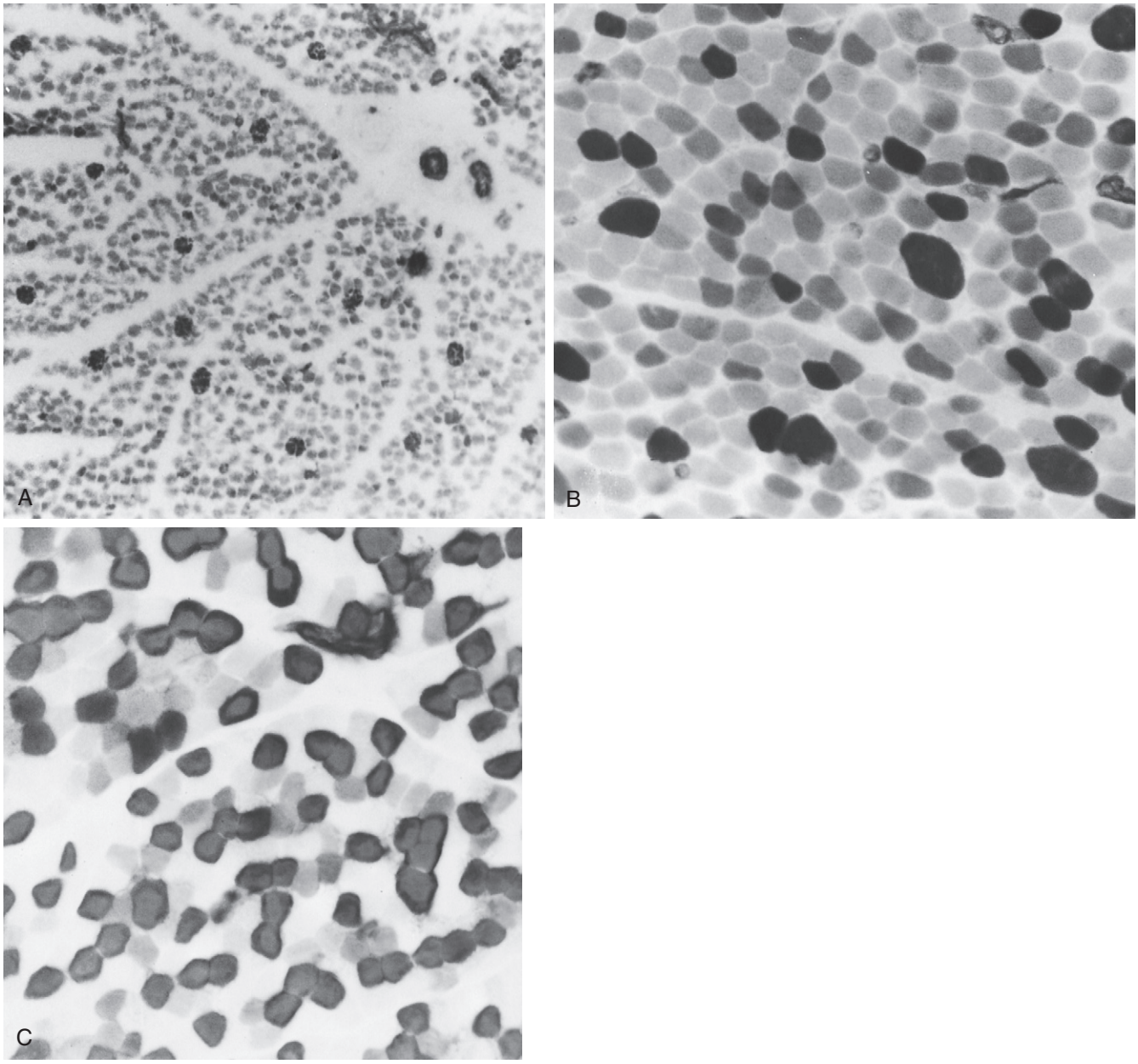


Figure 175-15 Myofibrillar ATPase stain, preincubated at pH 4.3, in human fetuses at 24 weeks gestation **(A),** 27 weeks gestation **(B),** and 40 weeks gestation (term) **(C).** In the early histochemical stage of development **(A),** approximately 10% of myofibers become larger than the others, are scattered in distribution and never grouped, and react as type I fibers, the "Wohlfart b-fibers." As maturation proceeds, more type II fibers become converted to type I **(B),** until a relatively equal number of the two types exist at term **(C).** Still a greater proportion of subtype IIc fibers (intermediate intensity of staining) exist at birth than in the adult. (Original magnification ×250.)

The early phase of histochemical differentiation, generally 20 to 24 weeks, coincides with the appearance of Wohlfart b-fibers, the 10% of total myofibers that are larger than the rest and that are scattered evenly throughout the fascicles without being grouped.[151,154] These Wohlfart b-fibers are uniformly type I (i.e., strong ATPase activity at acid pH of preincubation, strong oxidative enzymatic activity and weaker glycolytic activity, less glycogen than type II); the 90% remaining Wohlfart a-fibers react histochemically as type II (i.e., strongest ATPase activity at alkaline pH of preincubation, weaker oxidative and stronger glycolytic enzymatic activity, and more glycogen than type I) and correspond almost exclusively to subtype IIc of mature muscle (Figure 175-15).

As maturation proceeds, and especially during the later phase of histochemical differentiation from 24 to 30 weeks gestation, progressive conversion of many type II fibers (Wohlfart a-fibers) to type I fibers occurs; *however,* growth of the population of smaller fibers at a greater rate than growth of the Wohlfart b-fibers obliterates the distinction of these populations by either size or their reactivity as type I. By 30 weeks gestation, most muscles of the human body have relatively equal numbers of types I and II myofibers, and they are distributed in a mosaic or checkerboard pattern without large groups of either type, although small groups of three to five fibers of the same type may occur. Type II fibers may predominate in the single line of myofibers along the edges of fascicles.

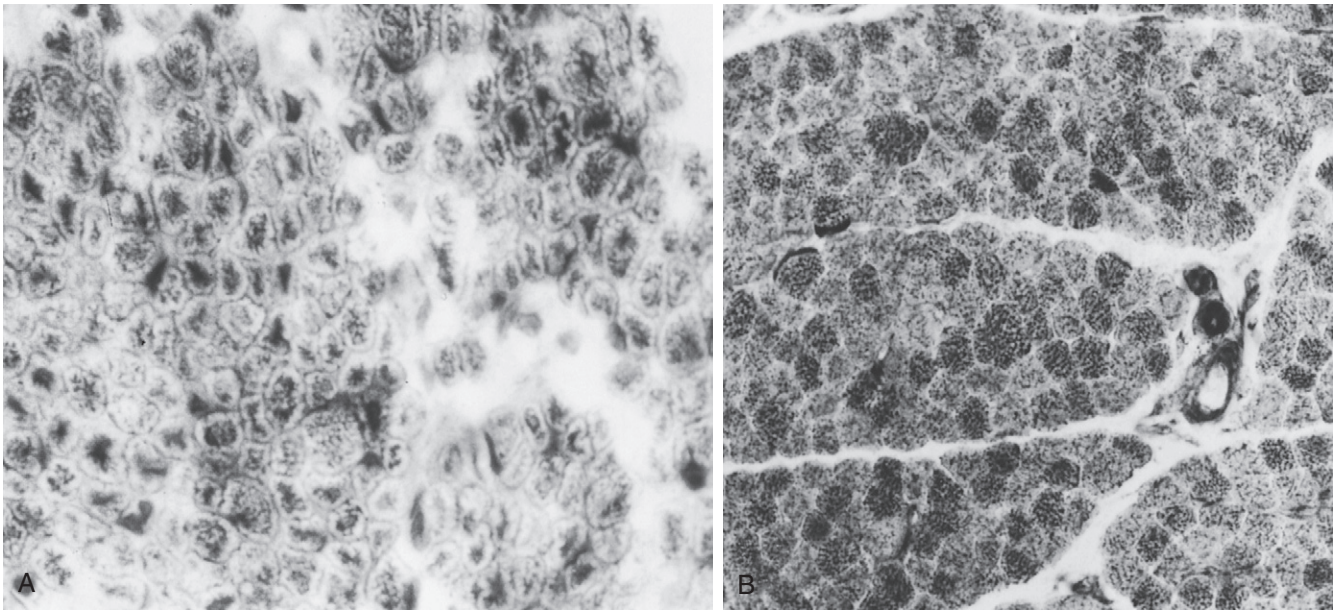


Figure 175-16 A, Oxidative enzymatic (mitochondrial) activity has a characteristic histochemical distribution in myotubes: intense staining confined to the central core of sarcoplasm and a thin rim in the subsarcolemmal region, the cylinder of myofibrils remaining unstained. Fiber types cannot be distinguished. **B,** In term neonatal muscle, the oxidative enzymatic activity is now redistributed uniformly in the intermyofibrillar sarcoplasm. Dark points of activity are mitochondria. The darker-staining fibers are type I, and the lighter-stained fibers are type II, as in the adult. (Nicotinamide adenine dinucleotide, tetrazolium reductase [NADH-TR]: **A**, original magnification ×1000; **B,** original magnification ×250.)

During the later period of histochemical differentiation (after 30 weeks gestation), type II fibers become distinguished by myofibrillar ATPase stains as subtypes defined by their degree of inhibition of reaction at varying pH of preincubation.[154] Thus, at high pH, such as 9.8 or 10.4, all type II fibers are uniformly stained darker than type I fibers. At pH 4.6 or 4.7, type I fibers are dark, whereas some type II fibers are intermediate in intensity and others are totally inhibited and unstained. The inhibited fibers are designated IIa, and the intermediate fibers are designated IIb and IIc. At a still lower pH of preincubation (4.3), IIa fibers remain completely inhibited, IIb fibers are either totally inhibited or very light, and IIc fibers are intermediate—darker than IIb fibers but not as dark as type I fibers. At maturity, approximately 65% of total type II fibers are designated IIa, 30% are designated IIb, and only 5% are designated IIc. In the term neonate, however, 15% to 20% of type II fibers are IIc or "undifferentiated" fibers (see Figure 175-15).[154-156] Differentiation of the soleus muscle, with a higher percentage of type I fibers, is not complete until 9 to 12 months of age in the human infant and is accompanied by slowing of contractile properties that correspond to the functional use of this muscle as the infant stands more and prepares to walk.[156]

The oxidative enzymatic activity of the mitochondria and SR has a characteristic histochemical distribution with use of stains of the electron transport chain (e.g., nicotine adenine dinucleotide [reduced form]–tetrazolium reductase) and of the tricarboxylic acid (Krebs) cycle (e.g., succinate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase). In myotubes, the activity follows the distribution of mitochondria and is seen only in the central core of sarcoplasm and in the subsarcolemmal region (Figure 175-16, *A*). As the myofiber matures, the mitochondria become redistributed in the intermyofibrillar sarcoplasm. Therefore type I fibers, which have more mitochondria than type II fibers, appear darker (see Figure 175-16, *B*). Oxidative enzymatic stains used to be regarded only in relative ratio of activity to glycolytic enzyme stains, but it is now recognized that each is a marker for the specific mitochondrial respiratory chain complexes, and they have assumed an even greater value in interpreting some muscle biopsy results, as well as in developmental studies of muscle.

Myophosphorylase activity also may be histochemically demonstrated in unfixed frozen sections of striated muscle. A fetal form of myophosphorylase is replaced by the mature form of the enzyme after birth. In addition to myophosphorylase, phosphofructokinase and other enzymes of the glycolytic Embden-Meyerhof pathway may also be demonstrated in fetal muscle as early as the late myotubular stage. Distinction between histochemical fiber types is not evident until after 24 weeks gestation, however. The myogenic genes may also play a role in muscle fiber–type differentiation, even though this phase of ontogenesis is considerably later than the primary period of expression of these genes. In transgenic mice, myogenin induces, or at least accompanies, a shift from glycolytic (type II fibers) to oxidative (type I fibers) metabolism.[157,158] With aging in adult life, changes in levels of expression of *myogenin* and *MyoD* proteins in muscle also occur.[159]

FASCICULAR ORGANIZATION OF DEVELOPING MUSCLE

As the paired longitudinal somitic plates on either side of the neural plate begin to segment into somites, the space between somites contains looser, less densely cellular mesenchyme than the more compact somites. The mesenchymal cells in this looser tissue differentiate into fibroblasts and soon secrete collagen into the intercellular space as the more compact mesenchymal cells of the somites predominantly become myoblasts and primordial dermal cells. During growth of the limb buds, the individual muscles of the extremities are derived from the somites, whereas the collagenous muscle capsules (i.e., epimysium), blood vessels, and tendons are somatopleural derivatives.

Individual muscles consist of loose arrays of presumptive and true myoblasts and differentiating myotubes without the

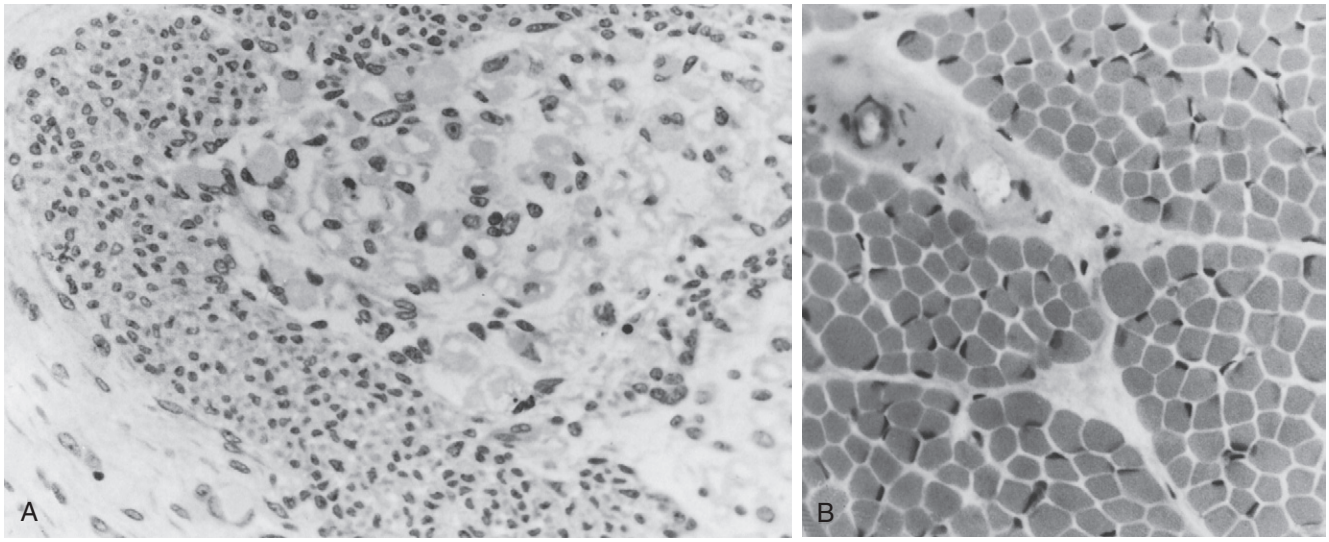


Figure 175-17 A, Soleus muscle of 12-week human fetus. Early myotubes and myoblasts tend to predominate at the periphery, and late myotubes are more frequent in the interior of the muscle. Perimysial connective tissue septa have not yet developed. **B,** Term neonate, quadriceps femoris muscle. The fascicular organization of the muscle is well developed, with a well-defined perimysium that includes arterioles, venules, and intramuscular nerves. (Hematoxylin-eosin; **A,** original magnification ×400; **B,** original magnification ×250.)

connective tissue septa that subdivide more mature muscle into fascicles. During the myotubular stage of myogenesis, myotubes and myoblasts cluster in small groups of three to as many as eight cells, but mostly three to five myotubes, surrounded by a single common basal lamina.[155,160] Single myotubes enclosed within their own individual basal lamina and isolated myoblasts without a basal lamina also appear.[78] Independent myotubes become more numerous in later stages of development, and, by the end of the myotubular stage, clusters of myotubes within a common basal lamina are no longer seen, although single myotubes continue to be closely associated with myoblasts and presumptive myoblasts.[160] Myoblasts and early myotubes tend to predominate at the periphery of the developing muscle, and maturation of the myotubes in the center of the muscle is more advanced (Figure 175-17, *A*).

Fibroblasts also are included within developing muscle and produce delicate reticular and collagen fibers. Thin collagenous septa (i.e., perimysium) divide the muscle into fascicles, particularly in association with the growth of blood vessels. This process begins in the late myotubular stage, occurring mainly as myotubes are converted to myocytes with peripheral nuclei and continuing during the early phase of histochemical differentiation. By 30 weeks gestation, the fascicular organization of muscle is well established. Perimysial connective tissue then thickens and includes all arterioles, venules, and nerves that penetrate the muscle (see Figure 175-17, *B*). Endomysial collagen fibers between individual myofibers within a fascicle are thinner, sparser, and much more delicate in the term neonate than in the older child; the endomysium mainly develops after birth.

Blood vessels within developing muscle are seen as early as 6 weeks gestation in humans. During the myotubular stage of development, vessels consist of delicate channels or sinuses with thin endothelial cells and wide lumina (Figures 175-18 and 175-19). With growth of the perimysium, the feeding vessels develop smooth muscular walls and a thicker endothelium. The capillary network becomes denser as myofibers mature and contraction becomes more forceful. By term, both capillaries and larger arterioles and venules are well developed (see Figure 175-17, *B*).

Several resident inflammatory cells are seen scattered in the perimysium of muscle. At least two distinct populations of macrophages (M1 and M2) have differing roles in necrosis and regeneration in development and in myopathies.[161] Mast cells and scattered T- and B-lymphocytes also are present, even in fetal muscle, and can increase when signaled to do so. In addition, hematogenous arrival of additional inflammatory cells in response to injury occurs.

MUSCLE SPINDLES AND TENDON ORGANS

Intrafusal fibers are already forming during the myotubular stage of development, but they are difficult to recognize histologically because they closely resemble extrafusal fibers at this time. The distinctive connective tissue capsule of the spindle does not form until later.[162-164] Spindles may be readily identified after 20 weeks gestation in humans, and they appear fully mature at 30 weeks, including well-developed nuclear bag and nuclear chain forms of intrafusal fibers (Figure 175-20) with their mature histochemical characteristics.

Because intrafusal fibers enclosed by a delicate collagenous capsule may be recognized as early as 14 weeks gestation in human fetuses, spindles actually begin forming late in the myotubular stages.

In the rat, the earliest detectable spindles in hind limb muscles are seen less than 2 days before birth and consist of a single myotube bearing simple nerve terminals of the large primary afferent axon from nearby unmyelinated intramuscular nerve trunks. The capsule forms by an extension of the perineurium of the supplying nerve fasciculus and is confined initially to the innervated zone.[162-165] Myoblasts are present within the capsule of the spindle throughout its development and fuse to form a smaller and less-differentiated myotube. This myotube matures in close association with the initial fiber, and by birth it has formed the smaller, intermediate nuclear bag fiber identified in the adult. Nuclear chain fibers develop in the same way, but myoblast fusion forms satellite myotubes in apposition to more mature fibers within the same basal lamina. By 4 days after birth, the adult complement of four intramuscular muscle fibers is present. Fusiform innervation begins at birth and is not complete until the 12th postnatal day (rat) when the myofibrillar ultrastructure is fully mature.[164] The periaxial space appears at the same time. The afferent innervation thus precedes the efferent nerve supply

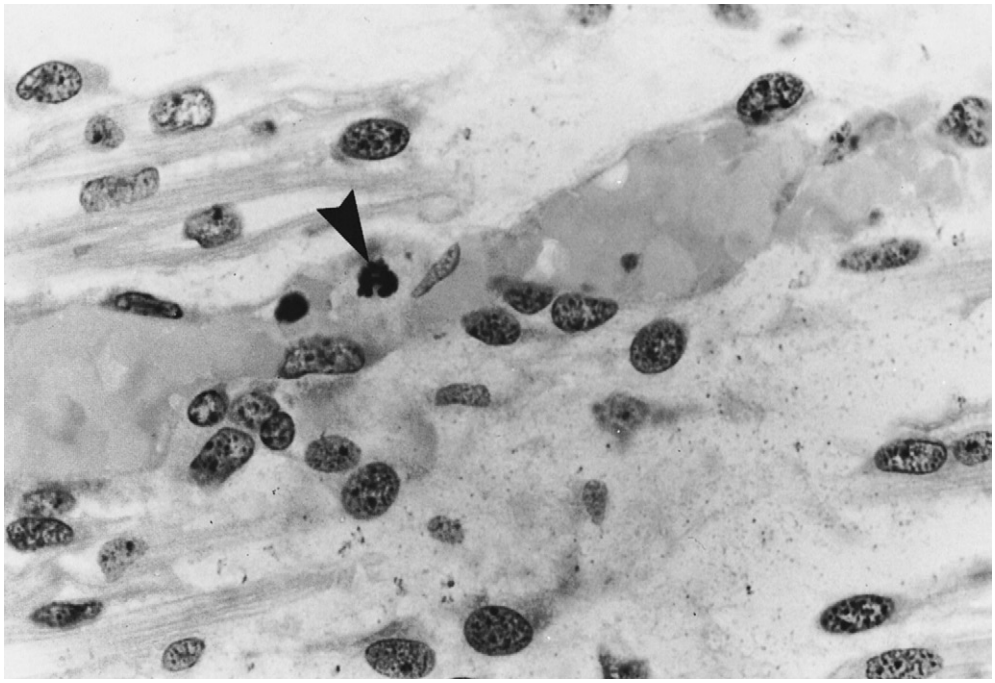


Figure 175-18 Intramuscular blood vessel in quadriceps femoris muscle of a 14-week fetus. The vessel is thin walled, and the lumen is wide and dilated. The mitotic figure *(arrowhead)* may be an endothelial cell or a presumptive myoblast. (Hematoxylin-eosin, original magnification ×1000.)

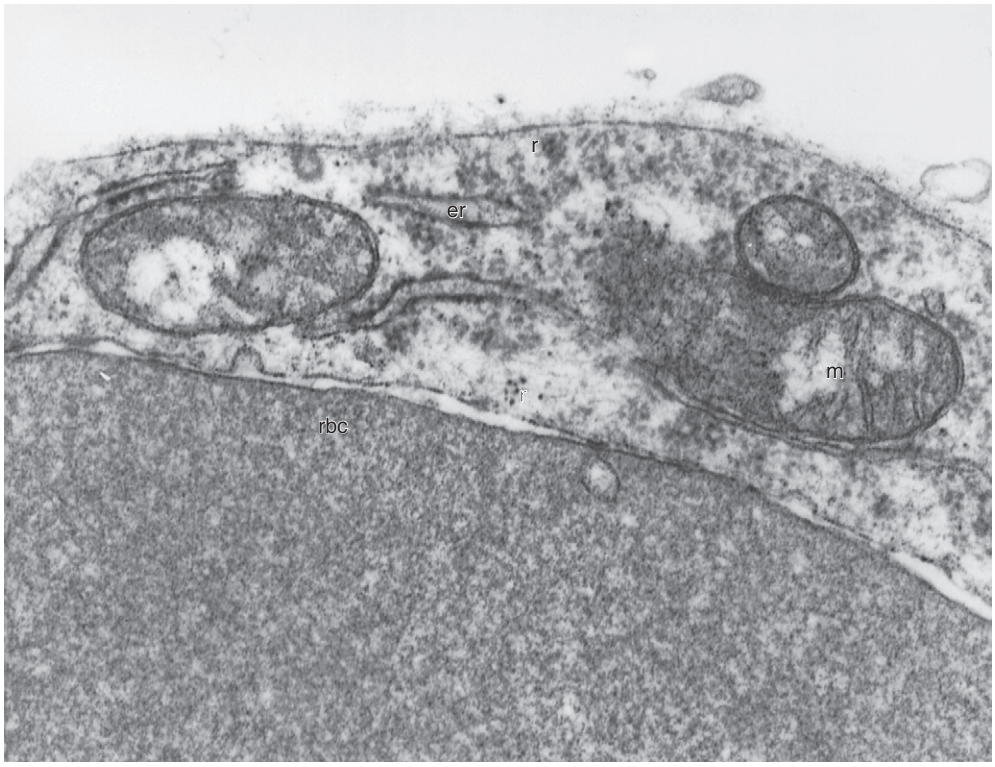


Figure 175-19 Endothelial cell of intramuscular blood vessel of a 14-week fetus. Mitochondria *(m),* rough endoplasmic reticulum *(er),* and free ribosomes *(r)* are seen in the cytoplasm. A red blood cell *(rbc)* is seen in the lumen of the vessel. (Electron micrograph, uranyl acetate and lead citrate, original magnification ×25,000.)

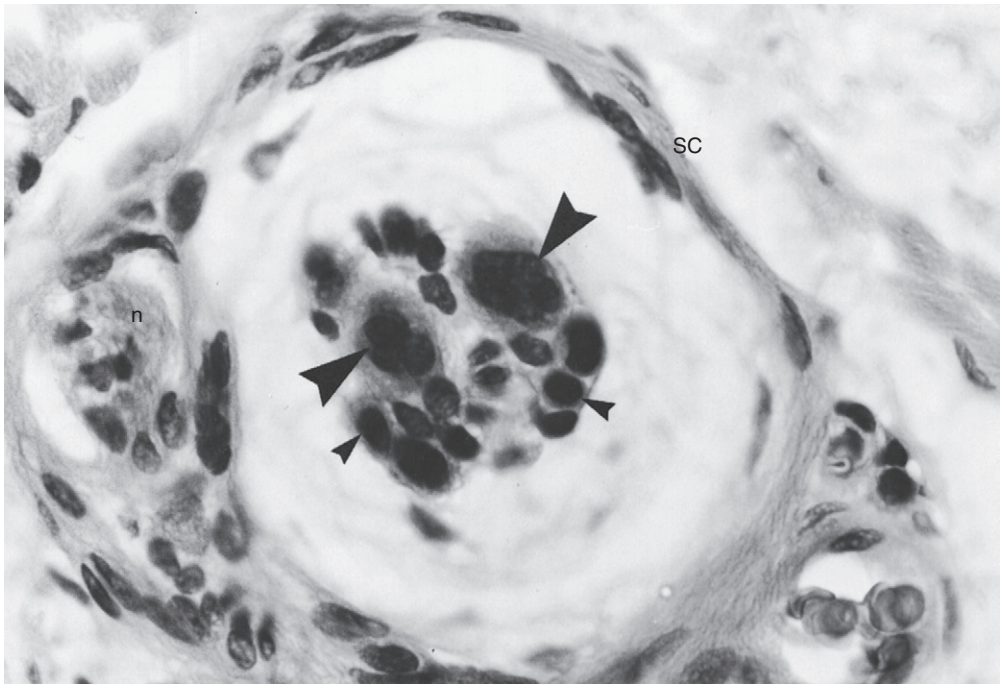


Figure 175-20 Muscle spindle within the quadriceps femoris muscle of a term neonate. Both nuclear bag fibers *(large arrowheads)* and nuclear chain fibers *(small arrowheads)*, as well as the spindle capsule *(SC)* and associated nerve *(n),* are well developed at birth. (Hematoxylin-eosin, original magnification ×1000.)

to the intrafusal spindle fibers. Morphogenesis of the rat muscle spindle is complete at 25 days after birth.[165]

As with the differing morphologic features between extrafusal and intrafusal muscle fibers, mature intrafusal fibers exhibit histochemical characteristics that are unique and do not correspond to the histochemical types of extrafusal fibers.[166-169] These histochemical subpopulations appear somewhat later than the differentiation of fiber types of extrafusal myofibers, with some overlap.

Golgi tendon organs are stretch receptors found between muscle and tendon and less frequently within the tendon itself. They also are distributed in the intermuscular connective tissue and in the capsules of joints. Tendon organs are almost as numerous as muscle spindles, although infrequently seen in muscle biopsy specimens.[170] Because tendon organs lie in series with the main mass of muscle, they are sensitive to any sudden change in muscle length, active or passive, or to a summation of active and passive stretch. Their afferent nerve fibers all belong to group Ib, with widespread central connections that allow for patterned and sequential engagement of synergistic and antagonistic muscles as integrated movements. Golgi tendon organs develop after the myotubular stage and are well formed by 28 weeks gestation. Numerous pacinian corpuscles similar to those of the dermis also occur near the myotendinous junction, mainly within the tendon (Figure 175-21), but they also occur in the perimysium between muscle fascicles at distal ends of muscles.

Unlike extrafusal myofibers that characteristically undergo atrophy and disappear after loss of their motor nerve supply, intrafusal spindle fibers continue to develop normally during the first 3 weeks after deefferentation of immature neonatal rat muscle, but only if their afferent sensory nerve supply and dorsal root ganglia are preserved (even if the spinal cord segment is extirpated). Intrafusal fibers then actually proliferate at 3 to 5 weeks after denervation with the supernumerary fibers forming from the fusion of myoblasts derived from activated intrafusal satellite cells and also from longitudinal splitting of preexisting intrafusal myofibers.[170-174] New intrafusal fibers originate from both nuclear bag and nuclear chain fibers but mainly from the latter.[171] The ratio of myosin heavy chain isoforms is altered under these circumstances.[167,174] The total efferent and afferent denervation of muscle spindles, by contrast, results in atrophy and numeric reduction of intrafusal myofibers.[174,175] In sum, intrafusal muscle fibers are much more dependent on sensory than on motor nerve supply to preserve their integrity, and they proliferate as a delayed response to deefferentation, rather than undergo atrophy.[166]

TENDON DEVELOPMENT

Tendons are dense fibrous bands that attach striated muscles to bones, as either the proximal origin or the distal insertion of the muscle. In *traction tendons*, in which the line of action corresponds to that of the muscle, few blood vessels are uniformly distributed within the tendon tissue. In *gliding tendons*, which change their direction of pull, an avascular zone normally forms in the region where the tendon wraps around the pulley.[176] The angiogenic peptide vascular endothelial growth factor is present in both endothelial cells and tenocytes (a fibroblast derivative) of human fetal tendons exposed to traction, but not in the avascular zone of gliding tendons that are predominantly exposed to compressive and shearing forces. The avascular zone of tendons might be caused by a mechanically induced down-regulation of vascular endothelial growth factor expression.[177] Tendons begin forming at the time of the differentiation of the fascicular organization of muscle. The transitional zone is a mixture of perimysial fibrous bands and myofibers that may show cytoarchitectural differences from the same fibers in the main belly of the muscle and hence can easily be misinterpreted as myopathic findings in muscle biopsy samples taken too near the insertion, as during "heel cord" (Achilles tendon)–lengthening procedures.

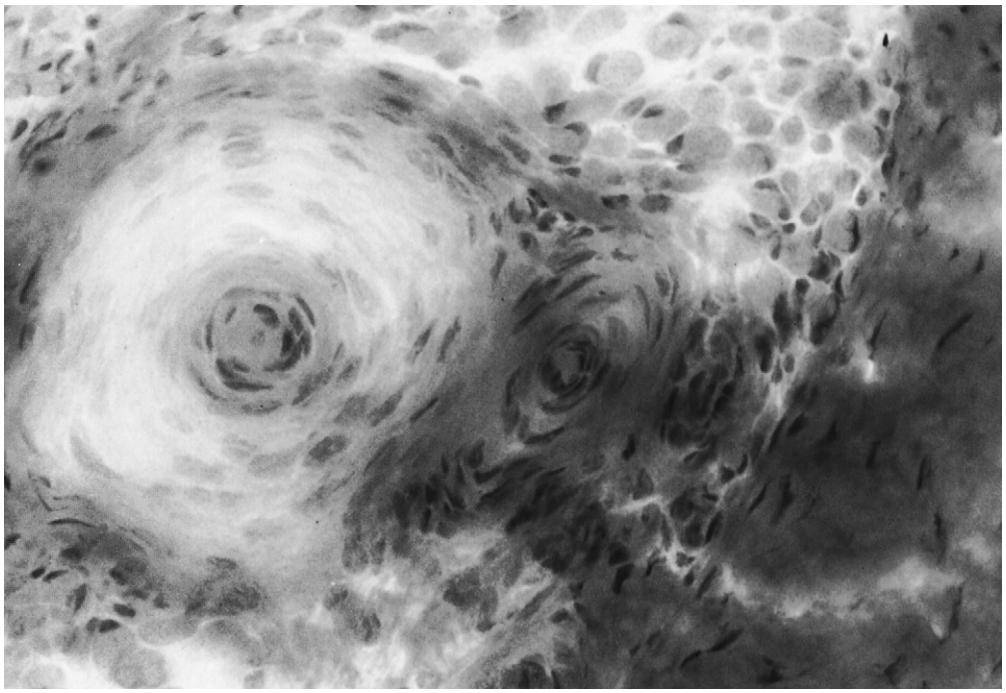


Figure 175-21 Pacinian corpuscle near myotendinous insertion of the quadriceps femoris muscle in a 36-week premature infant. The fibrous epimysial muscle capsule is seen on the *right.* (Gomori trichrome, original magnification ×1000.)

MEMBRANE EXCITABILITY AND INNERVATION OF FETAL MUSCLE

The resting membrane potential of mature muscle, approximately 90 mV, is actively maintained by continuous energy production derived from Na^+,K^+-ATPase (calcium-mediated ATPase and creatine kinase are the energy sources for the contractile mechanism of myofibrils). This energy-generating system for maintaining membrane excitability develops early in myogenesis; even early myotubes already have a continuously polarized membrane. Spontaneous subthreshold potentials may be recorded anywhere along the sarcolemmal membrane, but their site of origin is in the middle of the fiber at the point at which end plates are eventually formed.[177] They resemble the miniature end-plate potentials of adult fibers but occur at a lower frequency and with a slower time course. The plasma membrane of myoblasts also is excitable, although it may easily fatigue. The compound that most strongly and consistently provokes membrane depolarization is acetylcholine (ACh), the transmitter secreted at the neuromuscular junction of mature striated muscle in humans, all vertebrates, and some invertebrates.

ACh receptors are among the first membrane proteins to appear during the development of striated muscle. In the larval amphibian *Xenopus*, functional ACh receptors begin to form within 2 hours after the myotomes begin to appear, which is much earlier than nerve contacts muscle.[178,179] In the rat, peripheral nerves and functional ACh receptors both appear when developing muscle cells are first identified,[180] but functional innervation is not required for the development of ACh receptors, and myoblasts isolated in tissue culture continue to develop them.[181] The expression of ACh receptors in living rat embryos is unaltered after destruction of their motor neurons.[182]

Although innervation is not essential for the initial formation of functional ACh receptors, innervation of muscle leads to a major redistribution of receptors, eventually resulting in their aggregation at the points of nerve contact.[179,182,183] After the concentration of receptors at the synapse point, a decrease occurs in the density of receptors in portions of the sarcolemmal membrane not directly contacted by nerve endings.[179,184,185] The decreased density of ACh receptors is accompanied by a low sensitivity of nonsynaptic membrane to ACh, a characteristic of adult muscle fibers. Aggregates of synaptic junction receptors remain after denervation; removal of the nerve results in a return of high levels of extrajunctional receptors.[186,187]

Innervation of human striated muscle occurs in the middle of the myotubular stage of development, maximally at approximately 11 weeks gestation, but motor end plates begin to form as early as 9 weeks gestation and continue to develop and mature until 20 weeks gestation.[188] The primitive motor end plate contains a few axons always covered by one Schwann cell. It does not change either in number or in structure between 10 and 20 weeks gestation, although continuous modification of the postsynaptic membrane is observed throughout this period.[189] The axon contains a few synaptic vesicles and is in close contact with the unfolded plasma membrane of the primary myotube. The first morphologic change indicative of end-plate formation is seen at the junction of the sarcolemma with the axolemma, the former having a distinct thickening in the small junctional zone. This thickening of the postsynaptic membrane may already be evident when the axon is 20 to 30 nm away from the muscle cell.[189] At the mature myotubular stage, at approximately the 14th week of gestation, synaptic gutters and primary synaptic clefts form. Secondary synaptic clefts are first seen in the 16th week of gestation. The characteristic convolutions of the mature postsynaptic membrane are thus well established by midgestation and are fully mature at birth.

Myotubes initially are innervated from several nerves. The mature single-nerve supply to each myofiber results from retraction of all but one nerve as development proceeds. Fidziańska found several synaptic axonal terminals grouped together and filled with synaptic vesicles at the motor end plate of human fetuses of 10 to 20 weeks gestation; these axonal terminals did not seem to change in either ultrastructural appearance or number during this period and were always covered by a single

Schwann cell.[189] Silver impregnations of nerve fibers also show a single motor end plate on each fetal myofiber supplied by more than one axon.[190] It may be concluded from these data that each developing myofiber has just one end plate but that this end plate is supplied by two or more axons. This conclusion is consistent with other electrophysiologic and morphologic data indicating polyneural innervation of fetal muscle fibers. Scanning electron microscopy of nerve-muscle cultures also confirms that a single embryonic muscle fiber may be contacted by as many as five different nerves arising from different motor neurons.[191] Physiologic cell death of a spinal neuroblast is unrelated to the change from polyneuronal to mononeuronal innervation of muscle,[192] but functional neuromuscular innervation is probably one factor that regulates the natural process of cell death among embryonic motor neurons of the spinal cord, because the number of motor neurons undergoing natural cell death is closely related to muscular activity.[193]

"Myotube satellite cells" with long cytoplasmic processes extending beyond the basal lamina into the endomysial space are seen in developing extraocular muscles. They contact free cells of similar appearance, as well as axon-surrounding Schwann cells, often forming an extensive network. These myotube satellite cells resemble Schwann cells in all respects except for the lack of an associated axon, and they may be Schwann cells disposed to promote axonal growth toward differentiating myofibers not yet innervated.[194]

Spinal motor nerve roots begin their myelination cycle at approximately 16 weeks gestation, and roots and peripheral nerves are well myelinated by late gestation.[195] In the phrenic nerve, one and a half turns of myelin are seen around nerve fibers at 15 weeks gestation[196]; the vagal nerve begins its myelination cycle at approximately 24 weeks gestation in humans[197] and at a corresponding age in fetal lambs.[198] Myelination of axons innervating extraocular muscles is reported by some investigators as early as 18 weeks gestation.[199]

SUPRASEGMENTAL NEURAL INFLUENCES ON MUSCLE MATURATION

The importance of innervation to muscle fiber maturation beyond the myotubular stage is well demonstrated both in vivo and in vitro. Neonatal neurectomy in rats prevents further development of the myocyte.[200] In humans, the effects of perinatal denervation on the morphologic features of muscle are well demonstrated in progressive spinal muscular atrophy, a degenerative motor neuron disease beginning in fetal life and continuing after birth.[201]

Suprasegmental centers in the cerebrum, cerebellum, and brain stem may alter the discharge pattern of motor neurons and change the course of muscle maturation. Classic studies document that excessive nerve stimulation experimentally causes a conversion of type II fibers to type I in the rat,[202,203] and abnormal suprasegmental stimulation of lower motor neurons in the human fetus may have a similar influence on histochemical differentiation.

Histochemical abnormalities in the muscle biopsy specimens from infants with cerebral malformations have been noted by several researchers.[204-207] Similar aberrations in the sizes and ratios of histochemical fiber types occur in the muscles of neonates with congenital contractures after fetal immobilization (i.e., arthrogryposis multiplex congenita).[207] Prospective study of the muscles of infants with various types of cerebral dysgenesis documented by imaging procedures or by postmortem examination demonstrates that three categories of disorders in muscle maturation may be identified: (1) delayed maturation in term infants, the muscle being morphologically normal but incompletely differentiated histochemically; (2) fiber-type predominance of more than 80% of either type I or type II fibers; and (3) the classic "congenital muscle fiber-type disproportion," defined as a predominance and uniform hypoplasia of type I fibers, often with secondary hypertrophy of type II fibers.[208,209]

An equally dramatic finding is the presence of normal and histochemically mature neonatal muscle in infants with normal spinal cords but severely hypoplastic brains, or with such severe dysplasias that few if any descending pathways exist to relay suprasegmental impulses.[208] This finding in the human is supported by observations in the stage 13 or 14 (day 2) chick embryo that extirpation of several cervical segments of spinal cord does not alter the development of motor end-plate distribution or histochemical differentiation by ATPase of muscles innervated by more caudal segments.[210] The relatively isolated human spinal cord with normal muscle maturation demonstrates that the motor unit is capable of maturing normally without cerebral influence, and the aberrant patterns found in infants with cerebral dysgenesis suggest that abnormal descending upper motor neuron influences may alter the course of muscle ontogenesis, probably by interfering with the discharge pattern of the lower motor neuron.

The most common malformation of the brain associated with abnormal muscle development is cerebellar hypoplasia as an isolated anomaly or in conjunction with cerebral dysplasias.[208,209] Abnormal development of brain stem structures is also associated with aberrant muscle maturation, but congenital lesions confined to suprasegmental structures, even when severe, do not seem to affect muscle development. Descending brain stem pathways become myelinated during the period of histochemical differentiation of muscle, between 20 and 30 weeks gestation,[195] and are functional during this period. The corticospinal tract, by contrast, is unmyelinated until much later, after muscle maturation is complete, and probably subserves several functions in the term neonate.[211,212] It is therefore likely that suprasegmental influences on muscle maturation are mediated more by the numerous small bulbospinal (i.e., "subcorticospinal") pathways, such as the tectospinal, rubrospinal, olivospinal, vestibulospinal, and reticulospinal tracts, than by the much larger but late-myelinating corticospinal tract.

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SECTION XXIV

Endocrine Function

176

Adda Grimberg and Jessica Katz Kutikov

Hypothalamus: Neuroendometabolic Center

Enabled by its strategic anatomy, the hypothalamus uniquely serves the interface between the neural and endocrine systems. It is at the same time a part of the brain, an intrinsic component of neural pathways, and an endocrine gland, specially connected to the pituitary gland to form the "master gland" unit of the body. The multiple functions of the hypothalamus ultimately involve regulation of the body's energy status and the maintenance of *homeostasis,* or bodily equilibrium. It has been long understood that, to refine this regulation, the hypothalamus responds to information from the brain and to the levels of the peripheral hormones and body fluids it is controlling. An appreciation is growing that the hypothalamus also receives input from the gut and fat stores, in essence closing the loop of metabolic regulation. All these incoming factors are compared with intrinsic set points, and outgoing messages are then released to enact modifications that will match the body to the appropriate set point. We have reviewed pediatric disorders of the neuroendocrine system, both congenital and acquired, elsewhere[1]; this chapter focuses on normal anatomy, embryology, and physiology of the hypothalamus.

ANATOMY

Despite the importance of the hypothalamus for multiple homeostatic functions, the structure is less than 1% of human brain volume; its various parts total approximately 4 g of the 1200 to 1400 g of an adult human brain.[2] Many of the borders of the hypothalamus are difficult to distinguish and are semiarbitrary. The hypothalamus lies at the base of the third ventricle, immediately posterior to the optic chiasm.[2-4]

Because of its many eminences, the hypothalamus is an irregular structure that roughly forms a diamond. It is composed of four main structures[2]: the tuber cinereum, the median eminence, the infundibulum, and the mamillary bodies. The tuber cinereum lies centrally on the inferior aspect of the hypothalamus. The median eminence, a central swelling located on the tuber cinereum, forms the floor of the third ventricle. The infundibulum is a stalk that connects the median eminence to the posterior lobe of the pituitary, and the mamillary bodies are two round protuberances at the posterior end of the inferior surface of the hypothalamus.[4]

The hypothalamus can be difficult to describe because of its lack of landmarks. Of the many systems devised to divide the hypothalamus into discrete areas, two are particularly helpful. One describes nine zones, determined by their mediolateral and anteroposterior locations[2,3]; it is shown in Figure 176-1. The second system defines discrete clusters of cell bodies (nuclei) that have characteristic anatomic positions and functions (Table 176-1). Hypothalamic nuclei are not well circumscribed in adult brains. Examination of developing brains, in which cell groups are more discrete, has led to a greater understanding of hypothalamic architecture. Nonetheless, this schema is also semiarbitrary and is divided in many ways, depending on the source.

An area of particular controversy involves reports of sexual structural differences in several hypothalamic nuclei.[5] In both rats and human adults, the sexually dimorphic nucleus of the preoptic area is larger in males than in females.[6,7] Another preoptic cell group was found to be larger in men.[8-10] The bed nucleus of the stria terminalis is male predominant,[5,11] whereas the massa intermedia (interthalamic adhesion) is female predominant.[12] Age-related changes in neuronal health and sex hormone activity can affect these differences. For example, because the cell loss of human senescence in the sexually dimorphic nucleus of the preoptic area follows different time courses in men and women, the magnitude of the structural difference is age dependent.[5,13] Sexual functional differences have also been shown in the hypothalamus by immunocytochemistry staining for various neuronal products (e.g., somatostatin and vasopressin) and hormone receptors (e.g., androgen and estrogen receptors).[5] Speculation and controversy surround the implications for sexually dimorphic behaviors and psychological phenomena. Most relevant to neonatologists is the debate about imprinting of the brain by androgens in utero and its consequences for therapeutic outcomes in patients with various intersex conditions including ambiguous genitalia.[14]

A final anatomic feature that is integral to hypothalamic functioning is its blood supply. The hypothalamus communicates with the anterior pituitary gland through a special portal circulation that not only is fenestrated (the exception to the blood-brain barrier) but also transmits information bidirectionally. This feature enhances the ability of the hypothalamus to receive both signals from the general circulation and feedback from the pituitary. Furthermore, the hypothalamohypophysial portal system ensures that high concentrations of hypothalamic factors reach the pituitary, often at concentrations that far surpass those in the general circulation.[15]

EMBRYOLOGY

Early in nervous system development, three primary brain vesicles form from the neural tube: the prosencephalon or forebrain, the mesencephalon or midbrain, and the rhombencephalon or hindbrain.[16] Secondary vesicles then form from the prosencephalon during the fourth to fifth weeks of gestation. The secondary vesicles are termed the telencephalon, or endbrain, and the diencephalon. The telencephalon grows to cover all other brain structures and eventually becomes the cerebral hemispheres. The diencephalon also grows, forming a roof plate and two alar plates. During the sixth embryonic week, neuroblasts in the inferior portion of the alar plates of the diencephalon proliferate, forming the human hypothalamus.[16,17] Hypothalamic development occurs in two main stages: forebrain induction by early signals before or during gastrulation and then cell type differentiation by ventralizing and rostralizing signals from the axial mesendoderm. Axial mesendoderm induction of hypothalamic

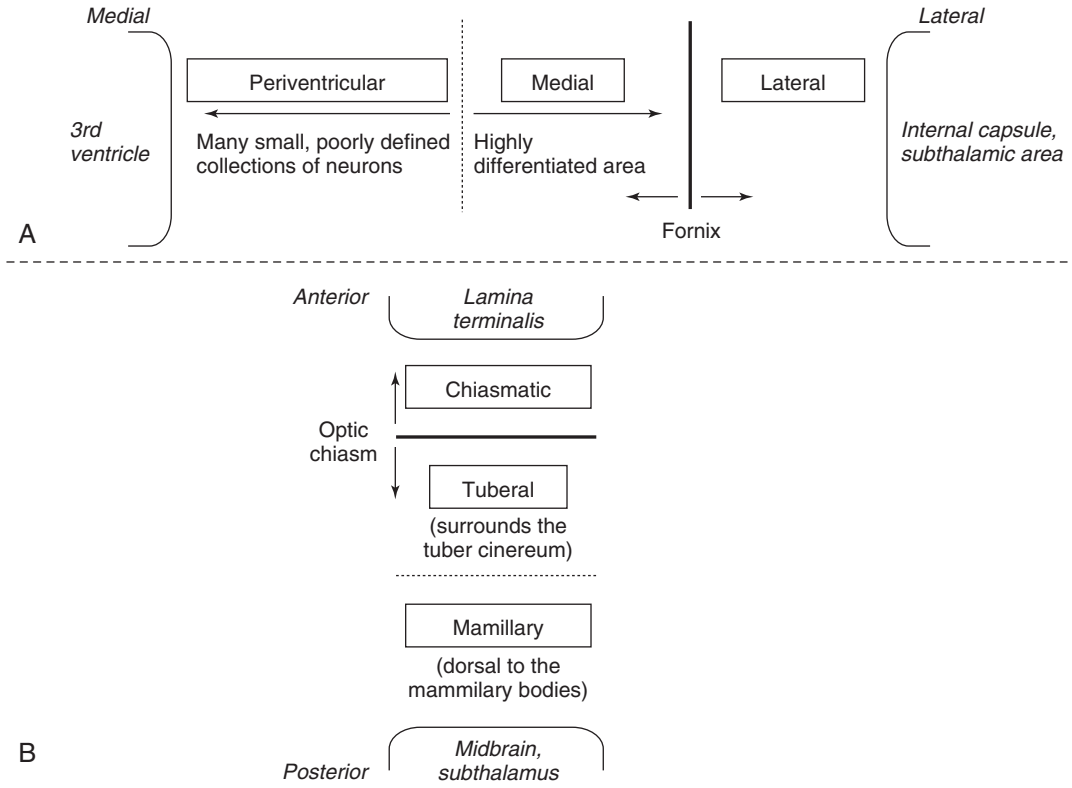


Figure 176-1 Nine anatomic areas of the hypothalamus. Three mediolateral **(A)** and three anteroposterior zones **(B)** form a grid that divides the hypothalamus into nine anatomic areas. Defining features and boundaries of each zone are shown.The chiasmatic region can be further subdivided into preoptic (anterior to optic chiasm) and supraoptic (superior to optic chiasm) areas.

TABLE 176-1

Hypothalamic Nuclei

Anteroposterior region	Nuclei	Anatomy	Function
Chiasmatic	Preoptic area	Small nuclear groups, anterior to optic chiasm	Controls blood pressure, body temperature, hormonal release, reproductive activity
	Suprachiasmatic nucleus	Midline nucleus above optic chiasm	Sets circadian rhythm and sleep-wake cycles, regulates body temperature, hormonal control
	Paraventricular nucleus	Medial half = parvocellular neurons; lateral half = magnocellular neurons	Produces oxytocin and antidiuretic hormone (magnocellular neurons); regulates anterior pituitary function (parvocellular neurons); regulates autonomic responses
	Supraoptic nucleus	Superior to optic chiasm; also contains magnocellular neurons	Produces oxytocin and antidiuretic hormone
	Anterior nucleus	Between the paraventricular and supraoptic nuclei	Less differentiated; role ill-defined
Tuberal	Dorsomedial nucleus	Dorsal part of medial zone; projects locally and to periaqueductal gray matter	Feeding, growth, maturation, reproduction; parvocellular neurons regulate anterior pituitary function
	Ventromedial nucleus	Ventral part of medial zone; projects locally and to periaqueductal gray matter	Feeding, growth, maturation, reproduction; parvocellular neurons regulate anterior pituitary function
	Arcuate nucleus	Basal portion of tuber cinereum	Parvocellular neurons regulate anterior pituitary function; dopaminergic neurons
	Lateral hypothalamic nucleus	Large neurons lateral to ventromedial nucleus	Signals hunger
Mamillary	Posterior nucleus	Prominent nucleus, medial	Controls autonomic function
	Mamillary nuclei	Complex of three or four nuclei	Hippocampus → mamillary nuclei → thalamus; Papez circuit possibly involved in emotion and memory

development is necessary for eye separation, and its failure causes holoprosencephaly.[18]

Distinct cell groups are easier to distinguish in the fetal hypothalamus than in the adult hypothalamus. The fetal hypothalamic nuclei become recognizable between 6 and 12 weeks gestation. At this same time, the hypothalamic fiber tracts develop, and many hypothalamic factors become detectable.[19] For example, somatostatin, dopamine, and thyrotropin-releasing hormone are detectable as early as the 10th week of gestation.[17] At 9 to 10 weeks gestation, little differentiation of the hypothalamic nuclei has taken place. By 24 to 33 weeks, the hypothalamus contains an increased number of better-defined structures and more closely resembles an adult hypothalamus. By the immediate postnatal period, the neonatal hypothalamic cell groups are remarkably similar to those structures in a mature adult.[20]

Information about the embryology of the hypothalamohypophysial portal system is limited. Evidence exists that the primary plexus begins to develop as early as 11.5 weeks gestation.[17] Capillary penetrance of the median eminence is not evident until more than 16 weeks gestation.[21] Further development throughout gestation is indicated by the greater number of capillary loops and the more mature vasculature present at birth.[17]

Our understanding of hypothalamic embryology has progressed from the morphologic to the genetic level. Development of the hypothalamus and pituitary gland is coordinated through parallel actions of genes expressed in both organs; extrinsic signals activate repertoires of transcription factors in a well-defined spatiotemporal pattern that lead to cellular differentiation into the various cell types that assume the distinct functions of the mature organs.[18,22,23] Some of these genes are summarized in Table 176-2.[18,22-25]

NEUROLOGIC FUNCTIONS

Like other brain centers, the hypothalamus participates in neural pathways by synthesizing and secreting neurotransmitters, chemical messengers used for synaptic transmission, signaling, and neuromodulation. Different pathways control various functions of the hypothalamus, although for some functions, discrete areas or nuclei are not identifiable as the control centers. Some of the neurotransmitters are summarized in Table 176-3. Other neurotransmitters are also involved in hypothalamic functioning, such as the excitatory amino acids in a pathway connecting to the hippocampus and limbic system, but their actions are less well understood.

Circadian Rhythms

The suprachiasmatic nucleus sets the clock for the body's circadian rhythms, adaptations to the reproducible 24-hour day-night cycle of life on earth.[26] Neurons of the suprachiasmatic nucleus oscillate in a synchronized fashion in vitro with a period close to 24 hours.[27] In vivo, these oscillations become entrained to the solar day-night cycle by light; signals from the retina are transmitted by the retinohypothalamic tract to the suprachiasmatic nucleus. In the suprachiasmatic nucleus, these signals affect gene expression for at least four transcription factors that act in a transcription-transduction feedback loop to determine the oscillations.[26,27] Suprachiasmatic neurons contain somatostatin, vasoactive intestinal polypeptide, vasopressin or antidiuretic hormone (ADH), and neurotensin. Efferent pathways, including one that controls pineal function, then regulate various endocrine, cardiovascular, temperature, and behavioral circadian rhythms.[2,26,28]

In humans, the suprachiasmatic nucleus is recognized by midgestation and the retinohypothalamic tract by 80% gestation.[26,29] Fetuses show 24-hour rhythms in hormones, cardiovascular function, and behavior. Rather than being synchronized with the time of day, their rhythms are set by maternal signals, including the maternal cortisol rhythm.[26] Although the circadian system is responsive to light at premature stages in human and other primate infants, the system progressively matures after birth to achieve full entrainment after 2 months of age.[30]

The daily sleep-wakefulness cycle is the most obvious behavioral manifestation of the suprachiasmatic nucleus's activity and circadian rhythms. Human evolution as hunter-gatherers has led to the coordination of sleep with metabolic homeostatic mechanisms; hunger, vigilance, and food-seeking behaviors occur primarily during daylight hours whereas satiety and somnolence occur together for periodic rest.[31,32] Hypocretin (also called orexin) neurons in the lateral hypothalamus respond to metabolic signals of the body's nutritional status and to dark-light cues from the suprachiasmatic nucleus pacemaker to stimulate both alertness and appetite and feeding behaviors. Hypocretin deficiency, in both humans and animal models, causes narcolepsy.[33] Disturbances of the circadian cycle and chronic sleep deprivation have been implicated in hyperphagia and obesity, mainly via the hypocretin system,[31] and, with further contributions from unbalanced autonomic nervous system activity,[34] in the metabolic syndrome (visceral obesity, type 2 diabetes mellitus, dyslipidemia, and hypertension).

Autonomic Nervous System Function

A topographic arrangement of autonomic function exists in the hypothalamus; parasympathetic control stems mainly from anterior and medial regions, whereas the lateral and posterior areas control sympathetic responses. Parasympathetic activity results in slowing of the heart rate, peripheral vasodilation, and increased gastrointestinal motility. Alternatively, sympathetic stimulation results in pupillary dilation, increased heart rate and blood pressure, and other responses associated with increased emotional stress.[2]

Heart rate, heart rate variability, and other cardiac measures are often used as markers when assessing fetal and neonatal autonomic function.[35,36] Fetuses at 20 weeks of gestational age demonstrate heart rate stability and differences among individuals. Fetal heart rate, beginning at 24 weeks of gestation, may be predictive of postnatal heart rate, but fetal heart rate variability does not become predictive of postnatal heart rate variability until 6 weeks later. This finding likely reflects a later maturation of the neuroregulatory system that controls heart rate variability.[35] The reduction in heart rate variability in fetuses with increasing gestational age also suggests that the autonomic nervous system continues to develop throughout gestation.[36]

Autonomic activity in fetuses varies over the course of a day. For instance, in normal fetuses, the fetal autonomic activity that controls heart rate follows a 12-hour cycle, as opposed to the maternal 24-hour rhythm. This 12-hour rhythm disappears immediately after birth, although the 24-hour circadian rhythm does not become established until 2 to 4 weeks later.[37]

Temperature Regulation

Like autonomic control, temperature regulation occurs regionally, rather than in discrete nuclei. The preoptic anterior hypothalamus contains thermosensitive neurons that detect increases in blood temperature and cool the body by heat dissipation, whereas the posterior hypothalamus conserves heat when decreased body temperature is detected.[2,38] The temperature control centers of the hypothalamus coordinate behavioral, autonomic, and endocrine responses of the body to regulate body temperature, including panting and peripheral vasodilation in response to increased body temperature, shivering and peripheral vasoconstriction in response to decreased body temperature,[39] and modulation of thyroid hormone–stimulated thermogenesis.[40,41] Hypothalamic injury can produce prolonged hypothermia resulting from

TABLE 176-2

Genes in Hypothalamic Development

Gene	Normal function	Consequences of mutation or deletion
Rpx/Hesx1	Expressed during gastrulation in the midline endoderm-mesoderm (prechordal plate precursor), to induce anterior head structures Afterward, expression is restricted to Rathke's pouch Ventral → dorsal down-regulation of expression coincides with pituitary cell differentiation	Deletions: Absence of infundibulum and Rathke's pouch (most severe); hypothalamic floor expansion and abnormal bifurcation of Rathke's pouch Homozygous missense mutations (R53C) cause septo-optic dysplasia, which is frequently associated with hypothalamic or pituitary dysfunction
Shh (sonic hedgehog)	Expressed by oral ectoderm Induces its own expression and that of other genes (e.g., *Nkx2.1*) in ventral midline neural cells Suppresses *Pax6* expression in the midline Required for resolution of the single retinal field into two separate primordia	Holoprosencephaly Hypothalamic or pituitary absence or dysfunction
Six3	Transcription factor Expressed in Rathke's pouch and hypothalamus Involved in early induction of the pituitary gland	Holoprosencephaly Hypothalamic or pituitary absence or dysfunction
Gli3	Represses *Shh* signaling	Pallister-Hall syndrome (hypothalamic hamartomas)
Nkx2.1/Tebp/TTF-1	Dorsoventral gradient of expression (higher in ventral region and posterior pituitary; none in Rathke's pouch) Required for development of Rathke's pouch	Absence of entire pituitary gland Absence of selective hypothalamic nuclei (premamillary nucleus, arcuate nucleus, mamillary body, supramamillary nucleus)
Brn-2	Coexpressed with *Brn-4* in presumptive paraventricular and supraoptic nuclei Required for terminal differentiation and survival of magnocellular and parvocellular neurons in these nuclei	Magnocellular and parvocellular neurons fail to project axons into the posterior pituitary Complete loss of posterior pituitary
Sim1	Expressed in paraventricular and supraoptic nuclei	Paraventricular and supraoptic nuclei fail to express *Brn-2,* oxytocin, antidiuretic hormone, thyrotropin-releasing hormone, corticotropin-releasing hormone, somatostatin Homozygous deletion: perinatal lethal from complete development defect of paraventricular nucleus Haploinsufficiency: isolated hyperphagia, early onset obesity, increased linear growth, no decrease in energy expenditure
Nhlh2	Expressed in developing hypothalamus Expressed in both embryonic and adult pituitary	Hypogonadism and progressive adult obesity Reduction of pro-opiomelanocortin–producing cells in the arcuate nucleus (increased leptin resistance)
SF-1/Ftz-F1	Expressed in developing adrenal glands, gonads, and diencephalon Regulates both androgens and müllerian inhibiting factor (i.e., male differentiation)	Absence of adrenal glands Absence of gonads Absence of pituitary gonadotropes (no luteinizing hormone, follicle-stimulating hormone, and gonadotropin-releasing hormone)
Gsh-1	Binds to growth hormone–releasing hormone promoter	Hypoplastic pituitary gland Dwarfism; growth hormone deficiency (from absence of growth hormone–releasing hormone in arcuate nucleus) Sexual infantilism and infertility
Necdin	Paternal monoallelic expression in developing hypothalamus	Decreased number of oxytocin-producing cells in paraventricular nucleus Decreased number of gonadotropin-releasing hormone–producing cells in preoptic region Mutant mice show neonatal lethality with reduced penetrance Maps to the critical region of Prader-Willi syndrome
Peg3	Paternal monoallelic expression in developing hypothalamus	Decreased number of oxytocin-producing cells in paraventricular nucleus Abnormal maternal behavior leading to perinatal lethality of offspring mice
Sox2	Expressed in the developing central nervous system and placodes	Heterozygous mutations in humans: bilateral eye defects, anterior pituitary hypoplasia and hypogonadotropic hypogonadism, variable defects affecting the corpus callosum and mesial temporal structures, hypothalamic hamartoma, sensorineural hearing loss, esophageal atresia
Sox3	Expressed in infundibulum	X-linked hypopituitarism, infundibular hypoplasia, variable learning difficulties

Data from Kioussi C, Carrière C, Rosenfeld MG: A model for the development of the hypothalamic-pituitary axis: transcribing the hypophysis, Mech Dev 81:23, 1999; Michaud JL: The developmental program of the hypothalamus and its disorders, Clin Genet 60:255, 2001; Kelberman D, Rizzoti K, Avilion A, et al: Mutations within Sox2/SOX2 are associated with abnormalities in the hypothalamo-pituitary-gonadal axis in mice and humans, J Clin Invest 116:2442, 2006; and Woods KS, Cundall M, Turton J, et al: Over- and underdosage of SOX3 is associated with infundibular hypoplasia and hypopituitarism, Am J Hum Genet 76:833, 2005.

TABLE 176-3

Hypothalamic Neurotransmitters

Category	Neurotransmitter	Pathway	Function
Monoamines			
Catecholamines	Dopamine	Incertohypothalamic tract Mesencephalic tract Tuberoinfundibular tract Median eminence	Influences secretion of pituitary hormones Primary inhibitor of PRL L-DOPA stimulates GH release
	Norepinephrine	Lateral tegmental system Dorsal tegmental system Can be localized to all hypothalamic nuclei; highest concentrations in the arcuate nucleus and median eminence	Regulates emotional and motivated behavior Stimulates GH and gonadotropin release Inhibits secretion of oxytocin, vasopressin, and ACTH
	Epinephrine	Cell bodies near the locus ceruleus	Inhibits secretion of vasopressin and oxytocin
Indolamines	Serotonin	Most of the hypothalamic nuclei	Involved in cyclic release of some hypothalamic hormones
		Highest concentrations in the arcuate and suprachiasmatic nucleus	Stimulates GH and PRL release
	Histamine	Tuberomamillary nucleus in the posterior lateral hypothalamus	May help maintain arousal Stimulates oxytocin, vasopressin, and GH release
Cholinergic	Acetylcholine	Large amount present in median eminence	Stimulates secretion of vasopressin, oxytocin, ACTH, and gonadotropin release Inhibits somatostatin; stimulates GH release Inhibits TSH release
Amino acids (inhibitory)	γ-Aminobutyric acid	Synthesized from glutamate Basal ganglial system	In anterior hypothalamus, helps induce sleep Inhibits oxytocin, vasopressin, ACTH, PRL, and TSH release Stimulates GH and gonadotropin release
Peptides	Angiotensin II	Renin (from kidneys) cleaves angiotensinogen (made by liver) to angiotensin I; angiotensin I cleaved by angiotensin-converting enzyme in lungs in angiotensin II	Stimulates secretion of vasopressin Stimulates ACTH and luteinizing hormone and inhibits PRL and GH release Induces thirst, drinking behavior Involved in regulation of water balance
	Atrial natriuretic peptide	Left atrium	Opposes vasopressin secretion
		Anterior tip of third ventricle Many neuronal endings in the median eminence	Involved in regulation of water balance
	β-Endorphins	Arcuate nucleus and basal tuberal area in the rat Project through the anterior hypothalamic area and innervate the median eminence	Released during stress Stimulate ACTH, PRL, and GH release

ACTH, Corticotropin; *GH,* growth hormone; *L-DOPA,* levo-3,4-dihydroxyphenylalanine; *PRL,* prolactin; *TSH,* thyroid-stimulating hormone.

alterations in the temperature set point or defects in heat production mechanisms. Fever results from an elevation of the temperature set point by inflammatory cytokines, such as interleukin-1, that raise prostaglandin E levels in the hypothalamus; normal physiologic mechanisms then maintain body temperature at this elevated set point.[38]

The fetal environment is stable at approximately 37° C. The temperature gradient, wherein fetal temperature is approximately 0.5° to 1.0° C higher than maternal temperature, results in fetal heat dissipation. Heat loss occurs through the placenta, fetal skin, amniotic fluid, and uterine wall. The hypothalamus most likely controls this higher fetal temperature in utero. After birth, the neonate has tremendous heat losses through the skin that require functioning thermoregulatory mechanisms to prevent hypothermia. Premature infants are often unable to maintain appropriate body temperature and behave like poikilotherms rather than homeotherms. How the hypothalamus matures to the environmental conditions after birth is still unknown.[42]

Emotional Expression and Behavior

Selective stimulation or lesions of various areas in the hypothalamus elicit different behavioral and emotional responses such as aggression, anger, fear, pleasure, and sexual gratification. Although the hypothalamus is important for integrating and expressing emotion, other areas of the brain, including the cortex, limbic system, and thalamus, interact with the hypothalamus to bring about the varied types of responses.[2,43,44]

The hypothalamus participates in the two physiologic stress response systems, the sympathetic-adrenal-medullary system and the hypothalamic-pituitary-adrenal system. The sympathetic-adrenal-medullary system causes the well-known stress-induced release of epinephrine. Sympathetic-adrenal-medullary activation, which is dependent on characteristics of both the situation and the individual, occurs in response to challenges with a faster onset than activation of the hypothalamic-pituitary-adrenal axis.[45] Hypothalamic-pituitary-adrenal axis function culminates in cortisol release, which is described in detail in Chapters 180 and 181. Stress induction of the hypothalamic-pituitary-adrenal axis is active in newborns. When neonates are exposed to noxious stimuli (e.g., circumcision, heel stick blood sampling), cortisol levels rise and can be associated with how much the infant cries. Once the noxious stimulus is removed, healthy neonates quickly return to basal cortisol concentrations. Soothing procedures such as the use of a pacifier can reduce crying in response to noxious stimuli, but they do not affect the hypothalamic-pituitary-adrenal response to the stimuli.[46]

ENDOCRINE FUNCTIONS

The hypothalamus also serves as an endocrine organ, synthesizing and releasing hormones into the circulation to effect changes in target tissues. Hypothalamic endocrine activity can be divided

into two distinct categories as established by the anatomy of the hypothalamopituitary unit. Because the posterior pituitary gland, or neurohypophysis, is composed of axonal projections from the hypothalamus, the hormones released into the circulation by the posterior pituitary are actually made in cell bodies of the hypothalamus. In contrast, the anterior pituitary gland, or adenohypophysis, is a distinct gland. The hypothalamus secretes various releasing and inhibiting factors that regulate anterior pituitary hormone synthesis and release. These factors are made in hypothalamic neurons and are carried down axons to the median eminence. They then reach the anterior pituitary by the special hypothalamohypophysial portal circulation, whereas the anterior pituitary hormones reach their target organs by the peripheral circulation.[28]

Hypothalamic hormone production and release are primarily regulated by humoral feedback. Three levels of feedback regulation to the hypothalamus exist. *First-order feedback* involves one hormone, as exemplified by oxytocin and ADH. These hormones act on target cells (breast, uterus, kidney) but do not stimulate the release of secondary hormones. Oxytocin and ADH levels, together with nonhormonal signals (e.g., plasma osmolality and hypovolemia or hypotension for ADH), directly regulate their own production and release. Close proximity of oxytocin and ADH neurons and co-localization of receptors for the hormones on the neurons that produce those hormones allow local concentrations of oxytocin and ADH within the supraoptic and paraventricular nuclei to refine further activity of the neurons that produce them.[47-49] *Second-order feedback* involves a second hormone produced by the pituitary, such as prolactin (PRL). Hypothalamic hormones stimulate or inhibit anterior pituitary release of a hormone, and the circulating levels of the pituitary hormone feed back to regulate hypothalamic hormone release. For example, increases in circulating PRL concentrations stimulate hypothalamic secretion of dopamine (PRL-inhibiting factor) into the hypothalamohypophysial portal circulation to prevent excessive PRL release by the pituitary; high PRL levels also inhibit hypothalamic release of vasoactive intestinal polypeptide, a hormone that stimulates PRL release.[50] In a *third-order feedback* loop, a third endocrine gland (e.g., adrenals, gonads, thyroid) is involved in addition to the hypothalamus and pituitary. Within a third-order feedback pathway, multiple types of feedback loops exist: long, short, and ultrashort feedback. Long-loop feedback occurs when hormones from peripheral endocrine target cells or substances from tissue metabolism influence the production of hypothalamic hormones. This type of feedback is most often negative, although it can be positive. Short-loop feedback is second-order feedback within a third-order feedback system. For instance, certain pituitary hormones (e.g., corticotropin, luteinizing hormone, thyroid-stimulating hormone) not only stimulate target gland hormone production, but also feed back to limit or terminate hypothalamic releasing hormone production (corticotropin-releasing hormone, gonadotropin-releasing hormone, and thyrotropin-releasing hormone, respectively). When hypothalamic hormone release affects its own synthesis, ultrashort feedback has occurred. This is similar to first-order feedback within a third-order system. The mechanism of this ultrashort feedback is either through neurotransmission between two cells in the hypothalamus or through pituitary tanycyte release of the hormone into cerebrospinal fluid, which can regulate hypothalamic activity because the hypothalamus is located at the base of the third ventricle.[28] The growth hormone pathway (reviewed in detail elsewhere[51,52]) is an excellent example of a third-order system and is shown in Figure 176-2.

Two neurosecretory systems produce hypothalamic hormones (summarized in Table 176-4): magnocellular and parvocellular. The *magnocellular system* consists of specialized, large-celled (magnocellular) neurons, the hypothalamoneurohypophysial tract, and the neurohypophysis (posterior pituitary). Its principal products are ADH and oxytocin. Most of the magnocellular neurons are located in the supraoptic and paraventricular nuclei, but additional small nuclei also contain these neurons.[3] Apart from projections to neurohypophysis, some magnocellular neurons project to the median eminence and some ADH-containing fibers project to the choroid plexus in the lateral ventricles and brain stem regions.[15]

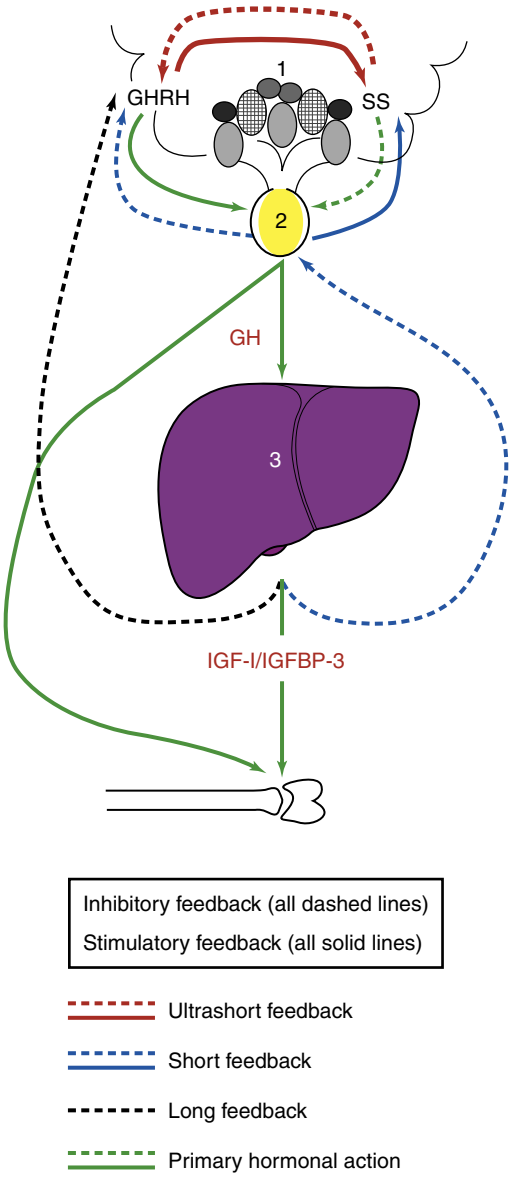


Figure 176-2 The growth hormone (GH) axis exemplifies a third-order feedback loop. A third-order feedback loop involves hormonal production by three endocrine glands: *(1)* the hypothalamus produces growth hormone–releasing hormone (GHRH) and somatostatin (ss); *(2)* the pituitary gland produces GH; and *(3)* the liver acts as an endocrine gland, producing insulin-like growth factor-I (IGF-I) and its binding protein (IGFBP-3). Stimulatory feedback is depicted as a *solid arrow,* inhibitory feedback as a *dashed arrow.* Ultrashort feedback is colored in *red,* short feedback in *blue,* and long feedback in *black.* Primary hormonal action is shown in *green.* Among the final end-organs of this system is the growth plate of growing bones.

TABLE 176-4

Hypothalamic Hormone Axes

Neurosecretory system	Hormone	Hormone gene locus	Hypothalamic nucleus of origin	Secondary pituitary hormone	Pituitary action	Final axis gland	Axis function
Magnocellular	ADH	20p13	Supraoptic and paraventricular	NA	V1b receptors: stimulate ACTH release	NA	Enhances renal free water retention; vasoconstriction
	Oxytocin	20p13	Supraoptic and paraventricular	NA	NA	NA	Stimulates uterine contractions at parturition; milk let-down reflex for lactation
Parvocellular	GHRH	20q11.2	Arcuate	GH	Stimulatory	Liver	Somatic growth
	ss	3q28	Paraventricular; also in neocortex, basal ganglia, limbic system; endocrine pancreas and gut (D cells)	GH, PRL, TSH, ACTH	Inhibitory	Liver	Regulation of pituitary function, especially somatic growth; neurotransmitter; gut function and pancreatic endocrine regulation (locally produced ss)
	CRH	8q13	Paraventricular	ACTH	Stimulatory	Adrenals	Maintenance of normal blood pressure and glucose; stress response
	TRH	3q13.3-q21	Paraventricular	TSH	Stimulatory	Thyroid	Regulates overall metabolic rate; effects on heart, bone, gut, and other organ functions
	GnRH	GnRH1 = 8p21-p11.2; GnRH2 = 20p13	Arcuate	LH, FSH	Stimulatory	Gonads	Reproduction
	Dopamine	—	Arcuate	PRL	Inhibitory	NA	Lactation

ACTH, Corticotropin; *ADH,* antidiuretic hormone or vasopressin; *CRH,* corticotropin-releasing hormone; *FSH,* follicle-stimulating hormone; *GH,* growth hormone; *GHRH,* growth hormone–releasing hormone; *GnRH,* gonadotropin-releasing hormone; *LH,* luteinizing hormone; *NA,* not applicable; *PRL,* prolactin; *ss,* somatostatin; *TRH,* thyrotropin-releasing hormone; *TSH,* thyroid-stimulating hormone (thyrotropin).

In contrast, the *parvocellular secretory cells* are small neurons, mostly in the region of the tuber cinereum below the third ventricle, that produce the hypophysiotropic hormones.[3] The parvocellular neurons are found in various hypothalamic nuclei, including the arcuate and paraventricular nuclei, and project to the median eminence.[28] Their hormonal products (all peptide hormones except dopamine) are carried by the hypothalamohypophysial portal system to the anterior pituitary, where they regulate adenohypophysial function. Because the hormonal systems of the anterior pituitary are reviewed in detail in other chapters of this book, we focus on the magnocellular system.

Adult numbers of oxytocin- and ADH-producing cells in the supraoptic and paraventricular nuclei exist as early as the second half of gestation, which is earlier than development of some other nuclei.[53] Oxytocin and ADH can be detected as early as the 10th gestational week. Vasopressinergic neurons appear 1 week earlier in the supraoptic than in the paraventricular nucleus. Although an adult organization of vasopressinergic neurons can be identified early in the second trimester, the number of those neurons seems to increase until the 26th week of gestation. The vasopressinergic system of neurons is also highly active in the perinatal period.[54]

The synthetic pathways of oxytocin and ADH are very similar.[55] Both are synthesized from protein precursors (prohormones) that are encoded by single-copy genes located near each other on chromosome 20. Both genes are composed of three exons; the entire mature hormone is encoded within the first exon. The remainder of the oxytocin gene encodes neurophysin I, whereas the longer ADH gene also encodes neurophysin II and the glycoprotein, copeptin.[56] The prohormones are transported down the axons and are stored in colloid-filled vesicles. Proteolytic enzymes in these neurosecretory granules cleave oxytocin and ADH from the neurophysins, which are presumed to primarily serve as their carriers during axonal transport. When the magnocellular cell bodies are properly stimulated, oxytocin, ADH, and the neurophysins are released from the axonal terminals by calcium-dependent exocytosis.[3] In addition to oxytocin and ADH, magnocellular secretory granules contain many other peptides but in much smaller amounts. These include dynorphin, enkephalins, galanin, and cholecystokinin, and, although they are secreted along with oxytocin and ADH, the role of many of these peptides is unknown.[57]

Oxytocin

Oxytocin secretion is prompted by mechanical vaginal distention and, in lactating women, by suckling and nipple stimulation; no recognized stimulus exists for its secretion in men. Cholinergic and α-adrenergic fibers carry the stimulatory inputs, whereas β-adrenergic fibers provide inhibition of oxytocin release. Oxytocin serves two physiologic functions: it aids in parturition by stimulating uterine contractions, and it facilitates nursing by stimulating contraction of myoepithelial cells in the lactating mammary gland (milk "let-down" reflex). In fact, nursing cannot occur without oxytocin, and maternal and fetal stress during labor and delivery can reduce oxytocin release, thereby impairing

lactation.[58] Oxytocin action at parturition is primed by two major changes. Oxytocin release, which occurs in brief secretory bursts, increases in both the fetus and the mother during labor.[59,60] Meanwhile, the increase in uterine oxytocin receptors at term makes the uterus particularly sensitive to the hormone.[59] Oxytocin secretion is suppressed by relaxin, an ovarian peptide that suppresses uterine contractions and relaxes pelvic connective tissue during parturition.[61] Therapeutically, oxytocin and its analogues are used to induce labor (or to augment labor in uterine dysfunction), to prevent postpartum uterine atony or hemorrhage, and to promote milk let-down in lactation.

Antidiuretic Hormone

The two names of this hormone, vasopressin and ADH, reflect its two main actions, which are mediated by two different classes of receptors. V1 receptors stimulate vasoconstriction, glycogenolysis, platelet aggregation, and vascular smooth muscle hypertrophy. A subclass of V1 receptors (V1b) is located in the anterior pituitary and stimulates corticotropin release. V2 receptors increase water permeability of the renal collecting ducts by inducing aquaphorins; this results in greater water retention and decreased urine volume. Neural input for ADH secretion can elicit newly synthesized hormone within 30 minutes, but it also exhibits fatigue with high frequencies or prolonged duration.[62] The main stimulus for the antidiuretic effects of ADH is an elevation in plasma osmolality, primarily the extracellular fluid concentration of sodium chloride, as detected by osmoreceptor cells within the hypothalamus. Baroreceptor (carotid sinuses, aortic arch, cardiac atria, and great veins) sensing of hypovolemia or hypotension leads to ADH levels superseding those required for maximal urine concentration and causes vasoconstriction.[15] In most cases, the two mechanisms act in concert; dehydration leads to both hypernatremia and hypovolemia. However, the two ADH responses may become dissociated in disease states involving intravascular volume depletion despite total body fluid overload and hyponatremia (e.g., congestive heart failure, cirrhosis, and nephrotic syndrome).[63] Deficiency of ADH hormone production and resistance to ADH action cause central and nephrogenic diabetes insipidus, respectively[64]; both disorders are characterized by polyuria, polydipsia, and a tendency to dehydration. In contrast, the syndrome of inappropriate ADH secretion is characterized by diminished urine output and free water intoxication.

During development, ADH aids in regulating the proliferation and morphogenesis of target cells in the brain, pituitary, kidney, and liver. Because the fetus contributes significantly to modulation of fluid fluxes among the maternal, fetal, and amniotic fluid compartments, fetal ADH action is an important component of adaptive responses to changes in the environment in utero.[65] The ADH system also plays an essential role in fetal adaptation to the stresses of labor.[54,65] In the perinatal period, ADH is integral in establishing body fluid homeostasis, by providing the brain, pituitary, heart, and adrenals with increased blood flow and aiding in regulation of the cardiovascular system, water reabsorption, gluconeogenesis, and glycogenolysis after delivery.[54]

METABOLIC REGULATION

The hypothalamus plays a crucial role in regulating the body's metabolic state by controlling both feeding behavior and energy expenditure rates. The regulation is so precise that, under reasonably steady-state conditions, deflections in the body weight and fat mass of mammals remain within 0.5% to 1%.[66] The importance of the hypothalamus to this system has long been appreciated because of the hyperphagia and obesity syndromes that result from hypothalamic lesions. The ventromedial nucleus of the hypothalamus has been identified as a satiety center, whereas the lateral hypothalamic area stimulates feeding.[2,19] More recent investigations have focused on the peripheral hormones that feed metabolic information back to the hypothalamus.

Leptin

The *OB* gene, located on chromosome 7q31.1 and encoding the hormone leptin, was cloned and sequenced in 1994.[67] Leptin is produced by white adipose tissue and is secreted into the circulation in proportion to the body's fat mass.[68,69] Circulating both freely and protein bound, leptin crosses the blood-brain barrier and binds to leptin receptors in the central nervous system. Many of these receptors are located in the hypothalamus and control pathways involved in the regulation of appetite and thermogenesis.[68] Thus leptin serves as the hypothalamic index of body fat mass.[69,70] Leptin has other neuroendocrine functions, including regulation of fertility and the secretion of growth hormone, corticotropin-releasing hormone, thyrotropin-releasing hormone, and PRL.[68,69]

The exact mechanism of leptin uptake into the central nervous system has yet to be elucidated, though it is inhibited by hypertriglyceridemia.[71] One possibility involves the neurons of the median eminence, which not only project outside the blood-brain barrier but also express leptin receptors. Once inside the central nervous system, leptin can bind to receptors present in many hypothalamic nuclei, especially the arcuate, dorsomedial, ventromedial, and ventral premamillary nuclei.[72] Leptin binding to its receptor signals the body's energy status to two opposing classes of neurons. Pro-opiomelanocortin (POMC) neurons (catabolic pathways) are stimulated by leptin, whereas neuropeptide Y (NPY) neurons (anabolic pathways) are inhibited.[70]

Leptin directly stimulates POMC neurons, which are located in the arcuate nucleus, as well as outside the hypothalamus in the nucleus of the tractus solitarius. POMC is a precursor to many polypeptides termed melanocortins, including α-melanocyte–stimulating hormone. When the hypothalamic melanocortin receptors MC3-R and MC4-R are stimulated by α-melanocyte–stimulating hormone, food intake is decreased and energy expenditure is increased.[66,70] Agouti gene–related peptide (AGRP) is an endogenous melanocortin system antagonist that is also expressed in the arcuate nucleus.[73]

AGRP messenger RNA is highly co-localized with messenger RNA of NPY,[74] the system opposing POMC. A member of the pancreatic polypeptide family, the anabolic, orexigenic hormone NPY is widely distributed in the human brain. NPY potently stimulates feeding, is capable of overriding the body's satiety and weight control mechanisms,[66] and reduces energy expenditure by decreasing nonshivering thermogenesis in brown adipose tissue.[66,68] Leptin inhibits NPY activity. NPY can inhibit POMC neurons directly at the cell body or postsynaptically through the release of AGRP.[66] Thus leptin stimulates POMC neurons both directly and indirectly, by eliminating the POMC inhibition by NPY,[70] and the two opposing systems are coordinated toward an overall negative effect on energy balance. Many other substances have been implicated in energy homeostasis, but not all are understood as well as the POMC-NPY systems. Those also under leptin regulation include orexins,[66] cocaine- and amphetamine-regulated transcript, glucagon-like peptide-1, melanin-concentrating hormone, and cholecystokinin.[68,72]

Leptin is secreted in a rhythmic pattern that correlates with meals (leptin levels are reduced by fasting). Leptin's actions on the hypothalamus probably serve a long-term regulatory system that sets a point of satiety; short-term regulatory systems that control single meal intake are more likely determined by leptin actions on the brain stem. Obesity more frequently results from leptin resistance than from leptin deficiency.[75] Leptin levels in females are almost double those in males with the same body mass index or fat reserve. This finding not only has implications for leptin physiology but also supports the role of leptin in

reproductive and gonadal function.[68] The gender difference in leptin levels is found at birth, which suggests that the process begins in utero.[68,76] Circulating leptin levels are also higher during gestation than in nongravid individuals, and disturbances in leptin production have been found in several pathologic pregnancy states that alter fetal growth.[77]

The role of leptin in the fetus is unknown. Leptin has been identified in fetal cord blood in the 18th week of gestation. Adipose tissue development occurs during the second trimester, and, consistent with this finding, leptin levels dramatically increase at 34 weeks gestation.[73] Leptin is also produced by the placenta but in very small amounts that are unlikely to contribute significantly to cord blood levels.[76] The level of leptin for body weight is disproportionately high in neonates.[68,76] The reason for this is unknown, but it normalizes toward adult levels around 2 weeks of life.[68] Neonatal leptin levels have been shown to predict weight gains in infancy.[68,78]

Ghrelin

Ghrelin is an orexigenic peptide that was first identified in 1999 as the endogenous ligand for the growth hormone secretagogue receptor (GHSR).[79] Its central role in the stimulation of feeding was not appreciated until 2001.[80] Ghrelin is produced primarily in the stomach (X/A-like cells of the oxyntic glands) but also in the duodenum, jejunum, pituitary, kidney, placenta, and hypothalamus.[81] Although it is accepted that ghrelin-producing neurons exist in the hypothalamus, it is still controversial whether ghrelin is produced there in physiologically relevant amounts.[80,81] Regardless of the source, ghrelin can reach the mediobasal and mediolateral hypothalamus through the general circulation. It is unknown whether peripherally produced ghrelin can reach areas of the hypothalamus protected by the blood-brain barrier. Once ghrelin reaches the hypothalamus, the peptide targets ghrelin receptors present in various hypothalamic nuclei. Ghrelin then exerts its energy homeostatic effects through a central mechanism involving NPY[81,82] and AGRP.[81]

The effects of ghrelin on body metabolism are opposite to those of leptin. However, ghrelin appears to be regulated more acutely, unlike the more chronic regulation of leptin. During food deprivation, leptin levels fall, POMC production decreases, NPY and AGRP levels rise, and ghrelin levels increase.[83] In this situation, ghrelin serves as a hunger signal, trying to restore the body to a state of positive energy balance. On eating, increases in lower intestinal osmolarity (rather than direct effects of nutrients in the foregut) and insulin surges suppress postprandial ghrelin release.[84] Ghrelin levels drop after feeding, are decreased in obesity (though high in Prader-Willi syndrome), are increased in anorexia nervosa, and are low after gastric bypass surgery.[81,84,85] Ghrelin suppresses reproductive function, both centrally and through direct gonadal effects, in times of nutritional stress,[86] whereas leptin plays a permissive role during times of nutritional plenty.[87]

Apparently independent of its orexigenic effects, ghrelin stimulates growth hormone release. Because they work through different receptors (GHSR and growth hormone–releasing hormone receptor, respectively), ghrelin and growth hormone–releasing hormone stimulation of growth hormone secretion are synergistic.[88] The GHSR type 1a is a G-protein–coupled receptor that is mainly expressed in the pituitary and hypothalamus and mediates ghrelin's endocrine functions. Various related receptor subtypes are widely distributed in the central and peripheral tissues and mediate ghrelin's nonendocrine actions.[89] Short stature in two unrelated families segregated with a missense mutation that decreased cell-surface expression of GHSR and selectively impaired GHSR constitutive activity without losing its ability to respond to ghrelin.[90] GHSR-null mice accumulated less body weight and adiposity than control mice when fed a high-fat diet,[91] similar to ghrelin knockout mice.[92]

Other Adipokines

Leptin has become the prototypic member of the adipokine family, a group of hormones produced primarily by adipose tissue that regulate energy homeostasis and affect other functions such as the reproductive, neuroendocrine, cardiovascular, and immune systems. Adipokines are reviewed extensively elsewhere.[93-96] Central actions, especially involving the hypothalamus, have been described as contributing mechanistically to adipokine effects on energy balance.[96] The most abundantly produced adipokine is adiponectin, a collagen-like molecule that decreases weight, fat, and eating; increases insulin sensitivity; and exerts antiatherogenic, antiangiogenic, and antitumor actions.[95-98] It was also found to regulate pituitary function, namely inhibiting growth hormone and luteinizing hormone secretion in vitro.[99] The role of adiponectin in modulating pregnancy-related insulin resistance, fetal growth, and the metabolism of newborns and children is now being studied.[100,101]

DETERMINANT OF FUTURE, AS WELL AS CURRENT, HEALTH

The current obesity epidemic has spurred keen interest in the identification of precedent risk factors and the interplay between genetic and environmental traits in influencing human physical and metabolic development throughout the life span. In particular, nutritional status during the in utero and neonatal periods has been found to affect adiposity and the metabolic syndrome later in life, even in adulthood.[102-104] A body of literature has emerged under the rubric of fetal origins of adult disease, extending beyond obesity and its comorbidities,[105,106] and the hypothalamus plays a prominent role. Because the hypothalamus integrates the various neuroendometabolic functions described in this chapter to maintain homeostasis, hypothalamic adaptations to in utero conditions mold the programming (i.e., set points and responsiveness) that will govern the homeostatic mechanisms throughout life.

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Growth Factor Regulation of Fetal Growth

Growth during fetal life is characterized by an early period of cell division and formation of the major organ systems, followed by a more prolonged period of growth and refinement of organ development. *Growth factors* are peptides or proteins that serve as key regulators of cell proliferation and differentiation. They exert their effects by binding to specific receptors, which mediate signal transduction across cell membranes. The binding triggers a cascade of responses, including those involving second messengers, which ultimately result in cell mitogenesis or differentiation. Over the past decade the scientific literature has witnessed an explosion of information regarding the physiology of these agents. It is evident that a variety of growth factors are expressed in diverse tissues during embryonic life, and they may have endocrine as well as autocrine-paracrine effects.

INSULIN-LIKE GROWTH FACTOR AXIS

The primary regulator of postnatal somatic growth is growth hormone (GH). In the liver and other target cells, GH induces the production of insulin-like growth factors (IGFs), IGF-I and IGF-II.[1] The IGFs, particularly IGF-I, have direct, endocrine effects on somatic growth and on the proliferation of many tissues and cell types. Both IGF-I and IGF-II are expressed during fetal development in a GH-independent fashion. In addition to their anabolic effects, IGFs also are thought to be significant autocrine-paracrine factors involved in cellular and tissue function.[2,3] Locally produced IGFs have been demonstrated in nearly all tissue types, where they are considered to be responsible for cell growth and differentiation. GH is thought to play only a minor role in fetal growth, limited to the third trimester. This chapter presents evidence that the IGFs and their regulatory proteins play a central role in the regulation of fetal growth.

Insulin-like Growth Factors

IGF-I and IGF-II are two closely related peptide hormones approximately 7 kDa in size that share a high degree of structural similarity with proinsulin.[3] Like proinsulin, they are composed of A, B, and C domains; however, they also include a D domain. Together, these components form the mature IGF peptide. The IGFs are synthesized with an additional extension peptide known as the *E peptide,* which is removed during posttranslational processing. Both IGF-I and IGF-II are encoded by a single gene each, which by alternative splicing results in several messenger RNA (mRNA) species. The *IGF1* gene spans 95 kilobases (kb) on the long arm of chromosome 12 and contains 6 exons; the *IGF2* gene has a total genomic size of 35 kb and is made up of 9 exons. *IGF2* is located on the short arm of chromosome 11 (near the insulin gene), in an area that is paternally imprinted in all mammalian species. This paternal imprinting phenomenon is related to the fact that IGF-II is a major fetal growth factor.

Insulin-like Growth Factor Receptors

The IGFs interact with specific receptors, designated type 1 and type 2 IGF receptors, as well as with the insulin receptor, which can bind IGF-I and IGF-II, but with much lower affinity than for insulin.[3] The type 1 IGF receptor binds IGF-I with high affinity, IGF-II with slightly lower affinity, and insulin with low affinity. This receptor mediates the known mitogenic effects of the IGFs. The type 1 IGF receptor and the closely related insulin receptor

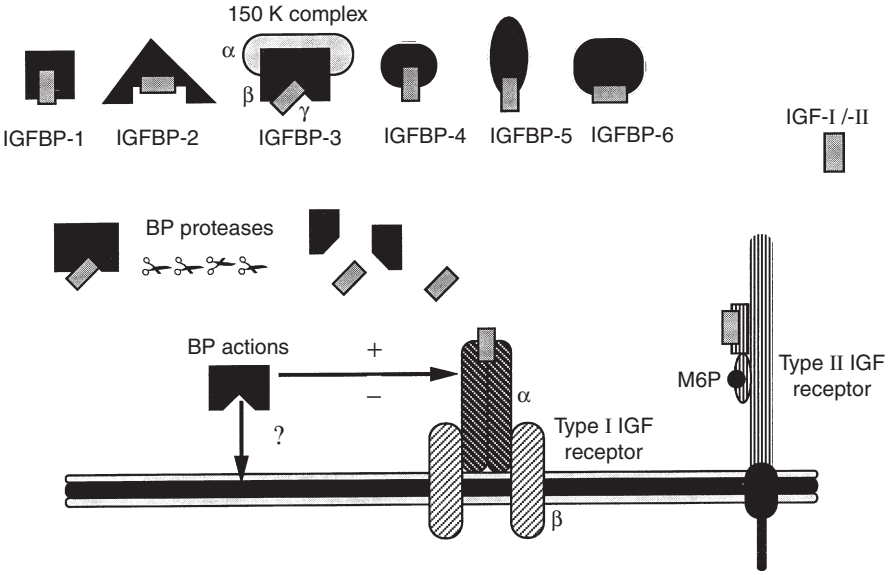


Figure 177-1 The insulin-like growth factor axis, including the IGFs, IGFBPs, and IGFBP proteases. The relationship of the components of the IGF axis is depicted in this figure. *IGF,* Insulin-like growth factor; *IGFBP,* IGF-binding protein.

are heterotetramers composed of two pairs of α and β subunits, the result of posttranslational processing of a single gene product of a single gene that encodes for the entire receptor. The two α subunits, which are involved in ligand binding, are linked by disulfide bonds and are primarily extracellular (Figure 177-1). The β subunits are connected to the α subunits by disulfide bonds and function as intracellular tyrosine kinases. On ligand binding, they undergo a conformational change that enables them to bind ATP and become autophosphorylated. Subsequently, these kinases phosphorylate cytoplasmic molecules known as the *insulin receptor substrates* (IRS-1 and -2), which are involved in mediating many of the effects of insulin and IGFs.[4] IRS proteins couple insulin and IGF receptors to the phosphoinositide 3-kinase (PI3-kinase) and extracellular signal-regulated kinase (ERK) cascades. Products of PI3-kinase, including phosphatidylinositol-3,4-biphosphate and phosphatidylinositol 3,4,5-triphosphate, attract serine kinases to the plasma membrane, including the phosphoinositide-dependent kinase (PDK1 and PDK2) and at least three protein kinases B (PKB)/AKT isoforms. During co-localization at the plasma membrane, PDK1 or PDK2 phosphorylates and activates PKB-1,-2, or -3. The activated PKB/AKT phosphorylates many substrates to control various biologic signaling cascades, including glucose transport, protein synthesis, glycogen synthesis, and cellular proliferation and survival in various cells and tissues.[5]

The insulin receptor itself exists in two isoforms in humans, produced by tissue-specific alternative splicing of exon 11 of the insulin receptor gene, *IR.*[6,7] Relative expression of the different insulin receptor isoforms, the type A insulin receptor (IR-A) and type B insulin receptor (IR-B), is not only tissue-specific but also developmentally regulated, with IR-A preferentially expressed in fetal tissues.[8] In addition, in cell transfection experiments, IR-A was found to bind IGF-II with an affinity equal to that for insulin. Whereas insulin binding to IR-A stimulated the metabolic effects of insulin receptor activation, IGF-II binding to IR-A more effectively stimulated mitogenic processes.[8]

The type 2 IGF receptor is structurally distinct from the type 1 receptor and the insulin receptor; it primarily binds IGF-II.[2] Unlike the insulin receptor and the type 1 IGF receptor, the type 2 IGF receptor is not a tyrosine kinase. Instead, it is a monomeric receptor with a large extracellular domain made up of 15 repeat sequences and a small region homologous to the collagen-binding domain of fibronectin. The type 2 IGF receptor is 270 kDa in size and functions also as the cation-independent mannose-6-phosphate receptor.[9] The receptor does not have a signaling domain and is thought to be recycled between the plasma membrane and intracellular compartments. It also has been suggested that the type 2 IGF receptor targets excess IGF-II for lysosomal destruction during fetal life. The type 2 receptor is maternally imprinted in mice but not in humans.[10]

Additional members of the class of receptors for the IGF-insulin family are less well characterized. One such receptor is composed of an insulin receptor α-β heterodimer binding to a type 1 IGF receptor α-β heterodimer and thus has been labeled the hybrid receptor.[11] Analysis of tissues in which this receptor appears to be common (such as placenta) and transfection experiments in cell lines have demonstrated two isoforms of this hybrid receptor—a hybrid receptor containing insulin receptor isoform A (Hybrid-Rs^A) and one containing insulin receptor isoform B (Hybrid-Rs^B). Hybrid-Rs^A has biologic properties intermediate between those of the insulin and the type 1 IGF receptor: It binds both insulin and IGFs with high affinity and stimulates cell proliferation and migration.[12] (By contrast, Hybrid-Rs^B binds IGF-I with high affinity but IGF-II with only low affinitiy, whereas insulin has little to no affinity at all.) The complementary DNA (cDNA) for an additional receptor has been cloned, and because of its high homology with the insulin receptor, it has been designated the *insulin receptor–related receptor* (IRR).[13] IRR is an orphan receptor of the insulin receptor family that does not bind insulin or insulin-like peptide and can be expressed as variably spliced isoforms. IRR may have a role in fetal development, because its expression occurs specifically in developing renal and neural tissues. IRR also is expressed in pancreatic islets, but the lack of this receptor in mice does not affect beta cell development and function.[14] An additional atypical IGF receptor with altered ligand binding also has been described.[15] This may represent a posttranslationally modified type 1 IGF receptor of unclear function.

Insulin-like Growth Factor Binding Proteins

The IGF-binding proteins (IGFBPs), a family of proteins with high affinity for the IGFs, have been shown to be involved in the modulation of the mitogenic effects of IGFs on cells. In vivo, the IGFs are bound to this family of six structurally and evolutionarily related IGFBPs (IGFBP-1 to IGFBP-6). In serum, a majority

(70% to 80%) of IGFs exist in a 150-kDa complex composed of one IGF molecule, IGFBP-3, and the acid-labile subunit (ALS). A smaller proportion (approximately 20%) of the IGFs are associated with other serum IGFBPs within a 50-kDa complex, and less than 5% of the IGFs are found in the free form of 7.5 kDa. ALS is a protein that binds to the IGF/IGFBP-3 binary complex, primarily in serum, prolonging the $t_{1/2}$ of serum IGFs and facilitating their endocrine actions. These IGFBPs appear to regulate the availability of free IGFs for interaction with the IGF receptors, as well as to interact directly with cell function.[16] They are part of a superfamily that comprises six high-affinity IGFBPs and at least four other low-affinity IGF binders, termed insulin-like growth factor–binding protein-related proteins (IGFBP-rPs).[17] In addition, limited evidence suggests that the cell surface proteoglycan glypican-3, mutations of which cause the overgrowth syndrome known as Simpson-Golabi-Behmel syndrome type 1, also binds IGF-II and may modulate its function.[18]

IGFBP-1 is a 25-kDa protein that is found in high concentrations in amniotic fluid; it also is secreted by hepatocytes under negative regulation by insulin. IGFBP-2 is a 31-kDa protein found in serum, cerebrospinal fluid, and seminal plasma. It is expressed in many fetal and adult tissues. IGFBP-3 is the major binding protein in postnatal serum and is synthesized by hepatocytes and other cells. In plasma, IGFBP-3 is found as part of a 150-kDa complex, which also includes an ALS and an IGF molecule, all of which are GH-dependent. IGFBP-4 is a 24-kDa protein that has been identified in serum, seminal plasma, and numerous cell types. IGFBP-5 appears to be an IGF-enhancing protein that is found in cerebrospinal fluid and serum. It also is observed in rapidly growing fetal tissues. IGFBP-6 has relative specificity for IGF-II over IGF-I. It is found in cerebrospinal fluid and serum and is produced by many cells. The six cloned cDNAs for the IGFBPs show a high degree of structural similarity and sequence conservation across species. IGFBPs are tightly regulated by various endocrine factors and are uniquely expressed during ontogeny.[19]

Three possible models for the interaction of the IGFBPs with the IGFs and their receptors have been proposed.[20] The first is that these molecules limit the availability of free IGFs for interaction with the IGF receptors. For example, the addition of IGFBPs to many cell cultures in vitro results in the inhibition of IGF actions within these experimental systems.[21] In other systems, however, IGFBPs have been demonstrated to enhance IGF action, under circumstances that may involve cellular processing of the IGFBPs.[16] Finally, as has been shown in several in vitro systems, IGFBPs may inhibit cell growth in an IGF-independent fashion, acting through their own receptors.[20,21]

IGFBP-3 is a mediator of apoptosis under the control of a number of cell cycle regulators.[22] In studies using specific antisense oligonucleotides and neutralizing anti-IGFBP-3 antibodies to block IGFBP-3 expression and action, IGFBP-3 was identified as the mediator of apoptosis induced by retinoic acid and transforming growth factor-β (TGF-β) in multiple cell types.[23-26]

Insulin-like Growth Factor–Binding Protein Proteases

A group of enzymes that are capable of cleaving IGFBPs have been recognized as potential modulators of IGF action.[27] First identified in pregnancy serum, IGFBP-3 proteolytic activity is responsible for the disappearance of intact IGFBP-3 from the serum of pregnant women, with no change in IGFBP-3 immunoreactivity and an increase in lower-molecular-weight fragments.[28,29] IGFBP proteases also have been reported in the serum of severely ill patients in a state of cachexia, in critically ill neonates, in patients with GH receptor deficiency, and in patients with in prostate cancer and other malignancies.[30-32] Prostate-specific antigen was the first kallikrein protein demonstrated to be an IGFBP protease.[33] Subsequently, it has been determined that other members of the kallikrein family, such as nerve growth factor (NGF), also serve as specific IGFBP proteases.[34] Cathepsins are another class of IGFBP proteases that have been shown to be active in acid pH.[35] Finally, matrix metalloproteinases, which are known to cleave collagen and other extracellular matrix components, are a group of IGFBP proteases.[36] It has been speculated that the IGFBP proteases are important modulators of IGF bioavailability and bioactivity through their modification of the IGF carrier proteins. The proteolytic activity may play a role in regulating IGF availability at the tissue level by decreasing the affinity of the binding proteins for the growth factors, thereby releasing free IGFs and allowing increased receptor binding.[33,36,37] Together, all of these interrelated molecules act in coordinated fashion to control growth and differentiation. This system of molecules has been termed the *IGF axis* and is summarized in Figure 177-1.

ADDITIONAL GROWTH-REGULATING PEPTIDES

Fibroblast Growth Factor Family of Peptides and Receptors

Fibroblast growth factors (FGFs) are a family of peptide cytokines that are important in the regulation of many tissues. To date, 23 different FGFs have been identified, including the best-characterized acidic FGF (aFGF), or FGF1; basic FGF (bFGF), or FGF2; and keratinocyte growth factor (KGF), or FGF7. The FGFs exert their actions by binding to one of a family of four membrane-bound FGF receptors, FGFR1 to FGFR4, and a heparin sulfate proteoglycan co-receptor to form a three-part signaling complex.[38] FGFs appear to have primarily autocrine-paracrine functions in organ growth and differentiation, bone formation, and cell differentiation and migration, as well as in carcinogenesis. The role of FGF in osteogenesis and growth initially was demonstrated by the discovery of a gain-of-function mutation in *FGFR3* as the cause of achondroplasia in humans.[39,40] Subsequently, other forms of chondrodysplasia as well as other skeletal dysplasias were found to be caused by mutations in the FGFRs, further emphasizing the importance of FGF signaling in bone formation.[41]

Nerve Growth Factors

The complex array of cells that make up the nervous system is under the regulatory influence of specific growth factors, such as nerve growth factor (NGF), neurotensin, and the neural-derived growth factors (NDGFs). NGF is synthesized in peripheral cells and transported in retrograde fashion up the neuronal axis to the cell body—a process which suggests that NGF is a neurotropic agent directing neurons to sites of innervation.[42] NGF exerts its effects on cells derived from the neural crest, including central nervous system and peripheral nervous system neurons, and some non-neurons, such as immune cells and endocrine cells. NGF stimulates mitosis in some cell types and is capable of inducing differentiation events in neural tissue. NGF synthesis is controlled in part by hormones, such as testosterone and thyroxine. It also has been demonstrated that the γ subunit of NGF serves as an IGFBP protease.[34] Further research will help elucidate the interactions between NGF and the IGF axis components in the regulation of cellular growth.

Epidermal Growth Factor System

Epidermal growth factors (EGFs) and their receptors are widespread in many tissues and participate in specific developmental processes such as neonatal eyelid opening and tooth eruption. EGFs have potent mitogenic actions that have been extensively explored in cell culture systems. The receptor for EGFs was characterized as a prototype model for signal transduction involving tyrosine kinases. EGFs have been identified in most body fluids of mammals, and extensive in vitro data indicate multiple cellular functions of EGFs. Members of the EGF family may have a role in

embryogenesis and fetal growth, because receptors have been identified in fetal tissues. The mouse fetus expresses EGF receptors from 12 days onward,[43] and targeted mutation of the EGF receptor demonstrated its significance in fetal development and growth.[44] EGFs can induce tracheal branching morphogenesis in chick lung rudiments and stimulate epithelial maturation in the lung and other tissues in rabbits.[45] Although EGF transcripts have not been detected in the fetus, it is maternal EGF that appears to play a role in fetal growth, because maternal EGF deficiency leads to hypoglycemia and intrauterine growth restriction (IUGR) in offspring—an effect that is corrected by EGF administration.[46,47] Transforming growth factor-α (TGF-α) is a peptide that displays 30% homology with EGF and also exerts effects through the EGF receptor.[44,48] It has been shown to be expressed in preimplantation mouse embryos and decidua of rat and may therefore be important in fetal development.[49-51]

Other Growth-Promoting Peptides

An increasing number of growth factors are being recognized as having growth-promoting activities in certain cell types. In general, these molecules appear to play important autocrine-paracrine as well as endocrine roles. Notable among these are groups of growth factors that have tissue-specific effects. Endothelin, platelet-derived growth factor (PDGF), and vascular epithelial growth factor (VEGF) regulate angiogenesis and other vascular processes, in addition to modulating the function of numerous cultured cells. PDGF acts in sequence with other growth factors to regulate entry into the cell cycle.[52] A variety of hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), erythropoietin, and thrombopoietin promote the growth of the different lineages of the precursor cells in the bone marrow. Growth of immune system cells is stimulated by an array of cytokines, including interleukins and interferons. Other growth factors, such as hepatocyte growth factor (HGF), that have been attributed to specific tissues are being recognized as having general growth-promoting effect in numerous tissues.

Growth Inhibitory Peptides

A particularly interesting class of cytokines that can negatively modulate cellular growth is the TGF-β family, members of which are structurally distinct from TGF-α. TGF-β can either act to mediate cellular growth and malignant transformation or act as a growth inhibitory substance with the potential for arresting the growth of normal and neoplastic cells. TGF-β transcripts have been detected in preimplantation embryos.[50] This peptide is involved in mesodermal differentiation and organogenesis. It may stimulate some mesenchymal mitosis while inhibiting the proliferation of epithelial cells.

TGF-βs have both autocrine and paracrine effects. The response depends on the origin of the cell: In some cell types (e.g., hepatocytes, hematopoietic cells), cell growth is stimulated, whereas in other cell types (e.g., osteoblasts, granulocytes), growth is inhibited. During pregnancy, TGFs are important in the regulation of trophoblast invasion, decidualization, and placentation.[53] Mutations in the TGF-β family ligands are responsible for a number of human diseases, including hereditary chondrodysplasia.[54] Retinoic acid and other steroid hormones also have been shown to have inhibitory effects on cell growth. Tumor necrosis factors (TNFs) and other compounds have been described to have similar effects. These molecules may regulate the entry of cells into programmed cell death (apoptosis).

GROWTH FACTORS IN FETAL GROWTH: ANIMAL MODELS

In this section, comparisons are made with corresponding human mutations when appropriate.

Insulin-like Growth Factor Axis Expression

The embryo establishes its own IGF axis very early in gestation, providing evidence that this axis plays a functional role in fetal growth. The mRNAs for IGF-II, the type 2 IGF receptor, and IGFBP-2, -3, and -4 are among the earliest of fetal mouse transcripts and are detectable in two-cell murine embryos by reverse transcriptase–polymerase chain reaction analysis.[55-57] These transcripts become more abundant as gestation progresses. The distribution of the type 2 IGF receptor in tissues corresponds to that of IGF-II mRNA. IGF-I expression occurs somewhat later, by 7 to 8 days gestation in mouse embryos.[51] The type 1 IGF receptor is expressed in the eight-cell rat embryo, and its mRNA is widely distributed in various tissues by midgestation.[58]

Regulation of IGF expression in fetal life is not fully defined. Postnatal IGF synthesis is controlled by GH and by nutritional status, but IGF regulation in intrauterine life is less well understood and does not appear to be GH-dependent. Furthermore, evidence suggests that IGFs not only stimulate proliferation but also can induce differentiation events in some cells during organogenesis in an autocrine or paracrine manner. With regard to organ differentiation, these actions, probably are tightly regulated within specific tissues and developmental stages during intrauterine life.

Overexpression of the Growth Hormone–Insulin-like Growth Factor Axis in Transgenic Animals

Overexpression of Growth Hormone and Insulin-like Growth Factor-I and -II

The creation of transgenic mice overexpressing GH-IGF axis molecules has furthered the understanding of the control of IGF-I secretion and actions in fetal and postnatal growth (Table 177-1). Mice with GH overexpression display increased postnatal linear growth and dramatic weight gain to as much as twice the normal body weight with selective organ hypertrophy.[59,60] Acceleration of growth correlates with the onset of GH-dependent

TABLE 177-1

Physical and Biochemical Features of Various GH/IGF-I Transgenic Mouse Models

Transgene Model	Postnatal Growth (% normal)	GH	IGF-I	IGFBP-3	Brain Size	Liver Size
GH	200	⇈	⇈	—	Normal	⇈
IGF-I	130	↓	↑	↑	⇈	↑
GH^-	60	⇊	⇊	⇊	↓	⇊
$GH^-/IGF\text{-}I^+$	Normal	↓	Normal	Normal or ↓	↑	↓
IGFBP-3	80	—	⇈	⇈	↓	↑

GH, Growth hormone; *GH^-,* growth hormone–deficient; *IGF-I,* insulin-like growth factor-I; *IGFBP-3,* IGF-binding protein-3.

IGF-I expression during the second to third postnatal week, suggesting that the growth-promoting properties of the transgene are mediated through the induction of IGF-I expression postnatally. IGF-I transgenic mice also are larger than normal, but their weight increase (to 30% over that of control mice), which occurs after 4 to 6 weeks of postnatal life, is more modest than that in the GH transgenic mice, which probably is related to the lower IGF levels seen with the former.[61] The brains and other organs of these animals demonstrate a large increase in weight relative to overall growth. IGF-I levels in these mice are elevated but are lower than those seen in the GH-transgenic mice; endogenous GH and hepatic IGF-I expression are suppressed by 60% to 75% (see Table 177-1).[62] IGF-II transgenic mice are not bigger than normal, but in the existing models, the major portion of IGF-II expression occurs postnatally.[63]

To separate out more completely the effects of endogenous GH and IGF-I expression, IGF-I transgenic mice were mated with a GH-deficient mouse strain.[64,65] The offspring displayed normal body weight and linear growth. Some non–GH-dependent effects of IGF-I were present, including a disproportionate increase in brain weight, implying that these are local effects of IGF-I. The livers of these mice were hypoplastic (an effect seen in GH-deficient mice), an abnormality that was only partially corrected with IGF-I. Overall, the transgenic animal data confirm the role of GH and IGF-I in postnatal growth and suggest that IGF-I mediates a majority of GH effects, but that variations in local expression IGF-I can result in altered growth of specific tissues.

Overexpression of IGFBPs

Mice overexpressing IGFBP-1 are similar in size to (or slightly smaller than) the wild type at birth.[66,67] Brain weights are decreased in these transgenic mice compared with wild type mice.[68] It is generally thought that IGFBP-1 functions to inhibit IGF-I action and thus may contribute to the impaired fetal brain development seen in these transgenic animals. Overexpression of IGFBP-2 in mice did not result in a different birth weight and was associated with a reduction of body weight gain only after the age of 3 weeks.[69]

In two different IGFBP-3 transgenic mouse models, transgene expression resulted in a 10% reduction in birth weight, as well as organomegaly in body regions different from the major expression sites.[70-72] Total body weight in transgenic mice overexpressing IGFBP-4 is indistinguishable from that in control animals.[73]

Insulin and Insulin-like Growth Factor Axis–Null Mutations

Studies of null mutations (knockouts) created by homologous recombination in the genes encoding insulin, IGF-I, IGF-II, and the IGF receptors have enabled investigators to explore the role of the IGF axis in fetal growth. In all of the null mutations, body proportions are qualitatively normal.

Insulin-like Growth Factors and Insulin-like Growth Factor Receptor Mutations

Mice in which the IGF-II gene has been eliminated by targeted disruption exhibit severe growth restriction, to 60% of normal size at birth, with small placentas.[74,75] After birth, however, survival is normal, and the mice grow at a near-normal rate, although they remain small relative to normal littermates. In the heterozygous state, the IGF-II knockout can manifest itself only if the paternal allele is targeted, elegantly demonstrating the paternal imprinting of the IGF-II gene.[76] IGF-I gene–targeted mice also are small (60% of normal) at birth, whereas the placentas are of normal size.[74,77,78] Furthermore, they display dramatic growth arrest postnatally (30% of normal). These mice also are more likely to die during the neonatal period; the increased death rate may be related to poor development of diaphragm and lung.

Postnatal development of surviving IGF-I–knockout mice has been analyzed in detail. At 2 months of age, IGF-I–deficient mice show extensive reductions in brain size, consistent with a role for IGF-I in axon growth.[79] Morphologic and morphometric analyses of long bones in mice lacking IGF-I indicate that IGF-I promotes bone development by increasing cellular proliferation, without affecting differentiation. The growth plates are uniformly affected, with reductions in the resting, proliferative, and hypertrophic chondrocytes. As a result, the formation of secondary ossification centers is delayed.[80] Other reports have described homozygous IGF-I mutations in humans, manifesting as severe prenatal and postnatal growth failure, sensineural deafness, and mental retardation.[81-83] Mice with null mutations of both IGF-I and IGF-II also displayed more severe IUGR (to 30% of normal size), and all of these mice died after birth from respiratory failure. Thus IGF-I and -II appear to have independent growth-promoting activities that are compounded when both are absent. The results of the studies confirm that both IGF-I and IGF-II appear to be critical for fetal growth, whereas IGF-I promotes postnatal growth as well. IGF-II signaling may dictate the size of the placenta, thereby affecting fetal size.

Liver IGF-I–deficient (LID) mice grow normally despite a 75% reduction in serum IGF-I levels.[84] In these mice, expression of IGF-I in nonhepatic tissues is normal, suggesting that the normal growth exhibited by these animals may be mediated by autocrine-paracrine actions of IGF-I, as well as by some nonhepatic sources of circulating IGF-I. Similarly, ALS-knockout (ALSKO) mice demonstrate only a 10% reduction in body weight despite a 65% reduction in circulating IGF-I levels.[85] More recent reports of human ALS mutations show similar findings: markedly reduced circulating total IGF-I levels, undetectable ALS, but only mildly affected prenatal and postnatal growth.[86,87] These findings are consistent with the idea that locally synthesized IGF-I plays a prominent role in somatic growth. LID + ALS double-knockout mice showed serum levels of IGF-I reduced to approximately 10% to 15% of normal levels.[88] In these animals, GH levels were markedly increased and linear growth was severely attenuated (for a 30% reduction in body length and weight starting at the age of 2 to 3 weeks). Thus the double-gene-disruption LID + ALSKO mouse model demonstrates that a threshold concentration of circulating IGF-I is necessary for normal growth.

Gene targeting of the type 1 IGF receptor results in severe IUGR, with a phenotype similar to that seen in double–IGF-I/IGF-II gene targeting.[77] These mice also die after birth, apparently from inadequate respiration. The results indicate that the mitogenic effects of IGF-I and IGF-II are mediated primarily through the type 1 IGF receptor. Although no humans have been identified with homozygous mutations, a handful of patients have been described with heterozygous mutations of the IGF-IR or the IGF-IR proreceptor, all with IUGR progressive postnatal growth failure.[89-91]

Although mice with disruption of the type 1 IGF receptor displayed growth failure, only the mice missing IGF-II displayed decreased placental growth. Indeed, placenta-specific IGF-II represents a major regulator of placental growth.[92] By controlling placental growth and ability to transport vital amino acids and glucose to the fetus, placental IGF-II in turn affects fetal growth, especially in late gestation.[93,94] Furthermore, the fact that genetic IGF-II deficiency has a phenotype independent of that associated with the type 1 and type 2 receptors suggests that an additional receptor mediates IGF-II actions in fetal life. Gene targeting of the type 2 receptor results in mice that are 30% larger than littermates—a finding compatible with its negative growth-modulating effect.[95] This gene is maternally imprinted in mice but not in humans. Thus those mice inheriting the maternal 2R allele lack functional 2R receptors and die shortly after birth. The levels of lysosomal enzymes in amniotic fluid are increased, and selective organ enlargement is apparent.

It has been hypothesized that mice lacking the type 2R accumulate toxic levels of IGF-II.[95]

These studies provide supportive evidence that the IGF-II/mannose-6-phosphate (Man-6-P) receptor may function primarily to degrade and remove IGF-II from the extracellular environment. The opposite imprinting of IGF-II and the type 2 IGF receptor may modulate fetal demand and maternal supply of nutrients.[96] The phenotype of mice lacking both type 1 IGF receptor and type 2 IGF receptor provides evidence for the ability of IGF-I to signal through the insulin receptor, because these mice exhibit normal embryonic and postnatal growth. It has been shown that mice lacking the type 2 IGF receptor are rescued from perinatal lethality and undergo near-normal postnatal development when they carry null mutations of the type 1 IGF receptor. Triple mutants lacking IGF-II and both IGF receptors exhibit only approximately 30% of normal weight, phenotypically indistinguishable from double mutants lacking IGF type 1 receptor and IGF-II (see Table 177-2).[97] Genetic evidence indicates that the receptor supporting somatic growth of the type 1– type 2 IGF receptor double mutants is the insulin receptor, because mice lacking all three genes (insulin receptor, type 1 IGF receptor, type 2 IGF receptor) are nonviable dwarfs that attain only 30% of normal size.[98]

To address the issue of the role of GH and IGF-I in growth, Lupu and colleagues[80] have generated mice lacking both IGF-I and GH receptor. Double-knockout mice exhibit a greater degree of growth restriction (to 17% of normal size) than that seen in mice lacking either gene alone, indicating that the two genes act both independently and synergistically to promote growth. Whereas IGF-I promotes both prenatal and postnatal growth, GH appears to be required exclusively for postnatal growth, because the growth defect in GH receptor–deficient mice becomes apparent only after postnatal day 20.

Insulin and Insulin Receptor Mutations

The generation of mice bearing insulin receptor mutations has been instrumental in delineating the pathogenesis of insulin resistance, diabetes, and obesity. In terms of somatic growth, mice lacking the insulin receptor are born at term with slight growth retardation (approximately 10%)[98]; inactivation of the two insulin genes also results in a slight impairment of embryonic growth, with a 15% to 20% decrease in birth weight.[99] The growth impairment observed in mice lacking the insulin genes indicates that the effect of insulin to promote mouse embryo growth is paltry compared with that of IGF-I and IGF-II. Mutations of the insulin receptor in humans are phenotypically heterogeneous in terms of the metabolic consequences. As in mice, lack of the insulin receptor in humans is compatible with embryonic development and term birth. Most strikingly, however, human infants lacking the insulin receptor demonstrate severe growth restriction at birth and gain little if any weight thereafter.[100] The most likely explanation of the difference between insulin receptor–deficient mice and children is that embryonic growth in humans and that in rodents follow different patterns. During the last trimester of human gestation, corresponding to the first weeks of postnatal life in mice, a significant increase in adipose mass is seen. The adipose organ is exquisitely sensitive to insulin, as demonstrated by the excessive adiposity of fetuses exposed to high insulin concentrations in utero who are born large for gestational age (LGA) as a result of maternal diabetes[101,102] or hyperinsulinism.[103,104] Thus, in contrast with its role in mice, insulin is a fetal growth factor in humans. Null mutations of the human insulin gene have not been reported.

Insulin-like Growth Factor–Binding Protein Mutations

When the knockout approach was applied to the IGFBPs, the results were surprisingly subtle: In IGFBP-2–knockout mice, a reduction in spleen weight of adult males was the only morphologic alteration.[104] IGFBP-4 mutants appear to be slightly smaller than their normal counterparts, and the genetic ablation of IGFBP-6 has not resulted in any apparent phenotypic manifestation.[105] A recently described IGFBP-3/IGFBP-4/IGFBP-5 triple-knockout mouse was shown to have a modest reduction in birth weight, similar to that for the IGFBP-4 single-gene mutant, and a moderate reduction in postnatal growth (78% of wild type).[106] It has been speculated that functional compensation by other members of the IGFBP family (including IGFBP-rPs) may prevent the appearance of dramatic phenotypes.[107]

Insulin Receptor Substrate and AKT Mutations

The two IRS proteins, IRS-1 and IRS-2, mediate many insulin and IGF responses, especially those associated with somatic growth and carbohydrate metabolism. The IRS-1 branch of the pathway plays a significant role in mediating the effects of IGF-I on growth. Deletion of the *IRS1* gene in mice reduces embryonic and neonatal growth,[108] whereas deletion of *IRS2* barely reduces prenatal and early postnatal growth by 10%.[109] The *IRS1*-knockout mouse has been described as having a phenotype of fetal growth restriction to 50% to 70% normal size in the homozygous state.[108] Postnatally, these mice remain small (at 50% to 70% of normal weight), and catch-up growth does not occur. The offspring display no obvious developmental abnormalities and have normal bone development. Gene targeting of *IRS1* results in impairment of both growth and carbohydrate metabolism, compatible with its role in mediating both insulin and IGF-I effects.[108,110] Growth is reduced 40% in *IRS2*-null mice that are also haploinsufficient for IRS-1, whereas growth is reduced 70% in *IRS1*-null mice also haploinsufficient for *IRS2*.[111] Thus IRS-2 cannot fully replace IRS-1 in this process. *IRS2*-null mice also exhibit a diabetic phenotype with peripheral insulin resistance and panceatic beta cell failure.[109]

Downstream from IRS, *AKT1* and *AKT2* gene deletions also result in different phenotypes. Although *AKT2*-null mice exhibit insulin resistance,[112] at birth *AKT1*-null mice have a 20% reduction in body weight compared with wild-type mice.[113] The decrease in body weight in AKT-1–deficient mice persists throughout postnatal development regardless of sex, and glucose tolerance is normal.[113] These data demonstrate that AKT-1 is more important to the growth of the organism, both in utero and after birth, whereas AKT-2 is critical to insulin-dependent control of carbohydrate metabolism.

IGF axis gene knockouts are summarized in Table 177-2.

GROWTH FACTORS IN HUMAN FETAL GROWTH

Pregnancy

During pregnancy, circulating levels of IGFs rise in maternal serum and significantly in fetal serum.[114,115] Placental GH, human placental lactogen (HPL), and nutritional factors may regulate maternal IGF-I levels during pregnancy.[116,117] Placental GH is not detected in the fetus, but HPL stimulates IGF-I and IGF-II production in fetal tissue.[118] Maternal IGFBP-1 levels rise 5- to 10-fold during gestation,[29,114] probably as a manifestation of the insulin resistance of pregnancy. IGFBP-1 levels are substantial in amniotic fluid, where they may have a fetal renal or hepatic source. A negative correlation exists between term IGFBP-1 levels in maternal serum or IGFBP-1 levels in amniotic fluid at 16 weeks gestation and fetal weight.[119] Thus the elevated IGFBP-1 concentrations (10 to 100 ng/L) in amniotic fluid may modulate fetal IGF action. It also has been suggested that elevated maternal IGFBP-1 levels may limit trophoblast invasion, thereby negatively affecting placental growth.

Pregnancy-associated IGFBP-3 protease activity, which has been shown to appear after 6 weeks gestation, is responsible for the decrease in and progressive disappearance of intact IGFBP-3 on Western ligand blot analysis (without a change in IGFBP-3 immunoreactivity) and the appearance of lower-molecular-weight

TABLE 177-2

Physical and Biochemical Features of the Various GH/IGF1–Knockout Mouse Models

Knockout Model	Birth Weight (% of normal)	Other Features	Postnatal Course
IGF-I	60	Normal placenta; decreased brain size	Slow growth; death before adulthood
IGF-II	60	Small placenta	Normal growth rate
IGF-I + IGF-II	30	Small placenta; no respirations	Immediate postnatal death
LID	Normal	75% reduction in serum IGF-I	Normal growth
ALSKO	Normal	65% reduction in serum IGF-I	10% reduction in body weight
LID + ALSKO*	Normal	85-90% reduction in serum IGF-I; increased GH levels	30% reduction in body weight and length
IGF-I receptor	45	No respirations	Immediate postnatal death
IGF-II receptor	130	Edema, large placenta	Death in utero
IGF-I receptor + IGF-II receptor	100	Large placenta	Normal growth rate
IGF-I receptor + IGF-II	30	Small placenta	
IGF-II receptor + IGF-II	80	Small placenta	
IGF-I receptor + IGF-II receptor + IGF-II	30	Small placenta	
IGF-I + GH receptor	17		Postnatal growth retardation
IRS-1	50-70	Insulin resistance	Survival with normal growth rate, but persistent small size (no catch-up growth)
IRS-2	90	Insulin resistance, pancreatic beta cell failure	Development of diabetes
AKT-1	80	Normal glucose tolerance	Neonatal death in some animals Persistent small size in surviving pups
Insulin genes (Ins1 + Ins2)	80-85	Diabetes	Normal growth rate; early death from diabetes
Insulin receptor	90	Diabetes; hypotrophy of subcutaneous fat	Normal growth rate; early death from diabetes

*Double-knockout model.
ALSKO, ALS-knockout mice; *GH,* growth hormone; *IGF-I*, insulin-like growth factor-I; *IRS-1,* insulin-receptor substrate-1; *LID,* liver IGF-I–deficient mice.

fragments of IGFBP-3 with a lower affinity for IGFs.[29,120,121] It has been demonstrated that IGFBP-2, IGFBP-4, and IGFBP-5 also undergo proteolysis during pregnancy.[29,122] The significance of the pregnancy-associated protease remains unclear. Some investigators have postulated that IGFBP proteolysis may play a role in facilitating trophoblastic invasion.[123] It has been demonstrated that the pregnancy serum protease may play a role in regulating IGF bioactivity by making IGFs more available to the cells.[124] It is possible, therefore, that this proteolytic activity could affect placental and, consequently, fetal growth by releasing free IGFs and allowing increased receptor binding.

Insulin-like Growth Factor Axis Expression by the Fetus

Available data regarding the earliest expression of the IGF axis in the human embryo are limited. In the trophoblast, however, *IGF2* mRNA is detected by days 12 to 18,[125,126] and soon after, mRNA encoding the type I and II receptors also can be found.[127] The animal data demonstrating IGF-II expression before that of IGF-I indicate the importance of IGF-II in early embryogenesis. Expression of IGFs is widespread in all human tissues during the second trimester.[128] Western immunoblotting techniques have demonstrated that type II IGF receptors are present in most human fetal tissues at 23 weeks.[129] The widespread expression and action of IGFs in embryogenesis are mostly autocrine-paracrine in nature and GH-independent. Although the control of IGF levels in the fetus is not well understood, IGF actions are thought to occur in concert with other hormones and growth factors and to be greatly influenced by the presence and distribution of IGFBPs. Techniques of immunocytochemistry, in situ hybridization, and Northern blot analysis have identified IGFBP-1 through IGFBP-6 in most fetal tissues.[130,131] Synthesis and secretion of IGFBPs are tissue-specific.[129]

In the third trimester, fetal serum IGF-I concentrations steadily increase, correlating with the time of peak fetal weight gain. Although fetal serum IGF-I levels are lower than those in adults, several investigators have reported that maternal and fetal cord serum IGF-I levels correlated with birth weight,[132-135] suggesting co-regulation by nutritional factors. This correlation, however, has not been a consistent finding in every study.[114] Insulin levels appear to affect IGF levels as well, although this effect may be secondary. Fetal IGF-II levels have shown a correlation with birth weight in some studies but not in others.[132,135] The animal studies presented in the previous section indicate that IGFs are not under endocrine control prenatally and that GH deficiency, although affecting postnatal growth, does not result in significant prenatal growth retardation. Nevertheless, data from humans with hypopituitarism do support a role of GH in third-trimester fetal growth, because these infants, although demonstrating overall growth still within the normal curves, often have mean birth lengths 0.8 to 1.7 standard deviation scores below the mean.[136,137] Furthermore, neonates with GH insensitivity (resulting in Laron syndrome) also are small at birth (–1.59 mean standard deviation score [SDS] for height), and their serum levels of IGF-I and IGF-II are low at birth as well.[138] The phenotype of severe IUGR in patients with mutations of the *IGF1* gene proves that IGF-I plays a critical part in human fetal growth, independently of GH.[81-83]

The IGFBP profile for cord serum differs from that for adult serum. IGFBP-3 is low, with relative predominance of IGFBP-2.[134] Furthermore, IGFBP-1 levels in cord serum are up to 10 times higher than levels seen in adults and decline markedly during the first week of life. IGFBP-1 levels show a negative correlation with birth weight. In addition, correlation between IGFBP-1 and insulin level has been demonstrated both in infants who are born LGA and those with IUGR. In LGA infants born to nondiabetic mothers, significantly elevated insulin levels are associated with decreased IGFBP-1 levels; an opposite trend is seen in IUGR infants.[139]

Intrauterine Growth Restriction

IUGR is defined by a birth weight or length 2 or more standard deviations below the mean (i.e., –2 SD) for gestational age and gender, a cutoff that corresponds approximately to the 3rd

percentile for gestational age. A total of 4,112,052 infants were born in the United States in 2004; of these, approximately 94,600 infants were affected with IUGR, as determined using an overall prevalence rate of 2.3%.[140]

IUGR may result from diverse genetic and environmental influences on the developing fetus: chromosomal defects, maternal nutrition, genetics, or decreased uteroplacental blood flow. Disproportional IUGR can result from circulatory changes in the fetus that favor redistribution of blood flow to the heart and brain. In severe IUGR, head size is compromised as well. Endocrine factors appear to play a role in the pathogenesis of IUGR. Decreased levels of placental GH and maternal IGF-I have been demonstrated in IUGR.[141] In both preterm and full-term infants, low birth weight is associated with low cord serum IGF-I, IGF-II, and IGFBP-3 and high IGFBP-1.[134,135,139,142,143] Some investigators, however, have demonstrated more variable IGF levels in infants with IUGR,[114] indicating that this single marker may underestimate the complexity of this condition and the heterogeneity of its causes. IGFBP-2 levels may be higher, similar to those seen in hypopituitary patients postnatally.[139] The increase in IGFBP-1 (mostly of hepatic origin) appears to be the result of inadequate nutrient supply, with resultant hypoinsulinemia.

It has been speculated that the elevated IGFBP-1 levels in these infants may induce sequestration of IGFs, thereby restricting fetal growth. Not only are alterations in IGF levels seen in children with IUGR, but the IGF type 1 receptor affinity also was reduced in a subgroup of patients with IUGR.[144] The finding of a homozygous deletions of *IGF1* in a handful of patients with severe IUGR[81-83] implicates IGF-I in the etiology of this disorder, but larger studies looking for *IGF1* mutations in larger groups of children born with IUGR have failed to identify them, suggesting that these mutations are not a common cause of IUGR.[145-147] Conversely, an association between a polymorphism of the *IGF1* gene and low serum IGF-I levels was found in a study involving children with short stature born small for gestational age (SGA), suggesting that genetically determined low serum IGF-I levels may lead not only to reduction in birth length, weight, and head circumference but also to persistent short stature.[148] TGF-β also has been implicated in the pathogenesis of IUGR. TGF-β cord blood levels were found to be significantly lower in pregnancies complicated by IUGR than those in "control pregnancies," in which children were born at a weight appropriate for gestational age (AGA).[53]

A majority of children born with IUGR will experience adequate catch-up growth in length during the first 2 years of life. Postnatal catch-up growth usually begins immediately after birth, and in fact, most of the increases in SDS for height occur by 6 months of age.[149,150] McCowan and co-workers[151] showed that 40 of 203 infants (20%) born SGA remained short and 31 (16%) remained light at 6 months. Around 2 years of age, most children born SGA tend not to exhibit further catch-up growth, and approximately 10% will remain at –2 SD or below for height throughout childhood and adolescence and into adulthood, indicating a persistent failure to achieve normal linear growth. In one study of a large population of neonates with follow-up to adulthood, height was measured at the age of 20 to 21 years. Compared with an AGA control population, the SGA subjects were 4 to 6 cm shorter as adults.[152]

In 2001, the use of recombinant human GH was approved by the U.S. Food and Drug Administration (FDA) for the long-term treatment of growth failure in children born SGA who fail to manifest catch-up growth by age 2 years. Multiple studies have demonstrated the efficacy of GH therapy in promoting catch-up growth in children born with IUGR.[153-157] Some of these studies also have demonstrated positive effects of GH therapy on body composition, blood pressure, and lipid metabolism.[158-161,137-139] Although decreased insulin sensitivity has been reported in SGA children on GH treatment, glucose tolerance remained normal,[162] and fasting insulin and glucose levels returned to the levels in control subjects after the discontinuation of treatment.[158] Thus far, the evidence suggests that GH therapy in short children born SGA is safe and efficacious.[158,162]

Growth restriction during the fetal period has been associated with a higher prevalence of such conditions as hypertension, cardiovascular disease, and type 2 diabetes in adult life.[163] These findings constitute the basis for the fetal origins of the adult disease hypothesis, which proposes that insults in utero lead to "reprogramming" of the endocrine system, which in turn predisposes infants born with IUGR to adverse health outcomes in adulthood. Lifelong programming of the IGF system by the intrauterine hormonal and nutritional environment has been suggested to be a key mediator of the link between small size at birth and cardiovascular disease in adulthood.[142] An increased prevalence of metabolic syndrome (consisting of obesity, cardiovascular disease, hypertension, and type 2 diabetes) has been reported among adults who were born SGA.[164] In addition to risk factors associated with IUGR, increasing evidence suggests that rapid postnatal catch-up growth further enhances risk for development of obesity and the metabolic syndrome.[165,166] Insulin resistance has been linked to thinness at birth followed by obesity in adulthood.[167,168]

Macrosomia

Alterations in the IGF axis also have been demonstrated in infants who are LGA, including those born to diabetic mothers (see Figure 177-2 and Table 177-3). In these infants, IGF-I, insulin, and IGFBP-3 levels are increased, whereas IGFBP-1 levels are significantly decreased.[139] Fetal hyperinsulinemia secondary to maternal diabetes mellitus results in increased body fat, with a more modest increase in body length. Insulin promotes fetal growth by permitting nutrient uptake, and increased IGF-I production may be a result of the nutritional state of these infants.[169] The alterations in IGFBP-1 also appear to be secondary to the higher insulin levels, but IGF-I levels and nutritional status also may play a role. It has been speculated that the lower IGFBP-1 levels result in less inhibition of IGF-I effects, resulting in greater growth.

Infants with the Beckwith-Wiedemann syndrome have a doubling in *IGF2* gene dosage as a result of alterations at chromosomal locus 11p15.5. These infants are macrosomic and often demonstrate evidence of congenital hyperinsulinism. Placental hypertrophy is seen as well.[170] In the limited studies that have been performed, levels of IGF-II are not increased in cord serum in these infants, but it is possible that *IGF2* gene dosage may increase fetal growth through the stimulation of placental growth.[123] These infants also are predisposed to the development of IGF-II–dependent neoplasms, such as Wilms tumor. In fact, in an analysis of birth weights for more than 1800 patients with Wilms tumor, the mean was significantly greater than the national average, especially for those with Beckwith-Wiedemann syndrome or hemihypertrophy, suggesting the presence of systemic growth effects of IGF-II on both fetal size and the development of Wilms tumor.[171] Of interest, single-nucleotide polymorphisms in *H19,* a maternally imprinted gene implicated in translational regulation of *IGF2,* when identified in either offspring or maternal DNA, have been associated with elevated cord blood IGF-II levels and higher birth weight.[172] This evidence suggests a role for *H19* in controlling fetal growth restraint through regulation of IGF-II.

SUMMARY

In recent years, the medical and scientific literature has witnessed an explosion of information regarding the physiology of growth and growth factors in prenatal life, and the various components of the IGF axis in particular. Local IGFs and IGFBPs are of prime importance in fetal and placental growth. Genetic manipulations of IGF axis components in fetal mice have further clarified the

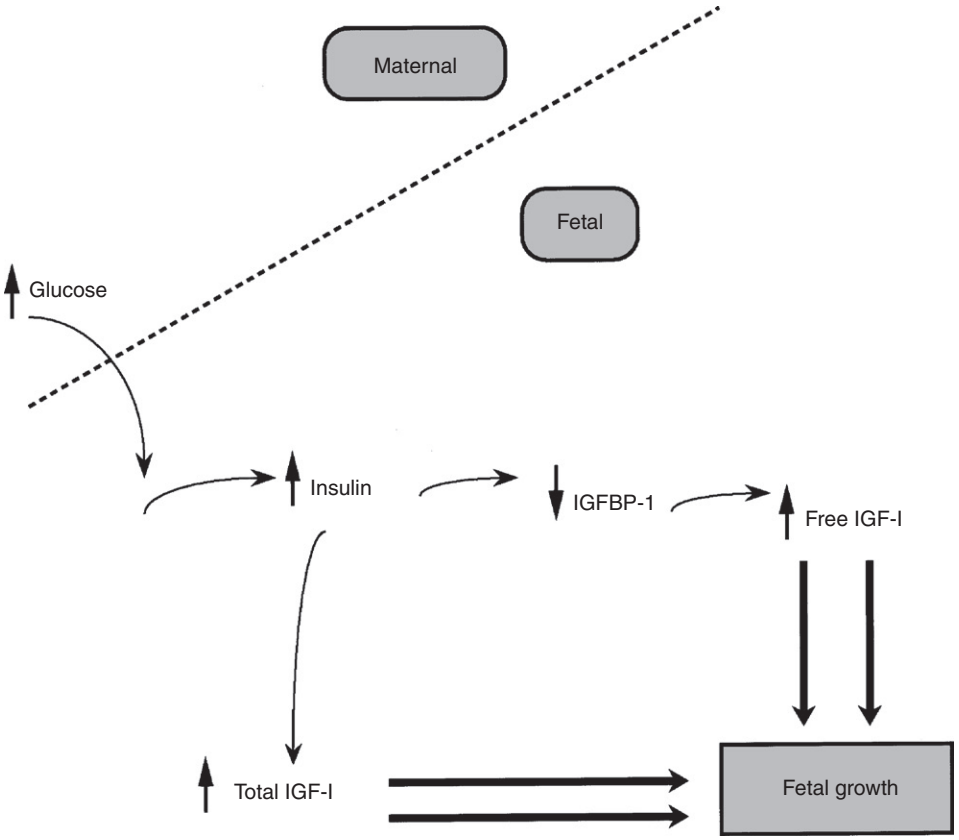


Figure 177-2 A schematic model illustrating the interrelationships among maternal and fetal glucose, insulin, IGF-I, and IGFBP-1 levels in the regulation of fetal growth and the evolution of macrosomia. *IGF-I,* Insulin-like growth factor-I; *IGFBP-1,* IGF-binding protein-1.

TABLE 177-3

Insulin-like Growth Factor (IGF) Axis Findings Seen in Extremes of Human Fetal Growth

Growth Pattern	IGF-I	IGF-II	IGFBP-1	IGFBP-3	IGFBP-2
Intrauterine growth restriction	↓	↓	↑	↓	↑
Large for gestational age	↑	Normal or ↑	↓	↑	Normal

IGFBP, IGF-binding protein.

roles of these agents. Undoubtedly, the coming years will bring even more new information on the physiology and pathology of these key cellular regulators. These discoveries are likely to lead to the increasing use of diagnostic tests and to the recognition of genetic mutations of the GH-IGF axis in cases of IUGR, as well as to therapeutic applications of these agents.

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Growth Hormone and Prolactin

This chapter examines the roles of the diverse and varied functions of growth hormone (GH) and prolactin (PRL) in fetal development and the perinatal period.These are evolutionarily related hormones of the lactogenic superfamily, and, although their concentrations are high in fetal life, the functional significance of these high concentrations remains uncertain.The placenta is also a rich source of somatogenic and lactogenic hormones secreted into the maternal circulation, where they are believed to play an important role in maternal metabolic adaptations to support pregnancy and in preparation of the mammary gland. Although high levels of circulating GH and PRL in the fetus and neonate are universal in mammals, much more diversity exists in the nature of placental hormone production, reflecting gene duplications of either the GH or placental lactogen (PL) ancestral genes (see later discussion). This chapter focuses on the roles of GH, PRL, and the PL hormones during fetal development and the perinatal period.

THE DEVELOPMENT OF HYPOTHALAMOPITUITARY CONTROL

The differentiation of adenohypophyseal cell lineages within Rathke's pouch is dependent on a series of transcriptional factors that generate spatial and temporal patterns of gene expression (for review, see reference 1). Gene deletions or mutations of some of these signaling molecules (i.e., Pit-1, Six6, and Pax6) have been shown to result in pituitary abnormalities or altered numbers of pituitary cell types. Pit-1, in particular, is required for the development of somatotrophs, lactotrophs, and thyrotrophs.[1] Dominant and recessive mutations in Pit-1 lead to

absent or diminished GH, PRL, and thyroid-stimulating hormone secretion. Pit-1 regulates the respective transcription of GH, PRL, and thyroid-stimulating hormone, acting as a gene activator or repressor, depending upon in which cell type these genes are expressed. Pit-1 interacts with cell type–specific transcriptional co-regulators, and this interaction may be determined by slight structural changes in their otherwise highly homologous 5′ flanking regions. A 2-bp sequence variation in the Pit-1 binding site of the GH promoter can induce a conformational change in the DNA structure that may be critical to the transcriptional machinery distinguishing between the GH and PRL regulatory regions. Thus several mechanisms are in place to ensure specificity of pituitary cell-type transcriptional control.

In mice, differentiation of the thyrotrope occurs around embryonic day 12.5 (E12.5),[1] making thyroid-stimulating hormone one of the first pituitary gene products observed. Somatotrophs and lactotrophs develop last (in mice, approximately E16), and can comprise up to half of the pituitary cell-type population.[1,2] In sheep, in which brain development is largely completed before birth, both somatotrophs and lactotrophs can be identified by 60 days postconception; term is 146 days).[3] In humans, GH is first detectable in the fetal pituitary by immunocytochemistry at 8 weeks postconception. PRL-secreting cells can be detected by 12 weeks postconception, but their number is low until after 26 weeks postconception, when a considerable hyperplasia of lactotrophs is observed.[4]

Some pituitary tumors secrete both GH and PRL from a single cell, and it has been suggested that there may be a common bihormonal-secreting precursor. In studies of primary pituitary cultures derived from neonatal rats[5] and cultured human fetal pituitary cells,[6] one third of the GH- or PRL-synthesizing cells secrete both GH and PRL simultaneously. As PRL secretion inevitably commences at a later stage of development than GH secretion, it has been suggested that lactotrophs may be derived from this putative somatomammotroph.[6] Indeed, it has been shown that an immortalized cell line—GHFT[7]—has properties of somatomammotrophs and is able to differentiate to PRL-secreting lactotrophs after treatment with FGF-2.[8]

The development of the hypothalamic-pituitary adenohypophyseal circulation has been reviewed elsewhere.[9,10] The portal circulation matures early in development and is not a rate-limiting step in the development of functional control of adenohypophyseal secretion in fetal life. Instead, the development of neuroregulatory control over hypothalamic release and inhibitory factors, as well as the development of hormone receptors, makes way for negative feedback loops to appear. Additionally, the unique milieu of the fetus exposes it to placental neuromodulators, such as prostanoids and estrogen, which play a part in the changing pattern of circulating fetal and perinatal hormone concentrations.

The mechanisms for GH release and inhibition develop progressively from early gestation. Somatostatin, which is inhibitory to GH release, is detectable in the human fetal hypothalamus by 11 weeks postconception,[11,12] and somatostatin-staining neurons terminate in the median eminence by 16 weeks.[13] GH-releasing hormone–staining neurons are detected in the median eminence by 18 weeks.[14] Less is known of the development of the other hypothalamic GH-releasing factor, ghrelin (discussed later). The dominant stimulator of PRL release is the tripeptide, thyrotropin-releasing factor. However, in contrast to GH release, which is low in the absence of hypothalamic influences, PRL release is under tonic inhibition by dopamine of hypothalamic origin.[15] Dopamine is present in the human fetal hypothalamus by 10 weeks gestation, at concentrations higher than those observed in the adult hypothalamus.[16,17] Dopaminergic regulation of PRL release can be demonstrated in the fetal sheep by the beginning of the third trimester. Additionally, dopamine inhibition may derive from nonneuronal sources.[15,18,19]

GROWTH HORMONE

Growth hormone (GH) promotes postnatal somatic growth by stimulating paracrine and endocrine production of IGF-1 and also has IGF-1 independent effects on metabolism. The pulsatile release of pituitary GH is primarily due to the opposing influences of hypothalamic GH-releasing hormone and somatostatin. Circulating GH and IGF-1, in turn, can influence their own expression by negative feedback mechanisms to the hypothalamus and pituitary.

GH is detectable in human fetal plasma by at least 10 weeks postconception.[20] In species with long gestations, such as the human and sheep, high levels of GH relative to postnatal concentrations are established by midgestation. The pattern of secretion can be shown to be pulsatile in utero with both elevated peaks and nadirs and sexually dimorphic.[21] Concentrations fall gradually in late gestation and precipitously within hours of birth.[21] This pattern appears to be due to early maturation of responsivity of the fetal somatotroph to GH-releasing hormone, plus later development of inhibitory control accelerated by endocrine and metabolic events surrounding birth. The release of somatostatin and GH-releasing hormone is under control of a variety of neuromodulators and neurotransmitters. These include dopamine, which is inhibitory[22]; opiates[23]; serotonin[24]; γ-aminobutyric acid (GABA)[25]; and β-adrenergic stimuli.[26] The development of these neurotransmitter controls appears progressively during the late phase of pregnancy.[21] Immaturity of inhibitory control by somatostatin and IGF-1[9,27] contribute to the high concentration of GH observed in the fetus. Both somatostatin and GH-releasing hormone influence the sheep fetal pituitary from at least 75 days postconception,[28,29] although the data suggest that in vitro and in vivo somatostatin is less effective in inhibiting GH secretion prenatally than it is postnatally.[30,31] IGF-I levels are low in the fetal circulation because of the relative immaturity of GH receptors (GHR), thus failing to induce the negative feedback loop that would prevent GH hypersecretion.[32] Hence the fetal pituitary is "gated" to remain in a hypersecretory state.

Ghrelin, an endogenous ligand for the GH secretagogue receptor, has potent GH-secreting properties.[33] Ghrelin is primarily produced in the stomach but is also detected in the pituitary, hypothalamus, and placenta.[33] Several reports suggest a role for ghrelin in pregnancy. Ghrelin levels are higher in women with pregnancy-induced hypertension than in normotensive pregnant women,[34] which is supported by observations of ghrelin-binding studies in heart and blood vessels.[35] Its effects on fetal development are less clear—knockout mice models show that it is not essential for embryonic or fetal development under normal nutritional conditions, suggesting that its role is largely related to postnatal regulation of energy balance via appetite modulation.[36] Neverthess, ghrelin levels are high in the neonate, in whom it may have a role in the initiation of feeding and protection against neonatal hypoglycemia.[37] Ghrelin also has been detected in fetal and infant lung, where its expression peaks in the first 18 weeks gestation and decreases thereafter.[38] The role of ghrelin in controlling fetal GH secretion, however, is unknown.

In humans, GH levels are high at birth but decline rapidly within days and then progressively during the following months.[39,40] The mechanism of this decline has been studied in detail in the sheep. Free fatty acid infusions inhibit GH secretion almost totally by a direct action on the somatotroph.[27] Free fatty acid concentrations are low until birth, when they rise rapidly within a few minutes owing to the onset of nonshivering thermogenesis. In a series of experiments in which the individual events of birth have been mimicked without delivering the fetus, it has been possible to show that the initiation of nonshivering thermogenesis is dependent on oxygenation of the fetus and removal of a presumed placental inhibitor.[41,42] Data in other species are limited, but in the human term infant, thermogenesis is initiated soon after birth[43]

and increases more slowly in the premature infant, offering one explanation for the slower GH decline in preterm infants.[44]

The functional significance of elevated GH concentrations in the fetus remains uncertain—the phenotype of the child with isolated GH deficiency is rather unremarkable: generally tending to have hypoglycemia, micropenis, and perhaps a small reduction in birth length. Indeed, it has generally been considered that GH had no significant role in promoting fetal skeletal growth, and recent work has also found that in the neonate, growth is dependent on increased bioavailability of IGF-1, which is probably directly regulated by nutrition.[45] It has been postulated that the low responsiveness is due to low levels of the GHR and related signaling components.[46] In line with this, the small effect of GH in late gestation on linear growth in clinical studies[47] has been linked to the late appearance of the GHR in musculoskeletal tissues.

The physiology of the GHR has been reviewed in other publications.[48-50] Data suggest that the concentration of GHR in hepatic tissue is low until after birth, as determined in sheep,[51,52] bovine,[53,54] pig,[55] and rat,[56] and GHR appears to be induced by the peripartum glucocorticoid surge.[57] If extrapolated to the human, it would suggest that GH-dependent growth first appears after the week 34 of pregnancy.

Pantaleon and colleagues[58] have reported that transcripts encoding GH, GHR, and GH-binding protein have been detected in mouse preimplantation embryos from E1 to E4, followed by detection of GH protein at the two-cell stage. GHR is presumed to be functional at this stage in that exogenous GH administration to blastocysts promotes glucose transport and glycogen breakdown and increases protein synthesis.[58] Waters and Kaye[59] suggest that the increased glucose transport and glycogen breakdown promote the formation of ATP that fuels the Na^+ pumps and ionic gradients required for blastocoele formation and resulting cell proliferation. Reports have shown immunoreactive GHR in the human fetus from as early as 8.5 weeks fetal age.[60,61] Whether GHR is fully functional at these early developmental stages remains to be determined. GHR can be alternatively spliced to give rise to transcript variants, although not much is known about their function.[62] One common variant is an individual-specific polymorphism that leads to deletion of exon 3.[63,64] This polymorphism is associated with increased postnatal growth velocity, but interestingly, it is also associated with decreased fetal growth.[65]

GH may play a role in fetal metabolism. The hypophysectomized fetal lamb retains excess adiposity, and this is reversed by the administration of GH.[66] Similarly, GH has been shown in the fetal lamb to affect glucose/insulin metabolism[67] in a manner analogous to its postnatal diabetogenic action. In the rabbit, GH has been suggested as a stimulator of fetal hepatic glycogen synthesis.[68,69] This may be the basis of the hypoglycemia seen in human neonates with isolated GH deficiency.[70,71] Based on rodent studies, a more speculative role for embryonic/fetal GH has been proposed in relation to neural development.[72,73] GH promotes neuronal differentiation, neurogenesis and gliagenesis in the fetal rat, possibly via IGF-1 induction.[74] In line with this, GH-deficient mice show hypomyelination and microcephaly. GH also displays antiapoptotic properties in neonatal rat brains subjected to hypoxic ischemic injury. However, human studies are more equivocal on the functional role of GH; individuals with congenital GH deficiency or an abnormal GHR (and hence GH insensitivity) have apparently normal cognitive function.

PROLACTIN

Prolactin (PRL) is synthesized by lactotrophs in the pituitary, but it may also be produced in other tissues (i.e., mammary gland, ovary, decidua, pancreas, kidney, osteoblasts, lymphocytes, and so on) where it is synthesized locally (for review, see references 75, 76, and 77). In the mother, PRL is best understood for its role in milk secretion, but it has also been associated with the development of maternal behavior in rodents.[19] In the fetus, PRL has been associated with lung maturation and may have osmoregulatory properties.[19] The *PRL* gene is present as a single copy on human chromosome 6.[19] Several transcriptional and translational modifications of PRL have been described that may contribute to its ability to mediate diverse functions.

PRL is detected in both the maternal and fetal circulation. In the first trimester of human pregnancy, maternal PRL levels begin to rise in response to increasing estrogen and by term reach a level ten times that of a nonpregnant woman.[78] As PRL does not cross the placenta, the fetus produces its own PRL from early on in gestation.[79,80] PRL is first detected by 12 weeks postconception,[81-83] when levels are low; after 26 weeks postconception, they rise sharply. PRL in cord blood is markedly elevated at 170 ng/mL compared with less than 20 ng/mL in adults. A positive correlation exists between cord blood PRL levels and gestational age.[84,85] PRL levels decline by more than 60% in the first week after birth,[86] and this decline is delayed in premature infants.[87]

A similar pattern is observed in experimental animals. In sheep, PRL levels rise by 135 days gestation (term = 146 days) and decline after birth.[88,89] Data are similar for the rhesus.[90] However, in most altricial species such as the rat, plasma PRL is low at birth, and levels do not rise until 15 days after birth and peak before puberty.[91,92]

Estrogen is the primary contributor to the prenatal rise in PRL levels. Estrogen acts directly on the pituitary to promote the synthesis and release of PRL.[19] It increases the number of TRH-binding sites on the lactotroph and reduces dopamine secretion and antagonism of dopamine action.[93-96] A temporal correlation between the rise in PRL and estrogen levels late in gestation shows a lag in the rise in PRL compared with estrogen in both humans[6] and sheep. Further, at least in sheep, the interanimal variation in PRL concentrations and the seasonal and diurnal variations are difficult to reconcile, with a dominant role for estrogen in short-term regulation of fetal PRL secretion. It seems likely that estrogen exerts a long-term tonic influence on the maturation of the lactotroph and synthesis of PRL, rather than having short- or intermediate-term regulatory effects. It may be that chronic exposure to estrogen is the mechanism by which the pituitary in the second half of gestation switches from secreting virtually no PRL to maintaining a significant and progressive hyperprolactinemia.

PRL levels are also influenced by seasonal patterns.[19] They fluctuate in fetal sheep according to the length of the photoperiod, with higher concentrations secreted in longer, rather than shorter, photoperiods. It has been determined that maternal melatonin during the dark phase plays a key role in relaying this information to the sheep fetus.

PRL exerts its biologic activity by binding to the PRL receptor (PRLR), which like GHR, is a member of the cytokine receptor superfamily.[75] PRLR protein can detected in the human fetus as early as 7.5 weeks gestation, and like PRL, it is distributed in nearly all tissues.[97] Structurally, the receptor transverses the cell membrane, once with a single, short transmembrane domain, and extracellular and intracellular domains that are conserved across the cytokine receptor superfamily.[75] A single gene *(PRLR)* encoding hPRL is localized to chromosome 5 in close proximity to the *GHR* gene.[75] Human GH and PL (to be described) also bind to the hPRLR with high affinity,[98] in contrast to many other species. The PRLR is alternatively spliced to generate several short (s) and one long (l) variant, differing by length and sequence in their cytoplasmic tail.[75] In general, binding to PRLR causes activation of the JAK/STAT cell-signaling pathways and STAT-1 and -5 are directly activated by PRL.[75] The l-PRL receptor transmits a complete intracellular signal, whereas the s-PRL receptor may be a clearance form.[75] It is interesting that s-PRLR may act in a dominant negative fashion over PRLR dimerization by forming inactive s- and l-PRL heterodimers.[99] PRLR expression is modulated by

glucocorticoids[100]—cortisol stimulates the expression of l- and s-PRLR in fetal sheep,[101] and corticosterone exerts the same effect on l-PRLR in pregnant and lactating mice.[102]

It has been suggested that PRL plays a role in the development of brown fat. In placentally restricted sheep, the l-PRL receptor is reduced in fetal adipose tissue,[103] which may have an impact on nonshivering thermogenesis after birth, whereas enhanced maternal nutrition results in increased levels of l-PRL receptor in adipose tissue, as well as an increase in uncoupling protein-1 (UCP-1), a protein involved in thermoregulation.[104]

Lower levels of fetal/neonatal PRL correlate with increased risk of respiratory distress syndrome, and PRL may play a role in enhancing fetal lung maturation.[84,85,105] Lactogenic receptors are present in the fetal lung,[106] although a group has reported an inability of labeled hPRL to bind human lung preparations.[107] Infusion of PRL alone to the fetal rabbit or lamb did not appear to alter surfactant production.[108] However, PRL is synergistic with cortisol and thyroid hormones in promoting lung maturation in the sheep in vivo,[109] and this is supported by in vitro data.[110]

PRL has long been recognized as an osmoregulatory hormone in teleost fish and amphibia.[19,111] Fetal PRL secretion is dramatically affected by changes in the osmolality of fetal cerebrospinal fluid,[112] and it has been suggested that the perinatal changes in PRL secretion relate to changes in sodium homeostasis.[113] Manipulation of PRL levels in the newborn rabbit suggests that PRL enhances water retention, and that the loss of total body water in the first few days after birth could be due to the decline in plasma PRL concentrations.[114] Decidual PRL has been suggested to play a role in the regulation of amniotic fluid volume and osmolarity,[115] and it can be demonstrated to alter the permeability of the human fetal membranes to water.[116,117]

It has also been suggested that in the rodent, PRL may regulate somatic growth in the early neonate,[118,119] but no comparable data exist in larger mammals.

PLACENTAL LACTOGENIC HORMONES

The placentas of all species secrete multiple members of the lactogenic family, suggesting an important role in maintaining pregnancy. However, this role is poorly understood. In humans, members of this family include a variant form of GH and an evolutionarily and structurally related class of hormones, the PLs. In humans, the genes encoding pituitary GH *(GH-N),* placental GH *(GH-V)* (see later), and PL (*PL-A, PL-B,* and PL-like *[PL-L]*) are clustered on the same chromosome, 17q22-24. This reflects their evolutionary origin from a single somatolactogenic gene, which had also duplicated to give rise to the *PRL* gene that is now located on chromosome 6.[120] *PL-A* and *PL-B* have slightly divergent cDNA sequences, but after processing of their respective prehormones, they give rise to an identical PL hormone product. *PL-L* codes for a variant of PL that is spliced into a much shorter form, and transcripts from all three PL genes can be detected at varying abundance in the placenta.[121]

However, many species have differences in relation to the evolution of this so-called GH gene cluster, and this in turn determines the species-specific presence of PLs. Most nonprimate mammals, and the prosimian primates, possess only one GH-like gene that encodes pituitary GH.[122] However, GH (and PRL) were subjected to a period of rapid, adaptive evolutionary change in the lineage leading to higher primates.[122] The rapid evolution of GH was followed by gene duplication events that cumulatively led to the development of a complex locus for GH genes, and in humans this process enabled the emergence of placental GH and PL genes.[123] Comparative genomic studies on primates have demonstrated that the GH cluster in New World monkeys and humans arose from independent gene duplication events, and the cluster shows high variability even among New World monkeys.[124] Among ruminants, GH gene duplication has been found in sheep and goats but not cattle.[125] With respect to PRL, no similar evidence for gene duplication is available for higher primates and humans. In contrast, rodents and ruminants possess a large family of highly divergent, PRL-like genes. These clusters are less well characterized, but are known to express PLs and a broad range of placental lactogenic hormones as such proliferins.[126]

PL is detected in both the maternal and fetal circulation, but its role in fetal development is largely unknown.[121,125] In humans, PL is first detected in the maternal circulation beginning at 6 weeks gestation where its levels progressively rise to a concentration range of 200 to 300 nM at term. In a species-specific fashion, PL is functionally similar to PRL or GH: In sheep, PL is primarily somatotropic; in humans, it is primarily lactogenic. PL stimulates mammogenesis and promotes maternal insulin resistance and, thus maternal lipolysis, to ensure adequate reserves of glucose and free fatty acids for the fetus.[121] PL may also enhance maternal appetite.[127] In sheep, PL levels rise in response to maternal starvation, leading some to suggest that it stimulates repartitioning of maternal nutrients to the fetus and stimulates the fetus to use its own substrates.[128-130] In the fetus, PL may indirectly promote growth by stimulating amino acid uptake, DNA synthesis, and IGF-1 production.[121]

Although hPL is functionally more like hPRL, its sequence is similar to that of hGH. In humans and primates, the PL and GH gene families are clustered on the same respective chromosome. Human PL has more sequence identity to hGH-N (85%) than to hPRL (17%).[121] In contrast, PL in rodents and ruminants is most similar to PRL. Analogous to humans, the mouse and ovine (o) PL genes are contiguous with their PRL counterpart and share more sequence similarity with the PRL coding region than with GH (oPL versus oPRL: 48% homology; oPL versus oGH: 27% homology).[131,132] Mice also have two variants of PL—mPL-1 and mPL-2—that can substitute for pituitary PRL secretion during pregnancy. mPL-1 is first detected after implantation, when pituitary PRL expression ceases, where it assumes maintenance of the corpus luteum. By midgestation, mPL-1 expression declines and is replaced by mPL-2 for the remainder of pregnancy.[133,134] This redundancy of function bears some resemblance to the onset of hGH-V, another unique placental hormone (see later discussion).

The search for a specific PL receptor has uncovered another example of redundancy of function within the GH-PL-PRL family. No evidence is definitive for a distinct PL receptor.[135] Instead, PL is capable of binding to either homodimers or heterodimers of GH and PRL receptors. In sheep, oPL binds the GH receptor with higher affinity than oGH.[51,136,137] It has been shown in mice that mPL-1 and -2 exert their biological activity by binding to PRLR,[138-140] whereas in sheep, oPL stimulates heterodimerization between GHR and PRLR.[141] It is unknown whether an analogous heterodimer of GHR and PRLR occurs in humans.

A second gene for GH encodes a variant form found only in the placenta. Placental GH (GH-V) is synthesized by syncytiotrophoblasts beginning at around 24 weeks postconception, and in the mother gradually replaces GH expression from the pituitary (for review, see references 142 and 143). GH-V is synthesized by the placenta until term and is secreted in a continuous fashion, unlike pulsatile pituitary GH secretion. However, it would appear that unlike PL, secretion of GH-V is solely into the maternal circulation.[142,143] GH-V is not subject to regulation by GH-releasing hormone but is suppressed by elevated maternal glucose.[142,143] GH-V exerts its biologic activity by binding primarily to the GH receptor, as it does not have the Zn^+ binding site required for interaction with the PRL receptor.[144] A clear definitive role for GH-V has not been determined,[145] but in pregnancies complicated by intrauterine growth retardation, GH-V levels are decreased.[142,143] GH-V may, like PL, have a part in inducing insulin resistance in the mother to ensure adequate glucose reserves for the fetus, thereby contributing indirectly to fetal growth.[142,143]

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179

Sumana Narasimhan, Mark R. Palmert, and Leona Cuttler

Luteinizing Hormone and Follicle-Stimulating Hormone Secretion in the Fetus and Newborn

The pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), influence the function of the differentiated gonad in utero and regulate gonadal function in later life. LH and FSH are secreted by the same pituitary cell, the gonadotroph, under the influence of hypothalamic and other hormones. Both LH and FSH are glycoprotein hormones, as are thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG). All are dimers composed of two noncovalently associated protein subunits α and β. The α subunits are identical, but the β subunits are unique and confer biologic specificity. The biologic specificity of hCG, a placental glycoprotein, is similar to that of LH.

Gonadotropin-releasing hormone (GnRH; also known as LHRH or LRF) is the main hypothalamic factor controlling the secretion of LH and FSH. The major role of LH is to control steroidogenesis in the gonad and that of FSH to control gametogenesis. Congenital abnormalities or mutations in the regulation of LH and FSH can therefore lead to abnormalities of phallic development, puberty, and fertility.

This chapter reviews the anatomic development of key components of the gonadotropin regulatory axis (including the hypothalamus, pituitary, and portal circulation), the role of gonadotropins in the fetus and neonate, the factors that control gonadotropin secretion, and disorders in the development of the gonadotropin regulatory system.

DEVELOPMENT OF THE HYPOTHALAMUS AND PITUITARY

Hypothalamus

The hypothalamus (Table 179-1) develops from the primitive forebrain (prosencephalon). By approximately the fifth week after conception, the forebrain differentiates into the cerebral hemispheres and the diencephalon; the ventral aspect of the diencephalon then develops into the hypothalamus. By 14 to 16 weeks gestation, hypothalamic nuclei and fiber tracts have differentiated.[1-5]

GnRH, the major secretagogue of pituitary LH and FSH is a decapeptide released by specific neurosecretory cells in the hypothalamus and is carried by portal circulation to the pituitary.

Most studies of fetal hypothalamic GnRH content have involved fetuses from a relatively brief developmental window during early gestation to midgestation, and thus comprehensive developmental trends have been difficult to establish. The hypothalamic content of GnRH has been reported by some investigators[6,7] to increase during the first half of gestation, although others report little relationship to gestational age during this period.[8-10] Hypothalamic GnRH content generally has been reported to be similar in male and female fetuses,[6-8,11] with some exceptions.[9] Little is known about the early postnatal development of hypothalamic GnRH in humans, although Bugnon and

TABLE 179-1

Luteinizing Hormone and Follicle-Stimulating Hormone in the Human Fetus and Neonate: Early Milestones in the Anatomic Development of the Hypothalamus and Pituitary Unit

	Weeks Gestation	Comment	References
Hypothalamus			
Forebrain differentiates into cerebral hemispheres and diencephalon; the latter then develops into hypothalamus	3-5		(81)
GnRH identified in whole brain extract	4.5		(66)
GnRH neurons found in olfactory placode	5.5		(19)
GnRH identified in hypothalamus	8-13		(72)
Pituitary			
LH and FSH identified within the pituitary	9.5-11	Pituitary secretes LH and FSH in vitro 5-7 weeks	
Pituitary releases gonadotropins into circulation	11-12		
Portal circulation			
intact	11.5-12		

FSH, Follicle-stimulating hormone; *GnRH,* gonadotropin-releasing hormone; *LH,* luteinizing hormone.

TABLE 179-2

Genes or Proteins Thought to Influence GnRH Neuron Migration

Molecules secreted that may influence GnRH neurons	GABA Norepinephrine, serotonin Cholecystokinin FGF Netrin-1 Gas-6 Hepatocyte growth factor
Proteins on GnRH neuron cell surfaces	$GABA_A$ receptor Glutamate receptor FGFR GnRH receptor
Proteins on cell surfaces other than GnRH neurons	Anosmin (Kal-1)
Extracellular matrix	Heparan sulfate
Proteins in GnRH neuron cytoplasmic compartment	MAPKinase galanin
Nuclear factors	Oct-1, SCIP, GATA4, Brn2, Estrogen receptor β

Modified from Tobet SA, Schwarting GA: Minireview: Recent progress in gonadotropin-releasing hormone neuronal migration. *Endocrinology* 147:1159, 2006.

FGF, Fibroblast growth factor; *FGFR,* fibroblast growth factor receptor; *GABA,* γ-aminobutyric acid; *GnRH,* gonadotropin-releasing hormone.

colleagues[12] noted an increase in immunoreactive GnRH neurons in hypothalami of human neonates compared with those of late gestational fetuses.

The GnRH gene is expressed and the protein found in the hypothalamus during early stages of development. GnRH protein has been detected by radioimmunoassay of whole brain extracts as early as 4.5 weeks gestation.[10] GnRH has been identified within the hypothalamus by 8 to 13 weeks gestation.[7,10-13] By 16 weeks gestation, GnRH-containing neurons are known to terminate on portal vessels.[14]

Several aspects of GnRH ontogeny are noteworthy. First, GnRH neurons originate in the embryonic olfactory placode as early as 5.5 weeks gestation[15] and undergo a unique pattern of axophilic migration across the terminal nerve to their ultimate location in the hypothalamus by midgestation.[16-18] Then they extend axonal processes into the median eminence to establish neurovascular connections.

Second, GnRH neuronal migration is complex and is influenced by many genes, proteins and nuclear transcription factors. Pax6, Otx1, Otx2[19,20] are a few of the transcription factors that have been implicated in this process. They may also activate pathways essential for cell survival, growth, and differentiation. Subsequently, transcription factors such as Pax6 and Gsh2 determine brain migration route, switch on GnRH gene expression, and determine final cell position in the brain. The chemical guidance for axonal routing and GnRH neuron migration is provided by various axonal guidance molecules, extracellular matrix proteins, growth factors and neurotransmitters. Molecular pathways involved in GnRH cell migration include the Rho GTPase family, PI3K, MAPK and Ras family. (For detailed review of this process, see references 19 and 20.) The various transcription factors and other molecules that influence GnRH neuronal cell migration are summarized in Table 179-2. Studies on human GnRH cells in long-term cell cultures have shown that GnRH can also modulate the differentiation and migration of GnRH-secreting neurons in an autocrine fashion.[21] Various genes that have been implicated in GnRH neuronal migration include the product of the Kallmann syndrome gene (KAL1) located on Xp22.3 region, KAL 2 (fibroblast growth factor receptor-1[FGFR-1]),[22-24] and GPR 54 (G-protein coupled receptor).

The KAL1 gene and its product Anosmin[25] are implicated in the X-linked form of Kallmann syndrome, whereas FGFR-1 is implicated in autosomal dominant hypothalamic hypogonadism seen in males and females.[22,23,26] FGFR-1 is a member of the receptor tyrosine kinase superfamily and also regulates cell proliferation, migration differentiation, and survival. Hence, mutations in FGFR-1 are associated with other congenital malformations in addition to hypogonadotropic hypogonadism and may explain some forms of Kallmann syndrome that are not associated with anosmia. GPR 54, a G-protein coupled receptor and its ligand Kisspeptin have been described as major neuromodulators of the gonadotropic axis.[26-28] Mutations in the GPR 54 gene have been found in consanguineous families with high incidence of hypogonadotropic hypogonadism.[27,28]

Third, complex molecular mechanisms are responsible for tissue-specific transcriptional activity of the GnRH gene. The human GnRH gene (GnRH-I), located on 8p21-p11.2 and comprised of three exons encoding a protein of 92 amino acids,[29,30] is the form found in hypothalamic neurons and regulates pituitary gonadotropins. A second gene, GnRH-II encodes a decapeptide neurotransmitter in the midbrain. Other GnRH genes have been described in mammals and non mammalian vertebrates.[31] Available evidence indicates that the GnRH-I gene is targeted to hypothalamic neurons by enhancer and promoter elements acting in concert with transcription factors Otx2, Brn-2, and Oct-1[32,33] and the POU domain of transcription factors, SCIP, Pct-6 and Tst-1. A variety of hormones and second messengers also influence GnRH gene expression.[34] Also evidence shows that the orphan nuclear receptors DAX-1 and SF-1 are important for the proper development and function of the entire hypothalamic-pituitary-gonadal axis.[35,36]

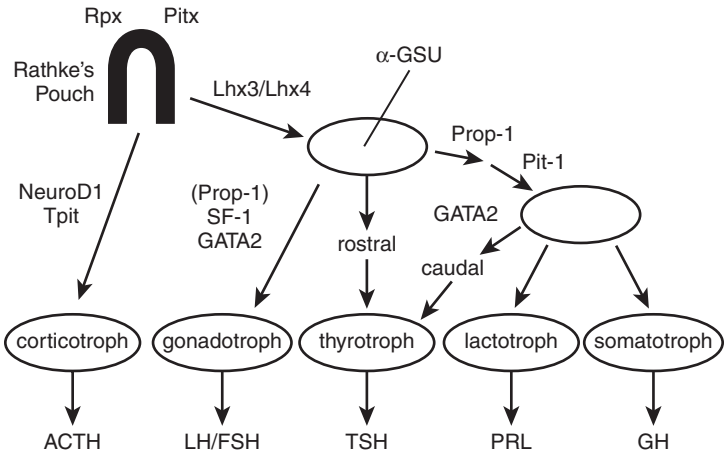


Figure 179-1 Schematic summary of pituitary-specific transcription factors involved in anterior pituitary development. RPX, also known as HESX1, is the earliest known specific marker for pituitary primordium. *ACTH,* Adrenocorticotrophic hormone; *FSH,* follicle-stimulating hormone; *GH,* growth hormone; *LH,* luteinizing hormone; *TSH,* thyroid-stimulating hormone; *PRL,* prolactin. *(From Cohen LE, Radovick S: Molecular basis of combined pituitary hormone deficiencies.* Endocr Rev *23:431, 2002.)*

Pituitary

The pituitary gland arises from ectoderm, with the adenohypophysis (anterior pituitary) originating from oral ectoderm and the neurohypophysis (posterior pituitary) deriving from the neuroectoderm.[37] Pituitary development starts on embryonic day 8.5 in the mouse. In humans, terminal differentiation of cells is completed in the first trimester, whereas in mice the process continues until after birth. Pituitary development has been identified in the human fetus by the third week of pregnancy with Rathke's pouch, the precursor of the anterior pituitary, developing as an evagination of the primitive stomodeum at the fourth week of gestation, with subsequent separation from the stomodeum by the fifth week of gestation.[38-40] The floor of the sella turcica forms by the seventh week of gestation, and, by the eighth week, the connection of the pouch with the oral cavity fully disappears.[41]

The anterior pituitary is eventually populated by at least five highly differentiated cell types that emerge in a temporally and spatially specific fashion and include the corticotrophs (which secrete adrenocorticotropic hormone), the somatotrophs (which secrete growth hormone), the lactotrophs (which secrete prolactin), the thyrotrophs (which secrete TSH), and the gonadotrophs (which secrete LH and FSH).[37,42] Intense investigation has focused on the molecular mechanisms responsible for the differentiation of the specific cell types, and two important principles have emerged from these ongoing studies: the differentiation of specific cell types in the pituitary follows a highly ordered sequence, and the coordinated expression and down-regulation of homeodomain transcription factors plays a critical role in controlling the differentiation.[37,42,43] For example, rodent studies indicate that differentiation of all cell types requires transient expression of Rpx (also called Hesx1) and Pitx, but then, on approximately embryonic day 10, the cells divide into Lhx3 dependent and independent lineages (Figure 179-1).[37,44] The main population of corticotrophs is Lhx3 independent and appears by embryonic day 12 or 13; the other cell lineages appear to be Lhx3 dependent. Under the influence of Prop-1, the Lhx3 dependent lineage further bifurcates into Pit-1 dependent and independent pathways. The Pit-1 dependent pathway leads to the differentiation of somatotrophs, lactotrophs, and thyrotrophs by embryonic day 16. The Pit-1 independent pathway, under the influence of other factors, including steroidogenic factor-1 (SF1), leads to differentiation of the gonadotrophs by embryonic day 17. In addition to intrinsic pituitary factors, evidence shows that, at distinct developmental stages, inductive processes from adjacent tissue are also important for proper cell differentiation and expansion of pituitary cell lineages.[41] In animal models, other signaling molecules such as GnRH2, ACVR2, BMP4, FGF, Wnt, and SHH have been found to be critical for pituitary development. (See references 37, 42, and 44 for detailed discussion of this complex developmental sequence.) The importance of these basic components of pituitary development to human physiology is emphasized by identification of human mutations that cause predictable phenotypes.[42]

Once formed, the gonadotroph secretes LH and FSH early in embryogenesis. The fetal pituitary secretes LH and FSH in culture at 5 to 7 weeks gestation.[45,46] LH and FSH have been detected in the pituitary by immunoassay and immunocytochemistry as early as 9.5 to 11 weeks gestation.[47-51] The pituitary content of LH and FSH then rises sharply, with pituitary LH and FSH content peaking at 25 to 29 weeks gestation in female fetuses and at 35 to 40 weeks gestation in males.[50] The pituitary content of LH and FSH is higher in female fetuses than in males between approximately 10 and 25 weeks gestation, as assessed by radioimmunoassay[48,50-52] and immunocytochemistry.[53] This sexual dimorphism may reflect testosterone production by the male fetus, with resulting inhibition of pituitary gonadotropin production.[54]

Early in gestation, the predominant pituitary glycoprotein fraction is the α subunit.[12,49,55] As gestation progresses, the relative amount of β subunit and intact gonadotropin appears to increase.[12,53,56] Relatively little is known about postnatal pituitary gonadotropin development. Kaplan and colleagues[8] found that the content and concentration of pituitary LH and FSH in 2- to 6-month-old infants were higher than in late gestational female fetuses.

It is not clear to what extent GnRH normally contributes to the functional development of the gonadotroph. In vitro, GnRH can stimulate differentiation of fetal rat gonadotrophs[57,58] and LH synthesis in cultured fetal human pituitary cells.[59] Human anencephalic fetuses have reduced pituitary gonadotropin content[8] as well as persistent predominant secretion of the α subunit of glycoproteins,[55,56] suggesting that GnRH normally contributes to gonadotroph development and function. The fetal pituitary also has potential for some differentiation in the absence of direct hypothalamic influences,[60] as suggested by studies on anencephalic fetuses[61-63] and transplanted rat pituitary.[64,65]

Portal Circulation

Work using vascular casts suggests that the hypothalamic pituitary portal system is intact by 11.5 to 12 weeks gestation,[66] earlier than originally estimated.[67-69] It has been suggested that

local diffusion may allow communication between the hypothalamus and pituitary before that time.[8]

ROLE OF PITUITARY GONADOTROPINS IN THE FETUS AND NEONATE

Fetal pituitary LH and FSH are not required for sexual and gonadal differentiation. Pituitary gonadotropins, however, are required for normal function of the differentiated gonad. Because hCG can activate LH receptors, placental hCG leads to production of testosterone early in gestation,[54,70] and this testosterone production leads to normal formation of male external genitalia by 12 to 14 weeks gestation. However, later in gestation, testosterone production by the fetal testes depends on fetal LH, and this LH-induced testosterone production is responsible for growth of the formed phallus during the later stages of pregnancy. The increased incidence of normally formed but small phallus (microphallus) and cryptorchidism in male infants with anencephaly or gonadotropin deficiency[71-73] supports the concept that pituitary gonadotropins influence testicular growth and function later in gestation, as does the finding that the testes of anencephalic fetuses are hypoplastic, with decreased numbers of testicular Leydig cells.[73-75] In females, anencephalic fetuses have been reported to show small ovaries with hypoplasia of the primary follicles.[75] Some reports suggest that the ovaries are relatively normal until 34 to 36 weeks gestation and only later show evidence of maldevelopment.[76]

REGULATION OF PITUITARY LUTEINIZING HORMONE AND FOLLICLE-STIMULATING HORMONE SECRETION IN THE FETUS AND NEONATE

Factors That Regulate Pituitary Luteinizing Hormone and Follicle-Stimulating Hormone Secretion in Adults

In adults, the secretion of pituitary LH and FSH involves the finely balanced interplay of several regulatory agents (Figure 179-2). The major elements involved in this interplay are the hypothalamus, pituitary, and gonad (a unit referred to as the hypothalamic-pituitary-gonadal axis). The main stimulus for pituitary gonadotropin secretion is the hypothalamic decapeptide, GnRH. More than 16 GnRH molecules and several GnRH receptors have been identified in mammals and nonmammalian vertebrates.[77,78] The function and tissue specificity of these various molecules is still being elucidated, but the regulation of LH and FSH secretion seems to reside primarily with the originally identified molecular species.[77] At least two forms of GnRH and two GnRH receptors are expressed in humans.

Many afferent neurotransmitter systems from the brain stem, limbic system, and other areas of the hypothalamus convey information to the GnRH neurons. They may contain norepinephrine, dopamine, serotonin, γ-aminobutyric acid (GABA), glutamate, endogenous opioid peptides, neuropeptide-Y, galanin, and other neuropeptides.[79-81] Glutamate and norepinephrine are stimulatory, whereas GABA and endogenous opioid peptides are inhibitory (see Figure 179-2). Secretions from surrounding glial cells such as transforming growth factor-α can also stimulate GnRH release.[79,82] Steroid hormones such as estradiol and testosterone can also alter the pattern of GnRH secretion by binding to steroid hormone receptors in the hypothalamus and pituitary, providing negative feedback. It is important to note that GnRH neurons themselves lack steroid hormone receptors; hence the negative feedback of steroids at the level of the hypothalamus is mediated via actions on other neural systems that, in turn, input to the GnRH neurons.[79] In addition, glucose, thyroxine, glucocorticoids, leptin, and exogenous factors such as stress, health, exercise, and nutritional factors can also influence GnRH secretion.[83]

The normal pulsatile secretion of LH is believed to be mediated directly by episodic release of GnRH. The pulsatile release of GnRH is critical for the maintenance of gonadotropin secretion in adults. Animal studies have shown that different pulse frequencies of GnRH can lead to differential ratios of FSH and LH.[79,84] Continuous administration of exogenous GnRH to adults leads to an initial increase in gonadotropin secretion, followed by prolonged suppression of gonadotropin secretion owing to down-regulation of the pituitary gonadotroph.[57,84,85] The rate of pulsatile GnRH secretion, along with ovarian hormone feedback, also influences the degree of glycosylation of LH and FSH, which can profoundly influence the physiologic function and stability of these hormones in circulation.[86,87]

GnRH travels through the hypophysial portal blood system to the anterior pituitary, where it binds to GnRH receptors on the cell surface of gonadotrophs to stimulate gonadotropin release.[79,88] The human GnRH receptor gene is localized on chromosome 4q21.2. The GnRH receptor belongs to a family of G-protein coupled receptors, and binding of the GnRH to its receptor initiates several intracellular signaling pathways including phospholipase C, inositol 1,4,5 triphosphate (IP3), protein kinase C, mitogen activated protein kinase-1 (MAPK1), calcium influx, and calcium release from intracellular stores.[89] The mobilization of intracellular calcium affects the initial burst of LH secretion and the influx of extracellular calcium maintains sustained gonadotropin secretion.[90]

Another G-protein coupled receptor, GPR54 and its ligand kisspeptin, have generated intense research.[91-95] Kisspeptin is encoded by the Kiss-1 gene. Current understanding from mouse studies indicates that Kisspeptin stimulates GnRH secretion by a direct effect on GnRH neurons, most of which express GPR54. Estradiol and testosterone inhibit Kiss-1 mRNA expression in the arcuate nucleus, whereas these steroids induce Kiss-1 mRNA expression in the anteroventral periventricular nucleus.[93,94]

Gonadal secretions are also major physiologic regulators of LH and FSH secretion in adults. Gonadal steroids exert negative feedback (inhibitory) effects on gonadotropin release. In mature premenopausal women, estrogens can also exert positive feedback, mediating the ovulatory surge of gonadotropins. In addition to steroids, the gonads produce peptides, such as inhibins A and B, that selectively modulate FSH-secreting cells. Although other regulators, such as activins and follistatin, are present in the peripheral circulation, these factors probably act in an autocrine or paracrine fashion, with activin stimulating FSH release and follistatin inhibiting FSH release by binding to activin. (For complete reviews of the regulation of gonadotropin secretion in adults, see references 79 and 96). In developing animals, the roles of the various components of this complex system are not fully understood. In the next section, each of these regulatory influences in the fetus and newborn infant is reviewed.

Factors That Regulate Pituitary Luteinizing Hormone and Follicle-Stimulating Hormone Release in the Fetus and Newborn Infant

Gonadotropin-Releasing Hormone (GnRH)

GnRH is present in the human fetal hypothalamus by 8 to 11 weeks gestation.[7,10-13,97] The secretory pattern of GnRH during human fetal and early neonatal development is not known. However, GnRH neurons from nonhuman primates secrete their peptide in a pulsatile manner in vitro, suggesting that early intrinsic pulsatile secretion of GnRH may be independent of non-GnRH neuronal synaptic input.[81,98]

GnRH appears to influence gonadotropin release early in gestation. The human fetal pituitary in vitro releases LH, FSH, or both, in response to GnRH by approximately 13 to 19 weeks

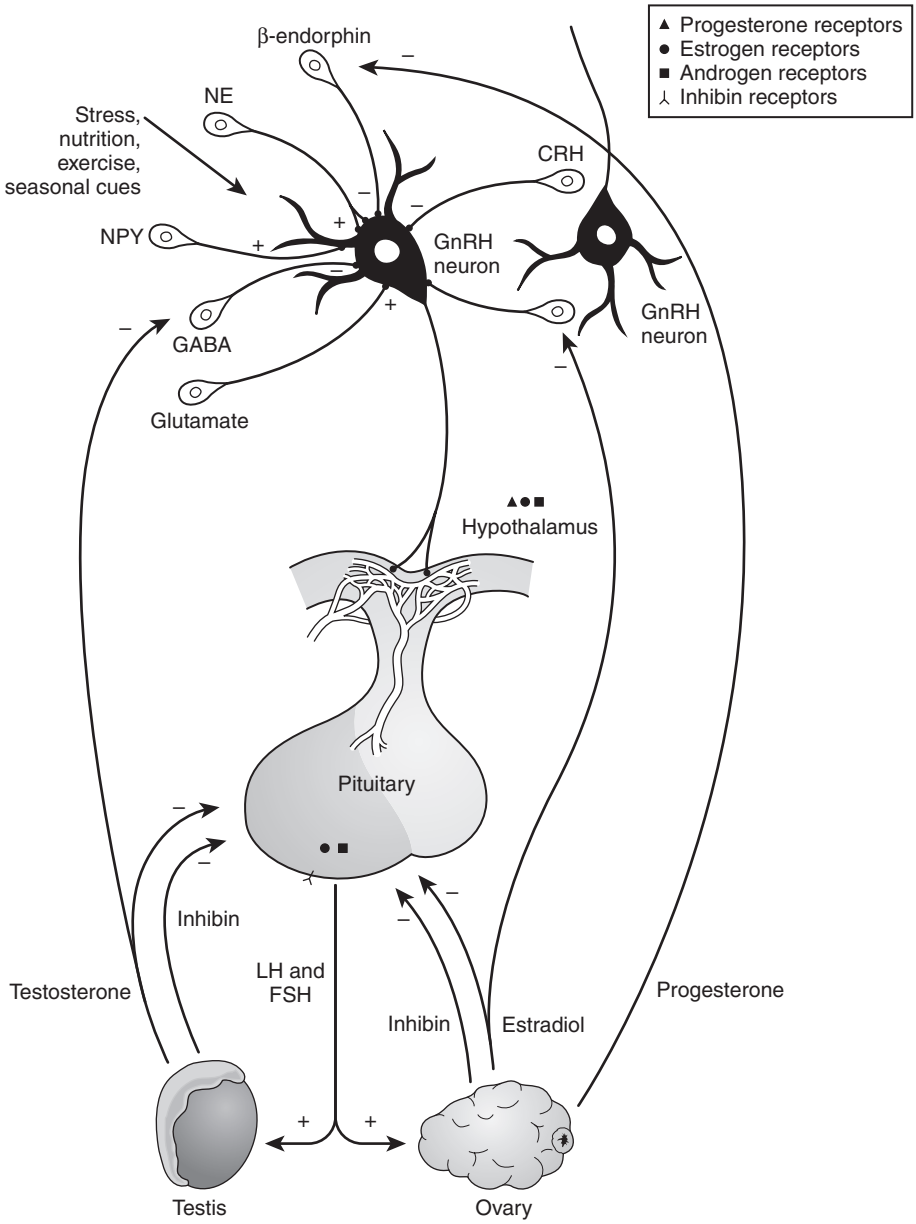


Figure 179-2 Schematic representation of the gonadotropin control system in adults. Feedback loops are indicated by *arrows;* + indicates positive feedback and – indicates negative feedback loops. The principal sex steroids in females are estradiol and progesterone; in males, the principal sex steroid is testosterone. *CRH,* Corticotrophin- releasing hormone; *GABA,* γ-aminobutyric acid; *GnRH,* gonadotropin-releasing hormone; *NE,* norepinephrine; *NPY,* neuropeptide Y. *(From Cone RD, Low MJ, Elmguist JK, Cameron JL: Neuroendocrinology. In Larsen PR, Kronenberg HM, Melmed S, Plonsky KS, [eds]:* Williams Textbook of Endocrinology, *ed 10. Philadelphia, WB Saunders, 2003, p 132.)*

gestation (Figure 179-3 to 179-6).[99-101] By the second trimester, pretreatment with estradiol enhances the sensitivity of human fetal pituitaries to GnRH.[102] GnRH appears capable of influencing LH synthesis as well as secretion at this period of development.[59]

The human fetal pituitary releases gonadotropins in response to exogenous GnRH in vivo by the second trimester, and the increment in LH and FSH levels after GnRH administration is greater in the second than in the third trimester.[103] These findings are supported by studies in monkeys[52,104,105] and sheep[106] that show the fetal gonadotroph to be responsive to GnRH, with a declining response as gestational age advances.[106,107]

In human neonates[102,103,108] and infants,[108-111] exogenous GnRH also stimulates gonadotropin release. Limited data suggest that the responsiveness of the gonadotroph to GnRH varies with age during infancy. Tapanainen and colleagues[111] administered GnRH (50 μg/m^2 intravenously) to infant boys who had undescended but palpable testes and who ranged in age from 3 to 390 days.[111] GnRH stimulated LH and FSH release in all age groups, but peak gonadotropin release occurred at 1 to 3 months of age, with a subsequent decline from 3 to 12 months of age. A similar age-dependent change in LH response to GnRH was found in infant monkeys.[104]

Gonadotropin secretion is episodic in rhesus monkey [112] and ovine[113] fetuses, as well as in human, ovine,[114] and rhesus[115] infants. In the ovine fetus, administration of a GnRH antagonist decreases LH pulse frequency.[113] Because pulsatile secretion of LH is believed to depend on pulsatile release of GnRH, these

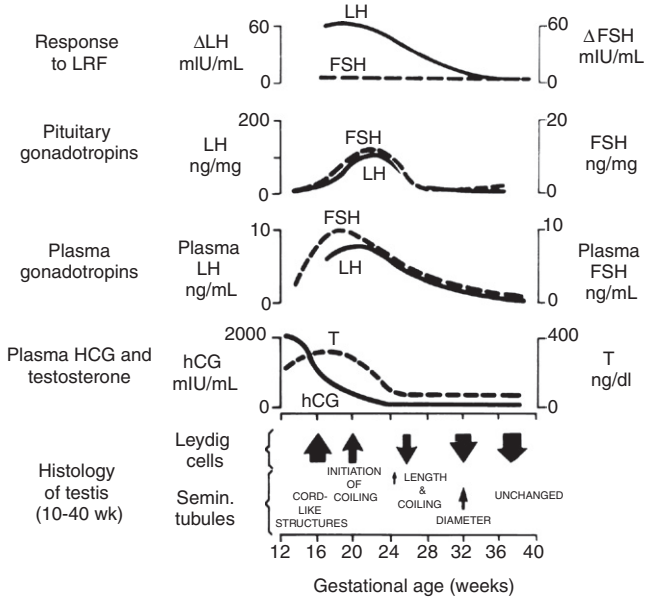


Figure 179-3 Graphic comparison of gonadotropin response to leutinizing hormone-releasing factor (LRF) (GnRH), pituitary and plasma gonadotropins, and plasma hCG and testosterone, with histologic changes in the testis of the human male fetus during gestation. *(From Gluckman PD, Grumbach MM, Kaplan SL: The human fetal hypothalamus and pituitary gland, In Tulchinsky D, Ryan KJ [eds]:* Maternal-Fetal Endocrinology. *Philadelphia, WB Saunders, 1980, p 217.)*

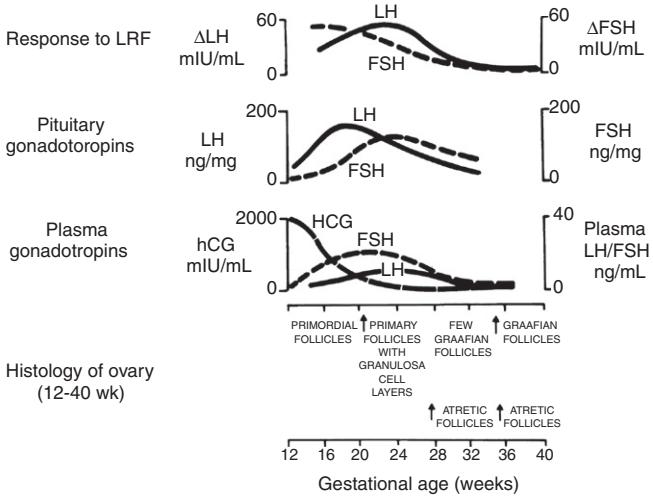


Figure 179-4 Graphic comparison of gonadotropin response to LRF (GnRH), pituitary and plasma gonadotropins, and plasma hCG, with histologic changes in the ovary of the human female fetus during gestation. *(From Gluckman PD, Grumbach MM, Kaplan SL: In Tulchinsky LD, Ryan KJ [eds]:* Maternal-Fetal Endocrinology. *Philadelphia, WB Saunders, 1980, p 218.)*

findings also suggest a role for endogenous GnRH in the regulation of LH release in the perinatal period.

The ontogeny of gonadotroph GnRH receptors in humans is not known. However, the observation that the fetal pituitary releases gonadotropins in response to GnRH by the second trimester[99,101,103,116] suggests the presence of hypophyseal GnRH receptors by that time. Animal studies on zebra fish indicate that GnRH receptors mRNA may be present as early as 36 to 56 hours postfertilization.[117] In rats, pituitary binding sites for GnRH have been detected by 13 days gestation (term = 22 days)[118] with the pituitary GnRH receptor number increasing in the late prenatal and early postnatal period.[119] Postnatally, the concentration of GnRH receptors in rodents is highest between 15 and 20 to 30 days of age and decreases thereafter to reach adult levels by approximately 60 days of age.[88] Although GnRH has been detected in the placenta,[77,120] the role of the placental material in the physiologic regulation of pituitary gonadotropin release is uncertain. Anencephalic and gonadotropin-deficient fetuses are deficient in hypothalamic—but presumably not in placental—GnRH. That these infants generally have subnormal quantities of pituitary gonadotropins,[8,121] suggests that placental GnRH may not be an important factor for the normal regulation of gonadotropin release in utero. The overall influence of neurotransmitters on gonadotropin release in the human fetus and neonate is not known, although some animal studies suggest that specific neurotransmitters such as endogenous opioids may be involved.[122]

Taken together, the available data indicate that the pituitary gonadotroph is responsive to hypothalamic GnRH during fetal life, and that endogenous GnRH stimulates gonadotropin release in the fetus and neonate.

Gonadal Steroids

From animal studies, evidence is indirect that the capacity of gonadal steroids, especially testosterone, to inhibit gonadotropin release is present during fetal development. For example, castration of male rhesus fetuses at approximately midgestation results in elevation of gonadotropin levels,[81,123,124] and early testosterone or dihydrotestosterone replacement prevents the increase of gonadotropins after castration.[124] These results suggest that functional negative feedback between the testes and the hypothalamic-pituitary axis is present by midgestation in rhesus fetuses. The role of fetal ovarian estrogen in regulating gonadotropin secretion is less clear, in part because both male and female fetuses develop in an environment that is rich in estrogens of fetoplacental origin. In female rhesus fetuses, gonadectomy has had variable results on gonadotropin secretion.[123-125] Estradiol infusions blunt LH release in the late gestational ovine fetus.[126] Gonadotropin levels, which increase in the early postnatal period, may reflect, in part, a release from negative feedback as sex steroids are removed from the fetal circulation.

By the newborn period, gonadal steroids appear to suppress gonadotropin secretion in both sexes. Castration of newborn female rhesus monkeys results in elevation of FSH and, to a lesser extent, LH levels.[127] Orchiectomy of male monkey neonates also results in elevation of LH and FSH, suggesting that testosterone may influence early postnatal gonadotropin secretion in male.[125,127-129] The specific gonadotropin secretion pattern differs between neonatal castrated male and female monkeys, and it has been suggested that this reflects intrinsic sexual dimorphism of hypothalamic GnRH secretion.[127] Similarly, gonadal failure in human neonates leads to elevated gonadotropin levels,[130] indicating that gonadal products normally suppress gonadotropin secretion even in the neonatal period. Further evidence for negative feedback by gonadal steroids derives from the observation that administration of estrogen and progesterone to extremely premature female infants suppresses LH and FSH secretion.[131]

Castration-induced elevation of circulating gonadotropins, however, is not fully sustained into late childhood, and gonadotropin levels in both experimental orchiectomy and in naturally occurring gonadal failure decline during infancy or early childhood (only to rise again in the peripubertal period).[125,128,130] However, the gonadotropin levels do not reach levels as low as those in children with normal gonads.[132] Together, these findings suggest that although gonadal steroids influence gonadotropin secretion in neonates, they are not the sole mediators of changing gonadotropin levels in infancy. The eventual decline in

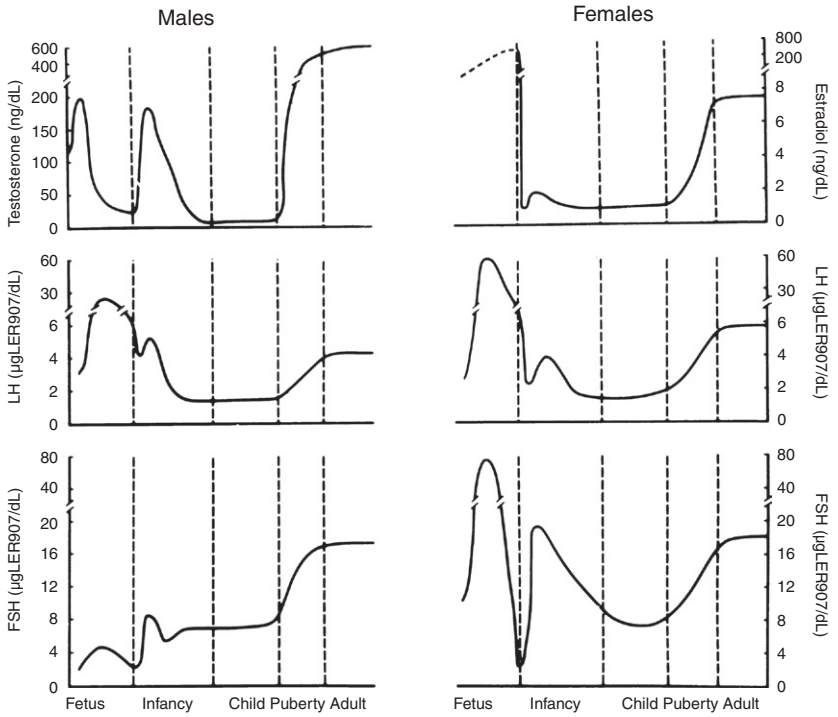


Figure 179-5 Mean concentrations of serum luteinizing hormone (LH), follicle-stimulating hormome (FSH), and estradiol during human pregnancy and postnatal development. *(From Winter JS, Hughes IA, Reyes FI, Faiman C: Pituitary-gonadal relations in infancy: 2. Patterns of serum gonadal steroid concentrations in man from birth to two years of age.* J Clin Endocrinol Metab *42:679, 1976.)*

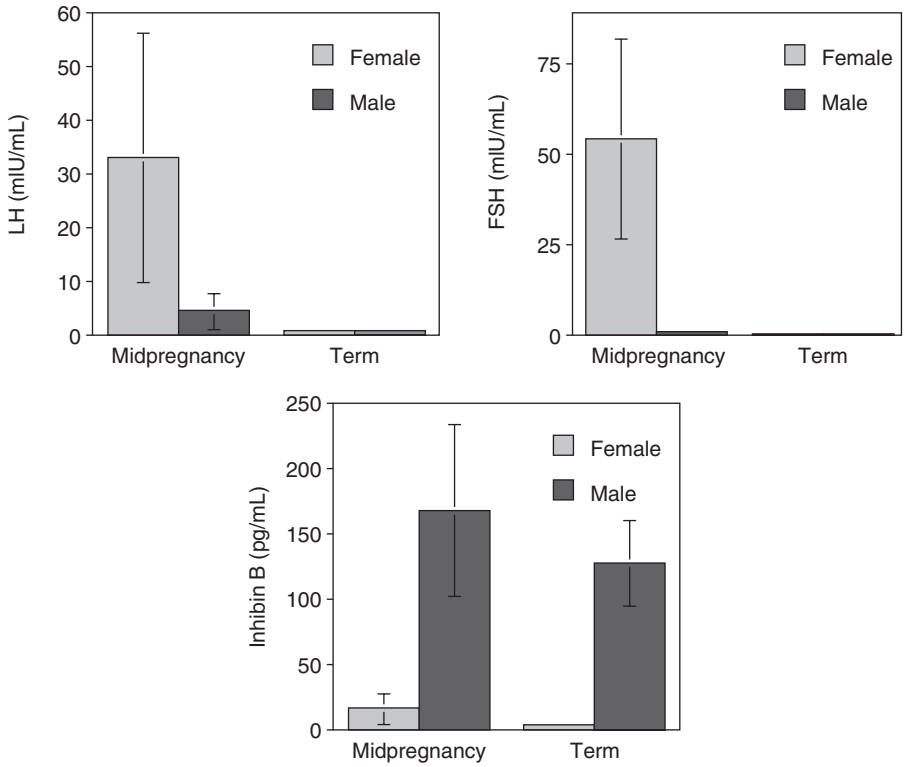


Figure 179-6 Luteinizing hormone (LH), follicle-stimulating hormone (FSH), and inhibin B levels in human fetal female and male serum at midpregnancy and term (mean ± SD). *(From Debieve F, Beerlandt S, Hubinont C, Thomas K: Gonadotropins, prolactin, inhibin A, inhibin B, and activin A in human fetal serum from midpregnancy and term pregnancy.* J Clin Endocrinol Metab *85:270, 2000.)*

gonadotropin levels in late infancy and early childhood is due primarily to extragonadal and presumably central hypothalamic-pituitary factors, in combination with some gonadal derived inhibition.[125,128,129]

Inhibin

Inhibins are polypeptides secreted by the Sertoli cells in the testis and by the granulosa cell in the ovary, which inhibit the release of FSH but not LH from the pituitary. Inhibin is also synthesized by the placenta.[133] Biologically active inhibin is present in the fetus, and administration of inhibin suppresses serum FSH.[134]

Studies have shown inhibin B in male and female fetuses at midgestation, with higher levels in males than females (Figures 179-6 to 179-8). Circulating inhibin levels are higher in premature than in term infants.[135] Inhibin B levels are higher in boys than in girls after birth.[136-138] In boys, levels peak at approximately 3 to 12 months of age and remain elevated until approximately 15 months of age.[137] The production of inhibin during the first month of life may be enhanced by the postnatal surge in gonadotropins.[50,54] Although the precise role of inhibins in the fetus and neonate is not clear, these findings suggest that endogenous inhibin may play a physiologic role in suppressing FSH in fetal and neonatal animals.[134]

PLASMA GONADOTROPINS IN THE FETUS AND NEONATE

General Pattern

Little is known about plasma gonadotropin levels in human fetuses before 10 to 14 weeks gestation. Circulating immunoreactive FSH is detectable by the 10th to 13th week of gestation in human fetuses and increases to peak levels at approximately midgestation (see Figures 179-3 to 179-6).[50,54] At that time, FSH concentrations are comparable with those of castrated animals. FSH levels then decline; by birth, FSH levels are low, similar to those of prepubertal children.[8,50,139,140] Circulating LH concentrations in the fetus follow a similar pattern.[50,51,54,140] Studies in premature infants are consistent with studies in fetuses and show a decline in serum LH and FSH levels with advancing gestational age.[135]

Soon after birth, LH and FSH levels rise (see Figure 179-5). Although reported patterns vary somewhat, this increase is sustained for weeks to months and is followed by a decline to prepubertal levels (see Figures 179-5, 179-7, and 179-8).[123,141,142] The specific patterns also differ between boys and girls, as discussed in detail later.[138,142-144]

Pulsatile secretion of gonadotropins is a hallmark feature of the mature hypothalamic-pituitary axis. Studies in the primate[112] and ovine[113,145] fetuses suggest that pulsatile secretion of gonadotropins may develop in utero. Evidence shows that gonadotropin secretion in the 1- to 3-month-old human infant is also episodic.[146,147]

Circulating hCG, the placental glycoprotein, declines from high levels at 13 to 17 weeks gestation to low levels at term (see Figures 179-3 and 179-4)[50,51,103] and falls to undetectable levels by the fifth postnatal day.[103,144] The α glycoprotein subunit is the predominant glycoprotein component in fetal plasma and cord blood and in the pituitary gland. Plasma levels of free α subunit are high at midgestation and decline near term.[55,56,148] The α glycoprotein hormone subunit may originate from cells that secrete LH, FSH, hCG, or TSH. The finding that α subunit declines postnatally[49,149] whereas plasma LH and FSH increase suggests that most of the α subunit that circulates in the fetus is derived from hCG.

Sexual Dimorphism

Sexual dimorphism characterizes both the prenatal and postnatal patterns of gonadotropin secretion.[150] In the fetus, FSH levels peak at midgestation in both sexes, but FSH levels at this time are higher in female fetuses than in male fetuses (see Figures 179-3to 179-6).* LH levels have also been reported to be higher at midgestation in females than in males[51,140,148] but not in all studies.[50,103] The subsequent decline in LH and FSH levels with advancing gestational age occurs in both sexes, and by term, the low FSH levels are similar in males and females (see Figures 179-3 to 179-6). Cord blood concentrations of both LH and FSH are low.[54,150,151]

Reports of gonadotropin levels in the postnatal period and early infancy have varied somewhat, but some generalization seems valid. After birth, FSH levels increase markedly in girls to peak at approximately 3 months of age and usually decline to prepubertal levels by 6 to 9 months,[81] although elevated levels can persist up to 2 years.[136] In contrast, FSH levels of male infants have been reported to rise less briskly, peak at 1 week to 3 months of age at levels lower than those of females,[81,98,138,144,152] and more uniformly decline to prepubertal levels by 4 to 9 months after birth.[136,144] LH peaks in boys at approximately 1 to 3 months of age and declines to prepubertal levels by 4 to 9 months.[136,144,153] Girls exhibit a similar pattern, but, in contrast to FSH, peak LH levels are generally lower than those of boys.[144] Similar patterns of gonadotropin secretion have been found in the ovine fetus[154] and in neonatal subhuman primates.[144,155,156]

Mechanisms Postulated to Be Responsible for Characteristic Patterns of Gonadotropin Levels in the Fetus and Neonate

The mechanisms underlying the characteristic changes in gonadotropin levels in the fetus and neonate are not fully elucidated. In the fetus, it has been suggested that rising levels of LH and FSH at midgestation reflect autonomous or unrestrained release of gonadotropins.[50,54] The later decline in gonadotropin levels with advancing gestational age may reflect increased sensitivity to the inhibitory effects of gonadal steroids (especially in males)[50,54]; increasing levels of circulating fetal estrogen in both sexes[54]; and reduced GnRH secretion owing to central neuroinhibitory influences, maturation of sensitivity to sex steroids, or both.[54] Data from the study of 46,XY infants with androgen insensitivity syndrome suggest that the withdrawal from the negative feedback of androgens during the neonatal transition may play a critical role in the postnatal LH and testosterone surge.[57] In a longitudinal study, infants with partial androgen insensitivity syndrome mounted the expected postnatal surges, but LH and testosterone levels failed to increase in infants with complete androgen insensitivity syndrome.[157] The early postnatal rise in gonadotropin levels had been postulated to reflect the loss of the negative feedback effect previously exerted by maternal estrogens. This mechanism now seems less likely in 46,XY infants, but the roles that estradiol and androgens play in regulation of the postnatal surge in 46,XX infants remains unclear.[158] After the postnatal surge, the reproductive endocrine axis enters a long period of relative, but not absolute, quiescence (often referred to as the "juvenile pause") until GnRH secretion re-emerges, leading to pubertal maturation. The factors causing the postnatal decline in gonadotropin levels are not clear. Several regulatory processes likely are involved, including central neuroinhibitory processes[128,129] and the potentially increased sensitivity of the hypothalamus and pituitary to the negative feedback effect of gonadal steroids.[54] Factors that may mediate the central inhibition include the relative predominance of γ-aminobutyric acid versus glutamate neurotransmitter activity and the inhibitory action of neuropeptide-Y.[81,159] Although not fully understood, it is becoming increasingly likely that the factors responsible for the juvenile pause are not simply the mirror image of the factors that lead to reactivation of GnRH secretion with puberty.

*References 8, 51, 54, 70, 103, 140, 148.

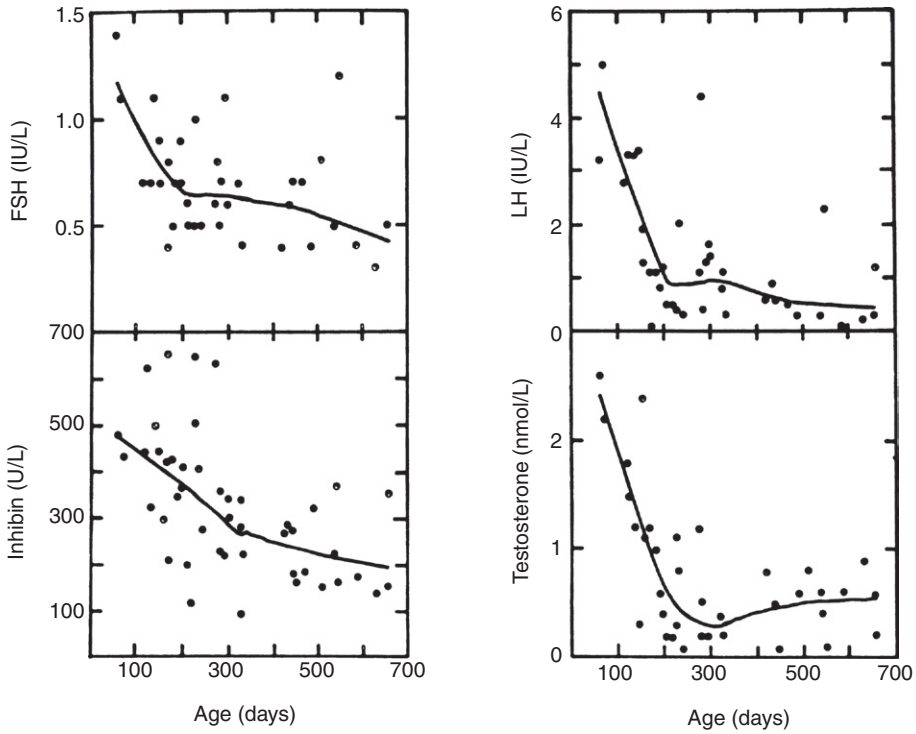


Figure 179-7 Scatterplot matrix of hormone changes with age in boys. The *continuous line* represents the robust, locally weighted regression. *(From Burger HG, Yamada Y, Bangah ML, et al: Serum gonadotropin, sex steroid, and immunoreactive inhibin levels in the first two years of life.* J Clin Endocrinol Metab *72:682, 1991.)*

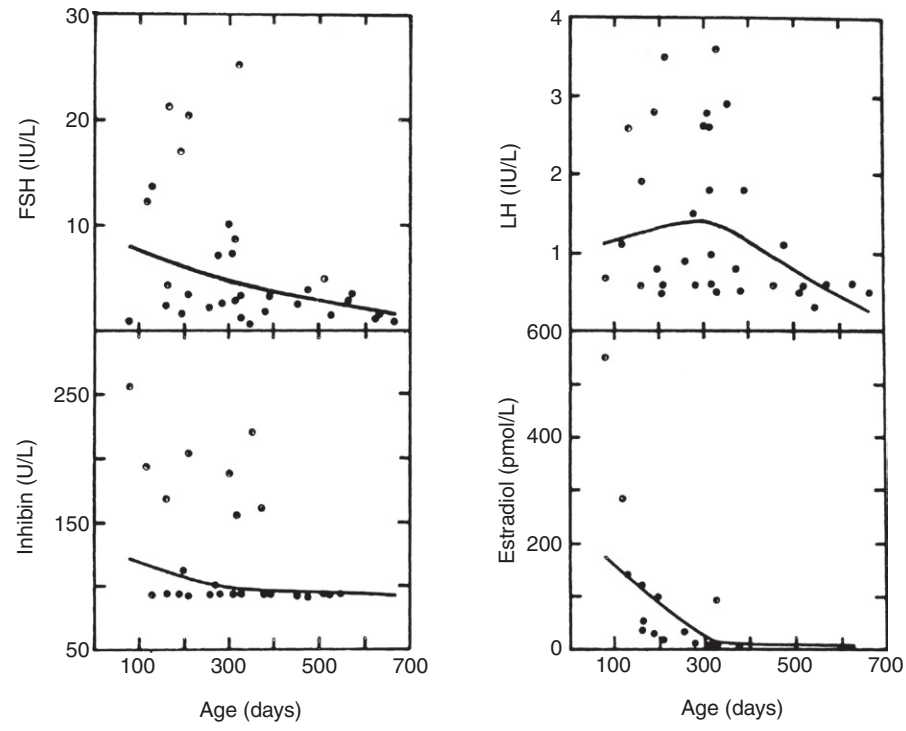


Figure 179-8 Scatterplot matrix of hormone changes with age in girls. The *continuous line* represents the robust, locally weighted regression. *(From Burger HG, Yamada Y, Bangah ML, et al: Serum gonadotropin, sex steroid, and immunoreactive inhibin levels in the first two years of life.* J Clin Endocrinol Metab *72:682, 1991.)*

CONDITIONS ASSOCIATED WITH ABNORMALITIES OF GONADOTROPIN SECRETION IN THE FETUS AND NEONATE

Anencephaly

Anencephaly is a severe defect of neural tube development that is incompatible with long-term survival. Major portions of the nervous system are absent, usually including the hypothalamus. The pituitary gland is present but small. Although the number and size of pituitary cells appear normal at week 17 or 18 of gestation, by term gonadotrophs are almost absent.[160] The pituitary content of LH and FSH appears reduced,[8,121] and is comparable with that of 10- to 20-week fetuses.[8] The α subunit appears to be the dominant glycoprotein factor.[56,121]

Anencephalic male infants often have normally formed but small external genitalia and undescended testes. This likely reflects reduced testosterone synthesis in the latter part of gestation owing to gonadotropin deficiency. The testes have reduced numbers of Leydig cells, and the epididymis is underdeveloped.[73,74] Anencephalic female fetuses have normal external genitalia, consistent with the concept that gonadotropins and ovarian secretion products are not essential for female sexual differentiation. The ovaries are small, with reduced numbers of primary follicles.[75] In anencephalic newborn infants, basal plasma LH and FSH levels are generally low compared with normal neonates,[8,151] and, in a limited number of studies, LH and FSH levels failed to increase after administration of exogenous GnRH.[8,161,162]

Together, these characteristics of infants with anencephaly suggest that pituitary differentiation may occur even in the absence of normal GnRH production, but that full activity and maintenance of the immature gonadotroph normally requires endogenous GnRH.

Congenital Hypothalamic and Pituitary Abnormalities

Hypogonadotropic hypogonadism can be caused by genetic defects at the level of the hypothalamus or pituitary. At the hypothalamic level defects of GnRH neuronal migration or defective hormone synthesis and secretion can cause disorders in hypothalamic GnRH function.

Kallmann syndrome is a genetically heterogeneous disorder of hypogonadotropic hypogonadism with hyposmia or anosmia. Various reports have indicated autosomal dominant, autosomal recessive and X-linked transmission.[23] Mutations in the KAL-1 gene have been found in 14% of familial and 11% sporadic cases of hypogonadotropic hypogonadism in males. Other phenotypic features in males with KAL-1 mutations include delayed puberty, variable degrees of anosmia or hyposmia, as well as mirror movements and unilateral renal agenesis.[30,163] Female carriers have no specific phenotypic abnormalities. Another gene implicated in Kallmann syndrome is the fibroblast growth factor receptor gene (also known as FGFR-1 or KAL2),[22,23] Heterozygous nonsense and missense mutations and small deletions of FGFR-1 have been described in familial (autosomal dominant) and in sporadic cases of Kallmann syndrome. Phenotypic features are variable and may include anosmia, delayed puberty, dental agenesis, and cleft palate.

The GPR54 gene and its ligand, kisspeptin, are important in the regulation of gonadotroph function.[28] At least four different mutations have been described in patients with autosomal recessive hypogonadotropic hypogonadism of this type, including homozygous deletion, missense, and nonsense mutations. The phenotypic features include normal sense of smell as well as other manifestations of hypogonadism.[30]

At the pituitary level, GnRH receptor mutations have been described in familial autosomal recessive, normosmic hypogonadotropic hypogonadism. They are predominantly missense mutations and result in loss of receptor function.[30]

Mutations in various pituitary transcription factors (Table 179-3) can cause gonadotropin deficiency which is usually associated with other pituitary hormone deficiencies. The reader is referred to references [42,72] for detailed descriptions.

Mutations in other genes such as the SNRPN (small nuclear ribonucleoprotein polypeptide), leptin, leptin receptor, and DAX1 have also been implicated in hypogonadotropic hypogonadism (see Table 179-3). In syndromes such as CHARGE[164] or Noonan, that are associated with hypogonadotropic hypogonadism, the exact molecular mechanism is not yet clear.

Many features of gonadotropin deficiency will not be apparent in the neonatal period. At birth, girls with congenital GnRH or gonadotropin deficiency have normal external genitalia because, as noted, female sexual differentiation does not appear to require GnRH, LH-FSH, or ovarian hormones. Males with congenital GnRH or gonadotropin deficiency (whether isolated or associated with other pituitary hormone deficiencies), tend to have microphallus and undescended testes[72] (see Table 179-3). Infants with Kallmann syndrome and other forms of gonadotropin deficiency have been reported to show low basal gonadotropin levels and subnormal gonadotropin responses to GnRH.[8,71,165] Male infants with LH receptor abnormalities are unable to fully stimulate testosterone production in response to LH or hCG signaling and can present with phenotypes ranging from complete failure of virilization to hypospadias and/or microphallus.[35] Of note, because

TABLE 179-3

Examples of Genetic Disorders Associated With Hypogonadotropic Hypogonadism

Gene	Phenotype (Inheritance; Clinical characteristics in addition to hypogonadotropic hypogonadism)
Hypothalamus	
KAL1	X-linked; Kallmann syndrome anosmia/hyposmia, renal agenesis
FGFR-1 (fibroblast growth factor receptor-1; KAL2)	Autosomal dominant; Kallmann syndrome with anosmia/hyposmia, cleft lip/palate
GPR54 (KiSS1 derived peptide receptor)	Autosomal recessive
Pituitary	
GnRH receptor	Autosomal recessive
HESX 1 (RPX)	Recessive; CPHD with septooptic dysplasia Dominant; IGHD
PROP 1 (POU 1 F1)	Recessive; CPHD
LHX3	Recessive; CPHD, rigid cervical spine
LHX4	Dominant; CPHD with defects in sella turcica and cerebellar tonsil
GLI 2	Dominant; CPHD with variable holoprosencephaly features
PHF 6	X Linked; CPHD; Borjeson-Lehmann syndrome, mental retardation
Other	
SNRPN (small nuclear ribonucleoprotein polypeptide SmN)	Lack of function of paternal 15q11-q13 region or maternal uniparental disomy; Prader Willi syndrome, obesity
Leptin	Autosomal recessive; obesity
Leptin receptor	Autosomal recessive; obesity
DAX1	X linked recessive; Adrenal hypoplasia

Modified from Grumbach MM: A window of opportunity: The diagnosis of gonadotropin deficiency in the male infant. *J Clin Endocrinol Metab* 90:3122, 2005, and from Zhu X, Lin CR, Prefontaine GG, et al: Genetic control of pituitary development and hypopituitarism. *Curr Opin Genet Dev* 15:332, 2005. Animal models of several of these genetic defects have been described.[30,42]

CPHD, Combined pituitary hormone deficiency; *IGHD,* isolated growth hormone deficiency.

neonates with congenital growth hormone and adrenocorticotropic hormone deficiency are prone to develop hypoglycemia, the association of hypoglycemia and microphallus—particularly in a full-term infant—should alert the physician to the possibility of congenital panhypopituitarism.[72]

Gonadal Failure

Gonadal differentiation and its abnormalities are described in another chapter. In this section, we refer to gonadal failure as absence of normal gonadal development or inability of the gonad to produce sex steroid, and we discuss the effects of such failure on gonadotropin secretion.

Elevated circulating levels of gonadotropins have been found in neonates and infants with gonadal failure.[130,132] In these conditions, the elevation of gonadotropins represents an appropriate response to the primary deficiency of gonadal products. For example, in the syndrome of gonadal dysgenesis (Turner syndrome), levels of FSH, and, to a lesser extent, LH are elevated above the normal range during infancy.[130] This finding is consistent with the concept that the negative feedback loop between the gonad and the hypothalamic-pituitary unit is intact in the newborn infant. In Turner syndrome, gonadotropin levels decline somewhat after age 4 years, even despite continuing gonadal failure,[130] suggesting that both central processes and gonadal factors contribute to the inhibition of gonadotropin secretion in the prepubertal child.

Similarly, elevation of basal and stimulated gonadotropin levels has been described in male infants with anorchia, rudimentary testes,[166] and other forms of primary testicular failure.[167] Interpretation of the normality of basal gonadotropin levels must be related to the age of the child. Boys with anorchia have elevated concentrations of basal gonadotropins, particularly FSH, from infancy to 4 years. The levels of gonadotropins then decrease somewhat until 9 years of age, at which time they rise and remain elevated during adulthood. The levels of FSH are lower than those seen in girls with gonadal dysgenesis. This may be attributable to the effect of fetal testosterone on the fetal hypothalamus.[168] Male infants with certain forms of androgen resistance may have elevation of LH.[169] Although many congenital disorders are associated with ambiguous genitalia or hypogonadism, information regarding associated perinatal gonadotropin secretion is often lacking.

Prematurity and Small-for-Gestational Age Status

The effects of prematurity on gonadotropin secretion are not fully understood. In preterm females, cord blood levels of LH and FSH were found to be elevated and declined with advancing gestational age to term.[170,171] For the first 10 postnatal weeks, serum levels of LH and FSH are higher in premature infant girls than in full-term girls; no difference in gonadotropin levels between premature and term male infants has been noted.[170,172] Hypersecretion of FSH has been reported in infants born small for gestational age.[172,173]

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James C. Rose, Luke C. Carey, and Jeffrey Schwartz

Development of the Corticotropin-Releasing Hormone–Corticotropin System in the Mammalian Fetus

Since the publication of the last edition of this chapter, advancements in understanding the importance of adrenocorticotropic hormone (ACTH) or corticotropin in the maturation of the adrenal cortex have been significant. In a large part, this has been due to the application of gene manipulation approaches that have enabled study of the effects of selective deletion of proopiomelanocortin (POMC; the precursor of ACTH) and the impact of replacement of ACTH on adrenal function. In addition, the impact of prenatal manipulations on the corticotropin-releasing hormone (CRH)-ACTH-adrenal axis in adulthood (i.e., "programming") has become an area of intense interest. Consequently, this chapter focuses on the CRH-ACTH system, and we refer readers to earlier versions of the chapter for information pertaining to β-endorphin during development. However, it should be noted that immunoreactive β-endorphin is frequently secreted with ACTH and appears to be under similar regulatory control. Also, when reviewing the information presented in this chapter, it is important to remember that antibodies used in the standard immunoassays employed for ACTH measurement frequently crossreact with other peptides derived from POMC, which contain similar amino acid sequences to corticotropin. Thus immunoreactive ACTH values reported in the literature may not always reflect only the fully processed and bioactive form of ACTH, namely ACTH[1-39].

A general scheme of the regulation of the brain-pituitary-adrenal axis is shown in Figure 180-1. The primary secretagogues that stimulate secretion of ACTH from corticotropes in the anterior pituitary are CRH and arginine vasopressin (AVP). These peptides are released from neurons in the paraventricular nucleus, nerve endings of which abut the hypothalamopituitary portal vessels. The peptides are carried in the portal blood to the anterior pituitary, where they stimulate the corticotropes. The release of CRH and AVP is governed by a variety of inputs into the paraventricular nucleus. From a developmental standpoint, an anatomical maturation of the system as well as maturation of the physiological regulation occurs. In general, corticotropes release ACTH in response to stimulation from early in development, but the ability of ACTH to stimulate adrenal glucocorticoid secretion appears relatively later in gestation.

The primary precursor of ACTH is POMC (Figure 180-2). This peptide undergoes a series of enzyme-induced cleavages resulting in the formation of several peptides that vary depending upon the site of origin. For example, in the anterior-pituitary corticotropes, both pro-ACTH and β-lipotropin are formed from POMC via the action of the enzyme prohormone convertase 1 (sometimes called prohormone convertase 3). Pro-ACTH is then further cleaved to yield other peptides as well as ACTH. The enzymatic processing of pro-ACTH to ACTH appears to require hypothalamic input to the pituitary as it is abolished by lesions

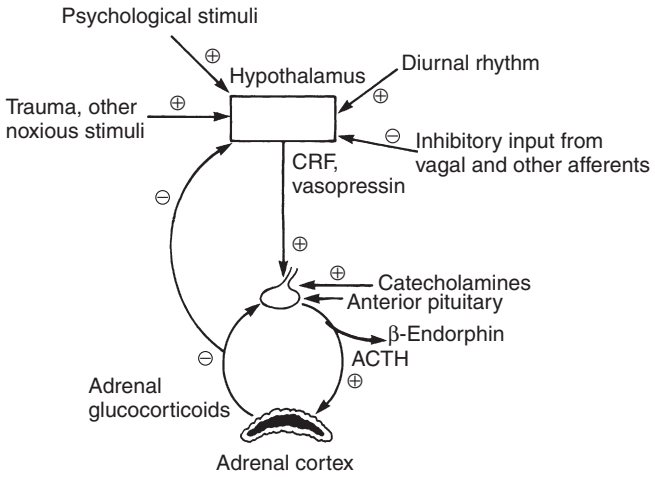


Figure 180-1 Diagram of regulation in the corticotropin-releasing factor *(CRF)*/corticotropin *(ACTH)* axis. *(Modified from Gannog W:* Review of Medical Physiology, *ed 13, Norwalk, CT, 1987, Appleton & Lange, p 314.)*

destroying the paraventricular nucleus in the hypothalamus.[1] In the anterior lobe, ACTH[1-39] is a terminal product, whereas in the melanotrophs of the intermediate lobe, ACTH is further cleaved to α-melanotropin, which can act on receptors distinct from the adrenal ACTH receptor (melanocortin-2 receptor). However, because the role of melanocortin in development is not clear at this time, it will not be discussed in this chapter.

DEVELOPMENT

During the course of pregnancy, significant changes occur in both the maternal and fetal CRH-ACTH systems. These changes play critical roles in the process of fetal maturation.

Corticotropin-Releasing Hormone

The neurovascular link between the hypothalamus and pituitary is first apparent at 12 weeks gestation (G; 0.30 G = 40 weeks) in the human fetus. By 16 weeks (0.40 G), nerve fibers in the rostral region of the median eminence are weakly stained by CRH antiserum. These CRH positive fibers increase in number between 18 and 30 weeks gestation and terminal dilatations in perivascular areas can be observed by midgestation. Cell bodies containing CRH are difficult to identify and have not been detected before 19 weeks (0.48 G).[2]

CRH-containing neurons have been identified in the ovine fetus at a stage comparable with that in humans (0.38 G versus 0.34 G). Both CRH peptide and mRNA levels are increased close to term in the fetal sheep hypothalamus. Further, specific changes in CRH mRNA expression within the paraventricular nucleus have been noted. In other regions of the fetal brain (hippocampal-amygdala complex and frontal cortex), CRH mRNA expression remains relatively constant, whereas peptide levels increase in late gestation. In the brainstem, where CRH is important for autonomic functioning, CRH mRNA expression does not change markedly with advancing gestation; however, increased polyadenylation of CRH mRNA in the hypothalamus is apparent.[2] The physiological significance of this increase in poly(A) tail length is not completely understood, but may relate to increased stability or translatability of the message.

Studies in fetal sheep indicate that hypothalamic CRH peptide and gene expression appears to be regulated by glucocorticoid-mediated negative feedback, as is the case in adults. However, the efficacy of this feedback may decrease with increasing gestational age. This would explain how, in spite of high prevailing plasma cortisol levels, the hypothalamic drive stimulating ACTH secretion does not decrease in the late gestation fetus.

CRH mRNA is present in the human placenta, where expression increases in the last 5 weeks gestation.[3,4] The CRH gene is found on chromosome 8 in humans. Substantial similarity between species related to conservation of the promoter region,[3,5] which contains regulatory elements for cyclic adenosine monophosphate (cAMP), half-estrogen response elements, and ecdysone. The cAMP element appears functional because both forskolin (which increases cAMP) and the cAMP analogue 8-Br-cAMP increase CRH expression in syncytiotrophoblast cells. Conversely, deletions or mutations of the consensus cAMP regulatory element in placental cells transfected with the CRH promoter lead to a decreased cAMP response.[3] Glucocorticoids, progesterone, and possibly estrogen may play roles in regulating placental CRH expression. Immunoreactive and bioactive CRH-like activity has also been detected in sheep placenta, where immunoreactive CRH is localized in the syncytiotrophoblast and intermediate trophoblast layers.[2,3]

In human maternal plasma, CRH concentrations remain at nonpregnant levels (<50 pg/mL) for much of gestation, before increasing dramatically (approximately 20-fold) close to term. This increase is a consequence of corresponding heightened placental, as well as decidual and fetal membrane secretion,[6] and decreased plasma CRH-binding protein levels. After delivery, maternal plasma CRH levels decline rapidly.[7] Maternal plasma CRH concentrations increase earlier in preterm delivery, intrauterine growth restriction, and preeclampsia. This suggests that maternal plasma CRH levels may reflect fetal well-being.

Human fetal plasma CRH concentrations are elevated compared with neonatal levels, but do not appear to change with advancing gestation, and are lower than maternal concentrations.[8,9] CRH concentrations are higher in the umbilical vein than artery. CRH in the fetal circulation is thought to be almost exclusively of placental origin.[8] The disparity between maternal and fetal plasma CRH concentrations during gestation suggests that differential regulation of CRH production and secretion in the maternal and fetal components of the placenta occurs.[8] Fetal

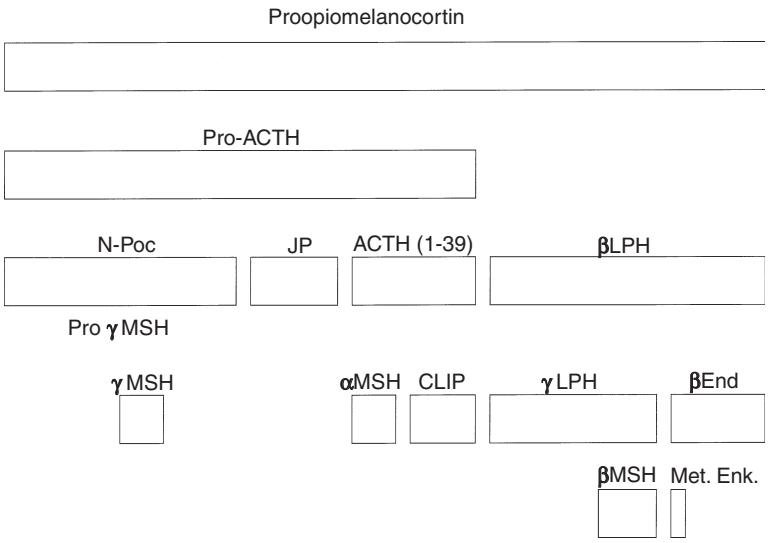


Figure 180-2 Schematic representation of proopiomelanocortin *(POMC)* and POMC-derived peptides. *N-Poc* (also called pro-γMSH) is the aminoterminal end of POMC. *CLIP,* Corticotropin-like intermediate lobe peptide; *JP,* joining peptide; *LPH,* lipotropic hormone; *Met Enk,* methionine enkephalin; *MSH,* melanocyte-stimulating hormone.

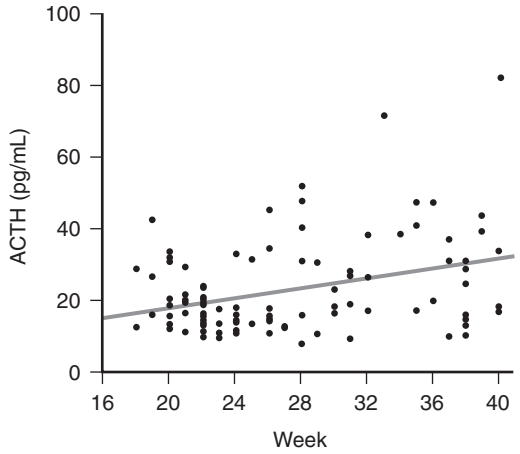


Figure 180-3 Human fetal plasma corticotropin concentrations with advancing gestational age. *(From Lockwood CJ, Radunovic N, Nastic D, et al: Corticotropin-releasing hormone and related pituitary-adrenal axis hormones in fetal and maternal blood during the second half of pregnancy,* J Perinat Med *24:243, 1996.)*

plasma CRH-binding protein levels decrease between midgestation and term.[10]

CRH exerts its effect by binding to CRH-R1 and CRH-R2 receptors, both of which have specific isoforms. As in the pituitary, CRH receptors are expressed in the placenta.[7] CRH-R2 receptors have also been detected in myometrium from pregnant, but not nonpregnant, individuals.[7] Cytokines, glucocorticoids, neurotransmitters, and peptides stimulate CRH release, but nitric oxide and progesterone inhibit release from placental tissues.[11]

Corticotropin

Human maternal plasma ACTH concentrations increase moderately in late gestation as do cortisol concentrations.[6,12] The extent of increase, however, is disproportional to the massive increase in circulating placental-derived CRH.

ACTH is detected in human fetal plasma by 16 weeks of gestation.[2] Some reports suggest that plasma concentrations tend to increase slightly with advancing gestation[9] (Figure 180-3); others have indicated that change is little or none. Given that fetal plasma cortisol concentrations tend to increase in the same period, it would seem logical for ACTH concentrations also to be elevated, at least to some extent. Indeed findings from comprehensive animal studies (see later discussion) have revealed that ACTH concentrations (specifically bioactive ACTH) increase significantly in late gestation. Fetal plasma ACTH levels are typically equal to, or higher than, maternal plasma concentrations.

Study of human anencephalic fetuses (in whom plasma ACTH is not detectable) suggests that all ACTH circulating in fetal plasma is of pituitary origin.[2] ACTH does not appear to readily cross the placenta from mother to fetus.[2]

In general, study of ovine fetuses has revealed that although plasma immunoreactive ACTH levels do not change markedly during gestation, an increase in bioactive ACTH$^{1\text{-}39}$ close to term is significant, coinciding with the plasma cortisol surge.[13] Moreover, the ratio of bioactive ACTH to ACTH precursors (including POMC and pro-ACTH) is elevated. This altered processing of POMC would appear to be related to changes in prohormone convertase 1 distribution and expression in the anterior pituitary. The percentage of anterior pituitary cells expressing prohormone convertase 1 increases significantly in the final 20 days gestation in keeping with the heightened bioactive ACTH concentrations evident at this time.[1]

Various manipulations in fetal sheep have demonstrated the importance of hypothalamic input in the control of ACTH secretion. Both surgical isolation of the fetal pituitary from hypothalamic influence (hypothalamic pituitary disconnection) and paraventricular lesioning interfere with the ontogenic increase in bioactive ACTH, the ratio of bio- to immunoactive ACTH, and cortisol.[1,14,15] These findings underline the importance of hypothalamic secretagogues in regulating POMC processing and ACTH secretion in the late gestation fetus. Further, it appears that AVP plays a critical role in mediating the late gestation increase in the ratio of bio- to immunoactive ACTH as pituitary responsiveness to AVP increases while responsiveness to CRH decreases close to term in the fetal sheep.[16,17]

Studies With CRH, CRH-R1, and POMC knockout mice

Mice genetically manipulated to lack expression of CRH, CRH-R1, and POMC (knockouts; CRH−/−, CRH-R1−/− and POMC−/−) offer insight into the importance of these peptides for general and HPA axis development in the fetus. When mice heterozygous for the CRH gene (CRH+/−) are mated, the resultant CRH−/− fetuses develop normally, and indeed when born are indistinguishable from CRH+/+ and CRH+/− littermates.[18] These mice do, however, exhibit impaired adrenal responsiveness to stress.[18,19] POMC expression is not different between CRH−/− and CRH+/+ fetuses in late gestation,[20] which suggests that CRH is not required for corticotroph development. CRH−/− fetuses in CRH−/− dams die soon after birth due to fetal glucocorticoid insufficiency unless exogenous glucocorticoids are provided to sustain fetal lung development.[18] For similar reasons, CRH-R1−/− progeny from CRH-R1−/− dams also die within 48 hours after birth.[21] These findings not only reflect the importance of glucocorticoids for fetal lung maturation, but also CRH (especially maternal) in mediating glucocorticoid synthesis during pregnancy.

Mouse POMC−/− offspring derived from POMC heterozygous parents are also viable and fertile, indicating that POMC and its derived peptide are not essential for fetal survival.[22,23] These mice are born with completely dysfunctional adrenal glands, as evidenced by the lack of corticosterone (and other adrenal steroid) production.[24] In adult POMC−/− mice, it has been demonstrated that chronic exposure to exogenous ACTH can fully restore adrenal functionality, morphology, and size.[25] In contrast, POMC alone exerts no such restorative effects. Although similar "replacement/rescue" interventions have not been applied during pregnancy, one would presume that exogenous ACTH should promote normal adrenal development in the POMC−/− fetus. Evidence favoring the singular importance of ACTH in regulating fetal adrenal maturation is also apparent in the findings of studies in which fetal sheep are subjected to hypothalamopituitary disconnection. These fetuses have low plasma levels of bioactive ACTH and their adrenals at term display reduced ACTH receptor expression and steroidogenic capacity, resembling adrenals in 120-day-old fetuses. Treatment of these fetuses with ACTH alone fully restores adrenal cortisol production, thereby demonstrating the primary importance of ACTH in fetal adrenal development.

REGULATION

A number of studies have examined the changing ratios of POMC-derived peptides, such as N-POMC$^{1\text{-}77}$ and pro-ACTH (as well as POMC itself) to ACTH$^{1\text{-}39}$ as a function of gestational age and stimulation by CRH or AVP. The increase in total immunoreactive ACTH, which may occur toward term, is paralleled by a small but definite increase in the concentration of ACTH$^{1\text{-}39}$ relative to either N-POMC$^{1\text{-}77}$ or the biosynthetic precursors POMC

and pro-ACTH. A complementary finding has been observed in vitro with fetal sheep anterior pituitary slices secreting increasing amounts of $ACTH^{1-39}$ relative to precursors as a function of gestational age. These findings suggest that the capacity of pituitary corticotrophs to synthesize, process, and secrete $ACTH^{1-39}$ increases as development progresses. Indeed, when a sensitive bioassay is used to measure ACTH activity in fetal plasma, levels increase between 100 and 140 days of gestation.[13]

A definite change in ACTH responsiveness to secretagogues occurs during development. Cultures of pituitary cells from immature sheep fetuses (100 to 115 days gestation) respond robustly to CRH, and very modestly to AVP. In contrast, cultured cells from mature fetal pituitaries respond less dramatically to CRH but markedly to AVP. This developmental change in the capacity of these secretagogues to stimulate ACTH release is not evident in cells from mature fetuses adrenalectomized at approximately 120 days gestation. These cells show a pattern of secretion resembling that of cells from immature fetuses. A probable explanation for these observations is that the increase in cortisol normally seen at the end of gestation promotes the change in ACTH secretory responses to CRH and AVP; when the cortisol increase does not occur late in gestation, corticotrophs maintain an immature profile of responsiveness.[16]

The developmental changes in the overall secretory pattern are universal at the single cell level. CRH causes more corticotrophs to secrete ACTH in pituitaries from immature fetuses, whereas AVP stimulates a greater number of cells to secrete ACTH in mature pituitaries.[17] Also, in the immature fetal pituitary, ACTH-secretory responses to CRH or AVP can be totally accounted for by the recruitment of erstwhile nonsecreting cells to secrete. However, in term pituitaries the response to AVP involves both recruitment and an increase in the average amount of ACTH secreted per cell.[17] This change in CRH-responsiveness is probably due to decreased pituitary CRH receptor expression considering that CRH-R1 receptor mRNA and protein decrease throughout late gestation and in response to cortisol treatment.[26]

Age related changes in responsiveness have also been detected in vitro, with the largest increments in ACTH release occurring in pituitary cells from earlier gestation fetuses. It is noteworthy that fetal sheep pituitaries isolated at 0.43 gestation exhibited ACTH responses to CRH. This approximates the period of development when CRH can first be shown to exert an effect on ACTH release in human fetal pituitary cells.

For glucocorticoids to affect activity of the CRH-ACTH axis, glucocorticoid receptors should be expressed in the fetal pituitary or in brain regions thought to be important for regulation of the system. The presence of specific glucocorticoid binding sites has been reported in cytosolic preparations of fetal pituitary, hypothalamus, and hippocampus. Receptors are expressed as early as midgestation in all three tissues, and the concentration of binding sites does not vary uniformly with gestational age. However, receptor levels are higher in the fetus than in the adult. The association constants calculated by Scatchard analysis of the data indicate a high affinity binding site and sucrose density gradient profiles of the ligand-binding site complex that are characteristic of a glucocorticoid receptor. Thus at least a portion of the biochemical machinery essential for glucocorticoid modulation of the CRH-ACTH system is present by 0.5 gestation.[2]

Incubation of pituitary cells in the presence of dexamethasone for 24 hours results in suppression of basal ACTH release and CRH-induced ACTH secretion. These observations suggest ACTH secretion in the early gestation human fetus is primarily regulated by CRH and that adrenal glucocorticoids exert negative feedback on ACTH secretion. Plasma immunoreactive ACTH levels in the fetal sheep remain relatively low during the last third of gestation. However, in the 24 to 48 hours preceding parturition, a marked increase in ACTH occurs. This increase in ACTH is unique because it occurs in spite of the high prevailing plasma cortisol concentrations apparent at this time. The explanation for this increment in ACTH is currently unknown but may be related to the aforementioned increase in pituitary responsiveness to AVP, and associated changes in postreceptor signal transduction. Specifically, evidence suggests that the amount of second message (inositol trisphosphate) generated upon AVP/AVP-R binding increases with advancing gestational age (Figure 180-4).

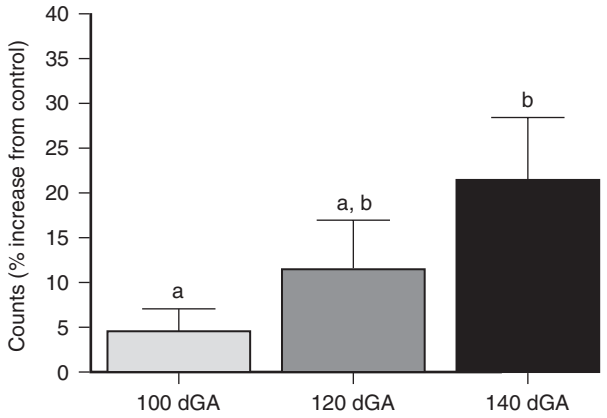


Figure 180-4 Ontogeny of inositol trisphosphate *(IP3)* formation in ovine pituitary cells after stimulation with arginine vasopressin *(AVP).* Cells were isolated from fetuses at 100, 120, and 140 days gestational age (dGA). Control cells were unstimulated. Groups with different letters are significantly different ($p < .05$), n = 5-7 fetuses per group. *(Modified from Carey LC, Tatter SB, Rose JC: Ontogeny and effects of hypothalamic pituitary disconnection on formation of inositol trisphosphate in fetal sheep pituitary cells,* Endocrinology *148:1440, 2007.)*

Consistent with the ability of glucocorticoids to inhibit ACTH secretion is the observation that cortisol infusion blocks CRH-induced ACTH release in the sheep fetus. The ACTH response to AVP has also been demonstrated to be impaired by synthetic glucocorticoids in the ovine fetus, and dexamethasone inhibits ACTH release after the simultaneous injection of CRH and AVP. The ability of glucocorticoids to inhibit CRH and vasopressin-stimulated release of ACTH in vivo has been confirmed in vitro.[2] These findings, however, appear inconsistent with the previously noted increase in pituitary responsiveness to AVP close to term that occurs in the presence of high endogenous plasma cortisol concentrations. Why such changes are not apparent after administration of synthetic glucocorticoids earlier in gestation is unclear, but this may be related to acute versus chronic exposure and/or naturally occurring as opposed to synthetic glucocorticoids.

FETAL PROGRAMMING AND THE HYPOTHALAMUS-PITUITARY-ADRENAL AXIS

Timely activation and inhibition of the hypothalamus-pituitary-adrenal (HPA) axis plays a key role in coordinating the development and maturation of various organs. Probably as a consequence of that role, the timing of HPA activity is an essential element of *fetal programming*. Programming is the process whereby early adaptation to adverse environments results in permanent physiological changes that may be beneficial during fetal life but are less advantageous postnatally. However, because programming is associated with both precocious exposure to glucocorticoids before birth and elevated HPA axis activity after birth, it remains an important aspect of investigation of the regulation of the HPA axis in fetuses. Accordingly, this chapter includes descriptions of some of the most relevant research models used to simulate the conditions associated with altered activation of the fetal HPA axis. Although a comprehensive review of all the research into

TABLE 180-1

Representative Studies Using Models of Fetal Programming That Demonstrate Altered Hypothalamus-Pituitary-Adrenal Axis Activity During Development or in Postnatal Life

Model	Species	Timing of the Finding(s)	Broad Finding(s)
Smoking	Human	Birth	Increased ACTH in umbilical blood of tobacco-exposed neonates[70]
Long term hypoxia	Sheep	Late gestation	Slightly elevated or unchanged plasma ACTH levels; increased adrenal sensitivity to ACTH[53-59]
Exposure to glucocorticoids	Rat	Birth	Decreased plasma ACTH and corticosterone; decreased mRNA for CRH in PVN[48]
	Sheep	105 days gestation	Previous exposure to dexamethasone blocks ACTH and cortisol responses to acute hypoxia[49]
		128 days gestation	2 days after exposure to glucocorticoids, basal plasma cortisol increased and an increased ACTH response to acute hypoxia[50]
		6 to 12 months postnatal	Prenatal exposure to betamethasone blunts the increase in the ACTH response to CRH and AVP that ordinarily occurs between ages 6 and 12 months[71]
		130 days gestation	Increased mRNA for GR and MR in the hippocampus[72]
	Guinea pig	52 days gestation	After exposure to dexamethasone on days 50 and 51, increased plasma cortisol and increased mRNA for GR and MR in the hippocampus of female fetuses; male fetuses have decreased plasma cortisol[51]
IL-6 exposure	Rat	As adult offspring	Increased basal plasma ACTH and corticosterone; decreased mRNA for MR and GR in PVN; increased mRNA for CRH receptor in pituitary[73]
Multiple pregnancy	Sheep	130 days gestation	Twins have lower basal cortisol and cortisol responses to acute hypoxia[38]
Undernutrition (periconceptional)	Sheep	Birth	Precocious activation of HPA axis; premature delivery[60]
Undernutrition early in pregnancy	Sheep	Age 85 days	Increased basal plasma cortisol[74]
		126-129 days gestation	Decreased ACTH, cortisol responses to acute hypoxia[61]
		113-127 days gestation	Decreased ACTH, cortisol responses to CRH and AVP at 113-116 days and decreased cortisol response at 126-127 days[40]
Undernutrition late in pregnancy	Rat	Neonate	Decreased adrenal weights and decreased mRNA for GR and MR in hippocampus[42]
Ethanol	Rat	Age 45 and 60 days	Increased HPA responses to immunological challenges[75]
	Rat	Age 22 days	Increased ACTH response to stress; possibly not mitigated by neonatal handling[76]

NOTES: This table is not meant to be comprehensive in terms of models or experimental findings. Rather, it is intended to illustrate the breadth of models and findings that relate changes in fetal HPA axis activity to programming.

Gestation in sheep is approximately 145-150 days, in guinea pigs 70 days.

ACTH, Adrenocorticotropic hormone; *AVP,* arginine vasopressin; *CRH,* corticotropin-releasing hormone; *GR,* glucocorticoid receptor; *HPA,* hypothalamus-pituitary-adrenal; *IL,* interleukin; *MR,* mineralocorticoid receptor; *PVN,* paraventricular nucleus.

the fetal HPA axis and programming is far beyond the scope of this chapter, it focuses on a few of the key, recent lessons learned from that research (Table 180-1).

Generally, in acute situations, the fetal HPA axis responds similarly to its postnatal counterpart. For example, hypoxia or hypoglycemia will be detected in the central nervous system leading to activation of the HPA cascade and resultant elevated glucocorticoid levels. Acute responses would appear to be part of the ordinary developmental process and not associated with programming. In contrast, adaptations to chronic situations may be different. It is possible that these are associated with programming not only because of the temporal element but because they potentially contribute to establishing a trajectory for growth. For example, prolonged hypoglycemia at a vulnerable time might signal a trajectory for growth in a nutritionally challenged environment, with the consequence of altering mechanisms for the uptake of glucose with increased efficiency. The HPA axis is a fetal organ system that also responds differently to chronic stimuli and, in doing so, contributes to the programming process.

Among the most important animal models developed for this research are those in which metabolic substrates, such as oxygen or protein, are restricted during gestation. For various, obvious reasons, numerous species have been used, with the most common being rats and sheep. These models include those of global substrate reduction, accomplished by placental embolism,[27,28] placental restriction through the surgical excision of attachment sites,[29-31] uterine artery ligation,[32,33] cord occlusion,[26,34] or multiple pregnancy (in monovulatory species).[35-38] In another important category of models of programming, specific substrates are limited. These include decreasing maternal food or protein intake,[39-42] insulin-induced hypoglycemia,[43] and decreased oxygen availability via gestation at altitude,[44] or decreasing the fraction of maternal inspired oxygen.[45,46]

In addition to limiting substrates during gestation, other models of programming include those that mimic ill-timed exposure of fetuses to elevated levels of glucocorticoids. This is generally accomplished by either direct administration of glucocorticoids to mother and/or fetus, inhibition of 11β-hydroxysteroid dehydrogenase (the placental enzyme that ordinarily shields the fetus from excursions in the levels of maternal glucocorticoids) or by stimulating endogenous production of maternal or fetal glucocorticoids, such as by maternal ethanol ingestion or smoking.[47-52] The administration of glucocorticoids has direct clinical relevance as well because glucocorticoid treatment is frequently used to stimulate lung development in cases of anticipated premature delivery.

Among the more interesting fetal adaptations are those that occur in response to prolonged hypoxemia. This has been typically studied in sheep and accomplished in a number of experimental ways. In contrast to the acute response to hypoxemia, which is an increase in HPA activity, both before and after birth, chronic fetal hypoxemia is associated with plasma ACTH levels that are indistinguishable from control levels or only slightly increased.[53-57] An additional, interesting adaptation to long-term hypoxia is that the adrenal apparently becomes somewhat more sensitive to increases in ACTH. Acute stimulation of the HPA axis by cord occlusion or

other means, when superimposed on chronic hypoxemia, tends to produce an attenuated ACTH response and potentiated cortisol response compared with the normal acute response.[55-59] The reasons for the increased adrenal responsiveness are not clear because many of the adrenal molecules involved in responses to ACTH are unchanged or decreased, although it should be noted that the plasma ratio of ACTH[1-39] to ACTH precursors (see previous discussion) is increased in with chronic hypoxia.[53]

Maternal undernutrition is another important model used to study programming and the role of the HPA axis therein. Of particular significance are studies that involve early or periconceptional undernutrition. In these cases, the undernutrition would be expected to have no or negligible impact on the fetus by virtue of limiting substrate; yet the effects can be quite profound. The effect on the developing HPA axis is therefore expected to be more likely due to altering the greater environment in early gestation and/or influencing the developmental trajectory. In two models of ovine periconceptional undernutrition, evidence shows precocious activation of the HPA axis. In one, periconceptional (−60 to +7 days relative to mating), but not subsequent, undernutrition was associated with elevated ACTH levels in twin, but not singleton, fetuses late in gestation.[36] In another study, more severe nutrient restriction from days −60 to +30 relative to mating resulted in premature delivery in half of the fetuses, associated with precociously elevated ACTH and cortisol levels.[60] In contrast, results from other studies in which maternal nutrient intake was restricted early in gestation, but not *before* mating, are consistent with negligible effects on the HPA axis, or possibly even some blunting of the adrenal response to ACTH.[40,61] Although the results are not consistent, they have shed an entirely new light on the regulation of the fetal HPA axis and the influence of the maternal environment around the time of conception thereupon.

Perhaps less surprisingly, undernutrition late in gestation, when fetal metabolism is nearing the limits of available substrate, is also associated with elevated plasma levels of ACTH. Interestingly, evidence suggests that the effects of early undernutrition in a sheep model is independent of maternal glucocorticoids,[62] whereas late undernutrition in another model in rats may be an indirect effect, mediated by maternal glucocorticoids.[42] If so, this could be considered counterintuitive because any periconceptional glucocorticoid effect would have to be maternal, whereas late in gestation, the fetal HPA axis is fully active and can act independently.

Evidence that alterations of fetal HPA activity have long-lasting, if not permanent, effects on the function of the axis come from studies in which animals subjected as fetuses to the above conditions have been followed after birth and from studies in which the direct effects of fetal exposure to glucocorticoids on various regulatory systems have been documented postnatally. For example, treatment of pregnant vervet monkeys with dexamethasone daily from gestation until delivery resulted in an enhanced cortisol response to stress in offspring at 8 months of age.[63] Although the precise mechanisms by which elevated or precocious activity in the fetal HPA axis leads to altered regulation of the axis postnatally have yet to be elucidated, much interest is focused on the setpoint of feedback regulation. During development it is likely that some increased aspects, representing HPA activity, trigger permanent changes to establish that level of activity as normal. Some evidence shows that elevated levels of glucocorticoids alter brain physiology, including the down-regulation of glucocorticoid receptors in the hippocampus, which may decrease sensitivity to negative feedback.[64,65] This remains an area of intense research.

The widespread use of antenatal glucocorticoid therapy in humans to enhance fetal lung maturation has also prompted investigators to assess whether prenatal steroid exposure programs changes in activity in the HPA axis after birth. The available data suggest this is the case. In one of the first prospective studies examining this issue, the plasma ACTH and cortisol values in response to stimulation by CRH were measured in 137 very-low-birth-weight infants at 7 and 14 days of age. Comparisons were made between infants whose mothers received betamethasone treatment before delivery and those whose mothers did not. Although the ACTH responses were similar in the groups that received steroid and those that did not, the plasma cortisol responses were significantly attenuated at 7 days in the infants whose mothers received antenatal corticosteroids. However, at 14 days of age the differences in cortisol responses to CRH had disappeared. These data suggest that one or two doses of antenatal corticosteroids are capable of attenuating the adrenal response to stimulation in the newborn.[66] Other studies have demonstrated that prenatal betamethasone treatment modulates the stress response in healthy premature infants. In a small study, salivary cortisol production after heelstick blood sampling was monitored in nine newborns whose mothers had antenatal betamethasone treatment and in nine infants from untreated mothers. All the infants were born at 33 to 34 weeks gestation and studied within 3 to 6 days of delivery. The cortisol response to heelstick was blocked in the infants exposed to betamethasone prenatally.[67] A similar comparison was subsequently made in infants 4 to 6 weeks after exposure to the steroid. At that time, the cortisol response to the stress of a heelstick remained blocked.[68] Similarly, the salivary cortisol response to immunization at 4 months of age is attenuated in preterm babies whose mothers received either betamethasone or dexamethasone before delivery.[69] These data are reasonably consistent and suggest that programming of the HPA axis in fetal life can occur as a result of prenatal glucocorticoid exposure. The impact of such programming persists for at least 4 months after birth.

The association of the fetal HPA axis in general, and regulation of ACTH secretion in particular, with both normal coordination of the maturation of various organs and with fetal programming places the axis in an intriguing place both physiologically and as a subject of current interest. Clearly, timing is critical and alterations to the environment that signal a less favorable progression thorough gestation are often associated with altered ACTH secretion or adrenal sensitivity to ACTH. However, the mechanisms involved in this altered regulatory function referred to as programming have not been identified.

SIGNIFICANCE

It now seems reasonably clear that a primary role for the CRH-ACTH system is related to its essential nature in promoting the maturation of adrenal steroidogenesis in fetal life. This can be seen in a variety of animal models that have as their end result impairment of CRH synthesis, the capacity of CRH to stimulate ACTH release, or the ability of the pituitary to synthesize ACTH. Thus targeted disruption of the CRH gene or the CRH-R1 receptor results in animals that lack the ability to secrete adrenal glucocorticoids and that cannot survive for prolonged periods after birth unless replacement steroid is provided. Likewise, animals lacking the POMC gene have dysfunctional adrenals and suffer the consequences of a profound lack of circulating glucocorticoids. Even if disruptions of the CRH-ACTH system occur in the last half of pregnancy, the ability of the fetal adrenal to secrete corticosteroids is almost completely lost. Because the normal development of multiple organ systems in the fetus, especially the lung, have an absolute requirement for glucocorticoids to be present in fetal circulation the normal development of the CRH-ACTH system is essential for preparing the fetus for extrauterine life.

The recognition that activity in the fetal CRH-ACTH-adrenal axis is susceptible to the effects of programming by various

stimuli adds a new layer of complexity for understanding the interactions among the factors that regulate normal development of this hormonal system. The observations in humans that prenatal events can program alterations in responses to stress by the brain-pituitary-adrenal axis after birth also suggest that the normal development of this system has important ramifications for well-being in adulthood. Clearly, additional research is needed on the normal development of this system to better understand its impact on prenatal and postnatal health.

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Kristi L. Watterberg

Fetal and Neonatal Adrenocortical Physiology

The adrenal cortex undergoes remarkable growth and unique metamorphosis during the fetal and neonatal period. Its morphology, functions, and secretory product profiles are distinctly different during fetal versus postnatal life. The major products of the fetal adrenal cortex, the weak androgens dehydroepiandrosterone (DHEA) and its sulfate (DHEAS), disappear almost completely after birth, reappearing years later at the onset of adrenarche. The functions of the adrenal cortex during fetal life—participation in the maintenance of pregnancy and initiation of parturition, as well as secretion of cortisol to promote organ maturation before birth—also change postnatally to maintenance of homeostasis, particularly in the presence of stress, and modulation of inflammatory challenges.[1]

The cumulative work of many investigators has demonstrated that the structural and functional organization of the fetal adrenal cortex of humans and higher primates is unique.[2] In addition, a major regulator of fetal adrenal development, placental corticotropin-releasing hormone (CRH), is present only in humans and closely related primates.[3] Thus, although insights can be obtained from other animal models, caution must be exercised in applying such findings to human physiology. Wherever possible, this chapter focuses on studies of humans and other primates.

DEVELOPMENTAL HISTOLOGY

The primordial adrenal gland is first evident in humans as a distinct cluster of cells at 33 days postconception.[4] By 50 to 52 days postconception, steroidogenic activity can be detected within the nascent fetal zone.[5] The gland then grows exponentially, increasing 10-fold between 8 and 10 weeks postconception,[5] equaling the kidney in weight at 20 weeks of gestation, then doubling in size between 20 and 30 weeks and doubling again by term, reaching a combined weight of about 8 grams (Figure 181-1).[6] After birth, the gland quickly involutes, shrinking to a combined weight of about 2 grams by 1 year of age before beginning to grow again.

This remarkable growth during fetal life occurs through a combination of hyperplasia, hypertrophy, and reduced apoptosis. During the embryonic period, mitotic activity has been observed throughout the fetal adrenal cortex.[6] The cortex then quickly differentiates into two distinct zones: an outer definitive zone, composed of a thin layer of tightly packed small, basophilic cells with ultrastructural characteristics consistent with cellular proliferation, and a much larger inner fetal zone, containing large, eosinophilic cells with a steroid secreting phenotype.[7-10]

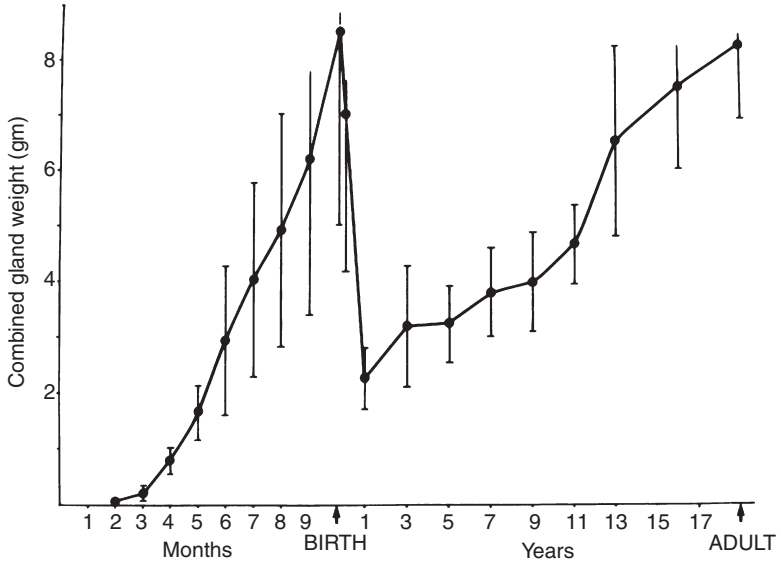


Figure 181-1 Adrenal gland weights during human development. *(From Neville AM, O'Hare MJ:* The Human Adrenal Cortex, *Berlin, 1982, Springer-Verlag, p 12.)*

The ultrastructural appearance of these two zones led investigators to conclude that cells in the fetal zone did not divide. Using newer techniques, however, investigators have identified mitotic figures throughout the fetal zone, suggesting that it grows by hyperplasia as well as hypertrophy.[8] Because the mitotic index in the definitive zone exceeds that in the fetal zone, however, proliferation appears to occur predominantly in the definitive zone, with new cells migrating toward the center, where they hypertrophy and terminally differentiate into steroid producing cells.[8]

At the intersection of these two zones, a transitional zone appears and begins to secrete cortisol at week 22 to 24 of pregnancy.[11] This transitional zone expands through the latter part of gestation and becomes the cortisol-secreting zona fasciculata by term, and the outer, definitive zone becomes the zona glomerulosa, which secretes the mineralocorticoid aldosterone.[11]

Apoptotic nuclei have been identified primarily at the center of the cortex.[8] These nuclei are present in low but increasing numbers during gestation.[8] After birth, maximal apoptosis is observed in the fetal zone, which subsequently disappears.[8] Several years later, the zona reticularis will appear at the site of the earlier fetal zone and begin to secrete DHEA and DHEAS, regulating the onset of adrenarche.[1]

ENZYMES AND SECRETORY PRODUCTS

The major secretory products of the adrenal cortex include weak androgens (DHEA and DHEAS), cortisol and aldosterone. As shown in Figure 181-2, steroidogenesis requires a series of enzymatic steps, including four cytochrome P450 oxidase enzymes.[1] Two of these are located in the mitochondria—cholesterol side chain cleavage enzyme (P450scc) and 11β-hydroxylase (P450C11)—and two are found in the endoplasmic reticulum: 17α-hydroxylase/17,20 lyase (P450C17) and 21-hydroxylase (P450C21).[12] The first, rate-limiting step in the synthesis of steroid hormones is the conversion of cholesterol to pregnenolone by P450scc, which is dependent on delivery of cholesterol to the mitochondrion by the steroidogenic acute regulatory protein.[12,13] Absence of this regulatory protein impairs adrenal and gonadal steroidogenesis and is clinically manifested as congenital lipoid adrenal hyperplasia.[14] The critical branch point enzymes regulating steroidogenesis are 3β-hydroxysteroid dehydrogenase (type 2 isozyme, 3βHSD2) and P450C17. These two enzymes compete for substrate.[15] In tissues in which 3βHSD2 is highly expressed, cortisol is produced preferentially; low 3βHSD activity in conjunction with high P450C17 promotes DHEA production.[15,16]

After the embryonic period, the enzymes and secretory patterns of each zone within the adrenal cortex are distinct, and the specific steroidogenic enzymes within each zone determine the type of steroid produced in that zone[2,9,11,17] (Figure 181-3). The fetal zone contains P450C17, but lacks 3βHSD2, and therefore secretes DHEA and DHEAS in massive amounts—up to 200 mg/day during the third trimester.[18] These adrenal androgens serve as substrates for placental aromatases, which convert them to the estrogens estriol, estradiol, and estrone[19,20] (Figure 181-4). This process appears to be fundamental to the normal maintenance of pregnancy, as reviewed later in this chapter.

The transitional zone, which appears at 22 to 24 weeks gestation, contains 3βHSD2 and can therefore synthesize cortisol de novo from cholesterol[1,17] (see Figures 181-2 and 181-3). Before the appearance of the transitional zone, however, cortisol may be produced by the fetus using placental progesterone as a substrate[9,21] (see Figure 181-4). The outer, definitive zone of the cortex also begins to express 3βHSD2 toward the end of gestation, but this zone lacks P450C17 and therefore secretes the mineralocorticoid aldosterone[9,11,17] (see Figures 181-2 and 181-3). The zona reticularis, which will not become morphologically apparent until adrenarche, appears to be analogous to the fetal adrenal cortex in that it lacks 3βHSD2 and secretes DHEA and DHEAS.[1]

The expression of these critical branch point enzymes very early in gestation and their effects on early development have been unclear. 3βHSD2 was generally believed to be absent from the human fetal adrenal cortex in the undisturbed pregnancy until 22 to 24 weeks gestation. However, one immunohistochemical study found transient staining for 3βHSD in numerous definitive zone and occasional fetal zone cells very early in development, which disappeared by 14 weeks postconception.[22] A recent study confirmed this transient expression of 3βHSD2, as well as its regulatory orphan nuclear receptor (nerve growth factor–induced clone B, or NGFIB), in human adrenal cortex during the embryonic period, and additionally documented a brief spike in adrenal cortisol content and secretion during this time.[5] Biosynthesis was maximal at 8 to 9 weeks postconception, after which 3βHSD immunoreactivity and cortisol content decreased quickly, declining by half at 10 weeks and to undetectable levels

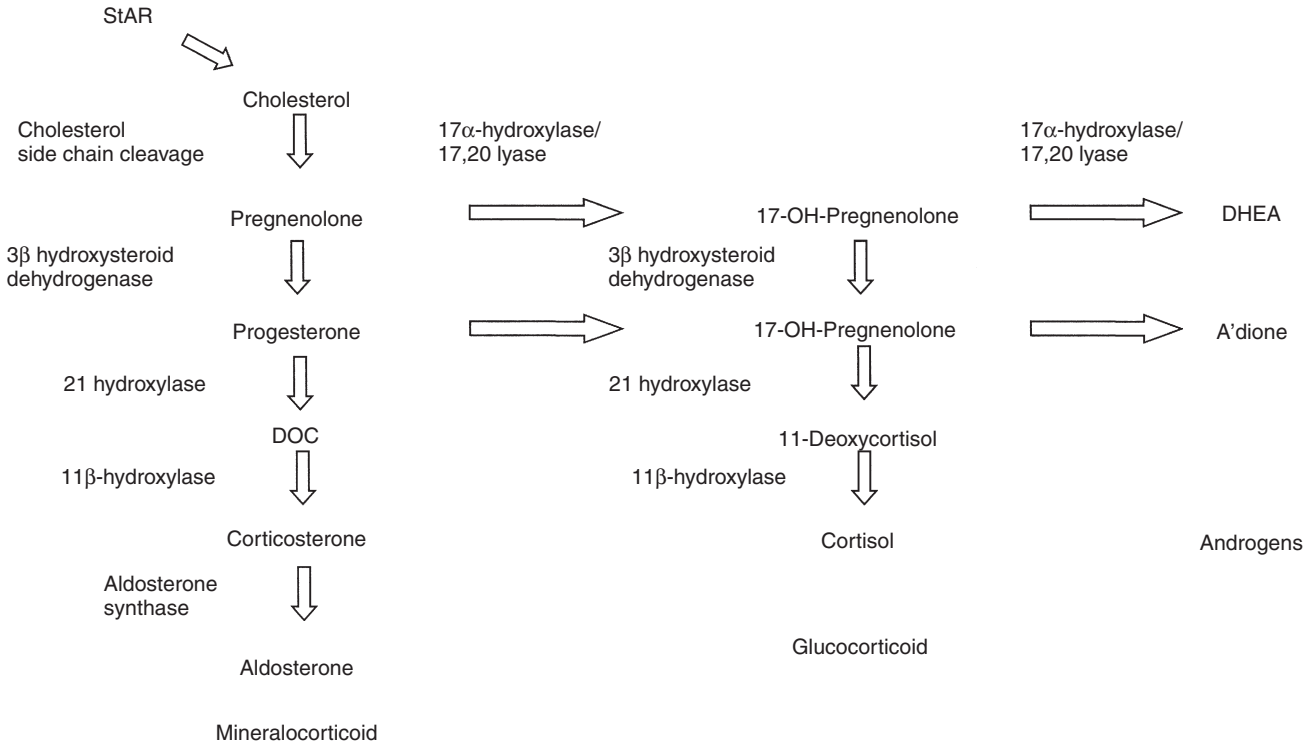


Figure 181-2 Adrenal steroidogenesis. After the steroidogenic acute regulatory *(StAR)* protein-mediated uptake of cholesterol into mitochondria within adrenocortical cells, aldosterone, cortisol, and adrenal androgens are synthesized through the coordinated action of a series of steroidogenic enzymes in a zone-specific fashion. *A'dione,* Androstenedione; *DHEA,* dehydroepiandrosterone; *DOC,* deoxycorticosterone. *(From Stewart PM: The adrenal cortex. In Larsen PR, Kronenberg HM, Melmed S, Polonsky KS, editors:* Williams Textbook of Endocrinology, *Philadelphia, 2003, Saunders, p 495.)*

by 14 weeks postconception. This early, transitory cortisol production was coincident with the detection of adrenocorticotropic hormone (ACTH) in the same fetuses. Additionally these adrenocortical cells were responsive to ACTH, strongly suggesting that even at this early stage adrenal cortical cells are regulated by ACTH secretion.[5]

This thought-provoking new work may solve the puzzle of how female fetuses affected by P450C21-deficient congenital adrenal hyperplasia become virilized very early in gestation, at a time before the fetus had been thought to produce cortisol. Virilization occurs in congenital adrenal hyperplasia when the cortisol biosynthesis pathway is stimulated by ACTH, but cortisol production is blocked by the absence of P450C21 (see Figure 181-2). This block in cortisol synthesis removes the normal negative feedback of cortisol to the pituitary and results in increased ACTH secretion. In this situation, continuing stimulation of the cortisol synthesis pathway leads to an accumulation of cortisol precursors, which are shunted into increased production of DHEA and DHEAS (see Figure 181-2). Later in gestation, these increased fetal androgens will be converted to estrogens by placental aromatases (see Figure 181-4), thus preventing accumulation in the fetus.[5,19] At this early stage of gestation, however, placental aromatase activity is low, resulting in an accumulation of androgens and virilization of the external female genitalia during differentiation, a process that occurs at 8 to 10 weeks postconception.[5]

REGULATION OF FETAL ADRENAL DEVELOPMENT

As befits an organ involved in the vital functions of maintaining the pregnancy, initiating parturition, and maturing fetal organs for extrauterine existence, the fetal adrenal cortex is regulated by an intricate web of factors from fetus, placenta, and mother. These factors combine to shield the fetus from premature exposure to excess cortisol early in gestation and then to stimulate the increased cortisol production essential for preparation for extrauterine survival. Normal fetal cortisol concentrations are low until well into the third trimester of pregnancy (Figure 181-5).[23] The profound effects of premature exposure of the fetus to increased glucocorticoid concentrations have been well documented in animal models, and include decreased body, brain, and organ weights.[24,25]

Fetal Factors

Adrenocorticotropic Hormone

Stimulation of the adrenal cortex by ACTH from the fetal pituitary is necessary for its normal morphologic development and steroidogenic function. The effects of a lack of ACTH in the human are demonstrated in the anencephalic fetus. Stereologic studies of adrenal cortex structure in anencephalic fetuses have shown a striking decrease in both cell number and total volume of the fetal zone and transitional zone (zona fasciculata), with less effect on the zona glomerulosa.[26,27] Although ACTH has been detected in placental tissue,[28] outcomes such as this would suggest that placental ACTH is not sufficient for normal human adrenocortical growth and development.

The specific effects of ACTH on adrenocortical cells have been documented in primate models. In vivo increasing fetal ACTH either by directly administering ACTH or by suppressing cortisol synthesis with metyrapone results in hypertrophy of the transitional zone and early appearance of 3βHSD message in both the transitional and definitive zones.[29,30] Conversely, administration of betamethasone to suppress fetal ACTH secretion eliminates

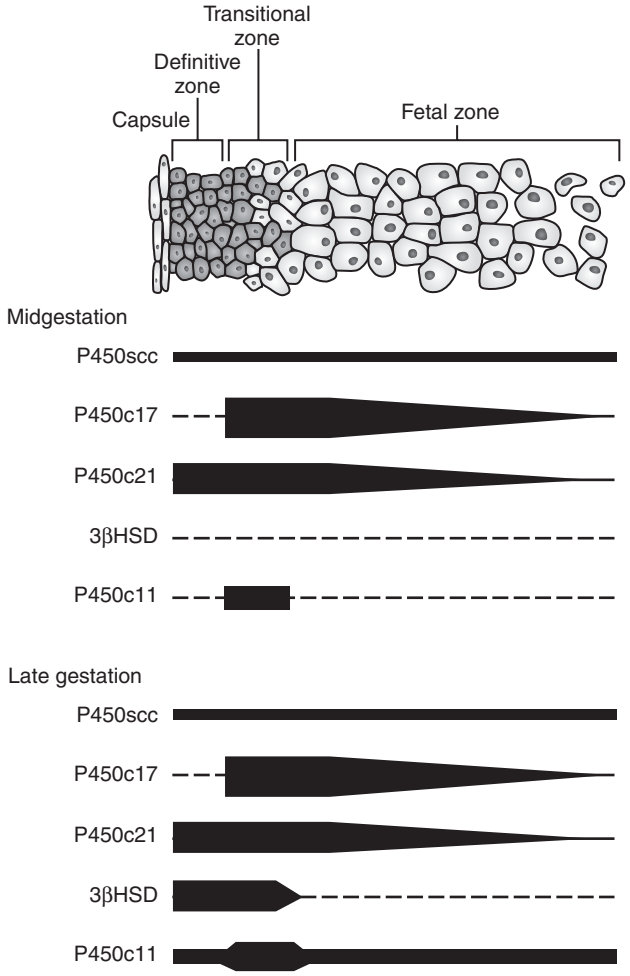


Figure 181-3 Schematic representation of the localization of expression of P450scc, P450c17, P450c21, 3βHSD, and P450c11 in the primate fetal adrenal cortex during mid- and late gestation. Thickness of the line indicates relative abundance of expression. *Dashed line* indicates lack of expression. Note the lack of P450c17 expression in the definitive zone at all stages of gestation and the ontogenetic expression of 3βHSD only in the definitive and transitional zones late in gestation. *(From Mesiano S, Jaffe RB: Developmental and functional biology of the primate fetal adrenal cortex,* Endocr Rev *18:385, 1997).*

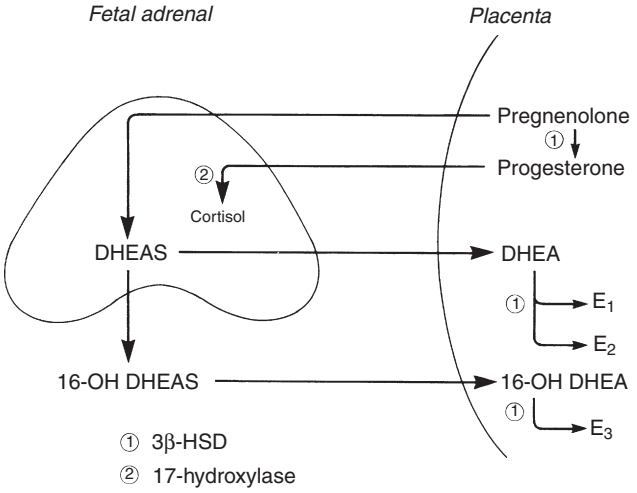


Figure 181-4 Diagrammatic representation of the fetoplacental unit composed of the fetal adrenal cortex and the placenta. The placenta is deficient in 17-hydroxylase activity and cannot synthesize estrogens from progesterone. The fetal adrenal cortex has low 3βHSD and Δ4,5 isomerase activity and cannot synthesize progesterone. Sulfokinase activity is high in fetal adrenal tissue, and steroid sulfatase activity is high in placental tissue. Thus the placenta produces progesterone, which is predominantly converted to DHEA by the fetal adrenal cortex; the DHEA can be sulfated to form DHEAS. Part of this is 16-hydroxylated by the fetal liver, and both DHEA and DHEAS are used by the placenta as substrates for estrone *(E_1)* and estradiol *(E_2)* synthesis, respectively. Placental sulfatase converts DHEAS and 16-hydroxy-DHEAS to DHEA and 17-hydroxy-DHEA. The 16-hydroxy-DHEA is used for estriol *(E_3)* synthesis. *(From Fisher DA: Endocrinology of fetal development. In Larsen PR, Kronenberg HM, Melmed S, Polonsky KS, editors:* Williams Textbook of Endocrinology, *Philadelphia, 2003, Saunders, 2003, p 813.)*

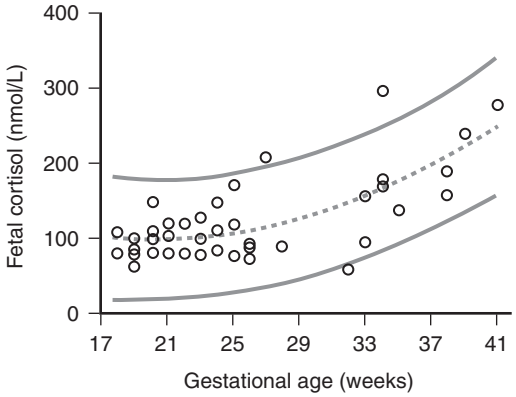


Figure 181-5 Individual values and reference range (mean and 95% confidence intervals) of fetal serum cortisol throughout pregnancy. Regression line: $y = 0.339x^{-2} - 13.5 + 231$; $r = 0.72$; $p = .0001$. *(From Donaldson A, Nicolini U, Symes EK, et al: Changes in concentrations of cortisol, dehydroepiandrosterone sulphate and progesterone in fetal and maternal serum during pregnancy,* Clin Endocrinol (Oxf) *35:447, 1991.)*

the transitional zone and decreases mRNA expression of both 3βHSD and the ACTH receptor.[29,31] Suppression of ACTH with betamethasone at midgestation also resulted in decreased adrenal weight and cell size, cellular disorganization, increased apoptosis, and reduction in expression of mRNA for the P450scc and P450C17 enzymes, effects that were reversed by concomitant treatment with ACTH.[32]

The expression of the ACTH receptor in the adrenal cortex increases through midgestation, then decreases substantially by term.[33] Additionally the responsiveness of the fetal and the developing transitional zones to ACTH appear to diverge during the second half of gestation. In the fetal zone, a selective decrease in ACTH receptor expression and a consequent decreased responsiveness to ACTH occurs; in the emerging transitional zone an increase in ACTH receptor density and 3βHSD expression is seen.[33,34] The decline in ACTH-responsiveness in the fetal zone may be due to suppression by increasing estrogen concentrations (see later discussion).[35] In contrast to the effects of ACTH on the fetal and transitional zones, the outer definitive zone, the future zona glomerulosa, may be less affected by exogenous ACTH or betamethasone.[29] This is consistent with findings in human anencephalic infants, in whom the growth of the zona glomerulosa is relatively unaffected by the absence of fetal ACTH.[26,27]

ACTH exerts its effects on adrenal cells at least in part through local growth factors, particularly insulin-like growth factor (IGF) II. A polypeptide growth factor important in the regulation of fetal growth,[36] IGF-II is highly expressed in the fetal adrenal cortex, and its expression is stimulated by ACTH.[37,38] In turn, IGF-II stimulates proliferation of fetal adrenal cortical cells, increases steroid production, and augments expression of steroidogenic enzymes.[38-41] IGF-II appears to act through the IGF-I binding protein, possibly in concert with other polypeptide growth factors that have been shown to be mitogenic for human fetal adrenal cells, particularly basic fibroblast growth factor and epidermal growth factor.[38,39,41-44]

Transcription of ACTH effect on the fetal adrenal cortex appears to be modulated by members of the nuclear receptor superfamily. Several nuclear receptors essential for human adrenal cortex development have been implicated in transcription of ACTH effect, and more will likely be identified. The orphan nuclear receptor steroidogenic factor (SF) 1 is expressed throughout the adrenal gland and participates in the activation of numerous enzymes in the steroid biosynthetic pathway.[45] Activation of ACTH-dependent signaling cascades has been shown to lead to the cyclical recruitment of SF-1 and multiple rounds of transcription.[45] Human mutations of SF-1 produce adrenal failure, indicating its importance in adrenal development.[46] Another orphan nuclear receptor responsive to ACTH stimulation is NGFIB. NGFIB shows differential expression in the different fetal adrenal zones, and may play a crucial role in functional adrenal zonation by up-regulating 3βHSD2 gene transcription in the transitional and definitive zones.[16] A third nuclear receptor, DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on X-chromosome, gene 1) appears to be an important negative modulator of ACTH-stimulated glucocorticoid synthesis, repressing SF-1–dependent activation.[47] ACTH has been shown to down-regulate DAX-1 as well as stimulate SF-1, a potential mechanism for modulating cortisol production by the adrenal cortex.[48] Deletion or mutation of DAX-1 results in X-linked adrenal hypoplasia congenita, a disorder with profound hormonal deficiencies.[49]

Angiotensin II

Angiotensin II has been implicated in apoptosis of fetal adrenal cells, acting through its type 2 receptor (AT_2).[50] This receptor is most strongly expressed at the center of the fetal zone, which is also the location with the highest density of apoptotic figures. In cells cultured from second trimester human adrenal glands, stimulation with angiotensin II produced changes consistent with apoptosis, including DNA fragmentation, interruption of the DNA repair mechanism, and reorganization of the actin cytoskeleton, leading to the hypothesis that angiotensin II–related apoptosis contributes to the involution of the fetal zone seen after birth.[50]

Local Microenvironment/Extracellular Matrix

The regulation of zonal expression of enzymes within the adrenal cortex has been a major area of curiosity. That is, if the cells primarily originate from the definitive zone and then migrate inward, why do they express different enzyme profiles in different zones, resulting in the functional zonation observed in the gland?[11,51] Comparing in vivo with in vitro cellular function has strongly implicated a local regulation of the phenotype. For example, human fetal zone cells do not express 3βHSD in vivo; however, these cells quickly begin to produce 3βHSD in response to ACTH in vitro.[52] This functional zonation of the cortex may be regulated in part by the extracellular matrix of the local microenvironment, in particular type IV collagen, laminin, and fibronectin. Type IV collagen is present throughout the adrenal cortex and has been shown to promote cellular proliferation, as well as increase cortisol secretion in response to ACTH.[53,54] Laminin is found primarily at the periphery of the gland and causes increased proliferation and decreased apoptosis in cultured human fetal adrenal cells.[54] In contrast, the fetal zone is rich in fibronectin, which does not promote proliferation, but facilitates migration and apoptosis, enhances DHEAS secretion, and decreases cortisol secretion in cell culture.[53,55] Additionally, type IV collagen and fibronectin both have been shown to modulate the intracellular localization of DAX-1, likely affecting the function of this negative regulator of steroidogenesis.[56]

Placental Factors

A unique interactive relationship between placenta and adrenal cortex in the primate pregnancy appears to be vital to the maintenance of pregnancy, the development and maturation of the adrenal gland—and consequently other organs, such as the lung—and possibly the initiation of parturition. In addition the interrelationship of the placenta and adrenal cortex serves to shield the fetus from premature exposure to excess cortisol.

Estrogen

Pepe and Albrecht[57-59] proposed a central integrative role for estrogen in regulating the maturation of both the placenta and the fetal hypothalamic-pituitary-adrenal axis (HPA axis). Their extensive studies of the baboon pregnancy demonstrated both the model's relevance to human pregnancy and the effects that estrogen exerts by (1) acting directly on the adrenal cortex, (2) regulating the expression of 11βHSD in the placenta, and (3) affecting placental progesterone production. Because fetal androgens are the primary substrate for placental estrogen production, the fetal adrenal cortex is central to these functions.

Estrogen appears to have a direct effect on the fetal adrenal cortex. In the baboon model, estrogen receptors-α and -β are expressed throughout the fetal adrenal cortex.[60] In this model, estrogen administration suppresses DHEA but not cortisol production in response to ACTH at midgestation.[60-62] Conversely, suppression of placental estrogen production during the second half of pregnancy significantly increases DHEA secretion and the size of the fetal zone, effects that are reversed by concomitant treatment with estradiol.[35] These findings are consistent with an inhibitory effect of estrogen on the fetal zone during the latter half of gestation. In these experiments, no such effect was observed in the transitional or definitive zone.[35] These results suggest that placental estrogen provides a feedback mechanism to restrain the growth of the fetal zone during the second half of pregnancy, thus providing a consistent, but not deleteriously high estrogen exposure.[35]

Numerous studies have shown that estrogen also modulates the maturation of the primate fetal adrenal cortex through its regulation of the expression of placental 11β-hydroxysteroid dehydrogenase (11βHSD)[59] (Figure 181-6). This enzyme is widely distributed in the body and is thought to modulate local exposure to cortisol in many tissues, including lung and kidney.[63] 11βHSD exists in two forms: 11βHSD1, which converts biologically inactive cortisone to active cortisol, and 11βHSD2, which transforms cortisol into cortisone.[59] At midgestation 11βHSD1 predominates in the placenta, allowing transfer of some maternal cortisol to the fetus, suppressing the fetal HPA axis.[64,65] Late in gestation, however, 11βHSD2 predominates, transforming most maternal cortisol into the biologically inactive cortisone, thus releasing the fetal HPA axis from negative feedback and stimulating its maturation.[65,66] Estrogen appears to regulate this increase in the relative concentration of 11βHSD2 to 11βHSD1 by late gestation; suppression of placental estrogen synthesis with an aromatase inhibitor significantly decreases 11βHSD2 expression.[67]

The two different forms of 11βHSD also appear to localize to different membrane fractions of the syncytiotrophoblast: 11βHSD1 is abundant in the membranes closest to the maternal circulation,

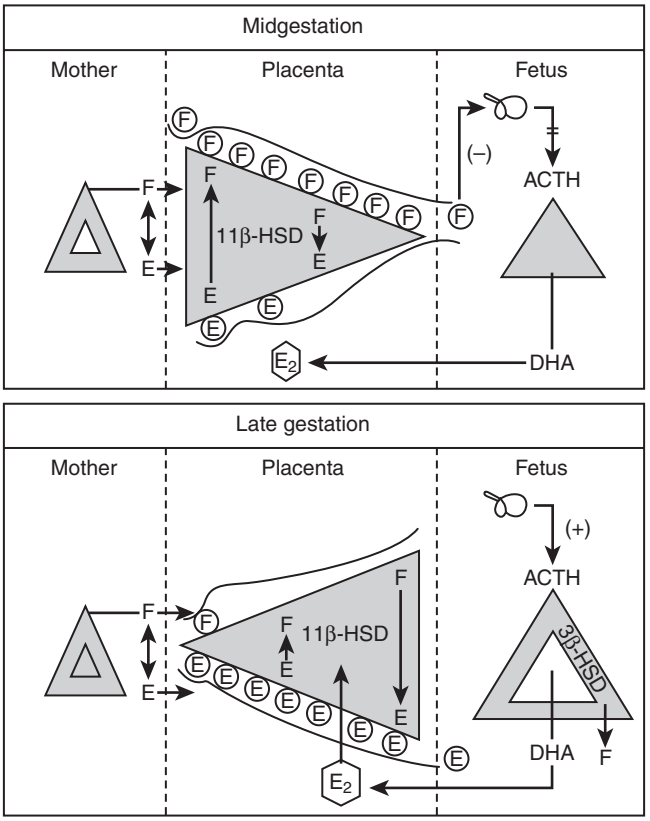


Figure 181-6 The role of estrogen *(E_2)* on placental 11βHSD-catalyzed metabolism of cortisol *(F)* and cortisone *(E)* and regulation of the fetal pituitary-adrenocortical axis at mid-and late gestation in the baboon. *(From Albrecht ED, Pepe GJ: Central integrative role of oestrogen in modulating the communication between the placenta and fetus that results in primate fetal-placental development,* Placenta *20:133, 1999.)*

and 11βHSD2 is concentrated in the membranes facing fetal vasculature.[66] This organization creates a gradient favoring metabolism of maternal cortisol to cortisone as gestation progresses. One group of investigators has reported that during the final few days of baboon gestation the expression of placental 11βHSD2 again decreased, possibly providing brief exposure to additional maternal cortisol to further mature the fetus for delivery.[68]

This changing pattern of placental 11βHSD expression would serve to first limit the exposure of the fetus to cortisol during most of the gestational period, permitting the necessary intense fetal growth and cellular proliferation, then subsequently stimulate the increased fetal adrenal cortisol production needed for maturation of vital organ systems before birth.[69,70] The importance of placental 11βHSD2 in limiting early fetal exposure to cortisol is supported by several findings in the human. First, placentas from pregnancies complicated by intrauterine growth restriction show decreased expression and activity of 11βHSD2, consistent with a role for placental 11βHSD2 in limiting exposure of the fetus to cortisol.[71-73] In addition, fetal growth restriction has been correlated with higher cortisol but lower ACTH concentrations in the fetus, as well as decreased response to ACTH stimulation in the preterm infant.[74,75] These findings are consistent with an increased exposure of the fetus to maternal cortisol in the face of decreased 11βHSD2 activity, with resulting passive suppression of the fetal HPA axis.

Estrogen also may play a part in maintaining pregnancy by increasing progesterone synthesis in the placenta. Estrogen promotes low density lipoprotein uptake and P450scc activity by the placenta, rate-limiting steps for progesterone production.[76-78] Suppression of estrogen production has been shown to decrease placental uptake of low density lipoprotein, placental P450scc activity, and peripheral progesterone concentrations in a dose-dependent manner.[76-78] However, when pregnant baboons were treated with a specific nonsteroidal aromatase inhibitor that substantially decreased maternal estrogen concentrations, maternal concentrations of progesterone were not different from untreated animals, leaving the effect of estrogen on progesterone concentrations in vivo open for further investigation.[79]

Placental Corticotropin-Releasing Hormone

Corticotropin-releasing hormone (CRH) is produced by the placenta and fetal membranes in increasing amounts during gestation, with exponentially increased production during the last 6 to 8 weeks of pregnancy.[80-83] A premature rise in CRH in maternal serum has been associated with preterm delivery, leading to its putative role as a "placental time clock" for human gestation, although the cellular basis for this mechanism is not known.[84] The magnitude of the increase in CRH during human pregnancy is much greater than in other primates,[85] which may limit the value of such models in describing this facet of human physiology.

Placental CRH stimulates the fetal HPA axis to increase fetal cortisol production.[86] However, in contrast to the hypothalamus, in which CRH production is inhibited by cortisol, placental CRH production is stimulated by glucocorticoids.[82,87] This cortisol-stimulated increase in placental CRH further stimulates fetal adrenal cortisol production, creating a "feed-forward" loop that may contribute to the onset of parturition and the maturation of the fetal adrenal cortex.[83,86] CRH has also been shown to stimulate DHEAS secretion, possibly increasing its availability for estrogen production.[88] In concert, increasing expression of placental 11βHSD2 late in gestation reduces the transmission of maternal cortisol to the fetus, releasing the fetal HPA axis from negative feedback, further stimulating increased production of fetal steroids and, in turn, estrogen and CRH production (Figure 181-7),[84] until the upward spiral ends in parturition.

FUNCTIONS OF THE FETAL ADRENAL CORTEX

The fetal adrenal cortex appears to partner with the placenta to maintain pregnancy and initiate parturition and to provide the late term cortisol surge necessary for maturation of organ systems in preparation for extrauterine life.

Maintenance of Pregnancy and Initiation of Parturition

As reviewed earlier, the fetal zone of the adrenal cortex secretes increasing amounts of DHEA and DHEAS through gestation, which serve as substrates for synthesis of placental estrogens. Estrogen appears critical to the maintenance of primate pregnancy. In humans, a 50% spontaneous abortion rate has been reported in women who have a mutation of the estrogen receptor gene,[89] and in baboons administration of an aromatase inhibitor to suppress placental estrogen production resulted in miscarriage in 50% of the pregnancies.[79] Although human pregnancies complicated by very low estrogen levels have resulted in term pregnancies, the authors of this study suggest that in such pregnancies estradiol is maintained in a low but bioactive range.[79]

In contrast to its actions in sheep, the increase in cortisol concentration at term does not initiate labor in the primate.[69,70] However, through its unique relationship with the placenta, the fetal adrenal cortex appears directly involved in the cascade of events leading to parturition. In the rhesus monkey, infusion of androstenedione increases placental estradiol synthesis and induces premature labor.[85,90] Conversely, when synthesis of estrogen from androstenedione is blocked by an aromatase

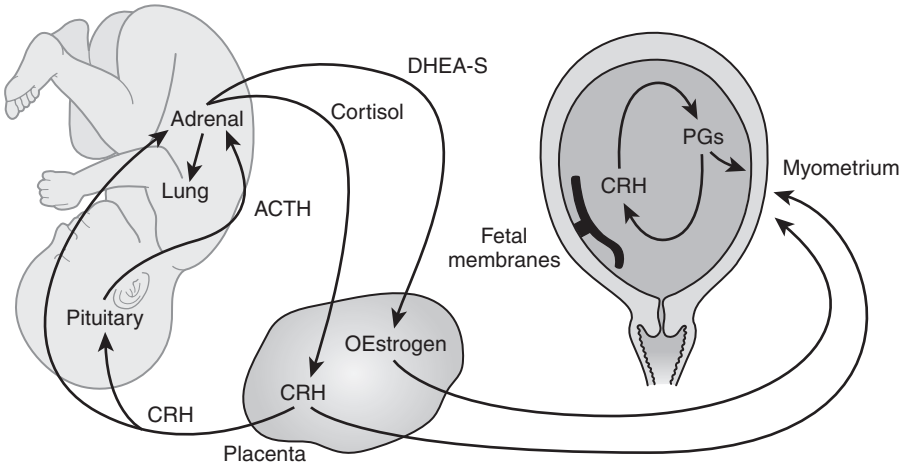


Figure 181-7 Interacting positive-feedback hormonal loops in the fetal, amniotic, and maternal compartments that may promote the progression of fetal maturation and initiation of parturition in humans. Each of the *arrows* represents a stimulatory action of the indicated hormone. *PGs,* Prostaglandins. *(From McLean M, Smith R: Corticotrophin-releasing hormone and human parturition,* Reproduction *121:496, 2001.)*

inhibitor, the androgen-induced preterm labor is prevented.[91] Interestingly in this model, peripheral infusion of estrogen to the dam increased myometrial activity but did not result in fetal membrane changes or preterm birth, implicating a paracrine function of placental estrogen in the initiation of parturition.[91] Cortisol may also participate in the initiation of labor indirectly by competing with progesterone for binding with placental glucocorticoid receptors. Progesterone binding of the glucocorticoid receptor inhibits placental CRH production; as fetal cortisol concentrations rise late in gestation, cortisol can displace progesterone from the glucocorticoid receptor, removing this inhibition.[92,93]

Maturation of Organs and Enzyme Systems

During most of gestation, low fetal cortisol concentrations permit necessary cellular growth and proliferation. Late in gestation, however, cortisol concentrations rise, promoting the cellular differentiation and organ maturation necessary to sustain independent existence[69] (see Figure 181-5). A large body of literature supports the role of cortisol in regulating numerous maturational processes, particularly lung structure and function, kidney function, glycogen accumulation and gluconeogenesis in the liver, triiodothyronine and catecholamine production, regression of lymphoid tissue in spleen and thymus, and increased β-adrenergic receptor density in numerous tissues.[69]

NEONATAL ADRENAL CORTICAL FUNCTION

The sudden loss of placental and maternal protection at birth requires that the newborn provide its own homeostasis, for which the hormones of the adrenal cortex are essential. Cortisol regulates protein, carbohydrate, lipid, and nucleic acid metabolism, maintains cardiac and vascular response to vasoconstrictors, regulates extracellular water and promotes free water excretion, suppresses the inflammatory response and decreases capillary permeability during inflammation, and modulates central nervous system processing and behavior.[94,95] Aldosterone regulates fluid and electrolyte homeostasis; deficiency produces hypovolemia, hyponatremia, and hyperkalemia.[96] In the absence of an enzyme deficiency that blocks glucocorticoid synthesis, the adrenal cortex of the newborn infant should be able to respond to ACTH stimulation with an appropriate rise in cortisol concentration. However, two circumstances may be exceptions to that statement: the first several days of transition to extrauterine life and prematurity.

Transition to Extrauterine Life

After the surge of cortisol normally engendered by parturition,[20] cortisol concentrations in well infants may fall to very low levels without apparent clinical compromise.[97] However, several small studies have suggested that in the first days of life, apparently normal term infants may be unable to respond to critical illness with production of sufficient cortisol to maintain cardiovascular stability, resulting in a relative adrenal insufficiency similar to that seen in other critically ill patients.[98-105] One preliminary report showed that critically ill term and near-term infants had low ACTH levels and low cortisol concentrations, but their cortisol response to exogenous ACTH stimulation was indistinguishable from that of well term infants.[106] These data suggest that the observed low cortisol concentrations may result from a lack of hypothalamic stimulation of the pituitary or a lack of pituitary response to CRH.

One possible reason for this finding of inadequate response to stress but adequate response to ACTH is the sudden withdrawal of the very high concentrations of CRH produced by the placenta at term. As reviewed earlier, CRH concentrations are low in nonpregnant individuals but reach very high concentrations in both maternal and fetal plasma before term or preterm delivery.[81,84] At birth, the major source of this CRH—the placenta—is removed, and maternal plasma concentrations again fall to undetectable levels within 24 hours.[80] A blunted ACTH response to CRH stimulation has been documented in women during the postpartum period.[107] It is not known whether the same phenomenon may exist in newborns, leading to a transient decrease in their ability to respond to stress.

Preterm Infants

From the review of fetal adrenal cortical development, it would seem reasonable to postulate that very preterm infants may not have sufficient functional adrenal capacity to respond to postnatal stress. In the undisturbed pregnancy, the transitional zone only begins to appear at 22 to 24 weeks gestation, and 3βHSD expression then increases gradually until term.[9] However, most preterm deliveries result from disordered pregnancy, which may alter adrenal development. For example, a large autopsy series

found that premature infants exposed to prenatal inflammation from chorioamnionitis had larger adrenal glands than those not exposed, suggesting an effect of inflammation on adrenal development.[108] Consistent with those findings, infants exposed to chorioamnionitis have elevated cortisol values early in life.[109]

In the baboon model, a significant percentage of extremely preterm neonates have decreased cortisol production early in life, with associated cardiovascular dysfunction that can be reversed by hydrocortisone supplementation.[110] In human infants, a developing literature has shown that although well preterm infants, similar to term infants, may have low serum cortisol values without apparent clinical compromise,[97] these infants also may not respond to critical illness with sufficient cortisol production. Sick preterm infants and those receiving vasopressor support for hypotension have lower cortisol concentrations than well infants, a reversal of the expected pattern of higher cortisol values with increasing illness.[111-114] Hydrocortisone treatment of infants with vasopressor-resistant hypotension results in amelioration of hypotension and weaning of vasopressor support.[115,116] Lower cortisol values have also been reported in sick preterm infants who died, compared to survivors.[117] Additionally, extremely preterm infants have elevated cortisol precursors, consistent with a limited enzymatic capacity to synthesize cortisol.[118-120] Several studies have linked lower cortisol values or decreased cortisol response to ACTH to the development of bronchopulmonary dysplasia, but others have not, likely due to differing populations and the multifactorial etiology of bronchopulmonary dysplasia.[113,121-126]

These data support the possibility of a developmental adrenal insufficiency in the extremely preterm infant with clinical consequences. The preponderance of these data might suggest a benefit to cortisol replacement in the extremely preterm infant; however, even low-dose hydrocortisone therapy in such infants was associated with a serious adverse effect: spontaneous gastrointestinal perforation.[127,128] Although this effect may have been due to an interaction with concurrent indomethacin or ibuprofen therapy, it emphasizes the need for caution in approaching the treatment of infants who, were they still in utero, would be exposed to a much lower level of cortisol. It is clear that extrauterine existence requires the extremely preterm infant to respond to significant stress and that both adrenal insufficiency and glucocorticoid excess are detrimental to homeostasis and neurologic development[94,95]; however, neither the most appropriate measures of adrenal function nor the most beneficial supportive therapies for preterm infants have been elucidated.

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Fetal and Neonatal Thyroid Physiology

EMBRYOGENESIS AND HISTOLOGIC DEVELOPMENT OF THE THYROID GLAND

The human *thyroid gland* is a derivative of the primitive buccopharyngeal cavity.[1-3] It develops from contributions of two types of anlagen: a midline thickening of the pharyngeal floor (median anlage) and paired caudal extensions of the fourth pharyngobranchial pouch (lateral anlagen). All these structures are discernible by day 16 to 17 of gestation; by 24 days gestation, the median anlage has developed a thin, flasklike diverticulum, extending from the floor of the buccal cavity down to the fourth branchial arch. At 40 days gestation, the median and lateral anlagen have fused, and, by 50 days gestation, the buccal stalk has ruptured. During this period, the thyroid gland migrates caudally to its definitive location in the anterior neck, helped in part by its relationship with developing cardiac structures.

The early histologic development of the thyroid gland has been divided into three phases: (1) precolloid (7 to 13 weeks gestation), (2) early colloid (13 to 14 weeks gestation), and (3) follicular (>14 weeks gestation) phases.[4] In the precolloid phase, the thyroid cells contain an intracellular canaliculus derived from

the smooth endoplasmic reticulum. As these canaliculi mature, they migrate toward the cell apex and begin to discharge their contents extracellularly. In the early follicular stage, these canaliculi fuse, and desmosomes define the borders of these spaces, thus allowing for the central accumulation of colloid. At this time (approximately 70 days gestation), iodide concentration and thyroglobulin synthesis can be demonstrated within the gland. During the final follicular phase, a further increase in the size of the colloid spaces occurs, as well as progressive cell growth and accumulation of thyroid hormones.

The *parathyroid glands* develop between 5 and 12 weeks gestation from the third and fourth pharyngeal pouches. The third pouches encounter the migrating thyroid anlage, and the parathyroid anlagen are carried caudally with the thyroid gland. They finally come to lie at the lower poles of the thyroid lobes as the inferior parathyroid glands. The fourth pouches encounter the thyroid anlage later and come to rest at the upper poles of the thyroid lobes as the superior parathyroid glands. The individual parathyroid glands increase in size from less than 0.1 mm diameter at 14 weeks to 1 to 2 mm at birth. The fifth pouches contribute paired ultimobranchial bodies that become incorporated into the developing thyroid gland as the parafollicular C cells, the calcitonin-secreting cells.[5,6]

Hypothalamic maturation proceeds from the fifth week through 30 to 35 weeks gestation.[2] Most of the hypothalamic nuclei and fibers of the supraoptic tract are visible histologically by 12 to 14 weeks. Thyrotropin-releasing hormone (TRH) is detectable in hypothalamic tissue by 10 to 12 weeks. Between 10 and 35 weeks, continuing histologic maturation of the hypothalamus takes place.

The anterior *pituitary gland* is a derivative of the primitive buccal cavity (Rathke pouch), and its embryologic development parallels that of the thyroid anlagen.[2] By the fifth week in the human embryo, the Rathke pouch makes contact with a funnel-shaped ventral diverticulum of the third cerebral ventricle, the infundibular process. By 12 weeks, the buccal connection is obliterated by the developing sphenoid bone, and the pituitary gland becomes partially encapsulated within a bony cavity, the sella turcica. Between 9 and 12 weeks, many pituitary cell types appear, each more or less specialized for secretion of one of the anterior pituitary hormones. Secretory granules can be identified within these cells at 10 to 12 weeks, and thyrotropin (TSH) is identifiable by bioassay and immunoassay at this time.

Abnormalities of hypothalamic or pituitary gland embryogenesis can be associated with TSH deficiency and secondary hypothyroidism. Developmental abnormalities of the thyroid gland usually represent defects in early morphogenesis resulting from aberrant thyroid tissue migration. The most common anomaly is the persistence of the thyroglossal duct. This is not usually associated with altered thyroid status in the newborn, but it may present in later life as an infected fistulous tract. Abnormalities of thyroid embryogenesis (thyroid dysgenesis) include agenesis or ectopic tissue in sublingual, cervical, mediastinal, or even intracardiac locations. Failure of fusion of median and lateral anlagen may produce cystic masses of cells in various locations in the neck. Although ectopic thyroid tissue may manifest some function, infants with these anomalies usually exhibit some degree of hypothyroidism. Calcitonin deficiency also is present in children with congenital hypothyroidism.[7] Hypoparathyroidism, however, is not associated with thyroid dysgenesis, although the parathyroid glands may be ectopic.

THYROID HORMONE SYNTHESIS

Circulating plasma iodide enters the thyroid follicular cells and is combined with tyrosine through a series of enzymatically mediated reactions to form the active thyroid hormones 3,5,3′-triiodothyronine (T_3) and thyroxine (T_4). The steps in synthesis and release of thyroid hormones include the following:

1. Active transport of inorganic iodide from plasma to thyroid cell.
2. Synthesis of tyrosine-rich thyroglobulin, which acts as the intermediate iodine acceptor.
3. Organification of trapped iodide as iodotyrosines.
4. Coupling of monoiodotyrosines (MIT) and diiodotyrosines (DIT) to form the iodothyronines, T_3 and T_4, with storage of iodotyrosines and iodothyronines in follicular colloid.
5. Endocytosis and proteolysis of colloid thyroglobulin to release MIT, DIT, T_3, and T_4.
6. Deiodination of released iodotyrosines within the thyroid cell with reuse of the iodine. These steps and their inhibitors are outlined in Table 182-1 and are pictorially represented in Figure 182-1.

Iodide Concentration

The first and rate-limiting step in the synthesis of thyroid hormones involves the concentration of iodide by the thyroid follicular cell. The thyroid iodide transporter is located in the follicular cell plasma membrane.[8,9] Extrathyroidal tissues (gastric mucosa, salivary gland, mammary gland, choroid plexus, placenta, and skin) also concentrate iodide but are not able to organify iodide. Iodide transport requires energy and is inhibited by dinitrophenol or ouabain, implying requirements for oxidative metabolism and phosphorylation. Iodide transport is linked to sodium (Na^+) transport and to Na^+, potassium–adenosine triphosphatase (Na^+,K^+-ATPase) activity, but the actual mechanism of transport remains uncertain. In basal conditions, the thyroid-serum ratio of iodide is 30 to 40:1. This ratio reaches several hundred-fold in circumstances of iodine deficiency and TSH stimulation.

Thyroglobulin Synthesis and Iodide Organification

Thyroglobulin contains the tyrosyl residues that provide the substrate for organification of iodide.[10,11] It is a large glycoprotein (molecular weight 660,000 daltons, sedimentation coefficient of 19 S) with two subunits, each of which is composed of several peptide chains. Thyroglobulin is the predominant protein in thyroid colloid, and approximately 3% of the thyroglobulin protein is composed of tyrosine residues. Iodination of these tyrosyl residues requires an activated, reactive iodine molecule. This is provided via a thyroid peroxidase enzyme complex that produces hydrogen peroxide as the oxidizing agent. The iodination occurs at the cell-colloid interface and is linked to the process of exocytosis of thyroglobulin. These processes of oxidation, iodination, and exocytosis are rapid; half the intracellular iodide is incorporated into thyroglobulin colloid within 2 minutes after the iodide

TABLE 182-1

Biochemical Steps to Thyroid Hormone Synthesis

Step	Inhibitor
1. Iodide transport	DNP, ouabain
2. Thyroglobulin synthesis	Protein synthesis inhibitors
3. Organization of iodide	PTU, MMI
4. MIT, DIT coupling	PTU, MMI
5. Thyroglobulin endocytosis and proteolysis	Lithium, colchicine, cytochalasin B
6. Deiodination	Dinitrotyrosine

DIT, Diiodotyrosine; *DNP,* dinitrophenol; *MIT,* monoiodotyrosine; *MMI,* methimazole; *PTU,* propylthiouracil.

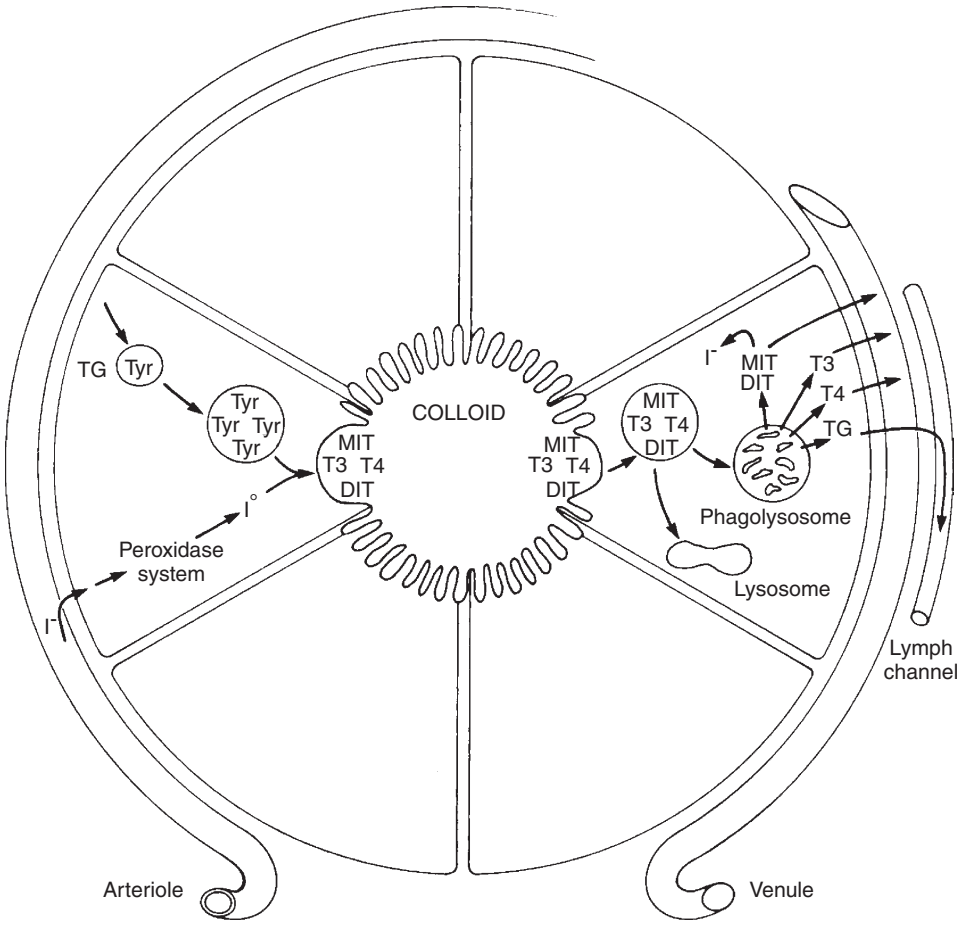


Figure 182-1 Biochemical steps in the production of thyroid hormone. *DIT,* Diiodotyrosine; *MIT,* monoiodotyrosine; *T3,* triiodothyronine; *T4,* thyroxine; *TG,* thyroglobulin; *Tyr,* tyrosine.

is trapped by the follicular cell.[10,11] Thyroid peroxidase also is involved in the coupling of MIT and DIT to form T_3 and T_4. The peroxidase enzyme complex is inhibited by the antithyroid drugs propylthiouracil and methimazole. A defect in the iodide trapping process, a defect in thyroglobulin synthesis, or a defect in the peroxidase enzyme complex can produce hypothyroidism.

Coupling of Iodotyrosines

Although it is not known whether T_4 formation in vivo involves predominantly intramolecular or intermolecular coupling of MIT and DIT residues, the coupling process itself has been well characterized. As indicated earlier, thyroid peroxidase also catalyzes the coupling of MIT and DIT residues, although nonenzymatic coupling is theoretically possible. The relative proportions of T_3 and T_4 formed depend on the influence of several factors, including TSH and iodine availability, which modulate the relative proportions of MIT and DIT. Under basal conditions, in the absence of iodine deficiency or excessive TSH stimulation, 70% of the total thyroglobulin iodoprotein is in the form of iodotyrosines, with the remainder as iodothyronines in a T_4 to T_3 ratio of 10 to 20:1.[10,11] Hypophysectomy and iodine deficiency are known to impair the efficiency of coupling, probably by decreasing the efficiency of thyroglobulin iodination. The thioureylene drugs (propylthiouracil, methimazole, carbimazole), through their action as thyroid peroxidase inhibitors, act to decrease both iodination and iodotyrosine coupling in the thyroid. Propylthiouracil also inhibits the peripheral metabolism of thyroid hormones. It is not clear whether specific defects in the coupling process lead to clinical disease. Most of the patients for whom coupling defects have been proposed are either iodine deficient or have organification defects that lead to inefficient coupling.

Thyroglobulin Proteolysis and Thyroid Hormone Secretion

Thyroglobulin, stored in colloid after iodination at the apical thyrocyte cell membrane, must reenter the cell and undergo proteolysis to release the bound and stored thyroid hormones.[10,11] This process, which is modulated to a great degree by TSH, involves endocytosis of colloid as phagosomes, fusion of phagosomes with lysosomes to form phagolysosomes, and subsequent proteolysis. Hydrolyzed free MIT, DIT, T_3, and T_4 are then released from the lysosomes into the follicular cell cytoplasm from which T_3 and T_4 can diffuse into thyroidal capillary blood. The follicular cell also contains an outer (phenolic) ring iodothyronine deiodinase that converts T_4 to T_3. Activity of this deiodinase is under TSH control such that the amounts of secreted T_3 and T_4 in relation to the ratio of T_3 to T_4 stored in thyroglobulin increase during TSH stimulation. Thyroid-stimulating antibodies may mimic these effects of TSH and contribute to the increased levels of T_3 secretion seen in patients with Graves' disease.

Microtubular function is necessary for proper transport of vesicles within the follicular cell.[11] Thus colchicine and cytochalasin B have inhibitory effects on the secretion of thyroid hormones. High doses of iodide also inhibit the release of thyroid hormones, at least in part via endocytosis and colloid resorption; in addition, an inhibiting effect on lysosomal protease activity may exist.

Evidence is ample to indicate that exposure of neonates to relatively high concentrations of iodide or organic iodine-containing compounds can produce transient hypothyroidism.

Iodotyrosine Deiodination and Recycling of Intrathyroidal Iodide

Although proteolytic cleavage of thyroglobulin results in the liberation of free iodotyrosines, as well as iodothyronines, only small amounts of MIT and DIT leave the follicular cell.[11] Thyroid follicular cells contain an iodotyrosine deiodinase, which catalyzes the rapid deiodination of MIT or DIT. This enzyme is inhibited by dinitrotyrosine. The iodide generated from deiodination of iodotyrosines is principally reused for iodination of thyroglobulin, although some diffuses from the thyrocyte (the so-called *iodide leak*).

Ontogenesis of Thyroid Hormone Receptors

The principal actions of thyroid hormones in target tissues are regulated principally through interaction with specific nuclear receptors functioning as transcription factors, both stimulating and inhibiting target gene expression.[12] Generally, the receptors show a much higher affinity for T_3 as a ligand than T_4. Four principal thyroid hormone receptors (TRs) have been identified and characterized: TR alpha 1 and 2, as well as TR beta 1 and 2. However, many more transcripts that bind T_3 as a ligand have been demonstrated, some of which also bind DNA. In humans, the gene encoding the alpha subtype has been mapped to chromosome 17 and that for the beta subtype to chromosome 3. Alternative splicing gives rise to the multiple subtypes. The interaction of these subtypes is complex. Several of the alpha variants indeed do not bind T_3 (TR alpha 2, as well as TRv alpha 2 and 3); these may act in a negative fashion to inhibit the binding of other TRs to their respective DNA sites. The TRs generally exist as monomers, homodimers, or heterodimers, typically associated with other nuclear proteins such as the retinoid-X receptors.[12]

As might be expected, the various TRs are differentially expressed in a wide variety of tissues, as well as being developmentally regulated. TR beta 1 expression is abundant in liver and kidney with some messenger RNA expression in a wide variety of other tissues. TR beta 2 expression is highest in pituitary tissue. The TR alpha variants are generally widely distributed.[13,14]

The maturation of TRs typically follows the pattern of thyroid hormone effects during development. Expression in pituitary, brain, and bone occurs before expression in liver and brown adipose tissue. The expression of the alpha family typically precedes that of the beta subtypes. This has been shown in rodent, ovine, and human species. Generally, brain and pituitary tissues exhibit the earliest expression of alpha 1 and 2 activity followed later by messenger RNA and protein products in liver, kidney, and brown adipose tissue.[14,15]

FETOPLACENTOMATERNAL THYROID INTERACTION

The relative independence of the maternal and fetal hypothalamic-pituitary thyroid hormone systems is suggested by several clinical observations. The placenta is impermeable to TSH and is largely impermeable to the thyroid hormones. These data are summarized in Table 182-2.[16] Direct evidence of placental transfer of thyroid hormones is provided by studies using various thyroid hormone analogues and tracers, as well as observations under various maternal and fetal conditions. Interspecies differences are important in the interpretation of these data. Prenatal and postnatal maturation varies widely between precocial and altricial species. Placental types differ among the most common species used for perinatal thyroid studies: rodents, sheep, and humans, each species having varying numbers of tissue layers separating maternal and fetal circulations, as well as differing degrees of monodeiodinase activities. Human studies have shown limited transfer from mother to fetus in the euthyroid state. Large doses of T_4 given to women produced only minor changes in cord serum concentrations of hormonal iodine.[17] Supraphysiologic doses of T_3 administered on a long-term basis to pregnant women several weeks before delivery significantly increased maternal serum T_3 levels but only minimally lowered fetal T_4 values.[18,19] Studies in rats have shown low but significant levels of thyroid hormones in fetal tissues before the time of fetal thyroid hormone production.[20,21] These observations have been elegantly reconfirmed in a transgenic rodent model.[22] However, maternally derived T_4 may have implications during pathologic states such as severe fetal hypothyroidism. For example, infants with certain iodide organification defects incapable of synthesizing thyroid hormones still have demonstrable albeit quite low levels of T_4 immediately after birth.[23] In areas of endemic cretinism resulting from low environmental iodide levels, a relationship between maternal thyroid function and subsequent fetal thyroid function can be shown.[24] The early ability of decreased fetal T_4 to markedly increase brain type 2 monodeiodinase levels,[25] as well as the timing and type of brain thyroid hormone receptors on ultimate tissue thyroid-dependent effects, have also been noted.[26,27] These complex and interrelated relationships likely underlie the relative neurodevelopmental sparing of athyrotic neonates. In addition, the inability to consistently diagnose congenital hypothyroidism on a clinical basis in the immediate neonatal period may reflect a maternal contribution to fetal thyroid system homeostasis in the hypothyroid state.

TABLE 182-2

Placental Permeability for Substances Affecting Thyroid Function

Substance	Placental Permeability
I^-	+ + + +
TRH	+ + +
Thioureylenes	+ + +
IgG antibodies	+ + +
T_3	0
T_4	+
TSH	0

I^-, Iodide; *IgG,* immunoglobulin G; *T_3,* triiodothyronine; *T_4,* thyroxine; *TRH,* thyrotropin-releasing hormone; *TSH,* thyrotropin.

The thioureylene antithyroid drugs cross the placenta and may compromise fetal and neonatal thyroid function.[28,29] The placenta also is permeable to selected synthetic thyroid hormone analogues such as 3′,5′-dimethyl,5-isopropyl thyronine. No rationale currently exists for use of these analogues, however, because of their low biologic activity. Finally, the placenta is permeable to the hypothalamic peptide TRH. Both primate and human fetuses early in the third trimester respond to pharmacologic doses of exogenous TRH with an increase in serum TSH.[30,31] Little endogenous TRH is normally detected in adult humans, because of the presence of TRH-degrading enzyme systems in the blood. Although the sera of pregnant women contain somewhat lower levels of these enzymes than do nonpregnant sera, the nearly immeasurable levels of TRH in the maternal circulation have little effect on fetal thyroid function.[28]

The unique fetal environment, by contrast, includes many extrahypothalamic sources of TRH. Fetal gut tissues, particularly pancreas, produce TRH, and this pancreatic TRH has been isolated and found to be identical to hypothalamic TRH. In newborn rat pups, pancreatectomy, but not encephalectomy, is associated with reduced circulating TRH levels.[32] The placenta also

produces an immunoreactive TRH, which appears to be identical to hypothalamic pyroGlu-His-ProNH_2.[33] Other forms of TRH that have TSH-releasing activity have been reported in the placenta.[34] These substances presumably derive from alternative cleavage of the prepro-TRH molecule. The high circulating level of TRH in the fetus results from increased production in extrahypothalamic tissues and from low or absent levels of TRH-degrading activity in fetal blood.[35,36] Human cord values of TRH are also elevated.[37] Administration of anti-TRH antibodies to neonatal rats does not alter serum TSH levels, a finding suggesting that TRH is not required for TSH secretion at this early stage of thyroid development.[38] The significance of TRH in human cord blood is not clear.

In addition to TRH, the placenta produces substances with TSH-like activity. The α-subunit of TSH is identical to that of human chorionic gonadotropin (hCG), and the β-subunit of hCG has structural homology with the β-subunit of TSH; thus, hCG has some TSH-like bioactivity.[39,40] The biologic potency of hCG, however, is only approximately 0.01% that of TSH, and hCG normally has little influence on fetal thyroid system development or function. The large increase in maternal serum hCG activity that occurs early in pregnancy may account for the slight, transient suppression of maternal TSH levels seen early in the first trimester.[39,40]

Placental permeability to maternal molecules may be a factor affecting fetal thyroid function as a result of maternal pathophysiologic states (acute iodide administration, autoimmune thyroid disease, pharmacotherapy of thyrotoxicosis). The fetal pituitary-thyroid axis, however, normally develops independently of maternal thyroid axis influence. The placenta and selected fetal tissues may serve as a source of TRH, but the extent of this influence on fetal thyroid function is uncertain. The demonstrated maternal-fetal transfer of thyroid hormones may be important for fetal development, particularly brain growth and differentiation, in circumstances of fetal hypothyroidism.

CONTROL OF THYROID HORMONE PRODUCTION

The pattern of human perinatal thyroid hormone secretion is shown in Figure 182-2. Maturation of thyroid system control can be considered in three phases: hypothalamic, pituitary, and thyroidal. Changes in these systems are complex and are superimposed on the increasing production and increasing serum concentration of serum thyroid hormone–binding globulin (TBG), as well as the changing pattern of fetal tissue iodothyronine deiodination during gestation. Maturation of these latter systems is described in the next section.

Although the fetal thyroid gland is able to concentrate iodide and to synthesize thyroglobulin at 70 to 80 days gestation, little thyroid hormone synthesis occurs until approximately 18 weeks gestation. At this time, thyroid follicular cell iodine uptake increases, and T_4 becomes measurable in the serum. Both total and free T_4 concentrations then increase steadily until the final weeks of pregnancy.[41,42] This pattern differs from the pattern of development of serum T_3 levels in the fetus. The fetal serum T_3 concentration is low (<15 ng/dL) until 30 weeks and then increases slowly in two distinct phases: a prenatal and a postnatal phase. Before birth, serum T_3 increases slowly after 30 weeks gestation to reach a level of approximately 50 ng/dL in term cord serum.[41-44] After birth, both T_3 and T_4 serum concentrations increase four-fold to six-fold within the first few hours of life, and they peak 24 to 36 hours after birth. These levels then gradually decline to adult values over the first 4 to 5 weeks of life. The prenatal increase in serum T_3 seems to be largely the result of progressive maturation of hepatic type I (phenolic) outer ring iodothyronine deiodinase activity and increasing hepatic conversion of T_4 to T_3, although other tissue sources of deiodinase such as brown fat and kidney may be involved.[44]

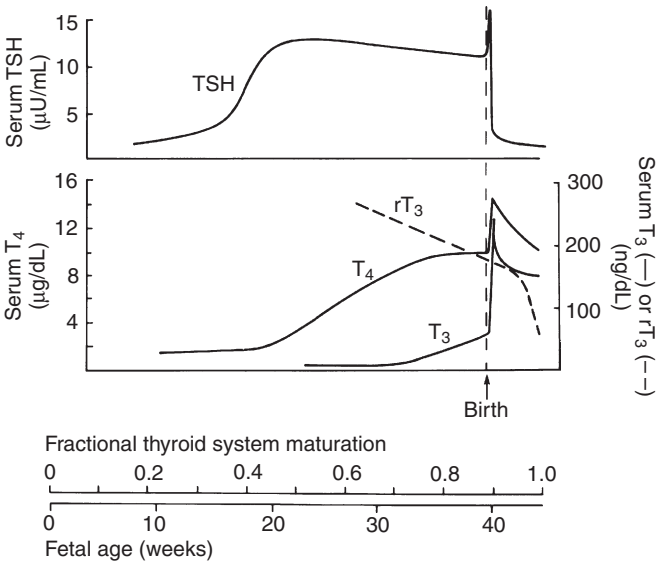


Figure 182-2 Patterns of circulating levels of thyroid-stimulating hormone *(TSH),* reverse triiodothyronine *(rT_3),* thyroxine *(T_4),* and triiodothyronine *(T_3)* in the human fetus.

Fetal serum TSH increases rapidly from a low level at 18 weeks to a peak value at 24 to 28 weeks gestation, and then it gradually declines until term.[41,42] At the time of parturition, partly in response to cold stress, an acute release of TSH (the TSH surge) occurs, resulting in an elevated level by 30 minutes of life.[45] The level of circulating TSH remains modestly elevated for 2 to 3 days after birth. The increases in thyroid hormone that occur immediately after birth are not totally dependent on TSH and may represent other influences on the thyroid gland at the time of parturition. The high postnatal T_3 level in the days after birth results from TSH stimulation of thyroidal T_3 secretion, as well as further rapid maturation of tissue outer-ring monodeiodinase activity in the neonatal period.[46,47]

Progressive increases in fetal serum T_4, free T_4, and TSH take place during the latter half of gestation, with an increasing free T_4–TSH ratio.[42,43,48] The pituitary gland contains a type II outer-ring iodothyronine deiodinase that converts T_4 to T_3, which, in turn, binds to the nuclear T_3 receptor to modulate TSH production. In most circumstances, it is circulating T_4 that is most important in TSH control. Thus, even when the circulating T_3 level is low (as in midgestation), negative feedback control (by T_4) of pituitary TSH secretion may be significant. The progressive increase in the free T_4–TSH ratio in the late-gestation fetus, however, results more from the increase in free T_4 than from a decrease in TSH; a progressive maturation of thyroidal response to TSH has been shown in fetal sheep and seems likely in the human fetus.[43,48] An additional cause of the progressive increase in the free T_4-TSH ratio with a minimal change in TSH is a progressive increase in feedback sensitivity of the pituitary to thyroid hormone inhibition of TSH production. The mechanism for this is not yet clear. Changes in pituitary thyrotroph T_3 or TRH receptors, a decrease in thyrotroph outer-ring iodothyronine deiodinase activity, or decreasing T_3 receptor action on TSH biosynthesis may be involved.

The ontogeny of TRH secretion and function in the fetus remains somewhat obscure. TRH immunoactivity is detectable in the hypothalamus by midgestation, and it increases markedly in the third trimester after the peak in serum TSH activity is noted. The premature infant (<30 to 32 weeks gestation) is characterized by low levels of T_4 and free T_4, a normal or low level of TSH, and a normal or prolonged TSH response to TRH, indicating a

state of physiologic TRH deficiency.[49,50] The term human fetus responds to pharmacologic maternal doses of TRH with a somewhat prolonged increase in TSH, a finding suggesting a degree of relative hypothalamic (tertiary) hypothyroidism.[31] Peripheral sources of TRH (placenta and pancreas) probably contribute to the elevated circulating levels of fetal and cord blood TRH and presumably account for the high circulating TSH level characteristic of the midgestation fetus. This TRH may be responsible for the high fetal serum TSH level before the increase in hypothalamic TRH concentration. The significance of ectopic TRH to the development of thyroid system control, however, remains to be investigated.

The control of fetal thyroid hormone secretion can be characterized as a balance among increasing hypothalamic TRH secretion, increasing thyroid follicular cell sensitivity to TSH, and increasing pituitary sensitivity to thyroid hormone inhibition of TSH release.[42,47,50] The fetus progresses from a state of both primary (thyroidal) and tertiary (hypothalamic) hypothyroidism in midgestation through a state of mild tertiary hypothyroidism during the final weeks of pregnancy and to fully mature thyroid function in the perinatal period.

FETAL THYROID HORMONE METABOLISM

The thyroid gland is the sole source of T_4, whereas most of the T_3 that circulates in the adult is derived from conversion of T_4 to T_3 via monodeiodination in peripheral tissues.[16,51,52] Deiodination of the iodothyronines is the major route of metabolism, and monodeiodination may occur either at the outer (phenolic) ring or the inner (tyrosyl) ring of the iodothyronine molecule. Outer-ring monodeiodination of T_4 produces T_3, the active form of thyroid hormone with greatest affinity for the nuclear receptor. Inner-ring monodeiodination of T_4 produces rT_3, an inactive metabolite. In mature humans, 70% to 90% of circulating T_3 is derived from peripheral conversion of T_4, and 10% to 30% results from direct glandular secretion. Nearly all the circulating rT_3 derives from peripheral conversion, with only 2% to 3% coming directly from the thyroid gland. T_3 and rT_3 are progressively metabolized to diiodinated, monoiodinated, and noniodinated forms of thyronine, none of which possesses biologic activity.

Two types of outer-ring iodothyronine monodeiodinase (5′-MDI) have been described. Type I 5′-MDI, predominantly expressed in liver and kidney, is a high-dissociation constant (K_m) enzyme, inhibited by propylthiouracil, and its activity is stimulated by thyroid hormone. Type II 5′-MDI, predominantly located in brain, pituitary, and brown adipose tissues, is a low-K_m enzyme insensitive to propylthiouracil, and its activity is inhibited by thyroid hormone.[51] Type I 5′-MDI activity in liver and perhaps kidney and muscle probably accounts for most of the circulating T_3. Type II 5′-MDI acts primarily to increase local intracellular levels of T_3 in the brain and pituitary and is important to brown adipose tissue function during the immediate postnatal period. The outer-ring iodothyronine deiodinase also deiodinates reverse T_3 to diiodothyronine.

Both type I and type II 5′-MDI are present in third-trimester fetuses.[25,52-54] In sheep, a species in which thyroid hormone system maturation closely resembles that in humans, hepatic type I 5′-MDI activity increases three-fold to four-fold, and brain type II 5′-MDI activity increases approximately 50% over the last third of gestation.[52] Both deiodinase enzymes are thyroid hormone responsive. Hepatic type I 5′-MDI activity, however, becomes thyroid hormone responsive (e.g., activity decreases with hypothyroidism) only during the final weeks of gestation. Brain type II activity, in contrast, is responsive (activity increases with hypothyroidism) throughout the final third of gestation.[25,55] Thus type II deiodinase probably plays an important role to provide a source of intracellular T_3 to those tissues (e.g., pituitary and, in some species, brown fat and brain) dependent on T_3 during fetal life, whereas the ontogeny of the type I enzyme (to provide increased serum T_3 levels) increases only during the final weeks of gestation and during postnatal life.[32]

TABLE 182-3

Average Iodothyronine Production Rates in Fetal Sheep

	PRODUCTION RATE (μg/kg per day)	
Iodothyronine	**Fetus***	**Newborn†**
T_4	20-50	35
rT_3	5	2
T_3	2	16
T_4S	10	0.6
rT_3S	12	8
T_3S	2	0.4

Data from Fisher DA, Polk DH, Wu SY: Fetal thyroid metabolism, a pluralistic system. *Thyroid* 4:367, 1994.

*120-135 days gestation; pregnancy 150 days duration in sheep.

†7-14 postnatal days.

rT_3, Reverse triiodothyronine; *rT_3S,* reverse T_3 sulfate; *T_3,* triiodothyronine; *T_4,* thyroxine; *T_3S,* T_3 sulfate; *T_4S,* T_4 sulfate.

An inner (tyrosyl)-ring iodothyronine monodeiodinase (type III 5′-MDI) has been characterized in most fetal tissues, including the placenta.[31,52] This enzyme system catalyzes the conversion of T_4 to rT_3 and T_3 to diiodothyronine. The maturation of these enzyme systems has been studied primarily in the rodent and sheep. Fetal thyroid hormone metabolism is characterized by a predominance of type III enzyme activity, particularly in liver, kidney, and placenta, and this accounts in part for the increased circulating levels of rT_3 observed in the fetus.[51-53] Placental type III deiodinase contributes to amniotic fluid rT_3 levels and presumably also contributes to circulating fetal rT_3.[16] The persistence of high circulating rT_3 levels for several postnatal weeks in the newborn, however, indicates that type III 5′-MDI activities expressed in nonplacental tissues importantly contribute to the maintenance of high circulating rT_3 levels.[16]

Studies have shown high concentrations of sulfated iodothyronine analogues in human and ovine fetal serum.[56-60] These include T_4 sulfate (T_4S), reverse T_3 sulfate (rT_3S), T_3 sulfate (T_3S), and T_2 sulfate (T_2S). Average production rates, calculated as the product of basal hormone concentrations and clearances, are shown in Table 182-3. The sulfate conjugates and rT_3 have low affinity for the nuclear T_3 receptors and minimal biologic activity.[61] Thus the production of inactive thyroid hormone analogues in the fetus (rT_3,T_4S, rT_3S = 29 μg/kg per day) greatly exceeds the production of active T_3 (2 μg/kg per day). Clearly, most of the T_4 in the fetus is metabolized to inactive metabolites via the type I and type III iodothyronine monodeiodinase and sulfotransferase enzyme systems in fetal tissues. Evidence is suggestive that T_3S can be metabolized in vivo to T_3.[62] T_3S production is quite limited, however, and the combined average production rate for T_3 and T_3S (4 μg/kg per day) in the fetal sheep is low. It appears from these data that peripheral thyroid hormone metabolism in the sheep fetus, in contrast to the adult, is shunted to inactivation, rather than to the production of active hormone.

The alanine side chain of the inner ring of the iodothyronines also may undergo decarboxylation or transamination.[63] Although the acetic acid derivatives of T_4 have some biologic activity in vitro, their rapid degradation in vivo precludes much physiologic significance. Pyruvate and lactate derivatives also have been isolated, both with minimal biologic activity. Thyroid hormones also are excreted as both free and glucuronide or sulfuronide conjugates in urine and stool; some of these forms can undergo hydrolysis and enterohepatic recirculation. In general, gut excretion accounts for less than 15% of the total amount of metabolized T_3 or T_4 after birth.

Both T_3 and T_4 in blood are associated with various plasma proteins, including TBG, T_4-binding prealbumin (TBPA), and albumin.[63] TBG serves as the primary transport protein for both T_3 and T_4; approximately 70% of the total T_4 and 40% to 60% of total T_3 are bound to TBG. The remaining thyroid hormones are distributed about equally between TBPA and albumin. The binding affinities of these proteins are such that adult free T_4 and T_3 concentrations are approximately 0.03% and 0.3% of the total hormone concentrations. TBG, TBPA, and albumin are produced by the liver, and production of these proteins increases progressively during the final half of gestation. Hepatic TBG production is stimulated by estrogen, and the increasing levels of estrogens during pregnancy account, at least in part, for the T_4 concentration, which increases progressively from midgestation until 34 to 35 weeks gestation.[16,43] The free T_4 level, however, also increases, because of progressively increasing T_4 production.[43,47] The T_3 concentration also increases, from a barely detectable level before 30 weeks gestation to 50 ng/dL in the term fetus, and the free T_3 concentration probably increases in parallel.

Fetal thyroid hormone metabolism is largely oriented to production of inactive metabolites via production of sulfate conjugates and predominant T_4 inner-ring monodeiodination to rT_3. The type 2 deiodinase activity in fetal brain and brown adipose tissues serves to protect these tissues in the event of fetal T_4 deficiency.

Thyroid System Effects and Adaptation to Extrauterine Life

In general, much of the fetal thyroid development is preparatory, providing for the relatively large amounts of thyroid hormones required for normal postnatal development. The production of active thyroid hormones is markedly increased in association with the events of parturition (see Figure 182-2). During the first hours after birth, an abrupt three-fold to six-fold increase occurs in circulating T_4 and T_3 levels, coincident with an increase in serum TSH concentration.[42] The initial increases in circulating thyroid hormone levels largely result from increased hormone secretion from the thyroid gland.[45,46] The TSH surge (which is likely a response to the thermal stimuli coincident with birth), however, can be obtunded in the neonatal sheep by warm bath immersion without complete inhibition of the increments in T_3 or T_4.[3] A postnatal increase in serum catecholamine concentrations also occurs at the time of parturition,[64] and the thyroid gland is adrenergically innervated. Stimulation of thyroidal adrenergic receptors may augment TSH-induced changes in the T_4-T_3 ratio of secreted thyroid hormones. Because TBG concentrations remain relatively constant throughout the newborn period, free T_4 and T_3 levels rise abruptly after birth in association with increases in hormone production rates. The cold-stimulated TSH surge is short-lived, and the decrease in TSH that follows during the 72 to 96 hours after birth results from feedback inhibition by T_4 at either the hypothalamic or the pituitary level (or both).[42,47] The serum TRH concentration is elevated in cord blood and falls in the days after birth. A clear increase in the serum TRH value coincident with the increase in TSH after parturition has not been reported, but the parallel increases in both TSH and prolactin levels in the early hours after birth support the view that the TSH surge is mediated by TRH.[42,47] Thyroid hormone levels in the newborn gradually return to adult levels by approximately 1 month of age. The high level of circulating rT_3 characteristic of the fetus persists after birth and gradually declines to the adult range by 4 to 6 weeks of age.[54]

The metabolic significance of the neonatal thyroid hormone surge is not entirely clear. Physiologic processes known to be modulated by thyroid hormone in adults, such as thermogenesis and cardiovascular responses, clearly are important in the transition from intrauterine to extrauterine life, and it is tempting to link these with the changes in thyroid hormone metabolism that occur during transition. Several studies have attempted to establish such a link. Brown adipose tissue is the major site for newborn thermogenesis.[65] Hypothyroidism induced during the final weeks of gestation is associated with impaired brown adipose tissue thermogenesis, largely because of the effects of T_4 on brown adipose tissue iodothyronine monodeiodinase activity and mitochondrial uncoupling protein (thermogenin).[66] Obtundation of the postnatal T_3 surge (by acute thyroidectomy in newborn sheep), however, does not accentuate transient neonatal hypothermia, a finding indicating that the prenatal T_4 level is more important in neonatal thermogenesis than is the early postnatal T_3 surge.[46]

A similar relationship between thyroid system function and neonatal cardiovascular adaptation has been described in neonatal sheep; normal fetal thyroid function in the final weeks of gestation is required for the increases in myocardial performance associated with the transition to extrauterine life. Obtundation of the acute increase in serum T_3 at birth, however, does not alter neonatal cardiovascular adaptation.[67] These observations support the view that, in the sheep, the level of thyroid function during the final weeks of gestation is more important than the neonatal increases in T_3 and T_4 for successful neonatal transition. The situation in the human neonate may be somewhat different. Neonates with congenital thyroid agenesis have few, if any, signs or symptoms of thyroid hormone deficiency, and their postnatal environmental adaptation usually is not impaired.[42,68] The fetal sheep is relatively more mature at birth, presumably accounting for this difference. The precise timing of maturation of thyroid hormone effects on thermogenesis and cardiovascular function in the human neonate has not been defined. It is clear that most (95%) infants with congenital hypothyroidism at birth have few manifestations of thyroid hormone deficiency and develop normally when replacement therapy is begun in the neonatal period. Serum TSH levels are elevated, and a 3- to 6-week delay in bone maturation may occur.[42] The posterior fontanelle may be relatively large, and the physiologic hyperbilirubinemia of the newborn may be more marked and prolonged. Growth is not impaired, however, and other metabolic alterations usually are not evident.

The ontogenesis of various thyroid actions has been characterized in rodents. These include tissue thermogenesis, carcass and muscle growth, skeletal maturation, skin and brain maturation, and hormone and growth factor production and action. Some of these effects represent thyroid hormone actions on specific gene products, mediated via thyroid hormone receptor control of gene transcription.[52,69] Other thyroid hormone actions represent complex events, the mechanisms of which are not yet understood, such as the effects on growth, skeletal maturation, and brain development.

The thyroid hormone dependency of these growth and metabolic events in rodents is manifest in the postnatal period, in general during the second and third postnatal weeks. Nuclear thyroid hormone receptors appear in most rodent tissues during the last week in utero and the first postnatal week.[70] Thus there is a discrepancy between the timing of appearance of selected thyroid hormone actions and the appearance of thyroid nuclear receptors, and it is possible that at least some of the variation in timing of appearance of thyroid hormone actions in the rodent relates to maturation of events beyond the step of T_3 binding to the nuclear receptors. Other thyroid hormone receptors have been identified, including mitochondrial and plasma membrane receptors, but the significance of these is not yet clear.[69] Most of the effects described previously are thought to be mediated via the nuclear receptors.

A similar delay in appearance of selected thyroid hormone actions has been observed in developing sheep.[52,69] The sheep, like the human and in contrast to the rodent, is a precocious

species and is relatively mature at birth. In fetal sheep, effects of thyroid hormones on various parameters of brain development appear as early as 0.5 to 0.6 of fractional thyroid system development. Thyroid effects on skin maturation and growth also appear relatively early in gestation, whereas effects on heart, lung, kidney, and liver are delayed until the final few weeks before birth. In the sheep, as in the rodent, the appearance of selected thyroid hormone actions late in gestation, such as the effects on hepatic malic enzyme activity and hepatic epidermal growth factor receptor concentrations, is not accounted for by the appearance of thyroid hormone nuclear receptors; hepatic receptors are present by 0.5 (on the fractional thyroid system maturation scale) of gestation.[52] The effect of thyroid hormone on lung β-adrenergic receptor binding also occurs well after the appearance of thyroid hormone nuclear receptors in the lung.[71] Thus the timing of maturation of thyroid hormone actions is variable in both rodent and sheep species and relates to the ontogenesis of nuclear thyroid hormone receptors, as well as to maturation of postnuclear mechanisms.

In humans, thyroid nuclear receptors have been reported in fetal lung, brain, heart, and liver at 13 to 19 weeks gestation.[15,72] The only thyroid hormone actions that have been characterized in the fetus are the effects of hypothyroidism on serum TSH and bone maturation. Most effects of thyroid hormone on perinatal developmental processes occur after birth (Figure 182-3).

Thyroid hormones affect important developmental processes, including growth, thermogenesis, and development. The largely successful transition of athyrotic infants to extrauterine life reflects the limited importance of fetal thyroid hormones in all but the final weeks of gestation. An exception to this may be the fetal brain, a major site of type II iodothyronine monodeiodinase activity in the fetus. The presence of this enzyme system in the brain early in development and its demonstrated response to fetal hypothyroidism in the rat and sheep suggest that intracellular conversion of T_4 to T_3 in the brain is important in these species for normal development and differentiation of the central nervous system. The critical period for this effect in the sheep is the last trimester of gestation; in the rat, the critical period is the first 3 postnatal weeks. Precise data are not available for the human fetus, but early treatment of congenital hypothyroidism in the neonate prevents subsequent mental retardation, a finding suggesting that the period of thyroid dependency of the human brain extends into the postnatal period. Early and adequate postnatal thyroid hormone replacement in athyrotic infants also normalizes growth and development.

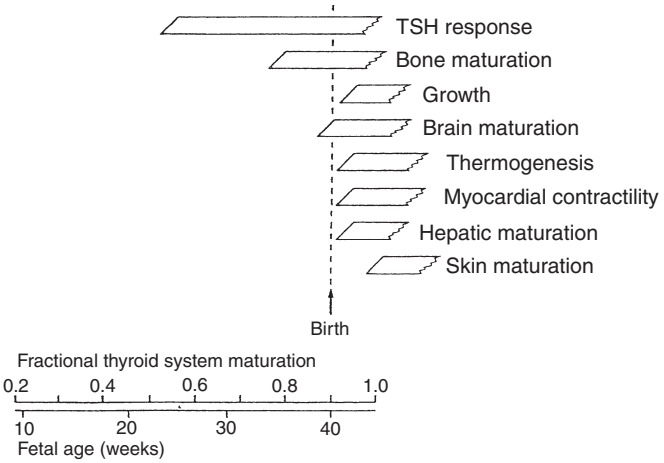


Figure 182-3 Onset of actions of thyroid hormone in the developing human. The *left edge of the bar* indicates the initiation of thyroid hormone responsiveness of the indicated parameter. *TSH,* Thyroid-stimulating hormone.

Low-T_3 Syndrome in Premature Infants

In the preterm infant, changes in thyroid function parameters during neonatal adaptation are qualitatively similar to those in term infants but are quantitatively obtunded.[42,43] The neonatal TSH surge and the neonatal T_4 and T_3 peak responses decrease with decreasing gestational age, and the transient low-T_3 state that follows probably is related to the state of relative undernutrition in the neonatal period.[42] Premature infants have an increased susceptibility to neonatal morbidity, including birth trauma, acidosis, hypoxia, hypoglycemia, hypocalcemia, and infection, all superimposed on feeding disorders and relative malnutrition. All these factors tend to inhibit conversion of T_4 to T_3 in the neonatal period and aggravate the extent of the low-T_3 state characteristic of prematurity.[42,73-75] Serum T_3 values may remain low in these infants for 1 to 2 months.

Features of the *low-T_3 syndrome* in premature infants include a low serum T_3 concentration resulting from a decreased rate of conversion of T_4 to T_3, variable but usually elevated serum rT_3 levels, and normal or low total serum T_4 concentrations.[42,43] Free T_4 levels usually are in the range of values for healthy premature infants of matched gestational age and weight. TSH values are low in these infants. Preterm infants presenting with low or borderline-low thyroid hormone levels and elevated TSH values should be carefully evaluated for hypothyroidism, including assays of thyroid-blocking immunoglobulins. A trial of T_4 replacement therapy may be warranted in these infants.

Concerns remain regarding ultimate cognitive function in preterm infants with low thyroid hormone values. Early (first week of life) low T_4 levels have been associated with neurologic dysfunction at 5 years of life and poor school performance at 9 years of age.[76] This finding has prompted several studies examining the effect of T_4 supplementation in very preterm infants. Randomized, placebo-controlled T_4 replacement in preterm infants less than 30 weeks gestation resulted in improved neurologic function in very preterm infants (25 to 26 weeks gestation) but worsened cognition and behavioral development in infants born after 29 weeks gestation.[77] Review of thyroid hormone augmentation for preterm infants fails to demonstrate improved neurodevelopmental outcome in treated infants.[78] Moreover, results from various antenatal TRH exposure trials (preterm fetuses exposed to maternal TRH treatment in an attempt to improve subsequent postnatal lung function) also suggest that early alterations in thyroid system function may be associated with deleterious intellectual outcomes.[79,80] Thus the use of thyroid hormones in nonhypothyroid preterm infants should be restricted to those infants enrolled in approved studies.

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SECTION XXV

Ovary and Testis

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Stan R. Blecher and Robert P. Erickson

Genetics of Sex Determination and Differentiation

The classical paradigm of mammalian, including human, sexual development postulates an initially "indifferent" (sexually undifferentiated) state that is transformed, in two major stages, into the sexually dimorphic male and female forms. The two stages are called *primary,* relating to the gonad, and *secondary,* referring to all other genital organs.[1]

In the first stage, *primary sex determination,* specific sex determining genetic factors, present from conception, drive the development of the gonad. The primordial, indifferent gonad is situated in the gonadal ridge region of the intermediate mesoderm of the fetus. In genetic males, the indifferent gonad is induced to become a testis, under the influence of (a) male-determining gene(s). In the female, with no such genes present, the gonad remains uninduced and an ovary develops. Mesenchymal cells of the gonadal (genital) ridge and the neighboring metanephros give rise, in males, to androgen-producing Leydig cells of the testis or, in females to the theca cells of the ovary and to connective tissue. The adjacent celomic epithelium grows into the mesodermal mass to produce the Sertoli cells of the testicular seminiferous tubules, or the follicle cells of the ovary. In addition, primordial germ cells migrate to the developing gonad to give rise to sperm or egg cells.

In the second stage,[2] *secondary sexual differentiation,* all other features of sexual dimorphism are established. In this concept, all features of both internal and external sexually dimorphic structures other than the gonads develop from the action of hormones produced in males by the testis, lack of these hormones (or production of other hormones) in females, and independently of the sex-determining gene(s).[1] This paradigm is known as the Jost Principle.

Although current understanding builds on this framework, our knowledge has advanced considerably in recent years, leading to some modifications of the original paradigm. It is now known that "genetic maleness" in mammals is normally dependent on the presence of a sex- determining region of the Y chromosome and "genetic femaleness" on its absence. The identity of the testis-determining gene on the Y chromosome is known, as is the fact that the effect of this gene in formation of the testis is not direct, but rather is mediated by other, so-called downstream genes. Some of the latter have been identified. Also, the action of the Y-chromosomal region is preceded by "upstream" gene action that is evidently prerequisite for the Y-chromosomal function in gonadal determination. Furthermore, some sex specific, including Y chromosomal (male-specific) gene action itself, appears to influence sexual phenotype, either before gonadal development (i.e., at a "pregonadal" juncture) or later, but independently of hormonal action. Finally, we now better understand the development of the ovary as being not merely a passive, uninduced state, but also dependent on specific gene action. These findings necessitate modification of the Jost paradigm.

A large number of genetic pathways interact in a complex and, in part, overlapping and redundant manner, to execute the genetic control of sexual development. In this chapter, we describe this dynamic, sequential program as occurring in four stages. These are: sex-chromosomal assignment of sex, established at conception; pregonadal sexual development and sexual dimorphism; gonadal development; and secondary sexual differentiation.

Y-CHROMOSOMAL SEX DETERMINATION

Sex-Determining Role of the Mammalian Y Chromosome

In 1923 it was demonstrated cytologically that humans have X and Y chromosomes.[3] By analogy with *Drosophila,* it was assumed that sex determination in mammals would be mediated by the ratio of the number of X chromosomes to the number of sets of autosomes (i.e., chromosomes other than the sex chromosomes). However, in 1959, it was discovered that an XO sex chromosome karyotype (i.e., absence of the Y chromosome) produced a basically female phenotype both in humans (Turner syndrome)[4] and mice,[5] and XXY produced predominant maleness in humans (Klinefelter syndrome).[6,7] These observations demonstrated that the presence of a Y chromosome, independent of the number of X chromosomes, resulted in the development of a predominantly male phenotype, and the absence of the Y chromosome resulted in the development of a predominantly female phenotype. Thus the Y chromosome appeared to possess a gene or genes the presence or absence of which determined the destiny of the bipotential gonad as a testis or ovary, respectively, and, through this, the development of the dimorphic secondary sex characteristics.[1] In the human, the hypothetical Y-chromosomal gene was named *TDF* (testis-determining factor), and in mice *Tdy* (testis-determining gene on the Y). (In accordance with standard convention, gene symbols are italicized; gene product symbols are not. Because of a high degree of homology [genetic equivalence] between mouse [and other mammals] and human and of accessibility to experimentation especially in mice, modern human genetic research relies extensively on murine studies, from which much can be extrapolated to humans. Human genes are indicated by uppercase letters whereas their mouse homologues are in lower-case, except that for dominant genes the first letter is capitalized.) The minor deviations of Klinefelter and Turner syndromes from normal male and female phenotype, respectively, are probably due to the influence of other (non-*TDF*) sex-chromosomal factors (see later section) on the sex differentiation pathway.

Early Y-Chromosomal Candidate Sequences: H-Y antigen, *Bkm* Sequences, and *Zfy*

Many candidate genes for *TDF* have been studied, taking into account biochemical differences between males and females and the evolutionary conservation that would be expected in genes involved in sex determination. It was proposed that the

highly conserved, male-specific molecule, H-Y antigen (originally identified in skin transplantation experiments), was the product of *TDF*.[8] For more than 10 years this proposal was considered valid. However, the findings of phenotypically male XX*Sxr* male mice that did not express H-Y[9] and of XY humans of female phenotype who were H-Y positive,[10] excluded this hypothesis. Another candidate for *TDF* was the repetitive sequence *Bkm* ("Banded krait minor") originally isolated from the snake *Bungarus fasciatus*[11] and which, in some vertebrates, was found only in the sex chromosomes. This hypothesis was rejected because in the human these sequences are dispersed in the genome and are not concentrated in the sex specific region of the Y chromosome.[12]

The discovery that 46,XX males usually have translocations (transfers of chromosomal material) of Yp (where p indicates the short arm of a chromosome and q the long arm) to one of their Xps was a major breakthrough in mapping *TDF* on the Y chromosome.[13,14] The X and Y chromosomes have homologous (genetically equivalent) segments at the ends of their respective p arms, and these segments pair at meiosis. Because of genetic exchange between these segments, genes located in this region produce phenotypes that appear to be neither X- nor Y-linked (i.e., appear to be inherited as if on autosomes). Accordingly, these regions are called *pseudoautosomal.* The occurrence of abnormal crossovers adjacent to the pseudoautosomal region can result in the illegitimate transfer of *TDF* to the X chromosome.[15,16] Molecular analyses in such patients permitted the construction of a deletion map of the Y chromosome[17,18] and showed that a small region of Yp adjacent to the pseudoautosomal region is essential for testicular differentiation. Finer mapping continued, and one candidate gene was mistakenly identified: Page and colleagues[19] found a highly conserved sequence that seemed to map with a male-determining Y fragment and named it *ZFY*. However, one of their XX males did not have *ZFY*, and the finding of more such patients[20,21] suggested that *ZFY* could not be *TDF*.

The *SRY* Gene

The resumed search for *TDF* was targeted on 35 kilobases (kb) of DNA between the proximal (i.e., toward the centromere of the chromosome) limit of the pseudoautosomal region of the Y chromosome and the breakpoint in XX males lacking *ZFY*. A subclone selected for its male specificity in mammals was found to encode a protein containing an 80-amino-acid segment that was homologous to the mating-type protein in *Schizosaccharomyces pombe* and to a conserved 80-amino-acid, DNA-binding motif present in the HMG1 (High Mobility Group 1) transcription-regulating proteins.[22] Further analyses of a variety of tissues disclosed a transcript of this gene, only in the testes. It was proposed that this gene, designated *SRY* (sex-determining region, Y chromosome), was *TDF*.[22] Soon thereafter, mutations in the *SRY* were found in some cases of XY, pure gonadal dysgenesis.[23–26] The mouse *Sry* gene was cloned at the same time as *SRY*.[27] Much information on human *SRY* has been obtained indirectly from studies on mouse *Sry*. In the following, these symbols are used specifically for their respective species, or *SRY/Sry* is used for situations applying to both.

Some mice transgenic (i.e., in which a gene has been artificially transferred) for a fragment containing *Sry* showed 40,XX sex-reversal—that is, mice otherwise destined to be female had a male appearance (although they were sterile).[28] Testes of these mice were histologically similar to those of human XX males. However, sex reversal did not always occur. This incomplete penetrance may be related to the influence of genetic background on *Sry* transgene expression.[29] Genetic modifiers may also be the explanation for the multiple phenotypes seen with particular mutations in *SRY*. Interestingly, the human gene *SRY* does not cause sex reversal in transgenic mice; it is not nearly as conserved in function as some human genes, a few of which can work even in *Drosophila*. The HMG boxes of *SRY* and its mouse homologue, *Sry*, are examples of so-called generalized HMG boxes.[30] It has been demonstrated that the SRY HMG box binds to synthetic DNA fragments of the sequence AACAAAG,[31] but also to cruciform DNA structures irrespective of their sequence. Both targets adopt similar conformations in solution, in which state binding of SRY induces a large conformational change in the DNA. Some mutations in the SRY HMG box alter the DNA contact sites in such a way as to preclude the usual deformation of the DNA.[32,33]

The principle evidence in favor of *SRY/Sry* being *TDF/Tdy* is the following:

1. It is localized in the smallest region of chromosome Y capable of inducing testicular differentiation in humans[22] and mice.[27]
2. Some cases of XY, pure gonadal dysgenesis present de novo mutations in the *SRY* gene.[23–26]
3. In the mouse, *Sry* expression correlates with the initiation of testicular determination in the gonadal ridge[34] and, in both mouse and man, its expression in the blastocyst corresponds to the more rapid growth of the Y-bearing blastocyst (see later discussion).[35–38]
4. A fragment of DNA that includes the mouse gene is able to induce male-type development in some transgenic XX mice.[28]
5. It is an evolutionarily conserved gene on the Y chromosome of metatherian and eutherian mammals.[22,39]

PREGONADAL SEXUAL DEVELOPMENT AND SEXUAL DIMORPHISM

In humans, although the sex chromosome complement, and therewith the sex-determining genes, are established at fertilization, it is not until about 42 days that differentiation of the indifferent gonads into testes in the male or, slightly later, their development into ovaries in the female, can be detected. However, very early male and female embryos, and possibly even zygotes, do differ phenotypically. In a previous edition of this book, we argued that differential gene expression at pregonadal stages influences or sets the scene for subsequent gonadal sex determination and differentiation and that this warrants the inclusion of a Pregonadal Stage in the sequence of sexual developmental events. Sex differences that are evident in early developmental stages, but for which the detailed genetic basis is not yet determined, include the time of occurrence of the first cleavage division, the rate of early embryonic cell division, the rate of accumulation of proteins, and differences in external genitalia. Now evidence exists that pregonadal expression of genes upstream to *Sry* influence the sex determining pathway. Some of these upstream genes are autosomal although, as discussed later, pregonadal Y-chromosomal gene expression, including expression of *Sry* itself, also occurs.

Phenotypic Pregonadal Sexual Dimorphism for Which Specific Genes Have not yet Been Identified

First Cleavage Division of the Zygote

In studying in vitro fertilized and cultured bovine embryos, Yadav and colleagues[40] found that early cleaving embryos were significantly more likely to be male than female. This sexual dimorphism suggests the possibility that differential expression of Y or X-linked genes is present in the zygote shortly after fertilization.

Rates of Cell Division in Early Embryos

Scott and Holson[41] reported that male rat fetuses are larger than female and have higher protein content at 12.5 days postcoitus. Preimplantation XY mouse embryos have been reported to be

more advanced than XX[42] as have cattle embryos.[43,44] Thornhill and Burgoyne[45] argued that the paternal X has a retarding effect on postimplantation mouse embryos. However, Burgoyne[46] found an accelerating effect of the Y chromosome on preimplantation mouse embryos, although the trait appeared to be polymorphic. Peippo and Bredbacka[47] found that in day 3 *in vivo* mouse embryos, females were more advanced; during a subsequent 24-hour *in vitro* culture period the increase in cell number was greater in male embryos. These authors proposed that previous results suggesting increased rates of cell division in male embryos might be an artifact of in vitro culture. Ursula Mittwoch has long maintained[48] that fast growth rate actually leads to testis formation and slow rate to ovary formation.

Pregonadal, Genetically Determined Development of Male External Genitalia

An important brick in the structure of our current understanding of pregonadal expression of sex-determining genes emerged from studies on some marsupials, such as the tammar wallaby. In these animals some features of sexual dimorphism, including the scrotum in the male and the mammary glands in the female, appear before development of the gonads and independently of hormones.[49] Although these organs develop mainly under hormonal influence in true (eutherian) mammals, evolutionary conservation of some pregonadal sex-determining gene expression has been shown to occur in mice. The XX*Sxr* pseudomale mouse is a chromosomal female (XX) that is masculinized by transposition of *Sry* and neighboring genes to an X, but which lacks the rest of the Y chromosome. The anogenital space, the size of which is a measure of masculinization (and which is also the region in which the scrotum develops) is smaller in prepubertal and adult pseudomales than in their normal, XY brothers.[50] Interestingly, XX*SXr* pseudomale mice, in which testosterone levels are as high as or higher than in normal males, have abnormal epididymides that lack the important initial segment,[51] and the abnormality is present before the pubertal androgen surge.[52] However, XO*Sxr* mice, with equivalent levels of testosterone, have normal epididymides. According to the Jost Principle, epididymal development should be solely dependent on androgens, but these data suggest an effect of a noninactivated X-chromosomal gene, independent of hormonal action, on this secondary sex organ.[51,52]

These examples in tammar wallabies and mice illustrate the existence of secondary sexually dimorphic gene expression patterns in pre- or postgonadal embryos that are independent of gonadal hormonal action, contrary to the Jost Principle. Some candidate genes that could account for such pregonadal gene activity[53] and a possible model molecular mechanism[54] is discussed later.

Genes Known to be Transcribed Pregonadally

Early Transcription of Zfy and SRY

Transcripts of *Sry* and *Zfy* are present in the mouse embryo at the blastocyst stage,[35] and, in the human embryo, *SRY* and *ZFY* have been found to be transcribed at the one cell stage—the earliest genes yet found to be transcribed in the human embryo.[36,37] These data suggest that *SRY/Sry* could be involved in the initiation of the genetic cascade that leads to sex determination.[55]

Expression of H-Y Antigen by Early Embryos

As mentioned earlier, evidence for the existence of (a) male-specific molecule(s), subsequently named H-Y, originally emerged from skin transplantation experiments in inbred mice. One epitope of human H-Y, called *SMCY*, has been cloned.[56] A serologically detectable counterpart of H-Y, now called SMA (serologically detected male antigen) has been extensively used as a method of exploring genetic sex. Studies on early embryos have shown that H-Y antigen is detectable as early as at the eight-cell stage in mice.[57] Although the genetics of sex-specific antigens is far from clear, this finding further underscores the fact that sex-specific gene expression commences at an early stage in embryogenesis.

Genes Functioning Upstream of SRY/Sry

For the orderly function of the sex-determining gene *SRY/Sry*, normal operation of two other sets of genes must first occur. These two sets are those genes controlling the establishment of the cells and tissues that are precursors of the gonads and those genes that directly influence *SRY/Sry*.

Genes That Act on Precursor Cells and Tissues

Several genes of this type are known. Some of these are expressed at early developmental stages, and others are expressed in the gonadal ridge shortly before the activation of *SRY/Sry* that immediately precedes gonadal differentiation.

Of genes that function at early stages of development, members of the *Lhx* family may be near the "top" of the cascade. *Lhx* refers to the LIM homeobox gene family, which contains transcription factors.[58] Genetic "knockout" (KO) (experimental inactivation) of the mouse gene *Lhx1*, which has a role in gastrulation, causes absence of kidneys and gonads.[59] KOs of *Lhx-9* also fail to form gonads because somatic cells of the genital ridge fail to proliferate. *Fgf-8* and *Wnt-4* are upstream of *Lhx-1*,[60,61] and *Lhx-9* and *Wt-1* both activate *Sf-1*[62] (see next section). Mice with KO of *Emx-2* lack kidneys, ureters, gonads, and genital tracts.[63]

Gene action in the gonadal ridge before sex-specific gonadal differentiation includes steroidogenic factor-1 (*SF-1/Sf-1*), which is homologous to the *Drosophila* gene Fushi tarazu 1 (*Ftz-F1*). A key regulator of steroidogenic enzymes in adrenocortical cells,[64] it is expressed in the urogenital ridge at embryonic day 9 to 9.5 in the mouse. This is before the time of expression of *Sry*[65]; this result was confirmed in humans.[66] KO of *Sf-1* resulted in mice that died 8 days after birth.[67,68] The knockout mice lacked adrenal glands and gonads and had a resulting deficiency in corticosterone that was the likely cause of death.

Erickson and colleagues[69] predicted that human mutations in *SF-1* would lead to "a phenotype associated with predominantly female appearance and severe neonatal problems due to lack of steroid hormones." Such a patient was reported in 1999: A 46,XY female with adrenal failure at two weeks of life was found to have a heterozygous, adjacent two-base pair mutation.[70] Three other such patients have now been described. The region of 9q33 to which *SF-1* maps[71] has not yet been associated with cytogenetic abnormalities and problems of sexual differentiation. Evidence has accrued that SF-1 may have a role in co-stimulation of the Müllerian-inhibiting substance (MIS) promotor.[72,73]

Other genes that are expressed in mouse gonadal ridge at day 9.5, before *Sry* and before sexual determination becomes evident, may have a role in preparing the gonad for sexual differentiation. These include *Dmrt-1* and *-2* and *Wt-1*. *DMRT-1* and *DMRT-2*, mammalian homologues of a gene shared between *C. elegans* and *Drosophila*, map to distal 9p (toward the tip of the chromosome arm).[74,75] It has long been known that deletions at distal 9p are frequently associated with 46,XY sex reversal. *Dmtr-1* is expressed in the mouse genital ridge at day 9.5.[76] Expression is initially equal in genital ridges of both sexes but gradually it becomes limited to expression in the 40,XY gonad. The avian homologue of *DMRT-1* is also expressed in the genital ridge and Wolffian duct (a precursor of internal sex organs) before sexual differentiation in the avian embryo; the same is not true of the *SRY* homologue.[76] Thus, as might be expected for a gene with homology to a *C. elegans* and *Drosophila* gene involved in sex determination, *DMRT-1* may have a more conserved role in sexual determination in vertebrates than does *SRY*. The association between the Wilms tumor 1 (*WT-1*) gene and testis development was recognized from investigations into the Denys-Drash and the WAGR syndromes. In Denys-Drash syndrome, caused by point

mutations of the *WT-1* gene, complex nephropathy and sexual ambiguity is present. Male (XY) patients with Denys-Drash syndrome may have female or ambiguous external genitalia with dysgenic gonads. WAGR syndrome, caused by deletions of chromosomal band 11p13, consists of Wilms tumor, aniridia, genitourinary anomalies, and mental retardation. Patients with the WAGR syndrome may have undescended testes and hypospadias, both of which are abnormalities of secondary sexual development. The occurrence of deletions suggested the presence in this region of a tumor suppressor related to Wilms tumor.[77] It has been proposed that the *WT-1* gene is involved in mesenchymal-epithelial interactions[78]—for example, between Sertoli cells and Leydig cells in the testis. Such interactions are crucial not only in the developing gonad but also the kidney. *WT-1* has at least 24 different isoforms (different forms of the gene) simultaneously present in individuals, which arise through alternative splicing of the messenger RNA of the gene. Of these many isoforms, particular interest has focussed around two that are highly conserved in evolution[79] and that are due to alternative splice donor sites at the end of exon 9 of the gene. The two isoforms respectively either possess, or lack, the three amino acids K (lysine), T (threonine) and S (serine). Children with Frasier syndrome, a variant of Denys-Drash, who frequently show partial or complete XY sex-reversal, mostly have a mutation in the splice site that creates the KTS insertion and thus a deficiency in the +KTS isoform.[80] Homozygous mice lacking the *Wt-1* +KTS isoforms also show a complete XY sex reversal due to a dramatic reduction of *Sry* expression.[81] Some conflicting data exist, however; thus, although interactions between *Sry* and *Wt-1* in the developing mouse gonad are clearly important, their relative roles in control of sexual determination are not yet clear.

Genes That Directly Influence *SRY/Sry*

Two known mutants in mice suggest the possibility that chromatin changes could be the initial stage in sex determination. One possible mechanism for this might be "release" of *SRY* from an autoinhibition that this gene may normally undergo through a receptor on the gene to which the gene's own product can bind. The gene *M33* is the mouse homologue of the *Drosophila* gene *Polycomb*, which regulates chromatin structure. KO of *M33* caused failure of growth of the primordial gonad at the normal time of *Sry* expression, suggesting that *M33* maybe upstream of *Sry*. The mutants show hermaphroditism with intersex genitalia or complete male-to-female sex reversal.[82] In addition, the gene for the syndrome ATR-X (*a*lpha-*t*halassemia [sometimes], mental *r*etardation, mapping to the *X*), which also shows sexual ambiguity,[83] interacts with heterochromatin proteins and modifies DNA. The Wt-l +KTS isoform could possibly also be involved in alterations of chromatin structure.

Fibroblast growth factor 9 (*Fgf-9*) may be an important mediator of *SRY* action.[84] The *Fgf-9* KO shows deficient mesonephric cell migration into the gonad, deficient mesenchymal proliferation, decreased Sertoli cell function, and frequent sex reversal. Male to female sex-reversal, with reduced expression of *Sry* (and *Sox-9*; discussed later) is found in triple KO of three insulin receptor genes.[85] Gonadal anlages were small, supporting the notion of a relation of gonadal cellular proliferation and *Sry* expression.

GONADAL DEVELOPMENT

The data on transcription of *SRY* in human and mouse blastocysts, discussed earlier, suggest the possibility that earlier events have set in motion biochemical pathways related to the visible event of gonadal ridge differentiation. Furthermore, for normal female sex-determination to occur and the primitive gonad to differentiate to an ovary, homozygosity of at least one X-chromosomal locus appears to be essential. Relatively little is known about genetic control of ovarian development. A possible candidate gene for a role is *DSS*[86] (discussed later).

The components of the human bipotential gonadal primordium were described in the introduction to this chapter. Migration of the primordial germ cells is completed in the sixth week of gestation, when the embryonic female or male gonads are indistinguishable. Absence of primordial germ cells is not compatible with ovarian differentiation but does not prevent testicular seminiferous tubule morphogenesis.[87] Evidence suggests that the initial stages of gonadal differentiation are realized in the supporting cells (pre-Sertoli or prefollicular),[88] but cell to cell interactions are critical for the function of *SRY* and, in general, for gonadal differentiation.[89] In the next section, we discuss the development of the testis and review recent data on genes involved in ovarian differentiation.

Development of the Testis: Genes Acting Downstream of *SRY/Sry*

Although *SRY/Sry* appears to be the Y-chromosomal sex-determining *switch*, recent work has made clear that it is not the ultimate, masculinizing gene of the sex determination pathway. A strong candidate for the latter is *SOX-9/Sox-9*. Because no evidence for a role of *SRY/Sry* in *SOX-9/Sox-9* expression has emerged, current thinking is that the masculinizing gene is inhibited by a hypothetical "gene *Z*,"[90] and that testicular induction occurs when gene *Z* is itself suppressed by *SRY/Sry*. In the following paragraphs, we develop this concept and consider candidates for gene *Z* and possible modifying factors of it.

That *SRY/Sry* is not the ultimate masculinizing gene is suggested by the following evidence. First, it is not essential in all mammals; the males of several species of voles[91] and spinous country rats[92] develop appropriately without it. Second, investigations into the XX true hermaphrodites frequently seen among South African Bantus have also not detected Y chromosomal sequences.[93] Thus mammalian male sex determination can certainly occur in the absence of *SRY*, and, in normal human development, several genes downstream of *SRY* are involved.

It has been proposed that the HMG domain of the gene product SRY binds to regulatory DNA sequences of the hypothetical negative regulator of male sex determination, gene *Z*.[90] Interaction of SRY with the *Z* gene is postulated to inhibit the regulatory effect of *Z,* resulting in a male phenotype. One possible inhibitor of male development, and target for *SRY*, is *SOX3*,[94] a member of the *SOX* gene family, to be described below. *Sox-3* is more abundantly expressed in female than in male genital ridges.[95]

Gene *Z* was postulated to be located on Xp21.[90] A number of 46, XY individuals with sex reversal have partial duplications of Xp and an intact *SRY* gene. They usually have female external and internal genitalia with partial gonadal dysgenesis.[86] Bernstein and colleagues[96] presented two cases and concluded that "testis-determining genes of the Y chromosome may be suppressed by regulatory elements of the X." The X-homologue of *ZFY*, *ZFX*, which escapes inactivation, may be one candidate.[97] XXY (Klinefelter syndrome) individuals do develop testes but they are not normal, and these patients do have partially feminized phenotypes. Furthermore, some XXY triploids have shown sexual ambiguity;[98] lack of X-inactivation in the triploids may be the cause.[99] Bardoni and colleagues[86] hypothesized a single gene in the Xp region of interest, and named it *DSS* (*d*osage *s*ensitive *s*ex reversal). This region includes *DAX-1* (*d*osage sensitive sex reversal, *a*drenal hypoplasia congenita gene on the *X* chromosome gene *1*), gene defects of which cause adrenal hypoplasia.[100,101] *DAX-1* is a candidate for *DSS*. Capel and colleages[102] have argued that an increased dosage of *DAX-1* would override the *SRY* signal for testis development. Indeed, *DAX-1* is normally down-regulated in the developing testis, whereas its expression persists in the developing ovary.[103] Overexpression in *Dax-1* transgenics delays testicular development and

can result in sex reversal in the presence of a weak-functioning (hypomorphic) *Sry*.[104] Another gene involved in dosage-dependent 46,XY sex reversal is *WNT-4*, which maps to distal 1p, duplications of which sometimes cause male pseudohermaphroditism.[105] The *Wnt-4* KO does not affect male sexual development but the females are masculinized.[106] Interestingly, overexpression of *WNT-4* (in transfection—i.e., transgenic—experiments with cultured Sertoli and Leydig cells) appears to result in up-regulation of *DAX-1*, linking these two dosage-dependent, female sexual development program-enhancing pathways.[107]

Genes that are functionally downstream of *Sry* in gonadal development, and possible mediators of its action, include nexin 1 and *Fgf-9*, involved in cell proliferation and migration. KO of the latter can lead to decreased function of Sertoli cells and sex reversal. Desert hedgehog (*DHH*) is expressed in Sertoli cells and is required for spermatogenesis.

A family of genes with homology greater than 60% to the HMG box of *SRY* was discovered, and designated *SOX* (*SRY* box-related) genes.[108,109] These genes are evolutionarily very conserved. The relationship between *SRY* and the *SOX* genes has aroused great interest from the point of view of the origin and evolution of *SRY* in mammals.[110] *SOX3* and *SOX-9* have been mentioned previously in this chapter, the former as a possible inhibitor of masculinization and target of *SRY*. *SOX-9/Sox-9* is a candidate for the ultimate masculinizing gene. This is supported by some evidence. In camptomelic dysplasia, due to haploinsufficiency of *SOX9*, partial sex reversal of XY individuals may occur.[111] Increased dosage of *SOX9*, due to a chromosomal duplication, strongly masculinized an XX fetus.[112] In mice, *Sox9* transgenics develop a normal male appearance in the absence of *Sry*,[113] and 40,XX, *Sry*− mice (i.e., genetic females) carrying the deletion *Odsex* (which maps about 1 million base pairs upstream of *Sox9*) up-regulate *Sox9* expression in fetal gonads and develop as phenotypic males.[114] *Sox-9* is only expressed in Sertoli cell precursors.[115] Prostaglandin D2 signaling, induced by *Sry*, may recruit Sertoli cells to express *Sox-9*.[116] Controlled KO of *Sox-9* in the gonadal anlage results in complete XY sex reversal.[117] *SOX8* is also expressed in the developing testis and may function redundantly with *SOX-9*.[118] Inhibition of nuclear export of SOX-9 that had been transfected into granulosa precursor cells of XX mouse gonadal anlage induced AMH production, a sex reversal phenotype (see later section).[119] Nuclear localization of this molecule normally occurs only in the male gonad.[120,121]

It has been shown that the HMG box of SRY recognizes the promoter elements of the gene for MIS (discussed in a later section), but this binding is insufficient to regulate transcription in in vitro systems. It also binds to the promoter of aromatase P450.[122] Thus SRY could contribute, with SOX-9, to transcription of these target genes. Because no evidence for a role of *SRY* in *SOX-9* expression has come to light, *SRY* is postulated to be the deinhibitor of a yet to be identified inhibitor of *SOX-9* in mammals. MIS is a presumptive target of *SOX-9* regulation in these species.[123] The relationships of *SOX-9/Sox-9* and MIS may be different in *SRY*-negative male mammals and in nonmammalian species. Co-stimulators, with SOX9, of MIS may include SF-1, WT-1, and other gene products. MIS and testosterone, both of which are produced in the testis, appear at this time to be the major, downstream masculinizers in the sex differentiation pathway. Other genes that are downstream of *SOX-9* include vanin 1.[124] However, no downstream target of *SOX-9* that has a specific function in testicular development has been identified.

Development of the Ovary

As indicated in the introduction for this chapter, the idea that the ovary arises solely as an outcome of an "uninduced state" is being replaced, as new data emerge that elucidate the existence of genes specifically involved in ovarian differentiation. As noted earlier, overexpression of *WNT-4* (in transfection experiments with cultured Sertoli and Leydig cells) causes up-regulation of *DAX-1*, thus suggesting a correlation between these two genes such that each, in various circumstances, appear to promote ovarian development. Lack of *Wnt-4* gives rise to masculinization of the XX gonad, though a recent study has shown that *Wnt-4* also has a specific but different role in male gonadal development.[125] Nef and colleagues[53] used microarrays to compare gene expression patterns of XX and XY Sf-1 positive gonadal cells and found that cyclin-dependent kinase inhibitors were overexpressed in XX gonads, a finding that could relate to the known difference in proliferation rates of XX and XY gonads, discussed in an earlier section. Jorgensen and Gao[126] demonstrated a six-fold ovary-specific enhancement of *Irx-3* (Iroquois homeobox gene family member 3) at days 12 to 13.5 of murine development. This finding was independent of the presence of germ cells.

The female to male sex reversal seen in polled (hornless) goats (Polled Intersex Syndrome [PIS]) has long been the subject of much interest, partly because the reversal trait occurs only in animals homozygous for the mutation (i.e., is inherited as an autosomal recessive), whereas the trait of absence of horns behaves as an autosomal dominant. Recent research reviewed by Baron and colleagues[127] has revealed some fascinating insights and brought to light the existence of another gene involved in ovarian development in mammals, including humans.

The polled mutation in goats turns out to be a ~12 kb deletion from a *cis*-regulating region, at band q43 on goat chromosome number 1, that controls the expression of three contiguous genes.[54] One of these three, *FOXL-2*, which is highly conserved in vertebrates, appears to be an ovary-determining gene. Another of the three—*PISRT-1* (*PIS*-related *t*ranscript, gene *1*)—is a regulatory element necessary for *FOXL-2* expression in the ovary. In XY males, *SRY* evidently blocks *PISRT-1* and thus prevents gonadal expression of *FOXL-2* (and hence ovarian induction). However, *PISRT-1* evidently also inhibits *SOX-9*, thus suppressing testis formation, as proposed for the hypothetical gene *Z*, discussed in a previous section. Fetal gonads of the Polled goat (i.e., animals carrying the *PIS* deletion) show complete repression of both *FOXL-2*, the ovarian inducer, and *PISTR-1*, the suppressor of testis inducing *SOX-9*,[54] thus accounting for the observed sex-reversal phenotype.

The genetics of the polled trait itself illuminates another intriguing feature of sexual development: the independence from hormonal influence of a feature of secondary sexual maturity. In wild-type fetuses the PIS cis-regulating element suppresses the expression of the three adjacent genes in skin surrounding the future horn buds. The function of at least one of these is evidently to limit horn growth.[54] Other examples, in the XX*Sxr* pseudomale mouse and in the tammar Wallaby, of such exceptions to the Jost Principle were given in an earlier section. The polled situation in which the PIS cis-regulating element controls the expression of adjacent genes[54] might be a model to account for those other Jost principle exceptions.[49–51]

In humans, band q23 on chromosome 3 is homologous to the *FOXL-2/PISRT-1* region (q43) of caprine chromosome 1. FOXL-2 protein has been detected in the nuclei of human fetal granulosa cells, but not in fetal or adult testes. Loss of function mutations in *FOXL-2* in humans can be responsible for a syndrome of eyelid dysmorphism and ovarian dysgenesis that causes primary amenorrhea in 46,XX women.[128]

The mutual interrelationships of the different genes recently shown to be involved in ovarian development remain to be elucidated, but it is clear that clarification of this central piece of the sex determination paradigm is imminent.

SECONDARY SEXUAL DIFFERENTIATION

The classical paradigm of sexual development introduced at the beginning of the chapter was formulated in principle by Alfred Jost[1] at the end of the 1940s to explain the results of his

observation that extirpation of the gonads (ovaries or testes) of rabbits before sexual differentiation had occurred invariably resulted in the development of a basic, female-type pattern in the fetuses.[129] This and subsequent research established that although sex-determining genes are evidently responsible for directing the development of the gonad, secondary sexual differentiation of internal duct systems and external genitalia is under hormonal control in eutherian mammals, including humans.

Indifferent (bipotential) external structures are initially present in males and females. The testis produces androgens, which masculinize the labioscrotal folds to form a scrotum, and the genital tubercle to form the penis. In the absence of the testis, and therewith of high levels of androgen molecules, the labioscrotal folds do not fuse and form labia, and the genital tubercle becomes the clitoris. Internally, the mesonephric (Wolffian) and paramesonephric (Müllerian) ducts will develop into male or female structures respectively. Testicular androgen promotes development of the Wolffian ducts into epididymides, vasa deferentia, and seminal vesicles, and absence of androgens leads to degeneration of the Wolffian ducts. Also produced in the testis is MIS, which causes disintegration of the Müllerian duct. In the female, in absence of MIS, the Müllerian ducts become the uterine tubes, uterus, and upper part of the vagina. The possible place of the gene for MIS in the sequence of events downstream of *SRY*[130] was discussed earlier.

Information on genes controlling female secondary sexual development can be obtained from studies on patients with pseudohermaphroditism. In this group of conditions the patient's primary and secondary sexual features are discordant. Thus in male pseudohermaphroditism a genetic male, having a testis, shows female or ambiguous external and/or internal genitalia.

For example, Androgen Insensitivity Syndrome (AIS), an X-linked condition also known as Testicular Feminization, occurs in XY individuals, and illustrates the above description of the main features of secondary sexual differentiation. In AIS the Y-chromosomal testis-determining mechanism is intact, and testes are present, but because the X-linked mutation renders tissues insensitive to androgens, the external genitalia fail to develop in masculine direction. Instead, they retain the basic female configuration, and the individual gets diagnosed as a girl at birth. The Wolffian ducts also do not develop and, because the testis produces MIS, the Müllerian ducts regress, so the individual does not have uterus, tubes, and the upper part of the vagina. At pubertal age menarche fails to occur.

In female pseudohermaphroditism, individuals have ambiguous or male-type external genitalia with female gonads and karyotype. Perloff and colleagues[131] and others[132–135] have described a distinct form of female pseudohermaphroditism associated with malformations of the internal genital, urinary, and gastrointestinal tracts. They may occur in the apparent absence of testosterone or *SRY*.[136] Possible causes include a disturbance of a specific caudal developmental field,[133] a primary defect in the cloacal membrane[135] or failed fusion of the urorectal septum with the cloacal membrane.[137] Some cases of penoscrotal transposition[138,139] and some cases of prune belly sequence and of bilateral renal agenesis[132,140] have similar patterns of caudal malformations. Furthermore, this pattern of dysmorphism, but without female pseudohermaphroditism, is found in the MURCS (*Mu*llerian duct aplasia-*r*enal aplasia, *c*ervicothoracic *s*omite dysplasia) association. This condition usually occurs sporadically in families. In most cases, the cause is unknown.

The role of growth factors in the differentiation of the genital tubercle has been explored in mice. The possible role of *Fgf-9* in gonadal differentiation was mentioned earlier. *Fgf-8* and *-10* are implicated in external sexual differentiation; KO of *Fgf-10* results in marked sexual ambiguity.[141] A pathway initiated by Sonic hedgehog (*Shh*), and interactions of its product with a number of these factors, has been studied in organ cultures of murine genital tubercles.[142] *Shh* KO embryos showed no external genitalia at 12.5 days gestation and showed down-regulation of *Fgf8*. Cultured genital tubercle explants treated with antibody to Shh also showed no external genitalia at 12.5 days gestation and marked down regulation of *Fgf-10*. Recently it has been shown that *Fgf-8* also has a role upstream of *Shh*[143] and downstream of androgens.[144]

These findings are also relevant to the hypospadias seen in Smith-Lemli-Opitz syndrome; the developmental defects seen in this cholesterol synthesis deficiency may in part be due to altered Shh signaling. Holoprosencephaly, sometimes seen in Smith-Lemli-Opitz syndrome, occurs with *Shh* haploinsufficiency, whereas hypospadias does not. Shh is also required for prostate development.[145] In contrast, Desert hedgehog is required to maintain germ cells in the testis.[146]

Although, as mentioned previously, *Wnt-4* is implicated in gonadal differentiation, *Wnt-5a* has also been implicated in genital tubercle development.[147] Furthermore, the role of MIS in development seems dependent on the signaling molecule *Wnt-7a*. In *Wnt-7a* KO males fail to undergo regression of the Müllerian duct, and females show abnormal oviduct/uterine development.[148,149] MIS-receptor is not expressed in the males[148]; thus *Wnt-7a* is essential for MIS function.

Multiple *Hox* genes, including the *Hoxa* and *Hoxd* groups, are involved in sexual differentiation. For example, compound double homozygous deficiency of *Hoxa-13* and *Hoxd-13* showed a complete absence of external genitalia.[150] Haploinsufficiency of the *HOXD* complex caused genital anomalies of a small phallus and no palpable testes in one case, and penoscrotal transposition with micropenis with multiple limb and other abnormalities in another.[151] Hand-foot-genital syndrome with male cryptorchidism and hypospadias is due to mutations in *HOXA-13*.[152]

CONCLUSION

The classical paradigm of Jost, formulated in the 1940s, provides a starting point for present-day consideration of sexual development in humans. However, recent research requires modification of that framework. Although Jost regarded the establishment of genetic sex that occurs at conception, and the determination of the fate of the gonad, to constitute a single step, it is now known that much transpires between these two events. Furthermore, the Jost Principle envisioned active induction of the testis only; the ovary developed by default in the absence of testis determining factors. However, the existence of ovary determining genes has now been established. The new paradigm, formulated here, proposes that primary sex determination comprises the three steps of sex chromosomal (X or Y) assignment of sex; pregonadal sexual development and sexual dimorphism; and gonadal (testicular or ovarian) development. Furthermore, the Jost Principle envisioned that all development of sexual dimorphism other than that of the gonads was due to hormones and independent of genetic sex. It is now clear that the situation is more complex. The new paradigm recognizes that some features of secondary sexual differentiation are directly influenced by gene action that is independent of testicular (or ovarian) hormones.

The sex determination pathway, like most developmental pathways, has a strong tendency to maintain its function (i.e., is homeostatic). Redundancy in pathways may be part of the mechanism by which the homeostatic continuation of sexual development occurs. One can surmise that a number of genes in the pathway might be up- or down-regulated to compensate for deficiencies in other members of the pathway. Further understanding of the sex determination pathway will come from studies of altered gene expression in deficient states and the location and identification of modifier genes that alter the pathway.

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Differentiation of the Ovary

DEVELOPMENT OF A FUNCTIONAL OVARY

The follicle—the functional unit of the ovary—consists of an oocyte in the late prophase of the first meiotic division surrounded by granulosa cells and enclosed by a basement membrane that in turn is surrounded by theca cells.

Three major events are prerequisites for the development of a functional ovary, namely, initiation of meiosis, formation of follicles, and differentiation of steroid-producing cells. In the human, as in many other mammalian species, these events begin during fetal life, at a time when germ cells and somatic cells have populated the ovarian anlage.

In both sexes, the human gonads are closely connected to the mesonephros throughout early differentiation. Mesonephric cells, as well as cells from the ovarian surface epithelium, migrate simultaneously with the primordial germ cells to form the future gonadal tissue at the ventrocranial aspect of the mesonephros.[1,2] In the fetal mouse, cells from mesonephros form an *adrenogenital primordium* characterized by activity for steroidogenic factor 1.[3] This primordium may be the progenitor of the hormone-producing somatic cells of the differentiated gonad of both sexes and of the adrenal gland. SF-1 depends on the action of the ribonuclease Dicer1. Interestingly, knockout of Dicer1 results in complete degeneration of the testis, whereas the ovary proceeds through differentiation in the mouse.[4]

Meiosis and follicle formation are interrelated processes, the first being the prerequisite for the second, which, conversely, secures survival of the oocyte. The steroid-producing theca cells form later, around the growing follicles, whereas differentiation of steroid-producing cells in the stroma may be unrelated to the foregoing two events.[1]

Within the follicle, a unique micromilieu forms that secures oocyte growth and maturation. However, intraovarian and interovarian regulatory mechanisms, as well as systemic interrelationships, participate in controlling the fate of female germ cells.

Gonadal Sex Determination

In contrast to some other vertebrate classes (e.g., amphibians and fish), spontaneous sex reversal has never been reported in mammals.[5] In most vertebrate classes, gonadal differentiation is the result of the genetic sex. In mammals, the testis is formed when a Y chromosome is present, and an ovary develops if this chromosome is absent, irrespective of additional sex chromosomes.[6] During the 1970s and 1980s, a testis-determining region of the Y chromosome was located in smaller and smaller parts of the chromosome. In 1990, the sex-determining region Y—a single gene—was found.[7-9] It is thought that if the *Sry* gene is not activated, ovarian development will take place (for review, Wilhelm and colleagues[10]). However, in addition to the *Sry* numerous other genes are involved in the control of sex determination and early gonadal and sex duct formation and differentiation.[11,12] Edson and colleagues[13] provided an excellent review on genes and other factors involved in gonadal sex differentiation.

Early Ovarian Differentiation

In the human, the migration of primordial germ cells from the primitive gut to the gonadal ridge is guided by autonomic nerves and completed around the ninth week of fetal life.[14,15] Simultaneously invading mesonephric cells accumulate in the central part of the ovary and form the intraovarian rete in the medulla (Figure 184-1). The expanding medulla gradually pushes the germ cells toward the periphery, forming in the cortex a high density of germ cells (Figure 184-2).

By morphologic criteria the ovary is usually recognizable at a later stage of gonadal differentiation than the testis. The first signs signifying that the indifferent gonad is becoming a testis are the formation of the testicular cords enclosing the germ cells[16] and the rounding up of the organ.[17] In the human testis, these events take place at the sixth week of fetal life.

Cords enclosing the germ cells are, however, also seen at early stages of ovarian differentiation in some mammalian species

†Deceased

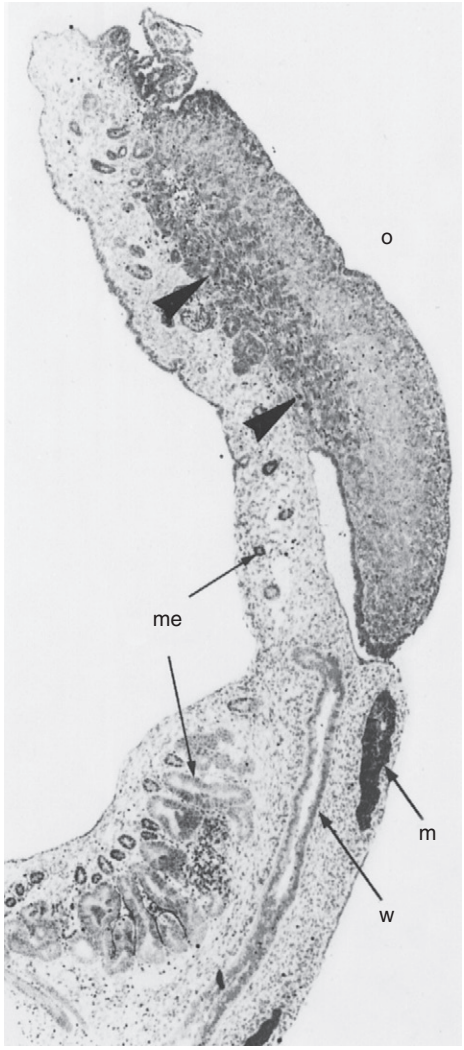


Figure 184-1 Ovarian-mesonephric complex of an 11-week-old human fetal ovary. The mesonephric cells that invade the ovary are recognized as the dark cell mass *(arrowheads)* in the basal part of the ovary *(o).* The Wolffian duct *(w),* the müllerian duct *(m),* and mesonephric tubules *(me)* are seen. (Original magnification ×200.) *(From Byskov AG, Høyer PE: In Knobil E, Neil J [eds]:* The Physiology of Reproduction. *New York, 1988, Raven Press.)*

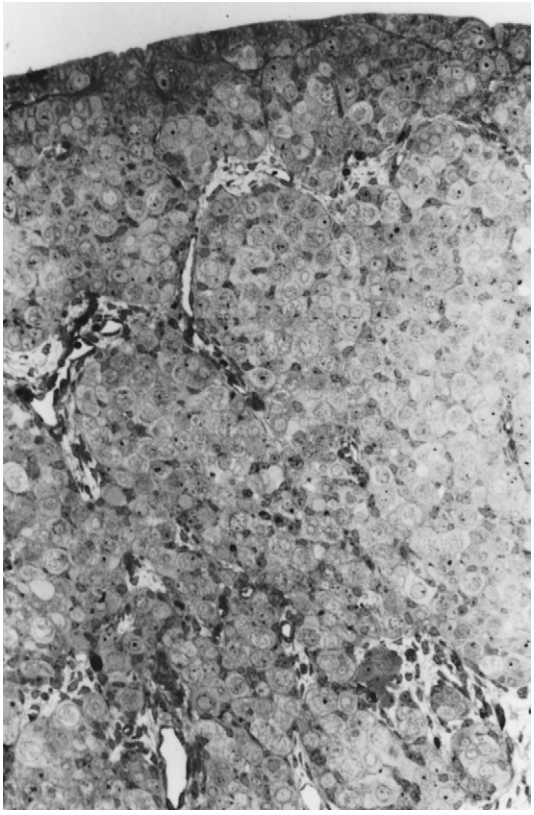


Figure 184-2 Human fetal ovary, 21st week, showing the cortex packed with oogonia in the outermost part of the cortex and oocytes towards medulla. (Original magnification ×300.)

(e.g., the pig and the sheep). In other species (e.g., the mouse and the hamster), the germ cells are more evenly distributed throughout the ovary. These different patterns of germ cell location within the ovary are closely related to the onset of meiosis. In species in which germ cell cords are formed, meiosis is delayed by a "delay period" (delayed meiosis) until the cords dissolve, whereas, in species without cord formation, meiosis proceeds immediately (immediate meiosis).[18] During the delay period, transitory ovarian steroidogenesis takes place (see later). This is analogous to early testicular differentiation, in which steroidogenesis begins immediately after testicular cords are formed.[19,20] It has been proposed that enclosure of germ cells into specific germ cell compartments, either as temporarily formed cords or as follicles, is a prerequisite for the start of steroid production in the developing gonads.[18]

Morphologically and functionally, the human fetal ovary represents an intermediate type between immediate and delayed meiosis. Although germ cells appear in clusters during fetal life, well-defined cords are not formed (Figure 184-3; see Figure 184-2).[21] Nonetheless, the delay period lasts for a relatively long time, from the onset of sex differentiation at approximately the 6th week of fetal life until the 9th week, when meiosis starts.[22,23] Although the aromatizing enzyme system is present by the sixth week of fetal life few or no steroids are produced by human ovaries during the delay period.[24] Interstitial cells with ultrastructural resemblance to steroid-producing cells have been recognized from the 13th week of fetal life (Figure 184-4).[25]

The origin of the somatic cell population of the cortex is uncertain, and different theories have been proposed. Generally, the following sources for their origin have been suggested: the surface epithelium,[26] the mesonephros, or a combination of the two.[21,27] In vitro experiments attempted to trace the origin of gonadal somatic cells by culturing fetal mesonephric tissue from one mouse strain carrying a cellular marker together with fetal gonads from another strain without a marker[28,29] and vice versa.[30] However, results of the first studies indicated that mesonephric cells almost entirely migrated to the testis, not to the ovary, whereas the later study showed that cells moved in both directions in both sexes. Thus the origin of the somatic cells of the gonads remains uncertain.

MEIOTIC PROPHASE OF OOCYTES

By the end of the 10th week of gestation, the human ovarian cortex contains numerous mitotically dividing *oogonia*. When the oogonia stop dividing and enter meiosis, they are termed *oocytes*. The first oocytes in the human ovary are seen during the 11th week of gestation.[23] The term *oogenesis* denotes the

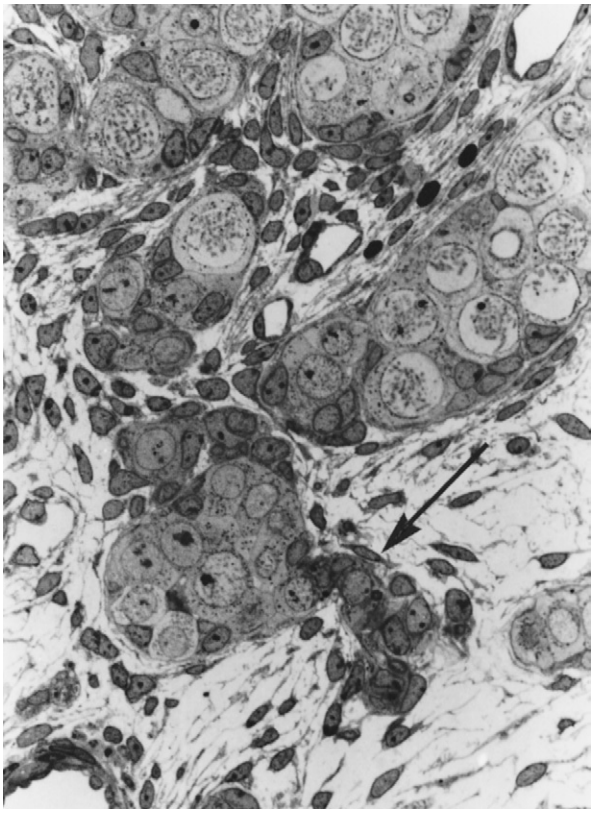


Figure 184-3 Human fetal ovary, 21st week, with clusters of oocytes in different stages of meiosis in the inner part of the cortex, in connection with an intraovarian rete cord *(arrow).* (Original magnification ×600.)

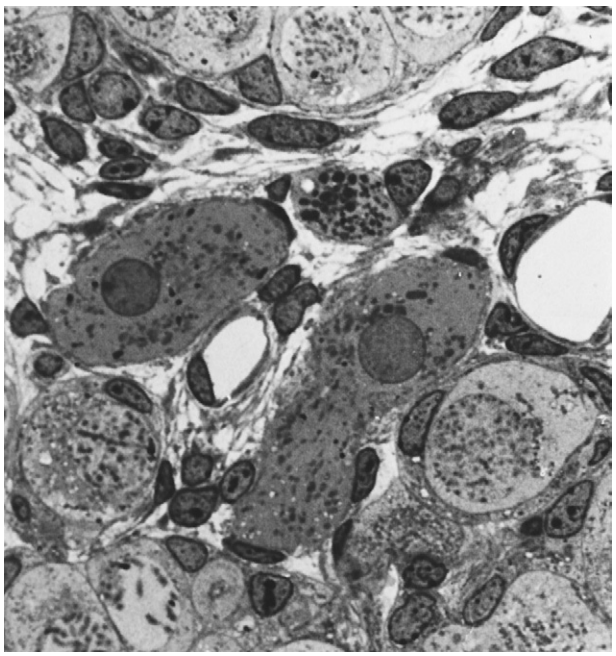


Figure 184-4 Two interstitial cells in the inner part of the ovarian cortex from a fetal human ovary, 21 weeks old. (Original magnification ×900.)

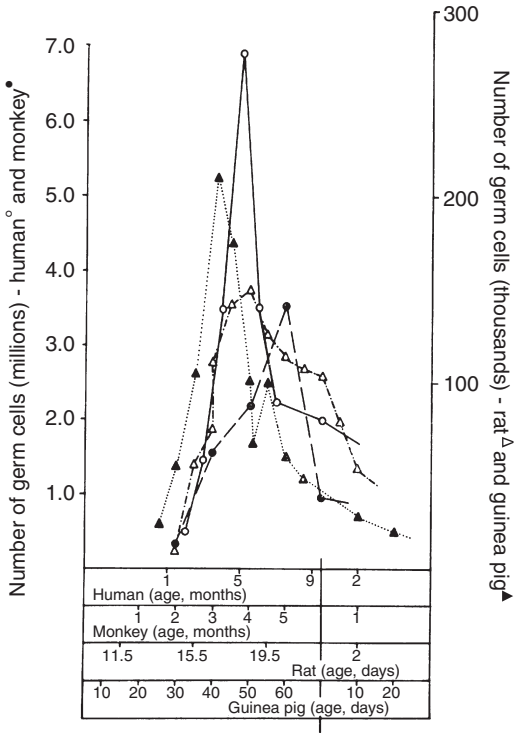


Figure 184-5 Number of germ cells in fetal and neonatal ovaries of human, monkey, rat, and guinea pig. *(From Baker TG: In Balin H, Glasser SR [eds]:* Reproductive Biology, *Amsterdam, 1973, Excerpta Medica.)*

transformation of an oogonium into an oocyte, and maturation into a fertilizable oocyte.

The number of oogonia increases exponentially by mitotic division from approximately 20,000 in the 6-week-old human fetus to approximately 250,000 in the 10th week,[23,31] and it reaches a maximum of 7 million in the 20th week (Figure 184-5).[32,33] Genes and transcription factors involved in multiplication of the oogonia include *DAX-1*[34] and expression of Kit protein.[35,36] Extensive degeneration, however, reduces the number of germ cells drastically during the rest of fetal life, leaving only 1 to 2 million oocytes in the ovaries of the newborn girl.[32,37]

Process of Meiosis

Meiosis begins with premeiotic DNA synthesis, followed by the transitory stages of the first meiotic prophase: preleptotene-leptotene-zygotene, and pachytene to arrest in diplotene stage. When the diplotene stage is reached, the process is arrested (Figure 184-6). During this first meiotic prophase, the maternal and paternal genes are exchanged between the homologous chromosomes. The diplotene stage, sometimes named the dictyate stage, is maintained until the oocyte resumes meiosis (just before ovulation) or degenerates. Some oocytes may thus last for the whole fertile life span. Oocyte degeneration may occur at any time during development. At resumption of meiosis, the first meiotic prophase is completed, and the oocyte proceeds through the first meiotic division, the reduction division, in which the homologous chromosomes are separated into the daughter cells, now each containing 1n chromosomes and 2c (i.e., copy) DNA. The second meiotic division, which in the human takes place by fertilization, is not preceded by the usual DNA synthesis, and the DNA content is therefore halved to 1c. In the human, the meiotic divisions result in gametes with 23 chromosomes (1n) and 1c DNA.

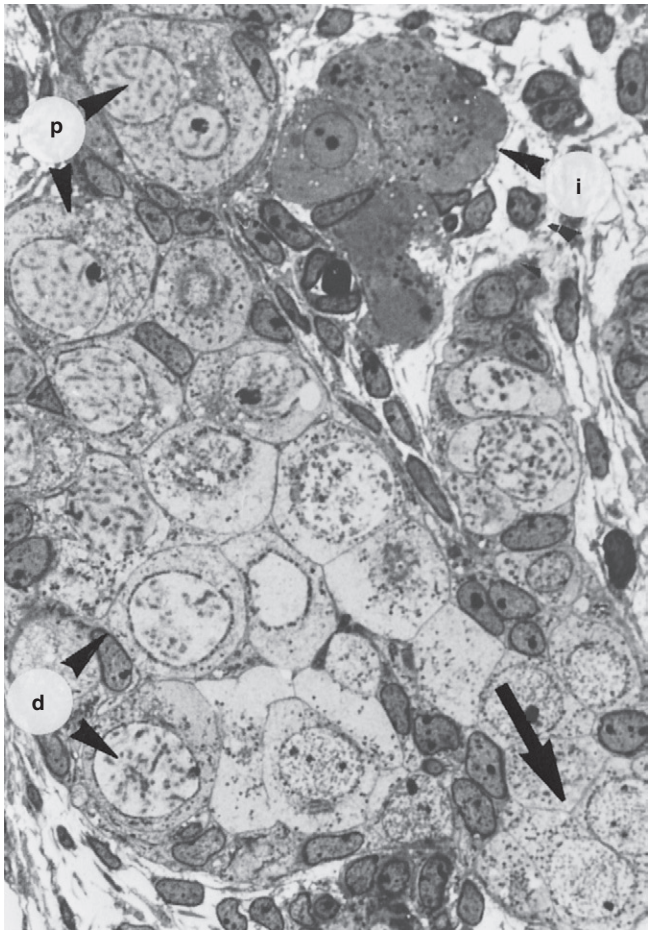


Figure 184-6 Inner part of the ovarian cortex of a human ovary, 21-week-old fetus, showing different stages of meiosis and interstitial cells *(arrow)*. *d,* Early diplotene; *i,* interstitial cells; *p,* pachytene. (Original magnification ×900.)

The Meiotic Prophase: Morphologic Features and Genetic Control

The different transitory stages of meiosis are identifiable by the configuration of the chromosomes. In the preleptotene and leptotene stages, the chromosomes condense and appear as thin, coiled threads, each thread consisting of identical sister chromosomes. The chromosomes coil increasingly during progression through the zygotene to pachytene stages. The synaptonemal complexes begin to form in the zygotene and are completed in the pachytene stage. These complexes represent the pairing of homologous chromatids. It seems that proper formation of the synaptonemal complexes ensures normal chromosome segregation in the reduction division of meiosis.[38] Passing into the diplotene stage, the chromosomes decoil, and chiasmata or crossing-over may be recognized. At this stage, the paternal and maternal chromatids exchange gene material. The amount of exchanged gene material can be evaluated in the metaphase of the second meiotic division after labeling of premeiotic oogonia in S phase with bromine deoxyuridine.[39] During the diplotene stage, the oocyte and its nucleus enlarge. The chromosomes decondense, thus making the nucleus faintly stainable.

Numerous genes with functions specific for the early meiotic processes are known. One of these is *DAZL 1,* which encodes for a protein with RNA-binding element and is found in human oogonia.[40] *Dazla*-deficient mice are infertile because the oocytes are lost when passing through meiosis.[41] Other genes such as *Scp1-3* and cohesin proteins are needed for the structural organization of chromosomes during assembly of the synaptonemal complexes and recombination.[42-45]

As discussed earlier, meiosis begins with premeiotic DNA synthesis and ultimately leads to the 4c DNA content of the leptotene stage.[46,47] This DNA must last until ovulation ceases, which in the human can occur up to 50 years later. In other mammalian species, it has been shown by DNA labeling that no neoformation of oocytes takes place later in life; that is, the entire pool of oogonia is transformed into oocytes before or around birth.[48-51] An exception to this rule was demonstrated in the ovaries of adult prosimians (lower primates) showing DNA synthesis in oogonia-like germ cells.[52,53] However, the concept that neoformation of mammalian oocytes does not occur later in life has recently been challenged and is still debated.[54-57]

In the early stages of fetal ovarian development, oogonia and oocytes are often interconnected by cytoplasmic bridges[58] forming so-called *germ cell cysts*.[59] These bridges may be important for exchange of intercellular signals and may explain why groups of germ cells divide synchronously and enter meiosis in clusters (see Figure 184-6).[60]

Initiation and Regulation of Meiosis

The time of onset of meiosis in the female varies among different mammalian species but occurs always at an early stage of development, often in fetal life. In the testis, by contrast, initiation of meiosis is postponed until puberty in all species. These discrepancies have led to the hypothesis that an internal clock of the germ cells determines the onset of meiosis, independent of external factors.[61] However, the male germ cells can be induced to enter meiosis prematurely, for example, if the cells are located in the adrenal gland of the human fetus[62] or if they are exposed to a meiosis-inducing substance before the testis is sex differentiated.[18] Thus it seems uncertain whether a germ cell clock governs the switch from mitosis to meiosis or it is triggered by paracrine or other external stimuli (see later).

Meiosis-Regulatory Substances

A characteristic feature of the developing mammalian ovary is that the germ cells first entering meiosis are those situated in the central part of the ovary,[63] in close connection with the mesonephric-derived rete ovarii.[27] It has therefore been proposed that the mesonephric-derived cells in the ovary trigger the onset of meiosis.[27] In vitro experiments with gonads of fetal mouse[64] and hamster[65] suggest that the mesonephros produces a substance that promotes and induces meiosis in male, as well as in female, germ cells. This substance was termed *meiosis-inducing substance* and is probably a lipid.[66,67]

The presence of meiosis-inducing substance in gonads, in which meiosis is initiated, and its role in this process have been questioned. One study showed that fetal male mouse germ cells are "passively" entering meiosis when they are mixed and cultured with lung cells.[68] However, recent research in fetal mouse gonads shows that retinoic acid, an active derivative of vitamin A, stimulates the *Stra8* gene (*Stimulated by Retinoic Acid Gene* 8), which is required for the transition into meiosis in both female and male germ cells.[69] Therefore the fact of retinoic acid being a lipid is in accordance with the original proposal 25 years ago that meiosis-inducing substance is a lipid.[67] Retinoic acid is secreted by mesonephros in both sexes, but in the developing testis it is transformed by the enzyme CYP26B1 to inactive products; in the ovary, this enzyme is inactive, and

retinoic acid can stimulate its receptor which, in turn, will result in meiotic entry.[69]

FOLLICULOGENESIS

Folliculogenesis is the process during which the diplotene oocyte becomes surrounded by a single layer of granulosa cells and enclosed by an intact basal lamina, thus forming a specific germ cell compartment (Figure 184-7). Whether a follicular arrangement of granulosa cells and basal membrane can occur without the presence of an oocyte has been disputed, because it has not yet been possible to create gonads completely devoid of germ cells, such as by selective destruction by drugs.[70]

The separation of the diplotene oocyte from the surrounding tissue secures its survival and creates a unique micromilieu, which sustains further growth and differentiation. If the oocyte in the diplotene stage fails to be enclosed in a follicle, it invariably degenerates. Some follicles die as a result of specific programmed cell death, or *apoptosis*.[71-73] In the fetal human ovary, *naked oocytes* (diplotene oocytes without a granulosa layer) are often seen, but they always appear to be degenerating. Such oocytes are never seen later in life.[74]

The first follicles are encountered in the human ovary at approximately the 14th week of fetal life, and folliculogenesis is completed immediately after birth.[75] The appearance and growth of the first follicles coincide with the increase of fetal gonadotropins. Whether these two events are causally related is uncertain. Recent research has shown that genes expressed in the germ cells, such as *Figla, Nobox, Kit,* and *Ntrk2,* as well as genes expressed in the surrounding cells, such as *Foxl2, Kitl,* and *Ngf,* are important for folliculogenesis.[76] A study on mouse reproduction reports that the gene PTEN (phosphatase and tensin homolog deleted on chromosome 10) has a profound influence on follicle growth activation.[77] In mice lacking PTEN, small follicles are induced to grow, resulting in: 1) depletion of the follicle pool early in life, and 2) sterility.

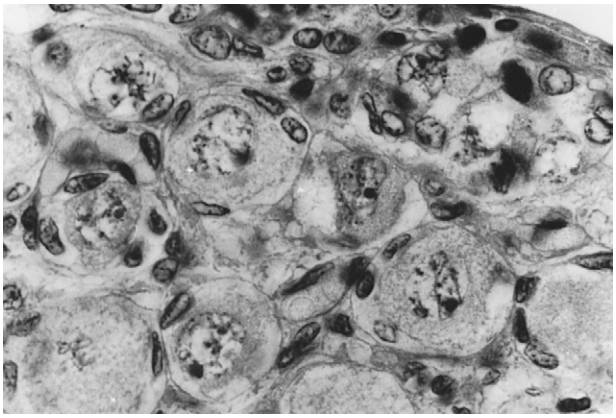


Figure 184-7 Primordial follicles in the ovarian cortex of a 30-week-old human fetus. (Original magnification ×560.)

Formation of the Granulosa Layer

The first follicles are formed in the inner part of the cortex, where oocytes first reach the diplotene stage.[78] In the newborn mouse ovary, programmed breakdown of the germ cell cysts may serve as a prerequisite for follicle formation.[79] It has been observed that the granulosa cells of the first and always centrally placed follicles are initially connected with the intraovarian rete cords.[80] This finding supports the assumption that granulosa cell precursors are of mesonephric origin. In fact, transplant experiments of fetal mouse ovaries showed that when invasion of mesonephric cells into the ovary is prevented, follicles are not formed.[81] It has also been proposed that the surface epithelium may give rise to granulosa cells, because proliferation of this epithelium into the cortex takes place during early stages of ovarian differentiation, before folliculogenesis.[27] One morphologic study of the formation of ovarian follicles during fetal sheep development suggested that almost all granulosa cells originate from the ovarian surface epithelium.[82] It is thus possible that mesonephric-derived cells, as well as cells of the surface epithelium, contribute to the granulosa cell population. In the mouse, the enclosure of the oocyte with the first granulosa cells is dependent on the action of FIGLA (factor in the germ line alpha).[83] In the human, *FIGLA* is only expressed in the oocytes from week 12 of fetal life, just before follicle formation takes place. However, a multitude of genes are probably involved in this important process.

Across mammalian species, the first generation of follicles appears to differ from later ones, exhibiting a different pattern of growth and differentiation (Figure 184-8).[84] The granulosa cells of these follicles seem to arise as a result of migratory processes of the extraovarian rete cells into the intraovarian rete that are

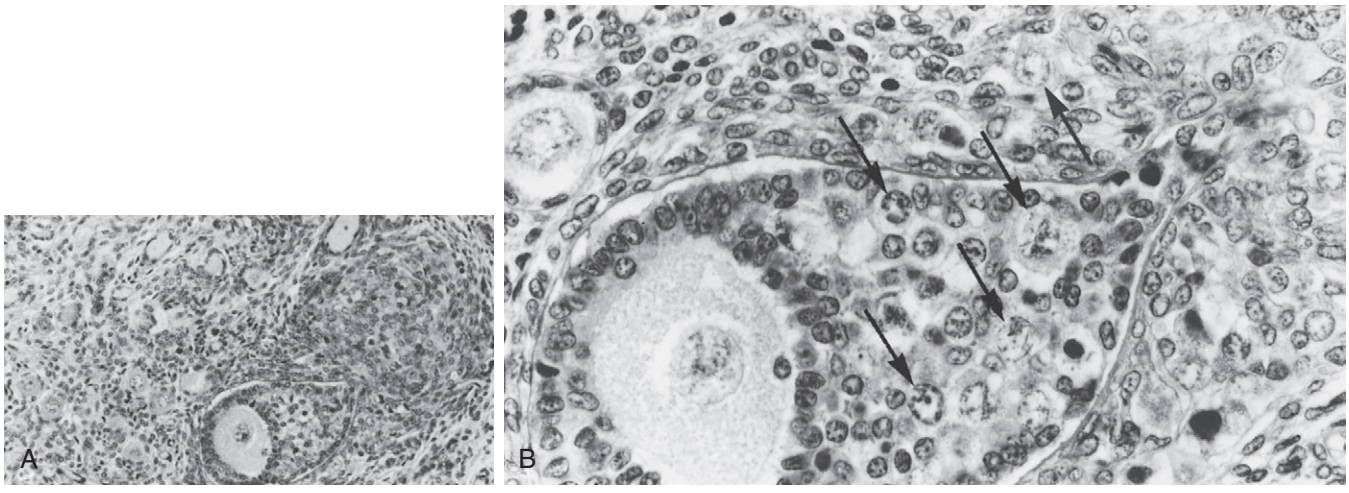


Figure 184-8 Abnormal follicle of a human fetal ovary, 33 weeks old. A, Besides a large oocyte, the granulosa layer contains numerous small oocytes and oogonia, some of which are degenerating. The granulosa layer opens up into a "nest" of small oocytes tightly packed with somatic cells. (Original magnification ×400.) **B,** Higher magnification of **A.** The arrows point at some of the small oocytes.

in contact with the oocytes.[81,85] Growth of later generations of follicles, which are no longer connected to the intraovarian rete, depends on proliferation of the fixed population of granulosa cells already enclosed in the follicle.[86,87]

The granulosa layer of the first generation of follicles that starts to grow in the inner part of the cortex and in the developing medulla often differs compared with later generations. The basal membrane does not always enclose the granulosa layer. It forms a tail-like extension (see Figure 184-8), which is sometimes connected to the intraovarian rete tubules. The first generation of follicles in the human fetal ovary may contain several oocytes of varying size and even oogonia that express genes characterizing primordial germ stem cells, such as CKIT, OCT4, and SSEA4.[57,88] Sometimes one oocyte is larger than the others (see Figure 184-8, *B*). Small oocytes—not enclosed in follicles—may also be seen in clusters densely packed with somatic cells (see Figure 184-8). In conclusion, it seems that early in development the interaction between the oocyte and the granulosa cells is not fully established, which results in abnormal follicles. Shortly after birth, however, these abnormal follicles disappear.

Initiation of Follicular Growth

As soon as follicles are formed, some of them begin to grow. From that time it has been estimated that approximately 10 to 20 primordial follicles are recruited daily for growth.[89] Thereafter initiation of follicular growth from the pool of small resting follicles continues until the pool is exhausted at menopause.[90] It is not certain whether follicular growth is initiated in the oocyte, in the granulosa cells, or simultaneously in both. In the immature mouse ovary, the number of granulosa cells of small follicles increases before the oocyte begins to grow.[91] At the same time, the oocyte nucleolar RNA polymerase activity increases,[91] and the nucleus enlarges.[92] Therefore it seems that initiation of growth in the oocyte and the granulosa cells is closely related.[93]

Once initiated, follicular growth continues until the follicle either ovulates or degenerates.[94] It is unknown what events trigger the initiation of growth. Exogenous gonadotropins have been reported to initiate follicular growth in the infant mouse ovary.[95] Follicular growth is also sustained by gonadotropins in vitro, as well as in vivo.[96-98] Moreover, retardation of follicular growth is seen in hypophysectomized fetal monkeys,[99] in anencephalic human fetuses,[75] and in infant mice treated with antigonadotropins.[100] Conversely, follicles begin to grow in cultures of fetal mouse[96,101] and hamster[97] ovaries, even in the absence of gonadotropins and steroid hormones.

It is therefore likely that factors other than gonadotropins control initiation of follicular growth. In the 1990s, numerous growth factors and other local acting factors or hormones, as well as their receptors, were identified in the mammalian ovary during early stages of folliculogenesis. However, folliculogenesis and early follicle growth seem to be controlled differently in different species. In primates, androgens stimulate insulin-like growth factor-I of the small oocyte and initiate follicular development, but this process does not occur in rodents.[102] Growth differentiation factor-9, a distant member of the transforming growth factor-β superfamily, is specifically expressed in the oocyte of all sizes in different species,[103-105] and mice with inactivated growth differentiation factor-9 are infertile and lack growing follicles.[106] Whether growth differentiation factor-9 is also needed for early follicular growth in other species is not yet clarified. In the human fetal and adult ovary, transforming growth factor-β and its receptor are expressed in granulosa cells of nongrowing follicles and may be involved in growth initiation.[107] It was recently found that the human ovarian medulla from early childhood through fertile life contains groups of small primordial and early growing follicles.[108] Thus, the dogma that all small, nongrowing follicles are confined to the cortex is invalid.

OVARIAN STEROIDOGENESIS

Early Steroid Production by the Ovary

In species with delayed meiosis, such as the sheep,[109] rabbit,[110] and cow,[111] considerable amounts of estradiol are secreted during a limited premeiotic period (the delay period) of fetal life. In the rabbit, it has been shown by quantitative cytochemistry that the intraovarian rete cells of the medulla exhibit activity of 3β-hydroxysteroid dehydrogenase during the delay period.[112] It is likely that these cells are responsible for the synthesis of the steroids.[113] However, in other species (i.e., those with immediate meiosis), steroid production is low or undetectable during early stages of development. In the human, no ovarian estradiol synthesis can be detected until late in fetal life.[114,115] This is striking considering the following: (1) the human fetal ovary possesses aromatizing enzyme systems for conversion of androgens to estrogens beginning with the eighth week of gestation[21]; (2) cells with ultrastructural characteristics of steroid production potentials are noted from the 12th week of gestation[1]; and (3) the activity of 3β-hydroxysteroid dehydrogenase is present in such cells from the 20th week of fetal life.[113] In the human, the early interstitial cells are located in the inner part of the ovarian cortex close to the developing follicles, but they never make contact with the oocytes (see Figure 184-4).

The mechanisms that trigger differentiation of the steroid-producing cells and initiation of steroid production are unknown. In the human fetal pituitary gland, the production of luteinizing hormone and follicle-stimulating hormone reaches a peak between the 16th and 20th weeks of gestation and then levels off and remains low until puberty.[116,117] Thus the peak production of gonadotropins occurs several weeks before the start of ovarian steroidogenesis. Moreover, once started, a slight increase of ovarian steroidogenesis (i.e., estrogen production) is seen throughout late fetal life when fetal gonadotropin secretion decreases. It therefore seems unlikely that pituitary gonadotropins control onset of fetal ovarian steroidogenesis.

Formation of the Theca Layer and Early Follicular Steroidogenesis

In the human ovary from late fetal life and until follicles are exhausted from the senile ovary, theca cells differentiate around the late preantral or early antral follicles (Figure 184-9).[86] Spindle-shaped

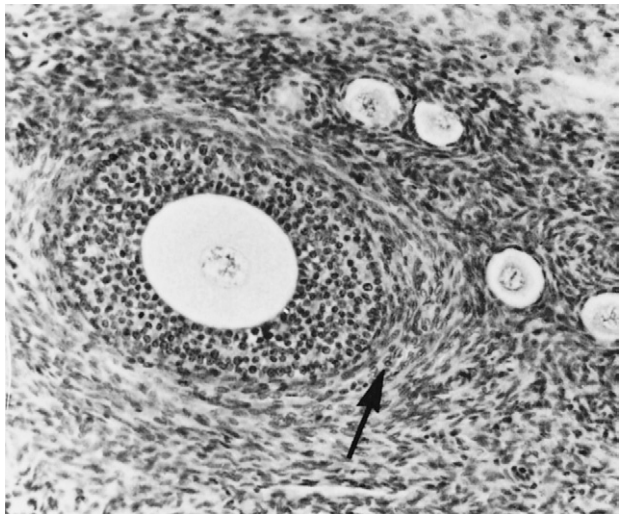


Figure 184-9 Inner part of the ovarian cortex of a 32-week-old human fetus showing a growing preantral follicle with a differentiating theca layer *(arrow)* and some primordial follicles. (Original magnification ×450.)



Figure 184-10 Cortex of an ovary from a 4-year-old child. Many small oocytes populate the area below the dense tunica albuginea. Remnants of a collapsing antral follicle occupy the lower part of the figure. (Original magnification ×280.)

hypotrophic cells encircle the basement membrane delineating the follicle together with blood vessels and nerves. Soon the theca cells acquire the ultrastructural and biochemical characteristics of steroid-producing cells. The origin of the theca cells is not established, but a pool of cells with potential for differentiating into theca cells must be present at all times, because every growing follicle sooner or later during its development requires a theca layer.

In the adult ovary, estrogen production by the follicle is the result of a synergism between the theca cells and the granulosa cells.[118] As soon as the theca cells differentiate around the follicle, the granulosa cells acquire 3β-hydroxysteroid dehydrogenase activity,[113] and by that time the follicular unit should be able to produce steroids, for example, by expressing P450c17 protein, the important steroidogenic enzyme involved in the conversion of pregnenolone and progesterone to androgens.[119] The late fetal ovary and the ovary during childhood contain numerous small antral follicles with well-differentiated theca cells. However, throughout childhood, serum levels of estrogens and other steroids remain low, probably because of lack of sufficient gonadotropic stimulation during this period.

FORMATION OF THE OVARIAN STROMA

During early fetal life, the main cell types that contribute to the ovarian stroma are mesenchyme and fibroblasts. Later in development, remnants of degenerating small follicles add to the stroma mass. Although some cells, for example hilus cells, are equipped with enzymes necessary for steroidogenesis, it is uncertain to what extent these cells participate in ovarian hormone production.[119] In fact, typical luteinized interstitial tissue is not seen until after the first ovulation has occurred.

FOLLICULAR GROWTH DURING CHILDHOOD

Birth itself is not a landmark in ovarian development and differentiation. Follicular growth starts in the early midtrimester, and antral follicles are often seen 1 month before birth. Therefore the ovaries of the female newborn infant contain preantral and antral follicles, at different stages of growth and atresia, in addition to a large pool of primordial follicles.[37] Follicular growth is the normal

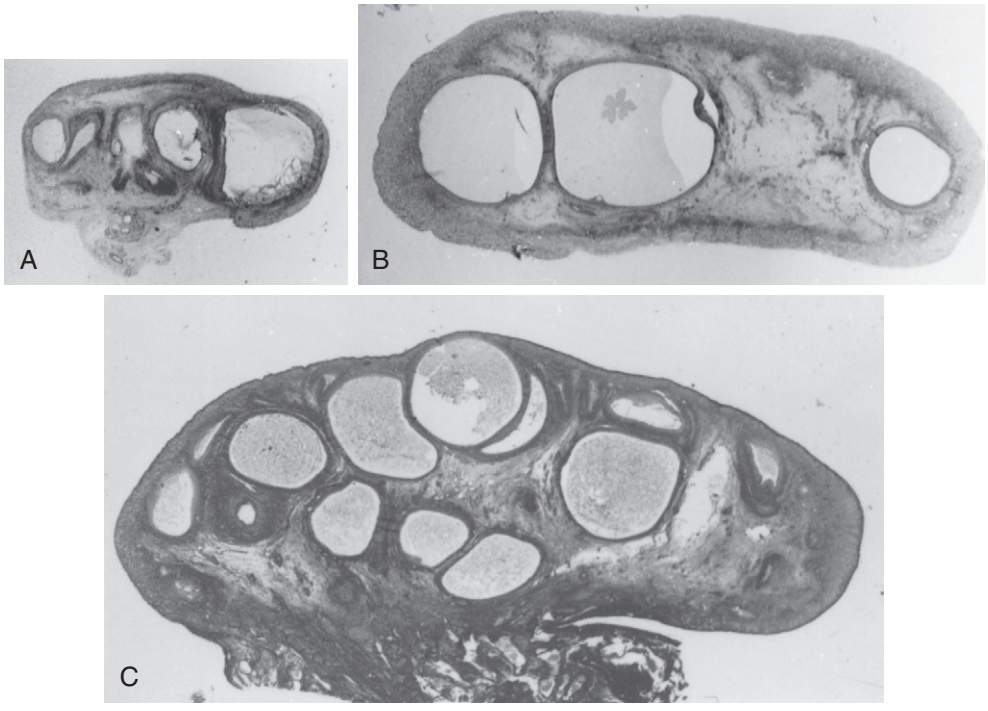


Figure 184-11 Human ovaries of young children, all with several antral follicles, none of which exceed 6 mm in diameter. (Original magnification ×5.) **A,** 2 months old. **B,** 4 years old. **C,** 7 years old.

finding in the ovary of the newborn infant (see Figure 184-8, *B*).[74,120] Absent or retarded growth of follicles at the time of birth is seen only in ovaries of children with disease.[121] The perinatal and postnatal increases in human gonadotropins, which decline only after the third month of age, sometimes cause development of ovarian cysts a few months later. If the cysts are larger than 5 cm in diameter, intervention may be needed to prevent adnexal torsion.[122] In addition to gonadotropins and steroids, small antral follicles accumulate anti-Müllerian hormone (AMH) in very high concentrations (twofold to threefold higher than in circulation). This suggests that these hormones also play important intrafollicular functions in follicles from the ovaries of young girls.[123]

All follicles that start to grow during childhood are destined to undergo atresia at some stage of development, because no ovulations occur before menarche (Figure 184-10). Although follicles may reach 6 mm in diameter during childhood, the size of the largest healthy, growing follicle does not generally exceed 10 mm.[124] The number of antral follicles also increases with age. In the newborn infant, three to five follicles larger than 1 mm in diameter may be present, whereas, in 8-year-old girls, the number is often 10 or more (Figure 184-11). This increase in the number of antral follicles is probably caused by the simultaneous increase in the basal levels of follicle-stimulating hormone and luteinizing hormone.[125]

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185 Testicular Development and Descent

Mary M. Lee

The testis originates as a bipotential gonad during sexual differentiation. Although chromosomal or genetic sex (46,XX or 46,XY) is established at the time of fertilization, the gonads and genitalia are initially similar morphologically with the capacity for differentiation to either a phenotypic male or female. The expression of *SRY* (the sex-determining region Y gene) is a key determinant for development of the embryo along a male-specific pathway. SRY activation initiates a series of transcriptional events that lead to testis determination, a critical step in sexually dimorphic sex differentiation.[1,2] Subsequently, the somatic cells of the testis secrete testosterone and Müllerian-inhibiting substance (MIS)—also known as anti-Müllerian hormone (AMH)—to elicit male-specific differentiation of the internal and external genitalia. This classic model for sexual differentiation was proposed by Professor Alfred Jost after he demonstrated in a series of grafting experiments that two discrete testicular hormones are needed for development of the male phenotype.[3] Jost hypothesized that testicular hormones direct differentiation along the male pathway; in the absence of a testis, female development was the default pathway.[4] This paradigm has been challenged in recent years with the recognition that specific gene signaling is also required for ovarian formation and development of female genitalia.[5]

TESTIS DETERMINATION

Three to four weeks after conception, primitive germ cells are visible in the dorsal endoderm of the yolk sac. These germ cells migrate to the hind-gut epithelium, then spread dorsally along the mesentery and the body wall mesenchyme in response to chemotactic signals to populate the primitive gonad in the urogenital ridge.[2,6,7] Epithelial cells delaminate from the coelomic epithelium, enter the urogenital ridge, and enclose the germ cells as they reach the urogenital ridge to form the primary sex cords. The germ cells actively divide during their migration from the yolk sac endoderm to the urogenital ridge. Although germ cells along the migratory pathway that fail to reach the ridge degenerate, residual primitive germ cells at these extragonadal sites are thought to be the origin of teratomas.[8] Transcription factors such as steroidogenic factor-1 *(SF1),* homeobox genes (*Lhx-9, Lim1,* aka *Lhx1,* and *Emx2*), and the Wilms tumor suppressor gene *(Wt1)* play key roles in early gonadal formation.[5,9-12] In mice with targeted mutations of these genes, an indifferent gonad is formed initially but fails to develop further and degenerates. Some of the genes critical for testis development also play a role in other aspects of fetal development. *WT1* gene mutations result in several syndromes associated with gonadal dysgenesis and renal impairment, whereas *SF1* mutations manifest as adrenal and gonadal dysgenesis.[9,11,13-15] At later developmental stages, *SF1* is a sexually dimorphic gene that is repressed in the ovary and up-regulated in the differentiating testis to control somatic cell differentiation and induce steroidogenic enzyme expression.[16]

The gonad remains undifferentiated until 6 weeks gestational age when activation of *SRY* in 46,XY embryos initiates the male developmental pathway[1,6,7] (Figure 185-1, *A*). *SRY* is a member of the SOX family of transcription factors that all share a high mobility group DNA-binding motif.[17] Activation of a transient but precise spatiotemporal pattern of expression of SRY in pre-Sertoli cells up-regulates the expression of *SOX9* (17q24.3-25.1), a key autosomal sex determining gene.[18,19] *SOX9* is initially expressed in the supporting cell lineage of both male and female gonads. After *SRY* activation, *Sox9* expression increases in males and shifts from a cytoplasmic to nuclear distribution.[20,21] In females, in the absence of SRY, *SOX9* expression is down-regulated. Patients with loss of function mutations in either *SRY* or *SOX9* genes have varying degrees of testicular dysgenesis.[22-26] In the mouse, the desert hedgehog gene *(Dhh)* and its receptor *Patched 1 (Ptch1)* are also expressed in male gonads soon after *Sry* is activated; *Dhh* localizes to Sertoli cell precursors and *Ptch1* to peritubular cells and Leydig cell precursors.[27,28] The expression of *Dhh* is not essential for initial testicular determination, but is critical for subsequent testicular differentiation and morphogenesis. Targeted deletion of *Dhh* disrupts early cord formation and severely impairs spermatogenesis and fetal Leydig cell differentiation. Other, as yet unidentified, autosomal genes located within chromosomal deletions, such as 9p24 and 10q that are associated with sex reversal or partial gonadal dysgenesis, may also play critical roles in testicular differentiation.

Sry expression in pre-Sertoli somatic cells stimulates their proliferation and differentiation to the Sertoli cell lineage denoted by expression of MIS and other Sertoli cell-specific genes.[6,7] *Sry* also stimulates migration of peritubular myoid cells and endothelial cells from the mesonephros into the ridge—an essential step in the formation of testicular cords.[29] Shortly after Sertoli cell differentiation, male specific vascularization of the gonad occurs with organization of the gonad into discrete cords (see Figure 185-1, *B*). The coelomic vessel is formed with a network of branching capillaries restricted to the interstitial space. These capillaries eventually drain into venules in the mesonephros and are thought to be important for rapid distribution of testicular hormones into the systemic circulation. Concurrently, fetal Leydig cells differentiate from the interstitial mesenchymal cells between the testicular cords. The interstitium also contains endothelial cells, macrophages, and fibroblasts. The Leydig cells are the steroidogenic cells of the testis that produce androgens necessary for virilization of the male genitalia and internal reproductive tract. The testicular cords start to separate from the overlying epithelium as a dense layer of fibroblastic cells forms the tunica albuginea. During this period, the primordial germ cells within the testicular cords continue to differentiate and proliferate, increasing from 3000 to 30,000 in number by 9 weeks gestation.[30] In contrast to ovarian development, however, the presence of germ cells is not necessary for testicular morphogenesis to proceed. By the ninth week, the rete testis, the anastomosing ends of the cords near the hilus of the testis, extends into the mesonephric ridge and later connects to the remaining mesonephric tubules to eventually form the ductus deferens.

INTERNAL REPRODUCTIVE TRACTS AND EXTERNAL GENITALIA

The urogenital ridge arises from the intermediate mesoderm as bilateral thickenings at the ventrolateral surface of the mesonephros. The wolffian ducts, the anlagen of the male internal

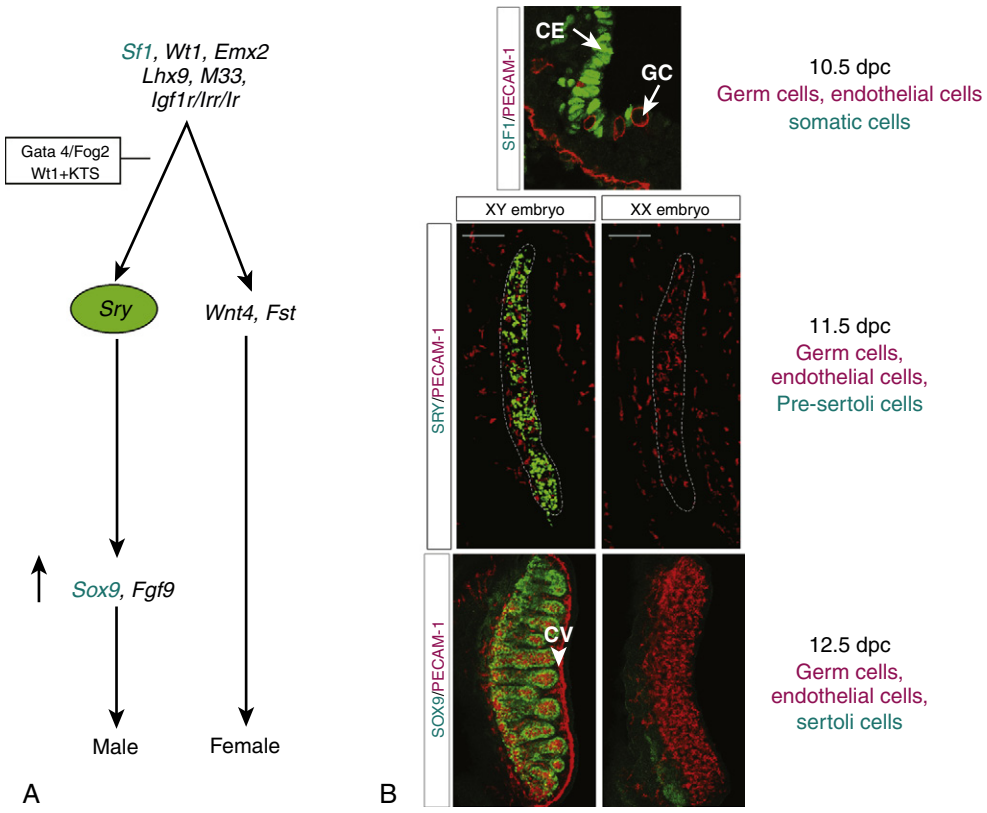


Figure 185-1 The mammalian sex determination pathway. A, The genes activated during gonad determination as a testis or ovary. **B,** Confocal images showing the sex-specific changes in gonadal morphology and gene expression during this early stage of gonadal development. *(From Polanco JC, Koopman P: Sry and the hesitant beginnings of male development,* Dev Biol *302:13, 2007.)*

reproductive tract, and the Müllerian ducts, the precursors of female structures, are present in all embryos.[4,5] The Wolffian duct arises as the caudal extension of the pronephric excretory duct. During gonadogenesis, the Müllerian (paramesonephric) duct develops in close proximity to the wolffian duct by invagination of the coelomic epithelium to form a tube surrounded by mesenchymal cells. The undifferentiated urogenital ridge has the capacity for developing male or female internal reproductive tracts depending on the local hormonal milieu. A number of genes that are critical for formation of the bipotential gonad are also essential for formation of the urogenital ridge. Mice with null deletions of *Lim1, Lhx9 and Emx2* manifest defects in urogenital ridge development as well as absent gonads.[10,12,31] In mice with *Pax2* deletions, the gonads form normally, but the genital ducts fail to develop, suggesting a specific role for *Pax2* in controlling differentiation of the intermediate mesoderm.[32] *Pax2* may also have a secondary role in wolffian duct differentiation as it localizes to the duct at later stages of genital tract development.

Paracrine hormones from the testis play key roles in mediating the sexually dimorphic differentiation of the wolffian (male) and Müllerian (female) internal genital ducts.[4,5,7] To achieve normal male differentiation of the internal and external genital structures, MIS and testosterone must be secreted by the fetal testis at the correct developmental stages and their downstream signaling pathways must be intact. A number of genes, including *SRY, SOX9s, SF1, GATA-4,* and *WT1* have been shown to act cooperatively or synergistically *in vitro* to transactivate MIS expression in Sertoli cells as an early event in testicular determination.[33,34] MIS, a 140 kDa glycoprotein in the transforming growth factor (TGF) β family of cellular growth and transcription factors, is produced as a prohormone requiring proteolytic cleavage to generate a bioactive hormone.[35-37] MIS binds to cell surface serine-threonine kinase receptors to signal through the Sma and Mad related proteins (SMAD) pathway. Although MIS receptors localize to the *mesenchymal* cells surrounding the Müllerian duct, epithelial-mesenchymal interactions culminate in apoptotic cell death of the adjacent epithelial cells.[38] MIS-induced regression of the Müllerian ducts starts by 8 weeks and is complete by 12 weeks gestation, leaving a vestigial remnant that persists as the prostatic utricle. Androgens have no independent effect on the Müllerian duct but act synergistically with MIS to promote full regression, whereas estrogens inhibit the actions of MIS on duct involution.

Testosterone-stimulated differentiation and growth of the Wolffian structures (epididymis, vas deferens, seminal vesicles) occurs between 9 and 14 weeks of embryonic life.[2,7,18] *SF1* induces the expression of the genes in the steroidogenic pathway: acute steroidogenic regulatory protein, the cytochrome P450 steroid hydroxylases, and 3β-hydroxysteroid dehydrogenase to initiate testosterone biosynthesis in Leydig cells.[16] Virilization of the external genitalia is also androgen dependant but requires additional reduction of testosterone to its more active metabolite, dihydrotestosterone by 5α-reductase, an enzyme present on genital skin. During the early stages of sex differentiation, fetal Leydig cell differentiation and synthesis of testosterone is gonadotropin-independent. Later in gestation, however, human chorionic gonadotropin or luteinizing hormone (LH) stimulation is needed for the continued synthesis of testosterone and completion of fetal masculinization.

LEYDIG CELLS

The Leydig stem cells are believed to migrate from either the mesonephrogenic mesenchyme or coelomic epithelium to the gonadal ridge where they localize to the interstitial space of the developing testis.[2,6,39] The initial differentiation of fetal

Leydig cells and onset of androgen production at 7 to 8 weeks occurs before the onset of pituitary LH production and testicular LH receptor expression, thus is considered LH independent.[40,41] Although the specific signal(s) triggering fetal Leydig cell differentiation remain uncertain, several genes are known to be critical for development of the Leydig cell lineage. Disruption of the GATA/FOG-2 interaction results in decreased expression of male-specific genes in the somatic cell lineages and in aberrant Leydig cell differentiation.[42] Sertoli cell secreted factors such as desert hedgehog (DHH) and genes expressed in Leydig cell precursors such as the Pdgf receptor-α and GATA-4 play key roles in modulating further differentiation of fetal Leydig cells.[6,18,27,28,39] *SF1,* a gene expressed downstream of Sry, has a pivotal role in gonadal and adrenal organogenesis as well as being a transcriptional activator of steroid hydroxylases and other genes that are essential for androgen biosynthesis.[16]

Fetal Leydig cells progressively increase in abundance to reach a maximum number at 48×10^6 cells per pair of testes at week 14, then remain stable until week 24 when they start to decline in number.[39,43,44] The increase in Leydig cell numbers is attributed to proliferation of the precursor cells because differentiated steroidogenic Leydig cells do not divide. From weeks 12 to 18, the Leydig cells appear hyperplastic and comprise the largest relative percentage of the testicular volume during fetal development. The fetal Leydig cells are at their most active phase of testosterone production and serum testosterone concentrations reach peak levels during this period (Figure 185-2). In the third trimester, fetal Leydig cells decline in number and mass through degeneration to reach their lowest numbers at 18×10^6 cells per pair of testes just before birth. The decrease in Leydig cell numbers during the latter part of gestation is accompanied by a parallel decline in androgen production.[41]

Androgen biosynthesis and secretion by fetal Leydig cells is essential for virilization of the internal and external genitalia. The hallmark of a terminally differentiated Leydig cell is the acquisition of steroidogenic capacity.[39,44] As the testis organizes into cords, 3β-hydroxysteroid dehydrogenase starts to be expressed in somatic interstitial cells that are identifiable as Leydig cells by ultrastructural features of steroidogenic cells. The synthesis of testosterone commences at weeks 7 to 8 and thereafter increases markedly to reach peak rates at 14 to 16 weeks. During the critical window of sex differentiation from week 8 to 12, the abundant production of testosterone and its conversion to the more active metabolite, dihydrotestosterone, are believed to be gonadotropin independent.[39-41,44] By the second trimester, androgen biosynthesis becomes gonadotropin-dependent as Leydig cells start to express the LH receptor, which is responsive to the high concentrations of placental human chorionic gonadotropin in the intrauterine environment. In concert with the induction of LH receptor expression, pituitary secretion of LH increases during the latter half of gestation.[41] Fetal Leydig cells are not subject to negative feedback by LH and have a greater response to its stimulatory effects, in contrast to robust negative feedback of the postnatal adult Leydig cells by LH. Thus the steroidogenic capacity per fetal Leydig cell is higher than that of adult Leydig cells.[44] Nevertheless, before birth the steroid content per fetal Leydig cell decreases (perhaps as a result of inhibitory paracrine hormones) and circulating testosterone concentrations are much lower because of the reduced numbers of Leydig cells.

Discrepancies exist in the data on the effects of other hormones and paracrine factors on steroidogenesis in fetal Leydig cells. Fetal Leydig cells have been shown to be insensitive to the inhibitory effects of estrogens, a property that would enable the fetus to circumvent the high concentrations of maternal estrogens that might be present in the intrauterine milieu.[45,46] Conversely, studies have also shown that estrogens inhibit testosterone production in cultured Leydig cells and decrease Leydig cell numbers. Other negative regulators of fetal steroidogenesis include two paracrine factors, MIS and TGF-β, that both have inhibitory effects on androgen biosynthesis.[44,47] In addition to hormones and growth factors, extracellular matrix components and structural interactions may have modulatory influences.[39]

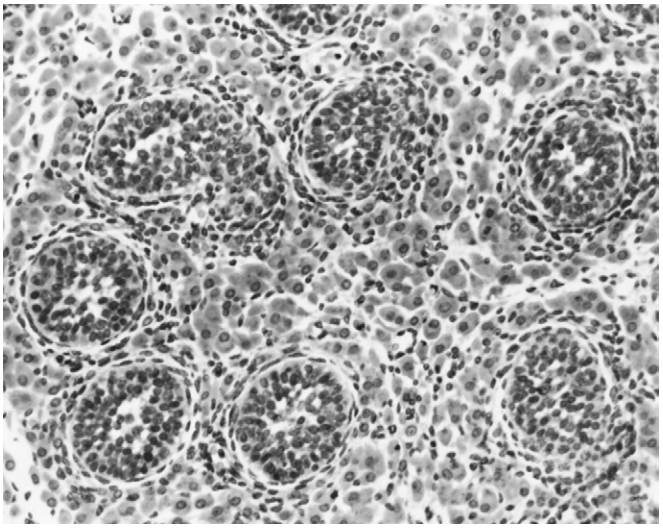


Figure 185-2 Human fetal testis histology. Section of testis at 12 weeks gestation showing the seminiferous cords and abundant Leydig cells in the interstitial spaces (×250). *(Reprinted with permission from Aslan AR, Kogan BA, Gondos B: Testicular development. In Polin RA, Fox WW, Abman SH [eds]:* Fetal and neonatal physiology, *ed 3, Philadelphia, 2004, WB Saunders, pp 1956-1960.)*

After birth, a transient neonatal activation of the hypothalamic-pituitary-gonadal axis at 2 to 4 months of age stimulates an increase in Leydig cell numbers and a robust rise in androgen production.[48] This is followed by dedifferentiation and/or degeneration of the fetal Leydig cells and a marked decline in androgen production to the low levels that persist until the onset of puberty.[43] Very few differentiated Leydig cells are present in the prepubertal testis. The interstitial space contains predominantly mesenchymal cells that are precursors of the postnatal adult Leydig cells. These "infantile" Leydig cells have a multilobed nucleus and minimal smooth endoplasmic reticulum. The mesenchymal cells proliferate and increase in number during childhood, then differentiate to adult steroid-secreting cells with a large polygonal configuration, eccentric nuclear position, abundant smooth endoplasmic reticulum, pleomorphic mitochondria, and well-developed Golgi apparatus at puberty.[39] LH, in concert with other hormones that stimulate androgen production, promotes maturation of the steroidogenic enzyme pathway to progressively increase androgen production. The secretion of testosterone during puberty is needed to achieve spermatogenesis and secondary sexual maturation. In the adult, the terminally differentiated Leydig cells produce testosterone abundantly. Morphologically, the adult Leydig cells can be distinguished from fetal Leydig cells by the presence of Reinke crystals, which are of unknown function but specific to mature Leydig cells. The number of mature Leydig cells in the adult testis is relatively static as the terminally differentiated cells do not divide, although a limited number of Leydig cells may arise de novo from mesenchymal precursors in the interstitial space.

SERTOLI AND GERM CELLS

As the primordial germ cells migrate to the urogenital ridge, they are enclosed by fetal Sertoli cells and organized into seminiferous cords (see Figure 185-1 and Figure 185-3). The Sertoli cells

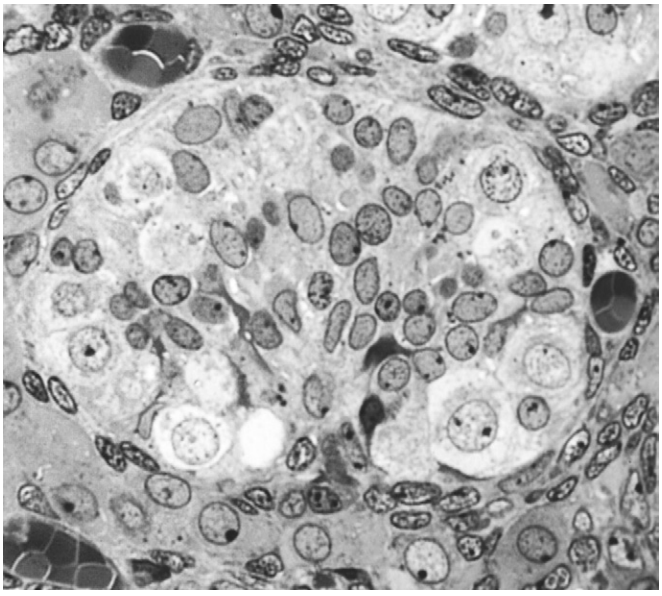


Figure 185-3 Seminiferous cords in human fetal testis with Sertoli cells and round immature germ cells at the periphery. (toluidine blue, ×500). *(Reprinted with permission from Aslan AR, Kogan BA, Gondos B: Testicular development. In Polin RA, Fox WW, Abman SH [eds]:* Fetal and neonatal physiology, *ed 3, Philadelphia, 2004, WB Saunders, pp 1956-1960.)*

are considered the "nurse" or supporting cells for the germ cells and regulate many germ cell functions. A deficiency in Sertoli cell number will impact the number and quality of sperm in the mature testis. The relative numbers of Sertoli and germ cells within the seminiferous tubules vary throughout the stages of reproductive development.[49] Initially predominantly Sertoli cells exist, but the high fetal testosterone concentration from weeks 8 to 22 gestation stimulates proliferation of the germ cells to reverse the ratio of germ cells to immature Sertoli cells. As testosterone production declines during the third trimester, however, germ cell mitotic activity also declines, resulting in a lower ratio of germ cells to Sertoli cells, which is sustained until puberty. After birth, germ cells proliferate transiently during the neonatal minipuberty, then stop dividing until the peripubertal years when a higher mitotic activity equalizes the number of germ cells and Sertoli cells in the pubertal testis. During early childhood, the Sertoli cells divide rapidly until the onset of puberty when they differentiate, form tight junctions, and no longer undergo cell division.[50]

In the immature testis, round to oval gonocytes are located centrally within the tubules. As they mature to prespermatogonia, they shift to the periphery of the tubules near the basal lamina. The prespermatogonia are connected by residual cytoplasmic bridges that remain after incomplete cytokinesis during mitosis. These interconnections between differentiating spermatogonia enable synchronized maturation of adjacent germ cells. Each germ cell division replenishes the spermatogonial pool as well as generates germ cells that undergo spermatogenesis. Spermatogonia are classified by their morphology, maturational stage, position within the germinal epithelium, and variations in nuclear chromatin precipitation.[51] More immature spermatogonia are found along the basement membrane whereas mature forms are present closer to the tubular lumen. Type A spermatogonia, divided into Ad (dark type A) and Ap (pale type A) groups are considered part of the spermatogonia stem pool. Progressive differentiation to B type spermatogonia represents the first step of spermatid formation. Sequential stages of spermatogenesis can be identified within a testis, including, from the least to the most mature forms: Ad spermatogonia, Ap spermatogonia, type B spermatogonia, primary spermatocytes (leptotene, zygotene, and pachytene), secondary spermatocytes, Sa, Sb1, Sb2, Sc, Sd1, and Sd2 spermatids, and mature spermatozoa. One group found that in men who had orchidopexy before the age of two years old, the risk for infertility correlates with the presence of Ad spermatogonia at the time of orchidopexy; they have proposed that increased testosterone during the minipuberty of infancy may be essential for transformation of germ cells to Ad spermatogonia.[52]

The development of germ cells occurs in parallel with the maturation of Sertoli cells. Fetal Sertoli cells have round to elliptical nuclei and small nucleoli. They have a minimal smooth endoplasmic reticulum, but abundant ribosomes and rough endoplasmic reticulum.[53] At puberty, the immature Sertoli cells develop tight junctions and acquire differentiated characteristics. The presence of tight junctions between adjacent cell membranes creates the blood-testis barrier. This unique characteristic enables the Sertoli cells to create a microenvironment for controlling the biochemical milieu within the cords as well as to facilitate the migration of germ cells within the seminiferous tubules.

Although the Sertoli cell number does not change during pubertal maturation, they occupy a relatively smaller area of the tubules as the tubules elongate and enlarge due to active germ cell proliferation.[53] The Sertoli cells have a critical role in the production and secretion of various proteins, some of which act as endocrine and paracrine hormones. During testis determination, the first hormone secreted by the Sertoli cell is MIS, the hormone responsible for inducing regression of Müllerian duct structures in males during normal embryogenesis. Mutations of MIS or its type II receptor cause retention of Müllerian structures in males, a condition termed the persistent Müllerian duct syndrome. Although Müllerian duct regression is complete by the second trimester, MIS continues to be abundantly produced throughout gestation and even after birth.[54] In addition to its role in Müllerian duct regression, MIS may also have paracrine modulatory actions on germ cell maturation and Leydig cell development in males.[44,55]

The Sertoli cells also secrete inhibin, a related hormone in the TGF-β family that acts via negative feedback on the pituitary to downregulate follicle-stimulating hormone secretion.[56] Like the other members of this gene family, inhibin consists of two subunits—inhibin A and inhibin B—and undergoes proteolytic processing. Inhibin indirectly regulates spermatogenesis through its modulation of follicle-stimulating hormone but is also believed to have direct paracrine effects on spermatogenesis.[57]

BLOOD-TESTIS BARRIER

The Sertoli cells develop tight junctions that create a physical barrier between the bloodstream and the testicular tubules.[58] In rats, the blood-testis barrier does not fully develop until puberty; in humans, the age at which the barrier becomes fully functional is not known. The blood-testis barrier helps maintain the microenvironment of the apical adluminal compartment of the seminiferous tubules. Experimental data show that the tight junctions prevent passage of large tracers from the basal compartment, that is, the interstitium to the apically located tubular lumen, whereas smaller tracers are not completely excluded.[58,59] This physical separation enables the Sertoli cell to control the special microenvironment of the tubular lumen to facilitate spermatogenesis. The blood-testis barrier is also thought to provide some immuno-protection and prevent autoimmune destruction of differentiating germ cells. Some of the mechanisms by which Sertoli cells maintain the barrier have been elucidated and a number of extracellular matrix proteins and ectoplasmic specialization proteins are critical for its structural and functional integrity. In the rat testis, inhibitors of protein phosphatase disrupt the barrier,

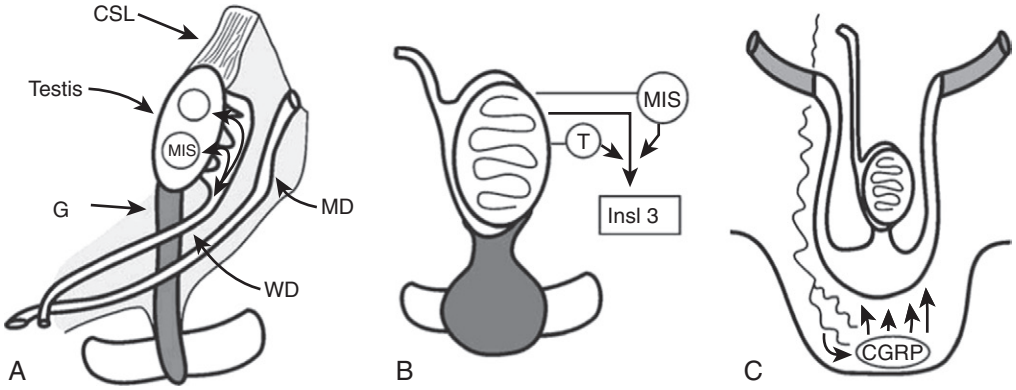


Figure 185-4 Schematic depicting the two phases of testicular descent. A, Initial intraabdominal position of the gonads, Wolffian duct *(WD),* Müllerian duct *(MD),* cranial suspensory ligament *(CSL),* and gubernaculum *(G).* **B,** The transabdominal phase of testicular descent is mediated primarily by INSL3. **C,** The inguinal scrotal phase of testicular descent is an androgen-dependent process. *(Reprinted with permission from Hutson JM, Hasthorpe S: Testicular descent and cryptorchidism: the state of the art in 2004,* J Pediatr Surg *40:297, 2005.)*

whereas pretreatment with protein tyrosine kinase inhibitors can prevent this effect. Similarly, both protein kinase A activator and protein kinase C inhibitors also perturb the Sertoli cell tight junctions, indicating a regulatory role for protein kinases. Formation of the barrier can be disrupted by administration of certain toxins, hormonally active compounds such as diethylstilbestrol, and heavy metals such as cadmium.

More immature spermatogonia (spermatogonia and preleptotene spermatocytes) are located close to the basement membrane, whereas more mature, differentiating spermatocytes and spermatids are found in the adluminal compartment. During spermatogenesis, at the time of meiosis, the intercellular junctions are remodeled to enable migration of the preleptotene and leptotene spermatocytes across the barrier into the adluminal compartment. From the apical side of the tubules, the spermatogonia complete maturation and are released from the seminiferous epithelium. The regulation of this process is not fully understood, but cytokines are known to have a role in the restructuring of the tight junctions that is essential during spermatogenesis.[60]

PERITUBULAR STRUCTURES

Although the organization of the interstitial cells varies among species, the peritubular structures uniformly help impart structural integrity to the seminiferous tubules. In humans, the lamina propria in humans contains five to seven layers separating the seminiferous tubules from the interstitium.[59] The outer one to two layers consist of fibroblastic cells that form the adventitia and provide structural support for the tubules. The middle layers contain myoid cells that have fibroblastic characteristics and help create the blood-testis barrier. These myofibroblasts have contractile ability and may therefore have a significant role in movement of spermatozoa through the tubules to the rete testis. In experimentally created cryptorchidism, there is thickening of the peritubular structures that may interrupt the movement of the differentiating germ cells and contribute to the associated infertility. The collagen-rich innermost layer is immediately adjacent to the basal membrane of the tubules. This cellular layer produces extracellular matrix proteins, which are needed for structural support of the basement membrane, and secrete paracrine growth factors, such as peritubular factor that modulates sertoli cell function (PModS) and TGF-α, that help regulate Sertoli cell function and differentiation.

A better understanding of testis development has revealed the complex cell-to-cell interactions and the diverse signaling pathways that are utilized to establish the organizational structure of the testes and achieve the maturational processes that are needed for virilization of the genitalia and reproductive competence. The descent of the testes from an intraabdominal to a scrotal position is a key developmental step to attain reproductive competence.

REGULATION OF TESTICULAR DESCENT

In conjunction with *in utero* testicular development and somatic and germ cell differentiation, the testis undergoes anatomic changes in its position during the second half of gestation. Insights on the process of testicular descent from an abdominal to scrotal position have been gained by investigating this event in animals. Differences among species in timing, anatomy and final testicular position, and hormonal milieu, however, limit the ability to fully extrapolate observations in animal models to human testicular descent. For example, in contrast to the late fetal timing of human testicular descent, testes in rats do not descend until puberty and move easily into the scrotum due to the formation of a pouch-like processus vaginalis. Aside from animal studies, clinical investigations that may include comprehensive hormonal and radiologic assessment and surgical or autopsy explorations with pathology specimens have helped clarify this process in humans.

The current model consists of a biphasic process comprising two distinct phases—a transabdominal and an inguinal-scrotal step[61] (Figure 185-4). When testes develop from the mesonephros, they are attached to the posterior abdominal wall by the cranial suspensory ligament and caudally by the genitoinguinal ligament (or the gubernaculum).[62,63] During the transabdominal phase of descent, the cranial suspensory ligament regresses, whereas the caudal end of the gubernaculum enlarges in a "swelling reaction" to form an outgrowth. The testes are anchored by the gubernaculum to the abdominal wall near the site of the future inner ring of the inguinal canal. Consequently, as the abdominal cavity enlarges, the testes remain in the lower abdomen and eventually assume a position lateral to the inferior pole of the kidneys.

The key hormone responsible for the transabdominal phase of testicular descent is insulin-like factor 3 (INSL3), also known as relaxin-like factor, a member of the insulin-like superfamily produced in Leydig cells.[64] INSL3 signals through its receptors (leucine-rich repeat-containing G protein–coupled receptor 8) on gubernacular cells to stimulate gubernacular differentiation and growth. In humans, the gubernaculum increases in size from 15 to 24 weeks gestation. This gubernacular outgrowth ("swelling") is characterized by hyperplasia and hypertrophy of the mesenchymal cells due to an increase in glycosaminoglycans and hyaluronic acid that promotes water retention and makes the

gubernaculum gelatinous.[63] Male mice with a null mutation of *Insl3* have a poorly developed gubernacular bulb, intact cranial suspensory ligament,[65,66] and undescended testes, whereas overexpression of *Insl3* in females leads to descent of the ovaries to the inguinal region.[67] Although mutations in *Insl3* and its receptor, leucine-rich repeat-containing G protein-coupled receptor 8, have been implicated in patients with cryptorchidism, the phenotype is more variable.[64] Moreover, the etiology of undescended testes in humans appears to be more complex and multifactorial because only a small subset of patients with cryptorchidism are found to have *INSL3* gene and promoter mutations.[68]

The cranial suspensory ligaments regress in males during the transabominal phase of testicular descent but remain intact in females, suggesting that their regression is needed for full abdominal descent. This process is thought to be under androgen control. In rodent models, prenatal antiandrogen treatment prevents complete regression of the cranial suspensory ligaments and prenatal androgen treatment induces regression of the ligaments and partial descent of ovaries.[69,70] In the absence of androgen action, such as with androgen-insensitive testicular feminizing mice and patients with androgen insensitivity syndrome, the cranial suspensory ligaments do not regress and the gubernacular bulb is smaller.[63,71,72] In patients with androgen insensitivity syndrome, the testes are located either in the inguinal region (suggesting that the transabdominal phase of descent occurs even in the absence of androgen action) or midway down the abdomen (suggesting that androgens may play a supportive role in this process).[73] These data confirm that androgens have only a minor role in the transabdominal phase of testicular descent; their predominant role is to mediate the movement of the testes from the inguinal region through the inguinal canal into the scrotum during the second inguinal-scrotal phase of testicular descent.

During the inguinal-scrotal phase of descent from weeks 25 to 32 of gestation, the testes move from within the abdominal cavity at the level of the anterior iliac spine through the internal and external rings of the inguinal canal into the scrotal sacs.[61-63,71,74,75] The period of gubernacular outgrowth during the transabdominal phase is followed by protrusion of the bulb through the developing scrotal sac and formation of the processus vaginalis that facilitates movement of the testes through the inguinal canal. Subsequent shortening of the proximal gubernaculum and involution of the caudal bulb also promotes movement of the testes from the external inguinal ring to the bottom of the scrotal sacs.

The actions of androgens are essential for the inguinal-scrotal phase of testicular descent. Cryptorchidism is a cardinal feature of a number of animal models and human disorders associated with either inadequate androgen action or perturbation of the hypothalamic-pituitary-gonadal axis.[61,71,73,74,76] Patients with hypothalamic hypogonadism or other forms of hypopituitary dysfunction have a much increased rate of inguinal testes. As described previously, cryptorchidism is also seen commonly in infants with androgen insensitivity syndrome or defects in testosterone biosynthesis that results in insufficient androgen action.

A number of animal studies have explored the dependency of testicular descent on androgens as well as the adjunct role of other hormones on this complex process. In rodents, testicular descent into the scrotum occurs at puberty rather than in utero; thus models manipulating the postnatal hormonal environment are not directly applicable to human testicular descent. For example, in postnatal rats, removal of the salivary glands, the major source of epidermal growth factor compromises testicular descent at birth and administration of epidermal growth factor reverses cryptorchidism induced by flutamide, but whether epidermal growth factor modulates testicular descent in humans is unknown.

Administration of estrogenic agents, endocrine disrupting compounds, and antagonists or antibodies that block LH, INSL3, and testosterone action can all inhibit testicular descent (reviewed in references 71, 74, and 75). Several models of experimentally induced cryptorchidism can be reversed by administration of exogenous testosterone, confirming its critical role for this developmental event.[77] Although 5α-reductase activity is present in the rat gubernaculum, finasteride, a 5α-reductase inhibitor, has little effect on testicular descent. The conversion of testosterone to dihydrotestosterone, therefore, does not appear to be essential, and testosterone itself is the primary hormonal mediator of inguinal-scrotal descent.

Clinical studies have examined differences in gonadal and pituitary hormones between children with cryptorchidism and those with normal testicular descent.[78] Boys with cryptorchidism have been reported to have lower MIS values, lower inhibin, and higher follicle-stimulating hormone values; normal, lower, or higher LH values; and lower or normal testosterone values than controls.[78-83] These discrepant findings may reflect differences in the patient population investigated and the heterogeneity in the etiology of cryptorchidism. Nevertheless, these data are consistent with the role of androgens as the primary determinant of the inguinal-scrotal phase of testicular descent.

The effects of androgens on testicular descent are mediated both directly through androgen receptors expressed on mesenchymal cells found between the smooth muscle cells and also indirectly via androgen actions on the genitofemoral nerve.[61,71] The key role of the genitofemoral nerve was recognized via experiments demonstrating that transection of the genitofemoral nerve early in gestation inhibited gubernacular development and left the testes in an inguinal position.[63] Although this was initially thought to be due to loss of motor innervation of the cremasteric muscle, later studies suggested that this manipulation was interfering with secretion of an androgen stimulated neuropeptide, calcitonin gene–related peptide (CGRP), by sensory neurons in the genitofemoral nerve. The role of the genitofemoral nerve and CGRP has subsequently been further clarified by this group.[61,63] They propose a model in which androgens masculinize the sensory neurons in the genitofemoral nerve to stimulate synthesis and release of CGRP into the gubernaculum. Receptors for CGRP are expressed on embryonic myotubules found within the gubernacular cells in the area of the developing cremasteric muscle. CGRP stimulates rapid rhythmic contractions of the gubernaculum that may be important for its migration to the scrotum. In vitro, CGRP has been shown to act as a chemotactic and proliferative signal for gubernacular cells at its caudal end. Exogenous treatment with a synthetic CGRP antagonist (CGRP 8-37) has been shown to delay testicular descent in rats, whereas administration of CGRP stimulated testicular descent in cryptorchid pigs. The data supporting a role of CGRP in human testicular descent are limited to evidence that CGRP is able to stimulate fusion of inguinal hernia sacs in organ culture.[84] Although indirect, this serves as a model for one of the events that occur during testicular descent, the obliteration of the processus vaginalis.

THE DIAGNOSIS AND INCIDENCE OF CRYPTORCHIDISM

Clinical disorders of testicular descent are the most common minor congenital abnormality in male newborns.[85] The term for this condition, *cryptorchidism,* is derived from the Greek words *kryptos* and *orchis* for "hidden gonad." Most commonly, the undescended testes are found in the inguinal region, stemming from a disruption of the more complex inguinal-scrotal phase of descent, especially involving the slower process of migration from the external ring to the scrotum. The testes, however, can be asymmetrically positioned and found anywhere along the path of embryologic testicular descent, even in ectopic locations (Figure 185-5).

The etiology of cryptorchidism is unknown in the majority of cases and appears to be multifactorial.[71,74,75,86] Only a minority

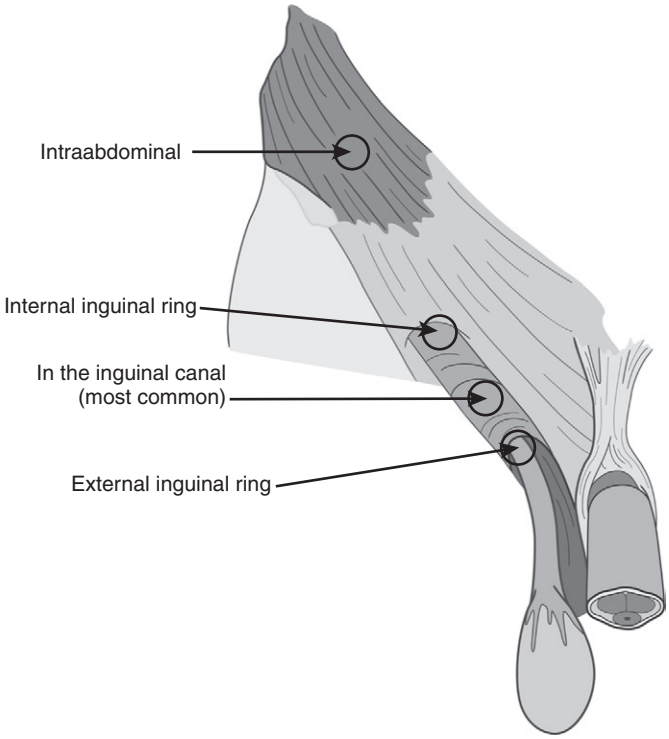


Figure 185-5 Common locations of the cryptorchid testis. *(From Christensen JD, Dogra VS: The undescended testis,* Semin Ultrasound CT MR *28:307, 2007.)*

of cases are identified as being caused by a specific genetic or endocrine disorder, although the likelihood of finding a specific etiology increases if associated anomalies are present. Epidemiologic studies have demonstrated geographic differences in the prevalence of cryptorchidism and other male reproductive disorders, such as hypospadias and decreased fertility, suggesting that underlying genetic, lifestyle, or environmental factors may play a causative role.[75,85,87] Some studies also report an increase in the incidence of these conditions in the past century.[75] A unified hypothesis of a testicular dysgenesis syndrome due to embryonic disturbance in testicular formation or function has been proposed to explain these findings.[88]

One of the major impediments to determining the exact prevalence of cryptorchidism is the lack of specificity in the diagnosis. Although the distinction between a completely nonpalpable, intraabdominal testis and a scrotal testis is readily appreciated, the difficulty lies in distinguishing inguinal undescended testes from those that are retractile. This becomes even more difficult later in infancy and childhood when the cremasteric reflex is more active and can have a high interobserver variability.[89] The cremasteric reflex is easily triggered during an examination when the scrotum is exposed to cold and the child is apprehensive, leading to potential misdiagnosis of a normally descended testis as being undescended. The erroneous classification of a retractile testis as cryptorchid and the different criteria used by investigators to define a cryptorchid testis may account for inconsistencies in the prevalence data among centers. Moreover, the lack of standardization in the diagnosis of cryptorchidism and the potential for misdiagnosis also contribute to the ambiguity on the efficacy of different modes of management.

A recent systematic review of the literature found a rate of undescended testes that varied from 1.0% to 4.6% in newborn infants with birth weight greater than 2.5 kg, and a rate of 1.1% to 45.35% in preterm infants weighing less than 2.5 kg.[85] At 1 year of age, the rate of undescended testes in the term infants had decreased to 1.0% to 1.5%, consistent with previous reports of spontaneous descent in the first year in 50% to 75% of cases, with most occurring in the first 3 months. Prevalence rates in school aged children were more variable—6-year-olds had a rate of 0% to 2.6% and 11-year-olds had a rate of 0% to 6.6%. In one longitudinal study, Scorer[90] reported that the majority of cryptorchid testes underwent spontaneous descent by 9 months of age with an incidence at birth of 2.7% decreasing to 0.8% by 1 year of age. An increase in prevalence, however, is noted in some school studies that has been attributed to acquired ascent of the testes during childhood as a result of inadequate elongation of the spermatic cord.[91,92] Prematurity and low birth weight are both significant risk factors for cryptorchidism, and a family history of cryptorchidism also increases the relative risk for cryptorchidism. A recent study showed that relative risk was 10.1% for twins and 3.52% for siblings.[93] Therefore familial as well as developmental or environmental factors may incur risk for the occurrence of cryptorchidism.

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